REVIEW OF LITERATURE
2.1. Tomato Crop

2.1.1. Crop history

Tomato (*Lycopersicon esculentum* Mill.) is one of the important ‘protective’ foods both because of its special nutritive values. It is the world’s largest vegetable crop produced after potato and sweet potato, but stands first when canned vegetables are considered (http://agridr.in/banking/PDF/Tomato.pdf). Cultivated tomato is thought to have originated in Peruvian or Mexican regions (Jenkins 1948). It was introduced into Europe by the Spanish explorers in the early sixteen century. Later, European migrants introduced tomato to USA and Canada. Tomato was perhaps introduced into India by Portuguese, though there is no definite record of when and how it entered India.

2.1.2. Production and importance of tomato

Tomato is the most important vegetable crop in India. It is grown in 865,000 hectares with an average productivity of 19.5 metric tonnes/ha in India (http://nhb.gov.in/area-pro/database-2011.pdf) mainly for fresh market consumption. In India, Karnataka is the largest producer of tomato with an annual production of 9.5 lakh tones from 46 000 hectares (Lokesh *et al.*, 2005). Tomato is cultivated in all the districts of the Karnataka but major area remains in Bangalore, Chikkaballapura, Doddaballapura and Kolar districts (Chowdappa *et al.*, 2013a).

Tomato is grown worldwide for its edible fruits, which are rich in vitamin A and C. Due to its high nutritional value (Table 2.1), tomato has been used as protective food and also exploited for production of different by-products like sauces.
Table 2.1. Nutrion value of tomato

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Nutritive value</th>
<th>Quantity/100g of edible fruit</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Moisture</td>
<td>93.1g</td>
</tr>
<tr>
<td>2</td>
<td>Protein</td>
<td>1.9g</td>
</tr>
<tr>
<td>3</td>
<td>Fat</td>
<td>0.1g</td>
</tr>
<tr>
<td>4</td>
<td>Minerals</td>
<td>0.6g</td>
</tr>
<tr>
<td>5</td>
<td>Fibre</td>
<td>0.7g</td>
</tr>
<tr>
<td>6</td>
<td>Other carbohydrates</td>
<td>3.6g</td>
</tr>
<tr>
<td>7</td>
<td>Calcium</td>
<td>0.2g</td>
</tr>
<tr>
<td>8</td>
<td>Magnesium</td>
<td>0.15g</td>
</tr>
<tr>
<td>9</td>
<td>Oxalic acid</td>
<td>0.2g</td>
</tr>
<tr>
<td>10</td>
<td>Phosphorous</td>
<td>0.036g</td>
</tr>
<tr>
<td>11</td>
<td>Iron</td>
<td>0.18g</td>
</tr>
<tr>
<td>12</td>
<td>Vitamin A</td>
<td>320 I.U.</td>
</tr>
<tr>
<td>13</td>
<td>Thiamine</td>
<td>0.07mg</td>
</tr>
<tr>
<td>14</td>
<td>Riboflavin</td>
<td>0.01mg</td>
</tr>
<tr>
<td>15</td>
<td>Nicotinic acid</td>
<td>0.4mg</td>
</tr>
<tr>
<td>16</td>
<td>Vitamin C</td>
<td>31 mg</td>
</tr>
<tr>
<td>17</td>
<td>Calories</td>
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</tr>
<tr>
<td>18</td>
<td>Sodium</td>
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</tr>
<tr>
<td>19</td>
<td>Potassium</td>
<td>114 mg</td>
</tr>
<tr>
<td>20</td>
<td>Copper</td>
<td>0.19 mg</td>
</tr>
<tr>
<td>21</td>
<td>Sulphur</td>
<td>24 mg</td>
</tr>
<tr>
<td>22</td>
<td>Chlorine</td>
<td>38 mg</td>
</tr>
</tbody>
</table>
2.1.3. Climate and Soil

Tomato is grown mainly in warm season as it does not survive frozen conditions. The optimum temperature for growth of tomato crop is 21°C- 23°C, but tomatoes are commercially grown at temperature ranging from 18°C to 27°C. Temperature and light intensity affect the fruit set, pigmentation and nutritive value of the fruit.

The best soil for growth of tomato is loam, with a little sand in the upper layer and good clay in the sub soil. Primary importance is soil texture and good early crops can also be produced in poor and medium quality lands, if managed properly.

2.2. Diseases of tomato

Tomato is susceptible to many fungal, bacterial and viral diseases. Among these, late blight, incited by *Phytophthora infestans* (Mont.) de Bary (Deahl *et al.*, 2006), is a major production constraint, causing losses up to 100 per cent (Fry *et al.*, 1993).

2.3. Late Blight

Late blight is one of the most devastating diseases of potato and tomato worldwide. Late blight was responsible for destruction of potato crop in Ireland in the 1840's, due to which 1 million people died and 2 million people emigrated (Martin *et al.*, 2013). The first occurrence of the disease was reported from the East coast of the USA around 1843. In 1845, the first outbreaks in Europe were discovered in Belgium. Later, the same year, it spread to Holland, Germany, England and Ireland (Bourke, 1991). Prior to 2006, late blight was not considered a
serious threat to tomato in India even though the disease was known since 1903 (Chowdappa et al., 2012). However, from 2006-2008, severe late blight epidemics have occurred both on potato and tomato throughout India (Chowdappa et al., 2012). Since the 2008 growing season, severe late blight epidemics have occurred on both tomato and potato crops in Karnataka, Tamil Nadu and Andhra Pradesh and have often caused 100% crop loss (Chowdappa et al., 2013).

2.3.1. Casual Organism

The late blight is caused by Phytophthora infestans (Mont.) de Bary. Heterothallic stramenopile Phytophthora infestans (Mont.) de Bary, is the most destructive disease of potatoes and tomatoes worldwide (Fry et al., 1993). It belongs to the oomycetes, a diverse group of eukaryotic microorganisms in a group called the Stramenopiles, clustering together with others in a super group, the Chromalveolata (Adl et al., 2005). The position of the oomycetes as a unique lineage of eukaryotes unrelated to true fungi, but closely related to heterokont (brown algae) and diatoms, is well established through molecular phylogenies and biochemical studies (Baldauf et al., 2000). It is characterized by the absence of chitin in the cell walls (true fungi contain chitin), and presence of β-glucan cellulose, zoospores with heterokont flagella (one whiplash and other tinsel) borne in sporangia, diploid nuclei in vegetative cells and sexual reproduction via oogonia and antheridia (Erwin and Riberio, 1996). This fungus is phylogenetically related to some algae (Erwin and Riberio, 1996). The asexual reproduction is through sporangia. The sporangia are caducous, semipapillate and limoniform to ellipsoid to ovoid shaped with short pedicels. Sporangia measure 29-36 µm in length and 19-22 µm in breadth. Sporangiophores formed in compound sympodia with characteristic swellings just below the sporangia. Each sporangium contain 7-12
zoospores. Zoospores are uninucleate. Hyphal swellings and chlamydomospores do not occur. *P. infestans* is a heterothallic fungus and forms oospores with A1 and A2 mating types. The oogonia are globose and average about 37 µm in diameter (Drenth *et al.*, 1995). The oospores are aplerotic with a diameter average about 30 µm. The antheridia are amphigynous and elongated with an average size of 23 x 18 µm (Erwin and Riberio, 1996).

The central highlands of Mexico are the center of origin and widespread diversity has been reported in virulence characteristics and neutral allozyme markers in the Mexican population (Grunwald *et al.*, 2001). Prior to 1980, the global population consisted of a single clonal lineage outside Mexico. This lineage was characterized by the A1 compatibility type and sensitivity to the phenylamide fungicide metalaxyl (Matuszak *et al.*, 1994). Isolates resistant to metalaxyl and belonging to the A2 compatibility type were detected in Europe during the 1980s (Schober-Butin, 2001). Since that time, numerous studies have reported the spread of the A2 compatibility type and metalaxyl resistance around the world. These ‘new’ populations have increased in frequency and replaced the ‘old’ populations in the Netherlands, Germany, Poland, United States, France and South East Asia. These clonal lineages were identified based on phenotypic markers such as mating type, virulence and metalaxyl sensitivity and genetic markers including RG57 probe generated RFLP patterns, mitochondrial DNA haplotypes, glucose 6-phosphate isomerase (*Gpi*) and peptidase (*Pep*) allozyme loci (Tooley *et al.*, 1985). The spread of the A2 compatibility type to areas where the A1 compatibility type has existed creates the possibility of sexual recombination. Oospores produced by sexual recombination may provide another source of initial inoculum for initiating epidemics and lead to offspring with characteristics different from the
parental types. Five genotypes are known to occur in *P. infestans* in USA, viz., US-1, US-6, US-7, US-8, US-14 of which, US-7 isolates are more aggressive on tomato than potato (Kirk *et al.*, 2004). This genotype belong to A2 mating type, but differs from European A2 genotypes by a number of features, such as alleles at *Gpi* and *Pep* loci (Fry *et al.*, 1992).

### 2.3.2. History of pathogen

The term *P. infestans* was coined by Anton deBary. The name is derived from the Greek: ‘Phyto’ = plant, ‘phthora’ = destroyer. *P. infestans*, which causes the late blight diseases of potato and tomato, has played a vital role in the history of mycology, plant pathology and mankind. This oomycete is best known for the human suffering that it caused during the Irish potato famine of the 1840s (Bourke, 1991).

Mexico is considered as the centre of origin of *P. infestans* because of presence of both A1 and A2 mating types and high genetic diversity within population in that region. In contrast, only A1 mating type and low genetic diversity was detected outside Mexico till 1980 (Gómez-Alpizar *et al.*, 2007). The first global migration of *P. infestans* occurred in the 1840s. The second migration including A2 mating type, probably occurred from Mexico during 1970s. The mechanism of migration was probably via infected potatoes and tomatoes. Apparently large quantities of potatoes and tomatoes for domestic consumption were imported from Mexico to Western Europe and USA respectively in the late 1970s (Gómez-Alpizar *et al.*, 2007).
2.3.3. Taxonomy

The prescribed name for this group of organisms is oomycota which have been assigned to the Kingdom Stramenopila of the eukaryotes. Oomycetes are no longer considered members of the Kingdom Fungi although they share many biological, ecological, and epidemiological characteristics with fungal plant pathogens (Volk, 2001).

Taxonomic classification of *P. infestans*

<table>
<thead>
<tr>
<th>Taxonomic Level</th>
<th>Taxon Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Domain</td>
<td>Eukarya</td>
</tr>
<tr>
<td>Kingdom</td>
<td>Stramenopila</td>
</tr>
<tr>
<td>Phylum</td>
<td>Oomycota</td>
</tr>
<tr>
<td>Class</td>
<td>Oomycetes</td>
</tr>
<tr>
<td>Order</td>
<td>Peronosporales</td>
</tr>
<tr>
<td>Family</td>
<td>Pythiaceae</td>
</tr>
<tr>
<td>Genus</td>
<td><em>Phytophthora</em></td>
</tr>
<tr>
<td>Species</td>
<td><em>Phytophthora infestans</em></td>
</tr>
</tbody>
</table>

2.3.4. Pathogenesis

Fundamental information of pathogen infection and defense response during *P. infestans* attack has been reviewed in many reports (Black, 1996). But recent reports have transformed our understanding of oomycete–host plant interactions with integration of cellular and molecular approaches. In particular, studies of immunocytochemistry and green-fluorescence protein (GFP)-tagging have revealed the site of plant receptors concerned in pathogen recognition and mechanisms underlying the basal defense (Cohen *et al.*, 1997). In particular, when tomatoes are part of the agroecosystem, severe late blight epidemics may occur when *P. infestans* grows and reproduces rapidly on the host plant (Cohen *et al.*, 1997).
1997). *P. infestans*, being a hemibiotrophs, adopts two-step infection mode. As an early phase of infection, pathogen obtains nutrients through development of haustoria from living host cells, pursued by extensive necrosis of host tissue resulting in colonization and sporulation. There are 11 different cell types involved in the life cycle of *P. infestans* which are highly specialized for life cycle stages concerned in sexual and asexual reproduction, propagule dispersal, spore germination, host penetration, and biotrophic or necrotrophic phases of infection (Avrova et al., 2003). The early stages of disease symptoms are easily missed as all affected plants do not exhibit symptoms simultaneously. Initial infection symptoms, including small lesions on leaf tips and plant stems, are visible only after 3 to 4 days, and in some cases reach only 1 to 2 mm in diameter. As the pathogen penetrates the plant tissue, lesions enlarge in size and white mold appears on the leaf underside (abaxial surface). The entire plant may collapse in 5 to 10 days.

### 2.3.5. Host range

The host range of *P. infestans* is mainly restricted to the family Solanaceae, of which potato (*Solanum tuberosum*) and tomato (*S. lycopersicum*) are the most important agricultural crops (Erwin and Ribeiro, 1996). In many places, potato and tomato are grown all year around. In colder climates, like, in Sweden, where only one growing season per year is possible, all commercial tomato cultivation is conducted in green houses which reduces the risks of late blight infections. There are a few wild *Solanum* spp. that are reported as hosts for *P. infestans* in Sweden (Cooke et al., 2002). The most widespread are *S. nigrum* (black nightshade, a common weed in potato crops), *S. dulcamara* (bittersweet) and *S. physalifolium* (hairy nightshade). However, attacks by *P. infestans* on *S. dulcamara* and *S.*
*nigrum* are very rare and must be considered to have no or very limited effect on late blight epidemics (Cooke *et al*., 2002). In contrast, another *Solanum* species, *S. physalifolium* has been found to be highly susceptible to *P. infestans* (Grönberg *et al*., 2012). Meanwhile, Erwin and Ribeiro (1996) listed 89 of *P. infestans* host species, among them, more than 25% were reported only on artificial inoculations. There is only a single report in some of the hosts mentioned and others may have been attacked by morphologically similar species, which have only recently been identified. *Ipomoea* and *Mirabilis* have been listed as hosts by Erwin and Ribeiro (1996), but disease on these may have been caused by *P. ipomoea* (Grünwald *et al*., 2001) and *P. mirabilis* (Legard *et al*., 1999), respectively. Although, *P. infestans* is probably an aggressive pathogen of all tuber-bearing genotypes in the genus *Solanum*, the large number of tuber-bearing *Solanum* species exaggerates the host range, as many of these species classifications could be the result of overzealous splitting by plant taxonomists (Spooner *et al*., 2003).

Of the non-tuber bearing *Solanum* hosts, tomato, *S. lycopersicum*, is the most important crop economically. Other domesticated *Solanum* species are also hosts of *P. infestans*, including pear melon (*S. muricatum*) (Turkensteen, 1978), tree tomato (*S. betaceum*) (Oliva *et al*., 2002) and Naranjilla (*S. quitoense*) (Adler *et al*., 2003). Eggplant has also been reported as an occasional host of *P. infestans* (Hooker, 1981), but lack of reports of the disease in this very cosmopolitan crop puts its host status in doubt. *P. infestans* is undoubtedly also a pathogen of the wild *Solanum* species closely related to tomato (Abad *et al*., 1995), but there are few references to field epidemics on these. A recent study in South America listed six wild non-tuber bearing *Solanum* species and one species complex attacked in nature by *P. infestans* (Adler *et al*., 2003). However, some isolates of the pathogen
associated with certain hosts are so unusual that they may be new species of *Phytophthora* (Adler *et al.*, 2003). A recent study in Canada showed that the nightshades, *S. dulcamara* and *S. sarrachoides*, were infected while black nightshade, *S. nigrum*, was not (Platt, 1999). In Europe, natural infections were found on *S. nigrum*, *S. dulcamara* and *S. sisymbriifolium* (Flier *et al.*, 2003). One study in California found that 11 Solanum species were infected by inoculation in a greenhouse, however, only one of these, *S. sarrachodes*, was infected naturally in the field (Vartanian and Endo, 1985).

There have also been references to infection in Solanaceous plants outside the genus *Solanum*. *Nolana* is apparently attacked on the coast of Peru during the wet winter season (Turkensteen, 1978). *Datura* has been reported as hosts of *P. infestans* (Sunita and Sen, 1997), but it is not clear if infections occur naturally in the field. Extensive sampling in Ecuador, were *Datura* spp. are common weeds, has not produced any indication that this genus is a host (Adler *et al.*, 2003). *Brugmansia sanguinea* is also attacked by *P. infestans* in South America, but lesions have only been found on flower petals (Adler *et al.*, 2003). *P. infestans* has also been reported to attack Petunia (Platt, 1999).

Africa is center of diversity of *Solanum* and several species from that continent have been cited as hosts of *P. infestans*. Kori (1972) reported that *S. indicum*, *S. incanum* and *S. aculosturm* were all infected in the field. Natural infection in *S. incanum* and *S. indicum* were confirmed by Nattrass and Ryan (1951), as well as infection in one unidentified species of *Solanum*. The cultivated crop, garden huckleberry (*S. scabrum*) was reported as a host of *P. infestans* in Cameroon (Nkengaka, 2000).
2.3.6. Social impact and economic importance of late blight

The potential economic and social impacts of late blight is best illustrated by the Irish Famine which occurred during the mid-nineteenth century. Irish peasants relied on the potato as a source of calories. Once blight arrived, it destroyed almost the entire potato crop in Ireland, either by eliminating foliage before the harvest or by inflicting large tuber rot in storage. The famine, which followed, resulted in the death of millions of people and also large scale human migration (Bourke, 1993).

In some cases, economic losses due to potato late blight were analysed consistently. For one epidemic occurring in 1995 within the Columbia Basin of the state of Washington within the America, the mean variety of fungicide agent applications per field varied from 5.1 to 12.3, counting on variety. Total per acre expenses (application costs plus fungicide material) ranged from $106.77 to $226.85 and also the total cost of managing blight was estimated to own approached $30 million (Johnson et al., 1997). During a national assessment, the economic impact of potato blight altogether of the USA was calculable to be regarding $210 million (Guenther et al., 2001). The International Potato Center (CIP) has created a world estimate currently blight injury in developing countries supported a mean production loss of 15%. This translates into a complete production loss in developing countries of roughly $2.75 billion (Anonymous, 1997).

*P. infestans* is also a very important pathogen of several other cultivated solanaceous hosts, including tomato (*Solanum lycopersicum*), pear melon (*S. muricatum*), naranjilla (*S. quitensis*) and tree tomato (*S. betaceum*).
Unfortunately, there are no quantitative accounts of the damage done to these crops, although global damage to tomato due to late blight is immense.

2.3.7. Disease cycle, infection and symptomology

The pathogen generally survives on infected plant debris. Primary infections initiate from debris in the soil. Secondary infections start from wind- or splash-borne sporangia from primary infections on tomato plant. Sporangia or zoospores can penetrate living cells within 2 hours when the conditions are favourable. After penetration, *P. infestans* establishes near biotrophic relationship and lesions visible within 3-5 days under optimal conditions (18-22°C) although disease can occur over a range of temperatures from 5 to 30°C. After one or two days of visible symptoms appearance, the pathogen produces sporangia under moderate temperatures (10-25°C) and wet conditions (leaf wetness or relative humidity > 95%). Sporangia are released during the day when relative humidity is lower and can be carried in air currents or dispersed through rain splashes. Sporangia can survive for many hours in atmosphere on cloudy days and are killed within an hour when higher solar radiation prevails. On cloudy days, the sporangia can be carried to hundreds of meters or kilometers. When the conditions are favourable, more than 1,000,000 sporangia can be produced in a single lesion. Historically, the disease has been associated with ‘cool wet weather’. Saturated air or leaf wetness is essential for sporangia to germinate and for zoospore motility (Fig. 2.1). Cool nights and warm days with moist weather, which prevail during July-December, are the best conditions for development of the disease. Rain, fog or heavy dew is also ideal.
The infection occurs on all above ground parts of the plant namely leaves, stems and fruits at all stages of plant growth. Late blight appears on tomato leaves as pale green, water-soaked spots, often beginning at leaf tips or edges. The circular or irregular leaf lesions are often surrounded by a pale yellowish-green border that merges with healthy tissue. Lesions enlarge rapidly and turn dark brown to purplish-black. During periods of high humidity and leaf wetness, a cottony, white mouldy growth is usually visible on lower leaf surfaces at the edges of lesions. In dry weather, infected leaf tissues quickly dry up and the white mold growth disappears. Infected areas on stems appear brown to black and entire stem may be killed in a short time when moist weather persists. *Phytophthora infestans* produces grey-green water-soaked spots on fruits, which enlarge, coalesce, and darken, resulting in large, firm, brown, leathery-appearing lesions (Fig.2.2).

### 2.3.8. Epidemiology

*P. infestans* is temperature and relative humidity dependent and these two are the most important environmental factors affecting late blight development. Relative humidity of < 90% supports the organism to colonize and sporulate on the lower leaf surfaces and infected stems. Sporulation can occur from 3-26°C (37-79°F), but the optimum range is 18-22°C (64-72°F). Sporangia germinate directly via a germ tube at 21-26°C (70-79°F). At temperatures below 18° C (65°F), sporangia produce six to eight zoospores which require water for swimming. Each zoospore is capable of initiating an infection, which explains why disease is more severe in cool, wet conditions. Cool nights, warm days and extended wet conditions from rain and fog can result in late blight epidemics in which entire tomato fields are destroyed in less than two weeks. Infected tubers can sporulate in poorly controlled storage areas where conditions are too humid. Condensation
produces water droplets on the surface of infected tubers which may then cause the pathogen to sporulate and contaminate neighboring tubers, leading to destruction of the entire pile by soft rot bacteria.

Fig.2.1. Life cycle of *P. infestans*
Fig. 2.2. Symptoms of late blight a) blighted leaf, b) white fungal growth on leaf, c) soft rot with brown lesion on stem, d) leathery symptoms on fruits, e) Sporangia of *P. infestans* f) Culture of *P. infestans*
2.4. Management of late blight of tomato

Late blight is one of the most destructive disease of tomatoes and a serious hazard to food security, which makes its control very important. The common processes employed to control infestation comprise of cultural practices, fungicide sprays and use of resistant cultivars (Black et al., 1996). Meanwhile, poor farmers are unable to afford the fungicides required to control the disease (Chycoski, 1996). Fungicides are applied at 7-8 days interval (Legard and Fry, 1995). Two areas of concern of fungicide application are development of resistance to the fungicide by the pathogen and the fungicide residue on fruits (Mulandi, 1998).

2.4.1. Host resistance

Since the incidence of the Irish famine in the 1840s, there has been an immense transaction of interest in developing late blight resistant potato and tomato cultivars. It has been observed that resistance against P. infestans could be classified into race-specific resistance and race non-specific resistance. Developing plants that exhibit disease resistance conferred by resistance (R) genes (i.e., vertical resistance) are initially effectual at avoiding infection and protecting the crop from late blight. However, due to the rapid evolution of pathogen effectors and sexual reproduction of P. infestans leading to more aggressive lineages (Klarfeld et al., 2009), the vertical resistance against the pathogen could ultimately fail (Klarfeld et al., 2009). In contrast, race-non-specific resistance is frequently proscribed by numerous genes or qualitative trait loci (QTLs), and potentially could be more durable (Brouwer et al., 2004). In both potato and tomato, vertical as well as horizontal resistances against late blight have been reported (Vleeshouwers et al., 2011). Race-specific major resistance genes, however, have reported to be
practically more useful in breeding for resistance against late blight especially when multiple \( R \) genes are pyramidized to increase resistance strength and durability. Thus, it is prudent to identify additional major resistance genes against late blight, a task that is underway in many potato and tomato breeding programs around the world (Merk and Foolad, 2012).

2.4.2. Genetics and Breeding of Late Blight Resistance in tomato

Genetic resistance against tomato late blight has been of interest for many years, and three major resistance genes have been identified in the red-fruited tomato wild species \( S. pinninellifolium \). These genes have been mapped to tomato chromosomes 7 (\( Ph-1 \)), 10 (\( Ph-2 \)), and 9 (\( Ph-3 \)) (Fig. 3). \( Ph-1 \) is a single dominant gene providing resistance against race T-0 (Peirce, 1971), but it has been rapidly overcome by new races of the pathogen (Conover and Walter, 1953; Gallegly, 1952; Peirce, 1971). The resistance habituated by \( Ph-2 \), a single incomplete-dominant gene mapped to the long arm of chromosome 10 (Moreau et al., 1998.), affords only partial resistance against several pathogen isolates (Peirce, 1971). \( Ph-2 \) provides only a decrease in the rate of disease expansion slightly than blocking the disease, and it often fails in the occurrence of more aggressive isolates (Black et al., 1996). A much stronger resistance gene, \( Ph-3 \), was found in \( S. pinninellifolium \) accession L3708 (\( a.k.a. \ LA1269 \) or \( PI365957 \)) by L. Black at the Asian Vegetable Research and Development Center in Taiwan (AVDRC, 1993). This gene confers incomplete-dominant resistance against a rather wide range of \( P. infestans \) isolates of tomato (Park et al., 2010). Several tomato-breeding programmes around the world, including NC State Tomato Breeding Program, Cornell Tomato Breeding Program, Penn State Tomato Breeding Program, AVRDC, and the World Vegetable Center have succeeded in transferring this
resistance gene to fresh-market or processing tomato breeding lines and commercial cultivars. But the breakdown or reduced effectiveness of known resistance genes in tomato and the appearance of new and more aggressive isolates of *P. infestans* have necessitated the identification, characterization, and utilization of new sources of resistance. Even though a few highly resistant accessions have been identified within wild tomato species, intensive searches have been carried out to identify new sources of late blight resistance in tomato (Foolad *et al.*, 2008). Since commercial cultivars do not possess sufficient resistance to leaf blights, cultural practices and fungicides applied at 5–7 days intervals form the basis for leaf blight management programs (Tumwine *et al.*, 2002).

### 2.4.3. Cultural management

For management of late blight, sanitation of preexisting inoculum of *P. infestans* and growing tomato far from potatoes is recommended (Sherf and Macnab, 1986). Sanitation has been attempted for late blight control in tomato and potato (Inglis *et al.*, 1996). Plastic shelters abridged the intensity of late blight, fungicide sprays were minimal, and tomato yields and fruit sizes were improved (Hanada, 1988). Green houses covered with polythene film significantly reduced *P. infestans* infection (Chee *et al.*, 1988). Growth parameters were considerably increased in tomato plant irrigated with shelter belts where warm and humid conditions were formed (Jebari, 1989). Late blight incidence was also reduced in tomato and potato on intercropping with faba beans and corn (Sharaiha *et al.*, 1989). Using the fungicide benlate in tomatoes at high plant densities, diseases including late blight were not aggravated (AVRDC, 1992). According to AVRDC (1987), yield responses of tomato to plant and row spacing could be expected. High tomato densities of 2 plants per 20 cm² (in Peto 86 and PT 4121) gave the
greatest numbers of green fruits and least unmarketable yields, compared with 1 plant per 15, 20 and 30 cm\(^2\) (AVRDC, 1992).

### 2.4.4. Chemical control

Farmers struggle to manage the disease through various methods including cultural methods and chemical methods but each of these control measures has substantial limitations. Late blight management has been profoundly based on fungicidal application and in recent years, exploitation of fungicides have been augmented due to prologue of new and aggressive genotypes and numerous fungicide applications (7-8 sprays) are needed (Mizubuti et al., 2007). Fungicides used for the management of late blight can be divided into contact fungicides and systemic fungicides. Contact fungicides includes copper based fungicide (Bordeaux mixture, Copper hydroxide, Copper oxychloride) (Mizubuti et al., 2007), Dithiocarbamates (zineb, manebe, mancozeb, metiram and propineb), Phthalimides (Folpet, captafol and captan), Phthalonitriles (Chlorothalonil), Pyridineamines (Fluazinam), Tin Fungicides (triphenyltin acetate and triphenyltin hydroxide) and systemic fungicides include Cyanocetamide-Oximes (Cymoxanil), Carbamates (Propamocarb HCl), Cinnamic acid derivatives (Dimethomorph), Phenylamides (Metalaxyl) and Phosphites (Fosetyl-aluminium) (Stein and Kirk, 2002). Namanda et al., (2004) demonstrated for management of late blight of potato with Mancozeb. Kirk et al., (2005) reported that utilizing host plant resistance fungicide application and frequencies of fungicide applications can be reduced for management of late blight of potato. Andreua and Caldiz (2006) successfully employed iprovalicarb+propineb for early management of late blight by applying to seed-potato tubers. Pre-pack mixtures with contact fungicides, accompanied by other measures, in accordance with the recommendations, can
also be found. In recent days, Metaxyl-based fungicides have been used extensively for the management of late blight of tomato (Fontem et al., 2001), which has resulted in resistance in population of Phytophthora infestans to metaxyl in many countries (Mukalazi et. al., 2001).

2.4.5. Disease forecasting

Appropriate timing of fungicide sprays can conserve resources and afford superlative disease control. Late blight epidemics take place within a particular temperature range when the environment becomes very moist and humid. By using accumulated severity values for weather parameters (rainfall, air temperature and relative humidity), fungicide applications should be undertaken occur at just the right time to protect the plants (Nelson, 2008).

Disease forecasting systems for tomato late blight allow farmers in a region to correctly time their fungicidal sprays. These computerized forecasting systems use regional weather monitoring stations to make fungicide recommendations for that region (Nelson, 2008). The forecasting systems assume that the pathogen inoculum is probably present and that an epidemic of the disease is highly likely if the right environmental conditions coincide. The systems predict disease development by the accumulation of various severity values for temperature, rainfall and/or relative humidity, and when a pre-determined threshold of severity values is reached, sprays are triggered. Regional modifications to the original systems have been developed.

- **BLITECAST system**

This is a regional disease forecasting system used by potato and tomato farmers in the Northeastern United States and is based on temperature, relative humidity
and rainfall,. Farmers in a region receive daily updates and spray advisories from their control centers.

- **NoBLIGHT system (Maine)**

  This is similar to BLITECAST, but differs in the calculation of severity values for relative humidity (Johnson 2005.)

- **HYRE system**

  This is based on temperature and rainfall.

- **WALLIN system**

  This is based on temperature and relative humidity.

### 2.5. Biological control

Due to imbalance created in nature by chemical practices in controlling late blight of tomato, biological control can be a safe alternative for chemical control since, in addition to controlling the disease, it can also promote growth and induce systemic resistance. The concept, mechanisms of biological control and its application in controlling late blight of tomato is discussed in following part, as it is a major focus of thesis.

Biological control is the decline of the quantity of pathogen or disease producing activity of a pathogen proficient by or through one or more organisms other than man (Cook and Baker 1983). Hartley (1921) made the first attempt of controlling plant pathogens by direct application of biocontrol agents. He applied 13 antagonistic fungi to control damping-off of pine seedlings (Cook and Baker, 1983). Meanwhile, during 1960s, significant interest arose in the area of biological control due to increase in use of chemical pesticides, development of fungicide
resistance, accumulation of residues in fruits and environment and reduction of beneficial phylloplane and soil microbes (Cook and Baker, 1983).

Antagonists are biocontrol agents possessing the potential to interfere in the life process of plant pathogens like fungi, bacteria, virus, nematodes, protozoa, viroids and seed plants. Major antagonists used as biocontrol agents are *Trichoderma*, *Bacillus* and *Pseudomonas*. Biocontrol agents inhibit pathogens by adopting many mechanisms including direct antagonism, antibiosis and siderophore production and induction of systemic resistance. (Yedidia *et al.*, 1999; Ahmed *et al.*, 2000; Compant *et al.*, 2005; Elad, 2000; Yang *et al.*, 2009; Babitha *et al.*, 2002). Apart from controlling diseases, these biocontrol organisms also promote plant growth by production of plant growth hormones like IAA and GA3 coupled with increased availability of nutrients (Harman, 2011).

### 2.5.1. *Trichoderma* as biocontrol agent

Among phytopathogenic fungal antagonists, 90% of applications have been carried out with different strains of the fungus *Trichoderma* which have been used to control plant diseases. Most of *Trichoderma* strains are classified as imperfect fungi as they have no known sexual stage (Monte, 2001). Though, some *Trichoderma* species are morphologically alike to the anamorph *Hypocrea*, their internal transcribed spacer (ITS) sequences have revealed their taxonomic proximity (Hermosa *et al.*, 2000). The most common biocontrol agents of the *Trichoderma* genus are strains of *T. virens*, *T. viride* and, above all, *T. harzianum*, which is a species aggregate that includes different strains used as biocontrol agent of phytopathogenic and viral vector fungi. Molecular characterization and phylogenetic analysis have allowed strains of *T. harzianum* originally identified as
the same species to be assigned to different species clustered into distinct sections and groups.

The success of *Trichoderma* strains as bio-control agents is due to their high reproductive capability, capacity to survive under unfavorable conditions, competence in the consumption of nutrients, ability to adapt the rhizosphere, sturdy aggressiveness against phytopathogenic fungi, and effectiveness in promoting plant growth and defense mechanisms. These characteristics have made *Trichoderma* an omnipresent genus in any habitat and at high population densities (Chet et al., 1997). *Trichoderma* spp. can control many phytopathogenic families viz., ascomycetous, deuteromycetous and basidiomycetous fungi; mainly of them soil-borne, while some are airborne pathogens (Monte, 2001). *Trichoderma* is more competent in acidic than alkaline soils.

2.5.1.1. Mechanisms of biological control

Biocontrol agents are living organisms whose behavior depends generally on the dissimilar physicochemical environmental conditions to which they are subjected to. For this cause, biocontrol exerted by *Trichoderma* strains is occasionally impulsive (Howell, 2003). Understanding the genetic diversity of strains within *Trichoderma* spp. and their mechanism of action will guide enhanced application of the diverse strains as biocontrol agents. The mechanisms of action are multifarious, and what has been distinct as biocontrol is the final result of different mechanisms performing synergistically to attain disease control (Howell, 2003). The mechanism of biocontrol results also from competition for nutrients and space or as a result of the capability of *Trichoderma* biocontrol agents to produce metabolites that obstruct spore germination (fungistasis), destroy
the cells (antibiosis) or adapt the rhizosphere, e.g. by acidifying the soil, so that pathogens cannot survive. Biocontrol may also result from a direct interaction between the pathogen itself and the biocontrol agents, as in mycoparasitism, which engages physical contact and synthesis of hydrolytic enzymes, toxic compounds and/or antibiotics that act synergistically with the enzymes. *Trichoderma* can even exert positive effects on plants with an increase in plant growth (biofertilization) and the stimulation of plant-defense mechanisms.

### i) Biocontrol by competition

#### a) Fungistasis

High-quality antagonists are generally capable to conquering the fungistatic consequence of soil that results from the occurrence of metabolites produced by other species, including plants, and to tolerate under very severe competitive circumstances. *Trichoderma* strains grow quickly when inoculated in the soil, because they are naturally resistant to many toxic compounds, including herbicides, fungicides and pesticides such as DDT, and phenolic compounds (Chet *et al.*, 1997), and because the strains improve very rapidly after the addition of sub-lethal doses of some of these compounds. Resistance to toxic compounds may be coupled with the presence in *Trichoderma* strains of ABC transport systems (Harman *et al.*, 2004). For this reason, preparations of *Trichoderma* strains are very proficient in controlling several phytopathogens, such as *R. solani*, *P. ultimum* or *Sclerotium rolfsii*, when alternated with methyl bromide, benomyl, captan or other chemicals (Vyas and Vyas, 1995).
b) Competition for nutrients.

Starvation is the most common cause of death for microorganisms, so that competition for limiting nutrients results in biological control of fungal phytopathogens (Chet et al., 1997). For instance, in most filamentous fungi, iron uptake is essential for viability (Eisendle et al., 2004), and under iron starvation, most fungi excrete low-molecular-weight ferric-iron specific chelators, termed siderophores, to mobilize environmental iron (Eisendle et al., 2004). Subsequently, iron from the ferrisiderophore complexes is recovered via specific uptake mechanisms. In Aspergillus fumigatus and A. nidulans, siderophore biosynthesis is negatively regulated by carbon source (Eisendle et al., 2004). In Ustilago maydis, gene products related to iron uptake affect the development of plant disease (McIntyre et al., 2004). Trichoderma spp. produce highly efficient siderophores that chelate iron and stop the growth of other fungi (Chet and Inbarm, 1994). For this reason, soil composition influences the biocontrol effectiveness of Pythium by Trichoderma according to iron availability. In addition, T. harzianum controls soil borne plant pathogens by competing for both rhizosphere colonization and nutrients, with biocontrol becoming more effective as the nutrient concentration decreases (Tjamos et al., 1992). Competition has proved to be particularly important for the biocontrol of phytopathogens such as Botrytis cinerea, the main pathogenic agent during the pre- and post-harvest in many countries (Latorre et al., 2001). The unusual genetic variability of this fungus makes it probable for new strains to become resistant to fundamentally any novel chemical fungicide it is exposed to (Latorre et al., 2001). The benefit of using Trichoderma to manage B. cinerea is the organization of numerous mechanisms at the same time, thus making it virtually impractical for resistant strains to emerge. Along with these
mechanisms, the most significant is nutrient competition, since *B. cinerea* is mainly sensitive to availability of nutrients.

*Trichoderma* has an advanced ability to assemble and take up soil nutrients compared to additional organisms. The proficient use of obtainable nutrients is based on the capability of *Trichoderma* to gain ATP from the metabolism of diverse sugars, such as those resulting from polymers widespread in fungal environments: cellulose, glucan and chitin amongst others, all of them rendering glucose (Chet *et al.*, 1997). The key mechanism of glucose metabolism comprises assimilation enzymes and permeases, jointly with proteins concerned in membrane and cell wall alterations. The role of the glucose transport system remains yet to be discovered, however, its competence may be vital in competition (Delgado-Jarana *et al.*, 2003), as supported by the isolation of a high-affinity glucose transporter, *Gtt1*, in *Trichoderma harzianum* CECT 2413 (referred to here as strain 2413). This strain is present in environment which is very poor in nutrients, and it relies on extracellular hydrolases for survival. *Gtt1* is only expressed at very low glucose concentrations, i.e. when sugar transport is predictable to be restrictive in nutrient competition (Delgado-Jarana *et al.*, 2003). In fact, glucose uptake is augmented three- to four-fold in a transformant imitative that carried a supplementary copy of the transporter gene. Only two other genes encoding glucose transporters have been explained in filamentous fungi (Franken *et al.*, 2002; Voegele *et al.*, 2001), one of which is a glucose transporter from *Uromyces fabae*. This basidiomycete has an ATPase and a proton-coupled glucose transport system that is expressed throughout infection of *Vicia faba*. This proposes an extra, antagonistic function for Gtt1, permitting the fungus to gain energy from hydrolyzed polymers and to convey sugar hastily into the cells. As a consequence, transformant able to transfer
glucose more quickly than the wild-type (Delgado-Jarana et al., 2003) should be more efficient bio-control agents. This would serve as a very useful mechanism of nutrient competition during mycoparasitism interactions. Promoter analysis of genes, allied to antagonism in Trichoderma strains, revealed the occurrence of sequences for transcription factors responsible for carbon (CreaA), nitrogen (AreA), Stress (Msn2/Msn4), pH (PacC) and mycoparasitism (MYC) regulation, among others (Mach and Zealander, 2003). Thus, suitable exploitation of these regulators would endow with an alternative to the isolation of more competitive bio-control agents. More information on these regulators has been described by Mach and Zealander (2003).

ii) Bio-fertilization and stimulation of plant defense mechanisms

Trichoderma strains are forever related with plant roots and root ecosystems. Various authors have defined Trichoderma strains as plant symbiont opportunistic avirulent organisms, with the capability of colonizing plant roots by mechanisms parallel to those of mycorrhizal fungi and to generate compounds that stimulate growth and plant defense mechanisms (Harman et al., 2004)

a) Plant root colonization.

Trichoderma strains have to colonize plant roots prior to stimulation of plant growth and protection against diseases. Colonization entails the capacity to adhere and recognize plant roots, penetrate the plant, and survive toxic metabolites produced by the plants in response to invasion by a foreign organism. Trichoderma strains are found in many root ecosystems. The extremely hydrated polysaccharides of the root-secreted mucigel layer and the mono- and disaccharides excreted by plant roots into the rhizosphere encourage growth of the
fungi. It has been observed that plant-derived sucrose is an important resource provided to *Trichoderma* cells to facilitate root colonization, the coordination of defense mechanisms, and increased rate of leaf photosynthesis (Vargas *et al.*, 2009). Solute transporters such as a di/tripeptide transporter and a permease/intracellular invertase system involved in the acquisition of root exudates have been described in *Trichoderma* (Vizcaíno *et al.*, 2006; Vargas *et al.*, 2009).

In *Trichoderma*, adherence to the root surface can be mediated by hydrophobins, which are small hydrophobic proteins of the outermost cell wall layer that coat the fungal cell surface, and expansin-like proteins related to cell wall development (Viterbo and Chet, 2006). *Trichoderma asperellum* produces the class I hydrophobin TasHyd1, which has been reported to sustain the colonization of plant roots, possibly by enhancing its attachment to the root surface and protecting the hyphal tips from plant defence compounds (Viterbo and Chet, 2006), and the swollen in TasSwo, an expansin-like protein with a cellulose-binding domain able to recognize cellulose and modify the plant cell wall architecture, assisting root colonization (Brotman *et al.*, 2008). Plant cell-wall-degrading enzymes, like endopolygalacturonase ThPG1 from *Trichoderma harzianum*, are also involved in active root colonization, (Morán-Diez *et al.*, 2009).

Seventy-two hours after root colonization, yeast-like cells of *Trichoderma* were observed together with an intensification of plant epidermal and cortical cell walls and the authentication of newly formed barriers containing large amounts of callose and infiltrations of cellulose (Chaco´n *et al.*, 2007). Characteristic host reactions to *Trichoderma* were established beyond the sites of latent fungal penetration and, unlike *P. indica* colonization, which does not stimulate plant cell wall strengthening, callose-enriched wall appositions were actually efficient in the
constraint of fungal growth to the intercellular spaces of the epidermis and cortex, preventing the entry of *Trichoderma* into the vascular stele (Yedidia *et al.*, 1999). Plants also respond adjacent to fungal invasion by synthesizing and accumulating antimicrobial compounds. The ability to colonize plant roots depends strongly on the capacity of each strain to tolerate them. In *Trichoderma*, this resistance has been associated with the presence of ABC transport systems, which are key factors in the multiple interactions established by *Trichoderma* biocontrol strains with other microbes in a potentially toxic or antagonistic environment (Ruocco *et al.*, 2009), with rapid degradation of the phenolic compounds radiated from plants (Chen *et al.*, 2011), and with the suppression of phytoalexin production, as detected in *Lotus japonica* during colonization with *Trichoderma koningii* (Masunaka *et al.*, 2011). In a proteome analysis, a small secreted cysteine-rich protein (SSCP) was identified in *T. harzianum* and *T. atroviride*, proving to be a homologue of the avirulence protein Avr4 from *Cladosporium fulvum* (Harman *et al.*, 2004). It has been proposed that the binding of Avr4 to chitin could protect *Trichoderma* against plant chitinases (Stergiopoulos & de Wit, 2009).

**b) Biofertilization.**

Root colonization by *Trichoderma* strains often increase root growth and development, crop productivity, resistance to abiotic stresses and the uptake and use of nutrients (Arora *et al.*, 1992). Crop productivity in fields can enhance up to 300% after the addition of *Trichoderma hamatum* or *Trichoderma koningii*. In experiments carried out in greenhouses, there was also a substantial yield increase when plant seeds were previously treated with spores from *Trichoderma* (Chet *et al.*, 1997). The same increase was observed when seeds were separated from *Trichoderma* by a cellophane membrane, which indicates that *Trichoderma*
produces growth factors that increased the rate of seed germination (Benítez et al., 1997). However, there are very few reports on strains that produce growth factors which have been noticed and recognized in the laboratory (auxins, cytokinins and ethylene) (Arora et al., 1992), even though the detection of many filamentous fungi that produce phytohormones, such as indol acetic acid (IAA) and ethylene, whose metabolic pathways have been identified (Arora et al., 1992; Osiewacz 2002). *Trichoderma* strains that produce cytokinin-like molecules, e.g. zeatyn and gibberellin GA3 or GA3-related, have been recently detected. The controlled production of these compounds could improve biofertilization (Osiewacz, 2002). Together with the synthesis or stimulation of phytohormone production, most *Trichoderma* strains acidify their surrounding environment by secreting organic acids, such as gluconic, citric or fumaric acid (Gómez-Alarcón and de la Torre, 1994). These organic acids result from the metabolism of other carbon sources, mainly glucose, and, in turn, are able to solubilize phosphates, micronutrients and mineral cations including iron, manganese and magnesium (Harman et al., 2004). Therefore, the addition of *Trichoderma* to soils where these cations are limited results in biofertilization by metal solubilization and an increase in crop productivity.

**iii) Mycoparasitism**

Mycoparasitism, the direct attack of one fungus on another, is a very complex mechanism that includes sequential actions, including detection, attack and ensuing penetration and killing of the host. *Trichoderma* spp. may be directly involved in parasitizing a range of fungi, detecting other fungi and growing towards them. The remote sensing is partially due to the sequential appearance of cell wall degrading enzymes (CWDE), mostly chitinases, glucanases and proteases
(Harman et al., 2004). The pattern of induction differs from one *Trichoderma* strain to another. It is believed that fungi secrete exochitinases constitutively at low levels. When chitinases degrade fungal cell walls, they release oligomers that induce exochitinases, and attack begins.

a) **Morphological changes**

Mycoparasitism involves morphological changes, such as coiling and formation of appressorium-like structures, which serve to penetrate the host and contain high concentrations of osmotic solutes such as glycerol (McIntyre et al., 2004). *Trichoderma* attaches to the pathogen with cell-wall carbohydrates that bind to pathogen lectins. Once *Trichoderma* is attached, it coils around the pathogen and forms the appresoria. Production of CWDEs and peptaibols (Howell, 2003) facilitate both the entry of *Trichoderma* hypha into the lumen of the parasitized fungus and the assimilation of the cell-wall content. The significance of lytic enzymes, reviewed by Viterbo et al. (2002), has been demonstrated by overexpression and deletion of the respective genes. Investigation on the responsible signal transduction pathways of *T. atroviride* during mycoparasitism have led to the isolation of key components of the cAMP and MAP kinase signaling pathways, such as α-subunits of G proteins (G-α), which control extracellular enzyme, antibiotic production and coiling around host hypha (McIntyre et al., 2004). In *Trichoderma*, there is biochemical evidence for the participation of G-α in coiling, since an increase in coiling around nylon fibers was detected after the addition of activators of G-protein (mastoparan and fluoroaluminate) (Omero et al., 1999). G-α gene (*tga1*) has been expressed either under the control of its own promoter or under the control of the promoter of the basic proteinase *prb1* in *T. atroviride* (Rocha-Ramírez 2002). Both types of
transformants showed an increase in coiling. Moreover, the capacity of *T. viride* overexpressing *tgal* to overgrow *Rhizoctonia* was also enhanced.

**b) Cell-wall-degrading enzymes**

**Chitinases.** The chitinolytic system of *Trichoderma* comprises many enzymes and the list of its components is rapidly being updated as new enzymes and genes are reported. Chitinases are divided into 1,4-β-acetylglucosaminidases (GlcNAcases), endochitinases and exochitinases. Many GlcNAcases and their genes, *exc1* (=*nag1*), *exc2*, *tvnag1*, and *tvnag2* from *T. harzianum* T25-1, *T. atroviride* P1 and *T. virens* Tv29-8, have been described (Harman *et al.*, 2004; Kim *et al.*, 2002). The 73-kDa Nag1 represents the main GlcNAcase in *T. atroviride*. Nag1-disruption strain lacks chitinase activity, and the endochitinase *chit42* mRNA is absent (Harman *et al.*, 2004). This indicates that *nag1* is essential for triggering chitinase gene expression. The pathogen cell wall and chitin induce *nag1*, but it is only triggered when there is contact with the pathogen (Carsolio *et al.*, 1999; Harman *et al.*, 2004; Howell, 2003; Mach *et al.*, 1999). GlcNAcases CHIT73 and CHIT102 were detected in *T. harzianum* TM and *Trichoderma asperellum* (Haran *et al.*, 1996). CHIT102 triggers the expression of other chitinolytic enzymes (Haran *et al.*, 1996). In addition, strain 2413 produces three extracellular endochitinases whose genes, *chit33*, *chit37* and *chit42*, have been cloned from this strain. Other genes coding for Chit42 chitinase viz., *ech42*, *cht42* and *ThEn4*, have also been cloned from *T. atroviride* IMI206040 (Carsolio *et al.*, 1999), Gv2908 (Howell, 2003) and *T. atroviride* P1 (Harman *et al.*, 2004), respectively. Chit37 shows 89% similarity to Chit36 from *T. harzianum* TM at the amino-acid level (Harman *et al.*, 2004). Chit36 inhibits *B. cinerea* spore germination and the growth of both *Sclerotium rolfsii* and *Fusarium oxysporum* (Viterbo *et al.*, 2001). Other
genes homologous to chit36 have been cloned from *T. harzianum* TM, *T. atroviride* P1 and *T. asperellum* T–203 (Kullnig et al., 2000). Endochitinases are regulated by a variety of mechanisms but induction by stress has been reported for chit33, chit36 and chit42. However, the induction under mycoparasitic conditions is not clear. Expression of ech42 is induced prior to any physical contact with *R. solani* (Kullnig et al., 2000). chit33 is expressed only during the contact phase and not before overgrowing *R. solani* (Dana et al., 2001) and chit36Y does not need the direct contact with the pathogen to be expressed. chit33, chit42 and chit36 have been overexpressed in *Trichoderma* spp. in order to test the role of these chitinases in mycoparasitism and the 42-kDa chitinase is believed to be a key enzyme (Howell, 2003). *T. virens* transformants overexpressing chit42 showed significantly enhanced biocontrol activity compared with the wildtype when assayed against *R. solani* in cotton seedlings experiments (Howell, 2003). Other *Trichoderma* sp. transformants overexpressing chit42 gave better antagonism than obtained with the wild-type (Carsolio et al., 1999; Limón et al., 2004). In greenhouse biocontrol tests, however, the activity of chit42 disruptants did not differ from that of the wild-type (Carsolio et al., 1999; Harman et al., 2004). *T. harzianum* transformants overexpressing chit33 chitinase constitutively inhibited the growth of *R. solani* under both repressing and derepressing conditions; the antagonist tests demonstrated that this chitinase also has an important role in mycoparasitism (Limón et al., 1999). *T. harzianum* Rifai TM transformants overexpressing chit36 inhibited *F. oxysporum* and *S. rolfsii* more strongly than the wild-type. Moreover, culture filtrates inhibited the germination of *B. cinerea* almost completely (Viterbo et al., 2001). The antagonism of chit33 and chit42 transformants has been improved by the addition of a cellulose-binding domain to
the chitinase genes. As a result, the strains producing the chimeric enzymes increased their specific chitinase activity (Limón, 2004).

**Glucanases.** It has been shown that β-1; 3-glucanases inhibit spore germination or the growth of pathogens in synergistic cooperation with chitinases (Benítez et al., 1998, El-Katatny et al., 2001) and antibiotics (Harman et al., 2004, Howell, 2003). Many β-1, 3-glucanases have been isolated, but only a few genes have been cloned, e.g., bgn13.1 (Benítez et al., 1998) and lam1.3 (Cohen-Kupiec et al., 1999) from *T. harzianum*, glu78 (Donzelli et al., 2001) from *T. atroviride*, and Tv-bgn1 and Tv-bgn2 (Kim et al., 2002) from *T. virens*. However, only strains overexpressing bgn13.1 from *T. harzianum* have been constructed. Transformants overexpressing BGN13.1 have been reported to inhibit the growth of *B. cinerea*, *R. solani* and *Phytophthora citrophthora*. Transformant T28, which had the highest BGN13.1 glucanase activity under both repressing and inducing conditions, showed the highest inhibition of pathogens. Antagonism was higher against *P. citrophthora*, oomycete with cellulose and glucans as the main cell wall components (Benítez et al., 1998) than against *Botrytis* or *Rhizoctonia*, which have chitin and glucan as their main cell-wall components (Benítez et al., 1998). In addition, three β-1, 6-glucanases have been purified from strain 2413 (Benítez et al., 1998; de la Cruz and Llobell, 1999; Elad et al., 2000). BGN16.2 exhibited antifungal properties alone or in combination with chitinases (Benítez et al., 1998) and reduced the growth of *B. cinerea* and *Gibberella fujikuroi* (De la Cruz and Llobell 1999). Transformants producing BGN16.2 controlled *R. solani* and *B. cinerea* growth (Benítez et al., 1998). Cellulases (β-1,4-glucanases), comprising cellobiohydrolases, endoglucanases (*egl1, egl2*) and β-glucosidases, have not been widely studied for biocontrol purposes, although cellulose is abundant in
oomycetes (Bartnicki-García, 1968). Migheli et al. (Howell 2003) obtained transformants with greater biocontrol activity than the wild-type against *P. ultimum* on cucumber seedling. *T. harzianum* T3 produces a variety of cellulases, which make this isolate very effective in the control of *P. ultimum*. Other hydrolases, such as α-1, 3-glucanases, have been purified from strain 2413, and their genes isolated and overexpressed, which resulted in increased biocontrol activity of the transformant strains (Ait-Lahsen et al., 2001).

**Proteases.** Biocontrol of *B. cinerea* by *T. harzianum* has been attributed in part to the action of proteases produced by the BCA that inactivate hydrolytic enzymes produced by this pathogen on bean leaves (Howell, 2003). Proteases involved in the degradation of heterologously produced proteins have been characterized (Delgado-Jarana et al., 2000). For example, alkaline protease Prb1 from *T. harzianum* IMI 206040 has been demonstrated to play an important role in biological control (Benítez et al., 1998) and *prb1* transformants showed an increase of up to five-fold in the biocontrol efficiency of *Trichoderma* strains against *R. solani*. Protease Pra1 from *T. harzianum* has affinity for fungal cell walls (Elad et al., 2000). The gene for an extracellular serine protease (*tvsp1*) has been cloned from *T. virens* (Pozo et al., 2004) and its overexpression significantly increased protection of cotton seedlings against *R. solani*. This gene shows great potential in improving biocontrol ability, as serine proteases are effective against oomycetes (Dunne et al., 2000) and nematodes (Bonants et al., 1995; Howell 2003). A serine protease of 28-kDa with trypsin activity isolated from strain 2413 also reduced the number of hatched eggs of root-knot nematodes and showed synergistic effects with other proteins produced during antagonistic activity of the strain (Suárez et al., 2004). Antal et al. (2000) screened cold-tolerant strains and found that most of
them antagonized phytopathogens and produced chitinases, β-glucosidases and trypsin-like, and chymotrypsin-like proteases, active at low temperatures. The role of proteases in mycoparasitism has been reinforced with the isolation of new protease-overproducing strains of *T. harzianum* (Szekeres et al., 2004). Mutants with new profiles and higher quantities of secreted proteases were obtained by UV-irradiation. Some of these mutants have proved to be effective antagonists against plant pathogenic fungi such as *Fusarium culmorum* and *R. solani*.

**Synergism.** Synergism among lytic enzymes and between enzymes and antibiotics suggests formulations to test mixtures of *Trichoderma* transformants that produce different enzymes, in order to improve the antagonistic effects of BCAs on phytopathogenic fungi. For instance, in experiments carried out using cellophane, which show the effect of enzymes and secondary metabolites secreted by BCAs, *T. harzianum* wild-type inhibited the growth rate of *B. cinerea* by 30% and transformants expressing either a β-1,3-glucanase, a chitinase, or a β-1,6-glucanase inhibited the growth rate of *B. cinerea* by 60%. Transformants were differently combined in order to test synergism among the enzymes secreted against several phytopathogens. The combination that overproduced chitinase and β-1, 3-glucanase was more effective than the individual transformants in inhibiting *Rhizoctonia meloni*, whereas using other combinations, the inhibition was not improved. Southern experiments showed that none of the transformants replaced the other transformant when grown together or had any advantage in growth or enzyme production (Benítez et al., 2004).

**iv) Antibiosis and secondary metabolites**

*Trichoderma* generate an excess of secondary metabolites with biological activity (Ghisalberti and Sivasithamparam, 1991; Sivasithamparam and
Ghisalberti, 1998). The term “secondary metabolite” comprises a heterogeneous cluster of chemically dissimilar natural compounds perhaps related to endurance functions for the producing organism, such as competition beside other micro- and macroorganisms, symbiosis, metal transport, differentiation, etc. (Demain and Fang, 2000). Antibiotics, are included in this group are which are natural products capable to inhibit microbial growth. Antibiotic production is frequently well associated with biocontrol capacity, and the application of purified antibiotics was devised to demonstrate its effects on the host pathogen comparable to those obtained by using the equivalent living microbe. Ghisalberti et al. (1990) demonstrated that the biocontrol efficiency of \textit{Trichoderma harzianum} isolates against \textit{Gaeumannomyces graminis} var. \textit{tritici} was related to the production of pyrone-like antibiotics. The production of secondary metabolites by \textit{Trichoderma} spp. is strain reliant and includes antifungal substances belonging to a diverse of classes of chemical compounds. They were classified by Ghisalberti and Sivasithamparam (1991) into three categories:

(i) Volatile antibiotics, i.e., 6-pentyl-a-pyrone (6PP) and most of the isocyanide derivates,

(ii) Water-soluble compounds, i.e. heptelidic acid or koningic acid, and

(iii) Peptaibols, which are linear oligopeptides of 12–22 amino acids rich in a-aminoisobutyric acid, N-acetylated at the N-terminus and containing an amino alcohol (Pheol or Trpol) at the C-terminus (Le Doan \textit{et al.}, 1986; Rebuffat \textit{et al.}, 1989).

Some of the secondary metabolites identified are reviewed below in \textbf{Table 2.2}. 

42
Table 2.2.: Secondary metabolites identified from *Trichoderma* Spp.

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Metabolite</th>
<th>Compounds</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Anthraquinones</td>
<td>pachybasin, chrysophanol and emodin</td>
<td>Slater <em>et al.</em> 1967; Donnelly and Sheridan, 1986; De Stefano and Nicoletti, 1999</td>
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<td></td>
<td></td>
<td>1,3,6,8-tetrahydroxyanthraquinone and 1-acetyl-2,4,5,7-tetrahydroxyanthraquinone</td>
<td>Betina <em>et al.</em>, 1986</td>
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<td></td>
<td></td>
<td>Trichodermaol</td>
<td>Adachi <em>et al.</em>, 1983</td>
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<td></td>
<td></td>
<td>dimeric xanthone</td>
<td>Manyu, 1980; Sivasithamparam and Ghisalberti, 1998</td>
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<td></td>
<td></td>
<td>Emodin</td>
<td>Jayasuriya <em>et al.</em>, 1992; Kumar <em>et al.</em>, 1998</td>
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<td></td>
<td></td>
<td>Trichodermaol</td>
<td>Adachi <em>et al.</em>, 1983</td>
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<td></td>
<td></td>
<td>Chrysophanol</td>
<td>Agarwal <em>et al.</em>, 2000</td>
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<tr>
<td>2.</td>
<td>Daucanes</td>
<td>carotane-type metabolite</td>
<td>Watanabe <em>et al.</em>, 1990</td>
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<td></td>
<td></td>
<td>oleic ester derivative</td>
<td>Lee <em>et al.</em> (1995a)</td>
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<td></td>
<td></td>
<td>Trichocaranes</td>
<td>Macias <em>et al.</em>, 2000</td>
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<td></td>
<td></td>
<td>6-(1¢-pentenyl)-2H-pyran-2-one</td>
<td>Claydon <em>et al.</em>, 1987; Parker <em>et al.</em>, 1997</td>
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<td></td>
<td></td>
<td>massoilactone and d-decanolactone</td>
<td>Hill <em>et al.</em>, 1995</td>
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<td>4.</td>
<td>Koninginins</td>
<td>Viridepyronone</td>
<td>Evidente et al., 2003</td>
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<td></td>
<td></td>
<td>koninginins A–E</td>
<td>Cutler et al., 1989; 1991</td>
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<tr>
<td></td>
<td></td>
<td>koninginin G</td>
<td>Cutler et al., 1999</td>
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<tr>
<td>5.</td>
<td>Trichoderamides</td>
<td>trichodermamides A and B</td>
<td>Garo et al., 2003; Liu et al., 2005a</td>
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<tr>
<td></td>
<td></td>
<td>aspergillazines A–E</td>
<td>Liu et al., 2005a; Capon et al., 2005</td>
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<tr>
<td>6.</td>
<td>Viridins</td>
<td>Viridin</td>
<td>Brian and McGowan, 1945</td>
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<td></td>
<td></td>
<td>C-3 alcohol viridiol</td>
<td>Moffat et al., 1969; Howell and Stipanovic, 1994</td>
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<td>Demethoxyviridin and demethoxyviridiol</td>
<td>Aldridge et al., 1975</td>
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<td></td>
<td>Wortmannin</td>
<td>Brian et al., 1957</td>
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<td></td>
<td>11-desacetoxywortmannin</td>
<td>Haefliger and Hauser, 1973</td>
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<td>Wortmannolone and virone</td>
<td>Blight and Grove, 1986</td>
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<td>Viridiofungins</td>
<td>viridiofungins A–C, A1, B2, Z</td>
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<td></td>
<td>trachyspic acid</td>
<td>Shiozawa et al., 1995</td>
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<td></td>
<td></td>
<td>citrafungin A</td>
<td>Singh et al., 2004</td>
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<td></td>
<td></td>
<td>L-731</td>
<td>Harris et al., 1995</td>
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<td></td>
<td></td>
<td>zaragozic acid A</td>
<td>Wilson et al., 1992</td>
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<tr>
<td>Nitrogen heterocyclic compounds</td>
<td>harzianopyridone</td>
<td>Dickinson et al., 1989</td>
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<tr>
<td>piercidin A</td>
<td>Takahashi et al., 1965; Jansen and Höfle, 1983</td>
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<td>Atpenins</td>
<td>Omura et al., 1988; Oshino et al., 1990; Kumagai et al., 1990</td>
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<tr>
<td>WF-16775 A2</td>
<td>Otsuka et al., 1992</td>
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<td>Harzianopyridone</td>
<td>Dickinson et al., 1989</td>
<td></td>
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<td>Harzianic acid</td>
<td>Sawa et al., 1994; Kawada et al., 2004</td>
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<td>demethylharzianic and homoharzianic</td>
<td>Kawada et al., 2004</td>
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<td>Melanoxadin and Melanoxazal</td>
<td>Hashimoto et al., 1995; Takahashi et al., 1996</td>
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<td>Trichodenones A–C</td>
<td>Amagata et al., 1998</td>
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<td>5-hydroxy-3-methoxy-5-vinylcyclopent-2-en-1-one</td>
<td>Strunz et al., 1977</td>
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<td>Pentenomycins I–IV</td>
<td>Umino et al., 1973</td>
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<td>epipentenomycin II and III</td>
<td>Sono et al., 1980; Elliott et al., 1983</td>
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</table>
v) Induction of plant defence responses

The induction of plant defence responses mediated by the antagonistic fungus has been well documented (De Meyer et al., 1998; Yedidia et al., 1999; Harman et al., 2004). Various plants, mono- and dicotyledonous species, showed increased resistance to pathogen attack when pre-treated with Trichoderma (Harman et al., 2004). Plant colonization by Trichoderma spp. reduced disease caused by one or more different pathogens, at the site of inoculation (induced localized acquired resistance or LAR), as well as when the biocontrol fungus was inoculated at different times or sites than that of the pathogen (induced systemic resistance or ISR). The induction of plant resistance by colonization with some Trichoderma species is similar to that elicited by rhizobacteria, which enhance the defence system but do not involve the production of pathogenesis-related proteins (PR proteins) (Stacey and Keen, 1999; Harman et al., 2004). In a recent work, Alfano et al., (2007) investigated at a molecular level the plant genes involved in Trichoderma hamatum 382 resistance induction by using a high-density oligonucleotide microarray approach. Interestingly, Trichoderma-induced genes were associated with biotic or abiotic stresses, as well as RNA, DNA and protein metabolism. In particular, genes that code for extensin and extensin-like proteins were found to be induced by the BCA, but not those coding for proteins belonging to the PR-5 family (thaumatin-like proteins), which are considered the main molecular markers of SAR. During the interaction of Trichoderma with the plant, different classes of metabolites may act as elicitors or resistance inducers (Harman et al., 2004; Woo et al., 2006). These molecules include: (i) proteins with enzymatic activity, such as xylanase (Lotan and Fluhr, 1990); (ii) avirulence-like gene products able to induce defence reactions in plants (Woo et al., 2004); (iii)
low-molecular-weight compounds released from fungal or plant cell walls by the activity of *Trichoderma* enzymes (Harman *et al*., 2004; Woo *et al*., 2006). Some of the low-molecular-weight degradation products released from fungal cell walls were purified and characterized and found to consist of short oligosaccharides comprised of two types of monomers, with and without an amino acid residue (Woo *et al*., 2006). These compounds elicited a reaction in the plant when applied to leaves or when injected into root or leaf tissues. Further, they also stimulated the biocontrol ability of *Trichoderma* by activating the mycoparasitic gene expression cascade. Recently, Djonovic´ *et al* (2007) identified a small protein (Sm1) elicitor secreted by *T. virens*, and demonstrated its involvement in the activation of plant defence mechanisms and the induction of systemic resistance. In addition to their innate antimicrobial effect, their action may also stimulate the biological activity of resident antagonistic microbial.

vi) **Influence of soil environment on *Trichoderma*–plant interaction**

The activities of BCAs are also affected by the presence of organic nutrients in soil (Hoitink and Boehm, 1999). Organic matter composition and the associated biotic and abiotic environment can affect the activities of *Trichoderma*, especially in relation to the conduciveness/receptivity of the soil to the strain (Simon and Sivasithamparam, 1989; Wakelin *et al*., 1999). So far, composts represent an optimal substrate for BCAs, thus encouraging their establishment into the soil environment (Hoitink and Boehm, 1999; Leandro *et al*., 2007). The mechanisms of action used by *Trichoderma* (competition, antibiosis, parasitism and systemic-induced resistance) are influenced by concentration and availability of nutrients (carbohydrates in lignocellulosic substances, chitin, lipids, etc.) within the soil organic matter (Hoitink *et al*., 2006). Krause *et al*. (2001) demonstrated
that *T. hamatum* inoculation of potting mix with a high microbial capacity, which supported high populations of BCAs, significantly reduced the severity of *Rhizoctonia* damping-off of radish or *Rhizoctonia* crown and root rot of poinsettia. Moreover, *T. hamatum* inoculated into the compost amended potting mix induced systemic acquired resistance on cucumber, reducing the severity of *Phytophthora* leaf blight (Khan *et al.*, 2004). This induction was more effective on plants grown in compost-amended media when compared to lower microbe carrying capacity sphagnum peat media (Hoitink *et al.*, 2006). A better understanding of the activities of *Trichoderma* strains in plant growth media high in organic matter could also help to select strains suitable for multiple acre field conditions associated with stubble retention practices and/or organic farming which are becoming increasingly popular world-wide.

vii) Pathogenesis related protein and its genes

PR-proteins are classified as proteins “encoded by the host plant but induced only in pathological or related situations” (Antoniw and White, 1980). These proteins are characterized by their chemical properties such as low molecular weight or their isoelectric point (pI, acidic or basic), their location either in the apoplast or in the vacuole, and more importantly the fact that they are induced and newly expressed (Van Loon *et al.*, 1994; Van Loon and Van Strien, 1999). Upto now, 14 PR-protein families are recognized, which are briefly summarized below: Unknown function (PR-1, PR-4); β-1,3-glucanase (PR-2); chitinase (PR-3, PR-8, PR-11); thaumatin- and osmotin-like protein (PR-5); proteinase inhibitor (PR-6); endoproteinase (PR-7); lignin-forming peroxidase (PR-9); ribonuclease-like protein (PR-10); 14 plant defensin (PR-12); thionin (PR-13), and lipid-transfer protein (PR-14) (Huang and Brasel 2001; Van Loon and
Van Strien, 1999). Since different plant families were used to identify PR-protein families, multiple PR-proteins families with the same biological function can occur.

In addition to pre-formed physical and chemical barriers, plants have an immune system that is able to detect motifs or domains with conserved structural traits typical of entire classes of microbes but not present in their host, namely the pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs, respectively). MAMP-triggered plant responses are elicited rapidly and transiently. Early MAMP responses involve ion fluxes across the plasma membrane, the generation of reactive oxygen species (ROS), nitric oxide, ET and also, but later, the deposition of callose and the synthesis of antimicrobial compounds. Many MAMPs have been identified for PGPR, such as flagellin or lipopolysaccharides, but also secreted compounds including antibiotics, biosurfactants and volatile organic compounds have been shown to elicit systemic resistance. Effective Trichoderma strains produce a variety of MAMPs, which to date are those most widely described among plant-beneficial fungi (Lorito et al., 2010). The first recognized Trichoderma MAMP was identified as an ET inducing xylanase (Xyn2/Eix), produced by Trichoderma as a potent elicitor of plant defence responses in specific tobacco and tomato cultivars (Rotblat et al., 2002). The Eix epitope recognized by plants consists of five surface-exposed amino acids that are not involved in enzyme activity. Trichoderma activated and heat-denatured cellulases also elicit melon defences through the activation of the SA and ET signaling pathways respectively (Martínez et al., 2001). Some Trichoderma proteins involved in root colonization can also act as MAMPs. Swollenin TasSwo (Brotman et al., 2008) stimulates defence responses in cucumber roots and leaves.
and affords local protection against fungi and bacteria, and the endopolygalacturonase ThPG1 (Mora´n-Diez et al., 2009) generates a response in Arabidopsis similar to the ISR triggered by PGPR. Orthologues of the SSCP cerato-platanin family – Sm1 from T. virens and Epl1 from T. atroviride – are accumulated in the hyphae during root colonization and act as MAMPs in cotton and maize (Djonovic´ et al., 2006; Seidl et al., 2006). A glycosylation mechanism keeps these proteins in a monomeric form necessary to elicit the ISR response (Vargas et al., 2008). Chitin oligosaccharides act as elicitors of defence responses in plants, and the scavenging of such oligomers is fundamental to the lifestyle of fungal pathogens upon colonization of their hosts (de Jonge et al., 2010). As a mechanism for perceiving chitin, plants probably developed chitinases to release the active polymers from the cell walls of invading fungi, thereby triggering defence responses. Thus, the mycotrophic activity of Trichoderma chitinases can also release chitoooligosaccharides and indirectly contribute to the induction of this defence mechanism. Certain secondary metabolites produced by Trichoderma exert an antimicrobial effect at high doses but act as MAMPs and as auxin-like compounds at low concentrations. At 1 ppm., 6-pentyl-a-pyrone, harzianolide and harzianopyridone activate plant defence mechanisms and regulate plant growth in pea, tomato and canola (Vinale et al., 2008), suggesting that plants’ defence mechanisms and their developmental responses to Trichoderma share common components. Peptaibols are linear peptide antibiotics of 5 to 20 amino acid residues generated by non-ribosomal peptide synthetase activity. Alamethicin, a 20mer peptaibol from T. viride, elicited JA and SA biosynthesis in lima bean (Engelberth et al., 2001), whereas 18-mer peptaibols from T. virens elicited systemic defences in cucumber against the leaf pathogen Pseudomonas syringae pv. lachrymans
(Viterbo et al., 2007). The protection of tobacco plants against tobacco mosaic virus by *Trichoderma* peptaibols was shown to involve multiple defence signalling pathways (Luo et al., 2010).

**viii) Defence signalling pathways induced by Trichoderma**

*Trichoderma* had received little attention as a potential inducer of plant resistance until the publication of studies describing that bean root colonization by *T. harzianum* was effective in inducing defence responses (De Meyer et al., 1998) and that penetration of *T. asperellum* in the root system triggered ISR in cucumber seedlings (Yedidia et al., 1999). As a consequence of *Trichoderma* root colonization and MAMP interaction the proteome and transcriptome of plant leaves are systemically affected (Shoresh et al., 2010). The ISR triggered by *Trichoderma* occurs through the JA/ET signalling pathway similarly to PGPR- ISR (Shoresh et al., 2005), as confirmed by several authors: (i) cerato-platanin Sm1 was required for *T. virens*-mediated ISR against *Colletotrichum graminicola* in maize (Djonovic et al., 2007), (ii) *Trichoderma* treatment of JA/ET-deficient *Arabidopsis* genotypes lead to enhanced susceptibility to *Botrytis cinerea* (Korolev et al., 2008), (iii) ISR triggered by PGPR and *Trichoderma* converged upstream from MYB72, an early key component of the onset of ISR (Segarra et al., 2009). However, other studies have shown that in *T. asperellum*–cucumber interaction, the induction of plant responses is a time- and concentration-dependent phenomenon, and in the first hours of contact, a SAR-like response is observed, with an increase in SA and peroxidase activity. In fact, a systemic increase in SA and JA levels was observed after inoculation of high densities of *Trichoderma* (Segarra et al., 2007). Gallou et al. (2009) also observed that the defence response
of *T. harzianum* challenged potato to *Rhizoctonia solani* was dependent on JA/ET and SA. Recent findings include:

(i) The colonization of *Arabidopsis* roots by *T. atroviride* induced a delayed and overlapping expression of the defence-related genes of the SA and JA/ET pathways against biotrophic and necrotrophic phytopathogens, both locally and systemically (Salas-Marina et al., 2011).

(ii) *Trichoderma* was able to trigger a long-lasting upregulation of SA gene markers in plants unchallenged by pathogens, although when plants are infected by a pathogen such as *B. cinerea*, the pretreatment with *Trichoderma* may modulate the SA-dependent gene expression and, soon after infection, the expression of defence genes induced through the JA signal transduction pathway occurs, causing ISR to increase over time (Tucci et al., 2011).

(iii) Colonization of *Arabidopsis* root by *T. asperellum* produced a clear ISR through an SA signalling cascade, and both the SA and JA/ET signalling pathways combined in the ISR triggered by cell-free culture filtrates of *Trichoderma* (Yoshioka et al., 2011).

### 2.5. Bacteria as biocontrol agent

Bacteria are the most copious microorganisms present in the soil and they influence the plant physiology to a significant extent, particularly considering their competitiveness in root colonization (Barriuso et al., 2008). Many researchers have reviewed the potential uses of plant allied bacteria as agents stimulating plant growth and managing soil and plant health (Glick, 1995; Hallman et al., 1997; Rovira, 1965; Sturz, et al., 2000; Welbaum et al., 2004). Plant growth-promoting bacteria (PGPB) (Bashan and Holguin 1998) are associated with many plant
species and are commonly present in many environments. The most extensively
studied group of PGPB are plant growth-promoting rhizobacteria (PGPR)
(Kloepper and Schroth, 1978) colonizing the root surfaces and the intimately
adhering soil interface, the rhizosphere (Kloepper and Schroth 1978, Kloepper et
al., 1999). As reported by Kloepper et al. (1999) and by Gray and Smith (2005),
most of these PGPR can also enter root interior and establish endophytic
populations. Many of them are able to transcend the endodermis barrier, crossing
from the root cortex to the vascular system, and subsequently thrive as endophytes
in stem, leaves, tubers, and other organs (Bell et al., 1995; Compant et al., 2005;
Gray and Smith, 2005; Hallman et al., 1997). The degree of endophytic
colonization of host plant organs these precise ecological niches (Gray and Smith,
2005; Hallman et al., 1997). Consequently, close associations between bacteria and
host plants can be formed (Compant et al., 2005; Hallman et al., 1997;,
Kloepper et al., 1999) without harming the plant (Hallman et al., 1997; Kloepper, 1992;
Kloepper et al., 1999; Lodewyckx et al., 2002; Whipps, 2001). Although, it is
generally assumed that many bacterial endophyte communities are the product of a
colonizing process initiated in the root zone (McInroy and Klopper, 1995; Sturz et
al., 2000; Van Peer et al., 1990), they may also originate from other source than
the rhizosphere like phyllosphere, the anthosphere, or the spermosphere (Hallman
et al., 1997). Even though their different ecological niches, free-living
rhizobacteria and endophytic bacteria use some of the same mechanisms to endorse
plant growth and control phytopathogens (Bloemberg and Lugtenberg, 2001;
Dobbelaeere et al., 2003; Glick,1995; Hallman et al., 1997;Lodewyckx et al., 2002;
Sturz et al., 2000). The widely recognized mechanisms of biocontrol mediated by
PGPB are competition for an ecological niche or a substrate, production of
inhibitory allelochemicals and induction of systemic resistance (ISR) in host plants to a broad spectrum of pathogens (Bloemberg and Lugtenberg, 2001; Glick, 1995; Haas et al., 2000; Haas et al., 2002; Ryan et al., 2001) and abiotic stresses (Mayak et al., 2004; Nowak and Shulaev, 2003).

2.5.1. Plant growth promotion

Plant growth promoting rhizobacteria are active group of bacteria which are present in rhizosphere, which represents the thin layer of soil surrounded by plant roots and soil occupied by roots. The PGPRs colonize the rhizosphere rapidly and inhibit soil-borne pathogens at the root surface (Rangarajan et al., 2003). These organisms also support the plant by stimulating growth (Bloemberg and Lugtenberg, 2001). PGPRs are known to produce secondary metabolities such as antibiotics, phytohormones, volatile compounds and siderophores. Moreover, PGPRs promote plant growth by inducing phytohormones like indole-3-acetic acid, siderophores and antibiotics. The genera of PGPRs include Azotobacter, Azospirillum, Pseudomonas, Acetobacter, Burkholderia, Bacillus, Paenibacillus and some members of the Enterobacteriaceae.

2.5.1.1. Mechanisms of plant growth promotion

According to Kloepper and Schroth (1981), PGPR mediated plant growth promotion happens by the amendment of the whole microbial community in rhizosphere niche through the production of various substances (Kloepper and Schroth, 1981). Generally, PGPR promote plant growth directly by either facilitating resource attainment (nitrogen, phosphorus and essential minerals) or transforming plant hormone levels, or indirectly by decreasing the inhibitory effects of various pathogens on plant growth and development in the forms of biocontrol agents (Glick, 2012).
i) Direct mechanisms

a) Nitrogen fixation

Nitrogen (N) is the most vital nutrient for plant growth and productivity. Although, there is about 78% N\textsubscript{2} in the atmosphere, it is unavailable to the growing plants. The atmospheric N\textsubscript{2} is converted into plant-utilizable forms by biological N\textsubscript{2} fixation (BNF) which changes nitrogen to ammonia by nitrogen fixing microorganisms using a complex enzyme system known as nitrogenase (Kim and Rees, 1994). In fact, BNF accounts for approximately two-thirds of the nitrogen fixed globally, while the rest of the nitrogen is industrially synthesized by the Haber–Bosch process (Rubio and Ludden, 2008). Biological nitrogen fixation occurs, generally at mild temperatures, by nitrogen fixing microorganisms, which are widely distributed in nature (Raymond et al., 2004). Furthermore, BNF represents an economically beneficial and environmentally sound alternative to chemical fertilizers (Ladha et al., 1997).

Nitrogen fixing organisms are generally categorized as (a) symbiotic N\textsubscript{2} fixing bacteria including members of the family rhizobiaceae which forms symbiosis with leguminous plants (e.g. rhizobia) (Ahemad and Khan, 2012; Zahran, 2001) and non-leguminous trees (e.g. Frankia) and (b) non-symbiotic (free living, associative and endophytes) nitrogen fixing forms such as cyanobacteria (Anabaena, Nostoc), Azospirillum, Azotobacter, Gluconoacetobacter diazotrophicus, Azocarui etc. (Bhattacharyya and Jha, 2012). However, non-symbiotic nitrogen fixing bacteria provide only a small amount of the fixed nitrogen that the bacterially-associated host plant requires (Glick, 2012). Symbiotic nitrogen fixing rhizobia within the rhizobiaceae family (α-proteobacteria) infect and establish symbiotic relationship with the roots of leguminous plants. The
establishment of the symbiosis involves a complex interplay between host and symbiont (Giordano and Hirsch, 2004) resulting in the formation of the nodules wherein the rhizobia colonize as intracellular symbionts. Plant growth-promoting rhizobacteria that fix \( \text{N}_2 \) in non-leguminous plants are also called as diazotrophs since they are capable of forming a nonobligate interaction with the host plants (Glick et al., 1999).

The process of \( \text{N}_2 \) fixation is carried out by a complex enzyme, the nitrogenase complex (Kim and Rees, 1994). Structure of nitrogenase was elucidated by Dean and Jacobson (1992) as a two-component metalloenzyme consisting of (i) dinitrogenase reductase, which is the iron protein, and (ii) dinitrogenase, which has a metal cofactor. Dinitrogenase reductase provides electrons with high reducing power while dinitrogenase uses these electrons to reduce \( \text{N}_2 \) to \( \text{NH}_3 \). Based on the metal cofactor, three different \( \text{N} \) fixing systems have been identified (a) Mo-nitrogenase, (b) V-nitrogenase and (c) Fe-nitrogenase. Structurally, \( \text{N}_2 \)-fixing system varies among different bacterial genera. Most biological nitrogen fixation is carried out by the activity of the molybdenum nitrogenase, which is found in all diazotrophs. The genes for nitrogen fixation, called \textit{nif} genes are found in both symbiotic and free living systems (Kim and Rees, 1994). Nitrogenase (\textit{nif}) genes include structural genes, genes involved in activation of the Fe protein, iron molybdenum cofactor biosynthesis, electron donation, and regulatory genes required for the synthesis and function of the enzyme. In diazotrophs, \textit{nif} genes are typically found in a cluster of around 20–24 kb with seven operons encoding 20 different proteins (Glick, 2012). The molybdenum nitrogenase enzyme complex has two component proteins encoded by the \textit{nif}DK and the \textit{nif}H genes. The NifDK component is a heterotetrameric
(a2b2) protein formed by two ab dimers related by a twofold symmetry. NifDK carries one iron molybdenum cofactor (FeMo-co) within the active site in each a-subunit (NifD) (Rubio and Ludden, 2008). The symbiotic activation of nif genes in *Rhizobium* is dependent on low oxygen concentration, which in turn is regulated by another set of genes called fix genes, which are common for both symbiotic and free living nitrogen fixation systems (Kim and Rees, 1994; Dean and Jacobson, 1992). Since nitrogen fixation is a very energy demanding process, requiring at least 16 mol of ATP for each mole of reduced nitrogen, it would be advantageous if bacterial carbon resources were directed toward oxidative phosphorylation, which results in the synthesis of ATP, rather than glycogen synthesis, which results in the storage of energy in the form of glycogen (Glick, 2012). For instance, treatment of legume plants with rhizobia having a deleted gene for glycogen synthase resulted in a considerable augmentation in both the nodule number and plant dry weight with reference to treatment with the wild-type strain (Marroqui *et al.*, 2001).

**b) Phosphate solubilization**

Phosphorus (P), the second important plant growth-limiting nutrient after nitrogen, is abundantly available in soils in both organic and inorganic forms (Fig. 3) (Khan *et al.*, 2009). Despite a large reservoir of P in nature, the amount of available forms to plants is generally low. This low availability of phosphorous to plants is due to the fact that the majority of soil P is found in insoluble forms, while the plants can absorb it only in two soluble forms, the monobasic (\( \text{H}_2\text{PO}_4^- \)) and the diabasic (\( \text{HPO}_4^{2-} \)) ions (Bhattacharyya and Jha, 2012). The insoluble P is present as an inorganic mineral such as apatite or as one of several organic forms including inositol phosphate (soil phytate), phosphomonesters, and
phosphotriesters (Glick, 2012). To overcome the P deficiency in soils, there are frequent applications of phosphatic fertilizers in agricultural fields. Plants absorb fewer amounts of applied phosphatic fertilizers and the rest is rapidly converted into insoluble complexes in the soil (Mckenzie and Roberts, 1990). But regular application of phosphate fertilizers is not only costly but is also environmentally undesirable. This has led to search for an ecologically safe and economically reasonable option for improving crop production in low P soils. In this context, organisms coupled with phosphate solubilizing activity, often termed as phosphate-solubilizing microorganisms (PSM), may provide the available forms of P to the plants and hence a viable substitute to chemical phosphatic fertilizers (Khan et al., 2006). Of the various PSM(s) inhabiting the rhizosphere, phosphate-solubilizing bacteria (PSB) are considered as promising biofertilizers since they can supply plants with P from sources otherwise poorly available by various mechanisms (Zaidi et al., 2009). Bacterial genera like Azotobacter, Bacillus, Beijerinckia, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Microbacterium, Pseudomonas, Rhizobium and Serratia are reported as the most significant phosphate-solubilizing bacteria (Bhattacharyya and Jha, 2012). Typically, the solubilization of inorganic phosphorus occurs as a consequence of the action of low molecular weight organic acids which are synthesized by various soil bacteria (Zaidi et al., 2009). Conversely, the mineralization of organic phosphorus occurs through the synthesis of a variety of different phosphatases, catalyzing the hydrolysis of phosphoric esters (Glick, 2012). Importantly, phosphate solubilization and mineralization can coexist in the same bacterial strain (Tao et al., 2008). Though, PSB are commonly found in most soils; their establishment and performances are severely affected by environmental factors especially under
stress conditions (Ahemad and Khan, 2012a, e; Ahemad and Khan, 2010a, b). However, the beneficial effects of the inoculation with PSB used alone (Ahemad and Khan, 2012e; Ahemad and Khan, 2011k; Ahemad and Khan, 2010d; Poonguzhali et al., 2008; Chen et al., 2008) or in combination with other rhizospheric microbes have been reported (Zaidi and Khan, 2005; Vikram and Hamzehzarghani, 2008). Besides providing phosphorus to the plants, the phosphate solubilizing bacteria also augment the growth of plants by stimulating the efficiency of BNF, enhancing the availability of other trace elements by synthesizing important plant growth promoting substances (Suman et al., 2001; Ahmad et al., 2008; Zaidi et al., 2009).

c) **Siderophore production**

Iron is a vital nutrient for almost all forms of life. All microorganisms known hitherto, with the exception of certain lactobacilli, essentially require iron (Neilands, 1995). In the aerobic environment, iron occurs principally as Fe3+ and is likely to form insoluble hydroxides and oxyhydroxides, thus making it generally inaccessible to both plants and microorganisms (Rajkumar et al., 2010). Commonly, bacteria acquire iron by the secretion of low-molecular mass iron chelators referred to as siderophores which have high association constants for complexing iron. Most of the siderophores are water-soluble and can be divided into extracellular siderophores and intracellular siderophores. Generally, rhizobacteria differs regarding the siderophore cross-utilizing ability; some are proficient in using siderophores of the same genus (homologous siderophores) while others could utilize those produced by other rhizobacteria of different genera (heterologous siderophores) (Khan et al., 2009). In both Gram-negative and Gram-positive rhizobacteria, iron (Fe3+) in Fe3+-siderophore complex on bacterial
membrane is reduced to Fe2+ which is further released into the cell from the siderophore via a gating mechanism linking the inner and outer membranes. During this reduction process, the siderophore may be destroyed/recycled (Rajkumar et al., 2010; Neilands, 1995). Thus, siderophores act as solubilizing agents for iron from minerals or organic compounds under conditions of iron limitation (Indiragandhi et al., 2008). Not only iron, siderophores also form stable complexes with other heavy metals that are of environmental concern, such as Al, Cd, Cu, Ga, In, Pb and Zn, as well as with radionuclides including U and Np (Neubauer et al., 2000; Kiss and Farkas, 1998). Binding of the siderophore to a metal increases the soluble metal concentration (Rajkumar et al., 2010). Hence, bacterial siderophores help to alleviate the stresses imposed on plants by high soil levels of heavy metals. Plants assimilate iron from bacterial siderophores by means of different mechanisms, for instance, chelate and release of iron, the direct uptake of siderophore-Fe complexes, or by a ligand exchange reaction (Schmidt, 1999). Numerous studies of the plant growth promotion vis-a-vis siderophore-mediated Fe-uptake as a result of siderophore producing rhizobacterial inoculations have been reported (Rajkumar et al., 2010). For example, Crowley and Kraemer (2007) revealed a siderophore-mediated iron transport system in oat plants and inferred that siderophores produced by rhizosphere microorganisms deliver iron to oat, which has mechanisms for using Fe-siderophore complexes under iron-limited conditions. Similarly, the Fe-pyoverdine complex synthesized by Pseudomonas fluorescens C7 was taken up by Arabidopsis thaliana plants, leading to an increase of iron inside plant tissues and to improved plant growth (Vansuyt et al., 2007). Recently, Sharma et al. (2003) assessed the role of the siderophore-producing Pseudomonas strain GRP3 on iron nutrition of Vigna radiate. After 45 days, the
plants showed a decline in chlorotic symptoms and iron, chlorophyll a and chlorophyll b content increased in strain GRP3 inoculated plants compared to control.

d) Phytohormone production

Microbial synthesis of the phytohormone auxin (indole-3-acetic acid/indole acetic acid/IAA) has been known for a long time. It is reported that 80% of microorganisms isolated from the rhizosphere of various crops possess the ability to synthesize and release auxins as secondary metabolites (Patten and Glick, 1996). Generally, IAA secreted by rhizobacteria interferes with the many plant developmental processes because the endogenous pool of plant IAA may be altered by the acquisition of IAA that has been secreted by soil bacteria (Glick, 2012; Spaepen et al., 2007). Evidently, IAA also acts as a reciprocal signaling molecule affecting gene expression in several microorganisms. Consequently, IAA plays a very important role in rhizobacteria-plant interactions (Spaepen and Vanderleyden, 2011). Moreover, down-regulation of IAA as signaling is associated with the plant defense mechanisms against a number of phyto-pathogenic bacteria as evidenced in enhanced susceptibility of plants to the bacterial pathogen by exogenous application of IAA or IAA produced by the pathogen (Spaepen and Vanderleyden, 2011). IAA has been implicated in virtually every aspect of plant growth and development, as well as defense responses. This diversity of function is reflected by the extraordinary complexity of IAA biosynthetic, transport and signaling pathways (Santner et al., 2009). Generally, IAA affects plant cell division, extension, and differentiation; stimulates seed and tuber germination; increases the rate of xylem and root development; controls processes of vegetative growth; initiates lateral and adventitious root formation; mediates responses to light,
gravity and florescence; affects photosynthesis, pigment formation, biosynthesis of various metabolites, and resistance to stressful conditions. IAA produced by rhizobacteria likely interfere the above physiological processes of plants by changing the plant auxin pool. Moreover, bacterial IAA increases root surface area and length, and thereby provides the plant greater access to soil nutrients. Also, rhizobacterial IAA loosens plant cell walls and as a result facilitates an increasing amount of root exudation that provides additional nutrients to support the growth of rhizosphere bacteria (Glick, 2012). Thus, rhizobacterial IAA is identified as an effector molecule in plant–microbe interactions, both in pathogenesis and phytostimulation (Spaepen and Vanderleyden, 2011). An important molecule that alters the level of IAA synthesis is the amino acid tryptophan, identified as the main precursor for IAA and thus plays a role in modulating the level of IAA biosynthesis (Zaidi et al., 2009). Strangely, tryptophan stimulates IAA production while, anthranilate, a precursor for tryptophan, reduces IAA synthesis. By this mechanism, IAA biosynthesis is fine-tuned because tryptophan inhibits anthranilate formation by a negative feedback regulation on the anthranilate synthase, resulting in an indirect induction of IAA production (Spaepen et al., 2007). However, supplementation of culture media with tryptophan increases the IAA production by most of the rhizobacteria (Spaepen and Vanderleyden, 2011). Biosynthesis of tryptophan starts from the metabolic node chorismate in a five-step reaction encoded by the trp genes. The branch point compound chorismate is synthesized starting from phosphoenolpyruvate and erythrose 4-phosphate in the shikimate pathway, a common pathway for the biosynthesis of aromatic amino acids and many secondary metabolites (Spaepen and Vanderleyden, 2011; Merino et al., 2008; Dosselaere and Vanderleyden, 2001). Starting with tryptophan, at least
five different pathways have been described for the synthesis of IAA, and most pathways show similarity to those described in plants, although some intermediates can differ (Spaepen and Vanderleyden, 2011; Patten and Glick, 1996):

- IAA formation via indole-3-pyruvic acid and indole-3-acetic aldehyde is found in a majority of bacteria like, *Erwinia herbicola*; saprophytic species of the genera *Agrobacterium* and *Pseudomonas*; certain representatives of *Bradyrhizobium, Rhizobium, Azospirillum, Klebsiella, and Enterobacter*.

- The conversion of tryptophan into indole-3-acetic aldehyde may involve an alternative pathway in which tryptamine is formed as in pseudomonads and azospirilla.

- IAA biosynthesis via indole-3-acetamide formation is reported for phytopathogenic bacteria *Agrobacterium tumefaciens, Pseudomonas syringae*, and *E. herbicola*; saprophytic pseudomonads like (e.g. *Pseudomonas putida* and *P. fluorescens*).

- IAA biosynthesis that involves tryptophan conversion into indole-3-acetonitrile is found in the cyanobacterium (*Synechocystis* sp.) and the tryptophan-independent pathway, more common in plants, is also found in azospirilla and cyanobacteria.

**ii) Indirect mechanisms**

The application of microorganisms to control diseases, which is a form of biological control, is an environment-friendly approach (Lugtenberg and Kamilova, 2009). The major indirect mechanism of plant growth promotion in rhizobacteria is through acting as biocontrol agents (Glick, 2012). In general, competition for nutrients, niche exclusion, induced systemic resistance and
antifungal metabolites production are the chief modes of biocontrol activity in PGPR (Lugtenberg and Kamilova, 2009). Many rhizobacteria have been reported to produce antifungal metabolites like, HCN, phenazines, pyrrolnitrin, 2,4-diacetylphloroglucinol, pyoluteorin, viscosinamide and tensin (Bhattacharyya and Jha, 2012). Interaction of some rhizobacteria with the plant roots can result in plant resistance against some pathogenic bacteria, fungi, and viruses. This phenomenon is called induced systemic resistance (ISR) (Lugtenberg and Kamilova, 2009). Moreover, ISR involves jasmonate and ethylene signaling within the plant and these hormones stimulate the host plant’s defense responses against a variety of plant pathogens (Glick, 2012). Many individual bacterial components induce ISR, such as lipopolysaccharides (LPS), flagella, siderophores, cyclic lipopeptides, 2,4-diacetylphloroglucinol, homoserine lactones, and volatiles like, acetoin and 2,3-butanediol (Lugtenberg and Kamilova, 2009).

2.5.2. Antagonism by Antibiosis

Some of the earliest studies on mechanisms of pathogen suppression by root-colonizing bacteria focused on their ability to produce specific compounds (siderophores) that efficiently sequester iron, thereby depriving the pathogen from this essential element during its deleterious activities in the rhizosphere (Kloepper et al., 1980ab). Over the past 20 years, numerous studies have demonstrated unequivocally that several other metabolites including antibiotics, enzymes, and volatiles produced by antagonistic bacteria play key roles in the control of various plant pathogens (Weller, 1988). Bacteria also can be beneficial to the host plant directly through the production of metabolites that either stimulate root development and plant growth or trigger the induction of systemic acquired resistance (Van Loon et al., 1998). Although most knowledge on mechanisms and
metabolites involved in biological control by bacteria are based on studies with fluorescent *Pseudomonas* spp., information on antibiotics produced by representatives of other bacterial genera will be discussed whenever possible.

**i) Role of antibiotics in biological control**

Most of the data on broad-spectrum activity of antibiotics produced by bacterial biocontrol agents are derived from assays performed *in vitro*. Several lines of evidence, however, have more conclusively demonstrated the role and function of antibiotics in *in situ* interactions between antagonistic bacteria and plant pathogens. The first line of evidence was the observation that culture filtrates or purified antibiotics provided similar levels of control as achieved by the producing wild-type strain (Howell and Stipanovic, 1979; Kang *et al.*, 1998; Nakayama *et al.*, 1999). Second, inactivation of antibiotic production by mutagenesis resulted, in many cases, in a reduced ability of the antagonistic bacteria to control the pathogen. Mutagenesis has been successfully used to demonstrate that antibiotics produced by *Pseudomonas* species (Thomashow and Weller, 1988; Keel *et al.*, 1990; Vincent *et al.*, 1991; Cronin *et al.*, 1997; Anjaiah *et al.*, 1998; Chin-A-Woeng *et al.*, 1998), *Burkholderia cepacia* (Kang *et al.*, 1998), and *Bacillus cereus* (Silo-Suh *et al.*, 1994) play an important role in the biological control of plant diseases. In several of these studies, complemented mutants with restored phenotypes and restored biocontrol activity were included (Thomashow *et al.*, 1997). The third line of evidence is provided by enhancement of antibiotic production in the producing wild-type strains via introduction or modification of antibiotic biosynthetic or regulatory genes. Suppression of *Pythium* root rot of cucumber was significantly improved by enhancement of the production of DAPG and pyoluteorin in *P. fluorescens* strain CHA0 (Maurhofer *et al.*, 1992).
Ligon et al. (2000) introduced multiple copies of the regulatory *gacA* gene into *P. fluorescens* strain BL915 resulting in a 2.5-fold increase in pyrrolnitrin production. Similar increases in pyrrolnitrin production in strain BL915 were obtained by PCR-based modification of the initiation codon of the chromosomal *gacA* gene or by replacing the native promoter of this regulatory gene with the strong *Ptac* promoter from *E. coli*. Relative to the wild-type strain, the genetically modified strains showed, in most cases, an increase in biocontrol activity against *Rhizoctonia solani* (Ligon et al., 2000). The fourth line of evidence is provided by introduction of antibiotic biosynthetic genes in heterologous, non-producing strains and subsequent evaluation of their ability to control plant diseases. For example, Fenton et al. (1992) introduced a 6-kb fragment from DAPG-producing *P. fluorescens* strain F113 into DAPG-nonproducing *Pseudomonas* strain M114; the derivative produced DAPG and was significantly more effective than its parental strain in controlling *Pythium ultimum* on sugar beet. Most studies on heterologous expression of antibiotic biosynthetic genes describe the use of multi-copy plasmid vectors. Recently, Timms-Wilson et al. (2000) introduced a disabled *Tn5* vector harboring the genes for phenazine-1-carboxylic acid production into the chromosome of PCA-nonproducing *P. fluorescens* strain SBW25. PCA-producing derivatives were significantly more protective than their parental strain against *Pythium ultimum* on pea seedlings. The authors suggested that single copy chromosomal insertions were less likely to affect the fitness of the heterologous strain than plasmid-based modifications. The latter two genetic strategies not only provide evidence for a role of antibiotics in biological control, but also clearly illustrate that knowledge about the genetics, regulation and biochemistry of
antibiotic biosynthesis provides powerful tools to improve the efficacy of bacterial biocontrol agents.

ii) *In situ* antibiotic production

A more direct line of evidence for the role of antibiotics in disease suppression by biocontrol agents is provided by detection of antibiotic production *in situ*, a strategy that complements indirect evidence provided by the above mentioned genetic approaches. The significance of antibiotics in biological control, and more generally in microbial antagonism, often has been questioned because of the perceived constraints to antibiotic production in natural environments (Gotlieb, 1976; Williams and Vickers, 1986). Because of the biotic and abiotic complexity of soils and plant associated environments, there are several inherent difficulties in detecting antibiotics produced by microorganisms *in situ*. Recovery and detection may be hampered by chemical instability of the compound, irreversible binding to soil colloids or organic matter, or microbial decomposition (Thomashow et al., 1997). Sensitive methods have been developed by which antibiotic production by biocontrol agents can be measured in natural environments.

One approach involves the use of reporter gene systems (Lindow, 1995; Chin-A-Woeng et al., 1998). Reporter gene systems are widely used as a marker to monitor populations of introduced strains but also may provide information on the transcriptional activity of specific antibiotic biosynthetic genes (Loper and Lindow, 1997). Unfortunately, reporter gene systems generally do not provide an accurate measure of the amount of the antibiotic produced *in situ*. Bioanalytical techniques like thin layer and high-pressure liquid chromatography are now being used to detect and quantify antibiotics produced by microorganisms *in situ*.
The versatility, resolving capability, and quantitative accuracy of HPLC make it one of the best direct methods to study the production of antibiotics in situ. In order to detect and quantify antibiotics by HPLC, however, the extracted and separated compounds need to be positively identified. Identification of antibiotic compounds on the basis of their retention time is not sufficient. Subsequent spectral analysis by photodiode array detectors is required to provide the necessary information about peak homogeneity and identity. In most cases, spectral analysis over a range of wavelengths will provide sufficient information on the compound. However, also spectral analysis may have its limits. Determining the identity of compounds in overlapping peaks may be difficult if the spectra of the compounds are similar and the peaks are poorly resolved (Dorschel, 1997). Also, deviations in the spectral characteristics of the compound can be induced by volatilization of the solvents, and may occur after derivatization. In that case, HPLC linked to a mass spectrometer provides a means to confirm the identity of antibiotics produced in situ (Raaijmakers et al., 1999). To date, HPLC has been successfully used for the in situ detection of a variety of antibiotics including phenazine-1-carboxylic acid, herbicolin A, pyrrolnitrin, pyoluteorin, surfactin, and DAPG (Thomashow et al., 1997). In almost all cases, these data stem from studies in which specific bacterial strains were introduced into soil or rhizosphere environments at relatively high densities. Recently, also antibiotic production levels by specific indigenous bacterial populations were determined. In a soil that is naturally suppressive to the soilborne pathogen Gaeumannomyces graminis var. tritici, the antibiotic DAPG was detected on roots of wheat at a concentration of approximately 20 ng per gram of root fresh weight (Raaijmakers et al., 1999). These results provided, for the first time, biochemical support for the conclusion of
other studies that DAPG-producing fluorescent *Pseudomonas* spp. are key components of the natural biological control that operates in several take-all suppressive soils in Washington State (USA) (Raaijmakers and Weller, 1998). For a wide variety of other antibiotics, *in situ* production levels may range from 5 to 5000 ng per seed or gram of dry soil or root fresh weight (Thomashow et al., 1997). Although detection of antibiotics *in situ* has been described as a strategy that complements the indirect evidence provided by genetic approaches, it in fact only confirms that antibiotic production has occurred. However, it does not address the question as to whether the amount of antibiotic is sufficient to inhibit the growth or metabolic activity of the pathogen *in situ*. The answer to this latter issue is difficult to give, because quantitative detection methods usually are not sensitive enough to determine spatial and temporal production patterns. The time and place of antibiotic production needs to be considered with respect to the efficacy of biological control of several plant pathogens. For example, an antibiotic may reach threshold concentrations for activity within certain microsites while remaining below this threshold level at other sites where pathogen infection also occurs. This may offer an explanation for the observation that, in spite of a significant positive linear relationship between *in situ* DAPG production levels and rhizosphere population densities of *P. fluorescens* strain Q2-87, increases in the rhizosphere population density of Q2-87 did not significantly improve the level of suppression of takeall of wheat (Raaijmakers and Weller, 1998; Raaijmakers et al., 1999). Recent advances in the use of different derivatives of the green fluorescent protein (Bloemberg et al., 2000) offer powerful tools to simultaneously visualize the pathogen and the biocontrol agent and, more specifically, to determine whether spatial colonization patterns of the pathogen coincide with spatial colonization and
antibiotic production patterns of the biocontrol agent. Also in situ PCR technologies (Hodson et al., 1995; Chen et al., 2000) provide powerful tools to detect single cells and target sequences in environmental samples and microsites on the plant root. These technologies not only allow detection of single cells but also enable visualization of gene expression inside the bacterial cells.

iii) Antagonism by Competition: Siderophore Production

Iron is important for plant health and metabolism. It is found in proteins such as nitrogenase, ferredoxins, cytochromes and leghemoglobin. PGPR bacteria could perform uptake of iron from soil and provide plant with this element. The most widely studied rhizospheric bacteria with respect to the production of siderophores are fluorescent pseudomonads. Siderophores are low-molecular-mass microbial compounds with high affinity for iron. They possess an iron uptake system (ironbinding ligand) able to chelate Fe3+ molecules. They are often induced under limiting Fe3+ concentrations to allow bacteria to partially fulfill their iron requirement. Siderophores represent a large biochemically diverse group produced by either plants or plant associated microorganisms (Loper and Buyer, 1991). They include pyoveridins produced by Pseudomonas; catechols produced by Agrobacterium tumefaciens, Erwinia chrysanthemi and Enterobacteriacea; hydroxamates produced by Erwinia carotovora, Enterobacter cloacae and various fungi; and rhizobactin produced by Rhizobium meliloti. Pyoverdine, which is a yellow-green, water-soluble fluorescent pigment is the major class of siderophores produced by fluorescent pseudomonad. However, strains of P. aeruginosa, P. syringae and P. putida could also produce pyoverdine. The chemical structure of pyoverdine has been elucidated and the presence of a chromophore consisting of a 2,3-diamino-6,7-dihydroxyquinoline derivative which is responsible for the
fluorescence was reported (Leong, 1986). Fluorescent *Pseudomonas* exclusively recognizes the ferric complex of its own PVD. Thus differences in PVD structure affect the biological activity of the siderophores (Hohnadel and Meyer, 1988). Suppressive soils to *Fusarium* wilts are known to have a very low solubility of ferric iron (Alabouvette *et al.*, 1996). Consequently, a strong iron competition occurs in these soils. In addition, the ability to produce siderophores is likely to contribute to the root-colonizing ability of *Pseudomonas* strains, their antagonistic properties, and their usefulness in biocontrol (Leong, 1986).

The role of these microorganisms in disease-suppressive soils, particularly to fusarium wilts, was shown to be related to siderophile-mediated iron competition. Addition of *Pseudomonas*, pyoverdine to soils conducive to *Fusarium* wilts and to *G. graminis* var. *tritici* confer them suppressiveness (Kloepper *et al.*, 1980a). In addition, when soil was treated either by *Pseudomonas* or its pyoverdine, reduced chlamydospore germination of pathogenic *F. oxysporum* was observed (Elad and Baker, 1985a), suggesting a possible role of pyoverdines in soil fungistasis and suppressiveness. In addition, some siderophores like pyocyanin and pyoverdin are essential for the induction of systemic resistance (Audenaert *et al.*, 2002). Actinomycetes have also been reported as siderophore producers (Khamna *et al.*, 2009). Endogenous siderophore (ferrioxamine) and exogenous siderophore (ferrichrome) have been studied in *Streptomyces pilosus* (Muller and Raymond, 1984). *S. lydicus* WYEC108 was found to produce hydroxamate-type siderophores (Tokala *et al.*, 2002). *Streptomyces violaceusniger* strain YCED9 was reported as able to chelate iron under limiting conditions (Buyer *et al.*, 1989).
2.6. Biocontrol of *Phytophthora infestans*

As discussed earlier, oomycetes form a diverse group of eukaryotic, fungus-like microorganisms containing a wide range of economically important pathogens of plants, insects, fish and animals (Kamoun, 2003). Among the plant pathogenic oomycetes, *Phytophthora infestans* is the most notorious, causing late blight of potato and tomato. In the disease cycle, zoospores are essential propagules in the preinfection process and a potential target to control *P. infestans* and other oomycete pathogens (Erwin and Robeiro, 1996). Late blight is traditionally controlled by a combination of cultural practices and chemical applications. To control late blight biologically, several antagonistic microorganisms have been tested for their activity against *P. infestans*, including nonpathogenic *Phytophthora cryptogea* (Stromberg and Brishammar, 1991) and endophytic microorganisms such as *Cellulomonas flavigena*, *Candida* sp. and *Cryptococcus* sp. (Lourenço Júnior et al., 2006). Although some effective fungal antagonists were identified, bacterial antagonists have shown by far the most promising results to date. Bacteria with antagonistic activities against *P. infestans* are mainly found in the genera of *Pseudomonas* and *Bacillus* (Sanchez, 1998; Yan et al., 2002; Daayf et al., 2003; Kloeper et al., 2004).

2.6.1. Antagonism by microorganisms

Formulations made of saprophytic, epiphytic and endophytic organisms have been evaluated as potential antagonists to manage late blight of tomato. Most of the mechanisms were used by the biocontrol agents to antagonize *P. infestans* like antibiosis, mycoparasitism, and competition for space and nutrients where direct antagonism is more involved in inhibiting the pathogen.
Following **table 2.3.** provides brief introduction about the use of biocontrol agents in management of late blight.

**Table 2.3.: Some of the biocontrol agents used against late blight of Potato and Tomato**

<table>
<thead>
<tr>
<th>Biocontrol agent used</th>
<th>Crop</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aurantiogriseum</em> and <em>Stachybotrys atra</em></td>
<td>Potato</td>
<td>Jindal <em>et al.</em>, 1988</td>
</tr>
<tr>
<td>Compost tea with microbes</td>
<td>Potato</td>
<td>Weltzien, 1991</td>
</tr>
<tr>
<td><em>P. fluorescens</em> and <em>B. subtilus</em></td>
<td>Potato</td>
<td>Jongebloed <em>et al.</em>, 1993</td>
</tr>
<tr>
<td>Unknown isolate</td>
<td>Potato</td>
<td>Clulow <em>et al.</em>, 1995</td>
</tr>
<tr>
<td><em>Serratia</em> sp., <em>Trichoderma</em> sp., <em>Fusarium</em> sp., and <em>Pencillium</em> sp.</td>
<td>Tomato</td>
<td>Garita <em>et al.</em>, 1998</td>
</tr>
<tr>
<td><em>Xenorhabdus</em> spp.</td>
<td>Tomato</td>
<td>Li <em>et al.</em>, 1995</td>
</tr>
<tr>
<td><em>Pseudomonas</em> sp.,<em>Bacillus</em> sp.</td>
<td>Tomato</td>
<td>El-Sheikh <em>et al.</em>, 2002</td>
</tr>
<tr>
<td><em>Pseudomonas</em> sp.,<em>Rahnella</em> sp., <em>Serratia</em> sp., and <em>Bacillus</em> sp.</td>
<td>Tomato</td>
<td>Daafy <em>et al.</em>, 2003</td>
</tr>
<tr>
<td><em>Pseudomonas</em> and <em>Bacillus</em></td>
<td>Tomato</td>
<td>Yan <em>et al.</em>, 2002; Daayf <em>et al.</em>, 2003; Kloeper <em>et al.</em>, 2004</td>
</tr>
<tr>
<td>Antibiotics from <em>Streptomyces</em> sp.</td>
<td>Tomato</td>
<td>Lee <em>et al.</em>, 2005</td>
</tr>
<tr>
<td><em>Aspergillus</em> spp.,<em>Cellulomonas flavigena</em>, <em>Candida</em> sp.,<em>Cryptococcus</em> sp.</td>
<td>Tomato</td>
<td>Lourenco Jr. <em>et al.</em>, 2006</td>
</tr>
<tr>
<td><em>Trichoderma</em> sp.</td>
<td>Tomato</td>
<td>Lozoya-Saldana <em>et al.</em>, 2006</td>
</tr>
<tr>
<td>Consortium of <em>B. cereus</em> with other unidentified cultures</td>
<td>Tomato</td>
<td>Lourenco Jr. <em>et al.</em>, 2006</td>
</tr>
</tbody>
</table>
2.6.2. Microbial formulations for plant tissues

The first report for using biocontrol agents against late blight was reported by Jindal et al. (1988)- conidial suspension (10^{4-5} spores/ml) of *Penicillium aurantiogriseum* and *Stachybotrys atra* (=*S. chartarum*) were applied to potato leaflets grown in green house, 12 h prior to the inoculation with *P. infestans*. Late blight incidence was reduced by 93% and 84% respectively. The reasons for the observed results were assumed to be antibiosis and competition for space and nutrients (Jindal et al., 1988). But the detailed mechanism of action of these biocontrol agents in managing late blight were not investigated. Since *S. atra* is a cellulose-decaying fungus (Chapman 2003), its cellulolytic activity could affect the cell wall integrity of *P. infestans*. But major drawback of using *S. atra* is its ability to produce trichotheccene, a mycotoxin that can affect human health (Chapman 2003).

Weltzien (1991) reported a series of experiments under field conditions in Germany which resulted in consistent and durable management of late blight. Adding microorganisms to the tea compost significantly enhanced disease control. Tea compost, made from horse or cow manure and amended with seven microorganisms, was applied to potato foliage to late blight. In plants treated with compost tea amended with microorganisms, 11% final late blight severity was recorded and the symptoms exhibited were on par with plants treated with a mixture of the fungicides metalaxyl and mancozeb.

Apparently, microflora may contribute to inconsistency in late blight management due to dissimilarity in the composition of compost. In The Netherlands, the two most effective isolates among more than 200 microorganisms isolated from compost extract (a mixture of compost + water 1:9 w/w) or from the
Phyllosphere of potato plants were used in field trials planned to evaluate their biological control abilities. The two bacterial isolates, one fluorescent *Pseudomonas* sp. and one *Bacillus* sp., did not control late blight under field conditions either when applied alone or in combination with compost extracts (Jongebloed *et al.*, 1993). Thus, addition of efficient biocontrol agents to the compost extract did not increase efficiency of the extract. The interactions amongst organisms inhabitant to the compost with those amended to it appear to play a role in disease management. Though, for practical reason is probable to be a complicated factor to be taken into account and controlled.

Biological control of blight infected tubers has also been assessed (Clulow *et al.*, 1995). Tubers grown in wet substrates (soil or compost) were less susceptible than those formed in dry conditions (Stewart *et al.*, 1993). Inhibition of tuber blight was not due to *Streptomyces* sp., *Penicillium* sp., *Trichoderma* sp., *Gliocladium* sp., or *Rhizoctonia* sp. however, bacteria isolated from the surface of tubers and then cultivated in compost were capable inhibiting growth of *P. infestans* *in vitro*. Higher numbers of bacterial isolates were recovered from tubers kept in wet conditions, mainly for the more resistant cultivar (Clulow *et al.*, 1995). Regrettably, no description of the bacterial isolates was done and no further developments were made towards using these agents under field conditions.

Fungal and bacterial isolates from the phylloplane and rhizoplane of cultivated and wild tomatoes were able to reduce late blight lesion size on detached leaflets and in whole tomato plants. *Serratia* sp. and isolates of *Trichoderma* sp., *Fusarium* sp., and *Penicillium* sp. were selected as potential biocontrol agents, but none were effective in reducing late blight severity in the field (Garita *et al.*, 1999).
Among the many trials conducted so far, the most consistent results of biological control of late blight have been achieved with the application of *Xenorhabdus* spp. (Li *et al*., 1995; Yang *et al*., 2001). The antagonist is a gram-negative member of the Enterobacteriaceae, commonly found in mutual relationship with entomopathogenic nematodes (Kaya *et al*., 2006). Formulations based on the complex of nematode and bacterial symbionts are available commercially and used against soil pests. Metabolites produced by species of *Xenorhabdus* have been evaluated against *Phytophthora* spp., including *P. infestans* induced by *Xenorhabdus* spp.: indole derivatives; xenorhab-dins, which are organically soluble dithiolopyrrolones; and the xenocoumacins, which are water soluble benzopyran-1-one derivatives (Li *et al*., 1995). The organic fraction of the supernatant of a tryptic soy broth culture of *X. bovienii* coming from the nematode *Steinernema feltiae* was tested against potato late blight. Both *in vitro* and *in vivo* trials were conducted and phytotoxicity of the metabolites to potato plants was evaluated. The organic fraction at 0.1 and 1.0 mg/ml completely prevented mycelial growth of *infestans in vitro* and the size of late blight lesions was reduced when detached leaflets were treated with 10 or 50 mg/ml (Ng and Webster, 1997). Leaflets treated with the compounds had alterations and slight phytotoxic effects were detected in those treated with 10 mg/ml and, more intensively, with 50 mg/ml. In whole plants, application of control level similar to that achieved the metabolites of *X. bovienii* at 10 mg/ml resulted in con with the protectant fungicide chlorothalonil (Ng and Webster, 1997).

Aminoglycoside antibiotic compounds have been reported to reduce the intensity of diseases caused by oomycetes (Xiao *et al*., 2002), including *P. infestans* (Lee *et al*., 2005). Four purified commercial aminoglycoside antibiotics: neomycin, paromomycin, ribostamycin, and streptomycin were tested against *P. infestans*.
Paromomycin was the most active compound against the pathogen in vitro and the estimated effective dose to reduce mycelial growth by 50% was approximately 10 µg/ml (Lee et al., 2005). Subsequent tests were done on tomato plants inoculated with P. infestans and then treated with either commercially available paromomycin or with the culture filtrate of a paromomycin-producing actinomycete Streptomyces sp. (strain AMG-P1). Outstanding tomato late blight control was reported when the concentration of paromomycin was adjusted to 100 µg/ml. Strain AMG-P1 produced this antibiotic at a rate of 25 mg/l. A freeze-dried culture extract used at the rate of 125 µg/ml gave effective disease control against tomato late blight. Nevertheless, even though no phytotoxicity was mentioned, tomato plants treated with 125, 250 and 500 µg/ml apparently had prominent chlorosis (Lee et al., 2005).

In another study, metabolites from the culture broth of X. nematophilus isolated from the nematode S. carpocapsae were also tested for the control of late blight in potted potato plants. Metabolites (the whole supernatant of culture broth) at 25 and 50 mg/l were effective in reducing late blight intensity (Yang et al., 2001). However plants were inoculated with sporangial suspension of P. infestans as shortly as 2 h after metabolite application. It would be interesting to assess longer time intervals between metabolite application and pathogen inoculation; also an assessment of the efficacy of treatment with bacteria, instead of the metabolites, could provide useful insights.

An interesting study demonstrated that a yeast-like fungus Pseudozyma flocculosa inhibited growth of P. infestans in vitro by means of cis-9 heptadecenoic acid (CHDA), a fatty acid molecule. Detailed biochemical analyses indicated that CHDA was incorporated in the membrane of P. infestans and affected its
permeability. The authors postulated that altered membrane permeability would lead to an increase in electrolyte and protein loss and even cytoplasmic disintegration of cells (Avis and Bélanger, 2001).

Fourteen of 83 bacterial isolates, mostly *Pseudomonas* spp. and *Bacillus* spp., were capable of preventing growth of *P. infestans in vitro* (El-Sheikh *et al.*, 2002). Overall *Pseudomonas* spp. isolates were more effective than *Bacillus* spp. isolates and both were more effective when applied preventively. Three isolates (2 *Bacillus* sp. and 1 *Pseudomonas* sp.) were found to possess good antagonistic properties, but caused tuber soft rot and were discarded. High levels of late blight control were reported in this study for application of antagonists, but no fungicide treatment was included for comparison.

Isolates of the bacterial genera *Bacillus*, *Pseudomonas*, *Rahnella* and *Serratia* contributed to a reduction in late blight severity in potato plants in controlled conditions. This study is noteworthy because a highly aggressive isolate of the US-8 clonal lineage of *P. infestans* was used for inoculations. Several mechanisms of inhibition were thought to jointly act to reduce late blight intensity (Daayf *et al.*, 2003). An isolate of *Pseudomonas putida* did not inhibit *in vitro* growth of *P. infestans*, but induced systemic resistance in potato plants, whereas an isolate of *Serratia plymuthica* inhibited *in vitro* growth of *P. infestans* by antibiosis, but did not induce systemic resistance to the pathogen. However, both bacteria were effective in controlling late blight (Daayf *et al.*, 2003).

2.6.3. Use of endophytic organisms

Another line of investigation in the general area of biocontrol is the use of endophytic organisms to control pathogen development. Control of late blight was
attempted with arbuscular mycorrhizal fungi (AMF) (O’Herlihy et al., 2003). Potato plantlets originating from tissue culture were transplanted to the field and commercial inoculum of AMF was applied in-furrow at planting. The authors claimed that the late blight epidemic on AMF treated potato plants was delayed, but careful analysis of the disease progress curves revealed that the major epidemiological effect of AMF application was a reduction of the disease progress rate. For polycyclic diseases such as late blight this is the most effective strategy to reduce crop losses. In this experiment, even though final late blight severity was high (around 80%), tuber yield in AMF-treated plots did not differ from the most effective treatment: application of fungicide plus chitosan (O’Herlihy et al., 2003).

2.6.4. Rhizobacteria

Crop growth, yield and disease resistance can be enhanced by plant growth promoting rhizobacteria (PGPR) (Pietersen et al., 2003). Two PGPR, Bacillus pumilus and Pseudomonas fluorescens, induced resistance to P. infestans and there was reduced zoospore formation and germination (Yan et al., 2002). The in vitro and in vivo tests have shown that species of Bacillus, Pseudomonas, Rahnella and Serratia can lessen late blight symptoms by a combination of antibiosis and induced resistance against P. infestans (Daayf et al., 2003). When phylloplane isolated organisms were tested in combination with B. cereus, a PGPR, late blight severity was significantly reduced compared with application of epiphytes alone. Curiously, application of the PGPR alone was not effective in reducing tomato late blight intensity, indicating an apparent synergistic effect (Lourenço Jr et al., 2006).
2.6.5. Combination of potential antagonists

As noted previously, combining antagonists with different modes of action can lead to better control (Punja, 1997). With the objective of developing several strategies to manage late blight in both conventional and organic production, potentially useful biocontrol agents for late blight management were isolated in Brazil. Many phylloplane microorganisms and rhizobacteria isolated from conventional or organically grown tomato plants were tested for antagonistic activity against *P. infestans*. Based on *in vitro* inhibition of sporangia germination and detached leaflet bioassays, four phylloplane microorganisms *Aspergillus* sp., *Cellulomonas flavigena*, *Candida* sp., and *Cryptococcus* sp. were selected (Lourenço Jr. *et al.*, 2006).

A strategy of selecting antagonists that could hamper distinct stages of *P. infestans* pathogenesis was implemented at the screening stage of candidate microorganisms *C. flavigena* and *Cryptococcus* sp. inhibited sporangia germination but did not reduce late blight severity in detached leaflets (Lourenço Jr *et al.*, 2006). *Aspergillus* sp. and *Candida* sp. reduced both sporangia germination and disease severity, probably, through reduced infection frequency due to low sporangial germination and/or inhibited zoospore germ tube formation.

The observation of limited infection of *P. infestans* on potato tubers from some susceptible varieties in the Toluca Valley incited researchers to search for microorganisms as possible antagonists to this pathogen. Isolates of *Pseudomonas* spp., *Burkholderia* spp., *Streptomyces* spp. and *Trichoderma* spp. were obtained from stems, leaves, tubers and rhizoplane of potato plants. The suppressive activity of these microorganisms to A1 and A2 mating type isolates of *P. infestans* was
assessed on potato leaves kept in a moist chamber, and also plants grown in a greenhouse and in the field (Lozoya-Saldaña et al., 2006). In the first experiment the microorganisms were evaluated individually or in combinations on detached potato leaves inoculated with zoospores and sporangia of *P. infestans*. Reduction of late blight severity occurred with *Burkholderia* spp. *Streptomyces* spp. and *Pseudomonas* spp., applied individually or in combination within and among species (Lozoya-Saldaña et al., 2006).

Strains of *Pseudomonas* spp. selected in the detached leaf assay were tested, along with several isolates of *Trichoderma* spp. for late blight control on potato plants in a greenhouse. The value of the area under disease progress curve (AUDPC) in the control plants, which were inoculated only with the pathogen, was 770, while that of the treatments with mixed bacterial strains, combined isolates of *Trichoderma* spp., and a commercial formulation of *Trichoderma* spp. (Biopack-F), were 313.3, 373.3, and 366.3, respectively (Lozo ya-Saldaña et al., 2006). Preparations based on the antagonists used in these experiments could be implemented as a control measure in greenhouses or in areas where the late blight epidemics are less severe, mainly when combined with other available methods of controlling *P. infestans* (Lozoya-Saldaña et al., 2006).

Under controlled conditions, tomato late blight severity was reduced when a combination of antagonists was used. The best results were achieved when roots of tomato plants were treated with the rhizobacterium *B. cereus* concurrently with the treatment of foliage with epiphytic microorganisms (Silva et al., 2004; Lourenço Jr. et al., 2006). *B. cereus* is postulated to have induced systemic resistance in tomato to *P. infestans*, since it has been shown to induce non-specific resistance to other pathogens in tomatoes (Silva et al., 2004).
The impacts of a potentially effective biocontrol agent against late blight would enhance disease management in organic cropping systems and its contribution would be of great relevance. Bordeaux mixture and other copper-based fungicides are still used to control late blight in organic crops in most countries. However, it is expected that copper-based fungicides will be reduced or banned in the near future. A combination of biocontrol agents with products such as neem oil, which was demonstrated to be effective in reducing tomato late blight severity (Diniz et al., 2006), could be another option to reduce crop losses caused by the disease.

### 2.6.6. Use of commercial products based on biological control agents

Several commercial formulations of biocontrol agents have been tested for efficacy against late blight. Of many trials involving different microorganisms, including *Trichoderma harzianum*, *Bacillus subtilis*, *Streptomyces* sp., *Coniothyrium minitans* and a pool of undetermined effective microorganisms (EM 5), the most effective was the *B. subtilis* based-product Serenade. Curiously, bacterial cells were not directly responsible for the inhibition of *P. infestans*. A cell-free culture extract contained metabolites that were active against *P. infestans* (Stephan et al., 2005). Caution must be exerted when using biocontrol agents capable of producing metabolites with antibiotic activity *B. subtilis*, an ubiquitous bacteria, can produce antibiotic compounds (Romero-Tabarez et al., 2006) and little is known about the persistence of these molecules on plant products or in the environment.

In a similar study, commercial formulations of three well-known antagonist species *Trichoderma harzianum* (Plant Shield HC), *Gliocladium virens* (G41), and
*Bacillus subtilis* (Rhapsody AS) were tested for the control of late blight on tomatoes and petunias, under greenhouse conditions. Biocontrol agents were not effective in controlling late blight on either host, but on petunias, which is less susceptible to late blight than tomato, the results were more promising (Becktell *et al.*, 2005). These results highlight the potential of integrated management by adjusting biocontrol agent applications as a function of host resistance and environmental conditions.

### 2.7. Biocontrol formulations

Research on biological control formulation research is increasing. Previously, the field recital of biocontrol agents has frequently been random and too uneven for large-scale use. Even though the number of registered biocontrol agents has augmented, only a small quantity of the isolates those are appraised, make a successful conversion from the laboratory to the field. Two frequent themes in these negative results are due to lack of knowledge of the biological control system and complexity in obtaining a successful formulation (Emmert and Handelsman, 1999). A good formulation is the key to the profitable success of biocontrol agents.

Research efforts from both industry and academia for development of microbial products have been accelerated due to increased market demand for substitutes to chemical pesticides. A formulated microbial product is composed of a biocontrol agent including carrier material to improve its endurance and efficacy (Schisler *et al.*, 2004). Formulation of biocontrol agents can be used for following purposes (Jones and Burges, 1998). The four basic functions of formulations are:
- stabilize the organisms during production, distribution and storage;
- aid in the handling and application of the product;
- protect the agent from harmful environmental factors
- enhance the activity of the organism

From a technical efficiency point of view, thorough knowledge of the biocontrol organism, pathogen, environment and interactions with other organisms (e.g. host plant, soil microflora) is essential to develop an effective formulation. From a customer stance, there is a requirement to recognize frequent application practices and apparatus, as well as the needs of clients for formulation handling. It is significant to understand biology of the biocontrol agent and the target, and to extend an apparent vision of the necessities of a given produce prior to formulation research is in progress.

Potential adjuvant must be secure and satisfactory to regulatory agencies in all areas where the product will be used. The practice of a broad spectrum of specialists can be consulted to recognize formulation constituents and assist in the expansion of a suitable formulation. Even though most of this analysis will focus on microbial biocontrol of plant pathogens, equivalents can be drawn to formulation necessities of inoculants and microbial biocontrol agents for insect and weed control.

**Table 2.4. : Biocontrol formulations available against Late blight of tomato**

<table>
<thead>
<tr>
<th>Trade name</th>
<th>Organism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serenade</td>
<td><em>B. subtilis</em></td>
<td>Stephan <em>et al.</em>, 2005</td>
</tr>
<tr>
<td>Plant shield</td>
<td><em>T. harzianum</em></td>
<td>Becktell <em>et al.</em>, 2005</td>
</tr>
<tr>
<td>Rhapsody AS</td>
<td><em>B. subtilis</em></td>
<td>Becktell <em>et al.</em>, 2005</td>
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</table>
2.7.1. Understanding the biocontrol agent

Biocontrol agents include bacteria, fungi, actinomycetes, protozoa and viruses. To develop stable formulations, understanding the organism is very important. The altitude of complexity associated with formulation of bacteria differs with diverse bacterial genera. Usually bacteria that generate spores endure better than those that do not (McQuilken et al., 1998). Sporulating Gram-positive microorganisms, such as *Bacillus* and *Streptomyces*, offer heat- and desiccation resistant spores. As these spores can be readily formulated into stable products (Emmert and Handelsman, 1999), fermentation protocols should be designed to maximize the production of efficacious spores rather than vegetative cells (Schisler et al., 2004). Optimizing the media components to alter C : N ratio, pH, type and amount of carbon and nitrogen content, and growth conditions, such as temperature aeration and incubation time, can contribute to the production of resistant stable spores (Schisler et al., 2004).

While understanding the genus is important and can help a researcher design a stable formulation for each species, each strain has unique characteristics that will affect its stability (Jones and Burges, 1998). For example, the survival and degree of biocontrol of two binucleate *Rhizoctonia* strains at 4°C in a pest formulation was isolate-dependent (Honeycutt and Benson, 2001). The decline in viability of formulated BNR621 and P9023 over time reduced disease control efficacy. Honeycutt and Benson (2001) found that the efficacy of isolate BNR621 in reducing pre-emergence damping-off of impatiens was greater with fresh formulation than with material that had been stored for four to six months. However, formulations of P9023 did not decline in efficacy after six months of storage. As the information on producing, stabilizing, and/or drying the biomass of
any biocontrol agent is commonly strain specific, it follows that each strain or isolate requires a separate research effort (Schisler et al., 2004). Understanding the biocontrol agent can lead to innovative ways of improving the efficacy of the biocontrol product. If the spores of the biocontrol agent have an exogenous dormancy, it may be possible to improve the agent’s speed of action by a formulation that breaks that dormancy after application (Jones and Burges, 1998). Formulation of biocontrol agents cannot be separated from the fermentation process, which can influence the efficacy, stability and desiccation tolerance of many biocontrol agents. It is also important to understand the environmental conditions that favour the germination and growth of the biocontrol agent. Biological organisms are sensitive to the pH and moisture in their surroundings and often have preferred ranges for some functions. Understanding these preferred ranges and the effect of formulation ingredients on them can allow researchers to improve efficacy. These adjuvants would, therefore, have pH values that would not favour the germination of *M. ochracea* and would reduce the effectiveness by reducing its ability to colonize the leaf. However, when the adjuvant solutions were applied to leaves they were buffered to a pH of about 5 and the ability of the fungus to colonize plant tissue was not affected. A fungal pathogen, *Colletotrichum gloeosporioides* (Penzig) Penzig et Saccardo has the potential to be used as a biocontrol for sicklepod but requires an immediate and lengthy dew period. Using an appropriate adjuvant can help to overcome this limitation. The duration of dew that was required to provide at least 60% control of sicklepod seedlings was reduced from 24 to 4 h when either an invert emulsion or unrefined corn oil/Silwet L-77 emulsion was used.
2.7.2. Understanding the pathogen and the host

Understanding the pathogen and its relation to the host is an essential step in determining the characteristics of the formulation and in developing a research programme. The choice of formulation type, characteristics and components often depend on the biology of the pathogen. Products used for the control of soil-borne pathogens can be seed-applied or in furrow applications – the goal is to place the active ingredient in direct contact with the roots. Wakelin et al. (2002) tested Bacillus mycoides Flügge MW27 as seed treatment in granular and prill formulations applied to the soil to control pea root rot caused by Aphanomyces euteiches Drechs. Their results suggest that seed treatments controlled diseases that were active in the spermosphere, as indicated by increased plant stand; whereas, the prill formulations which were placed beneath the seed were more able to colonize the rhizosphere. Cummings et al. (2009), however, proposed that seed-applied agents are able to control soil-borne as well as seed-borne pathogens. Seed treatments are a convenient, generally inexpensive, and familiar application method for farmers but they may not be able to deliver enough propagules to allow the biocontrol agent to successfully colonize the root. Inconsistency in performance is often a problem in the biological control of seed and soil-borne diseases (Cummings et al., 2009). Specific treatments may be more effective under certain conditions. Consistency in performance of seed treatments can be affected by crop species and variety, product formulation, pathogen biology (seed-borne or soil-borne), and soil conditions. One of the difficulties in developing formulations for soil applications is the extremely heterogeneous nature of soils and the resulting unpredictable and often hostile environments for introduced organisms. Biocontrol organisms should be assessed on target crop species using a range of
commercially relevant cultivars, across a broad spectrum of crop production conditions under which the product might be used (Cummings et al., 2009).

Formulation ingredients registered for use in Canada are listed in the PMRA List of Formulants and are categorized based on the level of concern with respect to human health and the environment. These lists provide a guidance tool for industry for the selection of formulants. The lists are updated when new formulants are added or when ingredients are no longer found in registered pest control products. They are reclassified and moved to another list if new information is received or if the formulant is reassessed.

In the USA, formulation information can be found in Inert Ingredients Permitted in Pesticide Products (http://www2.epa.gov/pesticide-registration/pesticide-registration-manual-chapter-8-inert-ingredients). This details which Non-Food Inert Ingredients are permitted for use in pesticide products applied to non-food use sites, such as ornamental plants, highway right-of-ways, rodent control, etc., as well as Food-Use Inert Ingredients. Many food-use inert ingredients have usage limitations and restrictions. Regulatory costs play an important role as different organisms and adjuvants have varying regulatory requirements. Researchers must remember that each isolate in a formulation has its own registration cost. The formulation needs of the various organisms may not be compatible. Cost considerations, formulation compatibility and regulatory body consultations should occur at the front end of experimental planning. Any formulation component must be acceptable in the target geography. Adding nutrients to a formulation may appear to offer a benefit, as these nutrients could stimulate the germination and growth of the biocontrol agent; however, care is required because these nutrients may also stimulate the pathogen and lead to crop
damage (Cook and Baker, 1983). In a greenhouse study of pre- and post-emergence damping-off, Cummings et al. (2009) discovered that drench treatments with several commercial biofungicides increased damping-off compared with other treatments, as did the compost tea drench in one trial. They hypothesized that the drench treatments may have exacerbated disease development because the increased moisture in the potting medium promoted the development of *P. ultimum*, which thrives under wet soil conditions (Cummings et al., 2009).

2.7.3. Understanding the environment and its effect on the biocontrol agent

Formulations must be developed that ensure biocontrol agents will be present in concentrations high enough to give effective and consistent control of the target disease. The organisms must therefore remain viable during the various conditions encountered throughout production, transportation, storage and application. Generally the biocontrol agent is produced, formulated, packaged and held in the producer’s warehouse until it is shipped to retailers. The warehouse can be adjusted for the optimum conditions to maintain a high concentration of viable cells. However, when the product leaves the warehouse, varying conditions in transit and storage until application will affect the stability of the product. Some global storage areas may reach 40°C, and formulation/product viability at these temperatures is vital to ensuring the efficacy of the biocontrol agent (Jones and Burges, 1998). Defining a limited range of storage conditions for the distribution channel (e.g. store only under refrigeration) may limit market penetration. Improved packaging can aid viability by limiting exposure of the formulation to fluctuations in moisture and oxygen. Fluctuating conditions may be as harmful as exposure to extreme temperatures. Multiple freeze-thaw cycles or other strong fluctuations in temperature can damage or destroy microbial cells and disrupt cell
aggregates (Sawicka et al., 2010). The next challenge to maintaining optimum
titres of viable spores occurs during application of the biocontrol agent. Biocontrol
agents must be formulated so that the organisms are viable when used in standard
farm equipment and remain alive to colonize the roots or leaves of the plant.
Awareness of the physical and chemical environment on the application target is
important as this dictates the choice of wetting agent (surfactant) and/or sticker
used in the formulation (Schisler et al., 2004). It can be impractical to produce a
range of fully formulated products that meet the needs of all situations experienced
during application. A few considerations are rain fastness, leaf wetting and soil
penetration. Often the best strategy is to produce a formulation compatible with the
range of standard adjuvants available to end users, and develop knowledge on the
efficacy of the formulation with those adjuvants across a range of control
requirements. Formulations applied to seed must remain viable on the seed, often
in conjunction with chemical pesticides. This allows the biocontrol organism to
germinate, multiply and colonize the rhizosphere. Seeds create a unique
environment (spermosphere), and seed exudates may be harmful to seedapplied
biocontrol agents.

2.7.4. Understanding the customer

Understanding customer need is a key factor in developing a commercially
successful biocontrol agent. Application of the biocontrol agent must fit in
conventional agricultural practices of farmers. The product must be compatible
with their machinery (Fravel et al., 1998). For instance, many farmers are
switching from high volume/low pressure sprayers to low volume/high pressure
sprayers. Using lower water volumes has many benefits, including reducing run-
off, biofungicide waste and applicator exposure. It also allows for a shorter application time as fewer tanks of water are used.

Formulation research for any biocontrol must be viewed as an ongoing challenge; larger farming operations with adoption of new practices and equipment may require the development of new formulations. Changes to the scale and planting equipment used by large North American soybean farmers have forced inoculant manufacturers to develop liquid formulations as alternatives to peat-based inoculants. Peat-based inoculants can be too cumbersome for large-scale field application and tend to plug precision air seeders. Awareness of regional differences is critical: many pea/lentil growers in Canada use peat-based, seed applied inoculants; United States growers prefer liquid inoculants. Producer preferences vary widely so having several formulations available is the best solution. Inoculant manufacturers routinely offer peat, liquid and granular formulations for the same active ingredient. It is important to understand all of a farmer’s practices and inputs for any given crop – a biocontrol may need to be applied with different organisms or with chemical pesticides (McQuilken et al., 1998). At a minimum, compatibility of the biocontrol agent with other chemicals must be determined and inoculant manufacturers can provide direction for chemical compatibility of seed-applied biocontrols. A method mimicking field application should be used when testing compatibility of the biocontrol agent with agrochemicals or other biologicals in laboratory bioassays.

Customers want products that are easy to use and biocontrol producers must test all their formulations using methods that simulate conditions on the farm. Test products can be given to growers who then provide feedback on the ease of application, as well as field performance of the product. For example, formulation
additives can prevent conidia from forming dry aggregates, which leads to mixtures that settle when added to water. As a consequence, this can obstruct nozzles in spray tanks (Sabuquillo et al., 2005) and produce irregular distribution or erratic control of applications. At this point, regulatory aspects of the formulation should be considered; liquid formulation is often preferred over dust formulations, especially in situations where allergenicity is a concern. Formulation ingredients can be used to alleviate some of the dusting problems. Many fungal biocontrol agents such as *Penicillium oxalicum* Currie and Thom (especially the dry conidia) are hydrophobic and do not disperse easily when mixed with water. For *P. oxalicum* and other fungi with hydrophobic spores it is essential to: enhance conidial dispersal in water; decrease dust hazard; reduce settling of conidia and aggregate formation; and thereby improve efficacy of root drenches (Sabuquillo et al., 2005). Adjuvants such as 1.5% sodium alginate, 60% sucrose, 60% D-sorbitol, 60% fructose and 20% PEG 8000 improved the dispersal of *P. oxalicum* when added to the formulation before drying the conidia. Only detergent solutions such as 1% Triton X-100 and 10% Agral improved dispersal once the conidia were dried (Sabuquillo et al., 2005). Understanding the financial constraints of the customer is also important. Some formulations that are available cannot be commercialized due to cost limitations. Choice of formulation also depends on the customer’s budget. Farmers with a high-value crop can opt for more expensive formulations than those with large acreages of a low-value crop. Understanding the cost of formulations and their impact on product acceptance is complex. Unfortunately, there are few examples to follow as literature references rarely incorporate a cost analysis (Jones and Burges, 1998). Market specialists evaluate the impact of a pest and estimate the value of a biocontrol product so researchers
can determine which additives could be incorporated without making the product too expensive to produce and use.

2.7.5. The research plan

Key steps can be followed in developing a concept of the formulation(s) that will have potential as a commercial biocontrol agent. Consideration includes the analysis of the biocontrol agent, the pathogen, the host plant and its environment, and the needs of the customer. This concept can then be a starting point for the development of a comprehensive research plan. The plan can identify possible formulation ingredients to increase stability, improve ease of application, improve the biocontrol agent’s resistance to environmental stresses, or increase the efficacy or consistency of the product. Researchers should not limit themselves to literature on biological control but also use the formulation experience of the inoculant, pesticide, food, pharmaceutical and cosmetics industries. These other industries can provide valuable information on the activity of formulation ingredients (Schisler et al., 2004). Emmert and Handelsman (1999) point out the substantial base of industrial experience with Bacillus and Streptomyces spp. that has been used for industrial enzyme and antibiotic production. Ingredients from the food industry may be more appropriate for biological formulations than those developed for chemical pesticides (Connick et al., 1991). The sclerotia of R. solani causing sheath blight in rice float to the surface and attach to the rice stems. The biocontrol formulation developed was able to float on the water surface, disperse into the rice hills, and remain floating in the rice hill to provide protection against sclerotial infection. Any formulation ingredient must be safe for all those producing and using the product. The complexity of formulation research and the need for product efficacy under a wide range of conditions calls for a thorough understanding, from
literature and experience, of the variety of interactions occurring among the biocontrol agent, formulation components and environmental conditions. This understanding can help in the selection, and therefore elimination, of many ingredients, thereby simplifying any optimization of the formulation. Often, experimental design procedures are used to identify the optimal formulation parameters when, for example, the interaction between components of the formulation and the surface to which it is applied is unknown. Sarais et al. (2009) used these procedures to determine the persistence and distribution of one commercial and two experimental limonoid formulations on the peach surface. They were able to uncover a mechanism by which surfactants improve penetration into the epicuticular wax layer and leave fewer residues on the surface. A fundamental understanding of the biology of the organisms must be used to select the parameters that will be tested and aid in the interpretation of results. Consequently, combining thorough understanding and experimental design will make formulation design less complex. This increases the chance of a successful outcome, and furthers the improvement and optimization of a formulation.

Developing effective formulations is a complex process. Formulating biological control agents should be done with a team that combines:

- Marketing personnel to understand the needs of the customer and agronomists
- Pathologists to understand interactions among host, pathogen and environment.
- Microbiologists to understand the physiology of the control agent.
- Chemists to understand the properties of formulation ingredients.
- Statisticians to help in the design and analysis of experiments.