CHAPTER - V

Summary and Conclusion
CHAPTER V

SUMMARY AND CONCLUSION

Plant diseases have been concerned with mankind since agriculture began and played an important role in the destruction of natural resources and contributing 13 to 20 per cent losses in crop production worldwide (Nene et al., 1996). In particular, soil-borne pathogens cause important losses, among pathogens, fungi being the most aggressive. The distribution of several phytopathogenic fungi, such as, Rhizoctonia, Sclerotium, and Fusarium have widely spread during the last few years due to changes introduced in farming with detrimental effects on crops of economic importance. Chemical control of plant diseases can be impressive but this is comparatively a short-term measure and additionally, the accumulation of harmful chemical residues sometimes causes serious ecological problems. In recent years, the increasing use of potentially hazardous chemicals in agriculture has been resulted in growing concern of both environment and public health properties. Moreover, use of such chemicals entails a substantial cost to the nation and developing country like India cannot afford it. By contrast, biological control is risk-free when it results in enhancement of resident antagonists. Many Trichoderma species are of great economic importance producing hydrolytic enzymes viz., cellulases, chitinases, and xylanases, biochemicals and antibiotic products which have been applied to fields such as food processing and pulp bleaching. In addition, some species produce heterologous proteins and others have been successfully used as biological control agents against phytopathogens. For about 70 years, Trichoderma spp. have been known to attack other fungi, to produce antibiotics that affect other microbes and to act as biocontrol microbes. Antagonists of phytopathogenic fungi have been used to control plant diseases and 90 per cent of such applications have been carried out with different strains of Trichoderma (Monte, 2001). The success of Trichoderma as biocontrol agents (BCAs) is due to their high reproductive capacity, ability to survive under very unfavorable conditions, efficiency in the utilization of nutrients, capacity to modify the rhizosphere, strong aggressiveness against phytopathogenic fungi and efficiency in promoting plant growth and defense mechanisms. These properties have made
Trichoderma, a ubiquitous genus present in any habitat and at high population density (Misra and Prasad, 2003).

The antagonistic potential of Trichoderma spp. against the major soil borne and yield limiting diseases, namely, wilt, dry root rot and collar rot in the major chickpea growing areas of the scarce rainfall zone of Andhra Pradesh with reference to its specialized agro-ecosystem for implementation of bio-control by exploration of native isolates of Trichoderma has not been carried out.

i. For achieving objectives in the present investigation, Survey conducted on dry root, wilt and collar rot incidence in major chickpea growing mandals of Kadapa, Kurnool and Anantapuram district, dry root rot incidence ranged from 5.6 to 22.5, wilt incidence recorded 5.2 to 20.4 in different mandals and collar rot incidence was observed 2.8 to 12.4 per cent disease incidence in different Madals of three District.

ii. Trichoderma spp. were isolated from Rhizosphere soils of chickpea growing areas of Kadapa, Kurnool and Anantapuram district of Rayalaseema Region and Identified T.asperellum (6 isolates), T.harzianum (5 isolates), T.viride (5 isolates) and T.longibrachiatum (4 isolates) based on morphological characters. Pathogens were isolated from infected root and purified by single hyphal tip. The colony characters and morphological characters of mycelium and sclerotia were in agreement with earlier reports Barnett and Hunter ((1972) and Sajeena et al. (2004). Thus, the fungi under present investigation were identified as R. bataticola, F. oxysporum ciceri and S. rolfsii.

iii. Characteristics Trichoderma isolates indicated that most of the T. asperellum and T. longibrachiatum isolates were fast growing with light green to dark green fluffy granular growth, mottled with white flecks and often with inconspicuous wefts of yellow hyphae whereas T. harzianum and T.viride isolates were relatively slow grower, with green to dark green coloured colony and effuse conidiation. The typical coconut odour was the characteristic feature of all the isolates of T. viride and some isolates of T. harzianum, T.longibrachiatum, whereas no such aroma was detected in case of T.asperelum when they were grown in PDA, OMA, and MEA media.
Furthermore, most of the isolates of *Trichoderma* were grown with dense mycelial mass in a concentric pattern when cultured in OMA and MEA media, but comparatively scanty mycelial growth of *Trichoderma* isolates were recorded in CZA medium.

Anamorphic characteristics of *T. harzianum* observed that, phialides shape varied from ampulliform to subglobose or lageniform divergent or crowded whorls of 2-5, shape of conidia were subglobose to ovoid, the conidiophores were highly branched pyramidal at nearly at right angles and the intercalary or terminal chlamydospores were subglobose to ellipsoid or pyriform. The size of phialides were varied from 3.9-6.8 x 2.6-3.4 \( \mu m \) (KNK1) to 7.8-9.7 x 3.3-4.3 \( \mu m \) (KNO9) while the size of phialospores were varied from 1.7-2.7 x 1.3-1.4 \( \mu m \) (KNK 1) to 3.5-4.0x 2.5-2.8 \( \mu m \) (ATPU 2). The conidiophores sizes were varied from 5.9-22.6 x 3.4-7.2 \( \mu m \) (KT 13) to 6.0-36.2 x 3.0-4.9 \( \mu m \) (KNK 1). The chlamydospores sizes were varied from 9.5-12.2 x 6.8-8.7\( \mu m \) (KT13 2) to 9.4-13.2 x 7.5-9.4 \( \mu m \) (KNK 1).

The shape of phialides of *T. viride* were of lageniform to subglobose, sometimes ampulliform to lageniform, verticillate with divergent whorls of 2-4. The shapes of phialosphores were varied from globose to ellipsoid or oblong with distinctive rough epispore walled. The conidiophores were comparatively narrow and flexuous, with primary branches at regular internodes, typically pyramidal branched and flex-uous. The size of phialides of *T. viride* isolates were varied from 5.3-8.2 x 1.2-1.6 \( \mu m \) (KNP 1) to 4.7-7.8 x 1.5-1.7 \( \mu m \) (KT 6) while the size of phialospores were varied from 2.1-3.5 x 1.6-2.0 \( \mu m \) (KJ 12) to 1.9-2.8 x 1.7-2.1 \( \mu m \) (KNP 1). The conidiophores sizes were varied from 4.9-33.4 x 2.9-3.7 \( \mu m \) (KNN2) to 7.2-39.7 x 3.0-4.1 \( \mu m \) (KJ12). The chlamydospores sizes were varied from 8.7 - 12.4 x 7.4 – 9.6 \( \mu m \) (KNN 2) to 9.5 - 13.3 x 8.2 - 9.4 \( \mu m \) (KJ12).

The shapes of chlamydospores of *T. asperellum* were varied from subglobose to ovoidal, pale green colour, born intercalary and terminal. The shape of phialides was of ampulliform to straight, slightly enlarged in the middle, cruciate whorls of 3 to more. The shapes of phialosphores were varied from subglobose to globose or ovoid to subglobose with fine incospicuos warts. The shape of conidiophores were subglobose to ovoidal. The size of phialides of *T. asperellum* isolates were varied
from 8.6-10.6 x 2.7-3.2 μm (ATPU 4) to 6.5-11.5 x 1.8-2.7 μm (ATPU 1) while the size of phialospores were varied from 4.5-6.5 x 3.2-4.2 μm (ATPU 4) to 3.5-4.5 x 3.0-4.0 μm (ATPU 1). The conidiophores sizes were varied from 5.6-17.3 x 2.8-4.7 μm (ATPU1) to 13.1-26.2 x 4.6-5.3 μm (KNO 2). The chlamydospores sizes were varied from 4.5-7.7 x 11.7-12.5 μm (KNK 9) 5.7-11.7 x 4.8-9.8 μm (ATPU 1).

The Anamorphic characteristics of *T. longibrachiatum* isolates has been observed that, phialides were of laginiform, cylindrical, slightly swollen near the middle. The shapes of phialospores were obovoid to ellipsoidal, oblong to ellipsoidal. The conidiophores have long central axis, phialides borne directly on the main branches and there was no secondary branches. The shapes of chlamydospores were varied from sugglbose, subglobose to ellipsoidal, born intercalary and terminal. The size of phialides of *T. longibrachiatum* isolates were varied from 6.0-12.7 x 2.3-3.3 μm (KT 7) to 5.2-8.8 x 2.0-2.6 μm (KP 10) while the size of phialospores were varied from 3.5-9.8 x 2.3-3.1 μm (KP 10) to 3.4-4.3 x 2.3-3.2 μm (APPE 6). The conidiophores sizes were varied from 10.9-28.1 x 2.3-3.4 μm (KP 10) to 16.7-35.6 x 3.2-3.4 (APPE6). The chlamydospores sizes were varied from 15.2-34.1 x 3.2-3.4 μm (KR 4) to 10.9-28.1 x 2.3-4.2 μm (KP 10).

Today, the use of morphological characteristics for identifying *Trichoderma* species is being progressively replaced by molecular tools, which provide a more robust and reliable form of species identification.

Sequence analysis of twenty isolates was done to confirm species identity, which initially has been done based solely on morphological parameters. Comparison of oligonucleotide fragments of rDNA sequences, which included the 5.8S gene and the flanking ITS1 and ITS2 regions, with reference sequences from public databases, showed that they were belonging to four different species such as *T. asperellum*, *T. longibrachiatum*, *T. viride* and *T. harzianum*.

The phylogenetic tree obtained by sequence analysis of ITS1 and ITS4 of 20 isolate sequences is prepared by using MEGA 6 software. Bootstrap analysis with 1000 bootstrap replications demonstrated three major branches. On the basis of the bootstrap values, 20 *Trichoderma* spp. could be divided into 9 groups. Group I, the *T. aspergillus*, Group II *T. harzianum* and *T. longibrachiatum* complex. Group III *T.
Summary and Conclusion

asperellum and T. viride, Group IV to Group IX belonged to T. asperellum. The phylogenetic analysis showed many solitary clades of T. asperellum, which indicates that more diversity among the T. asperellum. Further, this analysis showed less bootstrap values indicates instability of the clade.

The isolates ATPU 6, ATPU 4, ATPU 1, KNK9, KNO 2 and KNPG 3 were identified as T.asperellum on the basis of Morphological, as T.asperellum based on ITS region analysis. The isolates ATPPE 6, KR 4 and KP10 identified as T.longibrachiatum based on Morphological and ITS region, whereas KT 7 identified as T.longibrachiatum based on Morphological, as T.asperellum based on ITS region. KJ12 and KT1 isolates identified as T.viride based on two methods used in present study. Whereas KNP1and KNP3 identified as T.viride based on Morphology, as T.asperellum in ITS region. Isolate ATPP6 identified as T.harzianum through morphological and Molecular methods. Whereas KNK1, KNN4 and KNO 9 identified as T.harzianum based on Morphology, as T.asperellum in ITS region.

iv. Rapid screening of Trichoderma isolates from Kurnool, Anantapur and Kadapa Districts of Rayalaseema region, against three fungal pathogens revealed that there was a clear difference in four species of Trichoderma viz., T. harzianum, T. viride and T. asperellum and T.longibrachiatum with respect to their hyper parasitic potential against the pathogens tested. Kurnool and Anantapur district isolates showed high efficacy against three pathogens, attaining S1 stage at 4 days. Whereas, Kadapa District isolates showed least effective against three pathogens and attaining S1 stage in 5 days.

The dual culture test of twenty isolates of Trichoderma revealed that per cent inhibition in mycelial growth of pathogen like R. bataticola, F. oxysporum ciceri and S. rolfsii. T. harzianum (KNN 4) shown maximum inhibition of mycelia growth by 81.1% followed by T. harzianum (ATPP 6) with inhibition of 79.3% and the least was 66.3% inhibition by T. viride (KJ 12) isolate against R.bataticola. Whereas the isolate T.asperellum (KNPG 3) showed the maximum inhibition of S.rolfsii with 80.7% and it was found significantly superior over rest of the bioagents. Meanwhile T. asperellum (ATPU 1) was found to be most effective in inhibiting the growth of
Summary and Conclusion

F. o. f. sp ciceri with 84.1 per cent growth of the test fungus followed by T. asperellum (KNPG 3) with 83.7% inhibition.

The hyphae of antagonist grown, coiled around the hyphae of R. bataticola, S. rolfsii and F.o.f.sp.ciceri and penetrated into the hyphae of pathogen by producing hook or knob like structures. The mycelia of pathogens twisted and curled, often fragmented hyphae have been also observed due to intense coiling and secretion of antifungal substances including cell wall degrading enzymes.

Volatile metabolites produced by Trichoderma isolate having significant effect in reducing the radial growth of test pathogens. In case of R.bataticola, T.asperellum (KNO 2) inhibited the mycelial growth of test pathogen by 82.5% per cent. While, in case of S.rolfsii, T.asperellum (KNPG 3) was found most efficacious in reducing the mycelial growth of test pathogen by 86.7 % and isolate T. asperellum (ATPU 1) was found most efficacious in reducing the mycelial growth of F.o. f.sp.ciceri by 86.7per cent.

All Trichoderma isolates significantly inhibited test pathogens by production of non-volatile inhibitors at 10%, 15% and 20%. The pathogen, R.bataticola was significantly inhibited by Trichoderma spp. and ranged from 82.2% - 91.1% inhibition at 20 % concentration. The maximum zone of inhibition for non-volatile metabolites of T.longibrachiatum (ATPPE 6) While, in case of S.rolfsii, T.asperellum (ATPU 6) was found most efficacious in reducing the mycelial growth of test pathogen by 93.3%. The highest inhibition was recorded with T.viride (KNN 2) against F. o.f.sp. ciceri with 95.0 %.

v. The highest rhizosphere population recorded in sterilized soil compared to sundried soil. The rhizosphere population of Trichoderma increased at increased at an increasing rate from 15 to 30 DAS and 30 to 45 DAS and thereafter increased but at decreasing rate and finally declined at 75 DAS in all soil types. But there was appreciable more number of rhizosphere populations of antagonists in rhizosphere soil of test crop were observed at 75 DAS than at 15 and 30 DAS. The isolate KNN 2 was most efficient colonizer of chickpea with130.0x10³ cfu/g followed by ATPU 1 with 119.0x10³ cfu/g of soil at 60 DAS under natural soils. Highest germination
Summary and Conclusion

(%) of Chickpea seeds were noted with KNN 2 (96.7 %) followed by ATPU 1 (96.3 %), KNP 3 and KNPG 3 (93.3%). Highest rhizosphere population was observed at 60 DAS. Among the isolates, KNN 2 was most potent isolate with 314.3x10^8 cfu/g of soil, followed by ATPU 6(295.3 x10^8 cfu/g soil) under sterilized soil. The isolates KNK 1, ATPU 4, KNO 2 and ATPU 6 were most promising isolates with isolates with appreciable high rhizosphere population soil of chickpea.

Competitive parasitic ability of twenty Trichoderma spp. against sclerotia of S. rolfsii have been conducted in two different soil conditions, viz., natural, and steam sterilized soil and two different form of inoculaum used and data have been presented in terms of EID50 value (Effective inoculum density for 50% colonization of inoculum baits). An overview of the entire results clearly indicated that the nature of withdrawal of stasis barrier (fungistasis/mycostasis) from soil by means of either natural or steam sterilization gradually reduced the EID50 values of respective isolates. In general, all isolates of Trichoderma had different EID50 values for sclerotia of S. rolfsii, regardless of soil types and form of inocula used. The isolate ATPU 1(EID50 2.1) was most effective in parasitization of sclerotia of S. rolfsii used as live bait, under the natural soil of, when mycelial form of inoculum was used. This isolate was closely followed by KNO 2 and ATPP 6 with same (EID50 2.2) next best isolates were KNP 3 and ATPPE6 (EID50 2.3). The isolate KT 6 (EID50 5.7) was rated as poorest competitive colonizer, requiring comparatively highest inoculum level to colonize 50% sclerotia of S. rolfsii. whereas KNK1 (EID50 2.2) was most aggressive isolate required lowest inoculum dose for 50% colonization of sclerotia of S. rolfsii when conidial inoculum was used. When conidial form of inoculum (antagonists) was used, the isolate ATPP 1 (EID50 1.3) (EID50, 2.2) was the most efficient colonizer of sclerotia of S. rolfsii and the isolate KT 6 and KT 7 (EID50 5.6,5.7) was rated as least competitive colonizer, requiring comparatively highest inoculum level to colonize 50% sclerotia of S. rolfsii.

Test crop seeds were primed with twenty isolates of Trichoderma using both mycelial and conidial inocula under in vitro and pot culture to judge their potentialities in terms of improvement in per cent germination of seeds, vigour index
Summary and Conclusion

and seedling biomass. Highest per cent germination of seed was obtained (99.67%) for the isolate ATPU 1 of *T. asperellum* when crop seed was primed with mycelial form of inoculum. Highest vigour index was recorded in case KN0 9 (3627.0) and lowest vigour index (2011.1) for the isolate KJ 12 was recorded both condition. Highest vigour index was recorded in case ATPU 1 (2063.0) and lowest vigour index (1327.0) for the isolate KJ 12 was recorded in conidia form of inoculum. Among the two forms of inocula, the mycelial form gave better responses with respect to enhanced germination of seeds and increasing seed vigour index either in terms of calculated vigour index or estimated biomass on dry weight basis in two conditions.

vi. Potential isolates (with different treatments) significantly increase germination (%), reduced the disease incidence at all the dates observed and corresponding increase in yield of chickpea as compared to untreated control under *in vivo*. However, highest field emergence (%) in chickpea seeds was recorded with seed + soil application of antagonists even under the artificial infestation of field by *R. bataticola*. Lowest dry root rot disease incidence of chickpea (12.93%) was recorded with simultaneous soil and seed application of ATPU 1 (T6), followed by T9 (14.00%), T3 (16.87%) which were statistically insignificant under the same condition. Maximum plant height (45.97cm) was recorded in treatment T6 (seed treatment + soil application with potential ATPU 1). Least plant height was recorded in inoculated control T13 (25.60 cm). Maximum root, shoot length was recorded in treatment T6 (12.37, 33.60 cm) followed by treatment T11 (11.43, 24.37cm). *Trichoderma* significantly improved the germination, reduced the disease incidence and resulted into as much as 1751.00 kg/ha of seeds as compared to only 759.00kg/ha in control plot.