Summary
VI. SUMMARY

Capsicum (Bell pepper, *Capsicum annuum* L.) and egg-plant (Brinjal, *Solanum melongena* L.) are the important vegetable crops after potato and tomato belonging to the family solanaceae. In India, capsicum is grown in 4,783ha and production is 42,230 tones (Madhavi Reddy, 2003). Egg-plant area coverage is 2,99,770 ha and production is 31,24,487 tones (Sadashiva, 2003). Both capsicum and egg-plant are prone to many soil borne diseases among which the bacterial wilt (*Ralstonia solanacearum*) in combination with root-knot nematode (*Meloidogyne incognita*) takes heavy toll every year all over the world.

In this present investigations the special emphasis is given for molecular studies of a nematophagous fungus *Pochonia chlamydosporia* Zare (*Verticillium chlamydosporium* Goddarad) and molecular characterization of a broad spectrum bacterial bio-control agent *Pseudomonas fluorescens* thus achieving the integrated wilt disease complex management.

The fungus *P. chlamydosporia* Goddarad is found in soil and can colonize roots of certain plants. Nematophagous isolates of the fungus proliferate on the egg masses of pathogenic root-knot nematodes, where they form robust chlamydyospores. This fungus is a potential bio-control agent that destroys nematode life cycle.

To understand the interaction between *P. chlamydosporia* and its plant and nematode hosts, methods for *in situ* visualization of fungus are needed. This is possible by clubbing the two approaches viz, isolation of the filamentous fungi *P.
*chlamydosporia* using SSM followed by the polymerase chain reaction (PCR) based detection of marker gene (β-tubulin).

Successful isolation of a nematophagous fungus *Pochonia chlamydosporia* was done on semi-selective medium. Four different strains were isolated and numbered as

Vc-1, Vc-2, Vc-3 and Vc-4. Fungus produced verticillate nature of mycelium, which bears conidia and chlamydospores. The chlamydospores were visible after 21 days of fungus inoculation on corn meal agar medium.

Chlamydospore production by the different isolates of *P. chlamydosporia* confirmed that the isolated filamentous fungus was *P. chlamydosporia*. Isolate Vc-3 produced maximum number of chlamydospores (13x10⁸/g substrate) followed by Vc-1, Vc-2 and Vc-4 (i.e. 8x10⁸, 7.2 x10⁸ and 4.2 x10⁸ chlamydospores/g of substrate respectively).

The genomic DNA was successfully isolated using the modified CTAB method. DNA isolated from 2g of one week old culture of *P. chlamydosporia* yielded good quality of high molecular weight DNA and visualized by agarose gel electrophoresis. Further the purity of DNA was tested spectrophotometrically and by running on 1% agarose gel.

PCR was carried out to detect β-tubulin gene using the specifically designed primers. The gel picture analysis revealed that the PCR product obtained was of 270bp, which clearly indicated the detection of β-tubulin gene. β-tubulin, a marker gene exerts resistance against benzimidazole compounds which gives scope for the
easy isolation of the filamentous fungus *P. chlamydosporia* using selective marker fungicide and targeting marker gene (β-tubulin) using specific primers. In all four strains of *P. chlamydosporia*, the PCR product of 270 bp was obtained on 1% agarose gel. The sensitivity of PCR was tested, taking the DNA samples of *Paecilomyces lilacinus* and *Beauveria bassiana* using the tub1f and tub1r primer sets that were tested against *P. chlamydosporia*. There was no amplification of β-tubulin gene (270bp) in *P. lilacinus* and *B. bassiana*.

The 270bp region of β-tubulin gene was cloned and sequenced using the p-Drive vector. Sequencing was done with M13 forward and reverse primer. Using these sequences of Vc-1 and Vc-3 analysis was carried out. Blast result indicated that this sequence is β-tubulin gene, which has got 99% identity with *P. chlamydosporia* (AJ012713) and 92% identity with *Epichloe typhina* tubulinB gene (X52616). This tells about the presence of β-tubulin gene in *P. chlamydosporia*. β-tubulin of present study was similar and grouped under *Pochonia* (*Verticillium* (AJ012713)), thereby confirming the existence of tubulin gene, which is common in *Verticillium* spp.

In *Pochonia chlamydosporia* isolate Vc-1, there was glutamic acid (GAG) at position 162 and 180 codon region and in Vc-3 the glutamic acid was present at the codon region of 120 and 162. The role of β-tubulin gene is very important as far as bio-control agent and fungicides are concerned. To develop a benzimidazole resistant strain of *P. chlamydosporia* the alanine path way can be made switch-on which confers resistance to fungi. On the contrary if our aim is to combat the pathogen and development of new molecule of fungicides, then fungicide can be designed in such a
way that would switch-off the alanine pathway and switch-on glutamic acid pathway, which would make the pathogen susceptible to the fngicide.

The ITS primers generated a single band of about 600 bp. This size corresponded to the expected size according to the ITS sequence of other fungi. The variability within the ITS amplified region was also investigated by the restriction digestion of PCR products with Hinf I (Rsal) and Msp I. Both enzymes yielded 1-4 fragments. In all four isolates of P. chlamydosporia, Hinf I gave two distinct bands of size varying from 216 to 286bp while Msp I gave the band size of 250bp approximately. ITS-PCR digest patterns failed to generate sufficient polymorphism between the strains isolated from different parts of southern India.

Amplification of IGS region gave 500bp band size on 1% agarose gel. The variability within the IGS amplified region was also investigated by the restriction digestion of PCR products with Msp I. This enzyme yielded 3-7 bands (varying from 152 to 173bp) in Vc-1, Vc-2 and Vc-4 while in Vc-3 only 4 bands were present.

ERIC PCR yielded multiple distinct bands of size ranging from approximately 300 to 800bp. Differences among the strains were not found based on the migration pattern. In comparison of all four isolates of P. chlamydosporia it was found that all originated from the same geographical origin even though little variation was observed in isolate Vc-3.

PCR was carried out to detect the Phl-D gene using specifically designed primers. Five strains that were selected (based on good plant growth promoting effect, non-pathogenic nature and high disease suppression capacity) and subjected to antibiotic gene detection. Out of the five strains tested viz: 2+a, 2+b, 11+c, 11+b and 7,
the amplification of ~1 Kb and 745bp was observed in 11+b and 11+c respectively indicating that these two strains were positive to Phl-D gene.

Sequence analysis of strain 11+b, (Phl-D gene) revealed that it has got the similarity with the Phl-D gene of P. fluorescens. Approximately 1132 nucleotides were obtained as a full gene sequence of Phl-D gene. Nucleotide alignment of the Phl-D sequence and phylogenetic tree diagram analyses revealed that P. fluorescens strain 11+b shared the taxonomic relation between P. fluorescens strain WCS 365(AF281148) up to 90% identity and P. putida strain KT 2440(AE016789) up to 86% identity. This study gives the scope for isolation of gene based detection of Phl-D positive strains of P. fluorescens.

In our field experiment on the wilt disease management of capsicum we found different behaviour of P. fluorescens, which performed very well, in capsicum but not in egg-plant. However, in our study good correlation was found in glass house as well as field disease suppression in isolates 11+b and 11+c, which indicated the efficacy of Phl gene in the management of wilt disease complex.

The efficacy of P. fluorescens (11+c) on different media showed the difference in growth. Chitinase activity was more (confirmed by Western blotting) in the medium containing chitin with salts or chitin with peptone indicating the necessity of the other sources for bacterial growth. 11+c, which was known to contain more number of chitinase isoforms, followed by the strain 7 and 11+b.

11+c grown on chitin showed the increased activity of chitinase gene known to contain 4-5 polypeptides with molecular masses ranging from 17 to 48 kDa. Western
blotting analysis of conditioned media using chitinase antiserum detected 17, 20, 32 and 46.8-kDa chitinase.

Out of five strains tested for the plant growth promotion activity and chitin degradation against the naturally occurring soilborne diseases, 11+c found to be superior.

An attempt was made to standardize the liquid fermentation of bio-control agent followed by preparation of powder formulation. In our study the maximum cfu, root colonization and nematode egg parasitization was observed in Vc-3 when tested on both capsicum and egg-plant. Chlamydospore production by Vc-3 was $8.67 \times 10^8$. In capsicum and brinjal, the egg parasitization was 76.29% and 72.17% respectively by Vc-3. Based on all these criteria it was decided to mass-produce the isolate Vc-3 for further field efficacy test.

Effect on plant growth promotion, efficiency related to disease suppression, presence of \textit{Phl-D} gene for antibiotic production and chitinase activity are the important ones while screening for the best strain of \textit{P. fluorescens}. From our molecular approaches it was clear that 11+c strain is known to contain 2-3 chitinase isoforms with more chitinase activity against nematode. It is known to possess \textit{Phl-D} gene which is responsible for the production of a polyketide antibiotic 2,4-diacetyl phloroglucinol, a key factor in disease management of soil borne pathogens. 11+c strain performed very well with respect to disease inhibition, plant germination and plant growth.
Formulation made of 1:2 ratio of *Pseudomonas* broth: talc powder with 2% CMC, the CFU recovery was good even at the end of 6 months (32.19 x 10^3) which significantly differed from 0.2% CMC after treatment.

It was seen that in many cases the regular interference in liquid fermentation is common lab fungi *Aspergillus* spp. To eliminate this contaminant in the process of fermentation many chemicals were tested either alone or in combination with each other. Carbendazim @ 1% inhibited both fungi while Mancozeb @ 1% completely inhibited *Aspergillus* spp. but did not affect *T. harzianum*. We concluded that the liquid as well as solid fermentation of *T. harzianum* can be done using Mancozeb (@1g/l of liquid medium or 1g/kg of solid medium) which can completely avoid the contamination of *Aspergillus* spp. with out inhibiting the growth of *T. harzianum*.

As far as pathogen interaction is concerned in the wilt disease complex manifestation it was seen that in the presence of both nematode (*M. incognita*) and bacteria (*R. solanacearum*) the percent disease incidence was more

Experiments on the management of wilt disease complex in field level revealed that combination of two bio-agents performed very well compared to the single bio-agent. But same combination did not hold good in all crops.

In capsicum, based on the field performance of bio-agents it was found that combination of *T. harzianum* (TH) along with *P. fluorescens* (PF) performed very well in increasing the yield as well as decreasing the RGI (root gall index) (1.5) and wilt disease incidence (16.90%). RGI was less in combination of bio-agent treatment and it was more in individual bio-agent treatment. followed by organic cake treatment (neem cake and pongamia cake). The yield was also maximum in combination
treatment of TH and PF (3320g/3m²) followed by TH + P. chlamydospora (2960g/3m²). On the contrary the same combination did not perform very well in case of egg-plant, but the combination of T. harzianum and P. chlamydospora performed very well. The yield was 2024 g/3m² followed by combination of PF + VC where the yield was 1782.33 g. The performance of all three individual bio-control agents was on par with each other. RGI and wilt disease incidence was 1.8 and 22.18% respectively.

The overall performance of the combination formulation was good followed by individual application of bio-agents and application of organic cakes (neem, pongamia).

Experiments were conducted to evaluate the bio-efficacy of the formulations of bio-agents Pochonia chlamydospora and Pseudomonas fluorescens in the management of root-knot nematode of capsicum. These bio-agents were evaluated under field conditions for their efficacy against root-knot nematode Meloidogyne incognita infecting capsicum. Treatment of the nursery bed with the formulation of P. chlamydospora at 50g/m² was significantly effective than the treatment of nursery beds with bio-agent formulation at 25g/m² in reducing the galling index, number of nematodes in roots and soil, increasing the percent parasitization of eggs by bio agent and also yields. Seed treatment with P. fluroscence alone and the nursery bed treatment with P. chlamydospora alone were effective. However, their individual effect was maximized when both these bio-agents were integrated in the nursery bed stage. This could be because of combined effect of both bio-agents in the management of root-knot nematode (Meloidogyne incognita) on this crop, under field conditions.
Application of the formulation of the bio-agent *P. chlamydosporia* in the nursery beds of egg-plant proved significantly superior over control, in increasing the plant growth parameters (height and weight) of the seedlings. However, the role of *P. chlamydosporia* in increasing the growth of egg plant needs to be investigated further, mainly on its precise role in plant growth promotion.

Significant increase in the percent parasitisation of the eggs of root-knot nematode, by the bio-control fungus was observed when the formulation was applied at the rate of 50 g/m² and at 100 g/m². However, the dosage of 25 g/m² was not sufficient to effect an appreciable rate of egg parasitisation by the bio-control fungus and this could be due to low level rhizospheric competency at this dosage level. *Pochonia chlamydosporia* was reported to parasitise the eggs and egg masses of the root-knot nematodes.