Chapter 5

Discussion
DISCUSSION

Survey of Aligarh district revealed that 91.4% tomato fields were found to be infected with root-knot nematode *Meloidogyne* spp. and bacteria *Ralstonia solanacearum* and *Xanthomonas campestris* pv. *vesicatoria*. About 32.8% tomato plants were found concomitantly infected with all three pathogens. Moreover, 26% plants were found concomitantly infected with *Meloidogyne* spp. and *R. solanacearum* causing a bacterial wilt disease complex, while 9.4% plants were infected with *Meloidogyne* spp. plus *X. campestris* pv. *vesicatoria* causing a bacterial spot disease complex. Only 2.8% plants were found to be infected with *R. solanacearum* along with *X. campestris* pv. *vesicatoria*. Among *Meloidogyne* spp., *M. javanica* was found as the most dominant species with 49.50% frequency of occurrence followed by *M. incognita* (44.53%) and *M. arenaria* (5.97%). The dominance of *M. javanica* on tomato in Aligarh district has already been reported (Khan et al., 1984). Plants infected with these three pathogens were very poor in growth, showing galling and bacterial wilt and spot disease symptoms. Chlorosis and nutrient deficiency symptoms were also visible on their aerial parts. The synergistic interaction between these three pathogens cause severe damage to this important crop and is a major constraint in its successful cultivation. Therefore, attempts were made to isolate AM fungi and other phosphate solubilizing microorganisms from these soils for the management of bacterial wilt and spot disease complex of tomato.

Spore population in soil and percent colonization of tomato roots by these AM fungi were also assessed in the localities surveyed. Most of the plants from the fields were found mycorrhizal. Five species of AM fungi belonging to 3 genera were encountered in the survey with 66.2% frequency of occurrence. *Glomus intraradices* was predominant species with 32.0% frequency of occurrence followed by *G. fasciculatum* (20.4% frequency). The remaining 13.8% frequency of occurrence was shared by *Gigaspora* sp., *Glomus* sp. and *Scutellospora* sp. Akhtar (2007) also found *Glomus* as a predominant species in the survey of chickpea fields in Aligarh district. AM fungi are found to occur on the roots of the most of food, horticultural crops and tropical trees (Bagyaraj, 1992).

Seven isolates of *Bacillus* and *Pseudomonas* were isolated from the pathogen suppressive soils of tomato fields. These isolates were tested for their effect on
hatching and penetration of *M. javanica*. Root colonization and antibacterial activity of these isolates were also assessed. *P. fluorescens, P. putida* and *Bacillus subtilis* had maximum inhibitory effect on hatching and penetration of *M. javanica*, caused greater colonization of tomato roots and also showed antibacterial activity. Isolates having aggressive root colonization and inhibitory effect on hatching and penetration of nematodes are known to suppress diseases (Siddiqui et al., 2007). *P. fluorescens* followed by *P. putida* also caused greater IAA production, HCN production and increase in seedling growth. *Bacillus subtilis* has not shown HCN production but caused greater phosphate solubilization followed by *P. fluorescens* and *P. putida*. Therefore, one species of AM fungi (*Glamus intraradices*) and 3 species of bacteria collected from tomato fields (*Pseudomonas fluorescens, P. putida* and *Bacillus subtilis*) and 2 fungi obtained from culture collections (*Aspergillus awamori* and *A. niger*) were selected for the management of wilt and leaf spot disease complex of tomato under pot condition.

Pathogenicity test was conducted using different inoculum levels of all three tested pathogens (*Meloidogyne javanica, R. solanacearum* and *X. campestris pv. vescicatoria*) to determine the threshold inoculum of each pathogen. The effect of different inoculum levels of *Meloidogyne* spp. on tomato were earlier studied by several workers (Ogunfowora, 1977; Nassar and Mustafa, 1981; Stephan, 1983; Das and Sukul, 1984; Chindo and Khan, 1988; Daiber, 1990; Wonang and Akueshi, 1990; Hashmi et al., 1994; Fortnum et al., 1997; Lopez-Perez et al., 2006; El-Sherif et al., 2007; Olabiyi, 2008) but their findings have been at variance.

Das and Sukul (1984) found 1000 larvae / pot of *M. incognita* as damaging threshold level on tomato. Wonang and Akueshi (1990) reported 2000 eggs of *M. incognita* / pot as damaging threshold level on tomato. Similarly, 10.6 % yield loss of tomato at 1000-2000 larvae / kg soil of *M. incognita* race-1 was reported by Chindo and Khan (1988). Safiuddin et al., (2012) recorded 1000 J$_2$ / kg soil of *M. incognita* race-2 as threshold level. Khan et al., (2004) studied pathogenic effect of *M. javanica* on bitter gourd (*Momordica charantia*) and found 2000 J$_2$ / kg soil as damaging threshold level. My finding is in conformity with those of Wonang and Akueshi (1990) and Khan et al., (2004). The differences observed in damaging threshold levels by different workers can be attributed to the differences in experimental conditions, the cultivars used or the species and races of the nematode involved.
There was increase in the number of galls and final nematode population with the increase of inoculum levels as earlier reported by Stephan (1983); Kaul and Sethi (1982); Mani and Sethi (1984) on tomato, maize and chickpea respectively. Nematode population was found density dependent as also reported earlier (Chapman, 1959; Seinhorst, 1960; Oostenbrink, 1966; Dhawan and Sethi, 1976; Gupta and Yadav, 1979; Dhruj and Vaishnav, 1981; Salem and Eissa, 1981; Mishra and Gaur, 1981; Thakar and Yadav, 1985). The reduction of nematode reproduction rate with increasing nematode inoculum density has been reported for infections of several crops (Di Vito et al., 1986, 2004). The maximum multiplication at low inoculum level might have been due to less competition for food and space than at high inoculum level.

Increasing inoculum of *R. solanacearum* and *X. campestris* pv. *vesicatoria* caused increased bacterial wilt and bacterial spot indices and the resultant decrease in plant growth parameters. On the basis of my findings, it has therefore, been concluded that the damaging threshold levels of *M. javanica*, *R. solanacearum* and *X. campestris* pv. *vesicatoria* were respectively 2000 second stage juveniles of *M. javanica* and 10 ml culture of *R. solanacearum* / *X. campestris* pv. *vesicatoria*.

In another experiment, results showed that inoculation of test pathogens in combinations caused greater damage to plant growth parameters than caused by individual inoculation. Various combinations of variable inoculum of test pathogens caused decrease in plant growth parameters. Reduction in plant growth was directly proportional to the increase in inoculum of test pathogens. On the basis of my findings, the effect of interaction of test pathogens on plant growth was synergistic. Synergistic effects of nematode and bacterial interactions have been reported by other also (Sitaramaiah and Sinha, 1984a, 1984b; Vrain and Copeman, 1987; Swain et al., 1987; Chindo et al., 1991; Pathak et al., 1999; Stansbury et al., 2001; Rubio-Cabetas et al., 2001; Partridge, 2008; Mallesh et al., 2009). Inoculation of nematodes with plant pathogenic bacteria increased disease severity by pre-disposing plants to pathogenic bacteria. Wilt-inducing bacteria mainly depend on wounds as an infection court (Goodman et al., 1967) and these wounds are provided by nematodes feeding on roots (Sitaramaiah and Sinha, 1984a, 1984b). Similarly, inoculation of nematodes with *X. campestris* pv. *vesicatoria* caused less damage to tomato growth than caused by wilt inducing bacteria. This is possible because individually *X. campestris* pv.
*vesicatoria* is less pathogenic to tomato than *R. solanacearum*. Moreover, inoculation of both pathogenic bacteria together caused less damage to tomato growth than either of these bacteria was inoculated with nematodes. Nematodes aggravated bacterial disease by wounding the roots and allow bacteria to enter the plant because bacteria are less adapted for penetrating the host’s epidermis (Pitcher, 1965). Moreover, inoculations of both bacteria together also had inhibitory effect on each other and were unable to aggravate damage. Similarly, when nematode was inoculated with both *X. campestris* pv. *vesicatoria* and *R. solanacearum*, root-knot nematode induced physiological and / or biochemical changes in hosts. Modifications in the substrate due to nematode infestation provide an advantage to both bacterial pathogens. Creation of an infection court is one way in which nematode modifies a host to enhance infection by additional pathogens. Changes in biochemistry of the host are probably the most important factors favoring disease complexes involving nematodes (Slack, 1963).

My results also showed that *R. solanacearum* and *X. campestris* pv. *vesicatoria* adversely affected multiplication of *M. javanica*. Adverse effect of bacteria on nematode multiplication as observed in the present findings has also been observed by others (Lucas *et al.*, 1955; Johnson and Powell, 1969). The contents of giant cells degenerated following bacterial invasion, leaving virtually empty cells resulting into the death of root-knot nematodes. Similar to my findings, Swain *et al.*, (1987) observed inhibitory effect of *R. solanacearum* on *M. incognita*. Inoculation of *M. javanica* alone produces more galls and egg-masses compared to its association with *R. solanacearum*. It may be due to the reason that establishment of the bacteria induces certain changes in root system which are not favorable for nematodes (Bhagawati *et al.*, 1996; Hazarika, 2003; Hussain and Bora, 2009).

Interaction of root-knot nematode *M. javanica* with *R. solanacearum* and *X. campestris* pv. *vesicatoria* on tomato causes a disease complex under field conditions. Inoculation of these pathogens alone caused a significant reduction in plant growth over control. Root-knot nematode *M. javanica* have evolved strategies to induce feeding cell formation in many plants and in tomato also, probably by manipulating fundamental elements of plant cell development (Caillaud *et al.*, 2008) and caused significant yield loss on tomato (Reddy, 1985). Moreover, *X. campestris* pv. *vesicatoria* can severely devitalize plant by defoliation and it reduces yield and
quality of harvested fruit. Similarly, *R. solanacearum* had typical symptoms on the inoculated seedlings and by 4 weeks all plants show severe symptoms. Inoculations of these pathogens in combinations caused greater damage to tomato than that caused by individual inoculations but the effects of pathogens on the plant metabolism were not studied. Interactions between these pathogens may have both direct and indirect effects on disease severity. The direct effect includes the physical interactions of pathogens in the rhizosphere and also occupancy of same infection site inside the root. The direct interactions of pathogens inside host plant at same infection site generally had antagonistic effect on pathogen multiplication. On the other hand, indirect effects of interactions via plant response such as breaking of disease resistance and modification of host substrate had synergistic effect on disease severity. Plant parasitic nematodes cause physical damage that can allow secondary infection by other pathogens (Pitcher, 1963, 1965; Sitaramaiah and Pathak, 1993). Endoparasitic nematodes, such as *Meloidogyne* spp., wound roots and allow bacteria to become established (Stewart and Schindler, 1956).

Wounds created by nematodes apparently favor bacteria more than fungi, because bacteria are less adapted for penetrating the host epidermis (Pitcher, 1965). Disease symptoms similar to those which occur in nematode-bacteria wilt interactions were stimulated by substituting mechanical injury for nematode feeding (Libman et al., 1964; Lucas et al., 1955). When roots of tomato plants were mechanically injured by needle in the laboratory test and plants were inoculated with *R. solanacearum*, exhibited disease symptoms similar to those which occur in nematode-bacterial wilt interactions. Wounds created by nematodes leak nutrients allowing bacteria to multiply in the lesions and in the rhizosphere (Kurppa and Vrain, 1985). This was observed by the fact that rhizosphere soils revealed the presence of higher population of bacteria compared to non-rhizosphere soil. Moreover, root-knot nematode induces physiological and / or biochemical changes in hosts. These possible changes need additional study so that the interaction between the pathogens and host can be better understood. In this study, when inoculation with bacteria occurred prior to inoculation with nematodes, damage to plant growth was less than with simultaneous inoculations. This, in part, may be due to production of toxins by bacteria which adversely affected nematodes (Pitcher, 1963) or bacteria could not infect the roots effectively without the infection courts made by the nematodes.
Inoculation of bacterial pathogens with nematodes reduced nematode numbers. Adverse effects on nematodes may be due to these pathogens competing for the same host substrate. The unfavorable effect of bacteria on nematodes may be due to destruction of feeding sites reducing nutrition for nematodes which was observed on tobacco plant in Grainville wilt and root-knot nematode interaction (Lucas et al., 1955). Bacteria induce changes in root system which are not favorable for nematodes therefore galling and nematode multiplication was less in the presence of *R. solanacearum* and *X. campestris* pv. *vesicatoria*. Generally, bacteria had adverse effect on nematode multiplication but inoculation of nematodes with plant pathogenic bacteria increase disease severity by predisposing plants to pathogenic bacteria.

Chitosan exhibits a variety of antimicrobial activities. Its activity depends on the type of chitosan (native or modified), its degree of polymerization (oligomeric, polymeric), the host, the chemical and/or nutrient composition of the substrates, and environmental conditions (El Hadrami et al., 2010). Chitosan is reported to inhibit the growth of a wide range of bacteria. It is reasonable to hypothesize that application of chitosan could have inhibited the growth of *R. solanacearum* and *X. campestris* pv. *vesicatoria*. Similar effect of chitosan is possible on the *Meloidogyne* juveniles because laboratory test suggests that chitosan causes mortality of *Meloidogyne* juveniles *in vitro*, as has been done with insects (El Hadrami et al., 2010). Application of chitosan induces host defense responses in both monocotyledons and dicotyledons. These responses include lignification (Barber et al., 1989), ion flux variations, cytoplasmic acidification, membrane depolarization and protein phosphorylation (Felix et al., 1993, 1998; Kikuyama et al., 1997; Kuchitsu et al., 1997), chitinase and glucanase activation (Roby et al., 1987; Kaku et al., 1997) phytoalexin biosynthesis (Ren and West, 1992; Yamada et al., 1993) generation of reactive oxygen species (Kuchitsu et al., 1995) biosynthesis of jasmonic acid (Nojiri et al., 1996) and the expression of unique early responsive and defense-related genes (Minami et al., 1996; Nishizawa et al., 1999; Takai et al., 2001). In addition, chitosan was reported to induce callose formation (Köhle et al., 1985; Conrath et al., 1989), proteinase inhibitors (Walker-Simmons and Ryan, 1984), and phytoalexin biosynthesis (Hadwiger and Beckman, 1980) in many dicot species. Biocontrol of this disease complex of tomato achieved by application of chitosan can be attributed to above mentioned reasons.
Glomus intraradices improve plant growth of nematode infected plants by reducing nematode multiplication like other AM fungi (Bagyaraj et al., 1979). The wilt and leaf spot index of \textit{R. solanacearum} and \textit{X. campestris} pv. \textit{vesicatoria} inoculated plants was also reduced by \textit{G. intraradices} in the present study. Bodker et al., (1998) observed a reduction in root-rot of pea caused by \textit{Aphanomyces euteiches}, while Akköprü and Demir (2005) observed about 17 \% reduction in Fusarium wilt of tomato by inoculation of plants with \textit{G. intraradices}. In the present study, we presumed that disease inhibition by \textit{G. intraradices} might not be solely related to an increase in nutrient uptake mainly of phosphorus. Beside the plant nutrient uptake, the changes in the root system, mycorrhizosphere effect and activation of plant defense mechanisms are responsible for disease inhibition by \textit{G. intraradices} (Linderman, 1994; Demir and Akköprü, 2005). Moreover, treatment with \textit{Glomus} sp. is also reported to increase phenylalanine and serine in tomato roots (Suresh, 1980) and these amino acids have an inhibitory effect on nematodes (Reddy, 1974).

\textit{Pseudomonas putida} solubilizes chemically fixed soil phosphorus, rock phosphate, mineralizes organic phosphorus to soluble form by enzyme activity (Tilak, 1991) and increases yield of crops (Gaur, 1985). Increased phosphorus is known to improve plant growth of nematode infected plants by reducing their multiplication (Pant et al., 1983). \textit{P. putida} has been used in the biocontrol of \textit{Erwinia} spp. and \textit{Fusarium oxysporum} f. sp. \textit{cucumerinum} (Colyer and Mount, 1984; Simeoni et al., 1987). In addition, phosphorus is also useful in enhancing root growth and increasing disease tolerance (Hussey and Roncadori, 1982). Moreover, \textit{Pseudomonas} can also synthesize enzymes which may modulate the plant hormone levels, limit the available iron by production of siderophores and can also kill the pathogen by producing antibiotics (Siddiqi, 2006). An induced systemic resistance by \textit{Pseudomonas} is also considered as a mechanism for the biocontrol of plant pathogens (Wei et al., 1996). \textit{P. putida} was found to significantly reduce disease severity of bacterial spot in sweet pepper and bacterial wilt of tomato (Tsai et al., 2004; Jagadeesh, 2000).

\textit{Aspergillus} species are common, occur mainly in soils of warmer climates, compost, decaying plant material and stored grains (Domsch et al., 1980). \textit{A. niger} isolated from the rhizosphere of crop plants produced a number of secondary metabolites (Siddiqui et al., 2004). Moreover, \textit{A. niger} inhibits egg hatching, indicate the involvement of mechanisms other than parasitism (Eapen et al., 2005). This
species was not isolated from eggs or female nematodes, hence their effect is
exogenous. Moreover, enzymatic disintegration of the vitelline and chitin layers of the
nematode eggshell might have increased the permeability of the eggshell and
enhanced mycelial penetration, leading to total disintegration of the egg contents
(Eapen et al., 2005). Some Aspergillus species have also been reported for their
biocontrol potential against root-knot nematodes (Siddiqui et al., 2001).

Chitosan and P. putida when inoculated together improved plant growth better
and reduced nematode multiplication more than each inoculated alone, due to
combined mechanism of action. Chitosan is known to have eliciting activities leading
to a variety of defense responses in host plants. Based on these properties chitosan
strengthen host plant defense and reduce the negative impact of diseases. Combined
application of P. putida and chitosan, resulted in greater root colonization by P.
putida than individual application, which may be a reason for better plant growth. It is
important that chitosan showed no adverse effect on P. putida in combined treatments
unlike R. solanacearum and X. campestris pv. vesicatoria where it had adverse effect.
Reduced disease intensity in combined application of chitosan with P. putida was also
observed. The present study demonstrates that chitosan and P. putida may be used
concomitantly without exhibiting adverse effects on each other. The present study
also suggests that mixtures of biocontrol agents may protect better against a broader
range of pathogens. Mixtures of microorganisms may increase the genetic diversity of
biocontrol systems that may persist longer in the rhizosphere and utilize a wider array
of biocontrol mechanisms (Pierson and Weller, 1994).

Several B. subtilis strains have been successfully employed in pest and disease
management programmes (Bais et al., 2004; Stein, 2005; Nagorska et al., 2007;
Ongena and Jacques, 2008; Wulff et al., 2002b; Lemessa and Zeller, 2007; Ji et al.,
2008; Chen et al., 2009a, 2009b). In this work, I selected a strain of B. subtilis that
demonstrated strong biocontrol activities towards a bacterial pathogen R.
solanacearum in vitro assay. Some investigations provided evidence that production
of antimicrobial agents, biofilm formation and triggering of host systemic resistance
contribute to the biocontrol activities of B. subtilis (Bais et al., 2004; Nagorska et al.,
2007; Ongena et al., 2007). To better understand the interactions between
rhizobacteria and the tomato root surface, we estimated root colonization by B.
subtilis and P. fluorescens and found that P. fluorescens colonized tomato roots more
as compared to \textit{B. subtilis}. Study with CLSM or even phase-contrast microscopy suggests that tomato root surfaces are likely negatively charged (Chen \textit{et al.}, 2013). Since it is well-known that the surface of \textit{B. subtilis} cells (and many other gram-positive bacteria) are negatively charged (Weidenmaier and Peschel, 2008) one would predict that the electrical repulsion between \textit{B. subtilis} cells and the tomato root surfaces may prevent bacterial cell colonization. Therefore \textit{B. subtilis} colonized tomato roots less compared to \textit{P. fluorescens} which are gram-negative.

\textit{Pseudomonas} spp. commonly inhabits in soil and has been applied for biocontrol, promoting plant growth and bioremediation. 2, 4-diacyethylphloroglucinol (DAPG)-producing strains are major groups in biocontrol microorganisms, because of their easy colonization, good competition and broad antimicrobial spectrum. For example, \textit{P. fluorescens} F113 could inhibit \textit{Erwinia carotovora}, which is the agent of soft rot of potato (Cronin \textit{et al.}, 1997). It has been also reported that \textit{P. fluorescens} and 2, 4-diacyethylphloroglucinol (DAPG) that it produced could prevent \textit{Fusarium oxysporum}, \textit{Septoria tritici}, \textit{Thielaviopsis basicola}, \textit{Rhizoctonia solani} etc. (Bangera and Thomashow, 1996; Keel \textit{et al.}, 1992). \textit{P. fluorescens} 2P24 has strong inhibitory effect on \textit{R. solanacearum}, \textit{F. oxysporum} and \textit{R. solani} (Wei \textit{et al.}, 2004). The root colonization and biocontrol mechanism of it have been studied (Wei and Zhang, 2006; Yan \textit{et al.}, 2009; Wu \textit{et al.}, 2010) and it has been commercialized. \textit{Pseudomonas fluorescens} is also known to induce resistance against plant pathogens (Wei \textit{et al.}, 1996).

Inoculation of \textit{A. awamori} produced phenyl ethanol, phenyl acetic acid and phenoxy acetic acid (Nair and Burke, 1988) which may suppress \textit{Fusarium} spp., \textit{Sclerotium} spp., \textit{Phytophthora} spp. etc. (Palakshappa \textit{et al.}, 1989). \textit{A. awamori} apparently do not parasitize nematodes but enzymatic disintegration of the vitelline and chitin layers of the nematode eggshell may lead to total disintegration of the egg contents, therefore reduced galling and nematode multiplication.

When \textit{B. subtilis} and \textit{P. fluorescens} were inoculated together improved plant growth better and reduced nematode multiplication more than each inoculated alone. Selected strain of \textit{B. subtilis} demonstrated strong biocontrol activities towards \textit{R. solanacearum} in \textit{in vitro}. In addition, production of antimicrobial agents, and triggering of host systemic resistance contributed to the biocontrol activities of \textit{B. subtilis}. Combined application of \textit{P. fluorescens} and \textit{B. subtilis} resulted in greater
biocontrol efficacy than individual application, which may be a reason for better plant growth. The present study demonstrates that *P. fluorescens* and *B. subtilis* may be used concomitantly without exhibiting adverse effects on each other. Combinations of these biocontrol agents may increase plant growth and combat the adverse effect of plant pathogens.

Application of chitosan has broader role in disease control as discussed in previous paragraphs. The use of organic fertilizers results in several benefits, such as better soil structure, build-up of antagonistic organisms, increased supply of plant nutrients, and a more suitable medium for plant growth (Southey, 1978). The combined use of organic fertilizer with chitosan resulted in better plant growth. This in turn probably inhibited pathogen proliferation and consequently improved plant growth. Nutrient contents (NPK) were greatest in poultry manure, followed by goat dung, horse dung, and cow dung, and the better growth of tomato and greater reduction in nematode multiplication may be related to nutrient contents (Siddiqui et al., 2001; Siddiqui, 2004).

Chitosan and poultry manure when inoculated together improved plant growth better and reduced pathogens more than each inoculated alone due to combined mechanism of action. Reduced disease intensity in combined application of chitosan with poultry manure was observed which demonstrated that these two may be used concomitantly for the management of wilt-leaf spot disease complex of tomato. The present study also suggests that mixtures of chitosan and poultry manure may protect better against a broader range of pathogens.

*Pseudomonas putida* has been used in the biocontrol of many plant diseases and its role in the biocontrol of disease has been discussed in earlier paragraphs. The use of organic amendments also results in several benefits, as discussed earlier. The combined use of composted plant straw with *P. putida* resulted in better plant growth.

*Pseudomonas putida* and *Pennisetum typhoides* straw when inoculated together improved plant growth better and reduced pathogens more than each inoculated alone. *P. putida* colonized tomato roots better in *P. typhoides* amended soil which resulted in better protection of plants from pathogens thereby better plant growth. On other hand *P. typhoides* increased supply of plant nutrients, and provide more suitable medium for plant growth (Southey, 1978) and inhibit pathogen proliferation (Siddiqui, 2004). The present study also suggests that mixtures of *P.
*putida* and *P. typhoides* may protect better against diseases caused by multiple pathogens.

In future studies, therefore, more detailed investigations of the relationships and interactions between the microorganisms and the host plant are needed for developing biocontrol of related diseases.

The present study also suggests that greater emphasis on the development of mixtures of biocontrol agents is needed, because they may better adapt to the environmental changes that occur throughout the growing season and protect against a broader range of pathogens.