CHAPTER - I
INTRODUCTION

1.1 STATEMENT OF PROBLEM

Groundnut (*Arachis hypogaea* L.), a self-pollinated allotetraploid leguminous crop (2n = 4x = 40) with a DNA content of about 2813 Mbp/1C is a member of the family, Fabaceae (Arumuganatham, 1991). It originated in South America and is widely cultivated across 100 countries in tropical and sub-tropical regions of the world under rainfed conditions (Smartt, 1994). The allotetraploid *Arachis hypogaea* is believed to be evolved from hybridization of two diploid wild species, *A. duranensis* (A-genome) and *A. ipaensis* (B-genome), followed by a rare spontaneous duplication of chromosomes (Halward *et al.*, 1991; Kochert *et al.*, 1996; Seijo *et al.*, 2007; Koppolu *et al.*, 2010).

Groundnut is the 13th most important food crop, fourth largest oilseed crop and third most important source of vegetable protein in the world. Globally, it is grown on an area of 24.6 million hectare with an average production of 41.3 million tons. The largest producer of groundnut is China followed by India and USA. In India, it is grown on an area of 4.90 million hectare with an average production of 5.78 million tons (FAO stat, 2015). The state of Gujarat has the highest production of groundnut with 3.5 million tons followed by Tamil Nadu, Andhra Pradesh, Karnataka and Maharashtra (Anonymous, 2015).

Groundnut seeds are rich source of edible oil and constitute 40–60% oil, 20–40% protein and 10–20% carbohydrate. Additionally, it accounts for high nutritional value, possessing vitamin E, niacin, calcium, phosphorus, magnesium, zinc, iron, riboflavin, thiamine and potassium. It is generally used as a food and is considered as cash crop around the world for direct consumption, in the confectionary industry, for vegetable oil in cooking and also as a source for protein feed in the animal industry. These multiple uses of peanut make it more valuable crop for domestic as well as international trade (Pandey *et al.*, 2012).
Despite the high nutritional and economic implications; production and productivity of groundnut is constrained by various biotic and abiotic stresses. Among biotic stresses, stem and pod rot disease caused by *Sclerotium rolfsii* Sacc. is one of the significant factors contributing to yield losses. In India, stem and pod rot incidence occurs in all groundnut growing states. It is most severe in Maharashtra, Gujarat, Madhya Pradesh, Karnataka, Andhra Pradesh, Orissa and Tamil Nadu, where it is estimated that over 5,00,000 ha of groundnut are affected by the pathogen. Yield loss of over 25 per cent has been reported due to this disease. Irrigated groundnut crop grown in the post rainy and summer seasons in India is often infected by the pathogen (Mayee and Datar, 1988).

Control of stem rot disease mostly relies on cultural practices and fungicide treatment. However, cultural practices are not always effective due to the wide host range of the pathogen, and fungicides are often too expensive. Conventional crop protection against stem rot disease has been predominantly based on the use of chemical pesticides to control plant diseases and pests, a practice that can produce negative effects to the end user and the agro-ecosystem, including inhibition of pollinators, useful predators/parasitoid and beneficial microbial communities. Biological control has been proposed as a sustainable, affordable and supplementary measure to control *S. rolfsii*, but has not been explored and exploited in detail (Rodriguez *et al.*, 1975).

One of the implementation of biotechnology which can be exploited is the use of microbes (fungi, bacteria and actinomycetes) for biocontrol activity against plant diseases. In recent years, biological control of plant pathogens has received increasing attention as a promising supplement or alternative to chemical control. Among bacteria, *Pseudomonas* and *Bacillus* were widely used, and use of antagonistic fungi, especially *Trichoderma* and *Gliocladium* have been more extensive than their bacterial counterparts (Ganesan and Sekar, 2004).

*Trichoderma* - a genus under Deuteromycotina, Hypomycetes, Phialosporae, Hyphales, Dermatiaceae has gained immense importance as a biological control agent against several plant pathogenic fungi (e.g. *Aspergillus flavus*, *Botrytis cinerea*, *Sclerotium rolfsii*, *Fusarium culmorum*, *F. oxysporum*, *Rhizoctonia solani*, *Microdochium nivale*, *Pythium debaryanum*) (Kubicek and Harman, 1998; Lorito *et al*.)
Trichoderma species are free living fungi that are common in soil and root ecosystems. A recent discovery showed that they are opportunistic, avirulent plant symbionts as well as parasites on the other fungi. The researchers are interested in the genus *Trichoderma* because of its novel biological properties and biotechnological applications. Proposed mechanisms of antagonism included myco-parasitism by the action of cell-wall degrading enzymes, antibiosis by the production of antibiotics, competition for space and nutrients through rhizosphere competence, facilitation of seed germination and growth of the plants via releasing important minerals and trace elements from soil and induction of the defense responses in plants (Howell, 2003).

The genome size of filamentous fungi is usually small. They have a haploid nucleus. The estimated genome sizes and chromosome numbers of *Trichoderma* spp. range from 31 to 39 Mb and from 3 to 7, respectively. The size of chromosome differs among the species (Srivastava *et al*., 2014).

*Trichoderma* species are known mycoparasites on several plant pathogens especially against soil-borne plant pathogens. The effectiveness of biocontrol agents may respond differentially to varied soil conditions like temperature, pH, soil texture, water content, presence of pesticides, salt, metal ions and antagonist bacteria in the soil. Therefore, their application should be considered as the environmental stresses that could affect not only their survival in the soil, but also their ability to maintain their biocontrol capacity (Kumar and Sharma, 2011).

Another option for the control of these pathogens is chemical pest management, as various pesticides can be used against them successfully. However, degradation of these compounds can be slow and they may remain in soil for a long time, adversely affecting the soil microbial environment (Basamma, 2008). By the application of chemical fungicides at lower concentration combined with biocontrol *Trichoderma* strains, the same level of crop protection could be ensured by using fungicides alone, but with reduced environmental pollution. Therefore, there is an emerging need for biocontrol *Trichoderma* strains that can tolerate chemical fungicides, which would be a prerequisite for their application in an integrated pest management (IPM) strategy combining chemical and biological means of control.
Both classical mutagenesis and protoplast fusion have been shown to be appropriate tools for the improvement of biocontrol *Trichoderma* strains. Fusant with the ability of myco-parasitism (due to overproduction of extracellular metabolites and cell wall degrading enzymes) and having ability to tolerate pH, salt, temperature, fungicides is most effective to control wide range of pathogen in order to obtain *Trichoderma* strains with multi resistance (Hatvani *et al.*, 2006).

Various small non-coding RNAs (ncRNAs), about 20-24 nucleotides (nt) long, deriving from endogenous or extrinsic pathways could play crucial roles in the modulation of gene expression and genomes at different levels, which is known as post-transcriptional gene silencing (PTGS) (Matzke and Matzke, 1995), quelling (Romano and Macino, 1992) or RNA interference (RNAi). A variety of small RNAs (sRNAs) and its mediated RNA interference (RNAi) have been shown in filamentous fungi including *Trichoderma* to regulate the biocontrol activity (Mello and Conte, 2004). Recently, several groups reported that miRNA-like sRNAs (miRNAs) also exist in the filamentous fungi, including *Neurospora crassa* (Vazquez *et al.*, 2004), *Sclerotinia sclerotiorum* (Reinhart and Bartel, 2002) and *Metarhizium anisopliae* (Borsani *et al.*, 2005).

### 1.2 OBJECTIVES OF THE STUDY

In the context of above considerations, the present study was framed keeping in view following objectives

1. Screening of *Trichoderma* for multi tolerance with fungicides and abiotic stresses inhibiting *S. rolfsii* Sacc.
3. Antifungal and metabolomic study related to antagonistic activity of obtained fusants and parental *Trichoderma* strains.
4. Identification, characterization and expression profile of micro RNAs for biocontrol activity of *Trichoderma* fusants and parental strains.
5. Development and characterization of nanoformulations derived from best fusant and their bio-efficacy to control *S. rolfsii* infection in groundnut.
1.3 SIGNIFICANCE OF THE PROBLEM

Groundnut is a oilseed crop cultivated in Saurashtra region of Gujarat. The fungal pathogen *S. rolfsii* causing stem rot in groundnut is one of the major biotic constraints. Many chemical fungicides are used to control this disease which led to deteriorating human health, environmental pollution, and development of pathogen resistance to fungicides. The use of biocontrol agent to defeat this disease is eco-friendly. *Trichoderma* are used as biocontrol agent against *Sclerotium rolfsii*.

Data generated from this experiment may help to develop best *Trichoderma* antagonistic strain having multi stress tolerant capacities (*viz.*, fungicides tolerance, salt and drought tolerant) using protoplast fusion, which may have the potential to be applied in integrated disease management (IDM) strategies. Biochemical characterization of *Trichoderma* strain allow us to understand correlation between cell wall degrading enzymes, defense related substances and its biocontrol activities. Metabolomics study of antagonist in-depth demonstrated the biochemical behaviour and production of different novel bioactive compounds during biological interactions. Identification and characterization of miRNA, their targeted and novel genes, functional annotation and gene expression pattern are reported in this work which might be enhanced to understand miRNA regulatory mechanisms during antagonistic activities of parental strains and derived *Trichoderma* fusant against *S. rolfsii*. The study might be elucidated cellular, molecular and biological functions of miRNA and designed biochemical pathway analysis for specific functions of these regulatory miRNA for biocontrol activity of best *Trichoderma* fusant against *S. rolfsii*. The development and characterization of synergistic nanoformulations containing bioactive substances that can able to effectively defeat pathogen *S. rolfsii* causing stem rot in groundnut under pot culture and information derived from the experiments would be useful to farmers community for eco-friendly management of stem rot disease in groundnut.

1.4 ASSUMPTIONS OF THE STUDY

*Trichoderma* has been reported as biocontrol agent for many fungal pathogens of crop plants. All the *Trichoderma* strains could not work exactly to control plant disease. The potentiality of *Trichoderma* strains varies during interaction with specific phytopathogens. Hence, present study helped to identify best
Trichoderma isolates based on the antagonistic activities against S. rolfsii. The most efficient isolates identified in this study can be utilized to develop diverse Trichoderma fusants having multi stress tolerance (Fungicides, drought and salt) with mycoparasitic activity.

Trichoderma species are generally abundant on decaying wood and in soil because of their success in various heterotrophic interactions, including decomposition, parasitism, and even opportunistic endophytism. Many Trichoderma spp. have various important applications in industry and human life. Trichoderma species also have been adopted as agents of biological control of plant pathogenic fungi and as antibiotic producers. Trichoderma longibrachiatum is known as an opportunistic pathogen of immunocompromised mammals, including humans, and some species are common indoor contaminants. Given these properties, correct identification at the species level is highly desirable. With this intention, molecular marker technology was applied.

There are a number of techniques used to observe genetic variation within fungal biocontrol agents and pathogens. Now a day's attention has been focused on studying polymorphism at the DNA level for genetic characterization because different molecular markers, apart from elucidation of genetic variability, can also be used to study evolution and monitoring movement/shift of pathogen population over time.

The biocontrol related gene specific molecular markers (SSR), which is more repeatable can be used for studying molecular diversity in Trichoderma. SSR markers are more consistent and repeatable than RAPDs, and are less time-consuming compared to amplified-fragment length polymorphisms. The SSR combines simplicity, reliability, moderate throughput ratio and facile sequencing of selected bands. Further, it targets coding sequences in the genome and results in co-dominant markers (Rai et al., 2016). Besides that miRNA related sRNA identified from best Trichoderm fusants and parental strains which may regulate the biocontrol related pathways and identified the gene for biological activity.

Potential genetic variability available to conduct conventional breeding for resistance against the pathogen Sclerotium rolfsii has been found very limited in
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Groundnut. Therefore, a detailed investigation to understand the biochemical and molecular mechanism of pathogen and host tissue may prove to be fruitful.

1.5 LIMITATIONS OF THE STUDY

*Trichoderma* are act as biocontrol agent against phytopathogen. It adopts specific mechanism to act as biocontrol agent against specific pathogen causing disease in crops. Present study deals with antagonism of *Trichoderma* with *S. rolfsii* causing stem rot in groundnut. Several *Trichoderma* exist in soil rhizosphere. The present study helps to identify best *Trichoderma* strains for biocontrol activity and multi stress tolerance capacity among twenty isolates of *Trichoderma* belonging to different species viz., *T. harzianum, T. harzianum rifai, T. viride, T. atroviride, T. virens, T. hamatum, T. koningii, T. pseudokoningii, T. reesei and T. parceramosum*. Further, *Trichoderma* are screened for drought and fungicide tolerance limited to *Trichoderma* strains obtained from present study. The potent mycoparasitic and multi stress tolerant *Trichoderma* were subjected to protoplast fusion and about 36 stable colonies were observed in a three batches of fusants study.

The molecular markers studied during the research are biocontrol related gene specific SSR. Besides that, many markers are available to study diversity of antagonists. Limited numbers of SSR primers were used to amplify genomic DNA of *Trichoderma* fusants and their parental strain for analysis of genetic diversity and molecular heterozygosity of best *Trichoderma* fusants based on unique markers. This may be restricted to number of SSR primers and number of *Trichoderma* fusants used in the study.