BIOCHEMICAL AND MOLECULAR BASIS OF INNATE AND *Pseudomonas fluorescens* INDUCED STEM ROT TOLERANCE IN GROUNDNUT (*Arachis hypogaea* L.)

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IN
BIOCHEMISTRY
BY

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(Registration No-J4-01084-2012)
M. Sc. (Biochemistry)

DEPARTMENT OF BIOCHEMISTRY
COLLEGE OF AGRICULTURE
JUNAGADH AGRICULTURAL UNIVERSITY
JUNAGADH-362 001

OCTOBER - 2015

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ABSTRACT

Key words: 16s rRNA, tyloses, Ca-oxalate, gene expression, *pgip*

Groundnut (*Arachis hypogaea* L.), is an important legume crop and India is one among the leading producers of groundnut worldwide. *Sclerotium rolfsii* is the causal organism of stem rot in groundnut. Biological control of *S. rolfsii* using antagonistic fluorescent *Pseudomonas* is a potential management tool. Four groundnut genotypes such as CS19, GG16, GG20 and TG37A were selected to investigate the biochemical, molecular and histopathological basis of their varying degree of tolerance to stem rot disease. Eleven fluorescent *Pseudomonas* strains were isolated from different groundnut growing locations of *Saurasthra* region of Gujarat. To study the induced tolerance a potential antagonistic fluorescent *Pseudomonas* isolate (*SKPf 5*) belonging to species *Pseudomonas aeruginosa* was isolated and used as a treatment in the study. Scanning Electron Microscopy of stem tissues at five days post *S. rolfsii* infection revealed the formation of tyloses only in the tolerant genotype CS19 and the accumulations of Ca-oxalate crystals like structures only in the most susceptible genotype TG37A. Infection of *S. rolfsii* reported differential induction of antioxidative enzymes like Superoxide Dismutase, Catalase, Ascorbate Peroxidase and Glutathione Reductase; and pathogenesis related proteins such as chitinase and ß-1, 3-glucanases. In general a rapid, early and stronger induction was observed for the tolerant as compared to the susceptible genotypes. Application of *SKPf 5* as seed-priming and root inoculants reduced the disease incidence by promoting the overall growth of the plants and activating the innate defense system. Stem rot infection resulted decrease in ascorbic acid content and increase in oxalic acid content in all genotypes however the trend was higher in susceptible genotypes. The proteomic study using two-dimensional gel electrophoresis indicated change in protein expression as a response to fungal infection. The differential expression pattern of PR protein genes such as *PR2*, *PR4*, *PR5*, and *PR10* revealed an instant and stronger expression of those genes upon the fungal stress as compare to the weaker and/or delayed or no induction in the susceptible ones. A partial putative PGIP protein encoding gene was isolated and reported for the first time in groundnut (acc. no. *KP844637*). PGIP gene expression showed its higher induction in tolerant compared to susceptible genotypes as a response to *S. rolfsii* infection suggesting its possible role in stem rot stress tolerance in groundnut.

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CERTIFICATE-I

This is to certify that the thesis work report entitled **"BIOCHEMICAL AND MOLECULAR BASIS OF INNATE AND *Pseudomonas fluorescens* INDUCED STEM ROT TOLERANCE IN GROUNDNUT (*Arachis hypogaea* L.)"** submitted by **SUJIT KUMAR BISHI** in partial fulfillment of the requirements for the award of the degree of **Doctor of Philosophy (Agriculture)** in the subject of **BIOCHEMISTRY** to the Junagadh Agricultural University is a record of bonafide research work carried out by him under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma or other similar title. The candidate had fulfilled all prescribed requirements. The assistance and help received during the course of investigation have been fully acknowledged. He has successfully completed the comprehensive/ preliminary examination held on **Sept 9, 2014** as required under the regulation for post-graduate studies. He has submitted kachcha bound thesis on **July 28, 2015**.

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Date: 28.07.2015

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CERTIFICATE-II

Date: /10/2015

This is to certify that the thesis/project work report entitled **"BIOCHEMICAL AND MOLECULAR BASIS OF INNATE AND *Pseudomonas fluorescens* INDUCED STEM ROT TOLERANCE IN GROUNDNUT (*Arachis hypogaea* L.)"** submitted by **SUJIT KUMAR BISHI** to Junagadh Agricultural University, Junagadh in partial fulfillment of the requirements for award of the degree of **DOCTOR OF PHILOSOPHY (Agriculture)** in the subject of **BIOCHEMISTRY** after recommendation by the external examiners were defended by the candidate before the following members of the examination committee. The performance of the candidate in the oral examination was satisfactory. We, therefore, forward with recommendation.

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Place: Junagadh
Date: /10/15 *(Sujit Kumar Bishi)*

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LIST OF ABBREVIATIONS

ABBREVIATION		FULL NAME
%	:	Percentage
ΔC_T	:	Change in thermal cycle number
µg	:	Microgram
µl	:	Micro liter
3’	:	Carbon atom 3 of deoxyribose
5’	:	Carbon atom 5 of deoxyribose
A	:	Ampere
A_{260}/A_{280}	:	Absorbance ratio
bp	:	Base pair
C.V.	:	Co-efficient of variation
CAT	:	Catalase
cDNA	:	Complementary DNA
cm	:	Centimeter
C_T	:	Thermal cycle
CTAB	:	Cetyl Trimethyl Ammonium Bromide
DAI	:	Days after inoculation
DNA	:	deoxyribose nucleic acid
DNase	:	Deoxyribonuclease
dpi	:	Days post infection
e.g.	:	example gratia (For Example)
EDTA	:	Ethyl diamine tetra acetic acid
et al.	:	Co-workers
GR	:	Glutathione Reductase
H_2O_2	:	Hydrogen Peroxide
i.e.	:	id est. (That is)
kb	:	Kilo base pair
KB	:	King’s B medium
kDa	:	Kilo Dalton
L.S.D.	:	Least Significant Difference
M	:	Molar
mg	:	Milligram
Mha	:	Million Hectare
min	:	Minute(s)
mL	:	Milliliter
mM	:	Millimolar

N	Normal
NBT	Nitroblue tetrazolium chloride
ng	Nanogram
nm	Nanometer
^{O}C	Degree Celsius
OD	Optical density
P.	*Pseudomonas*
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
PEG	Poly ethylene glycol
PG	Polygalacturonase
PGIP	Polygalacturonase inhibitory Protein
pgip	PGIP encoding gene
POX	Peroxidase
PR	PR Protein encoding gene
PR Proteins	Pathogenesis Related Proteins
RNase	Ribonuclease
ROS	Reactive Oxygen Species
rpm	Revolution per minute
S	Sediment
S. rolfsii	*Sclerotium rolfsii*
S.E.	Standard Error
S.Em.	Standard Error of Mean
SA	Salicylic Acid
SOD	Superoxide Dismutase
Sr. No.	Serial Number
Taq	*Thermus aquaticus*
TBE	Tris Borate EDTA
TE	Tris EDTA Buffer
Tris –HCL	Tris (Hydroxy methyl) amino methane-hydrochloride
V	Volts
V	Volt
v/v	Volume per volume
w/v	Weight per Volume

CHAPTER – I

INTRODUCTION

Groundnut (*Arachis hypogaea* L.), is an important legume crop grown in over 100 countries. As a rich source of energy (564 kcal 100 g^{-1}), it contains about 48-50% oil, 25-28% proteins and 20-26% carbohydrates. Among oilseeds groundnut is unique in that it can be consumed directly as a foodstuff. The kernels contain many health enhancing essential minerals such as calcium, phosphorus, magnesium, zinc, iron and potassium and medicinally important compounds *viz.* *p*-coumaric acid and resveratrol, polyphenols, flavonoids and isoflavones; vitamins particularly vitamin E, folic acid and niacin; and antioxidants (Francisco and Resurreccion, 2008), hence groundnut has been recently recognised as a functional food. More than 60% of the total production of groundnut in India is used for the extraction of edible oil or industrial oil (Birthal *et al.*, 2010). The oil cake and haulm are being used for feeding the animals and industrial purposes. Being as a legume crop, it also improves the atmospheric N_2 fixation of the soil and thus increases the soil fertility (Janila *et al.*, 2013).

India is one among the leading producers of groundnut worldwide. With an annual production of about 9.67 million tonnes (MT), India ranks second in the world after China in production (ASG, 2014). As documented by DGCI&S (2014), in 2012-13, approximately 0.54 MT groundnuts has been exported estimating about 4065 crores rupees, ranking India the second largest exporter of the crop. Covering a cropping area of 5.5 million hector (Mha) which is about 2.66% of the national grossed cropped area with a production of 6.5 MT with productivity of 1182 kg ha^{-1}, groundnut contributes 22.3% of the total oilseed production in India, as recorded in 2013-14 by Directorate of Economics and Statistics, Govt. of India (DES, 2014). As documented by ASG (2014), the major portion of the groundnut produced in India is contributed by three states; Gujarat, Andhra Pradesh and Tamil Nadu. In groundnut production, Gujarat ranked first having a total area of 1.7 million ha with production of 4.92 MT, thereby sharing more than 50% of all India production as recorded in 2013-14 by DES (2014). The seven districts Amreli, Bhavnagar, Jamnagar, Junagadh, Kutch, Porbandar and Rajkot of Gujarat contribute more than 90% of production in Gujarat where Junagadh district is the largest producer of groundnut in Gujarat (DAGS, 2015). The major groundnut cultivars released for the cultivation in Gujarat were belonged to three groups Virginia bunch, Spanish and Valencia but in recent the most promising cultivars belong to Virginia bunch and the Spanish group.

Groundnut is a host to several fungal, bacterial, viral and nematode diseases, which are responsible for low productivity among which diseases like leaf spot, collar rot, stem rot, bud necrosis, etc., are very important. Stem rot, caused by *Sclerotium rolfsii*, is potential threat to this crop production in many warm, humid areas of the world and India, especially where irrigated groundnut cultivation is expanding (Janilla *et al.,* 2013). In Gujarat, especially in *Saurashtra* region, stem rot is a major concern among groundnut growers.

The pathogen *S. rolfsii,* is a soil-borne pathogen that commonly occurs in the tropics, sub-tropics and other warm temperate regions of the world. In India, it is wide spread in almost all the states especially in Gujarat, Maharashtra, Karnataka, Tamil Nadu and Andhra Pradesh (Kumar *et al.*, 2013). Groundnut crop grown in the post rainy and summer seasons in India are often infected by the pathogen. It is predominant especially in vertisols and relatively less severe in sandy loam soils. Yield losses due to stem rot usually range from 10 to 25%, but may reach upto 80% in severely infested fields in India (Mayee and Datar, 1988). It was also observed that about 20-60% of pod yield loss occur due to the disease in Karnataka and Andhra Pradesh (Anonymous, 1992)**.**

The primary symptoms are browning and wilting of leaves and branches which are still attached with the plant. The fungus preferentially infects stem by forming a whitish mycelia mat around the stem, but it can also infect any part of the plant including root, leaf and pod. In heavy soils, fungus damages groundnut plants near the soil surface but in light soils it can reach up to pod level, causing severe damage to pegs and pods (Mehan and McDonald, 1990). Infected pods are covered by mycelia usually rot. When bark is peeled off, the inner tissue shows a brown to yellow discoloration. Leaves of infected plants turn brown, dry and often remain attached to the dead stem. Drying or shrivelling of the affected branches ultimately lead to death of the complete plants after wilting.

S. rolfsii is a destructive soil inhabitant of worldwide significance which has a host range of over 500 species of plants (Punja *et al.,* 1985). Management of *S. rolfsii* through chemical methods leads to ill effects like residual toxicity, environmental pollution and fungicide resistance. Biological control has been proved to be a promising disease- management technology especially against soil-borne plant pathogens. Biological control using antagonistic fluorescent Pseudomonas appears to be a potential management tool for reducing the severity of groundnut stem rot; hence there is a need for identifying isolates specifically effective against *S. rolfsii*, for a planned regional deployment.

Several reactive oxygen species produced during infection by pathogen have been shown to be associated with the hypersensitive responses in plants. A strong correlation exists between the oxidative state of the host plant and its resistance against different stress. The oxidative state of the host plant is mediated through the production of ROS and their subsequent elimination by anti-oxidative enzymes. A coordinated defense response system is activated in plants and defense related proteins or pathogenesis related proteins (PR proteins) are produced in the host during the host-pathogen interaction. It has been shown that many antioxidative enzymes and PR proteins get induced upon fungal infection in different crop plants. However, the products of activated defense genes and antioxidative enzymes which are synthesized *de novo* during periods of plant pathogenic interaction have not been well documented in several host-pathogen interactions, especially in groundnut – *S. rolfsii* patho-system.

Stress induced gene expression has been studied extensively in many crops which are useful in potential future marker-assisted selection or transgenic disease control strategies. Hence, study of different genetic and biochemical pathways expressed early in the stem rot-groundnut infection process would potentially provide new targets to slow or stop infection by this significant groundnut disease-causing organism. The present study emphasizes on the expression of PR protein genes in the groundnut that are differentially expressed during the *S. rolfsii* infection using groundnut cultivars of varying resistance. The central hypothesis of this study is that the genes related to disease resistance would be up-regulated more in the tolerant genotypes compared to the susceptible genotypes while genotypes with intermediate tolerance would show intermediate levels of gene expression.

Presently unavailability of immune or absolute stem rot resistant groundnut genotypes stands as the reason for scarce knowledge about host–pathogen interactions at the plant surface and cellular level. A few studies have examined the interaction between a pathogen and a resistant groundnut genotype at the cellular or histological level. Though extensive study have been made on *Sclerotinia sclerotiorum,* a necrotrophic fungi similar to *S. rolfsii*, no information are available till date to describe the mechanism of tolerance in groundnut genotypes against the pathogen. The stem rot causing organism, *S. rolfsii*, seems to follow a similar mechanism of infection, hence the present study aimed at understanding the interaction of the pathogen during the initial entry and the subsequent structural defense mechanism happening in groundnut genotypes.

Keeping the above facts into consideration, following research objectives were made to understand the biochemical, histo-pathological and molecular basis of innate and fluorescent Pseudomonad induced stem rot tolerance in groundnut.

OBJECTIVES

1. Preliminary screening of groundnut genotypes for stem rot tolerance under high disease pressure in sick plot
2. To identify potent antagonistic *Pseudomonas fluorescens* isolate against *Sclerotium rolfsii* from groundnut growing areas of Gujarat
3. To investigate the histo-pathological basis of *S. rolfsii* infection in groundnut
4. To study the induced biochemical changes during host,-pathogen and-biocontrol agent interactions
5. To study the differential expression of defense related gene(s) in tolerant and susceptible genotypes

CHAPTER – II

REVIEW OF LITERATURE

This chapter covers the recent literature on biochemical, histo-pathological and molecular aspects innate and *Pseudomonas* induced stem rot tolerance in groundnut as well as to fill up the research gap, other related and relevant references have been briefly reviewed under appropriate sub-headings.

2.1 THE GROUNDNUT CROP: TAXONOMY AND ORIGIN

Groundnut (*Arachis hypogaea* L.) also known as peanut, is an important oil, food, and feed legume crop grown in over 100 countries. It is an important food as well as an oilseed crop of the tropical and sub-tropical world (Annonymous, 2011). The groundnut is believed to originate in South America specifically southern Brazil and northern Paraguay. The Portuguese introduced this crop into India in the 16th century. The cultivated groundnut, belonging to the family Fabaceae (Leguminosae), is classified into two subspecies, subsp. *fastigiata* Waldron and subsp. *hypogaea* Krap. *et*. Rig. The subsp. *hypogaea* contains two varieties, var. *hypogaea* and var. *hirsute* (Krapovickas and Gregory, 1994).

Groundnut is an allotetraploid (2n = 2x = 40) with “AA” and “BB” genomes. All species, except the cultivated species (*A. hypogaea* and *A. monticola*) in Section *Arachis*, and certain species in Section *Rhizomatosae*, are diploid (2n = 2x = 20). The diploid progenitors, *A. duranensis* and *A. ipaensis*, contributed “AA” and “BB” genomes, respectively, to the cultivated groundnut (Kochert *et al*., 1996). The phylogenetic analyses based on intron sequences and microsatellite markers also provide evidence for this hypothesis (Moretzsohn *et al*., 2012). The genetic diversity of the genus is classified into four gene pools (Janila *et al*., 2013): primary gene pool consisting of *A. hypogaea* and *A. monticola*, secondary consisting of diploid species from Section *Arachis* that are cross-compatible with *A. hypogaea*, tertiary consisting of species of the Section Procumbentes that are weakly cross-compatible with *A. hypogaea*, and the fourth gene pool consisting of the remaining wild *Arachis* species classified into seven other sections.

2.2 NUTRITIOUS GROUNDNUT

Groundnut (*Arachis hypogaea* L.) is unique in that it can be consumed directly as an item of food and also utilized in diverse ways. With about 26% protein, 48% oil, and 3% fiber and high in calcium, thiamine and niacin contents, it has all the potential to be used as a highly economical food-supplement to fight malnutrition that occurs due to deficiencies of these nutrients in the cereal grains like wheat and rice. As reported by NNDSR (2015), 100 groundnuts provide 570 calories and are an excellent source (defined as more than 20% of the Daily Value of several B vitamins, vitamin E, several dietary minerals, such as manganese (95% DV), magnesium (52% DV) and phosphorus (48% DV), and dietary fibres . The proximate composition of nutrients present in groundnut is given in the Table 2.2.1.

Table 2.2.1 Nutritional composition of raw groundnut

NUTRIENTS	Unit	Amonut in 100 g
Proximates		
Water	g	6.39
Energy	kcal	570
Protein	g	26.15
Total lipid (fat)	g	49.6
Carbohydrate, by difference	g	15.83
Fiber, total dietary	g	9.5
Minerals		
Calcium, Ca	mg	106
Iron, Fe	mg	3.91
Magnesium, Mg	mg	188
Phosphorus, P	mg	388
Potassium, K	mg	744
Sodium, Na	mg	22
Zinc, Zn	mg	2.12
Vitamins		
Thiamin	mg	0.675
Riboflavin	mg	0.135
Niacin	mg	15.925
Vitamin B-6	mg	0.348
Folate, DFE	µg	240
Lipids		
Fatty acids, total saturated	g	7.098
Fatty acids, total monounsaturated	g	22.325
Fatty acids, total polyunsaturated	g	17.238

2.3 PRODUCTION CONSTRAINTS

There is large gap between potential pod yield and the realized pod yield in most of the situations (Johansen and NageswaraRao, 1996). Potential yield is defined as the maximum yield obtainable by the best genotypes in a specified agro-climatic environment when the known biotic and abiotic constraints are overcome. The yield gap in the groundnut grown under water limiting conditions in rainfed areas is further aggravated by incidence of a host of diseases and insect pests. Therefore, tolerance/resistance traits that offer protection against losses caused by biotic and abiotic stresses are important target traits. The biotic and abiotic stresses limit the production during the rainy season. The major abiotic stresses are: soil moisture deficit stress at one or the other stage of crop during rainy season; low temperature prevailing during germination as well as vegetative stages followed by high temperature during the pod filling and maturation stages during summer season. In addition, build up of salinity and acidity and deficiencies of micronutrients in certain areas, lower the productivity.

Among the biotic stresses, the foliar fungal diseases (early leaf spot, late leaf spot and rust), viral diseases (peanut bud necrosis diseases and peanut stem necrosis disease), soil borne diseases (stem rot, collar rot, and pod rot complexes), and the insect pests like defoliators (red hairy caterpillar, tobacco caterpillar, gram pod borer and leaf miner) and sucking pests (jassids, aphids, and thrips) are the major ones (Janila *et al.*, 2013). Problems of nematodes and white grubs are also encountered in certain areas. In most breeding programs across the world, breeding for resistance to diseases has received more attention than breeding for resistance to insect pest except when they are vector of viral disease. Another important reason for this is the availability of the resistant sources for diseases in cultivated and wild *Arachis* species.

2.4 STEM ROT OF GROUNDNUT

Stem rot, caused by *Sclerotium rolfsii*, is a potential threat to groundnut production in many warm, humid areas, especially where irrigated groundnut cultivation is expanding. In Saurashtra region of Gujarat, groundnut is affected severely by soil borne diseases *viz.,* Collar rot, stem rot and pod rot. Stem rot has emerged as one of the most potential disease in groundnut in the last few years. Due to aberrant rainfall pattern, shifting cultivation and unavailability of varieties showing absolute resistance against this disease, has sometimes led to a heavy economic loss to the farmer growers.

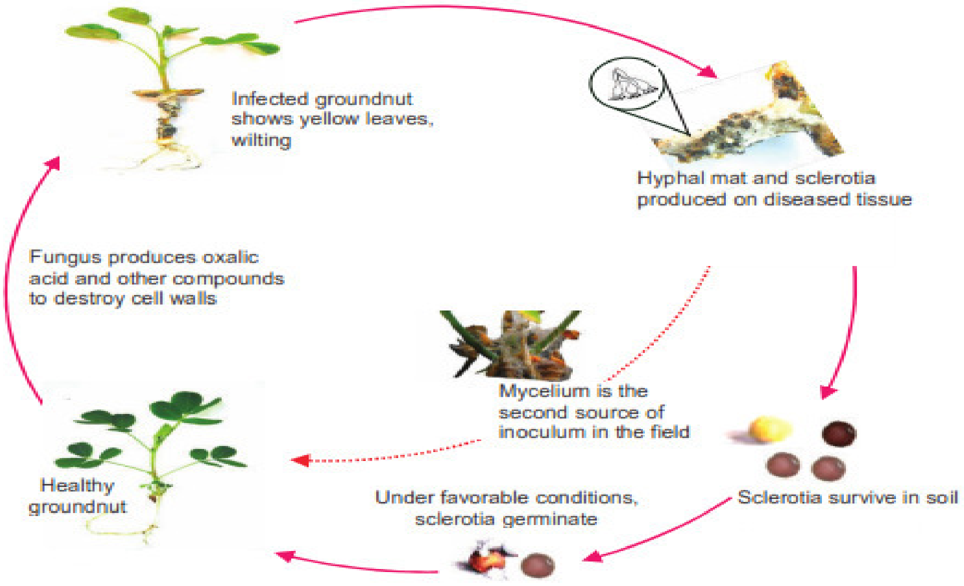
2.4.1 *Sclerotium rolfsii*

The pathogen *Sclerotium rolfsii* Sacc., is a soil-borne pathogen that commonly occurs in the tropics, sub-tropics and other warm temperate regions of the world causing root rot, stem rot, wilt and foot rot on more than 500 plant species including almost all the agricultural and horticultural crops (Farr *et al.,* 1989). *S. rolfsii* was first reported by Rolfs (1892), later the pathogen was named as *S. rolfsii* by Saccardo (1911). Higgins (1927) worked in detail on physiology and parasitism of *S. rolfsii*. The taxonomic classification of the pathogen has been given as under.

Kingdom	Fungi
Phylum	Basidiomycota
Class	Agaricomycetes
Order	Agaricales
Family	Typhulaceae
Genus	*Sclerotium*
Species	*Sclerotium rolfsii*

2.4.2 Disease Cycle

The pathogen survives as a saprophyte on plant debris, even debris from non-host crops. Sclerotia survive well (3-4 years) at or near the soil surface but survive poorly when buried deep because the fungus has a high oxygen demand (Mehan *et al.,* 1994). Infection starts from sclerotia that germinate eruptively in the presence of volatile compounds from decaying organic matter under warm and moist conditions. Any part of the groundnut plant that comes in contact with the soil is infected with fungus. In warm and high moisture condition, the occurrence of stem rot usually coincides with early stages of peg and pod development.



(Disease cycle of *Sclerotium rolfsii*)

Stem rot develops at all the growth stages (10-90 days) but disease development is slow in older (more than 40 days old) plants than the younger plants (Pande *et al.,* 1994). Sclerotia are the principal overwintering structures and the primary inoculum source for the disease (as shown in picture, Le, 2011). Under favourable conditions, sclerotia germinate and fungal hyphae grow towards and attack the lower part of the stem base. On diseased tissues, hyphal mat and sclerotia are produced.

2.4.3 Symptomatology

The primary symptoms are browning and wilting of leaves and branches which are still attached with the plant. The fungus preferentially infects stem by forming a whitish mycelia mat around the stem, but it can also infect any part of the plant including root, leaf and pod. In heavy soils, fungus damages groundnut plants near the soil surface but in light soils it can reach up to pod level, causing severe damage to pegs and pods (Mehan and McDonald 1990). Infected pods are covered by mycelia usually rot. When bark is peeled off, the inner tissue shows a brown to yellow discoloration. Leaves of infected plants turn brown, dry and often remain attached to the dead stem. Drying or shrivelling of the affected branches ultimately lead to death of the complete plants after wilting.

2.4.4 Epidemiology

Stem rot is a problem in warm and wet tropical and subtropical areas. The pathogen survives as mycelium on dead organic material when living susceptible plants are not present. It also survives as sclerotia in soil. Research has shown that fungal infection is enhanced when dead organic materials are available around susceptible plants. Temperature and moisture are very important factors in the spread and development of this pathogen. Hyphal growth occurs over a temperature range of 8-40ºC, but optimal growth and sclerotia production occurs between 27-35ºC. In addition to temperature effects, hyphal growth and sclerotia germination require a water-saturated soil. High humidity also favors fungal development. Host penetration and the fungal infection proceeds optimally at 27-30 ºC, when the moisture and high humidity are present (Mehan and McDonald, 1990). *S. rolfsii* survives adverse conditions as sclerotia or as mycelium in diseased plants or plant debris. Sclerotia formation is favored by temperature of 30ºC, ample moisture, soil pH below 7, and well-aerated, light soil. Sclerotia survive best when present at or near the soil surface in well-drained soil. Mycelium does not usually survive below freezing temperatures, but sclerotia are known to survive in locations where below freezing temperatures have occurred (Pande *et al.,* 1994).

2.5 PATHOGENICITY AND PRODUCTION OF PLANT CELL WALL DEGRADING ENZYMES

When a fungus encounters a potential host, the cell wall is an important barrier that plants use to limit pathogen attack. Plant cell walls are heterogeneous structures, composed of polysaccharides, proteins, and aromatic polymers. The composition and structure of the cell wall share basic principles: all contain cellulose microfibrils embedded in a matrix of pectin, hemicellulose, lignin, and structural proteins. To overcome the barrier of the plant cell wall, phytopathogenic especially necrotrophic fungal pathogens such as *S. rolfsii*, *Botrytis cinerea*, etc., produce toxins that inactivate the host cells, or cell wall degrading enzymes (CWDEs) that degrade the plant cell wall polymers prior to fungal penetration. Pectinases and cellulases break down pectin (compound gluing together cell walls) and cellulose, the two major polymers that maintain the firmness and structure of plant cell walls. Production of CWDEs often determines the pathogenicity of necrotrophic pathogens (Collmer and Keen, 1986).

Cellulase enzymes designated as C1, C2 and Cx are required to degrade cellulose. The C1 and C2 enzymes act upon native, insoluble cellulose to produce linear chain that is attacked by the Cx enzyme to produce cellobiose and glucose (MacHardy and Beckman, 1981). Polygalacturonase (PG) considered as important pectolytic cell wall degrading enzymes produced by phytopathogenic fungi during the process of infection and colonization of host plant. PG is a highly polymorphic enzyme and exhibits either an endo or exo mode of action (Posada *et al*., 2000). Production of oxalic acid and polygalacturonases are involved in the early stages of pathogenesis of *S. rolfsii* (Bateman and Beer, 1965).

2.6 MANAGEMENT OF STEM ROT

As with most fungal soil-borne plant pathogens, disease management involves exclusionary practices, plant removal, soil removal or treatment, plant treatment, crop rotation, use of resistant varieties, or a combination of these practices. The specific management practices used depends upon the crop and cropping situation. For control of this disease, growers prefer chemical applications followed by recently getting popular biocontrol agents.

2.6.1 Chemical control

Application of fungicides gives effective control of stem rot in groundnut. The effectiveness of fungicides use is dependent upon the method of application, time of application, optimum dose and weather factors. Stem rot is a seed and soil-borne disease so that it is important to treat the seed with vitavax @ 2 $g.kg^{-1}$ or tebuconazole @ 1.5 g kg^{-1} and spray tebuconazole @ 1 ml L^{-1} at 45 and 60 days after sowing for effective control of stem rot (Anonymous, 2011). Application of tebuconazole 2 % DS @ $g.kg^{-1}$ seed before sowing provide excellent control of stem rot of groundnut (Gururaj, 2012).

2.6.2 Growing of tolerant cultivars

Stem rot has wide host range, and is capable of producing several non-specific metabolites, oxalic acid and several cell wall degrading enzymes, hence it is logical to predict a low probability for success in finding useful levels of host resistance. This may be one of the reasons for the relatively low emphasis placed on breeding groundnuts for resistance to *S. rolfsii* (Mehan *et al*., 1994). At present there is no cultivars are known to be resistant to *S. rolfsii* in groundnut but some genotypes and breeding lines have been found to show lower than average susceptibility to stem rot under field conditions. Growing of tolerant groundnut varieties like GG 16 (JSP 39), Dh 8 and OG-52-1 may be found effective to stem rot under field conditions (Kumar *et al*., 2013). A tolerant line (CS19) for stem rot has been developed at Directorate of Groundnut Research, Junagadh (Bera *et al*., 2014).

2.6.3 Biological control

Management through chemical methods leads to ill effects like residual toxicity, environmental pollution and fungicide resistance. Although seed treatment with fungicides is recommended to minimize the infection at early stages, it does not give prolonged protection. Management of *S. rolfsii* through soil application of fungicides is difficult because of its broad host range as well as its worldwide distribution which precludes such strategy. Once established in the soil, it is difficult to eliminate the pathogen. Biological control has been proved to be a promising disease- management technology especially against soil-borne plant pathogens.

Application of beneficial microorganisms to soil, seeds or planting materials has been proposed as a sustainable and supplementary approach to control plant diseases (Cook and Baker, 1989). The most widely studied microorganisms with antagonistic activity against plant pathogens and with beneficial effects on plant growth, belong to the bacterial genera *Bacillus*, *Pseudomonas, Rhizobium,* or the fungal genus *Trichoderma* (Ongena and Jacques, 2008; Raaijmakers *et al.,* 2009; Lorito *et al.,* 2010).

Fungal biocontrol agents may directly or indirectly kill sclerotia or mycelium of *S. rolfsii*. Lectins produced by *S. rolfsii* were proposed to serve as recognition factors for fungal biocontrol agents. In a direct interaction, hyphae of *Trichoderma* penetrate the rind and the cortex of sclerotia and lyse the medullar tissue. Degraded sclerotia become dark, soft and disintegrate under slight pressure and it was shown that chitinase and ß-1,3-glucanase play a role in the interaction between *Trichoderma harzianum* and *S. rolfsii* (Prasad and Naik 2008). Next to *Trichoderma,* several other fungal genera have been tested for their ability to control diseases caused by *S. rolfsii* on bean, carrot, chilli, ginger, wheat, lentil, sesame, soybean, sugar beet, sunflower, tomato, or groundnut.

For biocontrol of *S. rolfsii,* among the identified bacterial genera and species, most of them belong to the genera *Pseudomonas* and *Bacillus* (Le, 2011). *Pseudomonas* strains can restrict *in vitro* hyphal growth or reduce germination of sclerotia of *S. rolfsii* (Ganesan and Gnanamanickam 1987; Tonelli *et al.,* 2010). Next to *Pseudomonas*, several *Bacillus* species and strains have been studied for their efficacy to control stem rot disease of groundnut. Pretreatment of groundnut seeds with *Bacillus subtilis* protected groundnut seeds against *S. rolfsii* and significantly increased the number of pods (Abd-Allah, 2005).

It is well known that all the isolates of biocontrol agents such as Pseudomonads are not equally antagonistic toward a particular species of pathogen. This emphasizes the need for identifying isolates specifically effective against *S. rolfsii*, for a planned regional deployment.

2.7 FEW SPECIES OF FLUORESCENT *PSEUDOMONADS* ARE BIOCONTROL AGENT

The *Pseudomonas* are motile (one or several polar flagella), non-sporulating rod shaped gram-negative bacteria, genome having 58–69 % GC content (Palleroni, 1973). They are catalase positive and chemo-organotrophic, with a strictly respiratory metabolism (using oxygen and in some cases nitrate as terminal electron acceptor). The fluorescent Pseudomonads include all *Pseudomonas* species with the ability to produce fluorescent pyoverdine siderophore(s), noticeably *P. aeruginosa, P. syringae, P. putida* and *P. fluorescens* (Bossis *et al*., 2000). *Pseudomonas* species are adapted to survive in soil and colonization of plant roots (Kiely *et al*., 2008).

2.7.1 Molecular identification of Pseudomonads through 16S sequencing

16S rDNA sequence has long been used as a taxonomic gold standard in determining the phylogenies of bacterial species (Woese, 1987). The current state of the *Pseudomonas* taxonomy makes it difficult to assess the phylogenetic distribution of biocontrol agents within *P. fluorescens* and closely-related fluorescent pseudomonads (Bossis *et al*., 2000). However, it seems clear that these taxa include both biocontrol agents and strains without any obvious biocontrol potential, regardless of whether only true *P. fluorescens* or also related fluorescent pseudomonads are considered (Sanguin *et al*., 2009).

2.7.2 Use of anatgonistic Pseudomonads as bio-control agent

One of the pre-requisites for the efficiency of biocontrol agents in controlling the plant diseases is their capacity to survive in the target sites. *P. fluorescens* has been shown to reduce soil borne diseases when applied as seed, soil or root inoculant (Weller *et al*., 2007). Various strains of *Pseudomonas fluorescence* showed known biological control activity against certain soil-borne phytopathogenic fungi and have a potential to produce known secondary metabolites such as siderophore, HCN and protease that showed antagonistic activity against *Macrophomina phaseolina*, *Rhizoctonia solani*, *Phytopthora nicotianae* var. *parasitica*, *Pythium* sp. and *Fusarium* sp. (Ahmadzadeh *et al.,* 2006). *Pseudomonas fluorescens* strains possess multiple mechanism of antagonism and stable production of antifungal agents under variable growth conditions to sustain antagonism during plant root development. They produce plant growth promoting traits like phosphatase and IAA along with fungistatic metabolites such as siderophores, HCN and proteases, hence can be explored as one among the best biocontrol agent against phyto-pathogens (Anand *et al.,* 2010). Patil *et al*., (1998) isolated *P. fluorescens* strain *FDP-15* from groundnut roots was most efficient and ecologically fit strain. This strain improved seed germination, nodulation, dry weight and pod yield as well as protected plants from sclerotial infection compared with captan. *FDP15* increased seedling emergence by 16%, nodulation 18%, dry weight 40%, total pod yield 65% and resulted in 18% greater survival of plants up to harvest. Sheela and Packiaraj (2000) studied the management of collar rot disease by the *Pseudomonas fluorescens* and found that *Pseudomonas* treatment was best for reduction of collar rot disease by 6.63 %. Leaf spot disease caused in groundnut by *Cercosporidium personatum* was reduced when the groundnut seeds were treated with *P. fluorescens* Pf 1, along with a foliar spray at seedling stage. The pod yield increased and maximum disease protection was observed 30 days after sowing by seed treatment with *P. fluorescens* (Meena *et al*., 2000). They further found that foliar application of *P. fluorescens* strain Pf1 significantly controlled late leaf spot and rust (*Puccinia arachis*) in greenhouse conditions (Meena *et al.,* 2001). Rangeshwaran and Prasad (2000) have shown that *Pseudomonas fluorescens* and *Pseudomonas putida* are capable of inhibiting *S. rolfsii* up to 60 to 63 per cent in sunflower. *Pseudomonas fluorescens* are highly effective in inhibition of seed carried *S. rolfsii*.

Further combine use of *Pseudomonas* and *Trichoderma* has proven to be effective against stem rot and collar rot disease in groundnut (Manjula, 2004). *Pseudomonas s*pp. have shown to reduce the seedlings mortality in groundnut by about 60% compared to the control as reported by Kishore *et al.* (2005). The same author has also shown that some *Pseudomonas* strains inhibit the *in vitro* activity of cell wall degrading enzymes (CWDE) like polygalacturonase and cellulase produced by *S. rolfsii* upto 55%. However, Paramageetham and PrasadBabu, (2012) demonstrated that some fluorescent *Pseudomonas spp.,* isolated from Eastern Ghats forest litter have antagonistic activity against *Sclerotium rolfsii* and are capable of inhibitiing radial growth of the fungus in *in-vitro*, which ranged from 44 to 74%. Evaluation of rhozospheric *Pseudomonas* spp., by Rakh, (2011) has also reported that the *Pseudomonas cf. monteilii* 9 inhibited the *Sclerotium rolfsii* up to 94% in terms of dry weight. The antifungal activity of *Pseudomonas* strains indicates the possibility of using *Pseudomonas fluorescens* as a biological control agent for some plant pathogenic fungi. However, this requires further screening of a large number of *Pseudomonas* strains from different regions of India. Hence the present study aimed at isolating and identifying fluorescent pseudomonads isolates from the groundnut growing areas of Saurasthra region of Gujarat, potentially capable of inhibiting *Sclerotium rolfsii,* the causal organism of stem rot in groundnut.

2.8 BIOCONTROL MECHANISM OF ANTAGONIST

Several methods of disease suppression have been implicated in biological control by *Pseudomonas spp*. The most frequently cited of these are competition, iron limitation through siderophore production, antibiosis, and induced systemic resistance (Haas and Defago, 2005).

2.8.1 Antibiosis/Antibiotic Production

One of the advantages of using *Pseudomonad* spp as biocontrol agents is their ability to produce numerous secondary metabolites which are potent antimicrobial allelochemicals (Raaijmakers *et al.,* 2002). The production of broad spectrum antibiotics has been determined to be the primary mechanism of disease suppression in classic suppressive soils (Haas and Defago, 2005). These antibiotics may also be involved in triggering induced systemic resistance. For six general categories of antibiotic including phenazines, phloroglucinols, pyoluteorin, pyrrolnitrin, cyclic lipopeptides and hydrogen cyanide (HCN) there was significant experimental evidence confirming their role in disease suppression (Raaijmakers *et al.,* 2002). All of these antibiotics are diffusible molecules with the exception of HCN, which is a volatile compound (Haas and Defago, 2005).

2.8.2 Competition for Nutrients and Siderophore Production

Competition is considered as a ‘classical’ mode of action of biological control. It involves competition between biocontrol antagonist and phytopathogen for space and nutrient (Kaur *et al.*, 2006). Siderophores are iron chelating molecules produced by microorganisms in response to low environmental iron concentrations. These molecules help the microorganism to acquire iron, which is often biologically unavailable in the environment, and create iron limiting conditions for competitors, including pathogenic microorganisms ((Haas and Defago, 2005). The earliest study by Kloepper and his coworker, (1980) showed strong evidence of siderophore production as a mechanism of disease suppression with increased yield and suppression of *Erwinia carotovora* in different crops. The primary siderophore molecule produced by *Pseudomonas fluorescens* is pyoverdine, the yellow-green pigment that gives them their characteristic fluorescence under UV light. Secondary siderophores include pyochelin and salicylic acid. In addition to producing their own siderophores, beneficial *Pseudomonas* can further compete for iron through the uptake of xeno-siderophores produced by other species (Kaur *et al.*, 2006).

It was noted that siderophore mediated biocontrol was strongly influenced by the bio-availability of iron, which varies with pH, and may not be a major factor in disease suppression. Haas and Defago, (2005) reported that there were many *Pseudomonas fluorescens* that produce pyoverdine but do not control plant pathogens. Mazumdar *et al.* (2007) reported that siderophore production of varying range in a king'sB medium by nine isolates of *Pseudomonas fluorescens* as per requirement for iron by specific isolates. They suggested that it could be their inherent attribute. Kandoliya and Vakharia, (2013) also stated the siderophore-mediated antibiosis is most often associated with fungal suppression by *Pseudomonas fluorescens* in the rhizosphere.

2.8.3 Mycoparasitism and production of mycolytic enzymes

One of the major mechanisms used by biocontrol agents to control soil-borne pathogens involves the production of cell wall degrading enzymes. Cell wall degrading enzymes such as β-1, 3-glucanase, chitinase, cellulase, and protease secreted by strains of biocontrol agents exert a direct inhibitory effect on the hyphal growth of fungal pathogens. Cell wall degrading enzymes of rhizobacteria affect the structural integrity of the walls of the target pathogen (Budi *et al*., 2000). Examples of protection from phytopathogenic infection as a result of the activity of cell wall degrading enzymes include control of *Sclerotium rolfsii* and *F. oxysporum* on beans (Felse and Panda, 1999). Among the successful biocontrol agents against *S. rolfsii*, next to *Trichoderma* species, fluorescent Pseudomonads are the most widely studied and accepted. Fluorescent pseudomonads are shown to produce lytic enzymes which are also abundant in many plant species after infection by different type of pathogens. Several studies indicated that the antagonistic potential of *P. fluorescens* against various soilborne plant pathogens is correlated with production of lytic enzymes (Haas and Defago, 2005).

2.8.3.1 Chitinases

Chitinase attack on chitin molecules which are the main structural component in fungal cell wall and insect's skeleton. Among the Gram-negative bacteria, *Serratia marcescens*, *Enterobacter agglomerans*, *Pseudomonas aeruginosa*, and *P. fluorescens* have been found to have chitinolytic activities (Nielson and Sorenson, 1999). Someya *et al*. (2000) reported that the mycelia of the fungal pathogens co-inoculated with biocontrol strain showed various abnormalities such as partial swelling in the hyphae and at the tip, hyphal curling or bursting of the hyphal tip. Ganesan and Sekar, (2004) observed that the among biocontrol agents against *Rhizoctonia solani* causing web blight disease of groundnut, potentially better strains produce higher amount of β-1,4-endoglucanase, indicating their vital role in inhibiting the *in-vitro* growth of the pathogen. In contrast to the above authors, Nagrajkumar *et al.* (2004) observed no significant relationship between the antagonistic potential of *P. fluorescens* strains and their level of chitinase production. However, evaluation of different isolates of *Pseudomonas fluorescens* for their biocontrol potential against the *A. niger,* in groundnut, Deshmukh *et al.* (2015)

observed a positive association between between the antagonistic potential of *P. fluorescens* strains and their level of chitinase production. Further, in particular, a strain of *Pseudomonas aeruginosa,* exhibited strong antagonistic activity against *Sclerotinia sclerotiorum*, *in vitro* and *in vivo* (Gupta *et al.*, 2006). This strain produced extracellular chitinase enzyme and its role was clearly demonstrated through Tn5 mutagenesis. Bacterization of groundnut seeds with GRC resulted in increased seed germination and reduced stem rot of groundnut in *S. rolfsii* infested soil by 97%.

2.8.3.2 β-1,3 Glucanases

The enzyme β-1,3-glucanase produced by the biocontrol agents like *P. fluorescens* have a capacity to hydrolyze the branched β-1,3-glucans found in the most fungal cell walls (Fridlender *et al.*, 1993). The β-1, 3-glucanase synthesized by many biocontrol strains lyse fungal cell walls. For instance, *Bacillus cepacia* synthesizes β-1, 3-glucanase, which destroys the cell walls of the soil-borne pathogens *R. solani*, *P. ultimum*, and *S. rolfsii* (Compant *et al.*, 2005). Similarly, Nagrajkumar *et al.* (2004) showed the significant relationship between the antagonistic activity of *P. fluorescens* strains and their level of production of β -1,3-glucanase. Gajera and Vakharia, (2012) showed β-1,3 glucanase inhibited the growth of pathogens in synergistic co- operation with chitinase during antagonism. While evaluating different isolates of *Pseudomonas fluorescens* for their biocontrol potential against the *A. niger,* the causal organism of collar rot of groundnut, Deshmukh *et al.* (2015) observed that some non potential isolates also produce higher amount of β-1,3-glucanase and on the contrary few potential isolates showing better *in-vitro* growth inhibition, produce less amount of the enzyme.

2.8.4 INDUCED SYSTEMIC RESISTANCE AND PRODUCTION OF ANTIFUNGAL METABOLITES AND GROWTH PROMOTING SUBSTANCES BY FLUORESCENT PSEUDOMONADS

2.8.4.1 Production of Salicylic Acid

Salicylic acid (SA) is a phenolic compound that affects a variety of biochemical and molecular events associated with induction of disease resistance. SA has been shown to play an important role in expression of both local resistance controlled by major genes and systemic induced resistance developed after an initial pathogen attack (Ton *et al.*, 2002). Under conditions of iron limitation, the Pseudomonad strains produce SA *in vitro.* This may explain the increased effectiveness of biocontrol strains upon iron limitation (Leeman *et al.*, 1996). This would suggest that induced systemic resistance (ISR) is triggered by biocontrol strains depends on SA signaling in the plants. The ability of *P. aeruginosa* 7NSK2 to elicit ISR against *Botrytis cinerea* in bean was linked to SA production (De Meyer and Hofte, 1997) where as induced resistance elicited by *P. aeruginosa* 7NSK2, and strains of *P. fluorescens* against the bacterial pathogen *Pseudomonas syringae pv. tomato* (Pst) was observed. In *Arabidopsis*, Ran *et al.* (2005) demonstrated that bacterially produced SA is not required. Salicylic acid is a precursor or intermediate in the biosynthesis of certain types of siderophores, (Bultreys *et al.*, 2006). Application of exogenous SA at a concentration of 1 to 5 mM has been long known to induce pathogenesis-related (PR) gene expression and acquired resistance against a variety of microbial pathogens (Meena *et al.*, 2001). Saikia *et al.* (2005) showed through *in-vitro* assay that all the isolates of *P. fluorescens* produce SA in synthetic medium but a significant negative correlation was observed in concentration of salicylic acid production and the respective mycelial growth inhibition of *Fusarium oxysporum* f. sp. *Ciceri*. However, Nagarajkumar *et al.* (2004) observed a significant relationship between inhibitory activity of *P. fluorescens* strains *in vitro* and their level of SA production. Similar findings were observed by Kandoliya and Vakharia, (2013) supporting the role of salicylic acid production *P. fluorescens* isolate in *in vitro* antagonism.

2.8.4.2 Production of Siderophores

Siderophores are fluorescent, yellow-green, water soluble, low molecular weight compounds (400–1, 500 Da) secreted by many *P. fluorescens* isolates under iron-limiting conditions (Sullivan and Gara, 1992), which preferentially chelate iron (Fe^{3+}) and transport it into the cell across the cell membrane (Wandersman and Delepelaire 2004). The siderophores bind most of the Fe^{+3} in the rhizosphere and effectively prevent the proliferation of fungal pathogens by depriving them of available iron (Sulivan and Gara, 1992). Suppression of the pathogens arises because iron deficiency causes growth inhibition, decrease in nucleic acid synthesis, inhibition of sporulation, and causes changes in cell morphology (Mathiyazhagan *et al.*, 2004). It was proved that fluorescent siderophore production as a mechanism of bio-control of the wilt disease by the *P. fluorescens* (Loper and Henkels 1999). It was further proved by Kandoliya and Vakharia, (2013), reporting that among the selected isolates of *Pseudomonas fluorescens,* the best antagonist produced the highest siderophore.

Siderophores show induced systemic resistance (ISR) in plants (De Vleesschauwer *et al.*, 2006). ISR is a state of enhanced defensive capacity developed by a plant when appropriately stimulated, and effective against a broad spectrum of both soilborne and foliar pathogens Whereas siderophore-mediated suppression of plant diseases is restricted to the site where biocontrol bacteria and pathogens contact each other, ISR is effective throughout the plant and reduces disease incited by pathogens also at sites distant from the inducing bacteria. Rhizobacteria-mediated induced resistance resembles pathogen-induced systemic acquired resistance (SAR), which is dependent on salicylic acid (SA) as a signalling compound (Van Loon *et al.*, 1999). Siderophores produced by certain strains of the *P. fluorescens-putida* group are also responsible for enhanced plant growth and biocontrol and are most often associated with fungal suppression in the rhizosphere of several crops (Battu and Reddy, 2009).

2.8.4.3 Production of Indole Acetic Acid like compounds

Rhizobacteria like *Pseudomonas fluorescens* establish positive interaction with host plant are called plant growth promoting rhizobacteria (PGPR). PGPR play important role in phytostimulation, phytoremediation and biofertilizaiton. These organisms also provide protection to plants against diseases by suppressing pathogenic microorganisms. *Pseudomonas* make up a dominant population in soil and rhizosphere and exert growth promoting influence on a variety of plant species because of their strong competitive behavior, colonization potential and sustainability (Glick, 1995). The intrinsic ability of *Pseudomonas fluorescence* to produce IAA was observed by many researchers (Glick, 1995; Caron *et al.*, 1995, Mazumdar *et al.,* 2007). It is assumed that plant growth regulators produced by *Pseudomonas* species could also influence plant growth.

Isolates of *P. fluorescens* of groundnut rhizosphere were shown to produce IAA in range of 3.6-11.8 ppm and enhance the plant growth (Pal *et al.,* 1999). Intrinsic ability of both *P. fluorescens* and *P. aeruginosa* isolates to produce IAA was reported by Karnwal, (2009). It was further suggested by Kandoliya and Vakharia, (2013), that the antagonistic behaviour of *Pseudomonas fluorescens* has a strong correlation with the capability of the isolates to produce IAA *in vitro*.

2.9 INDUCED BIOCHEMICAL CHANGES DURING HOST-PATHOGEN INTERACTIONS

Plants have developed a highly sophisticated antioxidative defense system to cope with many biotic and abiotic stresses (Heidari, 2009). Utilization of plant's own defense mechanism against stress is an attractive strategy that enables the plants to thrive well in hostile environment. After pathogen recognition, highly localized biochemical events are rapidly induced to inhibit the further development of the attacking pathogen. The rapid production and accumulation of reactive oxygen species (ROS), particularly the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) (Heller and Tudzynski, 2011), culminates with a hypersensitive response (HR) and localized programmed cell death (PCD) to impair the pathogen establishment and development inside the host tissues and deprive the pathogen of further access to nutrients. Concomitant with or following HR, several pathogen defense-related genes are translated into antioxidative enzymes and PR-proteins that confer resistance to the plant in defense against pathogen attack. The necrotropic pathogens normally launch the plant into cell-death program, thereby short circuiting a strong defense response and providing a ready source of pathogen nourishment (van Loon *et al.,* 2006). The products of activated defense genes which are synthesized *de novo* during periods of pathogenic stress have been well documented in several host-pathogen interactions but such information is really lacking in groundnut-*S. rolfsii* system.

2.9.1 Induction of antioxidant defense system

One of the earliest biochemical changes observed after pathogen recognition is the oxidative burst and generation of reactive oxygen species (ROS) (Nanda *et al.*, 2010). Excessive levels of ROS is potentially damaging to plant cell structure and function unless detoxified by antioxidative systems (Agarwal *et al.*, 2005). Prevention of ROS toxicity requires a large gene network, the so called "ROS gene network", which is composed of at least 150 genes in *Arabidopsis* (Mittler *et al.*, 2004). Plant antioxidant defense systems including enzymatic and non-enzymatic protection have co-evolved with aerobic metabolism to counteract oxidative damage due to ROS. The enzymic antioxidants include superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), enzymes of ascorbate-glutahione (AsA-GSH) cycle such as ascorbate peroxidise (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) where as Ascorbate (AsA), glutathione (GSH), carotenoids, tocopherols, and phenolics serve as potent nonenzymic antioxidants within the cell (Singh *et al*., 2013). Previous studies have shown that alteration in the expression/activity of ROS-scavenging enzymes could also be a key step in the activation of plant defence against phytopathogens. It has been reported that the activity of the enzymatic antioxidant system limits the propagation of oxidative processes allowing cells to maintain their viability against the penetration of *Aphanomyces euteiches* and *S. sclerotiorum* in plant tissues (Burhenne & Gregersen, 2001; Peluffo *et al*., 2010; Djebali *et al*., 2011).

2.9.1.1 Superoxide Dismutase (SOD)

SOD is a metalloprotein catalysing the first step in the scavenging system of active oxygen by the disproportionation of superoxide anion radicals to hydrogen peroxide and oxygen. Based on the metal cofactor used by the enzyme, SODs are classified into three groups: iron SOD (Fe SOD) located in the chloroplast, manganese SOD (Mn SOD) located in the mitochondria, the peroxisome and copper-zinc SOD (Cu-Zn SOD) in the chloroplast, cytosol and possibly in the extracellular space (Alscher *et al.,* 2002).

It was reported by Durner and Klessig (1995) that the increase in the activities of superoxide dismutase in plants can be correlated with increased susceptibility to pathogens. WeiBo *et al.,* (1998) observed that SOD activity increased in leaves of infected plants, and, susceptible genotypes have higher SOD activity than that of tolerant genotyped when three groundnut cultivars with different susceptibilities were inoculated with stripe potyvirus. SOD activity changed little when seeds of five *Aspergillus flavus* resistant cultivars and four highly susceptible cultivars of groundnut were inoculated with a suspension of *A. flavus* (Xuan, *et al.,* 2002). Davar *et al*., (2013) showed that the invasion of the necrotrophic *S. sclerotiorum* resulted in significant increase of the activity of SOD in the leaves of both tolerant and susceptible genotypes of sunflower. The SOD activity increased steadily up to five days after *S. rolfsii* infection and thereafter progressively decreased SOD upto 10 days post infection. It was proposed that the enhanced activities of SOD upto 5 DAI may have helped in scavenging of the reactive oxygen derivatives which in turn may have led to susceptibility of cowpea to *S. rolfsii* infection (Nandi *et al.*, 2013).

2.9.1.2 Catalase (CAT)

Catalase is a tetrameric heme containing enzyme that is found in all aerobic organisms and serves to rapidly degrade H_2O into water and oxygen. Much earlier it was proposed that there is proliferation of peroxisomes during stress, which might help in scavenging of H_2O_2, which can diffuse from the cytosol (Lopez-Huertas *et al.*, 2000). Induction of CAT upon fungal infection in crop plants was reported by many researchers (Garcia *et al.*, 2002; Nafie, 2003; Luhová *et al.* 2006; El-Khallal, 2007 and Naglaa and Heba, 2011). The H_2O_2 scavenging system represented by ascorbate peroxidase and catalase are more important in partitioning tolerance than SOD as reported in oxidative stressed wheat varieties (Lafitte *et al.*, 2007). Chandrashekar and Umesha, (2012) suggested the possible role in triggering the development of host resistance and observed higher induction CAT activity in resistant seedlings than in susceptible and highly susceptible seedlings of tomato. Similarly, Davar *et al.* (2013) observed that *Sclerotinia* infection greatly induced CAT activity in the leaves of infected resistant plants. The CAT activity decreased in susceptible line, in both stem and leaves, whereas in the resistant line, after pathogen invasion, CAT activity increased. This phenomenon was supported by the high level of susceptible line colonization by *S. sclerotiorum* in comparison with resistant line. Recently, Kobeasy, et al. (2011) also observed induction of CAT activity in groundnut upon Peanut mottle virus. They further suggested that tolerance to the pathogen can be improved by enhancing antioxidative enzyme activity.

2.9.1.3 Ascorbate Peroxidase (APX)

Ascorbate peroxidase is a central component of AsA-GSH cycle, and plays an essential role in the control of intracellular ROS levels. It reduces H_2O_2 to water by utilizing ascorbate as a specific electron donor and glutathione peroxidase oxidized to form oxidized glutathione (Noctor and Foyer, 1998). APX found in organelles scavenges H_2O_2 produced within the organelles, whereas cytosolic APX eliminates H_2O_2 produced in the cytosol, apoplast or that diffused from organelles (Sharma and Dubey, 2004).

Garcia *et al.* (2002) noted the induction of APX in both roots and stems of susceptible chickpea cultivars when inoculated with the highly virulent race of *Fusarium* wilt. However, non-significant induction of the APX was observed in highly tolerant genotype on the fungal infection. Pseudomonads induced APX activity was observed by many researchers (De Meyer, *et al.* 1997; Chen *et al.*, 2000; Sundaravadana, 2002; Saravankumar *et al.*, 2003; Karthikeyan, 2006), however the induction was comparatively at higher levels in the resistant cultivars as observed by De'Ascensao and Dubery, (2003). Infestation of *H. armigera* and *A. craccivora* in groundnut, showed an induction APX in groundnut genotypes as reported by War *et al.* (2013) however, the degree of the response differed across the genotypes .

2.9.1.4 Glutathione Reductase (GR)

GR is a flavo-protein oxidoreductase, found in both prokaryotes and eukaryotes. It is a potential enzyme of the ASH-GSH cycle and plays an essential role in defense system against ROS by sustaining the reduced status of GSH (Reduced Glutathione). It is localized predominantly in chloroplasts, but small amount of this enzyme has also been found in mitochondria and cytosol (Creissen *et al.,* 1994). GR catalyzes the reduction of GSH, a molecule involved in many metabolic regulatory and antioxidative processes in plants where GR catalyses the NADPH dependent reaction of disulphide bond of GSSG and is thus important for maintaining the GSH pool. Actually, GSSG consists of two GSH linked by a disulphide bridge which can be converted back to GSH by GR. GR is involved in defense against oxidative stress, whereas, GSH plays an important role within the cell system, which includes participation in the ASH-GSH cycle, maintenance of the sulfhydryl (eSH) group and a substrate for GSTs. GR and GSH play a crucial role in determining the tolerance of a plant under various stresses (Reddy and Raghavendra, 2006).

The induction of GR in response to biotic stress has been proven by many researchers (Gullner *et al.,* 1997; Bariya *et al.,* 2011; Kovacs *et al.* 2011) and further the higher induction was observed for tolerant genotypes compared to susceptible one (Garcia-Limones *et al*., 2002). However, Paciolla *et al.* (2008) observed no significant difference in GR activity when tomato plants treated with fungal mycotoxin. It implies the Gr mediated ASH-GSH cycle may not be operational for all types of stresses in all genotypes.

2.9.2 Induction of Pathogenesis Related (PR) Proteins

Plant resistance to pathogenic fungi involves multiple reaction pathways including the accumulation of Pathogenesis related proteins such as peroxidase, chitinases and β-1,3 glucanase.

2.9.2.1 Peroxidase (POX)

Plant peroxidase has been implicated in the last enzymatic step of lignin biosynthesis, that is, the oxidation of hydroxyl cinnamyl alcohols into free radical intermediates, which subsequently are coupled to lignin polymer. Two types of peroxidase have been reported in plant system: (1) cell wall peroxidase involved in oxidative burst, which leads to hypersensitive cell death, (2) cytosolic peoxidase involved in oxidative cross-linking and lignification (Shivakumar *et al.,* 2003). Furthermore, peroxidase is involved in the production or modulation of active oxygen species which may play various roles directly or indirectly in reducing pathogen viability and spread (Prasad *et al*., 2005). Earlier studies suggest that peroxidases are important PR proteins and the plant expresses POX activity during host-pathogen interaction (Saikia *et al*., 2004). Sudhagar *et al.,* (2000) studied changes in the activities of POX in groundnut against rust pathogen and found that the susceptible genotypes showed increased enzyme activity at 80th and 90th day unlike in the resistant genotypes where activity initially increased then later decreased. High level expression of POX was reported in *P. fluorescens* treated chilli plants challenged with *Colletotrichum capsici* (Bharathi *et al*., 2004). Similarly, upon infection of *Sphaceloma arachidis* (groundnut scab) in groundnut seedlings, ShuMin *et al*., (2007) observed increase activity of POX in the leaves following disease development. Increased activity of POX and polyphenol oxidase (PPO) was observed in the groundnut plants treated with biocontrol agents followed by challenge inoculation with *A. atlernata* (Chitra *et al*., 2006). Pudjihartati *et al*., (2006) studied peroxidase activity of *S. rolfsii* infected groundnut tissue. Infection of the pathogen resulted in the increased peroxidase activity and lignin content in the infected tissues. Regression analysis between POX and disease severity showed negative slope, indicating the more resistance the genotype, more peroxidase activity in tissues.

Nandi *et al.* (2013) observed in cowpea that POX activity reached at its peak at three days after inoculation and maximum reduction of its activity was observed 10 days after inoculation. Their studies on POX activity and isozymes indicate that the suppression of POX after 5 days after infection leads to the weakening of defense mechanisms in *S. rolfsii* inoculated cowpea plants. This helps in the further spread of the pathogen and eventually severe collar rot symptoms are expressed. The results suggest that suppression of peroxidase was found to be one of the important factors responsible for the successful pathogenesis of *S. rolfsii.*

2.9.2.2 Chitinases

It is known that chitinases degrade chitin in fungal cell wall by hydrolyzing β-1, 4 linkages between N-acetyl glucosamine residues and also act as synergistically with the β-1,3 glucanase for inhibiting fungal growth. They are categorised in to eleven groups based on their primary structures and immunological properties. The chitinases can either be induced by pathogens or elicitors/chemicals. They are constitutively expressed at low levels in leaves and high level in roots and seeds. Increased levels of gene expression or enzymatic activity have been observed after elicitation (van Loon *et al.*, 2006).

The role of chitinases in controlling *S. rolfsii* was proved by Manjula *et al.* (2004) has reported four isolates of *Pseudomonas fluorescens*, GB4, GB8, GB10 and GB27, and *Trichoderma viride* pq1 as potent antagonists of the pathogen which are capable of producing extracellular chitinases. Further, Saikia *et al.* (2005) isolated two chitinases from *Fusarium oxysporum* inoculated chickpea, which inhibit many other fungal pathogens. Gupta *et al.* (2006) used a strain of *Pseudomonas aeruginosa* which has a strong antagonistic activity against *Sclerotinia sclerotiorum*, *in vitro* and *in vivo*. This strain produced extracellular chitinase enzyme and its role was clearly demonstrated through Tn5 mutagenesis. Bacterization of groundnut seeds with GRC resulted in increased seed germination and reduced stem rot of groundnut in *S. sclerotiorum*-infested soil by 97%.

Chitinase have been known to be induced in the plant by fungal infection in many crop plants (Mauch *et al.*, 1988**;** Velazhahan *et al.*, 2000; Ji *et al.*, 2000). The induction of chitinase against phyto-pathogen systems is generally higher and stronger in the resistant varieties in comparison to susceptible varieties (Anguelova *et al.*, 2001). Transformation of chitinase gene has shown to enhance resistance to many fungal pathogens in groundnut (Rohini *et al.*, 2001 and Prasad *et al.* 2013). In groundnut, Jogi (2012), reported that two chitinases PR-3 (contig02763) and PR-4 (contig03022) were found to be up-regulated in the resistant groundnut cultivar compared to susceptible in response to *S. rolfsii*. Hence the present study aimed at evaluating the responses of groundnut genotypes to *S. rolfsii* infection in presence and absence of a biocontrol agent (*Pseudomonas aeruginosa*) in terms of chitinase activity at different stages of infection. Probably, the generated information will give some insight into the pathogenesis of *S. rolfsii.*

2.9.2.3 ß-1,3-glucanase

β-1,3-glucanases catalyze the cleavage of β-1,3-glucosidic bonds of β-1,3-glucan, another constituent of the fungal cell wall. Unlike chitinases, the substrate for the enzyme is widespread in plants and therefore these proteins are implicated in diverse physiological functions as well as in plant defense (van Loon *et al.*, 2006). β-1,3-glucanases and chitinases act synergistically and their optimal functions may be important in plant defense. Some β-1, 3- glucanase like the many other pathogenesis related proteins are acidic buffer extractable, have low molecular weights (25-36 KDa), resistant to proteases and secreted extra cellularly (Bowles *et al.*, 1990).

ß-1,3-glucanases have an indirect effect on plant defense by causing the formation of oligosaccharide elicitors, which elicit the production of other PR proteins or low molecular weight antifungal compounds, such as phytoalexins (Klarzynski *et al.*, 2000). The ß-1,3-glucanases are strongly induced in response to wounding, cold, ozone, UV and against a wide range of fungal pathogens attack in plants (Van Kan *et al.* 1992; Zemanek *et al.*, 2002). Nandini *et al.* (2010) reported substantial induction of the enzyme in groundnut plants using the fungal components of Sclerotium rolfsii in the form of fungal culture filtrate (FCF) and mycelial cell wall (MCW). Further Nandi *et al.* (2013) showed a significant increase of ß-1,3-glucanases upon *S. rolfsii* infection in cowpea. The enzyme activity reached at its peak (5.07 folds) at five days after fungal inoculation compared with the water treated control, and thereafter there was significant fast decline in its activity in the susceptible host-pathogenic interaction.

Pseudomonas aeruginosa reduced the pre-emergence rotting and post-emergence wilting of groundnut by >60% in *A. niger* infested potting mixture (Kishore *et al.,* 2006). Bacterial seed treatment induced the rapid accumulation of defense-related enzymes like chitinase, β-1, 3 glucanase in groundnut seedlings compared with the control. Thus, activation of host defense responses appears to play a significant role in the biocontrol activity of *Pseudomonas aeruginosa* in collar rot of groundnut. To further extent, recently, many researchers showed that the transgenic plants expressing ß-1,3-glucanases show resistance to fungal pathogens (Liu *et al.*, 2013; Wojtasik *et al.*, 2013 and Kheiri *et al.*, 2014).

2.9.3 Ascorbic acid: A non-enzymatic antioxidant

Ascorbic acid is found in the majority of plant cells. Under physiological conditions, ascorbic acid exists mostly in the reduced form (90% of the ascorbate pool) in chloroplast. The ability to donate electrons in a wide range of enzymatic and non-enzymatic reactions makes ascorbic acid as main ROS-detoxifying compound in the aqueous phase (Arrigoni and De Tullio, 2000). It acid can directly scavenge superoxide, hydroxyl radicals and singlet oxygen and reduce H_2O_2 to water via ascorbate peroxidase reaction and also regenerates tocopherol from tocopheroxyl radical providing membrane protection.

The regulation of ascorbate transport systems may be central in the regulation of different physiological processes including defense against abiotic and biotic threats (Horemans *et al.*, 2000). At a physiological level, the best studied phenomena involving ascorbate is its participation in an oxygen scavenging pathway in the chloroplast known as the ascorbate-glutathione cycle.

Prasad *et al.* (1976) reported decrease in the ascorbic acid content in *Puccinia carthami* infected leaves of four varieties of safflower, compared with healthy leaves. Chhabra *et al.* (2000) also studied leaf sample infected with (*Alternaria solani*), and found that at pre-infection stage, there was no significant difference in the ascorbic acid content of the leaves of different cultivars. After infection, drastic reductions in ascorbic acid content were observed, being highest (63.30%) in the highly susceptible cultivar and lowest (24.55%) in the resistant cultivar. Wang *et al.*, (2002) reported that the resistant cultivar (Kelunsheng) maintained higher content of ascorbic acid in root cells of watermelon cultivars than the susceptible (Zaohua) cultivar from their study after inoculation with *F. oxysporum* f.sp. *niveum* at the seedling stage. In general, the ascorbic acid content is reduced upon disease infection; however, the same was associated with disease resistance.

2.9.4 Oxalic acid: a fungal virulence factor

Oxalic acid has been detected in various organisms, including animals, plants and fungi. The occurence and the distribution of oxalate vary enormously among organisms. For instance, in plants the highest oxalate concentrations commonly occur in the leaves and the lowest in roots. Meanwhile, the oxalate content of plants can vary according to their age, the season, the climate and the type of soil (Caliskan, 2000).

It is well established that certain plant pathogenic fungi secrete oxalic acid as part of the process for invasion of plant tissues. Many other workers have implicated accumulation of oxalic acid in the infection process (Magro *et al.,* 1984) as the fungus produces relatively high level of oxalic acid in diseased plant tissues which act as a pathogenicity factor. For example, *Sclerotium rolfsii,* the stem rot pathogen, also produces good quantity of oxalic acid. Considerable quantities of oxalate have been detected in infected but not in healthy tissues. Oxalic acid produced by this pathogenic fungus played an essential role in its pathogenic capabilities (Maxwell and Bateman, 1968). *S. rolfsii* secretes several kinds of cell wall degrading enzymes that macerate the host tissue to provide nutrients for mycelial growth, and oxalic acid seems to play multiple roles. Bateman and Beer (1965) showed that oxalic acid acts synergistically with polygalacturonases, by lowering the pH and providing optimal conditions for the activity of the enzymes, and by chelating cell wall Ca^{2+} thereby providing polygalacturonases easy access to cell wall pectin.

It was suggested that oxalic acid produced by fungi played a key role in lignin biodegradation through its stimulation of lignin degrading enzymatic activities (e.g. Mn-peroxidase activity) (Kuan and Tien, 1993). The oxidative burst is also known to be suppressed at low pH and requires an increase in cytosolic Ca^{+2}. Because release of oxalate could conceivably both lower the p^H and chelate the Ca^{+2} ions, Cessna *et al.* (2000) have hypothesized that oxalic acid might enhance fungal pathogenicity by inhibiting the oxidative burst of the host plant. Oxalic acid interferes with defence mechanisms of host plants by inhibiting the activities of polyphenol oxidases by suppressing the oxidative burst and by manipulating the host redox environment. It is an elicitor of programmed cell death in plants and responsible for induction of apoptotic-like features in the plant during disease development (William *et al.,* 2011).

Nagrajkumar *et al.* (2005) observed that the virulent fungal isolates produced more oxalic acid. They have also reported that effective reduction of oxalic acid by *Pseudomonas fluorescens* strain pfMDU2 in a culture medium proves ability of *Pseudomonas fluorescens* as detoxification agent of oxalic acid produced by fungal pathogen in disease conditions. Heller and Witt-Geiges (2013) studied the multiple roles of oxalic acid in the plant–pathogen interaction involving *S. sclerotiorum* and postulated that oxalic acid is important in balancing calcium levels at the infection site to prevent toxic calcium concentrations from inhibiting growth *S. sclerotiorum.* As *S. rolfsii*, a necrotrophic fungi, similar to *S. sclerotiorum*, hence oxalic also may be playing such a role during pathogenesis of the fungi.

2.9.5 Proteomics analysis

Proteomic analysis reveals the translational products of gene expression of plant under stress condition and its physiological state under particular conditions. Analysis of proteins is a direct approach to define the function of their associated genes as it linked to genome sequence information, which is important for functional genomics. There are scanty reports of proteome analysis that focuses on the study of stress response of peanut genotypes against various stress conditions.

Wang, *et al.* (2010) analysed peanut seed proteins differentially expressed in resistance and susceptible peanut cultivars in response to *Aspergillus flavus* and reported expression of several disease resistance associated protein. Further, proteomic study of peanut cotyledons in response to atoxigenic and toxigenic *A. flavus* strains revealed aflatoxin-triggered immune response (Wang *et al.,* 2010). Proteomics analysis of seed proteins of four peanut cultivars by Kottapalli, *et al.* (2008) has revealed differential expression of storage, allergenic proteins. They also identified several physiologically significant candidate proteins associated with water-deficit stress tolerance mechanism in three peanut genotypes (Kottapalli *et al.*, 2009). Katam *et al.* (2010) carried out proteomic study in peanut leaf using a drought-tolerant variety and identified more than 200 proteins, predominantly carbohydrate metabolism and photosynthesis related proteins, which help to understand peanut leaf protein alterations under varied stress conditions.

Differential proteomic study of plant-pathogen interaction successfully reported in several plants. For example, Kaur *et al.* (2011) identified defence-related proteins, which are required for mounting a successful defence response in *Brassica juncea* against *Albugo candida*. Recently, Wu *et al.* (2013) analysed a plant-virus interaction in resistant and susceptible ecotypes of maize infected with sugarcane mosaic virus and identified several defence and stress related proteins during both compatible and incompatible interaction. The model plant, Arabidopsis thaliana differentially expressed proteins related to oxidative stress and metabolism in response to treatments with fungal elicitors in Arabidopsis cell cultures (Chivasa *et al.,* 2006). Castillejo *et al.,* (2011) analysed root pea proteome in response to *Orobanche crenata* inoculation and identified several proteins with protease activity which could play an important role in preventing the pathogen and some of metabolism and stress response protein.

Recently, Kumar and Kirti (2015), while investigating the molecular responses of the wild peanut, *Arachis diogoi* when challenged with the fungal pathogen, *Phaeoisariopsis personata*, found up regulation of several proteins associated with photosynthesis, metabolism, secondary metabolism, signal transduction and defence related proteins. They have successfully identified 16 protein spots with significant expression in wild peanut and 12 protein spots in susceptible groundnut, because the importance of the proteins involve in incompatible interaction from wild peanut. The fold changes of differentially expressed proteins upon pathogen challenge were found to be high in case of *A. diogoi* (resistant) in comparison to *A. hypogaea* (susceptible).

Till date, there is no report of proteome analysis that focuses on study of susceptible and tolerant groundnut genotypes against stem rot disease. Hence, the objective of this study was to investigate the proteins differentially expressed during the incompatible interaction in tolerant and susceptible genotypes by the 2D gel electrophoresis technique. These observations may possibly reveal genes and proteins, which might be useful in allowing the host plant to cope up with the invading pathogen and provide new insights into the molecular mechanism of plant-fungal interaction.

2.10 HISTO-PATHOLOGICAL BASIS OF *S. rolfsii* INFECTION IN GROUNDNUT

Hyphae from germinating sclerotia of *S. rolfsii* ramify over various host tissues within 24-48 hrs following inoculation as described by Smith *et al.* (1986). Further they have shown that the extensive hyphal growth within the tissues and the production of oxalic acid and cell wall degrading enzymes are major factors that contribute to the maceration of characteristics of plants infected by *S. rolfsii.* Presently unavailability of immune or absolute stem rot resistant groundnut genotypes stands as the reason for scarce knowledge about host–pathogen interactions at the plant surface and cellular level. A few studies have examined the interaction between the pathogen and a resistant genotype at the cellular or histological level in common bean, and in sunflower (Rodriguez *et al.,* 2004). The infection processes of *Sclerotium sclerotiorum* on cotyledon of a resistant genotype of *Brassica napus* was elaborated by Garg *et al.* (2010) using a moderately pathogenic strain. The histo-pathological study has detailed the infection processes i.e. impeded fungal growth, active suppression of infection cushion development, protoplast extrusion, and the hypersensitive response associated with resistance to *S. sclerotiorum* in *B. napus.* They have also shown that eight days after inoculation of the pathogen, hyphae continued growing in intercellular and intracellular spaces in susceptible genotype, and spongy mesophyll tissues were eventually invaded along with extensive damage to palisade mesophyll cells. In contrast, colonization by the pathogen was generally restricted to the epidermal layer in resistant genotype. Davar *et al.* (2012) has reported in sunflower that within 48 hour after inoculation, the host cells are completely colonized by *Sclerotinia sclerotiorum* fungal mycelium, leading to a tissue collapse in susceptible genotypes. The hyphae invaded the dead host tissues and emerged from the host tissue after extensive colonization. While studying the pathogenesis of *S. rolfsii* in cowpea, Nandi *et al.* (2013), through scanning electron microscopy, strengthened the presence of mycelial network in xylem vessel of infected collar region even at three days after inoculation. Though initial infection hampers the cortical tissues, the main machinery of solute transport was occupied by the pathogens at three days after infection. It was hypothesized that in case of groundnut, the stem rot causing organism, *S. rolfsii*, seems to follow a similar mechanism of infection as described by earlier researchers in other crops. Several attempts have been made to understand the mechanism of resistance of groundnut to S. rolfsii infection, however a very little or no informations are available toll date. Attempts have been going on to explain the pathogenesis of the pathogen at ICAR-Directorate of Groundnut Research, Junagadh, through histopathological basis.

Among the few sources available, CS19, an inter-specific derivative developed at ICAR-DGR, is a registered novel source of resistance. Through electron microscopy, it was observed that within 3 days after inoculation in seed, the fungus gained entry into the host cells of both tolerant (CS19) and susceptible variety (GG20). It was further revealed that CS19 has solid trichomes, wax deposits and compact arrangement of vascular cells which could be affording it resistance to stem rot. On the contrary, the susceptible variety lacks solid trichomes and wax deposits, and has loosely bound vascular cells which could be predisposing it for heavy incidence of stem rot (DAR, 2010). Since, stem rot is rampant not only in *kharif*, but also in irrigated *rabi* and summer production systems, the genetic resistance to the disease is very important. As there is lack of ample information in the above said aspect, it was realized that generation of information regarding innate resistance should be the research priority for better understanding of the mechanism of resistance.

2.11 EXPRESSION OF PATHOGENESIS-RELATED (PR) PROTEINS

Higher plants have a broad range of mechanisms to protect themselves against various threats including physical, chemical and biological stresses, such as wounding, exposures to salinity, drought, cold, heavy metals, air pollutants and ultraviolet rays and pathogen attacks, like fungi, bacteria and viruses (Agrios, 1997). Plant reactions to these factors are very complex, and involve the activation of set of genes, encoding different proteins. These stresses can induce biochemical and physiological changes in plants, such as physical strengthening of the cell wall through lignification, suberization, and callose deposition; by producing phenolic compounds, phytoalexins and pathogenesis-related (PR) proteins which subsequently prevent various pathogen invasion (Bowles, 1990). Among these, production and accumulation of pathogenesis related proteins in plants in response to invading pathogen and/or stress situation is very important. Phytoalexins are mainly produced by healthy cells adjacent to localized damaged and necrotic cells, but PR proteins accumulate locally in the infected and surrounding tissues, and also in remote uninfected tissues. Production of PR proteins in the uninfected parts of plants can prevent the affected plants from further infection (Delaney, 1997)**.**

Most PR proteins in the plant species are acid-soluble, low molecular weight, and protease-resistant proteins. PR proteins depending on their isoelectric points may be acidic or basic proteins but they have similar functions. Most acidic PR proteins are located in the intercellular spaces, whereas, basic PR proteins are predominantly located in the vacuole. Currently PR proteins were categorized into 17 families according to their properties and functions, including ß-1,3-glucanases, chitinases, thaumatin-like proteins, peroxidases, ribosome-inactivating proteins, defenses, thionins, nonspecific lipid transfer proteins, oxalate oxidase, and oxalate-oxidase-like proteins (van Loon *et al*., 1999). PR-proteins are specifically induced in response to infection by pathogens such as fungi, bacteria, and viruses, or to adverse environmental factors (van Loon *et al*., 2006). Also another report showed no difference in the induction timing or amounts of PR-protein in resistant and susceptible cultivars of cotton (McFadden *et al*., 2001). However, quick response in the resistant cultivars might affect the cell wall of germinating fungal spores, releasing elicitors leading to the expression of PR-genes and disease resistance. In the present study, the gene expression of few PR proteins were studied which includes ß-1,3-glucanase (*PR2*), chitinase type I, II (*PR4*), Thaumatin- like protein (*PR5*), and Ribonuclease like protein (*PR10*).

2.11.1 ß-1, 3-Glucanases (PR2)

ß-1,3-glucan is a another major structural component of the cell walls of many pathogenic fungi and ß-1,3-glucanases are able to catalyze the cleavage of the ß-1,3-glucosidic bonds in ß-1,3-glucan (Simmons,1994). Synthesis of these enzymes can be induced by pathogens or other stimuli. ß-1,3-glucanases have an indirect effect on plant defense by causing the formation of oligosaccharide elicitors, which elicit the production of other PR proteins or low molecular weight antifungal compounds, such as phytoalexins (Klarzynski *et al*., 2000). It has been suspected that ß-1,3-glucanases have direct effect in defending against fungi by hydrolyzing fungal cell walls, which consequently causes the lysis of fungal cells. In the plants, it has other functions such as cell division and cell elongation, fruit ripening, pollen germination and tube growth, fertilization, somatic embryogenesis, seed germination and flower formation (Akiyama *et al*., 2004).

The ß-1,3-glucanases are strongly induced in response to wounding, cold, ozone, UV and against a wide range of fungal pathogens attack in plants (Van Kan *et al.* 1992; Alonso *et al.*, 1995; Ignatius and Chopra, 1994; Lozovaya, 1998; Jung and Hwang, 2000; Hanselle and Barz, 2001; Zemanek *et al.*, 2002). Recently, many researchers showed that the transgenic plants expressing ß-1,3-glucanases show resistance to fungal pathogens (Liu *et al.*, 2013; Wojtasik *et al.*, 2013 and Kheiri *et al.*, 2014). As per information, till date no such reports are available for groundnut-*S. rolfsii* pathosystem, hence the present study included the expressional analysis of PR2.

2.11.2 Chitinases (PR4)

Chitinases (CHIs) act on the ß-1,4-glycosidic linkage of chitin. In plants, CHIs have also been implicated in the mechanisms of resistance against pathogens and insect pests, and other functions, such as nodulation, embryogenesis, and functions unrelated to their catalytic activity, including antifreeze proteins and inhibitors of a-amylases (Neuhaus, 1999). Moreover, at the cell wall, CHIs can release chitin oligomers, namely pathogen (microbe)-associated molecular patterns (PAMPs or MAMPs, respectively), which activate a variety of plant defense responses, including ROS generation, the production and export of anti-microbial compounds and the fortification of the plant cell wall (Segonzac *et al.*, 2011).

Chitinases and ß-1,3-glucanases are two important hydrolytic enzymes that are abundant in many plant species after infection by different type of pathogens. The amount of them significantly increase and play main role of defense reaction against fungal pathogen by degrading cell wall, because chitin and ß-1,3-glucan are also major structural components of the cell walls of many pathogenic fungi. Several CHIs inhibit the growth of many fungi through the lysis and disarrangement of the cell wall structure, particularly in combination with glucanases. Chitinase have been known to be induced in the plant by fungal infection in many crops (Mauch *et al.*, 1988**;** Velazhahan *et al.*, 2000; Ji *et al.*, 2000). Various studies have shown that chitinase expression against phyto-pathogen systems is higher and induction is stronger in the resistant varieties in comparison to susceptible varieties (Nielsen *et al.*, 1993; Lawrence *et al.*, 2000; Anguelova *et al.*, 2001). ß-1,3, glucanases appear to be co-ordinately expressed along with chitinases after fungal infection. This co-induction of the two hydrolytic enzymes has been described in many plant species, including pea, bean, tomato, tobacco, maize, soybean, potato, wheat, etc. The combined expression of CHI and GLU genes in rice (*Oryza sativa* L. subsp. indica variety Pusa Basmati 1) enhanced resistance against Rhizoctonia solani (Sridevi *et al*., 2008).

Transformation of chitinase gene has shown to enhance resistance to many fungal pathogens in various crops (Yamamoto *et al*., 2000; Datta *et al*., 2001; Grover *et al*., 2003; Fanta *et al*., 2003) including groundnut (Rohini *et al*., 2001 and Prasad *et al*. 2013). In groundnut, Jogi (2012), reported that two chitinases *PR-3* (contig02763) and *PR*-4 (contig03022) were found to be up-regulated in the resistant groundnut cultivar compared to susceptible in response to *S. rolfsii*.

2.11.3 Thaumatins Like Protein (TLP/ PR-5)

Members of the PR-5 proteins are called thaumatin-like proteins (TLPs) which have high sequence identity with thaumatins, a sweet-tasting protein isolated from the West African shrub *Thaumatococcus daniellii* (Cornelissen *et al*., 1986). TLPs are reported to be widely distributed PR proteins across kingdoms including gymnosperm, angiosperm, animal and fungal systems. About twenty-four PR-5 genes have been identified in *Arabidopsis.* PR5 family consists of TLP, osmotin, osmotinlike proteins (OLP) and zeamatin (Liu *et al*., 2010).

PR-5 family has diverse functions in plant disease resistance such as antifungal activity, antifreezing activity and osmotic stress tolerance. Overexpression of PR5 has reported to have antifungal activity against many fungal pathogens (Liu *et al*., 2003; Ho *et al*., 2007; Jami *et al*., 2007; Wang *et al*., 2011; Zamani *et al*., 2012; Acharya *et al*., 2013). Recently, transgenic tobacco plants expressing TLP gene from *Arachis diogoi* (wild peanut) (AdTLP) showed enhanced resistance to fungal pathogen *Rhizoctonia solani* along with enhance stress resistance in crop plants (Singh *et al*., 2013). However, the role of PR5 expression in stem rot tolerance in groundnut has not yet been reported as per the available information.

2.11.4 Ribonuclease-like protein (RLP/PR-10):

The PR10 family is one of the most important families among the 17 PR groups with more than 100 members reported so far from both angiosperms and gymnosperms, including the newly identified AmPR10 from *Astragalus mongholicus* (Yan *et al*., 2008). Unlike most other PR proteins with an extracellular destination, PR10 proteins are typically acidic, intracellular, and small (16–19 kDa). They have RNase and ligand-binding activities which protects plants during programmed cell death around infection sites or act directly on the pathogens. PR10 proteins have been reported to have various functions in both biotic and abiotic stresses (Liu and Ekramoddoullah, 2006) and plant growth and development through modulating the endogenous cytokinin level (Srivastava *et al.*, 2006). Therefore, it is proposed that PR10 proteins may play important roles in plant defense against pathogen attack.

Flores *et al.* (2002) identified a novel PR10 member (ocatin) from Oxalis tuberose accounting for 40–60% of tuber storage proteins, possessing antibacterial and antifungal activities. Then after, several lines of evidence corroborate and implicate a role of PR10 proteins during pathogen infection (Liu *et al.*, 2003, 2006; Chadha and Das, 2006; Fung *et al.*, 2007; Xie *et al.*, 2010). Many plant species have more than one PR10 protein (Liu and Ekramoddoullah, 2006) and there is a relationship between PR10 and other PR proteins (Colditz *et al.*, 2007). The possible relationship of PR10 and host resistant was first reported in groundnut by Luo *et al.* (2005), which showed up-regulation of PR10 gene in peanut cultivar A13 and responded to *A. parasiticus* attack. Jain *et al.* (2012) isolated PR10 gene from a callus cell line of *Arachis hypogaea*, and showed the involvement of PR10 across salinity, heavy metal, cold and mannitol-induced drought stress environments. Hence, the present study included PR10 for induce expression study with reference to *S. rolfsii* infection in groundnut.

2.11.5 Polygalacturonase Inhibitory Protein (PGIP)

Fungal pathogens produce a wide array of plant cell wall degrading enzymes, among which endo-polygalacturonases (PGs) are secreted at very early stages of the infection process (ten Have *et al.*, 1998). PGs cleave the α-(1–4) linkages between the D-galacturonic acid residues, the main component of pectin, and thereby causing cell separation and maceration of the host tissue. To counteract the activity of PGs, plants deploy the cell wall polygalacturonase inhibiting proteins (PGIPs) that inhibit the activity of PGs. No plant species or mutants totally lacking PGIP activities have been characterized so far (Kalunke *et al.*, 2015).

Several plants resistance gene products contain LRR domains, constituted by ten consecutive elements each containing 24 amino acid residues (Kajava, 1998). PGIPs belong to the large superfamily of proteins specialized in protein-protein interaction containing the leucine rich repeat (LRR) structural motif (Kobe and Kajava, 2001). PGIPs are glycoproteins with molecular masses ranging from about 37 to 54 kDa (Di Matteo**,** *et al*., 2006), known to be secreted in the extracellular space, being devoid of the classical intracellular and transmembrane domains (Protsenko, *et al*., 2008). In addition to PG inhibition, the interaction between PGs and PGIPs promotes the formation of oligogalacturonides (OGs), which are elicitors of a variety of defense responses (Ferrari *et al*., 2013).

Genome analysis has shown that PGIP genes (*pgip*) may exist as single genes, as in diploid wheat species (Di Giovanni *et al*., 2008), or organized into gene families, the members of which are organized in tandem and can vary from two, as in *Arabidopsis thaliana* (Ferrari *et al*., 2003), to sixteen, as in *Brassica napus* (Hegedus *et al*., 2008). The majority of PGIP genes are intron-less, however, some of them can contain a short intron as in *Arabidopsis* (Ferrari *et al*., 2003). Moreover, *pgip* genes can be inactivated by transposon elements as in cultivated and wild wheat (Di Giovanni *et al*., 2008).

Expression of *pgip* can be up-regulated in response to various biotic and abiotic stresses such as pathogen infection, mechanical wounding, cold, salinization, over-watering and jasmonic acid (Ahsan, *et al*., 2005). Moreover, under stress conditions, different expression patterns can be revealed for *pgip* belonging to the same family (Ferrari, *et al*., 2003). For example, in soybean, *GmPGIP1* and *GmPGIP3* are up-regulated following wounding and infection caused by *Sclerotinia sclerotiorum*. On the contrary, *GmPGIP2* is not induced by wounding and is only up-regulated following the same pathogenic attack (D'Ovidio, *et al*., 2004). In *Arabidopsis*, both *AtPGIP1* and *AtPGIP2* are up-regulated in response to *B. cinerea* but through separate signal transduction pathways (Ferrari, *et al*., 2003). Like other families of defense-related genes, *pgip* families show variation in the expression pattern of the different members, some of which are constitutive, others are tissue-specific and, in most cases, up-regulated following stress stimuli (Table 2.11.1). Early characterization of a polygalacturonase-inhibiting activity was reported in 1970s (Albersheim and Anderson, 1971) and the first *pgip* gene was isolated 20 years later in French bean (Toubart *et al*., 1992). Since then, several PGIPs and a large number of *pgip* genes (more than 170) have been characterized (Kalunke, *et al*., 2015).

Table 2.11.1 Treatments or stress stimuli affecting *pgip* expression in some plant species with a well characterized *pgip* family

Crop	Stress stimuli or Treatments	References
Rice	Abscisic acid (ABA), brassinosteroid, gibberellic acid (GA), 3-indole acetic acid (IAA), jasmonic acid (JA), kinetin, naphthalene acetic acid (NAA), salicylic acid (SA); *Rhizoctonia solani* (necrotrophic fungus)	Janni *et al*., 2006; Lu *et al*., 2012
Wheat	*Bipolaris sorokiniana* (necrotrophic fungus) and mechanical wounding	Janni *et al*., 2013
Bean	Oligogalacturonides (OGs); mechanical wounding; *Botrytis cinerea*, *Sclerotinia sclerotiorum* (necrotrophic fungi); *Colletotrichum lindemuthianum* (hemibiotrophic fungus)	Bergmann *et al*., 1994; Nuss *et al*., 1996; Devoto *et al*., 1997; D'Ovidio *et al*., 2004; Oliveira *et al*., 2010; Kalunke *et al*., 2011
Soybean	Mechanical wounding; *S. sclerotiorum* (necrotrophic fungus)	D'Ovidio *et al*., 2006; Kalunke *et al*., 2014
M. truncatula	JA, SA, ABA; *Colletotrichum trifolii* (hemibiotrophic fungus)	Song and Nam, 2005
Rapeseed	JA, SA, mechanical wounding; *S. sclerotiorum*	Hegedus *et al*., 2008
Pepper	SA, Methyl jasmonate (Me-JA), ABA, wounding, cold treatment	Wang *et al*., 2013
Arabidopsis	OGs; JA; *B. cinerea*; *Stemphylium solani* (necrotrophic fungus); aluminum, low-pH, cold;geminivirus	Ferrari *et al*., 2003; Ascencio-Ibanez *et al*., 2008; Sawaki *et al*., 2009; Di *et al*., 2012; Kobayashi *et al*., 2014

To the best of our knowledge, no reports are available for PGIP of groundnut. Realising the importance of this gene as a candidate for imparting stress tolerance in groundnut, the present study was aimed at isolating a member of *pgip* gene family, and further validate its induced expression at transcript level upon stem rot infection.

CHAPTER – III

MATERIALS AND METHODS

The present investigation on "Biochemical and Molecular Basis of Innate and *Pseudomonas fluorescens* Induced Stem Rot Tolerance in Groundnut (*Arachis hypogaea* L.)" was carried out at the Department of Biochemistry, Junagadh Agricultural University (JAU), Junagadh and ICAR-Directorate of Groundnut Research (ICAR-DGR), Junagadh.

3.1 EXPERIMENTAL SITE

The ICAR-DGR sick-plot for stem rot was used for field evaluation of genotypes *Kharif* 2013-14. Isolation of fluorescent Pseudomonads was carried out at the Microbiology Laboratory, Department of Biotechnology and Biochemistry, Junagadh Agricultural University, Junagadh. All biochemical and molecular study was carried out at ICAR-Directorate of Groundnut Research (ICAR-DGR). The glass house facility at ICAR-DGR was used to raise crop under controlled environmental conditions during 2013-14/14-15. Two-dimensional gel electrophoresis facility of Food Testing Laboratory (FTL), JAU was used for protein profiling study.

3.2 EXPERIMENTAL MATERIALS

Groundnut (*Arachis hypogaea* L.) seeds of twenty varieties developed at JAU and other State Agricultural Universities of Gujarat were obtained from Main Oilseed Research Station, Junagadh Agricultural University, Junagadh. Another two genotypes such as CS19 and TG37A were collected from ICAR-DGR, Junagadh.

3.3 GLASSWARES AND POLYWARES

The glass-wares and poly-wares used were of standard make such as Borosil. All glass-wares were scrubbed and washed thoroughly with the detergent then rinsed with tap water followed by distilled water. Finally, it was dried in an oven before use.

3.4 CHEMICALS AND CONSUMABLES

The chemicals used in this study were purchased from the following companies: Sigma-Aldrich (USA), Merck (India), Himedia (India), Amresco (Ohio), Fermentas (USA), SRL (India), Bangalore Genei (India), Qualigens (India) and Clonetech-Takara

(Japan). For two dimensional electrophoresis studies, the SERVA IPG Bluestrips and IEF markers were purchased from Genexy (India). The consumables were purchased from: Borosil (India), Sigma-Aldrich (USA), Eppendorf (Germany), Axiva (India), Genetix (India), Whatman (UK), Millipore (USA), Amersham Biosciences (UK), Axygen (USA) and Tarsons (India).

Enzymes and reaction kits

Taq DNA polymerase from Promega (USA) was used for routine PCR. DNase from Qiagen (Germany) was used for DNA removal during RNA extraction and RNase from Fermentas (USA) was used for RNA removal during DNA extraction and plasmid extraction.

Following kits were used for different purposes in the study: NucleoSpin®Plant II kit (MACHEREY-NAGEL GmbH & Co.KG, GmbH), QIAquick PCR purification kit (Qiagen, Germany), QIAquick gel extraction kit (Qiagen, Germany), RNeasy Plant Mini Kit (Qiagen, Germany), cDNA synthesis kit (Fermentas, USA), QuantiFast SYBR Green PCR reaction kit (Qiagen, USA).

Oligonucleotides used in the study

Oligonucleotides synthesis were outsourced from IDT (USA) and dissolved in sterilized nuclease-free milli-Q water. Oligonucleotide stocks were prepared with 100 pmols concentration and stored at -80 °C. Working concentration of oligonucleotides (20 pmols) was prepared by diluting oligonucleotides stocks and stored at -20 °C.

Media, buffers and solutions

Most of the solutions, buffers and media were prepared as described by Sambrook and Russel (2001) and unless supplied with the kits. The pH was adjusted with 1N NaOH, 1N HCl or with reagents specifically mentioned. Media for cultivating bacteria were sterilized by autoclaving (121 °C, 15 psi and 20 min). All the thermolabile solutions and components, such as antibiotics stocks, were sterilized by filter sterilization with 0.2 µm syringe filter (Axiva, India) and added to the medium after cooling to 50 °C.

3.5 MAJOR EQUIPMENTS AND INSTRUMENTS USED IN THE EXPERIMENT

The important equipments and instruments have been used are listed below:

Equipment/ Instrument	Model/Make
Autoclave	Synco (India)
Automated Gel Documentation System	FLA-5100 (Fujifilm Co., Ltd., Japan); Hybridization Oven: 6242 (Thermo Scientific, USA)
Centrifuges	7780 (Kubota, Japan), Allegra X22R (Beckman Coulter, USA)
Digital Balance	AT20, Mettler-Toledo USA; Precision: 1 μg
Digital Top Pan Balance	Startorious lab, GmbH; precision: 10 mg
DNA Gel Electrophoresis Apparatus	Mini cells for DNA agarose electrophoresis and power supplies (Bangalore Genei, India and Bio-Rad, USA)
Ice-Maker	Ziegra (Germany)
Incubator Shaker	C76 water bath shaker (New Brunswick Scientific, UK)
Ion Chromatography	Dionex, ICS 3000 (Thermo scientific, USA)
Laminar Air Flow Cabinet	Klenzaids (India), Reschlor (India)
Microwave	MagiCook (Kelvinator, India)
Nanodrop Spectrophotometer	ND1000 (Thermo scientific, USA)
PCR Thermocyclers	Eppendorf, Germany
p^H Meter	720 A Orion (Thermo scientific, USA)
Spectrophotometers	Specord 200, Analytikjena, Germany
Step One Real Time PCR System	Applied Biosystem, California, USA
Ultra Low Freezer (-80 °c)	Operon (Korea)
Vacuum Concentrator	DyNA vap (Labnet, USA)
Water Bath	RC 20 LAUDA (Brinkmann, Germany)
Water Purification System	Millipore, USA

3.6 EXPERIMENT I

PRELIMINARY FIELD SCREENINGS OF GROUNDNUT GENOTYPES FOR STEM ROT TOLERANCE UNDER HIGH DISEASE PRESSURE IN SICK PLOT

Screening of groundnut genotypes was carried out during *Kharif* 2013-14 in the sick-plot for stem rot pathogen (*Sclerotium rolfsii*) maintained at ICAR-Directorate of Groundnut Research, under normal field conditions. Each genotype was planted in rows of five meter length with a spacing of 45 cm between rows and 10 cm between plants and replicated thrice. The crop was raised as per the recommended package of practices except for the plant protection measures against stem rot and life saving irrigation was provided to maintain healthy growth of the crop. Disease pressure in the sick plot has been maintained by adding fungal inoculums every fort-nightly. Forty-day old plants were further challenged with the fungus by adding fungal inoculums on the soil surface in each line, closer to main stem. The progress of disease was monitored regularly. The per cent disease incidence in terms of mortality of plants was calculated by using the formula Per cent disease = (Number of infected plants/ Total number of plants) X 100.

Based on the above results which on accordance with the already available information, four groundnut genotypes were selected for further biochemical, histo-pathological and molecular study in glass house.

3.7 EXPERIMENT II

ISOLATION AND IDENTIFICATION OF A POTENT ANTAGONISTIC *Pseudomonas fluorescens* ISOLATE AGAINST *Sclerotium rolfsii*

Details of the experiments, materials used in the experiment, procedures followed and techniques adopted in the present investigation are described as below.

3.7.1 Isolation and Maintenance of Microbes

3.7.1.1 Isolation of fluorescent Pseudomonads

Soil samples were collected from plots of different areas of groundnut growing areas of *Saurasthra* region, Gujarat (details given below) and then fluorescent pseudomonads were isolated following the method of Kandoliya and Vakharia (2013). The procedure was started with one g of each soil sample, which was mixed by shaking for 2 h on a rotary shaker at 200 rpm in 10 ml of sterile water. Extracts were diluted and then plated on King's B (KB) medium. After incubation at 28 °C for 24 hr, colonies that showed fluorescence under UV light were selected. The representative types of colonies were further purified on KB agar medium and pure isolates have been maintained in 50% glycerol at -20 ^{0}C.

Sr. No.	Location	Isolate	Sr. No.	Location	Isolate
1	Amreli	*SKPf1*	**6**	Kodinar	*SKPf6*
2	Chotila	*SKPf2*	**7**	Kutiana	*SKPf7*
3	Jamnagar	*SKPf3*	**8**	Mendrda	*SKPf8*
4	Jetpur	*SKPf4*	**9**	Surendranagar	*SKPf9*
5	Junagadh	*SKPf5,SKPf11*	**10**	Una	*SKPf10*

3.7.1.2 Isolation of *Sclerotium rolfsii*

Stem of *Sclerotium rolfsii* infected groundnut plants from the stem-rot sick plot maintained at ICAR-DGR, Junagadh, were collected and cut into small pieces and surface sterilized with 0.1 % mercuric chloride solution for 30 seconds. To remove the excess mercuric chloride, 2 to 3 washing for 2 to 3 minutes each were given with distilled sterilized water. The pieces were then plunged in potato dextrose agar (PDA) medium in petriplates. These petri plates were kept for incubation at 26 ± 2^0 C in an incubator. A typical white mycelial growth was observed after 48 hours of incubation. This mycelial growth was further purified by "single spore isolation technique" and confirmed as *Sclerotium rolfsii*. Pure culture of the fungus has been maintained on PDA slant at 0-5^0 C.

3.7.2 Preparation of Media

3.7.2.1 Potato Dextrose Agar (PDA) Media

About 200g potato was boiled with 1L distilled water for 20 min and was filtered. To that 20g of dextrose and 30g of Agar-Agar powder was added and final volume was made 1L with distilled water. The medium was sterilized by autoclaving at 15 lb (PSI) for 20 min and the warm media was poured in aseptic condition to petri plates to get solidify at room temperature (Sinclair and Dhingra, 1985).

3.7.2.2 King's B (KB) Media

Accurately weighed 15g peptone, 1.5g magnesium sulphate, 1.5g potassium dihydrogen phosphate, 10ml of glycerol were dissolved in water properly and then

30g of Agar-Agar powder was added except in King's B broth media, and final volume was made 1L by adding distilled water. The mixture was boiled and autoclaved at 15 lbs (psi) for 20 min. (King *et al.,* 1954; Simon and Ridge, 1974). Twenty ml of warm liquid culture was then poured in a sterilized petri dish (9cm diameter) and allow for solidify at room temperature.

3.7.3 Experimental details

The isolated fluorescent Pseudomonads were used for *in vitro* antagonism study against the stem rot fungi of groundnut, as detailed below.

Treatments	**T_1**	*SKPf* 1 x *S. rolfsii*
	T_2	*SKPf* 2 x *S. rolfsii*
	T_3	*SKPf* 3 x *S. rolfsii*
	T_4	*SKPf* 4 x *S. rolfsii*
	T_5	*SKPf* 5 x *S. rolfsii*
	T_6	*SKPf* 6 x *S. rolfsii*
	T_7	*SKPf* 7 x *S. rolfsii*
	T_8	*SKPf* 8 x *S. rolfsii*
	T_9	*SKPf* 9 x *S. rolfsii*
	T_{10}	*SKPf* 10 x *S. rolfsii*
Control	**T_{11}**	*S. rolfsii* alone
Incubation period	:	6 days in incubator at 28-30 ^{0}C
No. of Replication	:	Three
Design	:	CRD
Observation recorded for antagonism at six days after incubation		
Morphological	:	Per cent growth inhibition of *Sclerotium rolfsii* (Reddy *et al*., 2008)
Cell wall degrading Enzymes	:	Cellulase (Collmer *et al*., 1988).
	:	Poly galacturonase (Collmer *et al*., 1988)
PR proteins	:	Chitinase (Boller and Mauch, 1988)
	:	ß -1, 3 Glucanase (Diby *et al.,* 2005)
	:	Protease Kandoliya and Vakharia, (2013)
Biochemical markers	:	Siderophores and Salicylic Acid (Reddy *et al.,* 2008) , IAA like compounds (Mazumdar *et al.,* 2007)
Molecular identification	:	16S rRNA sequencing

3.7.4 *In-vitro* per cent growth inhibition of *Sclerotium rolfsii*

To determine the *in-vitro* antagonistic effect of various fluorescent *Pseudomonad* isolates against *Sclerotium rolfsii,* 25 ml of King's B was poured aseptically in each of the petri dishes and allowed to solidify. Each isolates in round shape and mycelial disc of 4 mm diameter from test fungus (*Sclerotium rolfsii*) were placed in centre of the test medium in the same petri dish approximately 4 cm away from each other. All the inoculated plates were incubated at 28-30 ^{0}C temperature and observed after six days for growth of antagonist bacteria and test fungus (Reddy *et al.,* 2008). Index of antagonism was determined by following the method of as depicted below -

$$\% \text{ Growth Inhibition} = \frac{C - T}{C} \times 100$$

Where, C = colony diameter of pathogen in control, T = colony diameter of pathogen in inhibition plate

3.7.5 Cell wall degrading enzymes assay

Crude enzyme extract was prepared from the petri-dishes containing fungal pathogen and isolates (T_1 to T_{10}) as well as the test fungus alone (T_{11}) as a control (C) at 6 days after inoculation (DAI). Using 25 ml of 100mM phosphate buffer (pH-5.5) containing 50mM NaCl , the whole mycelia mat along with the bacterial growth was transferred to a conical flask. About 1% of each of carboxymethyl cellulose (CMC), sodium polypectate, chitin or casein was added into culture medium for enzyme induction and the pH was adjusted to 5.5 (Kishore *et al*., 2006), and then the cultures were shaken well in an orbital shaker at 120 rpm at 30 ^{0}C for about 6 hours (Sivan and Chet, 1989). After centrifugation 15,000 rpm for 10 min, the supernatant was collected and volume was made up to 100ml with the above $PO_4{}^{2-}$ buffer, and stored at -20 ^{0}C until used for protein estimation and enzymatic assay (Cellulase, Polygalacturonase, Chitinase, β-1, 3 Glucanase, Proteases). The assay values depicted in the text are normalised by deducting the respective C values obtained corresponding to the *S. rolfsii* alone during antagonism study.

3.7.5.1 Cellulase activity

Suitable aliquot (250 µl) of the culture supernatant was incubated with 250 µl of 100mM sodium citrate buffer, pH 5.2 containing 0.25 % carboxymethyl cellulose

(Collmer *et al*., 1988 with slight modification). After incubation at 55^0 C for 30 min. the glucose released was measured by the DNSA method (Sadasivam and Manickam, 1992).

3.7.5.2 Polygalacturonase activity

The culture supernatants (250 µl) were incubated with 500 µl of 50mM sodium acetate buffer, pH 5.2 containing 0.25% sodium polypectate (Collmer *et al*., 1988). After incubation at 37^0 C for 1 h, the galacturonic acid released was measured by the DNSA method (Sadasivam and Manickam, 1992).

3.7.6 Pathogenesis-related (PR) proteins assay

3.7.6.1 Chitinase activity

Reaction mixture contained 250µl of 0.5% chitin in 10mM sodium acetate buffer, pH 5.2 and 250µl of culture supernatants (Boller and Mauch, 1988) were incubated for 1 h at 50^0C. The formation of sugar N-acetylglucosamine was measured by DMAB method (Reissig *et al*, 1955).

3.7.6.2 β-1, 3 glucanase activity

The reaction system contained 250 µl of 0.4% laminarin in 50mM sodium acetate buffer, pH 5.2 and 250 µl of culture supernatants (Diby *et al.,* 2005). Reactions were carried out at 37^0C for 10 min. After incubation, the glucose released by enzyme β-1,3 glucanase was measured by DNSA method (Sadasivam and Manickam, 1992). Specific activity of the cell wall degrading enzymes (cellulase and PG) and pathogenesis related enzymes (chitinase and β-1, 3 glucanase) were expressed as Unit.mg^{-1} protein. However, Unit activity was defined as the amount of enzyme necessary to produce one µM of corresponding reducing sugar per min per ml of culture supernatants. Controls were also performed using boiled enzymes and were subtracted from the enzymatic values.

3.7.6.3 Proteases activity

The reaction system contained 500 µl enzyme solution and 500 µl of 0.36% bovine serum albumin (BSA) and 2.0 ml of 100 mM acetate buffer, pH 3.6. Reactions were allowed to proceed for 1 h at 50^0 C and stopped with 3 ml of 5% trichloroacetic acid (Malik and Singh, 1980). Blank was treated as zero time incubation. The reaction mixtures were then centrifuged at 5000 rpm for 10 min. to settle down precipitate and known volume of supernatants (500µl) were used for estimation of released free amino acids by ninhydrin method (Lee and Takahashi, 1966). Specific activity of protease was expressed as U.mg^{-1} protein and one unit of protease activity was defined as the amount of protein necessary to produce µg free amino acids per min per ml of culture supernatant.

3.7.7 Protein Assay

The method of Folin-Lowry (Lowry *et al*., 1951) was used to determine the protein content in the enzyme extracts. Suitable aliquot (0.2 ml) was taken and total 1 ml volume was made with distilled water. To that, 5.0 ml of reagent C (A: 2% Sodium Carbonate in 0.1 N Sodium Hydroxide, B: 0.5% Copper Sulphate in 1 % Sodium Potassium Tartrate (Prepared fresh) C: Prepared fresh by mixing 98 ml of reagent A with 2 ml of reagent B) was added and mixed properly. After keeping for 10 minutes at room temperature, 0.5 ml of reagent D (D: *Folin Ciocalteau* reagent diluted with distilled water (1:1 ratio) was added, thoroughly mixed and kept for 30 minutes at room temperature. The absorbance was then measured at 660nm. The standard curve for protein content was calculated by using Bovine serum albumin as standard, keeping the range of protein from 20-200µg. µl^{-1}. The protein content was calculated as below,

Protein content
(mg. ml^{-1} or mg. g^{-1}) = Sample O.D. x Graph factor x Dilution Factor

3.7.8 DNSA method for estimation of reducing sugars

The dinitrosalicylic acid (DNSA) method was used to estimate the glucose and galacturonic acid released by cellulase, polygalacturonase and β-1,3 glucanase enzymes (Sadasivam and Manickam, 1992). A known volume of aliquot was taken in test tube and final volume of 1.0 ml adjusted with distilled water. To this 0.5 ml DNSA reagent (1g DNSA + 200mg crystalline phenol + 50mg sodium sulphite in 100ml of 1% sodium hydroxide) was added and mixed properly. The content was heated in a boiling water bath for 5 min. When the contents of the tubes were still warm, 1.0 ml of 40% sodium potassium tartrate (Rochelle salt) solution was added. Cool it and final volume was made 5.0 ml with distilled water. After that the tubes were read at 540nm using spectrophotometer. Reagent blank was also performed by addition of 1.0 ml of distilled water in place of enzyme aliquot and treated it as the same way as above procedure. Standard curve was prepared by a known concentration of glucose (100-500 µg).

3.7.9 DMAB method for estimation of N- acetylglucosamine

Dimethylamino benzaldehyde (DMAB) method was used for determination of N-acetylglucosamine released by chitinase enzyme as the procedure described by Reissig *et al.,* (1955). Known aliquot of reaction mixture was taken into test tube and 0.1 ml, 120mM potassium borate buffer, pH 8.9 was added. The tubes were vigorously boiled in water bath for 3 min. and cooled. Then, 3 ml DMAB reagent (5.0 g DMAB dissolved in 500 ml of glacial acetic acid which contain 12.5% V/V 10 N HCl. It was stored at 20^0C as a stock and prior to use it was diluted with nine volume of glacial acetic acid) was added in each tubes and incubated at 38^0C for 20 min. Tubes were then cooled and absorbance was measured at 544nm in spectrophotometer. Standard for N-acetylglucosamine in a range of 2 to 10 µM was prepared in borate buffer and measured following the above procedure.

3.7.10 Ninhydrin method for estimation of free amino acids

Free amino acid content was estimated as described by Lee and Takahashi (1966). Suitable aliquots were taken and volume made up to 1 ml by adding distilled water. To this, 5 ml ninhydrin reagent (1% ninhydrin in 500mM citrate buffer, pure glycerol, and 500mM citrate buffer pH 5.5 in the ratio of 5:12:2) was added, mixed thoroughly and then, tubes were kept in a boiling water bath for 10 minutes. After that, the tubes were transferred to an ice bath for immediate cooling. The tubes were brought to room temperature and 5 ml 50% isopropanol was added. After 15 minutes, the absorbance was measured at 570 nm. The free amino acid content was calculated from reference curve prepared using glycine (10-50 µg) as standard and expressed as appropriate.

3.7.11 Estimation of secondary metabolites

3.7.11.1 Siderophores

Siderophores estimation was carried out as per method described by Reddy *et al.,* (2008). The Pseudomonads isolates were grown at 28 ± 2^0C with occasional shaking in King's B broth for 3 days and centrifuged at 10,000 rpm for 10 min and the supernatant was collected. The pH of the supernatant was adjusted to 2.0 with 1N HCl and equal quantity of ethyl acetate was added in a separating funnel, mixed well and ethyl acetate fraction was collected. Five ml of ethyl acetate fraction was mixed with 5 ml of Hathway's reagent (The reagent was prepared by adding 1 ml of 0.1 M ferric chloride in 0.1 N HCl to 100 ml of distilled water, and to this 1 ml of 0.1 M potassium ferricyanide was added). The absorbance for dihydroxy phenols was read at 700 nm in a spectrophotometer. A standard curve was prepared using dihydroxy benzoic acid. The quantity of siderophore synthesized was expressed as µ mol benzoic acid.ml^{-1} of culture broth.

3.7.11.2 IAA-like substances (IAA)

The Pseudomonads isolates were inoculated for determination of IAA like substances (Mazumdar *et al.,* 2007) in 50 ml of King's B broth supplemented with tryptophan 0.1 mg.ml^{-1} and incubated at 28±2^{0}C for 3 days (72 hr) with occasional shaking. Bacterial culture were centrifuged at 10,000 rpm at 4 C for 15 min. Supernatant acidified to pH 2.5 with 12 N HCl and extracted thrice with 10 ml of ethylacetate. Ethylacetate was evaporated at 40^{0}C and residue was redissolved in 2 ml of absolute methanol and mixed with 2 ml of Fe-$HClO_4$ reagent (1 ml 0.5 M $FeCl_3$ and 50 ml 35% $HClO_4$). After 25 min, O.D. was measured at 530 nm. A standard curve was prepared using different concentration of Indole-3-acetic acid .

3.7.11.3 Salicylic acid (SA)

The Pseudomonads isolates were grown at 28 ± 2^0 C for 72 hours on a rotary shaker in 250 ml conical flask containing 50 ml 0f the King's broth medium. Cells were then collected by centrifugation at 10,000 rpm 10 minutes and 4 ml of cell free culture filtrate was acidified with 1N HCl to pH 2.0 and SA was extracted in chloroform (2x2 ml). To the pooled chloroform extracts, 4 ml of distilled water and 5 ml of 2 M $FeCl_3$ were added. The absorbance of the purple iron – SA complex, which was developed in the aqueous phase, was read at 527 nm in a spectrophotometer (Reddy *et al.,* 2008). A standard curve was prepared with SA dissolved in King's B broth medium. The quantity of SA in the culture filtrate was expressed as mg.ml^{-1}. Three replications were maintained for each isolate.

3.7.12 Molecular characterization of fluorescent Pseudomonad isolates

3.7.12.1 Isolation of Bacterial genomic DNA

The isolation of genomic DNA of bacteria follows the standard phenol/chloroform method (Neumann et al. 1992), however in the present study the improved version of the above method as described by Cheng & Jiang (2006) was referred with slight modifications. One ml of the over-night grown bacterial culture in LB broth was centrifuged at 10,000g for 5 min. After removing the supernatant, the cells were washed with 400 µl STE Buffer (100 mM NaCl, 10 mM Tris/HCl, 1 mM EDTA, pH 8.0) twice.

Then the cells were centrifuged at 10,000g for 2 min. The pellets were resuspended in 200 µl TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0). Then 100 µl Tris-saturated phenol (pH 8.0) was added to these tubes, followed by a vortex-mixing for one minute. The samples were subsequently centrifuged at 13 000g for 5 min at 4^0 C to separate the aqueous phase from the organic phase. About 150 µl of the the upper aqueous phase was transferred to a clean 1.5 ml tube and 50 µl TE buffer was added to make 200 µl and mixed with 100 µl chloroform and centrifuged for 5 min at 13 000g at 4^0C. The process was repeated again to ensure the purification of nucleic acids. Then the upper aqueous phase was transferred to a clean 1.5 ml tube and RNAse treatment was given by adding 40 µl TE and 5 µl RNase (at 10 mg/ml) and incubated at 37^0 C for 10 min to digest RNA. Then 100 µl chloroform was added to the tube, mixed well and centrifuged for 5 min at 13 000g at 4^0C. About 150 µl of the upper aqueous phase was transferred to a clean 1.5 ml tube which contained the purified bacterial DNA and was stored at -20 ^{0}C for further use in PCR amplification for 16S rDNA gene.

3.7.12.*2* PCR amplification of 16S rDNA gene

The 16S rRNA gene was amplified using PCR with Taq-polymerase (Merck, Bioscience) (Saiki et al., 1988) and the universal primer pair of 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'), described by Weisburg et al. (1991). PCR amplifications were performed in 50 µl reaction mixture containing buffer containing required amount of PCR components along with and 100 ng bacterial DNA, by using a DNA thermal cycler (Fig. 3.7.1).

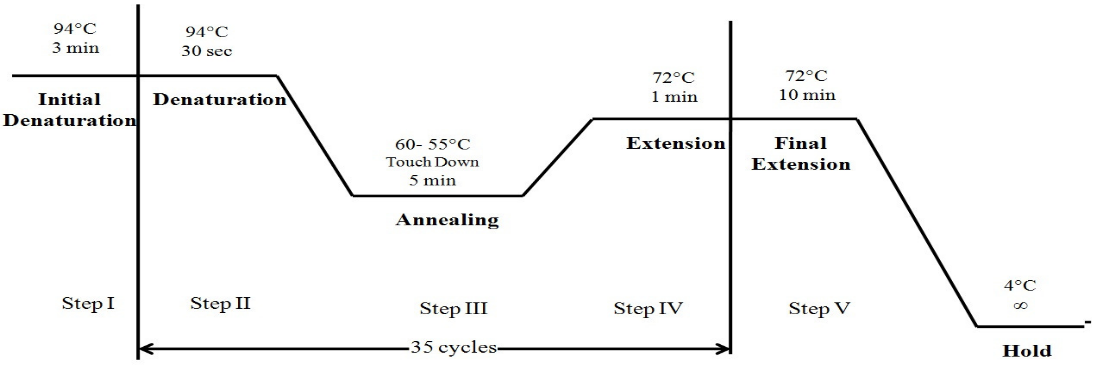


Fig. 3.7.1: Thermal cycler programme for 16S rDNA gene amplification

The expected size of the amplified PCR products was resolved on 1 % (w/v) agarose gel, visualized by ethidium bromide staining, and documented using a Fuji FLA 5200 imaging system. The amplicons were purified and sequenced directly using a Taq

DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems). Sequence reaction mixtures were electrophoresed and analyzed with an Applied Biosystems model 377A DNA sequencer at Department of Biotechnology, Junagadh Agricultural University, Junagadh. For reconfirmation, few samples were sequenced at SciGenome Facility, Cochin.

3.7.12.3 16S sequence analysis and identification of isolates

The amplified 16S DNA of all selected isolates were sequenced and all nucleotide sequences obtained were compared to the sequences available in NCBI GenBank. Sequence analysis of these isolates was performed using BLAST (Blastn) search tool (http://www.ncbi.nlm.nih.gov) available on the NCBI homepage. Based on maximum similarity of the 16S sequences of the isolates to the already available sequences in NCBI database, Pseudomonad species identification were made. The sequences were further checked for chimera sequences and submitted to the NCBI.

3.8 EXPERIMENT III

HISTO-PATHOLOGICAL BASIS OF *S. rolfsii* INFECTION IN GROUNDNUT

3.8.1 Experimental details

Location	:	Department of Biotechnology and Biochemistry, JAU, Junagadh
Host and Pathogen	:	Four groundnut genotypes and *S. rolfsii*
Infection	:	40 days old healthy plants were challenged with the pathogen under controlled conditions
Observations made: Anatomical differences and induced structural changes		

3.8.2 Sample Collections, preparation and Scanning Electron Microscopy

The healthy and infected plants , grown in controlled condition (already described in section 3.8.3) were collected 5 days post inoculation of *S. rolfsii*. The samples were selected from one cm above the collar region of both healthy and infected plants and proceed further following the steps described by Nandi *et at*, (2010) with slight modifications. The stem tissues were transversely and horizontally sectioned in 0.2 to 0.5 mm thick using a razor. The sections were fixed for 2 h in 25% (v/v) glutaraldehyde solution and then dehydrated in different strength of ethyl alcohol solutions (20% to 100%)

for 10 min in each. The sections were taken on SEM stubs and gold ionization was done through Ion coater prior to scanning electron microscopy (ZEISS-EVO-18 Special edition). Photographs were captured at different magnifications.

3.9 EXPERIMENT IV

INDUCED BIOCHEMICAL CHANGES DURING HOST,-PATHOGEN AND -BIOCONTROL AGENT INTERACTIONS

3.9.1 Experimental details

Location	:	Department of Biotechnology and Biochemistry, JAU
Pathogen	:	*Sclerotium rolfsii*
Induce Resistance	:	Seed priming with *SKPf 5*
Varieties	:	CS19, GG16, GG20 and TG37A
Replication	:	Three
Stages	:	Three
Treatments	**T_1**	Healthy plants as control
	T_2	Healthy plants raised from seed primed with *SKPf5*
	T_3	Healthy plants raised from seed primed with *SKPf5* challenged with *S. rolfsii*
	T_4	Healthy plants challenged with *S. rolfsii*
Design		3FCRD
Observations recorded		
Morphological	:	% disease incidence
Anti-oxidative Enzymes	:	Superoxide dismutase (Dhindsa *et al.*, 1981)
	:	Catalase (Aebi, 1984)
	:	Glutathione Reductase (Smith *et al.* 1988)
	:	Ascorbate Peroxidase (Nakano and Asada, 1981)
PR proteins	:	Peroxidase (Castillo *et al.,* 1984)
	:	β- 1,3-Glucanse (Kauffman *et al.,* 1987)
	:	Chitinase (Boller and Mauch, 1988)
Other Metabolites	:	Ascorbic Acid (Sairam and Saxena, 2005)
	:	Oxalic acid (Peñaloza *et al.*, 2002)
Protein expression	:	Protein profiling (Alam *et al.*, 2013)

3.9.2 Preparation of Pseudomonas formulation and seed priming

The *Pseudomonas* isolate (*SKPf* 5) which was stored in King's B broth amended with 20% glycerol at -80^{0}C prior to use, was revived and the re-suspension was prepared as described by NiranjanRaj *et al.* (2004) with slight modifications. Bacterial cell suspensions were prepared by first streaking the isolates onto King's B agar medium and incubating at 27-30 ^{0}C for 24 h to check the purity, then transferring single colonies to King's B plates. After 24 h, the bacterial cells were harvested from the plates in sterile distilled water (SDW), centrifuged at 6000 rpm for 5 min., and the pellet obtained was re-suspended in SDW. The optical density of the suspension was adjusted (1.2) using a UV-visible spectrophotometer following the method of Mortensen (1992) to obtain a final density of 10^8 cfu/ml. Matured and healthy seeds of groundnut genotypes as mentioned earlier were surface-sterilized with 0.02% mercuric chloride by shaking for 2 min and then soaked in the bacterial suspension amended with 0.2% carboxymethyl cellulose (CMC) to facilitate the adherence of the bacteria to the seeds and incubated at 27 ^{0}C in an incubator rotary shaker at 150 rpm for 12 h. After incubation the seeds were air-dried before use. Seeds soaked in distilled water amended with CMC served as the control.

3.9.3 Host and growing conditions

Four groundnut genotypes which were selected based on their varying degree of tolerance to the stem rot disease (section 3.6) such as CS19 (tolerant), GG16 (moderately tolerant), GG20 (susceptible) and TG37A (highly susceptible). Seed were sown in a mixture of black soil, farm yard manure and sand (2:1:1, v/v) in plastic pots in the glass house. Four seeds were sown in each pot, and the seedlings were later thinned to one per pot. Forty-eight pots for each variety were raised which included four treatments (described below) and three replications. The plants were grown in controlled condition in the glass house at Directorate of Groundnut Research, Junagadh (Plate.3.1). During the period of experimentation, the temperature inside the glass house was in the range of 30 ± 5 ^{0}C. The relative humidity was maintained 70-80% through out by intermittent fogging during the experimental period. The plants were watered as per the requirement to avoid water stress.

3.9.4 Multiplication of *Sclerotium rolfsii* inoculums

The *S. rolfsii* isolate was cultured in 90 mm petri-dishes containing standard potato dextrose agar (PDA) medium. The fungal pathogen, *S. rolfsii which* was isolated and maintained as described earlier in section 3.7.3 on PDA media, was further mass multiplied on sorghum grains. About 500g Sorghum grains were boiled in tap water for 30 minutes and autoclaved for 15 minutes under 121^0C and 15 lb pressure. Sterile sorghum grains were inoculated with mycelium of *S. rolfsii* taken from margin of actively growing cultures in PDA medium using crock borer of 10 mm in diameter. The inoculated bags were incubated for 8-10 days at room temperature for healthy growth of the fungus and for further use.

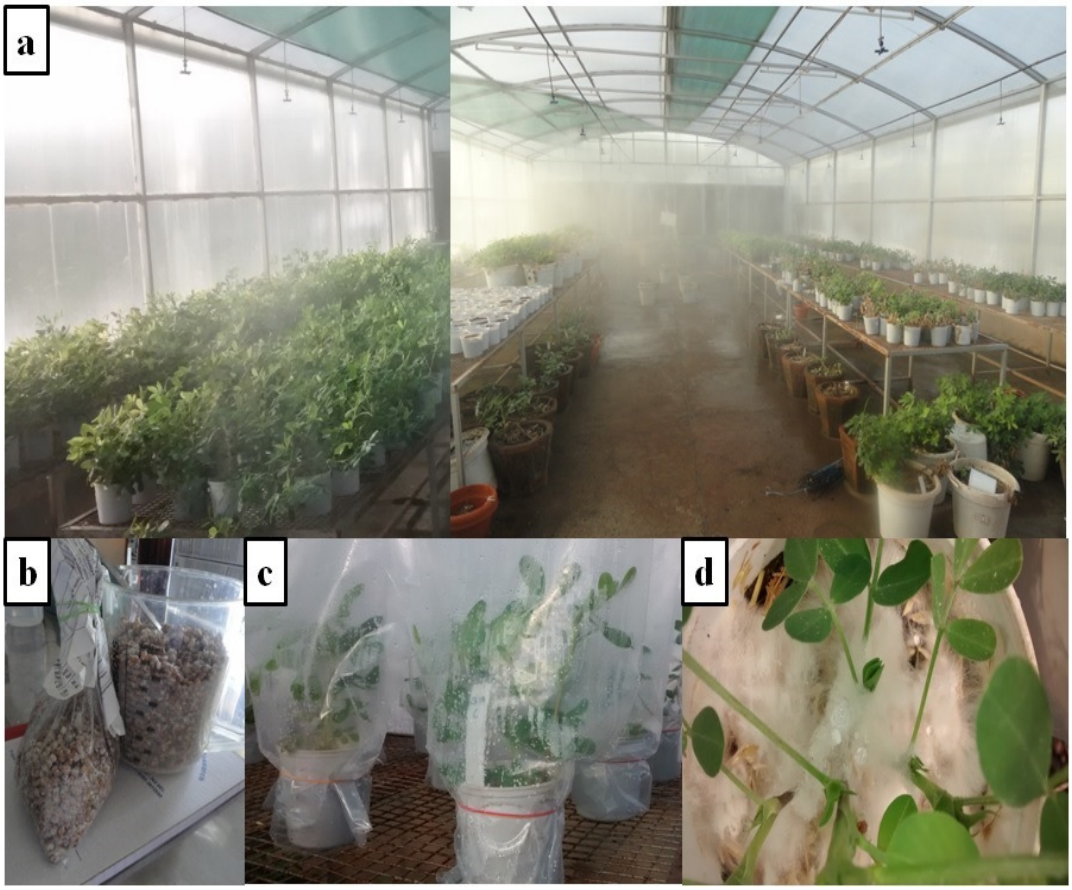


Plate 3.9.1: Plants growing conditions and *S. rolfsii* infection in glasshouse

(a) Plants grown in controlled conditions in glass house; (b) Fungal inoculums grown on sorghum; (c) Use of polythene bags for ensuring fungal infection ; (d) Mycelia of *S. rolfsii* covering the whole pot

3.9.5 Application of biocontrol agent and fungal inoculums

The Pseudomonads isolates were grown at 28 ± 2^0C with occasional shaking in King's B broth for 3 days and centrifuged at 10,000 rpm for 10 min and the supernatant was collected. The optical density of the supernatant was adjusted (1.2) using a UV-visible spectrophotometer following the method of Mortensen (1992) to obtain a final density of

10^8 cfu/ml. About 20 ml of the supernatant was added to the rhizosphere of each plant of T2 and T3.

The fungus multiplied in sorghum grain was applied to about 45-day old groundnut plants of T3 and T4. Each groundnut plants were inoculated by placing infested sorghum grains (about 2g) on soil surface nearer to the main stem. For ensuring the development of mycelia, the experiment was carried out in controlled conditions (Temperature 30 ± 5 ^{0}C and RH 70- 80%) in glass house.

3.9.6 Per cent Disease Incidence

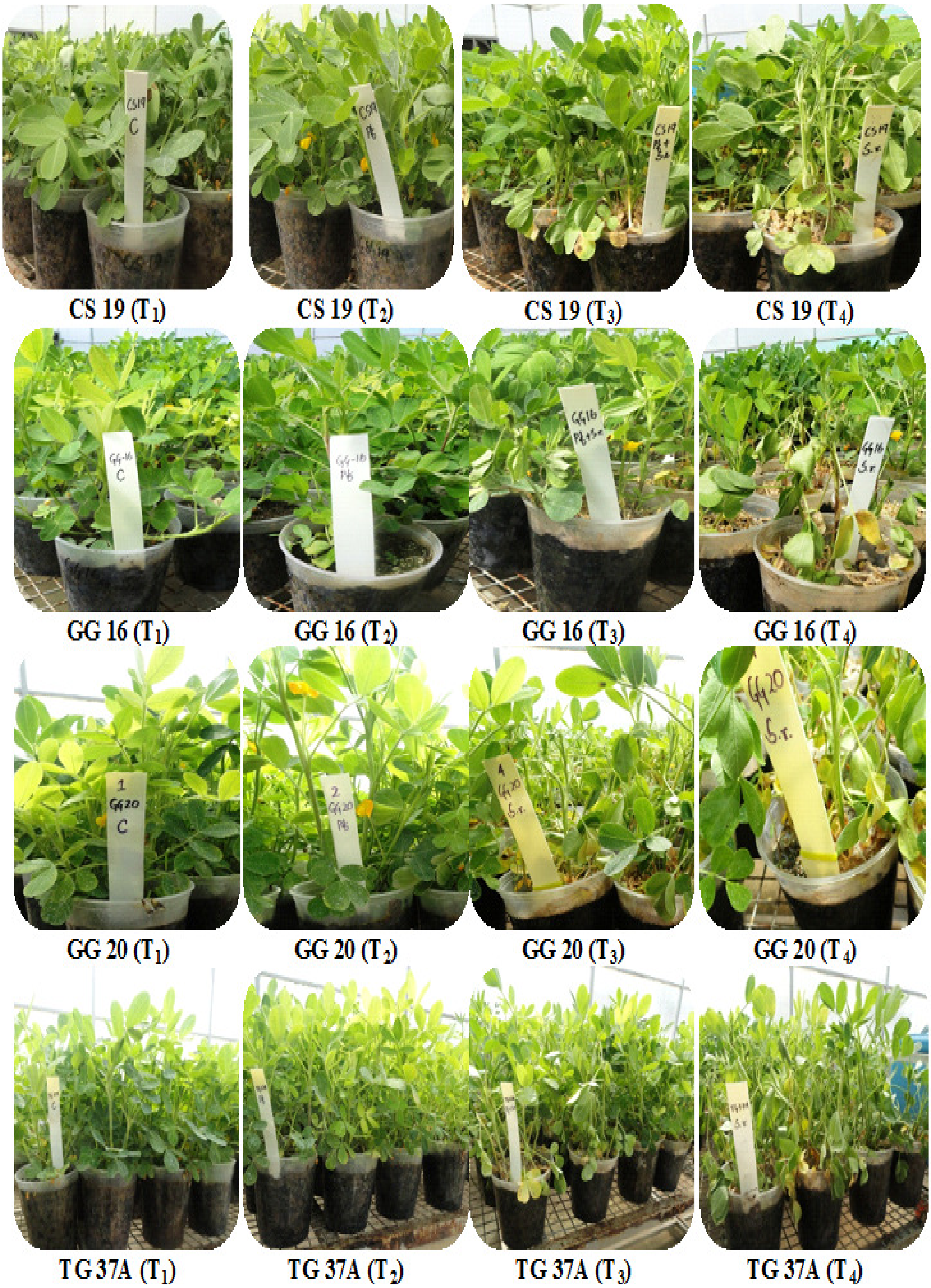
The incidence and progress of stem rot in each treatment were monitored regularly. The per cent disease incidence in terms of mortality of plants was calculated by using the formula "Per cent disease incidence = (Number of infected plants/ Total number of plants) X 100". The total number of plants for each treatment was sixteen individual plants (Plate 3.9.2 and Plate 3.9.3).

3.9.7 Sample Collections

The primary leaves were collected at 1, 5, and 10 days post inoculation (dpi) separately from all treatments and used for subsequent analyses. The harvested samples were separately packed in plastic bags and were brought to the laboratory in ice bucket. The samples were washed with distilled water and surface moisture was wiped out, and then used for further analysis. The experiment was repeated three times.

3.9.8 Estimation of anti-oxidative enzymes

Enzyme extracts for Superoxide dismutase, Peroxidase, Catalase and Glutathione reductase were prepared by first freezing the weighed amount of leaf samples (1g) in liquid nitrogen to prevent proteolytic activity followed by grinding with 10 ml extraction buffer (0.1 M phosphate buffer, pH 7.5, containing 0.5 mM EDTA). For extraction of ascorbate peroxidase, the above buffer containing 1 mM ascorbic acid was used. The crude extract was passed through 4 layers of cheesecloth and filtrate was centrifuged for 20 min at 15,000 g and the supernatant was used as enzyme extract (Dhindsa *et al*., 1981).



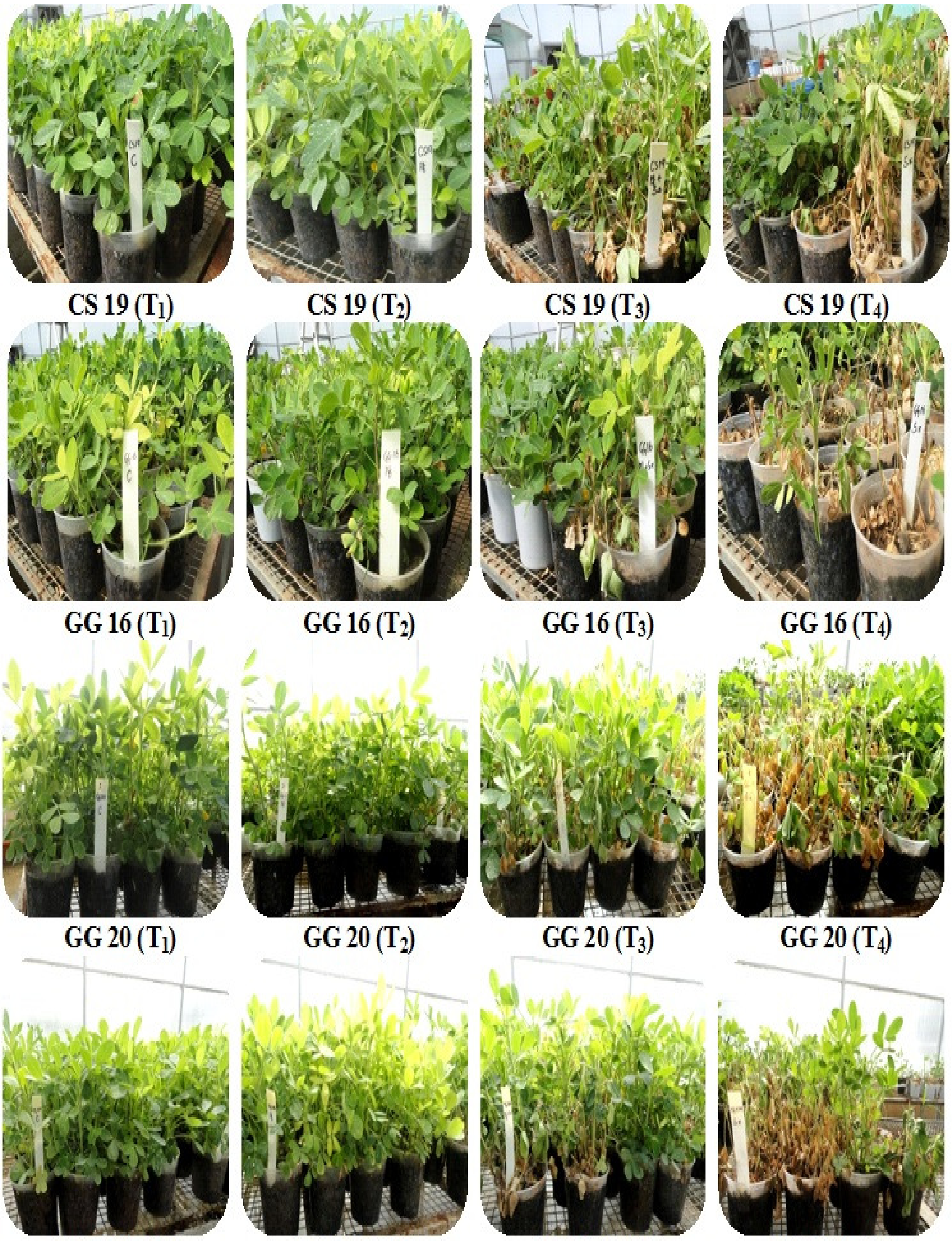
CS 19 (T$_1$) CS 19 (T$_2$) CS 19 (T$_3$) CS 19 (T$_4$)

GG 16 (T$_1$) GG 16 (T$_2$) GG 16 (T$_3$) GG 16 (T$_4$)

GG 20 (T$_1$) GG 20 (T$_2$) GG 20 (T$_3$) GG 20 (T$_4$)

TG 37A (T$_1$) TG 37A (T$_2$) TG 37A (T$_3$) TG 37A (T$_4$)

Plate 3.9.2: Effect of genotypes and treatments on stem rot incidence at 5 dpi under controlled conditions of temperature and humidity



CS 19 (T_1) CS 19 (T_2) CS 19 (T_3) CS 19 (T_4)

GG 16 (T_1) GG 16 (T_2) GG 16 (T_3) GG 16 (T_4)

GG 20 (T_1) GG 20 (T_2) GG 20 (T_3) GG 20 (T_4)

TG 37A (T_1) TG 37A (T_2) TG 37A (T_3) TG 37A (T_4)

Plate 3.9.3: Effect of genotypes and treatments on stem rot incidence at 10 dpi under controlled conditions of temperature and humidity

3.9.8.1 Superoxide (SOD, EC 1.15.1.1)

Total SOD activity was estimated by the inhibition of the photochemical reduction of nitrobluetetrazolium (NBT) by the enzyme by recording the decrease in optical density of formazone made by superoxide radical and nitro-blue tetrazolium dye by the enzyme (Dhindsa *et al.*, 1981).Three ml of the reaction mixture contained the following components and the final volume was made up by adding distilled water.

(i) 13.33 mM methionine (0.2 ml of 200 mM)
(ii) 75 µM nitrobluetetrazolium chloride (NBT) (0.1 ml of 2.25 mM)
(iii) 0.1 mM EDTA (0.1 ml of 3 mM)
(iv) 50 mM phosphate buffer (pH 7.8) (1.5 ml of 100 mM)
(v) 50 mM sodium carbonate (0.1 ml of 1.5 M)
(vi) 0.05 to 0.1 ml enzyme

Reaction was started by adding 2 mM riboflavin (0.1 ml) and placing the tubes under 15 W fluorescent lamps for 15 min. A complete reaction mixture without enzyme, which gave the maximal colour, served as control. Switching off the light and putting the tubes into dark stopped the reaction. A non-irradiated complete reaction mixture served as a blank. The absorbance was recorded at 560 nm, and one unit of enzyme activity was taken as that amount of enzyme, which reduced the absorbance reading to 50% in comparison with tubes lacking enzyme.

3.9.8.2 Catalase (CAT, EC 1.11.1.6)

Catalase assay is based on absorbance of hydrogen peroxide at 240 nm in UV range and decrease in absorbance by the enzyme due to decomposition of H_2O_2 to water (Aebi, 1984). The 3 ml reaction mixture consisted of 0.5 ml of 75 mM H_2O_2 and 1.5 ml of 0.1 M phosphate buffer (pH 7) in cuvettes, and the reaction was initiated by adding 50 µl of diluted enzyme extract. The decrease in absorbance at 240 nm was observed for 1 min in a UV- visible spectrophotometer (model Specord Bio-200, AnalytikJena, Germany). Enzyme activity was computed by calculating the amount of H_2O_2 decomposed. The initial and final contents of H_2O_2 were calculated by comparing with a standard curve drawn with known concentrations of H_2O_2.

3.9.8.3 Ascorbate peroxidase (APX, EC 1.11.1.11)

Ascorbate peroxidase was assayed by recording the decrease in optical density due to ascorbic acid at 290 nm (Nakano and Asada, 1981). The 3 ml reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0) (1.5 ml of 100 mM), 0.5 mM ascorbic acid (0.5 ml of 3.0 mM), 0.1 mM EDTA (0.1 ml of 3.0 mM), 0.1 mM H_2O_2(0.1 ml of 3.0 mM), 0.1 ml enzyme and water 0.7 ml (to make a final volume of 3.0 ml). The reaction was started with the addition of 0.2 ml of hydrogen peroxide. Decrease in absorbance for a period of 30 sec. was recorded at 290 nm in a UV-visible spectrophotometer. Activity is expressed by calculating the decrease in ascorbic acid content by comparing with a standard curve drawn with known concentrations of ascorbic acid. It was calculated by using the extinction coefficient 2.8 mM^{-1} cm^{-1} and expressed in enzyme units (mg protein). One unit of enzyme is the amount necessary to decompose 1 µmol H_2O_2 min^{-1} at 25 ^0C.

3.9.8.4 Glutathione Reductase (GR, EC 1.8.1.7)

Glutathione reductase was assayed following the method described by Smith *et al.* (1988). The reaction mixture contained: 66.67 mM potassium phosphate buffer (pH 7.5) and 0.33 mM EDTA (1 ml of 0.2 M buffer containing 1mM EDTA), 0.5 mM DTNB in 0.01 M potassiumphosphate buffer (pH 7.5) (0.5 ml of 3.0 mM), 66.67 µM NADPH (0.1 ml of 2.0 mM), 666.67 µM GSSG (0.1 ml of 20 mM), 0.1 ml enzyme extract and distilled water to make up a final volume of 3.0 ml. Reaction was started by adding 0.1 ml of 20.0 mM GSSG (oxidized glutathione). The increase in absorbance at 412 nm was recorded spectro-photometrically. The activity is expressed as total absorbance (ΔA_{412}) per mg protein per min (Smith *et al.,* 1988). It was calculated by using the extinction coefficient 6.2 mM^{-1} cm^{-1} and expressed in enzyme units (mg protein). One unit of enzyme is the amount necessary to decompose 1 µmol NADPH min^{-1} at 25 ^0C.

3.9.9 Estimation of Pathogenesis Related (PR) proteins

Enzyme extract for chitinase and ß-1,3-glucanase was prepared by following the method as described by Madhaiyan *et al.,* (2006) with slight modifications. The weighed amount of leaf samples (1g) was frozen in liquid nitrogen to prevent proteolytic activity followed by grinding with 10 ml extraction buffer (100mM sodium acetate buffer, pH-4.8 containing 0.2% (w/v) sodium sulphate and 1% (w/v) soluble polyvinylpyrolidone (PVP) and 10% sodium chloride. The crude extract was passed through 4 layers of cheesecloth and filtrate was centrifuged for 20 min at 15,000 g and the supernatant was used as enzyme extract. For peroxidise estimation, the enzyme extract already described in section 3.8.8 was used.

3.9.9.1 Peroxidase (POX, EC 1.11.1.7)

Peroxidase activity was assayed as per the method of Castillo *et al.,* (1984). The 3 ml reaction mixture consisted of the following components.

Phosphate buffer (50 m M, pH 6.1)	1.0 ml of 100 mM
Guaiacol (16 mM)	0.5 ml of 96 mM
H_2O_2 (2 mM)	0.5 ml of 12 mM
Enzyme	0.1 ml
Water	0.4 ml, to make final volume of 3.0 ml.

Absorbance due to the formation of tetra-guaiacol was recorded at 470 nm and enzyme activity was calculated as per extinction coefficient of its oxidation product, tetra-guaiacol ($\varepsilon = 26.6\ mM^{-1}\ cm^{-1}$) and expressed as µmol tetra-guaiacol formed per min per mg protein.

3.9.9.2 *ß-1,3 glucanases* (EC 3.2.1.6)

Reaction mixture consisted 0.4 ml of 4% laminarin (dissolved in boiled water) and 0.4 ml of enzyme extract. The mixture was incubated at 40°C for 10 min (Kauffman *et al.,* 1987). The reaction was stopped by adding 0.5 ml of DNSA reagent followed by steps described under sub heading ***3.7.8*** under this chapter to measure glucose released by enzyme. Blank was treated as zero time incubation. The specific activity of the enzyme was expressed as the U.mg^{-1}protein and unit activity was defined as µM glucose released.10min.$^{-1}$g.$^{-1}$ fresh wt. tissues under assay conditions.

3.9.9.3 Chitinase (EC 3.2.1.14)

The enzyme activity was assayed following the method of Boller and Mauch (1988). Reaction mixture consisted 0.3 ml enzyme, 0.3 ml of 10 mM sodium acetate buffer and 0.3 ml of 0.05% chitin (Sigma) (Dissolved in boiled water). The mixture was incubated at 50°C for 1 h. After that, tubes were centrifuged at 2000 rpm for 2 min. Blank was treated as zero time incubation. The formation of sugar N- acetylglucosamine was measured by DMAB method (Reissig *et al,* 1955) as described under subheading 3.7.9. The specific activity of the enzyme was expressed as the U.mg^{-1}protein and unit activity was defined as µM N-acetylglucosamine released.h.$^{-1}$g^{-1}Fresh wt. tissues under assay conditions.

3.9.10 Estimation of other metabolites

3.9.10.1 Ascorbic Acid

Ascorbic acid was estimated using the method as described by Sairam and Saxena (2005) with slight modifications. Fresh leaf sample (0.250 g) was extracted with 20 ml of 6% trichloroacetic acid. Four ml of the extract was mixed with 2 ml of 2% dinitrophenylhydrazine (in acidic medium) followed by the addition of 1 drop of 10% thiourea (in 70% ethanol). The mixture was boiled for 15 min in a water bath and after cooling to room temperature, 5 ml of 80% (v/v) H_2SO_4 was added to the mixture at ^{0}C (in an ice bath). The absorbance was recorded at 530 nm. The concentration of AA was calculated from a standard curve plotted with known concentration of Ascorbic acid.

3.9.10.2 Oxalic acid

Organic acids were extracted from the stem tissues of both healthy and infected groundnut genotypes according to the procedure of Peñaloza *et al.* (2002) with slight modifications. About 500mg fresh leave tissues were finely ground and treated with 10 ml of 2% (v/v) acetic acid by mixing well by gentle vortex. The sample was allowed to incubate at room temperature for 30 minutes with intermittent shaking and then spun at 10,000g for 10 minutes. The supernatant was collected and the organic acids were extracted by phase separation using chloroform in proportion 2:1 (supernatant: chloroform). The upper aqueous phase was collected after spinning at 10,000g for 10 minutes and referred as organic acid extract and stored at -20 °C until further analysis by ion chromatography. The organic acid extract was diluted five times, membrane filtered and 25 µl of samples were used for separation of organic acids by Ion Chromatograph (Dionex, ICS 3000). AS11HC Analytical column equipped with suppressor ASRS 300 was used for separation of organic acids using 150 mM NaOH as eluent with a flow rate of 1 ml min^{-1}. Oxalic acid was identified by comparison with the elution time of standard of the same using a conductivity detector, the amounts of each one in the samples were determined from the peak areas in the samples with that for the oxalic acid standard using the Chromeleon software provided with the Ion chromatograph.

3.9.11 Protein profiling through 2-D electrophoresis

3.9.11.1 Protein extraction

Method described by Alam *et al.* (2013) and GE Health care protocol was used for the sample extraction with some modifications. Leaf samples of groundnut genotypes were collected and stored at -20^0 C till further analysis. About 1.0 g frozen leaf samples were ground in a mortar pre-chilled with liquid nitrogen and incubated with extraction buffer (10 ml/g tissue) consisting of 0.5 M Tris-HCl (pH 7.6), 2% (v/v) Triton-X 100, 20 mM $MgCl_2$, 2% (v/v) ß–mercaptoethanol and 1mM PMSF. After vortexing for 1 min, each sample was centrifuged at 5000 g for 15 min. The supernatant was collected and the proteins in the supernatant were subjected to PEG fractionation.

3.9.11.2 PEG fractionation and phenol extraction

The buffer-extracted sample solution was adjusted to a final concentration of 15% (w/v) PEG by adding a 50% (w/v) stock solution; it then was incubated on ice for 30 min. Following centrifugation at 5000g for 15 min at 4 °C, the supernatant was collected. Four volumes of 100% acetone then were added to the recovered supernatant fraction and precipitated for overnight at −80 °C followed by centrifugation at 5000g for 20 min. The recovered pellet was dissolved in 10 ml of extraction buffer containing 0.7 M sucrose and was agitated at room temperature and mixed with an equal volume of Tris-saturated phenol. After vortexing, was centrifuged at 5000 g for 15 min. The upper phenolic phase was collected and the proteins were precipitated by adding four volumes of methanol containing 0.1 M ammonium acetate at −80 °C for 3 h, and then centrifuged at 5000 g for 10 min. The proteins were washed three times with methanol containing 0.1 M ammonium acetate. The pellet containing the proteins was precipitated as white flakes. Now the pellet was washed twice with ice-cold methanol (5 ml) to remove ammonium acetate and phenol, lipids and pigments. Later the above step was repeated using equal volume of acetone instead of methanol to achieve faster and more effective drying. After 30 minutes of drying, the pellet was dissolved in 500µl rehydration buffer without ampholytes and kept at 4 ^{0}C over-night to ensure the solubilisation. The protein amount was quantified using Folin-lowry method and the quality was checked by running a SDS-PAGE using 12% acryamide gel. The protein was stored in -20 ^{0}C till further use in 2-dimensional electrophoresis.

3.9.11.3 Rehydration of IPG strips

Method described by Kumar and Kirti, (2015) was followed for protein separation using GE Health care protocol. The pellet was then dissolved in 400 µl of rehydration buffer where 1% bromophenol blue and 0.2% (w/v) ampholytes were added separately. Rehydration was carried out for 12 hours in rehydration tray. Total volume of sample 400 µl was pipetted out along edge of the rehydration tray and evenly distributed except for about 1 cm at each end. All the protein samples were loaded into the rehydration or equilibration tray. By using forceps coversheet was peeled from Ready IPG linear strips of 24 cm and of 4-7 pH. Strip was gently placed gel side down onto the sample in rehydration tray. The “+” and “pH 4-7” of strip positioned at the left of the tray. 2 to 3 ml of mineral oil was overlaid on each strip to prevent the evaporation during rehydration process. Then rehydration or equilibration tray was covered with the plastic lid provided and kept overnight for 12 hours on a level bench to rehydrate the immobilized pH gradient (IPG) strips and load the protein sample.

3.9.11.4 First dimension (Isoelectric focusing)

Dry and clean Ettan IEF focusing tray (the same size as the rehydration tray) placed onto Ettan IEF cell, in which rehydrated IPG strip was placed. Paper wicks were wetted with double distilled water and adjusted their position by using forceps at both ends of the Ettan IEF channels covering the wire electrodes. Cover removed from the rehydration or equilibration tray containing the IPG strips. Using forceps strip hold vertically for about 7-8 seconds to allow the mineral oil to drain off. Then IPG strip was transferred to the corresponding channel in the focusing tray (maintained gel side up). Again cover the IPG strip with 2-3 ml of fresh mineral oil and checked for any air bubble beneath the strip and then closed the cover. Appropriate 6 step protocol was programmed for the Ettan IEF cell was as follow.

Steps	Voltage (V)	Current (µA)	Volt-hours (HH:MM)
Step 1	200	75	01:00
Step 2	500	75	07:00
Step 3	1000	75	01:00
Step 4	8000 (Gradient)	75	08:00
Step 5	8000	75	05:00
Step 6	500	75	04:00
Total Run time (volt hours)			26

Default cell temperature of 20 ^{0}C with maximum current of 75 µA per strip was maintained during the gel run.

3.9.11.5 IPG equilibration and Second dimension (SDS-PAGE)

Strip was incubated in 10 ml of equilibration buffer-I and kept for 15 minutes for equilibration on shaker with continuous shaking. Equilibration buffer-I containing Urea (6M), 2% SDS, Tris buffer (75 mM, pH- 8.8), 87% glycerol and DTT (100 mg per 10 ml buffer) was used. After 15 minutes, equilibration buffer-I was drained off and again 10 ml of equilibration buffer-II containing Urea (6M),2% SDS, Tris buffer (75 mM, pH- 8.8), 87% glycerol and iodoacetamide (250 mg per 10 ml buffer), was added to the strip and kept on shaker for 15 minutes with continuous shaking. Using forceps, IPG strip was placed on a 12% SDS-PAGE running gel and low melting agarose sealing solution was overlaid on it and kept for 5 minutes to solidify. Separation of proteins in 2-dimentional gel electrophoresis was similar as that of the SDS-PAGE. After completion of electrophoresis, gel was stained in coomassie stain containing commassie blue R-250 (0.1 g) was dissolved in 100 ml solution of Methanol (40): Acetic acid (10): Water (50). Gel was incubated for overnight at room temperature and destained in destaining solution containing 100 ml solution of Methanol (40): Acetic acid (10): Water (50).

3.9.11.6 Identification of spots

Gel was scanned through Typhoon FLA 7000 scanner and photographs were used for further analysis through Image master 2-D platinum software powered by Melanie.

3.10 EXPERIMENT V

DIFFERENTIAL EXPRESSION OF DEFENCE RELATED GENE(S) IN TOLERANT AND SUSCEPTIBLE GENOTYPES

3.10.1 Host and growing conditions

Groundnut genotypes were grown and treatments were given following the same method and conditions as described earlier in section 3.8. To ensure proper infection by the pathogen, the temperature inside the glass house was maintained at 30 ± 5^{0}C and the relative humidity was 70-80% throughout the period of experiment. The plants were watered as per the requirement to avoid water stress. Appropriate treatments were given in 40 days old plants.

3.10.2 Experimental details

Location	:	Department of Biotechnology and Biochemistry, JAU, and Directorate of Groundnut Research, Junagadh
Varieties and pathogen	:	Four groundnut varieties (CS19, GG16, GG20, TG37A) and *S. rolfsii*
Infection	:	40 days old healthy plants were challenged with the pathogen under controlled condition
Stages	:	Three
Replications	:	Three

3.10.3 Designing of primers

3.10.3.1 Gene specific primers for Polygalacturonase Inhibitory Protein (PGIP) gene

The gene sequence of PGIP for groundnut was not available in the NCBI database except for a sequence (gi|300517491|gb|HO115743.1) representing somewhat sequence similarity to PGIP of other crops. Hence an attempt was made to design the gene specific primers from that EST sequence using online softwares such as NCBI-Primer Blast software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and IDT-PrimerQuest (https://eu.idtdna.com/Primerquest/Home/Index). Some critical considerations in primer designing were 40-60% GC, avoidance of sequences with potential internal secondary structure formation, avoidance of primer dimer formation due to complementarity in the 3'-ends and also avoidance of three G or C in a row near the 3'ends of the primer. Wherever possible, care was taken for both primers to have similar Tm and length. The primer used for the study was as follow with an expected amplicon size of 600 bp.

Forward Sequence (5’-3’):	GCGCTTCTCCAAATCAAGAAGGAC
Reverse Sequence (5’-3’):	ATGGAAGCGTCACCCTCCAGC

3.10.3.2 Primers for Real Time PCR

The primers were designed by using NCBI-Primer Blast software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and IDT-PrimerQuest (https://eu.idtdna.com/Primerquest/Home/Index). Some critical considerations in primer designing were: amplicon size 100-200bp, ideal Tm should be 60°C (57-63 °C with a maximum difference of 3°C in the Tm’s of the two primers), GC content ~ 50-60%, primers should end with a C or G residue to avoid non-specificic amplification, with least self complementarity to avoid the possibility of primer-dimer formation, having a near random mix of nucleotides, no long stretches for single nucleotide sequence. The details of the primer sequences used in the present study were given in Table 3.1.

Table 3.10.1 Primer sequences used for gene expression analysis

Gene	Primer		Sequence (5'-3')	Amplicon Size (bp)
β-Actin	*Ah*-A	F:	TTGGAATGGGTCAGAAGGATGC	180
		R:	AGTGGTGCCTCAGTAAGAAGC	
PR 2	*Ah-2*	F:	CCCATTCTCTGGGAGTTCTG	173
		R:	AGGCACTTTCTTTGGGGAATCTC	
PR 4	*Ah-4*	F:	TGACACCACAAGGGAATAAGC	161
		R:	AGTTTGTGTTGGCACCTTTCC	
PR 5	*Ah-5*	F:	CAACCACCTTGAATGCAGTG	165
		R:	ACGTTACAGGCACCAGATCC	
PR 10	*Ah-10*	F:	ACAGCGTGGTTGGAGGAGTG	171
		R:	CCTTGGCCTTACCCTTCTTC	
PGIP	*Ah*-PGIP	F:	TCAACCTCACCAACCTCAAAG	179
		R:	CTGTAAGTCGAGGGATTGGAG	

3.10.3.3 Collection of sample

The primary tender leaves were collected at 1, 5, and 10 days post inoculation (dpi) separately from all treatments and used for subsequent analyses. The harvested samples were collected separately in silver foil and were brought to the laboratory in liquid nitrogen without allowing it to thaw. The samples were either directly proceed for RNA isolation or kept at -80 ^{0}C till further analysis.

3.10.3.4 Isolation of RNA from leaf tissues

Total RNA from leaf tissue of healthy and stem rot infected plants were isolated using RNASure Plant Kit (Genetix Brand, Cat.# NP-84905 India) according to manufacturer's protocol with minor modifications. Leaf tissues were homogenized in liquid nitrogen to fine powder. Nitrogen was allowed to evaporate and about 100 mg of powder was used for RNA isolation. The powder was suspended in 350 µl RLB1 buffer containing 3.5µl β-mercaptoethanol and incubated at 55 °C for 10 min. The lysate was then transferred to a 1.5 ml centrifuge tube (henceforth refers to RNAse free) and centrifuged at 15,000g for 2 min to remove cell debris. The cleared lysate was then transferred to an RNASure Shredder in collection tubes (2 ml), and centrifuged at 11,000g for one minute. To the filtrate, 350µl of 70% chilled ethanol was added and mixed well. The mixture was passed through RNASure Plant Column to a collection tube at 11,000g for 1 minute. The residual salt was removed by adding 350µl RDB to the above column and centrifuged at 11,000g for 2 minutes. For complete drying of the column, another spin for one minute was given. Salt removal helps the following rDNase digestion more effectively. For on column DNase digestion, DNase reaction mixture was prepared (by mixing 10µl DNase to 90µl of DNase reaction buffer) and applied about 95µl of it directly onto the centre of the silica membrane of the RNASure Column, followed by 15 minutes incubation at the room temperature. Then, to deactivate the rDNase activity, 200µl of buffer RWB2 was added to the above column and centrifuged for one minute at 11,000g and the filtrate was discarded. Now, 600µl of buffer RWB3 was added to the column and centrifuged for one minute at 11,000g to discard the filtrate. To dry the membrane completely, 200µl RWB3 buffer was added to the column and centrifuged for 2 minutes at 11,000g. Now the column was placed in a new nuclease free 1.5ml collection tube (supplied) and 40µl RNase-free water (supplied) was added at the centre of the column. After 10 minute incubation at room temperature, it was centrifuged at 11,000g for one minute. To avoid even little probability of genomic DNA contamination in the RNA, the eluted product was further rDNAse digestion in solution following the manufacturer's protocol followed by ethanol precipitation. To the above eluted solution, the already prepared rDNase reaction mixture was added (about 1/10th volume of the extracted RNA) and was incubated for 20 minutes at 37^{0}C. For repurifying the RNA, to one volume of the sample, about 1/10th volume of 3M Sodium Acetate, pH 5.2 (prepared in autoclaved DEPC-treated water) and 2.5 volume of absolute ethanol was added, and incubated at -20^{0}C for 30 minutes. Then, it was centrifuged for 10 minutes at 11,000g and the supernatant was discarded. The RNA pellet was washed with 70% ethanol and then allowed to dry at 55^{0}C for 10 minutes. The RNA was then re-suspended in 20µl of RNase-free water and stored at -20^{0}C till future use. About 2-3µl of the eluted RNA was checked in 1 % (w/v) agarose gel 1, prepared with DEPC-treated TBE buffer. The integrity of the RNA was confirmed by the observing intact two bright bands (the brightness of the 28S band was almost two times higher than that of the 18S band). Absorbance of the isolated RNA was recorded by Nanodrop Spectrophotometer.

3.10.3.5 cDNA synthesis

Approximately 2 µg of total RNA was used for cDNA synthesis using First strand cDNA synthesis kit (Thermo SCIENTIFIC). The reaction was performed in ice by taking about 2 µg RNA in a sterile, RNase-free PCR tube and was mixed with 1 µl oligo(dT)$_{18}$ primer, the final volume was adjusted to 11 µl and the mixture was incubated at 65 °C for 5 minutes. This reaction was immediately chilled for 5 min on ice, spun for 10 sec and the other reaction constituents were added. The other reaction constituents include 5X reaction buffer (4 µl), 1 µl of RiboLock RNase Inhibitor (20 U/µl), 10 mM dNTP mixture (2 µl), 2µl of M-MuLV Reverse Transcriptase, making the final volume to 20 µl. The mixture was mixed gently, spun down, and incubated for 60 minutes at 37^{0}C. The reaction was terminated by heating at 70 °C for 5 min. These synthesized cDNAs were subsequently used for amplification of genes or for RT-PCR analysis.

3.10.3.6 Checking of cDNA stock using actin as a primer

The reverse transcriptase PCR was carried out using all cDNA samples (about 100 ng) as template using β-actin as primer**.** The amplification was carried out following the following programme depicted in Fig 3.10.1. The PCR products were run on 2 % (w/v) agarose gel and photograph was saved.

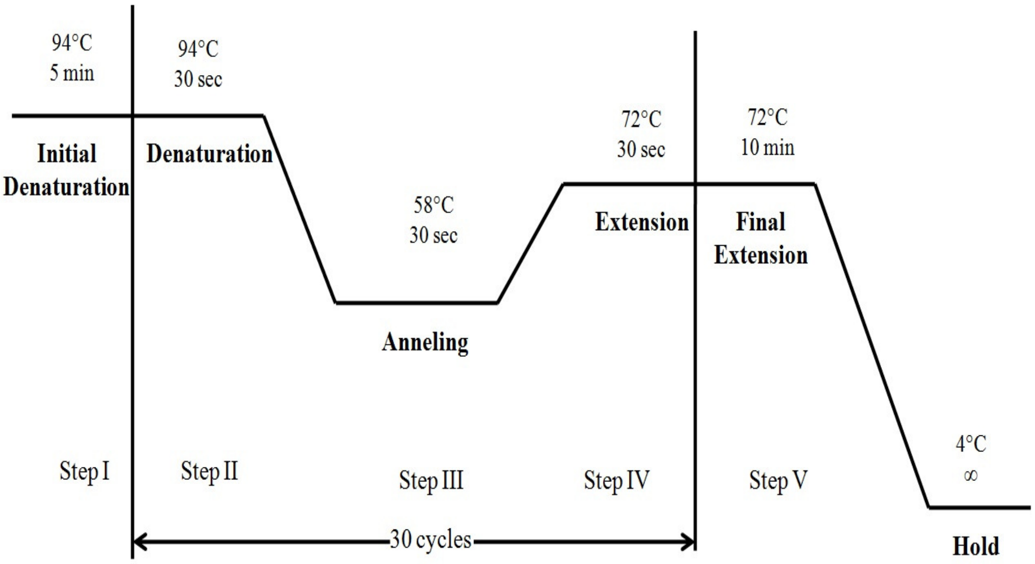


Fig. 3.10.1 PCR amplification programme used for cDNA checking

3.10.3.7 Amplification of PGIP using cDNA as template

PCR amplification was usually carried out in a 0.2 ml PCR tube (Axygen Inc., USA) containing 50 ng template DNA, 10 µM each of forward and reverse primers, 200 µM dNTPs (Fermentas), 1.5 mM $MgCl_2$ and 2.5 U *Taq* DNA polymerase (Fermentas) in 50 µl of reaction volume in a thermal cycler (Eppendorf, Germany). The programming of thermal cycler for the PCR amplification followed the same steps except for the annealing temperature which was kept 55 ^{0}C .

3.10.3.8 Agarose gel electrophoresis

PCR product was mixed with DNA loading dye and loaded along with 100bp DNA ladder (Genei, India) onto 1% agarose gel containing ethidium bromide (1.0 µg/ml). The gel electrophoresis was carried out at 60-100 volts and the gel was photographed using gel documentation system.

3.10.3.9 Purification of DNA fragment from agarose gel

The desired fragment from agarose gel was rescued by using Qiagen gel elution column following the manufacturer's guidelines. The desired bands was excised from the gel after visualizing on the UV-transluminator. Excised gel was weighed and amplicons were purified from the gel using QIAquick PCR purification kit according to manufacturer's protocol. Gel was dipped in 3 vol QG buffer and heated at 50 ºC till completely melted. This mixture was passed through the purification column. Column was washed with 750 µl of PE buffer; excess PE buffer was removed by centrifugation and finally eluted into 50 µl of sterile milli-Q water. Concentration of the purified DNA fragment was determined by Nanodrop spectrophotometer.

3.10.3.10 Competent cell preparation and transformation

Escherichia coli (E. coli) strain DH5α competent cells were prepared by chilled $CaCl_2$ treatment according to Sambrook and Russel (2001) with minor modifications. A flask containing 50 ml of LB medium was inoculated with 1% over night grown culture of DH5α. It was allowed to grow at 37°C at 220 rpm till OD_{600} was 0.4-0.6. The culture was chilled on ice for 15-20 min and centrifuged at 5000 rpm for 5 min at 4°C. The supernatant was removed and the pellet was gently re-suspended in 10 ml of ice- cold 100 mM $CaCl_2$

by gently swirling the tube. The re-suspended pellet was incubated on ice for 2 hs. The competent cells in the form of loose pellet were collected by centrifugation at 3000 rpm for 10 min at 4°C and were re-suspended in 1 ml of ice-cold 100 mM $CaCl_2$ by gently swirling the tube. It was further stored on ice (in cold room) for 14-16 h and then 1 ml, 50% sterile glycerol (made in 100 mM $CaCl_2$) was added and mixed gently. The competent cells (200 µl) were aliquoted into sterile, prechilled eppendorf tubes and were stored at -80°C.

3.10.3.11 Cloning in pGMET-Easy vector system and transformation in DH5α competent cells

Ligation of gel eluted product in pGMET-Easy vector

The ligation was carried out following the supplier's guidelines (Promega, Cat. No. A1360). All the components were given a short spin before use. The supplied 2X Rapid Ligation buffer was votexed well for ensuring proper mixing. The ligation reactions was set as per manufacture's guidelines where the insert to vector molar ratios was kept at 3:1. The reaction was properly mixed by pipetting and incubated overnight at 4°C to get maximum transformants.

To calculate the appropriate amount of PCR product (insert) to include in the ligation reaction, use the following equation.

$$\text{Required ng of insert} = \frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{Insert: Vector molar ratio}$$

Transformation

For transformation of DNA into DH5α competent cells, the eppendorf tube containing competent cells was removed from -80°C and kept on ice for 10 min to thaw the cells. An appropriate quantity of plasmid or ligation mix was mixed with the cells and incubated on ice for 45 min, followed by heat-shock exactly for 90 sec at 42°C. The tubes were rapidly chilled on ice for 10 min and 800 µl of LB medium was added to it. The tubes were incubated for ~1 h at 37°C with shaking (at 180 rpm). The cells were harvested at 5000 rpm for 10 min. The supernatant was discarded and the pellet was re-suspended in 100 µl of fresh LB and plated on LB agar plates containing specific antibiotics. The plates were incubated overnight at 37°C. The observed colonies on the plates were screened for the cloned insert by colony PCR.

3.10.3.12 Colony PCR

Visible colonies were picked by a sterile toothpick and suspended in 16.5 µl of sterile water for 25 µl of total reaction mixture volume. The reaction mixture tube containing colonies were kept in boiling water bath for 10 min to lyse the bacterial cells. To this, 10 µM each of vector specific forward and reverse primers (flanking the cloning sites), 200 µM dNTPs, 1.5 mM $MgCl_2$ and 1U *Taq* DNA polymerase was added and the reaction was amplified by PCR.The programming of thermal cycler for the PCR amplification was as follows: First cycle consisted of initial denaturation for 10 min at 94°C. Then, 30 cycles were carried out with each cycle having denaturation at 94°C for 30 sec, annealing at 60 °C for 30 sec and extension at 72°C for 60 sec. The last cycle was run for a final extension at 72°C for 10 min.

3.10.3.13 Isolation of plasmid DNA (mini-prep)

The protocol for mini preparation of plasmid DNA (mini-prep) was followed according to Sambrook and Russel (2001) . A single bacterial colony was grown in 2 ml of LB medium containing the appropriate antibiotic(s) in a loosely capped 15 ml tube which was incubated at 37°C with vigorous shaking for 15-16 h to allow good growth of cells. The cells were pelleted by centrifugation at 5000 rpm for 10 min at 4°C. The supernatant was removed and the bacterial pellet was re-suspended in 100 µl of ice cold Solution-I [(50 mM glucose; 10 mM EDTA, (pH 8.0) and 25 mM Tris-Cl, (pH 8.0)]. The cells were lysed by the addition of 200 µl of freshly prepared Solution-II (0.2 N NaOH and 1% SDS). The content of the vial was mixed gently by inverting tube for 4-6 times followed by storage on ice for 5 min. Then, 150 µl of ice-cold Solution-III (5 M potassium acetate, (pH 4.8); 11.5% v/v glacial acetic acid) was added and the contents were mixed by inverting around twenty times. The vial was incubated on ice for 5 min and centrifuged at 13000 rpm for 2 min at 4°C. The supernatant was transferred to a fresh tube. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed thoroughly followed by centrifugation at 13000 rpm for 2 min at 4°C in an eppendorf tube. The aqueous phase was transferred to a fresh tube. Plasmid DNA was precipitated by addition of two volumes of chilled ethanol followed by incubation for 30 min at -20°C. The precipitate was collected by centrifugation at 13000 rpm for 5 min at 4°C. The supernatant was removed and the pellet was washed with 70% ethanol and air dried briefly (15 min) under laminar air flow.

The pellet was dissolved in 50µl milli Q water. Once the pellet was completely dissolved, the quality and quantity was checked in naanodrop (ND 1000). The required amount of plasmid DNA was dried in a centrifugal evaporator (Labmate, DyNA Vap) and sent for sequencing.

3.10.3.14 Sequencing of DNA

The cloned DNA fragment was sequenced by Sanger's sequencing method using gene specific primers at SciGenom, Cochin, India.

3.10.3.15 Sequence homology search and NCBI submission

Homology search of the DNA sequences was performed against different DNA related databases (NCBI) using BLASTN programs. The DNA sequence was submitted to the NCBI database.

3.10.3.16 Expression study of PR proteins gene

Changes in transcript expression of the above genes were studied by Real-Time quantitative PCR. Real-time quantitative PCR was set using QuantiFast SYBR Green PCR reaction kit (Qiagen,USA). The reaction mixture includes about 100 ng of cDNA, 0.16 µM of primers and 12.5 µl of QuantiFast SYBR Green PCR mix. The volume of reaction was maintained to 25 µl by sterile nuclease free water. Reactions were run in StepOnePlus^{TM} Real-Time PCR System (Applied Biosystem) and conditions were set as follow: 95 °C-5 min for 1 cycle; 95 °C-10 sec and 60 °C-30 sec for 40 cycles. At the end of the PCRcycles, the products were put through a melt curve analysis to determine the specificity of amplification. The fold changes in transcript in fungus-infected plants compared to healthy plants were analysed by comparative $2^{-\Delta\Delta Ct}$method (Livak and Schmittgen 2001; Schmittgen and Livak 2008). The *Ah-actin* gene was used as internal control to normalize the PCR reactions.

3.11 Statistical analysis

Analysis of variance (ANOVA) was performed using DSAASTAT 1.1 and the significance of differences among means was carried out using Duncan's multiple range tests (DMRT) at $P < 0.05$ (Onofri, 2007). The correlation between different traits was studied using PAST v1.89 software (Hammer *et al*., 2001). Sequence homology analyses against the nucleotide and protein databases of GenBank were done using the programmes BLASTN and BLASTX, respectively.

CHAPTER – IV

RESULTS AND DISCUSSION

The experiment was conducted to understand the beneath mechanism of pathogenesis of stem rot causing pathogen, *S. rolfsii* in groundnut through biochemical, molecular and histological studies. The main aim of the experiment was to decipher the basis of innate immunity or susceptibility governed by the tolerant or susceptible genotypes respectively, towards the pathogen and further to study the induced mechanism of tolerance by application of fluorescent Pseudomonads.

The results are presented in five parts, such as, (1) To screen groundnut genotypes for stem rot tolerance in sick plot, (2) Isolation and *in-vitro* antagonism study of antagonistic fluorescent Pseudomonads isolates towards *S. rolfsii*, (3) Histo-pathological basis of stem rot tolerance, (4) Induced biochemical responses during the infection of *Sclerotium rolfsii* and interaction of the pathogen with the *Pseudomonads* isolate in groundnut, and (5) Stem rot induced differential expression of defense related genes.

4.1 EXPERIMENT I

TO SCREEN GROUNDNUT GENOTYPES FOR STEM ROT TOLERANCE IN SICK PLOT

Twenty-two groundnut genotypes (listed in Table 4.1.1) were raised during *Kharif* -2013 in the sick-plot maintained at ICAR-Directorate of Groundnut Research, for screening of stem rot pathogen, under normal field conditions as per the recommended package of practices except for the plant protection measures against stem rot (Plate 4.1.1).

The fungal load (*cfu*: $50x10^3$) was maintained regularly to ensure fungal pressure during the experimental period. Forty-day old plants were further challenged with the fungus by adding fungal inoculums on the soil surface in each line, closer to main stem. The progress of disease was monitored regularly and the per cent disease incidence (Table 4.1.1) in terms of mortality of plants was calculated at harvest by using the formula as below,

Per cent disease = (Number of infected plants/ Total number of plants) X 100.

Plate 4.1.1: Screening of groundnut genotypes for stem rot tolerance in sick plot

a. Stem rot plot at ICAR-DGR, Junagadh, maintaining high stem rot disease pressure for screening of groundnut genotypes for stem rot tolerance; **b.** Screening of genotypes in the sick plot following recommended practice; **c.** Stem rot infected Groundnut with visible symptoms; **d.** Stem rot infected dead groundnut at harvest in the field.

It was observed that the per cent disease incidence was in the range of 19 to 79%. Among the tested genotypes, both CS19 and GG16 has shown least per cent disease incidence with 19 and 23% respectively. The susceptible check, TG37A has shown maximum incidence of disease (79%) at harvest. Although some plants of genotype GG20 escaped from the disease, but overall, the recorded per cent incidence was quite high i.e. about 63%, confirming GG 20 as susceptible genotype to stem rot. Screening in both concrete and glass house conditions it was earlier reported genotypes CS19 and GG16 to be tolerant and TG37A to be a susceptible check variety for stem rot infection (Thirumalaisamy *et al.,* 2014).

The results indicated that the response of groundnut genotypes to stem rot disease was variable which was supported by the observation made by Bera, *et al.* (2014), who stated that the response of groundnut genotypes to the disease depend upon the seasons and years, and resistance of a genotype to *S. rolfsii* varies with the growth stages of the plant. In other words resistance could be controlled by different mechanism at different growth stages of the plant. Hence, the tolerance nature of genotypes recorded during the experiment may also vary.

Based on the above results, four genotypes were selected with varying of degree of tolerance to the stem rot pathogen such as CS19 (tolerant), GG16 (moderately tolerant), GG20 (susceptible) and TG37A (highly susceptible). Further to understand the beneath mechanism of tolerance and reason for susceptibility of groundnut genotypes to stem rot pathogen, *S. rolfsii*, a detail experiment was conducted to explain the above in terms of biochemical, histopathological and molecular basis.

Table 4.1.1 Screening of groundnut genotypes for stem rot tolerance in sick plot

S. N.	Genotypes	Habit Group*	Disease Incidence (%) ± SE**
1	CS 19	VB	19.0 ± 2.02
2	GAUG 1	VR	26.1 ± 1.87
3	GAUG 10	SB	52.0 ± 1.49
4	GG 11	VB	50.9 ± 2.34
5	GG 12	VR	34.1 ± 0.99
6	GG 13	SB	67.8 ± 2.69
7	GG 14	VR	29.4 ± 3.53
8	GG 15	VR	41.8 ± 5.56
9	GG 16	SB	22.7 ± 4.83
10	GG 17	SB	28.4 ± 5.44
11	GG 2	SB	34.8 ± 1.47
12	GG 20	SB	62.5 ± 1.62
13	GG 21	VR	27.8 ± 1.62
14	GG 3	VR	42.1 ± 8.87
15	GG 4	SB	53.8 ± 4.83
16	GG 5	VR	38.7 ± 2.47
17	GG 6	SB	29.4 ± 3.33
18	GG 7	VR	35.1 ± 0.46
19	GG 8	SB	51.4 ± 1.19
20	GJG 31	VB	34.6 ± 2.59
21	GJG-HPS-1	VR	41.3 ± 8.65
22	TG 37A	SB	79.1 ± 1.60
	S.Em. ±		3.88
	L.S.D (p<0.05)		11.06
	C.V. (%)		16.4

Per cent disease incidence is the average of three replications (n); * SB: Spanish Bunch; VB: Virginia Bunch; VR: Virginia Runner; **Standard Error: Standard deviation/√n

4.2 EXPERIMENT II:

ISOLATION AND *in-vitro* ANTAGONISM STUDY OF ANTAGONISTIC FLUORESCENT PSEUDOMONAD ISOLATES TOWARDS *S. rolfsii*

4.2.1 Isolation of fluorescent Pseudomonads

Eleven isolates of fluorescent pseudomonad were isolated from groundnut rhizosphere collected from different locations of *Saurasthra* region of Gujarat (described earlier in section 3.7.3.1) where the crop has been grown. The selections of the isolates were based on their capability to give prominent fluorescens under UV light and to form unique colony. Arbitral name were assigned such as *SKPf 1* to *SKPf 11* and hence forth the names have been used in the text. Antagonism study was carried out using the above isolates with the stem rot causing pathogen, *Sclerotium rolfsii* in culture medium of King's B at 6 days after inoculation (DAI).

4.2.2 Molecular identification of fluorescent Pseudomonad isolates

The genomic DNA was isolated and 16S rRNA gene was amplified (Fig 4.2.1). As expected, an amplicon of size ~1500 bp was observed on the gel confirming the amplification of 16S. The amplified product was purified and sequenced.

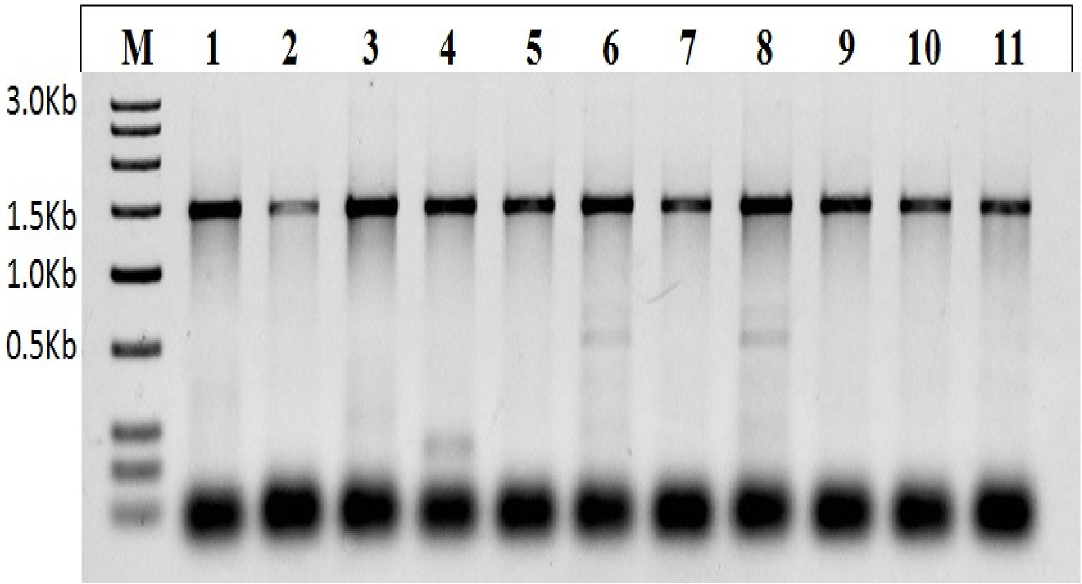


Figure 4.2.1: Amplification of 16s rRNA gene (~1500bp) in fluorescent Pseudomonad isolates, M: 100bp ladder, Lane 1 to 11 represents isolates *SKPf 1* to *SKPf 11*.

The 16S sequencing followed by their sequence similarity search analysis revealed that all the fluorescent Pseudomonad isolates used in experiment were belonging to the genera Pseudomonas (Table 4.2.1 and Appendix I) but none of them are *Pseudomonas fluorescens*. Among the 11 isolates, five (*SKPf 1, SKPf 4, SKPf 8, SKPf 10,* and *SKPf 11)* were *Pseudomonas putida*, three (*SKPf 2, SKPf 3,* and *SKPf 6)* were *Pseudomonas monteilii*, two (*SKPf 7* and *SKPf 9)* were *Pseudomonas plecoglossicida* and one (*SKPf 5)* was belonging to *Pseudomonas aeruginosa.* The fluorescent Pseudomonads is a large and heterogeneous group comprises, most notably, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Pseudomonas fluorescens* and *Pseudomonas syringae* etc. (Bossis *et al.,* 2000). All the members of this group give fluorescence under UV light as they are capable of producing the fluorescent pigment *Pvd* (also known as pseudobactin). The taxa Pseudomonas include both biocontrol agents and strains without any obvious biocontrol potential, regardless of whether only true *P. fluorescens* or also related fluorescent Pseudomonads are considered (Sanguin *et al.,* 2008). Hence, the selected fluorescent pseudomonad isolates were tested further *in vitro,* for their antagonistic potential against the stem rot causing pathogen in groundnut i.e. *S. rolfsii.*

Table 4.2.1: Fluorescent Pseudomonad isolates with their NCBI accession number and size of submitted sequences

Sl. No.	**Isolate Name**	**Sequences producing significant alignments (% identity)**	**NCBI Acc. No.**	**Size of sequence (bp)**
1	*SKPf 1*	*Pseudomonas putida* (100%)	KR422298	898
2	*SKPf 2*	*Pseudomonas monteilii* (100%)	KR422299	1386
3	*SKPf 3*	*Pseudomonas monteilii* (98%)	KP859615	731
4	*SKPf 4*	*Pseudomonas putida* (100%)	KR492887	1320
5	*SKPf 5*	*Pseudomonas aeruginosa* (97%)	KP859614	728
6	*SKPf 6*	*Pseudomonas monteilii* (100%)	KP859616	731
7	*SKPf 7*	*Pseudomonas plecoglossicida* (100%)	KP859617	741
8	*SKPf 8*	*Pseudomonas putida* (100%)	KR422300	1383
9	*SKPf 9*	*Pseudomonas plecoglossicida* (94%)	KR492888	715
10	*SKPf 10*	*Pseudomonas putida* (100%)	KR422301	1393
11	*SKPf 11*	*Pseudomonas putida* (100%)	KR492889	1260

4.2.3 *In-vitro* antagonistic potential of fluroscent Pseudomonad isolates against *S. rolfsii*

The *in-vitro* growth inhibition of *S. rolfsii* by fluorescent Pseudomonas isolates (*SKPf 1* to *SKPf 11*) was observed at six days after inoculation (6 DAI) revealed that all the isolates were not capable of inhibiting the growth of the fungus. The per cent growth inhibition was significantly different among the potential and non-potential antagonist (Table 4.2.2 and Plate 4.2.1). Based on the *in-vitro* antagonism study, the maximum inhibition was brought by *SKPf 5* against *S. rolfsii*, followed by *SKPf 2*, *SKPf 4,* and *SKPf 11* which were at par. Based on BLASTN results of partial 16S sequence, the *SKPf 5* was identified as *Pseudomonas aeruginosa,* where as both *SKPf 4* and *SKPf 11* were found to be *Pseudomonas putida.*. Previously, it was reported that some strains of *Pseudomonas aeruginosa* also inhibit the *S. rolfsii* of groundnut (Kishore *et al.,* 2005), which supports the finding and suggests the potential of some *Pseudomonas aeruginosa* isolates as biocontrol agent in groundnut.

It was observed that isolates, irrespective of their species showed different antagonistic activity. The isolates belonging to the same species reported their ability to inhibit the *in vitro* growth of the pathogen differently, for instance, although both *SKPf 1* and *SKPf 4* belong to *P. putida,* only *SKPf 4* showed growth inhibition activity. It is a fact that the ability to produce antifungal secondary metabolites and other lytic enzymes, decide the *in vitro* antagonism potential of an isolates, which could be reason for different antagonistic potential of isolates belonging to same species.

In an attempt to develop effective biocontrol system for management of stem rot disease in groundnut, few Pseudomonas spp. isolated from rhizospheric soil, were evaluated for their antagonistic activity against *S. rolfsii*. The most potential soil bacterium showing highest antagonistic activity against *S. rolfsii* was identified as, *Pseudomonas monteilii* (Rakh *et al.,* 2011). However, in the present experiment, none of the isolated *Pseudomonas monteilii*, did show any antagonistic activity against the same test fungus. Biological control using antagonistic Pseudomonads appears to be a potential management tool for reducing the severity of groundnut stem rot (Ganesan and Gnanamanickam, 1987). Many researchers proved that selected Pseudomonas strains can restrict *in vitro* hyphal growth or reduce germination of sclerotia of *S. rolfsii (*Ganesan and Gnanamanickam 1987; Kishore *et al.,* 2005; Ganesan *et al.*, 2007; de Curtis *et al.*, 2010; Pastor *et al.,* 2010 and Tonelli *et al.,* 2010*).*

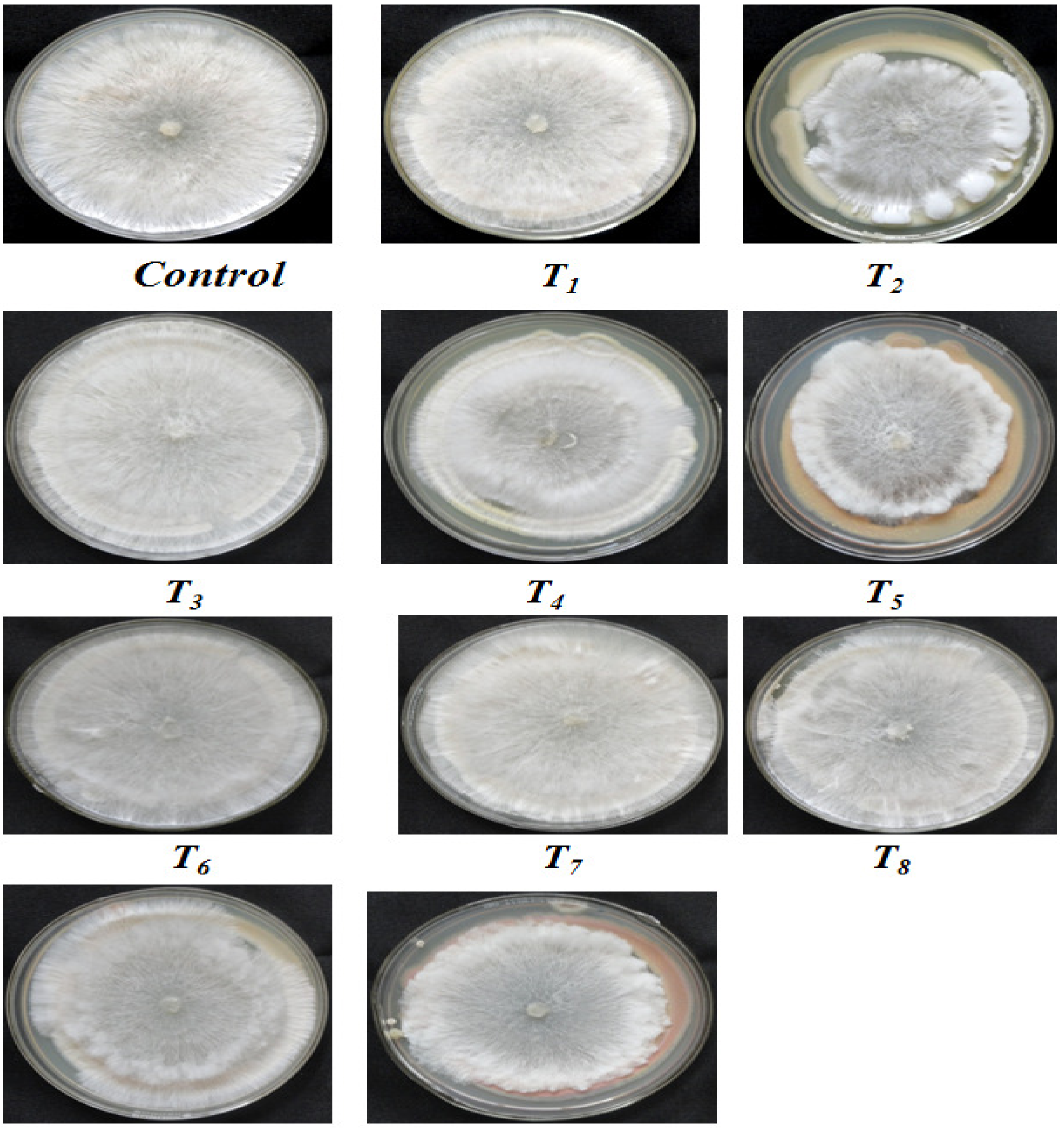
Table 4.2.2: *In vitro* per cent growth inhibition of *S. rolfsii* by fluorescent Pseudomonad isolates

Treatments	Species of Fluorescent Pseudomonad Isolates	Per cent Growth Inhibition ± SE
S. rolfsii alone	-	0.0 ± 0.0 c
SKPf 1 X *S. rolfsii* (T_1)	*Pseudomonas putida*	1.90 ± 1.4 c
SKPf 2 X *S. rolfsii* (T_2)	*Pseudomonas monteilii*	22.2 ± 2.0 b
SKPf 3 X *S. rolfsii* (T_3)	*Pseudomonas monteilii*	2.30 ± 1.2 c
SKPf 4 X *S. rolfsii* (T_4)	*Pseudomonas putida*	16.6 ± 3.5 b
SKPf 5 X *S. rolfsii* (T_5)	*Pseudomonas aeruginosa*	33.7 ± 2.4 a
SKPf 6 X *S. rolfsii* (T_6)	*Pseudomonas monteilii*	1.92 ± 1.4 c
SKPf 7 X *S. rolfsii* (T_7)	*Pseudomonas plecoglossicida*	3.50 ± 1.4 c
SKPf 8 X *S. rolfsii* (T_8)	*Pseudomonas putida*	1.10 ± 0.7 c
SKPf 9 X *S. rolfsii* (T_9)	*Pseudomonas plecoglossicida*	1.90 ± 1.4 c
SKPf 10 X *S. rolfsii* (T_{10})	*Pseudomonas putida*	2.30 ± 1.8 c
SKPf 11 X *S. rolfsii* (T_{11})	*Pseudomonas putida*	17.2 ± 4.5 b
	C.V. (%)	14.68
	L. S. D. ($p<0.05$)	6.68
	S. Em.	2.29

Values in the column followed by the same letters are not significantly different according to DMRT at $P < 0.05$ and are mean of three replications, SE refers to Standard error

4.2.4 Lytic Enzymes

Fluorescent Pseudomonads are shown to produce lytic enzymes which are also abundant in many plant species after infection by different type of pathogens. The main roles of these lytic enzymes for defence reaction against fungal pathogen are evident from the fact that they degrade the cell wall of pathogens because chitin and ß-1,3-glucan are also a major structural component of the cell walls of many pathogenic fungi. Among the successful biocontrol agents against *S. rolfsii*, *Trichoderma* species are the most widely studied. They may directly or indirectly kill sclerotia or mycelium of *S. rolfsii* through lysis. It was further shown that chitinase and ß-1,3-glucanase play a role in the interaction between *Trichoderma harzianum* and *S. rolfsii* (Prasad and Naik 2008). Hence it was hypothesized that the ability of an isolate to produce higher amount of fungal cell wall degrading enzymes such as chitinase, ß-1, 3-glucanase and protease could be marker of potential antagonist. The present experiment was to investigate the basis of biocontrol mechanism of fluorescent Pseudomonads and also to evaluate the stated hypothesis.



Control T_1 T_2

T_3 T_4 T_5

T_6 T_7 T_8

T_9 T_{11}

Plate 4.2.1.: Antagonism between isolates of fluorescent Pseudomonads and *S. rolfsii* at 6 DAI in King's B medium

Control: *S. rolfsii* alone ; T_1: *SKPf 1* X *S. rolfsii ;* T_2: *SKPf 2* X *S. rolfsii ;* T_3: *SKPf 3* X *S. rolfsii ;* T_4: *SKPf 4* X *S. rolfsii ;* T_5: *SKPf 5* X *S. rolfsii ;* T_6: *SKPf 6* X *S. rolfsii ;* T_7: *SKPf 7* X *S. rolfsii ;* T_8: *SKPf 8* X *S. rolfsii ;* T_9: *SKPf 9* X *S. rolfsii ;* T_{10}: *SKPf 10* X *S. rolfsii ;* T_{11}: *SKPf 11* X *S. rolfsii*

4.2.4.1 Chitinase activity

Chitinase produced by biocontrol agents like fluorescent Pseudomonads degrade the chitin, which is the major integral part of fungus. In the present study, all isolates have shown to produce the chitinase and there was a significant difference among the isolates for their capability to produce the enzyme against *S. rolfsii in-vitro* (Table 4.2.3 and Fig.4.2.2). The mean of specific activity of chitinase was ranged between 0.58 U mg^{-1} protein (*SKPf 3*) to 4.94 (*SKPf 5*) which was in accordance with their *in-vitro* growth inhibition ability against *S. rolfsii.* Although *SKPf-6* could not show *in-vitro* growth inhibition against *S. rolfsii,* a significantly more amount of chitinase (2.74 U mg^{-1} protein) was produced by the isolate than others. It was also observed that mostly, those isolates having no or little antagonistic behaviour were associated with less amount of chitinase production *in-vitro.* It further supported the findings of Gupta *et al*. (2006) suggesting the potential of *P. aeruginosa* as a potential biocontrol agent against *S. rolfsii,* capable of producing extracellular chitinase enzyme.

Our finding were supported by Ganesan and Sekar, (2004) observed that the among biocontrol agents potentially better strains produce higher amount of chitinase, indicating their vital role in inhibiting the *in-vitro* growth of the pathogen. It was also in agreement with the findings of Deshmukh *et al.* (2015) who reported a positive association between the antagonistic potential of *P. fluorescens* strains and their level of chitinase production

Table 4.2.3: Production PR proteins and inhibition of fungal lytic enzymes by fluorescent Pseudomonad isolates

	Pathogenesis related enzymes (U mg^{-1} protein)			**% Inhibition of cell wall degrading enzymes**	
Treatments	**Chitinase**	**β-1,3 Glucanase**	**Protease**	**Cellulase**	**Poly-galacturonase**
***SKPf 1* X *S. rolfsii* (T$_1$)**	0.95	65.40	25.07	37.87	48.29
***SKPf 2* X *S. rolfsii* (T$_2$)**	1.63	108.75	9.19	30.51	39.70
***SKPf 3* X *S. rolfsii* (T$_3$)**	0.58	37.71	16.81	57.41	40.43
***SKPf 4* X *S. rolfsii* (T$_4$)**	1.84	5.88	11.44	49.17	58.64
***SKPf 5* X *S. rolfsii* (T$_5$)**	4.94	200.35	30.89	62.26	62.63
***SKPf 6* X *S. rolfsii* (T$_6$)**	2.74	99.54	1.31	37.22	50.75
***SKPf 7* X *S. rolfsii* (T$_7$)**	1.15	83.62	2.71	36.38	45.27
***SKPf 8* X *S. rolfsii* (T$_8$)**	0.67	32.53	19.46	42.93	50.48
***SKPf 9* X *S. rolfsii* (T$_9$)**	0.96	68.66	24.33	33.90	46.04
***SKPf 10* X *S. rolfsii* (T$_{10}$)**	1.32	46.65	1.89	25.29	52.72
***SKPf 11* X *S. rolfsii* (T$_{11}$)**	1.99	16.08	18.81	23.83	56.14
S.Em.	0.14	6.79	1.23	2.39	2.87
LSD (p<0.05)	0.42	19.8	3.56	6.96	5.91
CV (%)	11.99	0.42	10.98	11.34	7.64

4.2.4.2.4.2 β-1, 3-glucanase activity

The enzyme β-1,3-glucanase produced by the biocontrol agents like *P. fluorescens* have a capacity to hydrolyze the branched β-1,3-glucans found in the most fungal cell walls (Fridlender *et al.*, 1993). All the fluorescent Pseudomonad isolates produced significant amount of the enzyme against *S. rolfsii*, and the specific activity of β-1,3-glucanase was in the range of 5.88 to 200.35 U mg^{-1} protein. The maximum specific activity was reported for by *SKPf 5* followed by *SKPf 2* (Table 4.2.3 and Fig.4.2.2). Nagrajkumar *et al.* (2004) reported a significant relationship between the antagonistic activity of *P. fluorescens* strains and their level of production of β-1, 3-glucanase. We found the isolate *SKPf 4* showed better *in-vitro* growth inhibition potential against *S. rolfsii*, but produced the least amount of β-1, 3-glucanase. Some of the inefficient strains like *SKPf 6, SKPf 7* and *SKPf 9* also produced significant amount of the enzyme. These finding were supported by Deshmukh *et al.* (2015) who observed that some non potential isolates also produce higher amount of β-1,3-glucanase and on the contrary few potential isolates showing better *in-vitro* growth inhibition, produce less amount of the enzyme. Based on the finding of chitinase or β-1, 3-glucanase, it can be hypothesized that fluorescent pseudomonad isolates adapt differently to inhibiting the fungal pathogens by modulating their lytic enzymes production. Hence, selection of potential *in-vitro* antagonistic isolates of fluorescent Pseudomonads against fungal pathogens such as *S. rolfsii* should be avoided only considering their efficiency to produce chitinase or β-1, 3-glucanase.

4.2.4.3 Protease activity

Filamentous fungal cell wall also contains lipids and proteins, hence it was therefore expected that antagonistic bacteria like *Pseudomonas fluorescens* synthesizes proteases which may act on the cell wall of fungal pathogen. Ahmadzadeh *et al.* (2006), reported that some strains of fluorescent Pseudomonads having potential to produce protease showed known biological control activity against certain soil borne phytopathogenic fungi. He hypothesized that the resulted antagonistic activity against such pathogenic fungi might be by degradation of cellulolytic enzymes produced by the fungi.

In present study, higher protease activity (30.89 U mg^{-1}protein) corresponded to the best antagonist isolate (*SKPf 5)* and the least was observed for *SKPf 6* and *SKPf 10* which were non-antagonist, thereby supporting the earlier hypothesis. However, few non potential isolates such as *SKPf 1* (25.07 U mg^{-1}protein) and *SKPf 9* (24.33 U mg^{-1}protein)

(Table 4.2.3 and Fig.4.2.2) also produced significant amount of proteases. Hence it can be concluded that the *in vitro* bio-control potential of Pseudomonads isolate are not solely depend upon their capacity to produce protease.

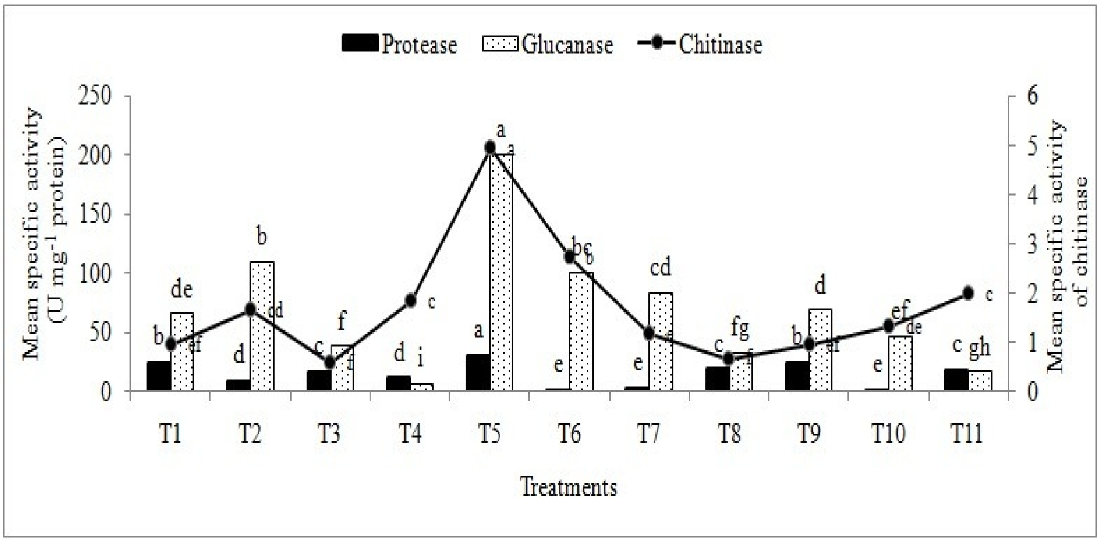


Fig. 4.2.2: Production of Protease, β-1, 3-glucanase and Chitinase during *in-vitro* antagonism between fluorescent Pseudomonads and *S. rolfsii*

4.2.5 Inhibition of cell wall degrading enzymes

Plant fungal pathogens especially necrotrophic fungus degrade the structural polymers in plant host cell wall and colonize in the intercellular spaces facilitated by the production of cell wall degrading enzymes (CWDEs) such as cellulase, and polygalacturonase. However, potential biocontrol agents, such as bacteria like Pseudomonas spp. prevent the production of these CDWEs and thereby reducing the cell wall damage to the host tissues. In the present study, the inhibition of fungal CDWEs was observed (Table 4.2.3 and Fig. 4.2.3) for all the isolates, however it was variable.

The per cent inhibition of cellulase activity of *S. rolfsii* was in the range of 23.83 % to 62.26% corresponding to *SKPf 11* and *SKPf 5* respectively. The inhibition of cellulase production by *SKPf 3* was at par with that of the best antagonist (*SKPf 5)*, however all the rest isolates inhibited cellulase activity of the fungus significantly less. Similarly, all the isolates showed the inhibition of polygalacturonase production of the test fungus *in vitro*. The maximum inhibition was recorded for *SKPf5* where as the least was for *SKPf 2*. Potentially better antagonist other than *SKPf 5* such as *SKPf 4* and *SKPf 11* also significantly inhibited the production of the enzyme which was higher than other isolates.

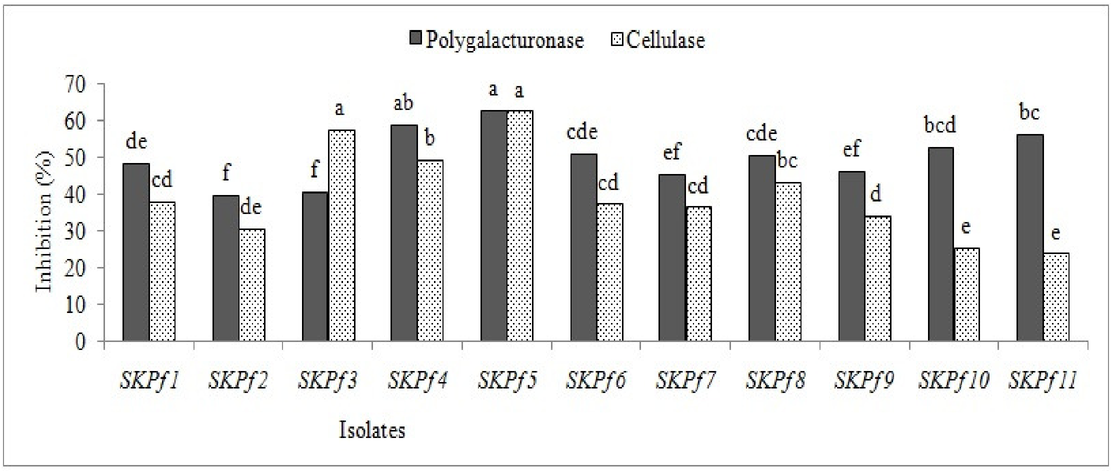


Figure 4.2.3: Inhibition of fungal Polygalacturonase and Cellulase activity by fluorescent Pseudomonad isolates

Kishore *et al.* (2005) reported that cell- free culture filtrates of *P. aeruginosa* GSE 18 and GSE 19 inhibited the activity *in vitro* of the cell wall-degrading enzymes (CWDE) polygalacturonase and cellulase by *S. rolfsii* up to a maximum of 55% and 50%, respectively, when measured 6 days after inoculation. As the finding of the present investigation also supported the earlier reports, it can be proposed that inhibitory activity of fluorescent Pseudomonad isolates on production of CWDEs activity of fungus plays an important role in *in vitro* antagonism.

4.2.6 Production of antifungal metabolites and growth promoting substances by fluorescent Pseudomonads

4.2.6.1 Production of siderophores

Siderophores are fluorescent, yellow-green, water soluble, low molecular weight compounds iron chelators secreted by many *P. fluorescens* isolates under iron-limiting conditions (Sullivan and Gara, 1992). The secretion of siderophores was observed by many researchers in King's B medium by isolates of fluorescent Pseudomonads and was further correlated with their corresponding antagonistic potential. In the present study siderophore production was also observed for all isolates of Pseudomonads (Figure 4.2.4), the maximum production was recorded for *SKPf 11* (15.13 µg benzoic acid.ml^{-1}), where as lowest was for *SKPf 1* (0.99 µg benzoic acid.ml^{-1}). Although *SKPf* 5 produced siderophores (12.75 µg benzoic acid.ml^{-1}) less than the above two, it was significantly higher than all other isolates. Our findings were not in agreement with the earlier reports of Mazumdar *et al*. (2007) and Kandoliya and Vakharia, (2013) who stated that higher siderophore production is associated with greater antagonistic activity of the same isolates. Although siderophore production is most often associated with fungal suppression by fluorescent *Pseudomonas* in the rhizosphere, but the result here revealed that it may not hold true for all isolates and all pathogens.

4.2.6.2 Production of Salicylic Acid (SA)

Under conditions of iron limitation, the many Pseudomonad strains produce SA *in vitro,* which is a phenolic compound that affects a variety of biochemical and molecular events associated with induction of disease resistance through Induced Systemic Resistance (ISR). Salicylic acid (SA) production by the fluorescent Pseudomonad isolates was in the range of 2.61 µg. ml^{-1} (*SKPf 9*) to 17.77 µg. ml^{-1} (*SKPf 5).* The highest record of SA was corresponding to the best antagonist (*SKPf 5*) which was significantly higher than that of all other isolates (Figure 4.2.4), suggesting a vital role of SA in *in vitro* antagonism. A similar type of observations were also made by Nagarajkumar *et al.* (2004) and Kandoliya and Vakharia, (2013), supporting the role of salicylic acid production of Pseudomonad isolates in *in-vitro* antagonism.

4.2.6.3 Production of IAA like compounds (IAA)

The intrinsic ability of *Pseudomonas fluorescence* to produce Inole acetic acid (IAA) was observed by many researchers (Glick, 1995; Caron *et al.*, 1995, Mazumdar *et al.,* 2007). Isolates of *P. fluorescens* of groundnut rhizosphere were also shown to produce IAA and enhance the plant growth (Pal *et al.,* 1999). This study also revealed and supported the fact that fluorescent Pseudomonads have an intrinsic ability to produce IAA like compounds.

The IAA production capacity of the tested isolates showed a greater variation (Figure 4.2.4) and was in the range of 59.32 (*SKPf 2*) to 282.84 ng.ml^{-1} (*SKPf 5*). Although the production of IAA by *SKPf 6, SKPf 10,* and *SKPf* 11 were at par, significantly less than that of *SKPf 5,* which was corresponding to the best antagonist among all the tested isolates. The capability of *P. aeruginosa* isolates to produce IAA was earlier reported by Karnwal, (2009). The results depicted here did not support the findings of Kandoliya and Vakharia, (2013), that the antagonistic behaviour of *Pseudomonas fluorescens* has a strong correlation with the capability of the isolates to produce IAA *in vitro*. From the above findings, it can be concluded that the most potential antagonist (*SKPf 5)* among the tested isolates was capable of producing all the antifungal metabolites and growth promoting compounds like IAA, always at a higher amount. However, some inefficient antagonists also produced a significant amount of one and/or the other secondary metabolites, but except *SKPf 5*, all isolates are not equally good in producing siderophores, SA and IAA. The capacity of *SKPf 5* to produce those secondary metabolites constantly better than the average and the possible synergistic effects of those metabolites on in vitro antagonism would have made the isolate better than the others.

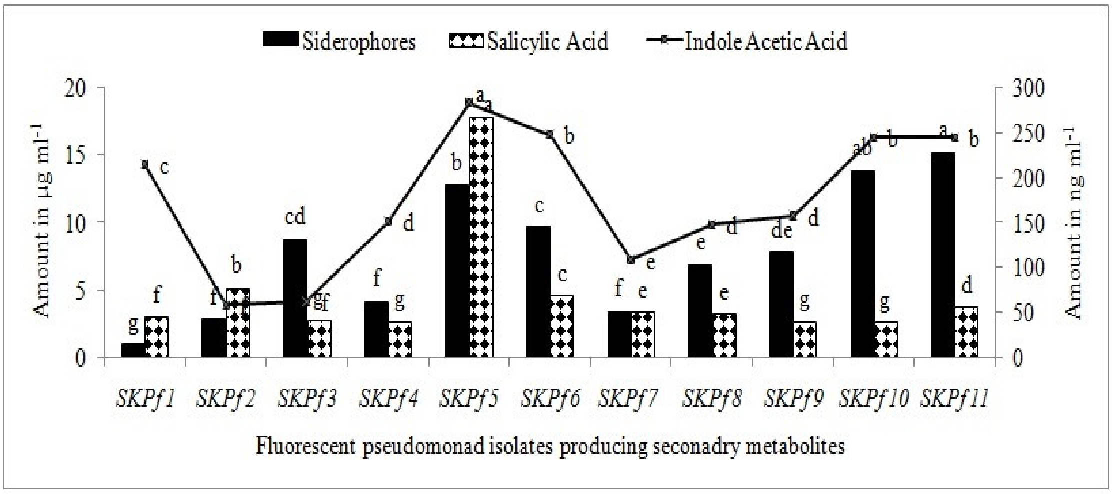
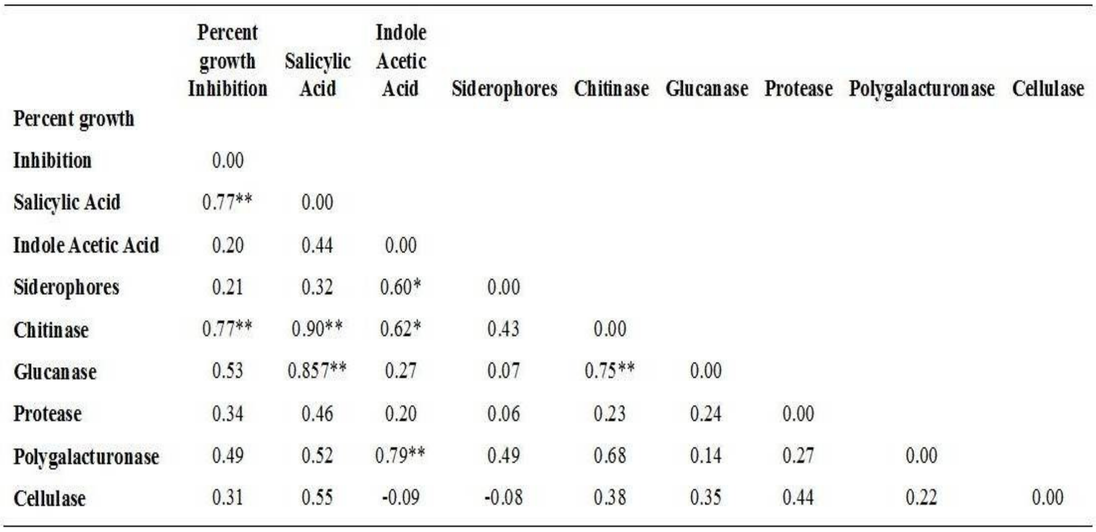


Figure 4.2.4: Production of secondary metabolites by Pseudomonads isolates

A similar logic can be put forth to justify the antagonistic potential of *SKPf 5* and its capacity to produce PR proteins and to inhibit the fungal cell wall degrading enzymes. In *in-vitro* condition, *SKPf 5* produced the maximum amount of pathogenesis related enzymes which logically made it the best antagonist against the *S. rolfsii* among all isolates. Although some isolates were individually good at producing one or the other enzymes to degrade the fungal cell wall, but not equally good as *SKPf 5,* for instance, growth inhibition potential is concerned. Similarly, *SKPf 5,* also is the best among the studied isolates for inhibiting the ability of *S. rolfsii* to produce cell wall degrading enzymes. Irrespective of the ability to inhibit the fungal growth *in vitro*, some isolates were individually also good at producing one or more of these enzymes. Hence from this study, it is deciphered that a potential antagonist, may be superior in production of both PR proteins as well as capable of inhibiting the concerned fungus to produce cell wall degrading enzymes.

4.2.7 Association between different traits

Correlation study among all the observed traits (Table 4.2.4) revealed that *in-vitro* growth inhibition potential of a fluorescent isolate depends on its capability to produce SA acid and Chitinase. Positive association between SA production and fungal cell wall degrading enzymes suggests the SA as a marker to identify antagonistic Pseudomonads. A positive association was observed between IAA production and siderophore production, suggesting an antagonistic isolate of Pseudomonad could be equally potential for growth promotion in plants. Further there was a highly significant association between IAA production and capacity of an isolate to inhibit the production of polygalacturonase by the fungus, *S. rolfsii*. Hence, it can be hypothesized that a plant growth promoting bacteria works both by providing growth promoting hormone as well as protecting the plant cell wall maceration by inhibiting the production of fungal PG, which otherwise degrade the plant cell wall. A significant association between production of IAA and chitinase, further extends the hypothesis that growth promoting effect of some IAA producing Pseudomonads may be enhanced by their capability to produce chitinase, which degrades the degrades rhizospheric fungal pathogens.



	Percent growth Inhibition	Salicylic Acid	Indole Acetic Acid	Siderophores	Chitinase	Glucanase	Protease	Polygalacturonase	Cellulase
Percent growth Inhibition	0.00								
Salicylic Acid	0.77**	0.00							
Indole Acetic Acid	0.20	0.44	0.00						
Siderophores	0.21	0.32	0.60*	0.00					
Chitinase	0.77**	0.90**	0.62*	0.43	0.00				
Glucanase	0.53	0.857**	0.27	0.07	0.75**	0.00			
Protease	0.34	0.46	0.20	0.06	0.23	0.24	0.00		
Polygalacturonase	0.49	0.52	0.79**	0.49	0.68	0.14	0.27	0.00	
Cellulase	0.31	0.55	-0.09	-0.08	0.38	0.35	0.44	0.22	0.00

Table 4.2.4: Correlation matrix between different biochemical parameters

A significant association was noticed between production of chitinase and glucanases, both of which are associated with degrading the fungal cell wall. Hence, the experiment can be summarised as selection of Pseudomonads isolates which are capable of producing higher amount of SA and IAA with better efficiency to degrade fungal cell wall through production of more chitinase and glucanase, could be a better choice.

4.3 EXPERIMENT III

4.3.1 HISTO-PATHOLOGICAL BASIS OF *S. rolfsii* INFECTION IN GROUNDNUT

Genotype CS19 is one of the tolerant genotypes toward *S. rolfsii* in groundnut available, and has been registered for a novel source of resistance. Through electron microscopy, it was earlier reported (DGR annual Report, 2007) that within 3 days after inoculation of *S. rolfsii* in seed, the fungus gained entry into the host cells of both tolerant (CS19) and susceptible variety (GG20). It was also reported that CS19 has solid trichomes, wax deposits and compact arrangement of vascular cells which could be affording it resistance to stem rot. On the contrary, the susceptible variety lacks solid trichomes and wax deposits, and has loosely bound vascular cells which could be predisposing it for heavy incidence of stem rot. However, till date very little information is available about mechanism of resistance of groundnut to *S. rolfsii* infection. Hence, the present study aimed at differentiating the tolerant and susceptible genotypes at histological level in more details.

It was found that the fungus has infected in all the genotypes and the symptoms like drooping of leaves and tender stems has started visible after three days post inoculation (dpi). The primary symptoms were more prominent in genotype TG37A than other genotypes. At 5 dpi, infection was clearly observed in all genotypes, however comparatively less disease severity was observed for the genotype CS19. The genotypes such as GG16 and GG20 showed less infection even up to 5 dpi. At 10 dpi, all the genotypes except few plants of genotype CS19 were heavily infected. Even the seeds primed with *Pseudomonas aeruginosa*, also followed the same pattern of disease development. Hence it was thought that CS19 must have some innate immunity system which makes it to withstand the stem rot disease to a better extent. To understand the beneath mechanism of tolerance and susceptibility, Scanning electron microscopy was conducted taking stem tissues sections from both infected and non infected genotypes at 5 dpi which represented the stage at which all genotypes had shown distinct responses to the pathogen, *S. rolfsii.* Two major findings were observed in the present study, which has differentiated the tolerant and susceptible genotypes, which are described in details as below.

S. rolfsii in groundnut is a necrotrophic pathogen show typical symptoms of other vascular wilt pathogens. Recognition of vascular wilt pathogens are mediated by either extracellular or intracellular receptors leads to the activation of defence responses in the xylem vessels. These comprise physical defence responses which halt or contain the pathogen from further spread in the xylem vessels, and chemical defence responses that kill the pathogen or inhibit its growth (Yadeta and Thomma, 2013). Currently, little is known about the interaction between vascular wilt pathogens and their hosts. As this interaction takes place in xylem vessels which are located deep in the plant interior, the molecular basis underlying the interaction between vascular wilt pathogens and their hosts remains largely obscure.

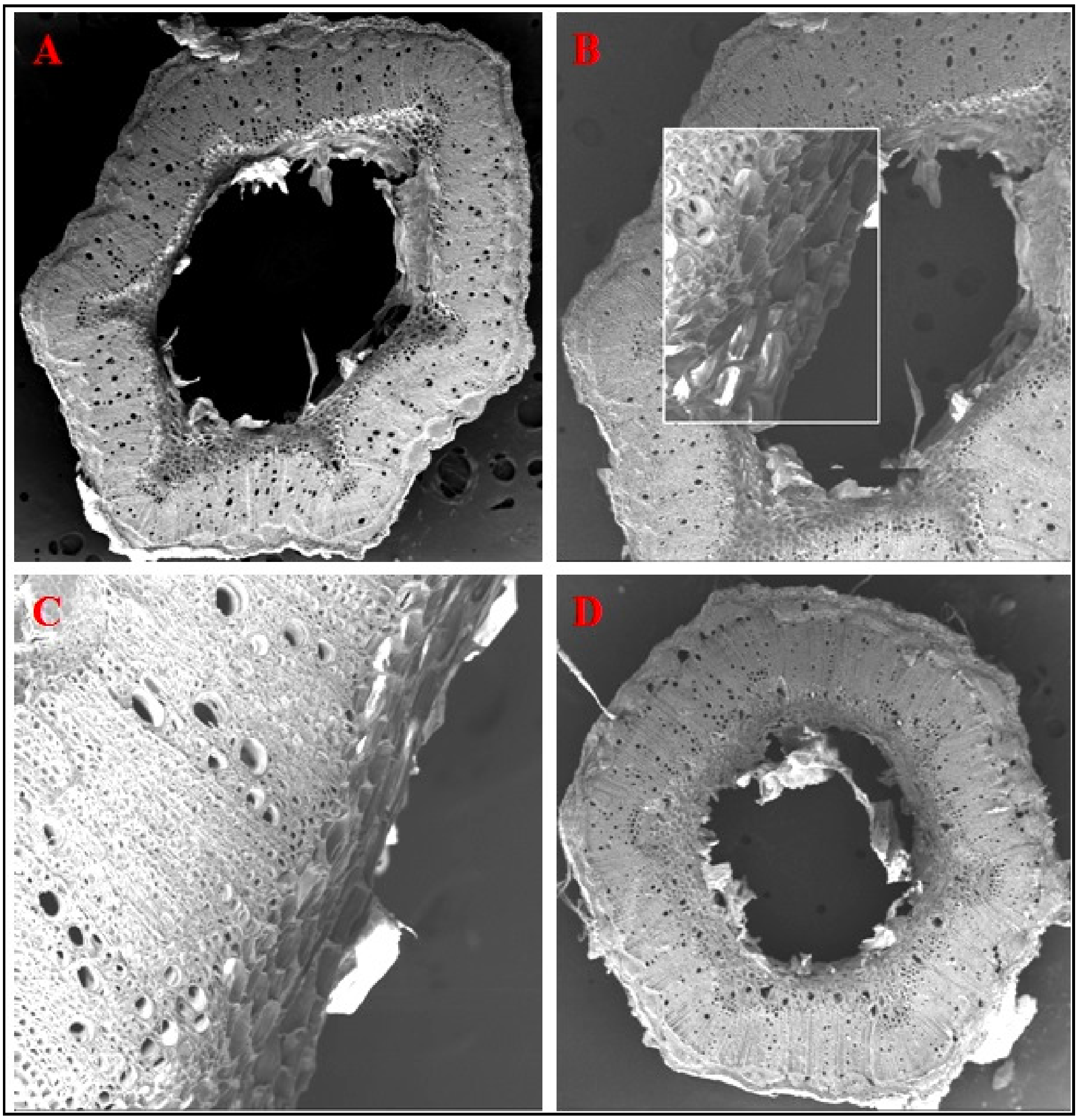


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Plate 4.3.1: Scanning Electron Microscopy images of healthy and infected stem anatomy and features of cellular responses of CS19 at 5 dpi

Cross section of healthy stem tissue showing the compactness of xylem vessels, and no traces of *S. rolfsii* near the xylem vessels, magnifications at about 100X (A) and 400X (B & C). *S. rolfsii* infected stem anatomy showing distorted structures both from inside and outside (D).

A common defense mechanism in xylem vessels against vascular wilt pathogens is the formation of tyloses (Fradin and Thomma, 2006). Tyloses are outgrowths of vessel-associated parenchyma cells which protrude into the xylem vessel through pits and block the spread of pathogens (Agrios, 2005). They are formed during both compatible and incompatible interactions between the host and vascular wilt pathogens, although the time and extent of tylose formation significantly differs (Yadeta and Thomma, 2013). Tyloses form much faster and more extensively in resistant plants when compared to susceptible plants (Grimault et al., 1994; Fradin and Thomma, 2006).

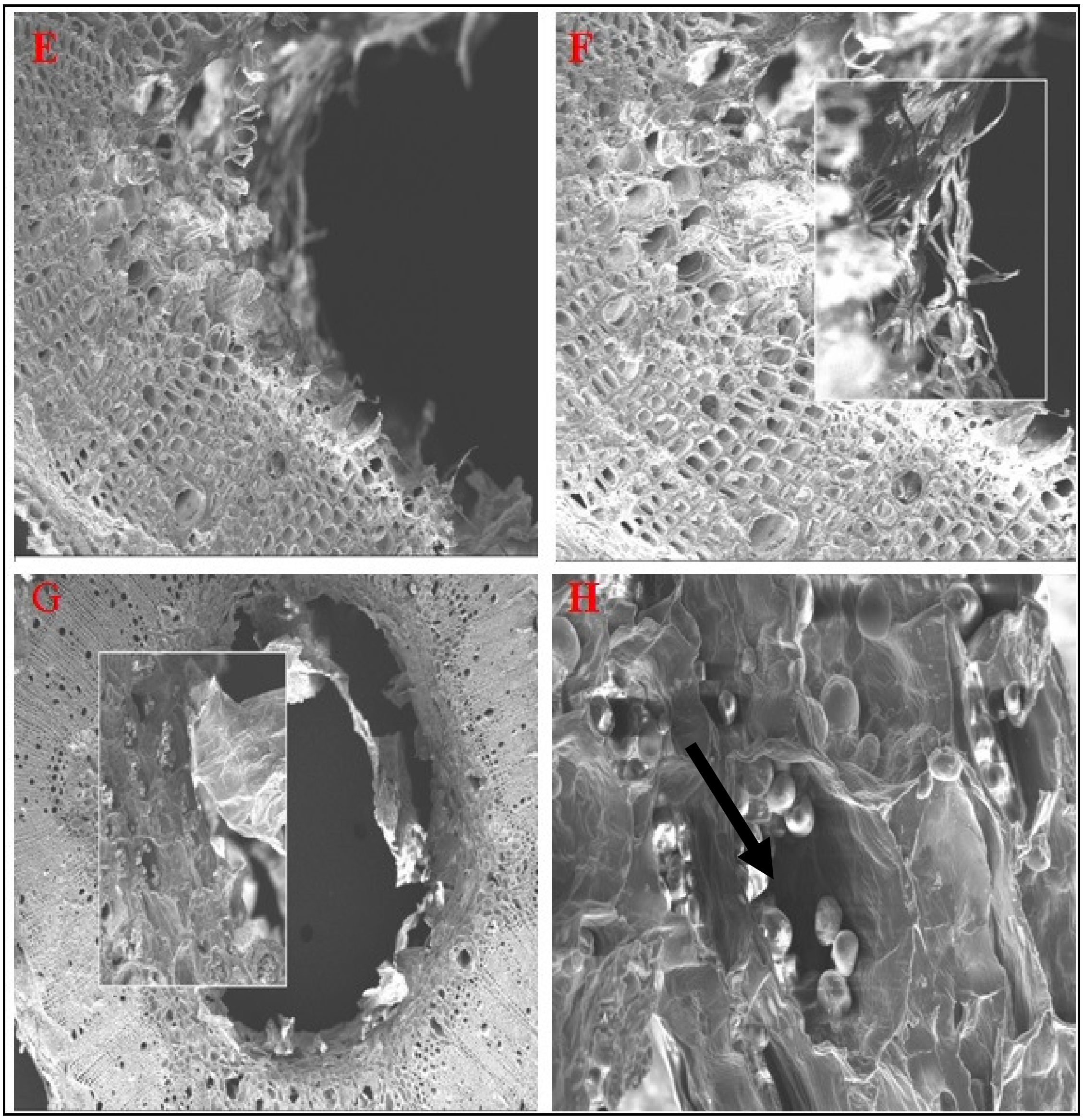

Plate 4.3.1: Scanning Electron Microscopy images of healthy and infected stem anatomy and features of cellular responses of CS19 at 5 dpi

Presence of *S. rolfsii* mycelia covering the xylem tissue (E); A closer view of fungal mycelia attached to the xylem tissue (F); An undistorted stem of CS19 (G) showing deposition of tyloses at 800X magnification(H).

Formation of tyloses was observed only in the tolerant genotype CS19 at 5 dpi (marked with arrow in Plate 4.3.1 H) where as no such cellular responses were seen in other genotypes. This typical response of CS19 could be the reason for affording tolerance by preventing the easy spreading of the stem rot pathogen. The lack of tylose formation at proper time in the susceptible genotype TG 37A or comparatively less tolerant genotypes (GG16 and GG20) could be the cause for their susceptibility to the pathogen (Plate 4.3.2, 4.3.3, 4.3.4).

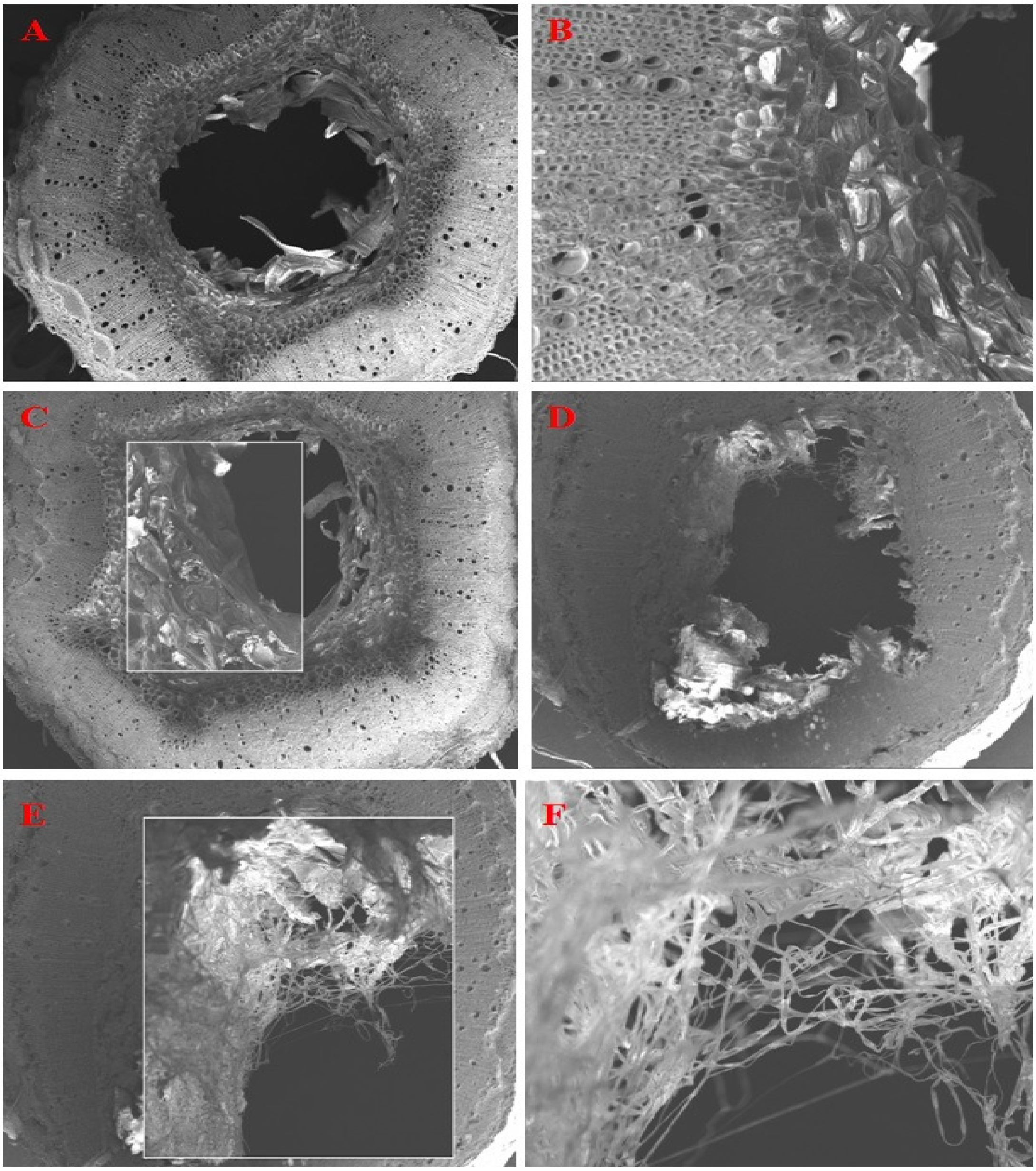

Plate 4.3.2: Scanning Electron Microscopy images of healthy and infected stem anatomy and features of cellular responses of GG16 at 5 dpi

Cross section of healthy stem tissue showing the compactness of xylem vessels with no traces of *S. rolfsii*, magnifications at about 100X (A) and 400X (B & C). Infected stem anatomy showing distorted xylem vessel (D) occupied by *S. rolfsii* hyphae (E); A closer view of fungal mycelia attached to the xylem tissue (F).

Ca-oxalate crystal was reported to be an integral component of necrotrophic pathogen-host system (Uloth *et al.,* 2015). It has been postulated by Heller and Witt-Geiges that oxalic acid produce by the necrotrophs is used to precipitate the Ca^{+2} ions released during degradation of plant cell wall, thereby preventing the pathogen from the toxic effect of Ca^{+2} ions.

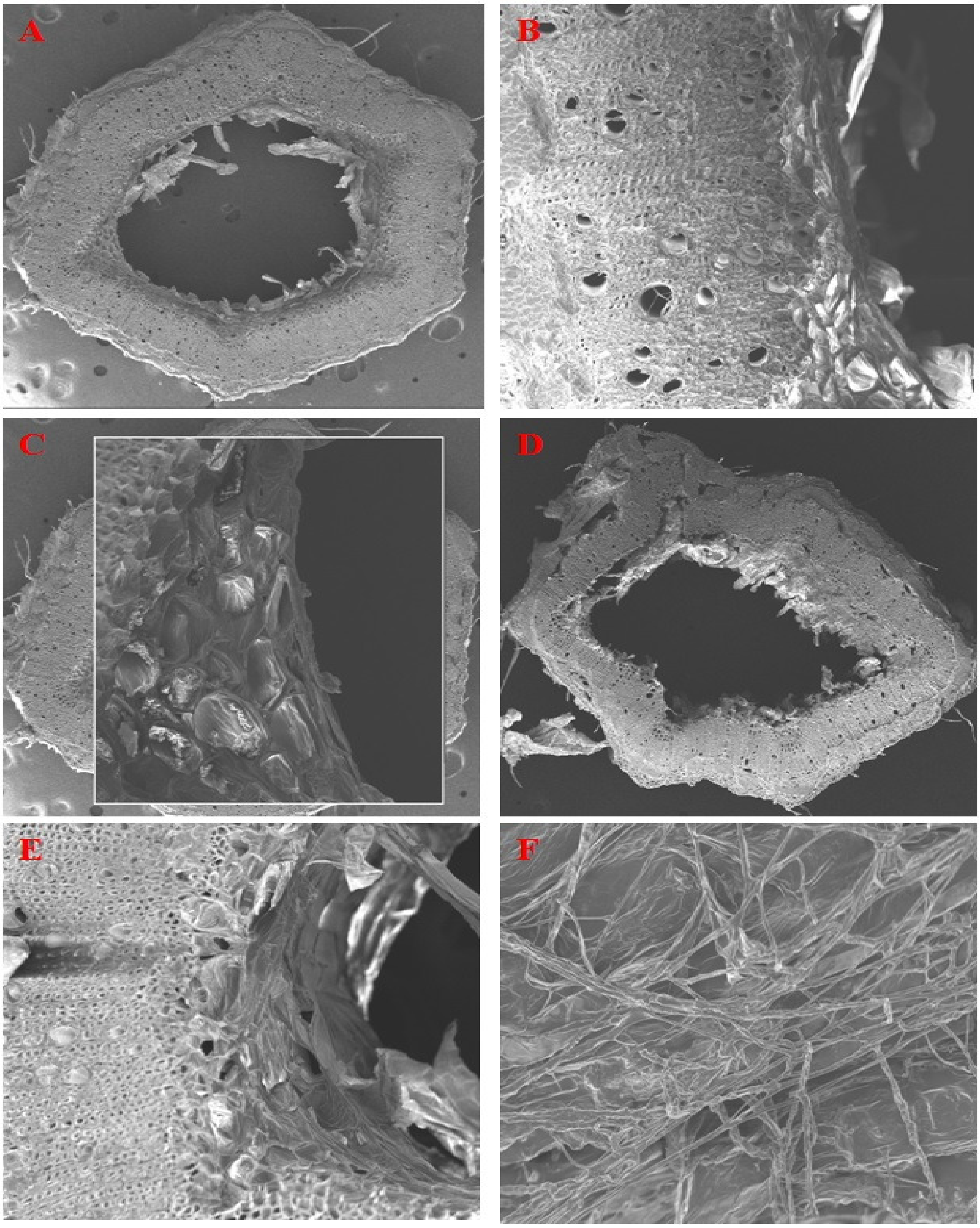

Plate 4.3.3: Scanning Electron Microscopy images of healthy and infected stem anatomy and features of cellular responses of GG20 at 5 dpi

Cross section of healthy stem tissue showing the compactness of xylem vessels with no traces of *S. rolfsii*, magnifications at about 100X (A) and 400X (B & C). Infected stem anatomy showing distorted xylem vessel (D); Some outgrowth near the vascular tissue (E); A closer view of *S. rolfsii* mycelia mat spread over the xylem tissue (F).

Ca Oxalate crystals were observed in the infected stems of TG37A at 5 dpi (marked with arrow in Plate 4.3.4 D & E) where as such structures were not detected in other genotypes (Plate 4.3.4, 4.3.2 and 4.3.3).

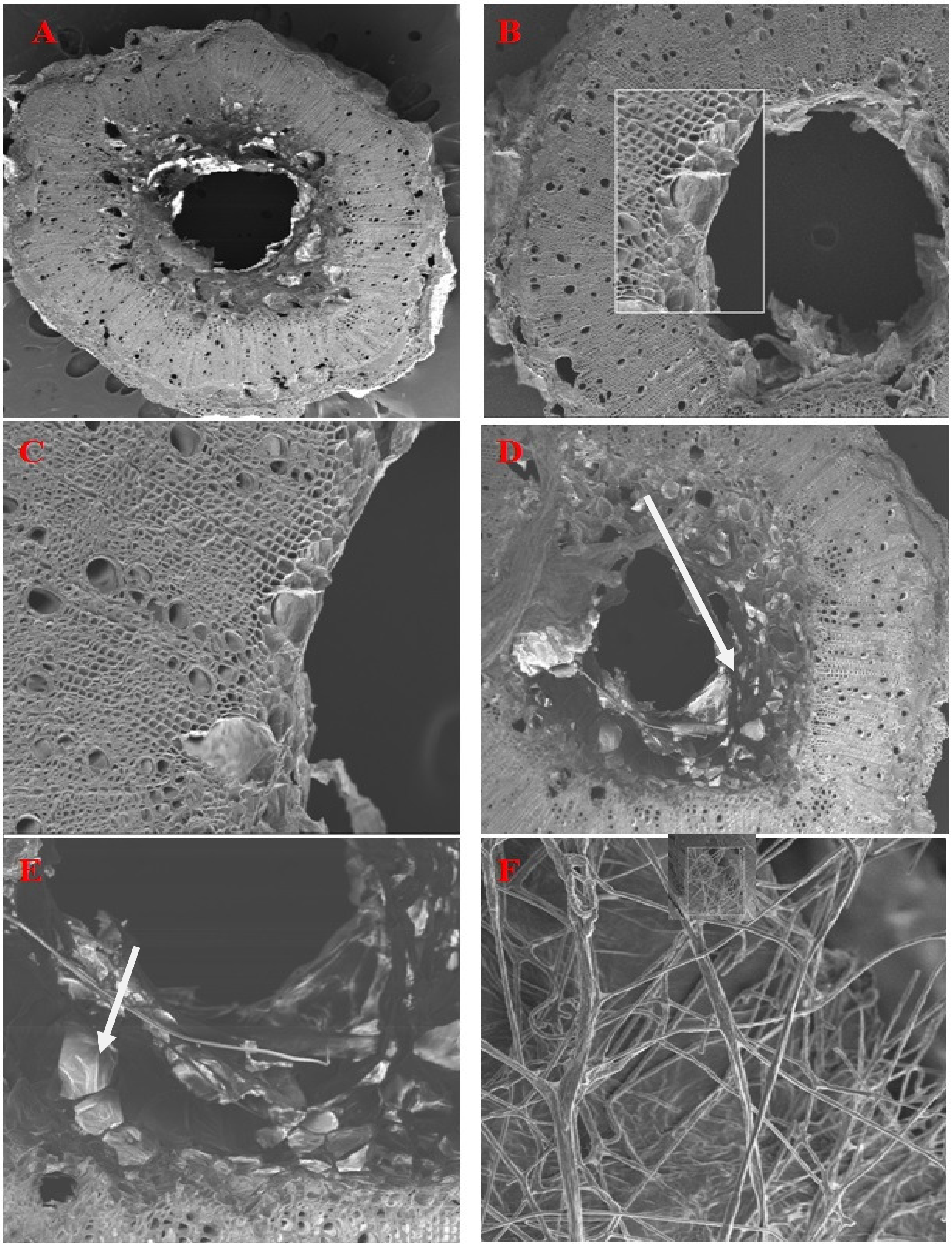

Plate 4.3.4: Scanning Electron Microscopy images of healthy and infected stem anatomy and features of cellular responses of TG37A at 5 dpi

Cross section of healthy stem tissue showing the compactness of xylem vessels with no traces of *S. rolfsii*, magnifications at about 100X (A) and 400X (B & C). *S. rolfsii* infected stem anatomy showing extensive damaged and distorted xylem vessel (D); Deposition of some crystal like structures, may be Calcium oxalate near xylem vessels (E); Closer view of *S. rolfsii* mycelia mat spread over the xylem tissue (F).

This finding appeared to be due to greater growth of the fungus within the stem tissue of the susceptible genotypes, thereby higher accumulation of oxalic acid led to synthesis of Ca Oxalate. The absence or undetectable Ca Oxalate in tolerant or moderately tolerant genotypes suggests that the fungus could not spread and produce oxalic acid as it did in case of TG37A. The above hypothesis was supported by the earlier reports of Garg *et al.* (2010) who detected higher accumulation of Ca Oxalate in cross section of susceptible infected *Brassica napus* cotyledons than that of resistant genotypes.

4.4 EXPERIMENT IV

INDUCED BIOCHEMICAL CHANGES DURING HOST PATHOGEN INTERACTION

4.4.1 Per cent Disease Incidence

Genotypes showed a wide range of per cent disease incidence (Table 4.4.1) upon the fungal infection both in presence and absence of the biocontrol agent. When the Pseudomonas untreated plants were exposed to fungal stress, TG37A showed the earliest symptoms of the disease within 1 dpi, whereas other genotypes were free from the same. At 5 dpi, all genotypes reported the disease incidence which was in the range of about 20 % (CS19) to 50% (TG37A). At the same time genotypes GG16 and GG20 showed relatively lesser incidence than TG37A. The application of the biocontrol agent significantly reduced (about half) the disease incidence in all genotypes at 5 dpi. At the later stage of disease development, all the genotypes showed their maximum disease incidence, for instance genotype TG37A being the susceptible one, reported more than 80 % disease incidence whereas both genotypes CS19 and GG16 showed less than 50 % (31.3 and 43.8% respectively). The effect of the biocontrol agent on disease incidence was evident at 10 dpi. Even at the highest load of the fungal pathogen, the bacterial isolate probably inhibited the entry of the fungus to the plant or made the plant tolerant enough to counteract the pathogen and hence brought down the disease incidence to almost 50 %.

4.4.2 Induction of antioxidative enzymes

4.4.2.1 Induction of Superoxide dismutase *(SOD, EC 1.15.1.1)*

The SOD constitutes the primary step of cellular defence and dismutates superoxide radical to H_2O_2 and O_2. Hence, higher accumulation of SOD may correspond to a higher stress in the plant. The susceptible genotypes are more prone of infection; hence an early induction and more accumulation of SOD can be expected compared to tolerant genotypes. The proposed hypothesis was supported by Durner and Klessig (1995), who stated that the increase in the activities of SOD in plants can be correlated with increased susceptibility to pathogens.

Table 4.4.1: Effect of genotypes and treatments on disease incidence (%) under controlled conditions

Genotypes	Treatments	Percent Disease Incidence		
		1dpi	**5dpi**	**10 dpi**
CS19	**T1**	0.0	0.0	0.0
	T2	0.0	0.0	0.0
	T3	0.0	6.3	18.8
	T4	0.0	18.8	31.3
GG20	**T1**	0.0	0.0	0.0
	T2	0.0	0.0	0.0
	T3	0.0	12.5	31.3
	T4	0.0	25.0	56.3
GG16	**T1**	0.0	0.0	0.0
	T2	0.0	0.0	0.0
	T3	0.0	6.3	18.8
	T4	0.0	18.8	43.8
TG37A	**T1**	0.0	0.0	0.0
	T2	0.0	0.0	0.0
	T3	6.3	25.0	43.8
	T4	18.8	50.0	81.3

T1: Healthy plants grown normally; T2: Pseudomomonas treated plants; T3: Pseudomonas treated + *S. rolfsii* infected plants; T4: *S. rolfsii* infected plants; 1 dpi: 1 day post *S. rolfsii* inoculation; 5 dpi: 5 days post *S. rolfsii* inoculation; 10 dpi: 10 days post *S. rolfsii* inoculation

S. *rolfsii* infection significantly increased the SOD activity in all genotypes at all stages; however, the higher induction of the enzyme was observed at 5 dpi corresponding to the peak stage of infection (Table 4.4.2). At early stage of infection i.e. at 1 dpi, both tolerant and susceptible genotypes followed a similar trend of SOD activation, whereas at 5 dpi, the specific activity of SOD was relatively higher in CS 19 (8.22 U mg protein^{-1} min^{-1}) than GG16 (6.51 U mg protein^{-1} min^{-1}), GG20 (5.24 U mg protein^{-1} min^{-1}) and TG37A (6.36 U mg protein^{-1} min^{-1}). Irrespective of the genotypes, the maximum induction of SOD was observed at 5 dpi which was significantly higher than that at 1 dpi and 10 dpi. The higher induction at 5 dpi might be due to the higher disease intensity which was corresponding to the most vulnerable stage of infection. However, irrespective of genotypes, at later stage of infection (10 dpi), a significant reduction of SOD activity was observed, suggesting a possibility of breaking down of the SOD mediated dimutation machinery due to cellular degradation by the fungus leading to unavailability of the enzyme.

Table 4.4.2: Effect of genotypes and treatments at different stages of disease development on SOD activity (U mg protein^{-1})

Stages	Treatment	CS19	GG16	GG20	TG 37A
1dpi	**T1**	1.73 ± 0.07 b	1.65 ± 0.06 c	1.69 ± 0.01 b	1.68 ± 0.06 b
	T2	2.52 ± 0.04 a	2.47 ± 0.19 b	2.69 ± 0.06 a	2.47 ± 0.18 a
	T3	1.76 ± 0.20 b	2.43 ± 0.12 b	2.44 ± 0.16 a	2.63 ± 0.03 a
	T4	2.32 ± 0.12 a	2.93 ± 0.06 a	2.74 ± 0.08 a	2.67 ± 0.18 a
5 dpi	**T1**	2.31 ± 0.12 d	1.93 ± 0.11 c	1.77 ± 0.02 c	2.05 ± 0.25 d
	T2	3.27 ± 0.06 c	3.47 ± 0.12 b	3.66 ± 0.15 b	3.64 ± 0.11 c
	T3	4.63 ± 0.30 b	3.76 ± 0.25 b	3.92 ± 0.15 b	5.76 ± 0.18 b
	T4	8.22 ± 0.14 a	6.51 ± 0.16 a	5.24 ± 0.04 a	6.36 ± 0.11 a
10 dpi	**T1**	1.95 ± 0.17 b	2.02 ± 0.15 c	2.25 ± 0.15 c	2.27 ± 0.18 b
	T2	2.45 ± 0.18 a	3.78 ± 0.05 a	3.49 ± 0.16 b	3.07 ± 0.20 a
	T3	1.18 ± 0.05 c	2.99 ± 0.11 b	3.90 ± 0.18 a	1.89 ± 0.31 b
	T4	1.37 ± 0.13 c	1.37 ± 0.09 d	1.11 ± 0.06 d	0.35 ± 0.07 c

	L.S.D. ($P<0.05$)	S.Em. ±
Stages (S)	0.10	0.04
Variety (V)	0.12	0.04
Treatment (T)	0.12	0.04
S X V	0.20	0.07
S X T	0.20	0.07
V X T	0.23	0.08
S X V X T	0.41	0.14
CV (%)	8.66	

Values are mean of three replications, Value in each column followed by the same letters are not significantly different according to DMRT at $P < 0.05$.

T1: Healthy plants grown normally; T2: Pseudomonas treated plants; T3: Pseudomonas treated + *S. rolfsii* infected plants; T4: *S. rolfsii* infected plants; 1 dpi: 1 day post *S. rolfsii* inoculation; 5 dpi: 5 days post *S. rolfsii* inoculation; 10 dpi: 10 days post *S. rolfsii* inoculation

One unit of SOD activity was taken as that amount of enzyme that reduced the absorbance reading to 50% in comparison with tubes lacking enzyme per unit time.

A similar type of results were obtained by Nandi *et al.* (2013) who observed gradual increase in SOD activity up to five days after *S. rolfsii* infection and thereafter progressively decreased up to 10 days of post infection. It was proposed by the author that the enhanced activities of SOD up to 5 dpi may have helped in scavenging of the reactive oxygen derivatives which in turn may have led to susceptibility to the pathogen. Our results also revealed non-significant genotypic differences within stages for SOD activity (Table 4.4.2) which were supported by Xuan, *et al.* (2002) who also observed a little change in SOD activity when seeds of five *Aspergillus flavus* resistant cultivars and four highly susceptible cultivars of groundnut were inoculated with a suspension of the fungus.

Application of *Pseudomonas aeruginosa* (*SKPf 5*) as seed-priming showed a higher induction of SOD activity in all genotypes as compared to their respective control plants. This phenomenon could be a result of growth promoting activity of the bacteria as the Pseudomonad isolate used in this experiment was a good producer of growth promoting hormone (IAA) *in vitro*. Further the capability of the isolate to produce significant amount of Salicylic acid could be a reason for induced systemic resistance in the treated groundnut genotypes leading to induction of SOD. Hence, when the primed genotypes were challenged with the fungus, although a higher induction of the enzyme was observed in all genotypes, but the activity of SOD was comparatively lesser than the corresponding non-primed genotypes. The lesser induction of in primed genotypes could be reflection of lesser extent of infection compared to their corresponding counterpart.

It can be summarized that SOD plays a very important role in defence against fungal infection in plants which was evident from its induction upon *S. rolfsii* inoculation. Higher susceptibility to the fungus at even 1 dpi could be the reason for relatively higher induction of SOD observed in susceptible compared to tolerant genotypes. The reduction of infection in presence of the Pseudomonad isolate led to the reduce SOD induction in the respective treated plants.

4.4.2.2 Induction of Catalase (CAT, EC 1.11.1.6)

Downstream to SOD, Catalase and peroxidase are the most important enzymes involved in regulation of intracellular levels of H_2O_2, leading to stress tolerance. An induction of CAT was observed for all the genotypes infected with *S. rolfsii* as compared to their respective control; however a significantly higher induction was recorded for the tolerant genotypes (Table 4.4.3). The genotype CS19 reported to have a specific activity of 1.47 (U mg protein^{-1}) at 1 dpi which was raised up to about 2 U mg protein^{-1} at 5 dpi and reduced to the basal level at 10 dpi. The GG16 showed a comparatively higher induction of CAT than that of CS19 at 1 dpi and 5 dpi, however at 10 dpi, the specific activity of the enzyme was quite low (0.44 U mg protein$^{-1)}$.

Table 4.4.3: Effect of genotypes and treatments at different stages of disease development on Catalase activity (U mg protein^{-1})

Stages	Variety	CS19	GG16	GG20	TG37A
1 dpi	T1	0.62 ± 0.04 c	1.07 ± 0.10 b	0.80 ± 0.03 c	0.79 ± 0.07 b
	T2	0.99 ± 0.05 b	1.22 ± 0.10 b	1.49 ± 0.12 a	0.97 ± 0.11 ab
	T3	1.33 ± 0.08 a	1.07 ± 0.07 b	0.98 ± 0.04 bc	0.81 ± 0.03 b
	T4	1.47 ± 0.18 a	1.68 ± 0.05 a	1.15 ± 0.06 b	1.21 ± 0.08 a
5 dpi	T1	0.87 ± 0.04 b	0.65 ± 0.04 c	1.17 ± 0.05 b	0.85 ± 0.06 b
	T2	1.70 ± 0.29 a	1.38 ± 0.17 b	1.46 ± 0.15 ab	1.17 ± 0.14 b
	T3	1.81 ± 0.09 a	1.21 ± 0.07 b	1.29 ± 0.14 b	1.74 ± 0.10 a
	T4	1.98 ± 0.12 a	2.01 ± 0.09 a	1.70 ± 0.16 a	1.55 ± 0.06 a
10 dpi	T1	0.98 ± 0.13 b	0.78 ± 0.13 b	0.74 ± 0.07 b	0.88 ± 0.07 b
	T2	1.32 ± 0.08 a	1.31 ± 0.08 a	1.27 ± 0.13 a	1.67 ± 0.13 a
	T3	0.64 ± 0.05 c	0.93 ± 0.05 b	0.95 ± 0.13 b	0.74 ± 0.13 b
	T4	1.03 ± 0.10 b	0.44 ± 0.10 c	0.30 ± 0.06 c	0.26 ± 0.06 c

Effects	L.S.D. (P<0.05)	S.Em±
Stages (S)	0.07	0.03
Variety (V)	0.08	0.03
Treatment (T)	0.08	0.03
SXV	0.15	0.05
SXT	0.15	0.05
VXT	0.17	0.06
SXVXT	0.29	0.10
C.V. (%)	9.95	

Values are mean of three replications, Value in each column followed by the same letters are not significantly different according to DMRT at $P < 0.05$.

T1: Healthy plants grown normally; T2: Pseudomonas treated plants; T3: Pseudomonas treated + *S. rolfsii* infected plants; T4: *S. rolfsii* infected plants; 1 dpi: 1 day post *S. rolfsii* inoculation; 5 dpi: 5 days post *S. rolfsii* inoculation; 10 dpi: 10 days post *S. rolfsii* inoculation

One unit of CAT activity was taken as that amount of enzyme required to reduce 1 μmol of H_2O_2 per minute.

On the contrary, both susceptible genotypes were unable to induce the enzyme to the same extent as the tolerant genotypes did. Both GG20 and TG37A reported to have relatively lesser amount of specific activity of CAT on fungal infection at all stages, which was even extremely low at 10 dpi. The higher induction CAT in tolerant genotypes might have helped them to avoid the building of H_2O_2 to the toxic level, thereby preventing the cellular losses to the extent the susceptible genotypes would have experienced. The seed priming by the biocontrol agent showed a significantly higher induction of CAT activity in healthy groundnut genotypes, which was probably due to the occurrence of induced systemic resistance. When those bio-primed plants were challenged with the *S. rolfsii*, although there was an induction of CAT activity was observed, but the extent was lesser than that happened with non-primed infected plants. The lower induction of CAT in those plants might have due to lower infection of the pathogen in presence of the biocontrol agent. Hence it can be hypothesised that induction of CAT and bio-priming with the *Pseudomonas aeruginosa* isolate prevent the groundnut from oxidative stress damage.

4.4.2.3 Induction of Ascorbate Peroxidase (APX, EC 1.11.1.11)

The enzymes APX and GR complete the detoxification cycle of ROS inside cell via the Halliwell-Asada pathway and the activity of these enzymes determine the rate of dissipation of toxic substances (Mittler, 2002). The hypothesis of the experiment was that the higher induction of APX in required quantity at the time of infection with *S. rolfsii* may bring tolerance to the groundnut plants and hence it is more likely that the tolerant genotypes should have better capacity to produce the enzyme.

The study revealed a higher activity of APX in tolerant as compared to the susceptible genotypes, for instance CS19 and GG16 reported 37.40 and 41.39 U mg protein^{-1} respectively whereas GG20 and TG37A showed 29.43 and 23.46 U mg protein^{-1} at 1 dpi (Table 4.4.4). Similarly, at 5 dpi, both tolerant genotypes produced a higher amount of APX than that of TG37A. GG20 showed a higher accumulation of the enzyme both at 5 dpi and 10 dpi, explaining its better tolerance to the stem rot fungal pathogen. The results supported the observations made by De'Ascensao and Dubery, (2003) suggesting the higher induction of APX in tolerant genotypes.

Use of the bacterial isolate, enhanced the activity of APX at all stages in all the genotypes, which was probably due to its positive effect on plant growth and induced systemic resistance. When the plants primed with the bacterial isolate (*SKPf 5*) were exposed to the fungal stress, they showed a lower induction of APX than those without primed suggesting a lesser extent of infection in those former plants.

Earlier Pseudomonads induced APX activity was observed by many researchers (De Meyer, *et al.* 1997; Chen *et al.*, 2000; Sundaravadana, 2002; Saravankumar *et al.*, 2003; Karthikeyan, 2006).

Table 4.4.4 Effect of genotypes and treatments at different stages of disease development on Ascorbate Peroxidase activity (U mg protein^{-1})

Stages	Treatment	CS19 ± SE	GG16 ± SE	GG20 ±SE	TG 37A ± SE
1 dpi	T1	22.14 ± 0.46 c	19.70 ± 0.62 d	19.44 ± 1.00 c	21.31 ± 0.38 b
	T2	25.55 ± 0.53 c	29.80 ± 0.78 c	23.26 ± 0.77 b	24.31 ± 0.58 b
	T3	31.39 ± 0.56 b	34.63 ± 0.82 b	31.16 ± 1.04 a	30.96 ± 0.93 a
	T4	37.40 ± 1.32 a	41.39 ± 0.73 a	29.43 ± 0.73 a	23.46 ± 0.97 b
5 dpi	T1	25.30 ± 1.10 d	22.41 ± 0.89 d	21.49 ± 1.33 c	21.65 ± 1.16 d
	T2	42.70 ± 1.28 b	36.65 ± 1.42 c	23.60 ± 0.80 c	26.44 ± 1.32 c
	T3	39.17 ± 1.68 c	46.82 ± 0.96 b	45.80 ± 1.47 b	30.85 ± 0.96 b
	T4	58.02 ± 0.95 a	59.39 ± 1.40 a	54.37 ± 0.58 a	45.73 ± 1.56 a
10 dpi	T1	26.13 ± 1.77 c	24.96 ± 1.11 c	23.72 ± 1.79 c	29.36 ± 0.93 a
	T2	41.08 ± 1.29 a	28.64 ± 1.32 b	23.97 ± 0.69 c	20.31 ± 1.18 c
	T3	40.13 ± 1.04 a	34.29 ± 1.88 a	35.00 ± 1.45 b	23.84 ± 0.68 b
	T4	36.18 ± 1.50 b	29.48 ± 0.85 b	39.26 ± 1.94 a	27.99 ± 0.78 a

	L.S.D. (P<0.05)	S.Em.±
Stages (S)	0.80	0.28
Variety (V)	0.92	0.33
Treatment (T)	0.92	0.33
S X V	1.59	0.57
S X T	1.59	0.57
V X T	1.84	0.66
S X V X T	3.19	1.14
CV (%)	6.17	

Values are mean of three replications, and value in each column followed by the same letters within a stage are not significantly different according to DMRT at $P < 0.05$.

T1: Healthy plants grown normally; T2: Pseudomonas treated plants; T3: Pseudomonas treated + *S. rolfsii* infected plants; T4: *S. rolfsii* infected plants; 1 dpi: 1 day post *S. rolfsii* inoculation; 5 dpi: 5 days post *S. rolfsii* inoculation; 10 dpi: 10 days post *S. rolfsii* inoculation

One unit of APX activity was calculated as that amount of enzyme that can reduce 1 µmol of ascorbate in one minute.

4.4.2.4 Induction of Glutathione Reductase (GR, EC 1.8.1.7)

Glutathione reductase (GR) is a potential enzyme of the ASH-GSH cycle (Halliwell-Asada pathway) and plays an essential role in defense system against ROS by sustaining the reduced status of GSH (Reduced Glutathione). GR and GSH play a crucial role in determining the tolerance of a plant under various stresses (Reddy and Raghavendra, 2006). In the present investigation, specific activity of GR in leaves of four groundnut varieties were estimated (Table 4.4.5) at different stages of *S. rolfsii* in both presence and absence of a Pseudomonas isolate agent (*SKPf 5*).

Table 4.4.5 Effect of genotypes and treatments at different stages of disease development on Glutathione Reductase activity (U mg protein^{-1})

Stages	Treatment	CS19	GG16	GG20	TG37A
1 dpi	T1	4.19 ± 0.04 a	4.14 ± 0.13 b	4.43 ± 0.08 ab	3.94 ± 0.06 b
	T2	4.20 ± 0.04 a	4.64 ± 0.18 a	4.77 ± 0.06 a	4.51 ± 0.08 a
	T3	4.39 ± 0.06 a	4.52 ± 0.10 ab	4.35 ± 0.04 ab	4.39 ± 0.03 a
	T4	4.56 ± 0.05 a	4.76 ± 0.15 a	4.26 ± 0.13 b	4.12 ± 0.09 ab
5 dpi	T1	4.53 ± 0.43 b	4.38 ± 0.21 c	4.61 ± 0.08 d	4.31 ± 0.13 d
	T2	5.52 ± 0.10 a	5.48 ± 0.23 b	5.81 ± 0.04 b	5.89 ± 0.14 b
	T3	5.32 ± 0.08 a	5.26 ± 0.22 b	5.26 ± 0.13 c	4.75 ± 0.09 c
	T4	4.02 ± 0.19 c	6.04 ± 0.06 a	6.56 ± 0.11 a	6.30 ± 0.25 a
10 dpi	T1	3.97 ± 0.10 ab	3.58 ± 0.21 c	4.56 ± 0.09 a	3.18 ± 0.04 b
	T2	4.31 ± 0.08 a	4.33 ± 0.12 b	3.80 ± 0.06 b	4.00 ± 0.06 a
	T3	3.69 ± 0.15 b	3.37 ± 0.09 c	4.58 ± 0.10 a	3.85 ± 0.37 a
	T4	3.22 ± 0.06 c	4.91 ± 0.11 a	4.27 ± 0.05 a	2.43 ± 0.20 c

Effects		L.S.D. (P<0.05)	S.Em.
Stages	**(S)**	0.10	0.04
Variety	**(V)**	0.12	0.04
Treatment	**(T)**	0.12	0.04
S X V		0.20	0.07
S X T		0.20	0.07
V X T		0.23	0.08
S X VX T		0.40	0.14
CV %		5.51	

Values are mean of three replications, and value in each column followed by the same letters within a stage are not significantly different according to DMRT at P < 0.05.

T1: Healthy plants grown normally; T2: Pseudomonas treated plants; T3: Pseudomonas treated + *S. rolfsii* infected plants; T4: *S. rolfsii* infected plants; 1 dpi: 1 day post *S. rolfsii* inoculation; 5 dpi: 5 days post *S. rolfsii* inoculation; 10 dpi: 10 days post *S. rolfsii* inoculation

One unit of GR activity was taken as that amount of enzyme required to reduce 1 µmol of oxidised glutathione per minute.

The result depicted a quick induction of GR in tolerant genotypes upon the fungal infection within 24 hr, where as the response was quite late for susceptible genotypes. At 5 dpi, all genotypes showed a higher induction of the enzymes compared to their respective control plants (Table 4.4.5). The maximum activity of GR was recorded for GG20 (6.56 U mg protein^{-1}) where as the lowest was observed for CS19 (4.02 U mg protein^{-1}) at the peak stage of disease infection i.e. at 5dpi.

In case of the most susceptible genotype TG37A, there was a steep rise in the activity of the enzyme at 5 dpi followed by a quick fall at 10 dpi, where as for the most tolerant genotype CS19, resulted an instant induction of the enzyme at the onset of infection, followed by a comparatively stable induction throughout. Further, GG16, being moderately tolerant, reported to have a higher induction of GR activity even than CS19 and maintained the highest activity at 10 dpi compared other infected plants. All genotypes reported a reduction in GR activity at 10 dpi compared to 5 dpi, suggesting that at later stage of infection, the plants might not able to sustain the ROS detoxification through the ASH-GSH cycle.

Induction of GR in both tolerant and susceptible genotypes as a response to fungal stress seems to be a complicated phenomenon. This is obvious from the earlier reports stating that sometimes higher induction in tolerant genotypes (Garcia-Limones *et al.*, 2002) or no significant difference in GR activity is observed upon fungal stress (Paciolla *et al.*, 2008). This is probably due to the fact that the various factors that affect the GR activity can be differently affected in plant–pathogen interactions, both due to different plant species-specific sensitivity and because, in different plant pathogen interactions, the suppression or strengthening of ROS detoxification could be obtained in different ways.

4.4.3 Induction of Pathogenesis related (PR) proteins

4.4.3.1 Induction of Peroxidase (POX, EC 1.11.1.7)

Peroxidases are important PR proteins and the plant expresses POX activity during host-pathogen interaction. Plant peroxidase has been implicated in the last enzymatic step of lignin biosynthesis, Furthermore, it is involved in the production or modulation of active oxygen species which may play various roles directly or indirectly in reducing pathogen viability and spread (Prasad *et al.*, 2005). We also noticed a prominent role of the enzyme as a PR protein, imparting tolerance to groundnut against *S. rolfsii.*

All genotypes showed significant induction of POX as an early response to the fungal pathogen; however the genotypic responses were varied with treatments and stages (Table 4.4.6). It was observed that with the progress of the disease, there was a gradual increase in POX in all genotypes which reached its maxima at 5 dpi followed by a steady decrease in activity towards 10 dpi.

Table 4.4.6 Effect of genotypes and treatments at different stages of disease development on Peroxidase activity (U mg protein^{-1}) in groundnut

Stages	Treatment	CS19	GG16	GG20	TG37A
1 dpi	T1	24.21 ± 1.73 c	24.28 ± 0.84 c	26.33 ± 1.30 c	23.56 ± 0.99 d
	T2	50.91 ± 1.18 b	44.52 ± 0.55 b	48.98 ± 0.99 a	33.33 ± 0.60 c
	T3	48.41 ± 1.35 b	55.84 ± 1.33 a	48.75 ± 1.14 a	46.40 ± 0.52 b
	T4	54.67 ± 1.00 a	57.48 ± 1.04 a	39.04 ± 0.66 b	53.49 ± 0.43 a
5 dpi	T1	25.04 ± 0.75 c	22.85 ± 1.19 c	23.58 ± 2.24 c	26.92 ± 1.79 c
	T2	86.90 ± 0.74 a	70.50 ± 0.86 b	65.65 ± 1.29 a	54.00 ± 1.70 b
	T3	76.19 ± 0.95 b	73.66 ± 1.26 b	58.31 ± 0.49 b	51.50 ± 1.52 b
	T4	86.82 ± 1.85 a	84.18 ± 0.97 a	65.24 ± 0.59 a	83.54 ± 0.76 a
10 dpi	T1	28.34 ± 1.17 c	27.34 ± 1.07 b	29.97 ± 1.53 b	22.74 ± 2.75 c
	T2	19.75 ± 0.86 d	30.33 ± 0.69 b	23.31 ± 1.59 c	18.64 ± 1.98 d
	T3	47.43 ± 1.68 b	54.52 ± 1.37 a	57.61 ± 1.72 a	49.05 ± 1.20 a
	T4	70.45 ± 0.55 a	56.18 ± 1.34 a	56.54 ± 1.51a	44.96 ± 0.94 b

Effects	L.S.D. (P<0.05)	S.Em.±
Stages (S)	0.89	0.32
Variety (V)	1.03	0.37
Treatment (T)	1.03	0.37
S X V	1.79	0.64
S X T	1.79	0.64
V X T	2.06	0.74
S X VX T	3.57	1.27
CV %	4.66	

Values are mean of three replications, and value in each column followed by the same letters within a stage are not significantly different according to DMRT at $P < 0.05$.

T1: Healthy plants grown normally; T2: Pseudomonas treated plants; T3: Pseudomonas treated + *S. rolfsii* infected plants; T4: *S. rolfsii* infected plants; 1 dpi: 1 day post *S. rolfsii* inoculation; 5 dpi: 5 days post *S. rolfsii* inoculation; 10 dpi: 10 days post *S. rolfsii* inoculation

One unit of POX activity was taken as that amount of enzyme required to form1 µmol of tetraguaiacol per minute.

However at all point of time, the infected tissues showed a higher activity of the enzyme compared to their respective control. Among the susceptible genotypes, GG20 showed a lower induction compared to TG37A. Although TG37A was a highly susceptible genotype, it was capable of producing higher amount of the enzyme as a disease response which was almost at par with the tolerant genotypes. A contrasting behaviour was observed between genotypes at 10 dpi, which differentiated the most tolerant genotype (CS19) from rest of the genotypes. Even at the later stage of infection, CS19 could able to retain a higher activity of POX, where as none of the other genotypes made it. Hence it can be summarized that POX induction upon fungal infection is a common strategy followed by both tolerant and susceptible groundnut genotypes, but the ability of the tolerant genotype to maintain the higher production of the enzyme for a longer period during the disease development might be the reason for imparting tolerance.

It was also noticed a significant induction of POX in all genotypes when treated with the biocontrol agent (*SKPf 5*). When those treated plants were challenged with the fungal pathogen (T3), comparatively a higher induction of POX was observed, which was however lower than that recorded for plants without treatment (T4). As with other antioxidative enzymes, the induction of POX by the biocontrol agent might be through Induced Systemic Resistance (ISR). Further, comparatively lower activity of POX in infected plants already treated with *SKPf 5* might be corresponding to lower disease incidence in presence of the biocontrol agent.

Our results was backed by the findings of Nandi *et al.* (2013) who observed the maximum activity of POX at three days after *S. rolfsii* inoculation and maximum reduction at 10 days after inoculation. They hypothesized that the suppression of POX after 5 days after infection leads to the weakening of defense mechanisms in *S. rolfsii* inoculated cowpea plants. The similar trend of the POX activity in our study also supports the earlier hypothesis. The reduction of the POX activity at the later stage of the disease probably helps in the further spread of the pathogen and eventually severe stem rot symptoms are expressed. Hence this can be concluded that the suppression of peroxidase is found to be one of the important factors responsible for the successful pathogenesis of *S. rolfsii*.

4.4.2.2 Induction of Chitinase (EC 3.2.1.14)

It is known that chitinases degrade chitin in fungal cell wall by hydrolyzing β-1, 4 linkages between N-acetyl glucosamine residues and also act as synergistically with the β-1,3 glucanase for inhibiting fungal growth. They are constitutively expressed at low levels in leaves and high level in roots and seeds. Increased levels of gene expression and enzymatic activity have been observed after biotic stress elicitation (van Loon *et al.*, 2006).

A significant difference of chitinase activity among genotypes for different treatments at different time interval was observed as a response towards the *S. rolfsii* infection (Table 4.4.7). Comparatively a higher induction of the enzyme was observed for the tolerant genotypes than that for the susceptible genotypes. As a response of tolerance, an early (at 1 dpi) and stronger induction (3.4 fold compared to control) of chitinase was noticed for CS19, which was significantly higher than GG16 (2.62 fold). The weaker induction of the enzyme in GG20 (1.35 fold) and TG37A (1.97 fold) at the early stage of infection, might be responsible for higher incidence of the disease in those susceptible genotypes. It was further observed that except for CS19, all genotypes showed a gradual increase in chitinase activity after the fungal infection, and reached the maximum at 5 dpi.

Table 4.4.7 Effect of genotypes and treatments at different stages of disease development on Chitinase activity (U mg protein^{-1})

Stages	Treatment	CS19	GG16	GG20	TG37A
1 dpi	T1	0.46 ± 0.06 c	0.37 ± 0.05 c	0.52 ± 0.06 b	0.38 ± 0.06 b
	T2	0.47 ± 0.02 c	0.49 ± 0.04 c	0.62 ± 0.01 ab	0.43 ± 0.07 b
	T3	0.74 ± 0.04 b	0.66 ± 0.07 b	0.47 ± 0.02 b	0.42 ± 0.08 b
	T4	1.57 ± 0.03 a	0.96 ± 0.04 a	0.71 ± 0.04 a	0.76 ± 0.05 a
5 dpi	T1	0.51 ± 0.01 c	0.44 ± 0.02 d	0.42 ± 0.07 c	0.47 ± 0.09 c
	T2	0.64 ± 0.02 c	0.75 ± 0.06 c	0.86 ± 0.11 a	0.68 ± 0.10 b
	T3	1.31 ± 0.05 b	1.11 ± 0.03 b	0.64 ± 0.04 b	0.56 ± 0.04 bc
	T4	1.47 ± 0.05 a	1.41 ± 0.07 a	0.89 ± 0.06 a	1.03 ± 0.02 a
10 dpi	T1	0.53 ± 0.03 a	0.45 ± 0.04 b	0.46 ± 0.03 b	0.43 ± 0.02 a
	T2	0.41 ± 0.02 a	0.70 ± 0.01 a	0.72 ± 0.03 a	0.40 ± 0.03 a
	T3	0.44 ± 0.04 a	0.42 ± 0.04 b	0.65 ± 0.04 a	0.15 ± 0.01 b
	T4	0.21 ± 0.03 b	0.47 ± 0.01 b	0.48 ± 0.03 b	0.15 ± 0.03 b

Effects	L.S.D. (P<0.05)	S.Em. ±
Stages (S)	0.034	0.012
Variety (V)	0.04	0.014
Treatment (T)	0.04	0.014
S X V	0.069	0.024
S X T	0.069	0.024
V X T	0.079	0.028
S X V X T	0.138	0.049
CV (%)	9.42	

Values are mean of three replications, and value in each column followed by the same letters within a stage are not significantly different according to DMRT at P < 0.05.

T1: Healthy plants grown normally; T2: Pseudomonas treated plants; T3: Pseudomonas treated + *S. rolfsii* infected plants; T4: *S. rolfsii* infected plants; 1 dpi: 1 day post *S. rolfsii* inoculation; 5 dpi: 5 days post *S. rolfsii* inoculation; 10 dpi: 10 days post *S. rolfsii* inoculation

One unit of Chitinase activity was taken as that amount of enzyme required to form 1µmol of N-acetylglucosamine per minute.

At 5 dpi both GG16and CS19 reported to have almost similar amount of the enzyme activity, GG16 maintained the highest induction among all. There was a significant decline in the activity of chitinase at the later stage of the disease in all genotypes. The effect of the application of the *Pseudomonas aurigenosa* isolate (*SKPf 5*) was significant in terms of chitinase activity (Table 4.4.7). However the induction was prominent at 5 dpi in all genotypes. When the *SKPf 5* treated plants were challenged with the *S. rolfsii*, comparatively a higher induction of chitinase was observed for all the genotypes, however the induction was weaker than that of the plants without the treatment with fungal infection. The lesser induction of the enzyme might be corresponding to lesser incidence of disease in presence of the biocontrol agent.

The results were in agrrement with the earlier reports of Anguelova *et al.* (2001) who suggests the higher and stronger induction of chitinase in the resistant varieties in comparison to susceptible varieties against phyto-pathogen system. Further, the vital role of chitinase in imparting stem rot tolerance in groundnut was evident from the results shown in this experiment which was in accordance with the recent transgenic experimental findings by Rohini *et al.* (2001) and Prasad *et al.* (2013). The researchers showed transformation of chitinase gene has shown to enhance resistance to many fungal pathogens in groundnut. Further the higher induction chitinase in tolerant groundnut genotypes compared to susceptible in response to *S. rolfsii* was supported by Jogi, (2012), who reported higher and stronger induction of two chitinases genes in groundnut upon the fungal infection.

4.4.2.3 Induction of β 1, 3 Glucanase (EC 3.2.1.6)

β-1,3-glucanases catalyze the cleavage of β-1,3-glucosidic bonds of β-1,3-glucan, another constituent of the fungal cell wall. ß-1,3-glucanases are believed to have an indirect effect on plant defense by causing the formation of oligosaccharide elicitors, which induce the production of other PR proteins or low molecular weight antifungal compounds, such as phytoalexins (Klarzynski *et al.*, 2000). We observed an early and stronger induction of ß-1,3-glucanases in tolerant genotypes at 1dpi compared to the susceptible genotypes when the plants were infected with *S. rolfsii* (Table4.4.8). However the induction was significantly higher in CS19 than all other genotypes irrespective of stages of infection. The maximum activity of the enzyme was noted for infected CS19 (2.32 U mg protein^{-1}) where was the lowest was for TG37A (1.04 U mg protein^{-1}) at 1 dpi. The activity of ß-1, 3-glucanases gradually increased and reached its maximum at 5 dpi for GG16 (2.0 U mg protein^{-1}) and TG37A (1.0 U mg protein^{-1}) corresponding to moderately tolerant and highly susceptible genotype respectively.

Table 4.4.8 Effect of genotypes and treatments at different stages of disease development on ß-1, 3-Glucanases activity (U mg protein^{-1})

Stages	Treatment	CS19	GG16	GG20	TG37A
1 dpi	**T1**	0.96 ± 0.03 d	0.88 ± 0.04 d	0.79 ± 0.04 c	0.86 ± 0.02 c
	T2	1.56 ± 0.06 c	1.17 ± 0.05 c	1.24 ± 0.02 b	1.49 ± 0.04 a
	T3	1.98 ± 0.04 b	1.40 ± 0.07 b	1.57 ± 0.03 a	1.11 ± 0.13 b
	T4	2.32 ± 0.11 a	1.77 ± 0.12 a	1.61 ± 0.05 a	1.04 ± 0.04 bc
5 dpi	**T1**	0.84 ± 0.07 b	0.82 ± 0.068 c	0.69 ± 0.03 c	0.67 ± 0.01 b
	T2	0.97 ± 0.05 b	1.03 ± 0.19 c	0.94 ± 0.02 b	0.88 ± 0.12 b
	T3	2.18 ± 0.04 a	1.57 ± 0.10 b	1.09 ± 0.09 b	1.17 ± 0.05 a
	T4	2.22 ± 0.13 a	2.00 ± 0.11 a	1.40 ± 0.06 a	1.33 ± 0.08 a
10 dpi	**T1**	1.01 ± 0.09 b	0.96 ± 0.07 b	0.68 ± 0.05 a	0.99 ± 0.05 b
	T2	1.02 ± 0.10 b	1.23 ± 0.09 a	0.86 ± 0.08 a	1.30 ± 0.03 a
	T3	1.05 ± 0.04 b	1.40 ± 0.06 a	0.66 ± 0.12 a	1.19 ± 0.11 ab
	T4	1.33 ± 0.02 a	0.73 ± 0.04 b	0.73 ± 0.10 a	0.75 ± 0.09 c

Effects	L.S.D. (P<0.05)	S.Em. ±
Stages (S)	0.06	0.02
Variety (V)	0.06	0.02
Treatment (T)	0.06	0.02
S X V	0.11	0.04
S X T	0.11	0.04
V X T	0.13	0.05
S X V X T	0.22	0.08
CV (%)	11.39	

Values are mean of three replications, and value in each column followed by the same letters within a stage are not significantly different according to DMRT at P < 0.05.

T1: Healthy plants grown normally; T2: Pseudomonas treated plants; T3: Pseudomonas treated + *S. rolfsii* infected plants; T4: *S. rolfsii* infected plants; 1 dpi: 1 day post *S. rolfsii* inoculation; 5 dpi: 5 days post *S. rolfsii* inoculation; 10 dpi: 10 days post *S. rolfsii* inoculation

One unit of β 1,3 Glucanase activity was taken as that amount of enzyme required to release 1μmol of glucose per minute.

For both CS19 and GG20, the maximum activity of the enzyme was attained at 1 dpi. Hence, our experiment suggested that the activity of ß-1,3-glucanases may vary with genotypes and time of induction. Genotypes with varying degree of tolerance may follow different pattern of the enzyme expression pattern and this hypothesis may be explained by the fact that β-1,3-glucanases are implicated in diverse physiological functions along with plant defence (van Loon *et al.*, 2006). Further β-1,3-glucanases and chitinases act synergistically and their optimal functions may be important in plant defense. Irrespective of the genotypes, the enzyme activity was significantly reduced at 10 dpi. Although the reduction was observed for both CS19 and GG20, but they somehow maintained the basal level of expression of ß-1,3-glucanases, where as GG16 and TG37A reported lowest activity of the enzyme which was even less than that of the corresponding control plants. Observations made by Nandi *et al.* (2013) supported our findings who showed a significant increase of ß-1,3-glucanases upon *S. rolfsii* infection in cowpea. They also noted that the enzyme activity reached at its peak (5.07 folds) at five days after fungal inoculation compared with the water treated control, and thereafter there was significant fast decline in its activity in the susceptible host-pathogenic interaction. Further, the induction of ß-1,3-glucanases upon *S. rolfsii* infection in groundnut got supports from Nandini *et al.* (2010) who reported substantial induction of the enzyme in groundnut plants using the fungal components of the pathogen in the form of fungal culture filtrate (FCF) and mycelial cell wall (MCW).

Overall it can be summarised that the PR proteins like Peroxidase, Chitinases and ß-1, 3-glucanases were induced upon *S. rolfsii* infection in groundnut. Presence of the biocontrol agent also caused induction of those enzymes possibly though ISR. The higher and rapid induction of PR protein at the early stage of infection might be responsible for providing tolerance in groundnut genotypes against the stem rot pathogen.

4.4.4 Induced changes in other metabolites

4.4.4.1 Changes in Ascorbic acid content

Under physiological conditions, ascorbic acid exists mostly in the reduced form in chloroplast. The ability to donate electrons in a wide range of enzymatic and non-enzymatic reactions makes ascorbic acid as main ROS-detoxifying compound in the aqueous phase (Arrigoni and De Tullio, 2000). Under both biotic and abiotic stresses high amount of ROS are produced and there the ascorbic acid participate in an oxygen scavenging pathway in the chloroplast known as the ascorbate-glutathione cycle. Hence, it can be hypothesized that upon induction of fungal stress, to detoxify the ROS, a part of the ascorbic acid pool will get exhausted and then the capacity to maintain the amount of ascorbic acid will decide the tolerance nature of a genotype.

Table 4.4.9: Effect of genotypes and treatments at different stages of disease development on Ascorbic acid content (mg g^{-1} Fresh weight)

Stages	Treatment	CS19	GG16	GG20	TG37A
1 dpi	**T1**	6.31 ± 0.14 a	5.64 ± 0.16 a	5.91 ± 0.09 ab	6.30 ± 0.12 a
	T2	5.87 ± 0.15 b	5.69 ± 0.15 a	5.99 ± 0.06 a	6.37 ± 0.18 a
	T3	5.79 ± 0.14 b	4.19 ± 0.10 b	5.59 ± 0.14 b	4.39 ± 0.04 b
	T4	5.74 ± 0.15 b	4.55 ± 0.09 b	4.51 ± 0.08 c	4.08 ± 0.04 b
5 dpi	**T1**	6.02 ± 0.25 a	6.09 ± 0.10 a	5.59 ± 0.07 b	5.45 ± 0.10 a
	T2	6.25 ± 0.10 a	6.26 ± 0.20 a	6.11 ± 0.12 a	5.72 ± 0.08 a
	T3	5.22 ± 0.06 b	4.67 ± 0.10 b	4.78 ± 0.33 c	4.44 ± 0.13 b
	T4	5.03 ± 0.09 b	3.90 ± 0.15 c	3.45 ± 0.12 d	3.46 ± 0.05 c
10 dpi	**T1**	6.12 ± 0.08 b	6.45 ± 0.04 b	5.77 ± 0.11 a	5.57 ± 0.12 b
	T2	6.73 ± 0.15 a	6.81 ± 0.08 a	5.76 ± 0.06 a	6.10 ± 0.08 a
	T3	4.41 ± 0.11 c	4.17 ± 0.16 c	4.19 ± 0.07 b	4.14 ± 0.15 c
	T4	4.44 ± 0.13 c	3.50 ± 0.08 d	3.08 ± 0.06 c	2.66 ± 0.10 d
Effects		**L.S.D. (P<0.05)**		**S.Em. ±**	
Stages (S)		0.09		0.03	
Variety (V)		0.10		0.04	
Treatment (T)		0.10		0.04	
S X V		0.17		0.06	
S X T		0.17		0.06	
V X T		0.20		0.07	
S X V X T		0.34		0.12	
CV (%)			8.05		

Values are mean of three replications, and value in each column followed by the same letters within a stage are not significantly different according to DMRT at P < 0.05.

T1: Healthy plants grown normally; T2: Pseudomonas treated plants; T3: Pseudomonas treated + *S. rolfsii* infected plants; T4: *S. rolfsii* infected plants; 1 dpi: 1 day post *S. rolfsii* inoculation; 5 dpi: 5 days post *S. rolfsii* inoculation; 10 dpi: 10 days post *S. rolfsii* inoculation

We observed the reduction of ascorbic acid content in all genotypes in response to *S. rolfsii* infection (Table 4.4.9). However, the reduction was comparatively higher in susceptible than tolerant genotypes. With the progress of the disease intensity, the ascorbic acid content gradually decreased in all infected plants, whereas the content remained all most stable in leaves of healthy and biocontrol treated plants. There were significant changes in ascorbic acid content observed between treatments, suggesting the effect of the biocontrol agent for maintaining the pool of ascorbic acid, thereby providing oxidative stress tolerance in groundnut plants. Although an exact mechanism is not known regarding the agent, but the induction of ascorbic acid pool might be due to the better physiological conditions of the seed primed plants which is contributed by the plant growth promoting effect of *SKPf 5*.

A number studies, reported the decrease in ascorbic acid content in infected leaves (Prasad *et al.*, 1976; Chhabra *et al.*, 2000) compared to healthy leaves. Further Wang *et al.* (2002) found that the resistant genotypes maintain higher content of ascorbic acid than the susceptible genotypes upon fungal infection. In general, the ascorbic acid content is reduced upon disease infection; however, the capacity of a genotype to maintain the ascorbic acid pool is the deciding factor for its tolerance to any oxidative stress, in particular fungal stress.

4.4.4.2 Changes in oxalic acid content

S. rolfsii is a necrotrophic fungus, capable of producing a substantial amount of oxalic acid which is sufficient enough to start the pathogenesis in various crop plants. It secretes several kinds of cell wall degrading enzymes particularly polygalacturonases that macerate the host tissue by degrading pectin to provide nutrients for mycelial growth, and oxalic acid assist the activity of the enzyme by lowering the pH and providing optimal conditions for the activity of the enzymes. Further, oxalic acid produced by the fungus chelates the cell wall Ca^{2+}, which otherwise at higher concentration have toxic effect on the fungus. Hence, it is more likely that upon fungal infection, more oxalic acid accumulation will occur in the infected tissues compared to healthy tissues. Moreover, as the infection proceeds, more oxalic acid will be detected in the infected tissue. As the accumulation of oxalic acid can be associated with the disease severity, hence a higher quantity of the metabolite can be expected from the susceptible compared to tolerant genotypes.

It was also observed higher amount of oxalic acid in the infected stem compared to that of the healthy tissue (Table 4.4.10). At 1 dpi, no significant change in oxalic acid content was observed in CS19, where as in the infected tissues of other genotypes, higher amount of the metabolite was found compared to their respective healthy tissues. The infected stem tissue of TG37A reported have about 1.5 fold increase in oxalic acid content compared to its control. The hypothesis stated earlier was supported by the findings of our experiment, which recorded a gradual increase in oxalic accumulation in the infected tissues from 1 dpi to 10 dpi.

Table 4.4.10: Effect of genotypes and treatments at different stages of disease development on oxalic acid content (μg g fresh weight^{-1})

Stages	Treatment	CS19	GG16	GG20	TG37A
1 dpi	**T1**	40.93 ± 0.75 b	43.13 ± 0.75 c	49.80 ± 0.99 c	46.70 ± 0.44 c
	T2	41.50 ± 1.01 b	46.00 ± 0.46 bc	42.87 ± 0.53 d	45.10 ± 0.38 c
	T3	50.83 ± 0.84 a	53.90 ± 0.76 a	56.00 ± 0.86 b	59.20 ± 1.21 b
	T4	40.77 ± 1.12 b	49.23 ± 1.34 b	62.57 ± 0.60 a	71.10 ± 0.72 a
5 dpi	**T1**	56.40 ± 0.64 c	55.77 ± 0.90 c	63.27 ± 0.78 c	59.00 ± 1.16 c
	T2	49.77 ± 1.13 d	49.70 ± 0.80 d	65.27 ± 1.53 c	58.17 ± 0.59 c
	T3	61.70 ± 1.41 b	63.47 ± 0.93 b	69.83 ± 1.16 b	71.77 ± 1.45 b
	T4	71.57 ± 1.45 a	77.27 ± 1.16 a	73.63 ± 0.63 a	84.77 ± 0.43 a
10 dpi	**T1**	60.27 ± 1.16 c	62.20 ± 1.86 c	68.40 ± 1.76 c	63.07 ± 0.91 d
	T2	55.37 ± 1.43 d	58.63 ± 0.64 c	67.03 ± 2.24 c	74.57 ± 1.23 c
	T3	67.63 ± 1.67 b	72.63 ± 1.48 b	72.23 ± 1.58 b	85.80 ± 2.17 b
	T4	88.43 ± 1.74 a	97.53 ± 2.00 a	93.33 ± 2.36 a	103.40 ± 3.03 a

Effects	L.S.D. (P<0.05)	S.Em. ±
Stages (S)	0.91	0.32
Variety (V)	1.05	0.37
Treatment (T)	1.05	0.37
S X V	1.82	0.65
S X T	1.82	0.65
V X T	2.11	0.75
S X V X T	3.65	1.3
CV (%)	5.57	

Values are mean of three replications, and value in each column followed by the same letters within a stage are not significantly different according to DMRT at P < 0.05.
T1: Healthy plants grown normally; T2: Pseudomonas treated plants; T3: Pseudomonas treated + *S. rolfsii* infected plants; T4: *S. rolfsii* infected plants; 1 dpi: 1 day post *S. rolfsii* inoculation; 5 dpi: 5 days post *S. rolfsii* inoculation; 10 dpi: 10 days post *S. rolfsii* inoculation

Further, the accumulation was higher in susceptible genotypes. Almost 1.5 fold increase in accumulation of the metabolite was found in all the disease tissues at 10 dpi. Although use of the Pseudomonad isolate (*SKPf 5*) altered the accumulation pattern in both healthy and infected plants, but a particular trend was lacking in the healthy tissues. However, when the *SKPf 5* treated plants were challenged with the fungus, a significantly lower oxalic acid accumulation was reported (Table 4.4.10), suggesting the fungal inhibitory activity of the biocontrol agent. The present findings further supported the vital role of oxalic acid in pathogenesis for *S. rolfsii*-groundnut pathosystem.

4.4.5 Protein expression profiling through 2D gel electrophoresis

Proteomics approach was made to analyse the changes in protein profiling during the interaction between *S. rolfsii* and groundnut genotypes. As the genotypes used have varying degree of tolerance to the pathogen, the result also depicted the variations during compatible and incompatible interactions in the above pathosystem. Incompatible interaction between tolerant genotypes and pathogens, leads to induction of hypersensitive responses (HR), production of pathogenesis related proteins and other cellular changes to prevent the spread of the disease, whereas in compatible interaction between susceptible genotypes and pathogen, do not induce HR, resulting in disease development.

The main aim of this experiment was to see the differential expression of proteins isolated from the healthy and their corresponding *S. rolfsii* infected plants. The present experiment included four groundnut genotypes with varying degree of tolerance to the pathogen and hence the comparative analysis of protein expression was carried out between the healthy and their corresponding infected plants after 5 days post fungal infection (5dpi). As expected the proteomic study indicated distinct changes of protein expression in all the genotypes between the healthy and their corresponding infected ones, which were evident from their spot matching analysis (Plate 4.4.1, 4.4.2, 4.4.3, and 4.4.4). There were distinct variations among and within genotypes in response to the fungal infection. In most of the cases, the numbers of spots identified were more in control than the stressed leaves (Table 4.4.10). For instance, in healthy leaves of CS19, about 412 protein spots were identified where as only 260 spots were seen in that of the fungal infected plants. Gel match analysis showed 71 proteins were expressed both under control and stress condition in CS19. Similarly, out of 357 spots in healthy, and 216 spots in stressed leaves, about 131 spots showed matching expression in GG16, suggesting a major fraction of the proteins were commonly expressed (Appendix IIa & IIb). In contrast to the above two, the number of spots identified in GG20 were almost similar i.e. 227 in control and 216 in infected leaves, and out of them commonly expressed proteins number was about 44. In the most susceptible genotype, i.e. TG37A, 383 spots were identified under healthy condition which reduced to 268 under the fungal infection. About 94 spots matched between the gel in TG37A leaves suggested that a good number of proteins were commonly expressed under the above both conditions.

Table 4.4.11: Effect of *S. rolfsii* infection on leaf proteomes and its differential expression

	Total number spots identified				
Genotypes	**Control**	**Stress**	**Commonly expressed**	**Expressed only in control**	**Expressed only in stress**
C19	412	260	71	341	189
GG16	357	271	131	226	140
GG20	227	216	44	183	172
TG37A	383	268	94	289	174

While studying the proteins which were expressed specific to control or the stress, a significant differences were observed among the genotypes. For instance, number of protein expressed only when the plants were healthy were 341, 226, 183 and 289 in CS19, GG16, GG20 and TG37A respectively. On the contrary, 189, 140, 172 and 174 numbers of proteins were found to be fungal induced in CS19, GG16, GG20 and TG37A respectively. Comparing the above data, it was realised that comparatively a higher number of proteins were induced in CS19 under the fungal stress compared to that of the susceptible genotypes. However, the lowest number of protein induction in GG16, could not be explained by this set of experiment. Comparing the relative fold change (≥2) in protein expression, it was observed that under fungal stress condition the number of up-regulated proteins were highest in GG16 (33), followed by CS19 (25), TG37A (22) and GG20 (11). Similarly there were some proteins which were up-regulated during controlled conditions. Counting the numbers of proteins whose fold change expression were ≥2 under control condition, the highest number was reported by TG37A (31), followed by GG16 (21), CS19 (16) and GG20 (12). Recently, Kumar and Kirti (2015), found up regulation of several proteins associated with photosynthesis, metabolism, secondary metabolism, signal transduction and defence related proteins while investigating the molecular responses of the wild groundnut, *Arachis diogoi* (accession number ICG-8962) when challenged with the fungal pathogen, *Phaeoisariopsis personata*. They have identified 16 protein unique spots with significant expression in wild groundnut where as 12 protein spots in susceptible groundnut cultivar (variety JL 24). The fold changes of differentially expressed proteins upon pathogen challenge were found to be high in case of *A. diogoi* (resistant) in comparison to *A. hypogaea* (susceptible). Hence, it can be hypothesised that those up-regulated proteins identified in the present study may also be corresponding to some defence related proteins, however further a detail investigation may be required to identify those differentially expressed proteins induced during the *S. rolfsii* and groundnut interaction.

4.5 EXPERIMENT V

DIFFERENTIAL EXPRESSION OF DEFENSE RELATED GENE(S) IN TOLERANT AND SUSCEPTIBLE GROUNDNUT GENOTYPES

Pathogenesis-related (PR) proteins are induced in response to pathogen attack. The PR proteins accumulate at the site of infection and are also systemically induced via Systemic Acquired Resistance (SAR) thus reducing further infection (van Loon *et al*., 2006). Most PRs are induced through the signalling compounds salicylic acid, jasmonic acid, or ethylene. Pathogenesis Related (PR) proteins may have antimicrobial activities through cell wall hydrolytic activity, toxicity, and defence signalling. This suggests that they play an important role in defence to pathogen attack (van Loon et al., 2006).

Very few related reports are available regarding the induced gene expression analysis in groundnut under biotic stresses (Prasad *et al.*, 2013, Rohini *et al*, 2001 and Jogi, 2012). However for *S. rolfsii*-groundnut pathosystem, till date no such expression study has been carried out in India and hence there was a need to decipher the nature of PR proteins expression to know their roles in stem rot tolerance in groundnut. In the present experiment, the differential expression of PR protein genes such as *PR2*, *PR4*, *PR5*, and *PR10* were studied in both healthy and infected groundnut genotypes having varying degree of tolerance to *S. rolfsii* such as CS19, GG16, GG20 and TG 37A. The Polygalacturonase Inhibitory Protein (PGIP) is also a type of defense related gene, whose extensive study has been made in many crops (reviewed in Section 2.11.5), but the gene has been remained unidentified in groundnut. Therefore the study aimed to identify and study its differential expression during *S. rolfsii* infection in groundnut

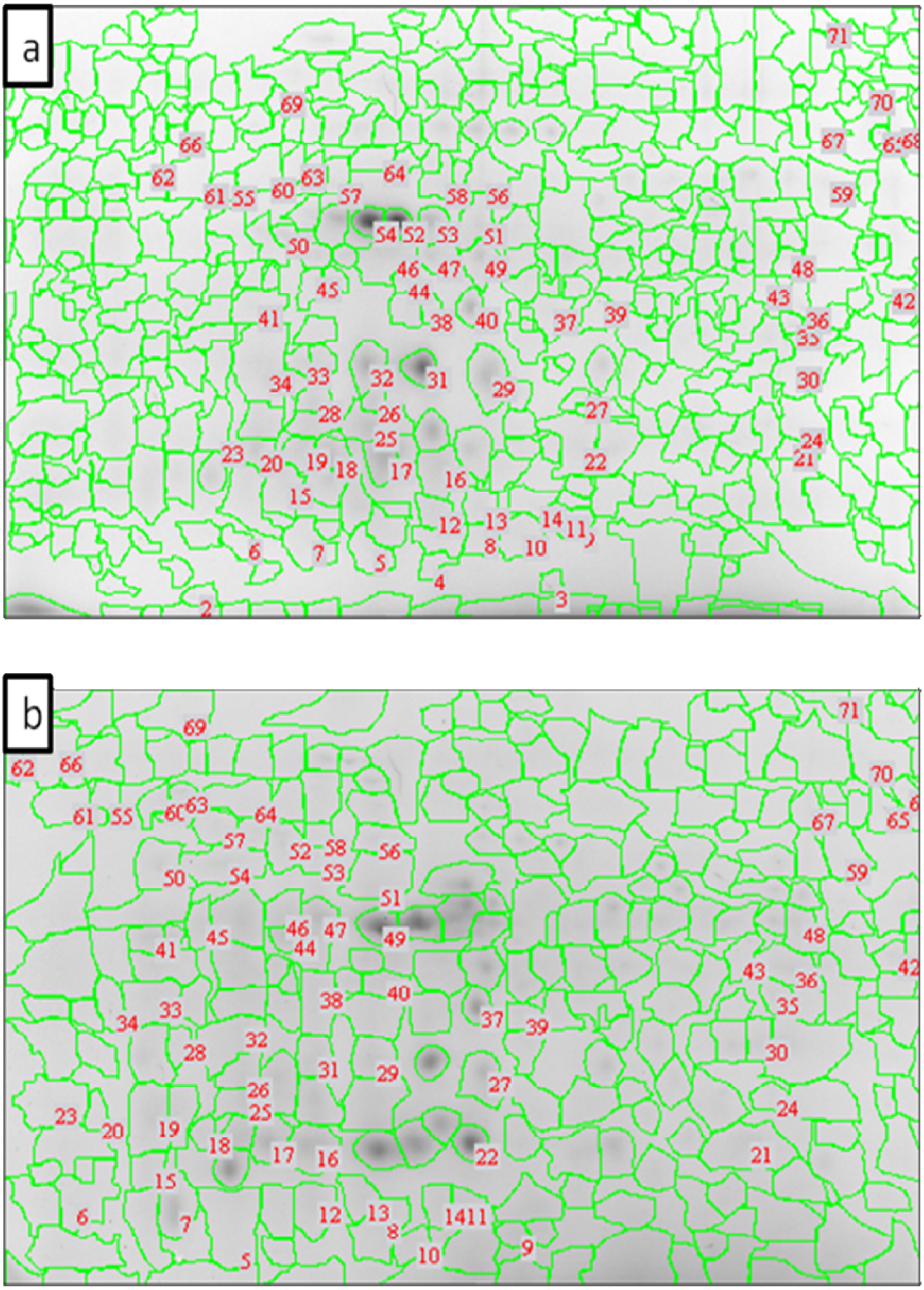

Plate 4.4.1: Leaf proteome of genotype CS19 expressed under control (a) and infected with *S. rolfsii* (b) conditions

Plate 4.4.2: Leaf proteome of genotype GG16 expressed under control (a) and infected with *S. rolfsii* (b) conditions

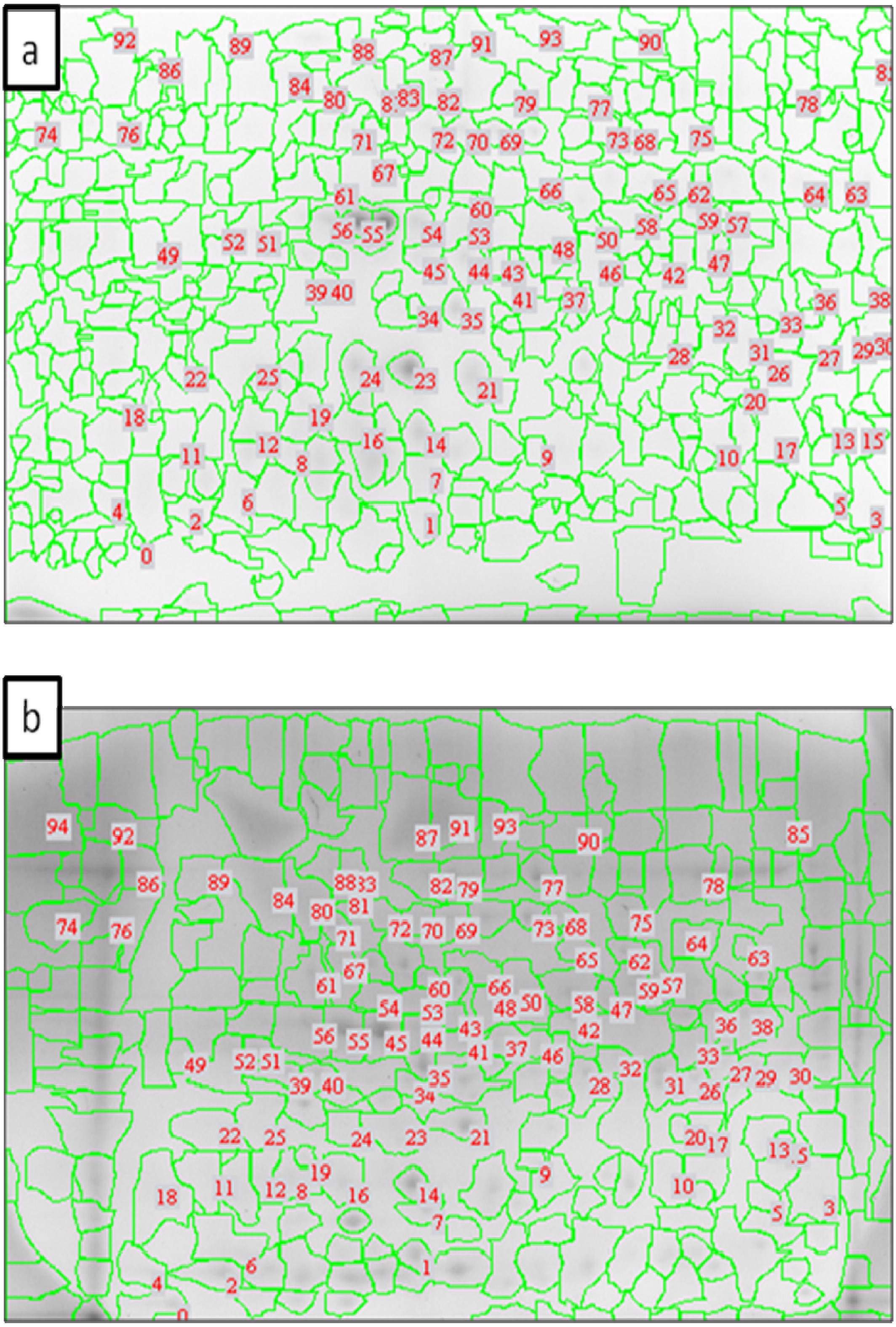

Plate 4.4.3: Leaf proteome of genotype GG20 expressed under control (a) and infected with *S. rolfsii* (b) conditions

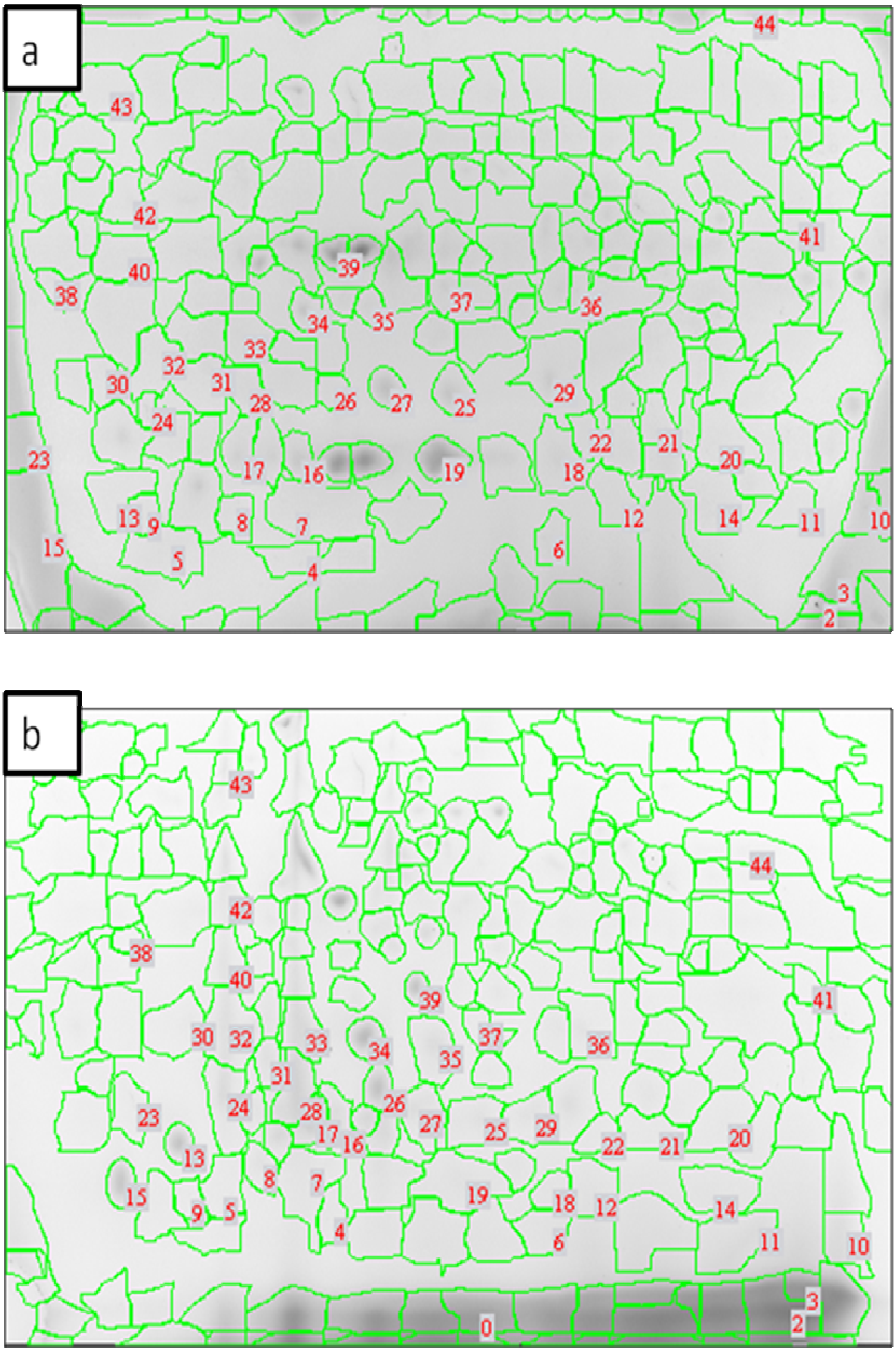

Plate 4.4.4: Leaf proteome of genotype TG37A expressed under control (a) and infected with *S. rolfsii* (b) conditions

The primary objective of this study was to study some PR proteins that are differentially expressed during groundnut *S. rolfsii* interaction using cultivars of varying resistance. The central hypothesis of this study was that the plant genes related to disease resistance would be up-regulated in the most resistant cultivar as compared to the most susceptible cultivar. A second tier hypothesis was that genotypes with intermediate resistance would show intermediate levels of gene expression for resistance.

4.5.1 Relative gene expression analysis of Pathogenesis Related Protein-2 (PR2)

PR2 represents β 1, 3 glucanase, which plays a very important role in disease resistance. Expression analysis of PR2 gene (*PR2*) showed a higher induction in tolerant genotype as compared to susceptible genotypes (Figure 4.5.1). At 24 hours after fungal infection, relatively a stronger induction was observed in tolerant genotype CS19 (1.74 fold) as compared to moderately tolerant genotype GG16 (1.58 fold). As in contrast to the above, almost no induction was observed in the highly susceptible genotype TG37A where as a weaker induction was recorded for susceptible genotype GG20 (1.38 fold).

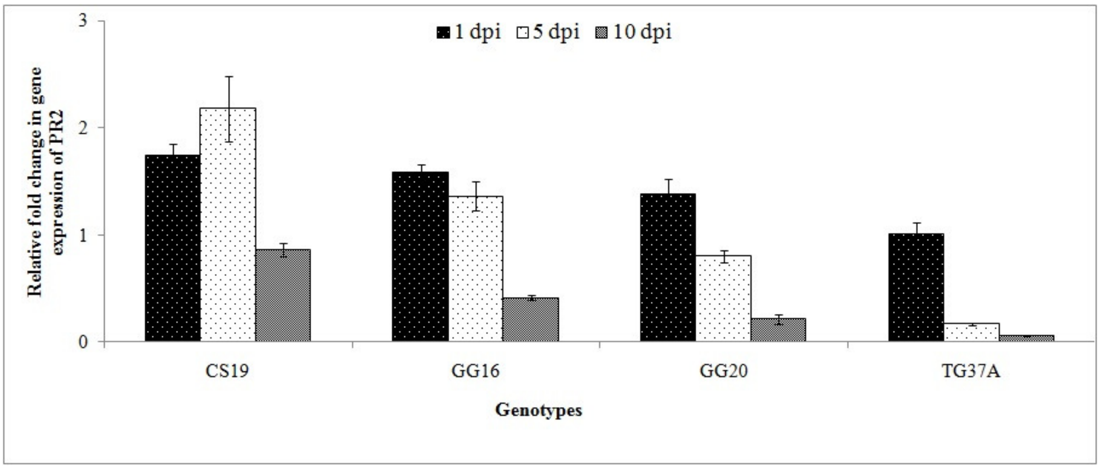

Figure 4.5.1: Relative fold change in PR2 gene expression in groundnut genotypes upon *S. rolfsii* infection at different time intervals

Bar above each column represents SE (Standard Error) for three replications, 1 dpi: 1 day post *S. rolfsii* inoculation; 5 dpi: 5 days post *S. rolfsii* inoculation; 10 dpi: 10 days post *S. rolfsii* inoculation

At 5 dpi, the induction of *PR2* was almost 2 fold in genotype CS19 than the respective control; whereas the induction in genotype GG16 was about 1.36 fold. However, at the same stage no relative induction was noticed in both susceptible genotypes, suggesting that at peak infection stage, the mechanism of tolerance through over expression of PR2 protein did not support. The relatively better and stable expression of *PR2* observed in tolerant genotypes especially in genotype CS19 even at 5 dpi, suggests the possible role of β 1, 3 glucanase in stem rot tolerance in groundnut. As the plants advanced towards severe disease infection stage at 10 dpi, the expression of *PR2* was reduced. For instance in both genotypes TG37A and GG20 the expression was almost nil. In genotype GG16 the induction was below the basal level, however the tolerant genotype CS19 still maintained its expression at basal level. Several studies revealed that the expression levels of these enzymes increased after infection in various crops (Ignatius and Chopra, 1994; Lozovaya, 1998, Jung and Hwang, 2000; Hanselle and Barz, 2001; Zemanek *et al.*, 2002). Our data revealed that in groundnut PR2 protein plays an important role in imparting tolerance to stem rot. It has been proposed that PR2 have direct effect in defending against fungi by hydrolyzing fungal cell walls, which consequently causes the lysis of fungal cells (van Loon et al., 2006). The higher and early induction of *PR2* in tolerant genotypes as compared to the susceptible genotypes possibly explains the tolerance nature of the former.

4.5.2 Relative gene expression analysis of Pathogenesis Related Protein-4 (PR 4)

Chitinases belong to PR4 family, are thought to provide the plants with anti-fungal activity since their substrate, chitin, is an integral component of fungal cell walls. Various studies have shown that chitinase gene expression against phyto-pathogen systems is higher and induction is stronger in the resistant as compared to susceptible varieties (Nielsen *et al.*, 1993; Lawrence *et al.*, 2000; Anguelova *et al.*, 2001).

Our expression data supported the earlier reports of higher induction of chitinase gene in tolerant genotypes as compared to susceptible genotypes upon *S. rolfsii* infection in groundnut. The induction of PR4 gene (*PR4*) in the tolerant genotype CS19 was late, which touched its maximum level (about 18 fold) at 5 dpi and brought down to about 10 fold at 10 dpi, and it was significantly higher than any other genotypes.

The moderately stem rot tolerant genotype GG16, showed an early induction of *PR4* at 1 dpi (about 7 fold), which was substantially decreased to 3.21 fold at 5 dpi and to basal level at 10 dpi. In contrary to the tolerant genotypes, a weak induction was observed in susceptible genotypes. In susceptible genotype GG20 a late induction of *PR4* at 10 dpi (2 fold) was observed where as 3 fold rise was recorded for the genotype TG37A at 5dpi.

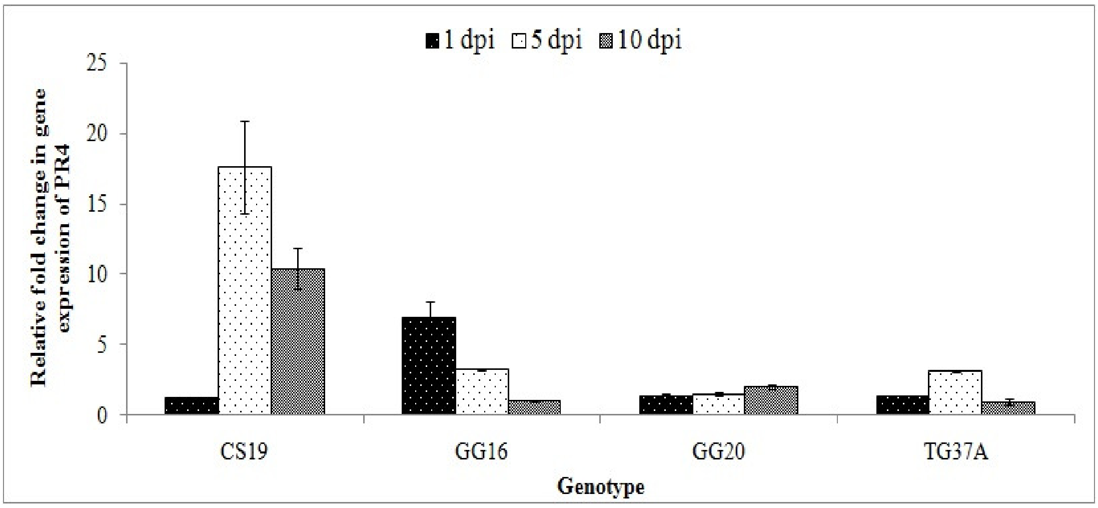

Figure 4.5.2: Relative fold change in PR4 gene expression in groundnut genotypes upon *S. rolfsii* infection at different time intervals

Bar above each column represents SE (Standard Error) for three replications, 1 dpi: 1 day post *S. rolfsii* inoculation; 5 dpi: 5 days post *S. rolfsii* inoculation; 10 dpi: 10 days post *S. rolfsii* inoculation

The present findings were supported by the observations made by Jogi (2012), who reported that two chitinases *PR*-3 (contig02763) and *PR*-4 (contig03022) were found to be up-regulated in the resistant groundnut cultivar as compared to susceptible in response to *S. rolfsii.*

4.5.3 Relative gene expression analysis of Pathogenesis Related Protein-5 (PR5)

Among the Pathogenesis Related Proteins, PR5 family has diverse functions in plant disease resistance such as antifungal activity, antifreezing activity and osmotic stress tolerance. Differential expression study of PR5 gene (*PR5)* revealed that the induction was comparatively higher in tolerant as compared to susceptible genotypes. The maximum induction of the gene was observed i.e. about 15 fold for the tolerant genotype CS19 followed by 11.83 fold for the moderately tolerant genotype GG16 at 1 dpi. Although the genotype TG37A is susceptible to stem rot, but a significant induction (about 5 fold) was observed even at 1 dpi which on progress of the disease intensity was reduced to the basal level.

No induction of *PR5* was observed for genotype GG20 at any point of time, suggesting a possible reason for its susceptibility. Further, the relatively higher induction of *PR5*gene in genotype TG37A seems to contradict the proposed hypothesis and it was thought that co-expression of PR proteins at required time and in required amount may provide tolerance to pathogens.

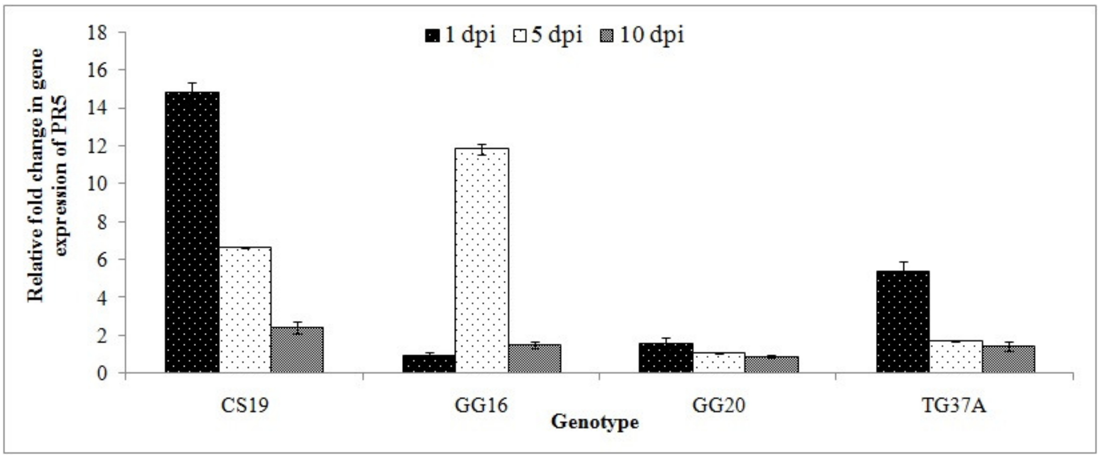

Figure 4.5.3: Relative fold change in PR5 gene expression in groundnut genotypes upon *S. rolfsii* infection at different time intervals

Bar above each column represents SE (Standard Error) for three replications, 1 dpi: 1 day post *S. rolfsii* inoculation; 5 dpi: 5 days post *S. rolfsii* inoculation; 10 dpi: 10 days post *S. rolfsii* inoculation

The present study supported the earlier transgenic studies regarding the involvement of PR5 in fungal disease tolerance (Liu *et al.*, 2003; Ho *et al.*, 2007; Jami *et al.*, 2007; Wang *et al.*, 2011; Zamani *et al.*, 2012; Acharya *et al.*, 2013). Recently, transgenic tobacco plants expressing a Traumatic Like Protein coding gene from *Arachis diogoi* (wild peanut) (AdTLP) showed enhanced resistance to fungal pathogen *Rhizoctonia solani* along with enhance stress resistance in crop plants (Singh *et al.*, 2013).

4.5.4 Relative expression analysis of gene Pathogenesis Related Protein-10 (PR10)

Unlike most other PR proteins with an extracellular destination, PR10 proteins are typically acidic, intracellular, and small. They have RNase and ligand-binding activities which protects plants during programmed cell death around infection sites or act directly on the pathogens. PR10 proteins have been reported to have various functions in both biotic and abiotic stresses (Liu and Ekramoddoullah, 2006) and plant growth and development through modulating the endogenous cytokinin level (Srivastava *et al.*, 2006). Therefore, it is proposed that PR10 proteins may play important roles in plant defense against pathogen attack. The possible relationship of PR10 and host resistant in groundnut was first reported by Luo *et al.* (2005), which showed up-regulation of PR10 gene (*PR10*) in response to *A. parasiticus* attack. The present study has supported the role of PR10 in fungal infection in groundnut.

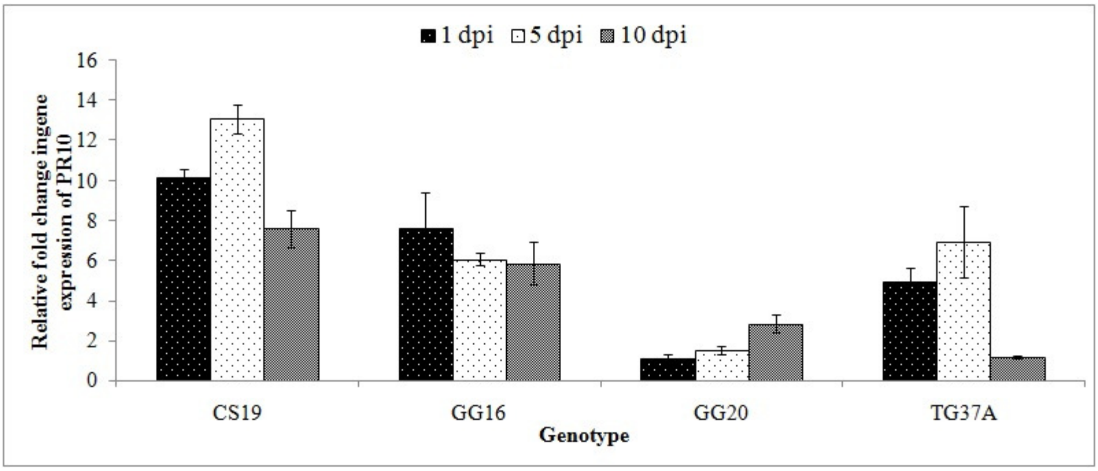

Figure 4.5.4: Relative fold change in PR10 gene expression in groundnut genotypes upon *S. rolfsii* infection at different time intervals

Bar above each column represents SE (Standard Error) for three replications, 1 dpi: 1 day post *S. rolfsii* inoculation; 5 dpi: 5 days post *S. rolfsii* inoculation; 10 dpi: 10 days post *S. rolfsii* inoculation

Upon fungal infection, a rapid, stronger and stable expression of *PR10* was observed in the tolerant genotype CS19 as compared to all other genotypes (Fig 4.5.4) where early induction of about 10 fold increase at 1 dpi in *PR10* expressions was seen which was increased up to 13 fold at 5 dpi suggesting its role against the stem rot tolerance. Similarly, a higher induction of *PR10* (about 8 fold) was reported for the genotype GG16 at 1 dpi which remained stable up to 10 dpi.

A late response towards the pathogen in terms of PR10 induction was seen for GG20, which remained almost at basal level at 5 dpi and reached its maxima at 10 dpi (3 fold). On the contrary, the susceptible TG37A, showed an early induction at 1 dpi (about 5 fold) which raised up to 7 fold at 5 dpi and then dropped down to basal level at 10 dpi. It can be summarized that a higher and stable expression of *PR10* is indeed needed for providing tolerance to groundnut genotypes against *S. rolfsii.* Lower and late induction of *PR10* explained the possible susceptibility of GG20 towards *S. rolfsii*, however the expression pattern showed by TG37A contradict the central hypothesis. Hence it can be hypothesised at this point of time that expression of one or few PR proteins at required amount and in time may provide tolerance to plants against fungal pathogens.

4.5.5 Isolation and differential gene expression study of a novel gene for Polygalacturonase inhibiting protein (*pgip*) in groundnut

To counteract the activity of fungal Polygalacturonases (PGs), plants deploy the cell wall Polygalacturonase Inhibiting Proteins (PGIPs) that inhibit the activity of PGs. Almost all plant species or mutants so far have been characterized reported to have PGIP activities (Kalunke *et al*., 2015). Till date more than 170 complete or partial *pgip* genes from dicot and monocot plants have been deposited in NCBI databases however in groundnut the gene has not yet been identified. Most of these genes have been identified as pgip genes on the basis of sequence identity but only a few of them have been shown to encode proteins with PG-inhibitory activity (Kalunke, *et al*., 2015). Like other families of defense-related genes, *pgip* families show variation in the expression pattern of the different members within a family. To the best of our knowledge, no reports are available for groundnut PGIP protein encoding gene(s) (*pgip*). Realizing the importance of this gene as a candidate for imparting stress tolerance in groundnut, in the experiment attempts were made to isolate a member of *pgip* gene family, and to examine its induced expression at transcript level upon stem rot infection.

4.5.5.1 Isolation of PGIP gene (*pgip*) in groundnut

The gene sequence of *pgip* for groundnut was not available in the NCBI database except for a sequence (gi|300517491|gb|HO115743.1) representing somewhat sequence similarity with PGIP of other crops. Hence primers were designed using the above sequence and used for amplification of *pgip* from cDNA prepared from stem rot infected leaf tissue (described in section 3.10.3.5) of groundnut (Variety GG20). The primer used for the study was as follow with an expected amplicon size of about 600 bp.

Forward Sequence (5’-3’)	GCGCTTCTCCAAATCAAGAAGGAC
Reverse Sequence (5’-3’)	ATGGAAGCGTCACCCTCCAGC

4.5.5.2 RNA isolation and cDNA synthesis

RNA was isolated from both healthy and stem rot infected leaf tissue of groundnut at different intervals (1, 5 and 10 days post infection). After ensuring the good quality of RNA by the observing intact two bright bands (Plate 4.5.1 a) brightness of the 28S band was almost two times higher than that of the 18S band), the RNA was quantified using a Nanodrop Spectrophotometer (ND 1000). Approximately 2 µg of total RNA was used for cDNA synthesis using First strand cDNA synthesis kit (Thermo SCIENTIFIC). Using all cDNA samples as template the reverse transcriptase PCR was carried out using β-actin as primer. The PCR products were run on 2 % (w/v) agarose gel and as expected about 180 bp amplicon was observed in all reactions (Plate 4.5.1 b) confirming the proper synthesis of cDNA.

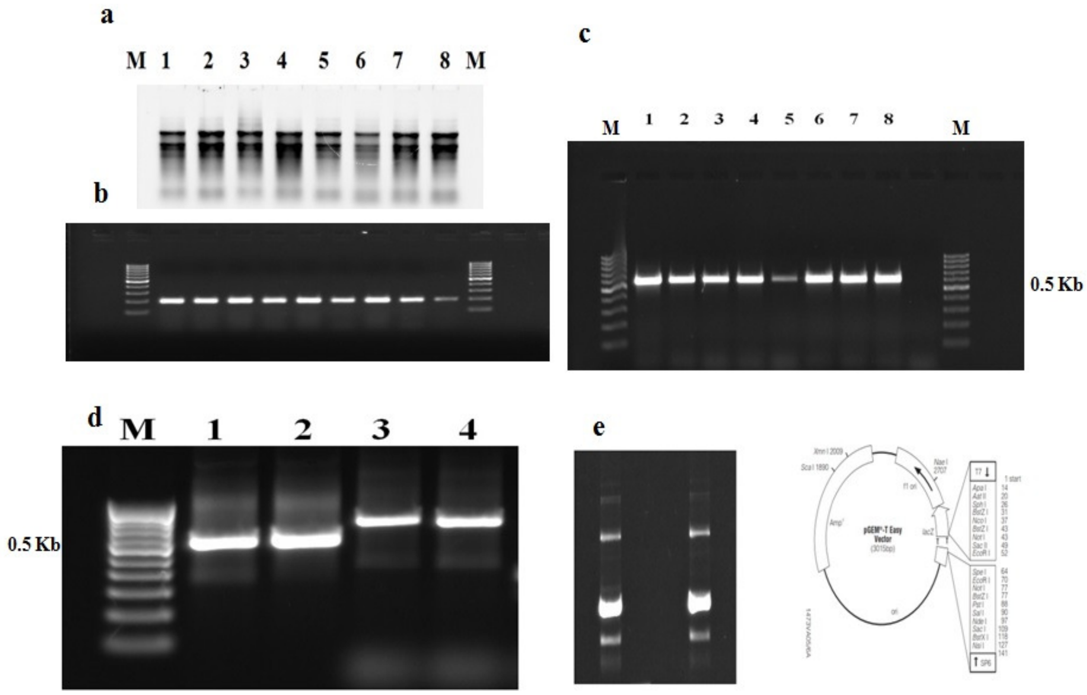

Plate No 4.5.1: Isolation of Polygalacturonase Inhibitory Protein encoding gene (*pgip)*

Quality check of RNA isolated from groundnut leaf tissue (a); cDNA checking using actin as a primer (b); Amplification of *pgip* (~600 bp) checked in 1% agarose gel (c); PCR confirmation of *pgip* transformation using gene specific (1 & 2) and vector specific (3 & 4) primers (d); and Isolation of transformed plasmid (e)

Note: 1, 3, 5 & 7 correspond to healthy CS 19, GG16, GG20 & TG37A respectively, where as 2, 4, 6 & 8 correspond to infected genotypes in the same order. M refers to 100bp gene ruler

4.5.5.3 Amplification of putative *pgip*

PCR amplification was made using the above cDNAs as template, following the protocol as described in the section 3.10.3.7. As expected an amplicon of about 600 bp size was observed in agarose gel for all the templates used corresponding to both normal and infected samples of all genotypes (Plate 4.5.1 c). The desired amplified fragment from agarose gel was rescued by using Qiagen gel elution column following the manufacturer's guidelines and cloned in pGMET-Easy vector system and transformed in DH5α competent cells following the methods as described in the section head 3.10.3.11. The selection of transformed colonies were initially selected through blue white screening method and was further reconfirmed by colony PCR using gene specific and vector specific primers (T_7 and SP_6). As expected an amplicon of 600bp size was observed for gene specific primers whereas about 800bp was noticed for vector specific amplification, confirming the transformation of PGIP gene (Plate 4.5.1 d). Now plasmids having the gene insert were isolated from the confirmed transformed colonies (Plate 4.5.1 d) and the quality and quantity (154 ng/µl) was checked in naanodrop (ND 1000) (Plate 4.5.1 e). The required amount of plasmid DNA (about 3000 ng) was dried in a centrifugal evaporator (Labmate, DyNA Vap) and sent for Sanger sequencing at SciGenom, Cochin, India.

4.5.5.4 Sequencing and BLAST analysis

Using gene specific primers sequencing was made. Homology search of the DNA sequences was performed against different DNA related databases (NCBI) using BLASTN programs. The BLASTN results showed the sequence similarity to many *pgip* in both related and unrelated crops, confirming the sequence to be a partial gene encoding for PGIP. The DNA sequence was submitted to the NCBI database (Accession no. KP844637, Annexure III)).

4.5.5.5 Differential gene expression of *pgip* upon *S. rolfsii* infection

To confirm its possible role in fungal infection groundnut, gene expression study was carried out using gene specific real time PCR. The primer sequence used for the study was as follow,

Forward (5’-3’): TCAACCTCACCAACCTCAAAG
Reverse (5’-3’): CTGTAAGTCGAGGGATTGGAG

Higher induction of *pgip* in leaf tissue of tolerant compared to susceptible genotypes was observed. In susceptible genotype, TG 37A, there was no induction of *pgip* throughout the period of infection and the expression was nil at 10 dpi. In GG20, though an early induction (2.6 fold) of *pgip* was observed at 1dpi, and then after the expression reduced to basal level at the following stages. A nil or transient expression of *pgip* explained the incapability of TG37A and GG20 to restrict the entry of *S. rolfsii.*

On the contrary higher expression of the gene was observed in both tolerant genotypes. Though a late induction of *pgip* was observed for CS19 at 5 dpi (~ 2 fold), the maximum induction was seen at 10 dpi (5.56 fold). On the other hand, GG16 showed a different pattern of expression where an early induction of 3.30 fold was seen which at 5 dpi brought down to 1.4 fold and then the induction was raised up to about 5 fold. The results of the present study suggest the possible role of PGIP plays in stem rot tolerance in groundnut. The induction of *pgip* expression upon *S. rolfsii*, was supported by the earlier findings of Ahsan, *et al.* (2005) which suggest that its expression can be up-regulated in response to various biotic and abiotic stresses such as pathogen infection, mechanical wounding, cold, salinization, over-watering and jasmonic acid stimuli.

Like other families of defense-related genes, *pgip* families show variation in the expression pattern of the different members, some of which are constitutive, others are tissue-specific and, in most cases, up-regulated following stress stimuli (Kalunke, *et al.*, 2015). In this study also, genotypic and stage specific differential expression pattern of *pgip* was observed (Figure 4.5.5).

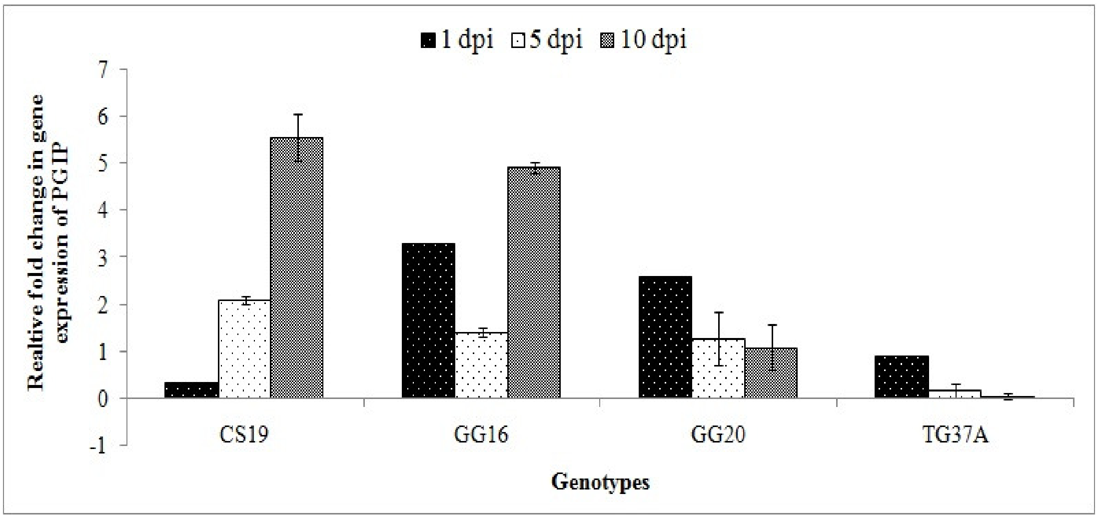

Figure 4.5.5: Relative fold change in *pgip* expression in groundnut genotypes upon *S. rolfsii* infection at different time intervals.

Bar above each column represents SE (Standard Error) for three replications, 1 dpi: 1 day post *S. rolfsii* inoculation; 5 dpi: 5 days post *S. rolfsii* inoculation; 10 dpi: 10 days post *S. rolfsii* inoculation

The tolerant genotype CS19 possibly having a tough and thick cell wall barrier, might have needed the later expression of the gene, hence a late induction of *pgip* was observed. On the contrary, the same hypothesis can be a possible explanation of early induction of the gene in case of GG20. GG16 being a moderately tolerant genotype, showed a different but intermediate expression pattern of *pgip*. The incapability of TG37A to induce the expression of *pgip* at any point of time confirmed its highly susceptible nature towards the pathogen. Hence it can be concluded by putting a hypothesis that consistently higher induction of *pgip* in tolerant genotypes at the time of *S. rolfsii* infection compared to susceptible genotypes, suggest a vital role of the gene expression in groundnut to stem rot tolerance. Further till date a number of complete or partial *pgip* genes from dicot and monocot plants have been deposited in NCBI databases, most of them have been identified as *pgip* genes on the basis of sequence identity but only a few of them have been shown to encode proteins with PG-inhibitory activity (Kalunke, *et al.*, 2015). In the present experiment, the identified *pgip* gene sequence was partial and its NCBI submission was purely based on its sequence similarity to related *pgip* gene in other crops. Hence in future, efforts should be made to identify and study the complete sequence of the gene, its functional validations and characterization of its up/down stream sequences, so that it can be used as a candidate gene to enhance fungal stress tolerance in groundnut and related crops.

It has been reported earlier that PGIPs belong to the large superfamily of proteins having different specificity towards different stimuli. For instance under stress conditions, different expression patterns can be revealed for *pgip* belonging to the same family (Ferrari, *et al.*, 2003). For example, in soybean, GmPGIP1 and GmPGIP3 are up-regulated following wounding and infection caused by *Sclerotinia sclerotiorum*. On the contrary, GmPGIP2 is not induced by wounding and is only up-regulated following the same pathogenic attack (D'Ovidio, *et al.*, 2004). Hence, in the above line of work, a more extensive research on *pgip* is needed in future to decipher in more details about the functions and specificities of the members of *pgip* gene family, if any in groundnut.

4.6 GENERAL DISCUSSION

Groundnut (*Arachis hypogaea* L.), is a important legume crop grown in over 100 countries. India ranks second in production, is one among the leading producers of groundnut worldwide. *Sclerotium rolfsii* is a major soil borne necrotrophic fungal pathogen causing stem rot in many crops, which employs a very complex but successful mechanism of pathogenesis, hence it has a host range of over 500 species of plants (Punja *et al.,* 1985). Till date, no or very little studies have been made on the host-pathogen interaction for this pathogen, especially in groundnut.

Management of *S. rolfsii* through chemical methods leads to ill effects like residual toxicity, environmental pollution and fungicide resistance. Presently no immune or absolute stem rot resistant groundnut genotypes are available. The only and the best option left with the growers of this crop is use of biocontrol agent against the pathogen. Biological control using antagonistic fluorescent *Pseudomonas* appears to be a potential management tool for reducing the severity of the disease (Ganesan and Gnanamanickam 1987; Tonelli *et al.,* 2010); however there is a need for identifying isolates specifically effective against S. rolfsii, for a planned regional deployment. Many researchers proved that some selected *Pseudomonas* strains can restrict in vitro hyphal growth or reduce germination of sclerotia of *S. rolfsii* (Ganesan and Gnanamanickam 1987; Kishore *et al.,* 2005; Ganesan *et al.,* 2007; and Tonelli *et al*., 2010). The importance of the P. aeruginosa as a potential biocontrol agent against the stem rot pathogen was earlier proved by Kishore *et al*., (2005) in groundnut. We too have isolated a bacterium belonging to the species Pseudomonas aeruginosa which was capable of inhibiting the growth of S. rolfsii in vitro.

Several methods of disease suppression have been implicated in biological control by *Pseudomonas* spp. The most frequently cited of them are competition, iron limitation through siderophore production, antibiosis, and induced systemic resistance (Haas and Defago, 2005). Cell wall degrading enzymes such as β-1, 3-glucanase, chitinase, and proteases secreted by strains of *Pseudomonas* inhibit the hyphal growth of fungal pathogens by affecting the structural integrity of the cell walls (Budi *et al*., 2000). The antagonistic potential of *P. fluorescens* against various soilborne plant pathogens has been correlated with production of lytic enzymes (Haas and Defago, 2005). We also observed a positive correlation between the antagonistic potential of the isolates and their ability to produce lytic enzymes. The most potential antagonist isolate i.e. *SKPf* 5 reported to have significantly higher amount of lytic enzymes and other antifungal metabolites in vitro.

In general plants defend themselves against pathogens by two ways: structural or morphological characteristics that act as physical barriers and biochemical reactions that take place in cells and tissues that are either toxic to the pathogen or create conditions that inhibit the growth of the pathogen in the plant. The structural defense mechanisms may be pre-existing, which exist in the plant even before the pathogen comes in contact with the plant or induced, i.e. even after the pathogen has penetrated the preformed defense structures, one or more type of structures are formed to protect the plant from further pathogen invasion.

The pre-existing structural defense structures include the amount and quality of wax and cuticle that cover the epidermal cells and the size, location and shapes of natural openings (stomata and lenticels) and presence of thick walled cells in the tissues of the plant that hinder the advance of the pathogen. According a report (DAR, 2010), the tolerant genotype CS19 possess solid trichomes, wax deposits and compact arrangement of vascular cells where as the susceptible variety (GG20) lacks the same, suggesting the possible reason for heavy incidence of stem rot in the later.

The post-inflectional structural defense mechanisms/Induced structural barriers may be regarded as histological defense barriers (cork layer, abscission layers and tyloses) and cellular defense structures (hyphal sheathing). A common defense mechanism in xylem vessels against vascular wilt pathogens is the formation of tyloses (Fradin and Thomma, 2006). Tyloses are the overgrowths of the protoplast of adjacent living parenchymatous cells, which protrude into xylem vessels through pits. Tyloses have cellulosic walls and are formed quickly ahead of the pathogen and may clog the xylem vessels completely blocking the further advance of the pathogen in resistant varieties. In susceptible varieties, few or no tyloses are formed ahead of pathogen invasion (Grimault *et al.*, 1994; Agrios, 2005; Fradin and Thomma, 2006). Recently observations made by Yadeta and Thomma (2013) suggest that these are formed during both compatible and incompatible interactions between the host and vascular wilt pathogens, although the time and extent of tylose formation significantly differs. We observed the typical formation of tyloses in tolerant genotype CS19 blocking the vascular tissues at 5 days after infection, which were absent in other genotypes. This typical response of genotype CS19 could be the reason for affording tolerance by preventing the easy spreading of the stem rot pathogen. Further, the lack of tylose formation in proper time in the susceptible (TG 37A) or comparatively less tolerant genotypes (GG16 and GG20) could be the cause for their susceptibility to the pathogen.

Once the pathogen crosses the physical barrier of the host, it has to overcome the biochemical barriers which remain either pre-induced or get induced rapidly after the pathogen recognition to inhibit the further development of the attacking pathogen. The rapid production and accumulation of reactive oxygen species (ROS), particularly the superoxide anion (O^{2-}) and hydrogen peroxide (H_2O_2) (Heller and Tudzynski, 2011), culminates with a hypersensitive response (HR) and localized programmed cell death (PCD) to impair the pathogen establishment and development inside the host tissues and deprive the pathogen of further access to nutrients. Plants possess a number of antioxidant enzymes that eliminate ROS. Superoxide dismutase (SOD) catalyzes the conversion of the superoxide free radical (O^{2-}) to molecular oxygen and H_2O_2 which is subsequently is eliminated by catalase (CAT), ascorbate peroxidase (APX), peroxidase (POX) and other scavenging enzymes, leading to oxidative stress tolerance and pathogen resistance. Concomitant with or following HR, several pathogen defense-related genes are translated into anti-oxidative enzymes and PR proteins that confer resistance to the plant in defense against pathogen attack. The necrotropic pathogens normally launch the plant into cell-death program, thereby short circuiting a strong defense response and providing a ready source of pathogen nourishment (van Loon *et al*., 2006). The *S. rolfsii* being a necrotroph, must be preferring to high load of ROS. Hence it was hypothesised that the induction of antioxidative machinery and PR proteins would be rapid and stronger in tolerant genotypes as compared to the susceptible to nullify the toxic effect of ROS, and to prevent the spread of the pathogen.

Our study revealed that there was a variable response of genotypes to fungal infection in terms of intensity and time of induction of the antioxidative enzymes. In general a rapid, early and stronger induction was observed for tolerant genotypes as compared to the susceptible ones. With the progress of the disease, the activity of anti-oxidative enzymes increased up to certain time, then after decreased. It was presumed that the plants tried to detoxify the ROS load to a threshold level and then it stopped or reduced. The antioxidative machinery being stronger continued to detoxify the ROS load for a longer time, hence the intensity of the disease was relatively less in the tolerant genotypes.

Infection by fungal necrotrophs is a complex process that includes conidial attachment, germination, host penetration, lesion formation and expansion, and tissue maceration followed by sporulation. Penetration may be achieved by degrading the external cuticle through the action of cutinases and lipases. Once penetrated the cuticle, necrotrophs have a spatial and temporal strategy of attacking the plant cell wall by producing several cell wall degrading enzymes (CWDEs) belonging to multiple families (Bellincampi *et al*., 2014). Polygalacturonases (PGs) are pathogenicity factors produced at the earlier stages of a microbial infection that depolymerize the homogalacturonan (HG), i.e., the main component of pectin in dicots but also present in monocots.To counteract the activity of PGs, plants deploy the cell wall polygalacturonase inhibiting proteins (PGIPs) that inhibit the activity of PGs. PGIPs are glycoproteins with molecular masses ranging from about 37 to 54 kDa (Di Matteo, *et al*., 2006), known to be secreted in the extracellular space, being devoid of the classical intracellular and transmembrane domains (Protsenko, *et al*., 2008). In addition to PG inhibition, the interaction between PGs and PGIPs promotes the formation of oligogalacturonides (OGs), which are elicitors of a variety of defense responses (Ferrari *et al*., 2013).

PGIPs expression can be up-regulated in response to various biotic and abiotic stresses such as pathogen infection, mechanical wounding, cold, salinization, over-watering and jasmonic acid (Ahsan, *et al.*, 2005). A number of plant species or mutants have been studied for PGIP activities. Like other families of defense-related genes, pgip families show variation in the expression pattern of the different members, some of which are constitutive, others are tissue-specific and, in most cases, up-regulated following stress stimuli (Kalunke *et al.*, 2015). We have identified a putative PGIP gene for the first time in groundnut which was also shown to be induced by fungal infection. The differential expression of the gene was evident from its higher and stable induction in tolerant genotype CS19 as compared to the susceptible genotype TG37A where almost no induction was observed.

Strains of fluorescent *Pseudomonas* spp. and *Bacillus* spp. stimulate plant growth by different traits like nitrogen fixation, phosphate solubilization, production of organic acids and IAA (Indole Acetic Acid) (Pal *et al.*, 2001) are called growth promoting rhizobacteria (PGPR). The intrinsic ability of many strains of fluorescent Pseudomonas spp. to produce IAA was observed by many researchers (Glick, 1995; Caron *et al.*, 1995, Mazumdar *et al.,* 2007). In groundnut, isolates of *P. fluorescens* were shown to produce IAA and enhance the plant growth (Pal et al., 1999). We observed the application of a P. aeruginosa isolate (*SKPf 5*) having the potential to produce IAA in vitro, reduced significantly the stem rot disease incidence in groundnut. The plant growth promoting and disease reducing abilities of P. aeruginosa were earlier reported by Karnwal (2009). Hence it was assumed that the fact of disease reduction by *Pseudomonas spp* may also be mediated through growth promoting activity because a healthy plant can better manage the stresses.

The induced resistance by fluorescent *Pseudomonas* spp has been reported by many researchers (Gupta *et al.*, 2006) which is mediated through Salicylic acid (SA). SA is a phenolic compound that affects a variety of biochemical and molecular events associated with induction of disease resistance. For instance, the ability of Pseudomonas aeruginosa (strain 7NSK2) to elicit ISR against *Botrytis cinerea* in bean was linked to SA production (De Meyer and Hofte, 1997). Further, a strain of *P. aeruginosa*, exhibited strong antagonistic activity in vitro and in vivo, when used for bacterization of groundnut seeds result in increased seed germination and reduced the infection of stem rot of groundnut (Gupta *et al.*, 2006). We also observed significant reduction of stem rot disease incidence by bio-priming the groundnut seeds with P. aeruginosa (*SKPf 5*) before sowing and application as root inoculants.

Induced Systemic Resistance (ISR) is a defense response to the local presence and activity of a variety of biotic and abiotic agents by plants. PGPR elicit defensive response by the plant to the pathogen presence. SA has been shown to play an important role in expression of both local resistance controlled by major genes and systemic induced resistance developed after an initial pathogen attack (Ton *et al.*, 2002). During the plant–pathogen interaction, the elicitors release either from the plants or pathogen known to induce the plant defence genes that ultimately lead to broad-spectrum resistance (Ricci, 1997). The defence responses in *Arachis hypogaea* were provoked using fungal components of *Sclerotium rolfsii* as elicitors (Nandini *et al.*, 2010). In groundnut exogenous application of SA has shown to induce significantly higher amount of anti-oxidative enzymes and pathogenesis related proteins (Kobeasy *et al.*, 2011) against the groundnut mottle virus. We also observed the induced resistance as an effect of groundnut seed priming and rhizosphere inoculation with fluorescent *Pseudomonas* isolate (*SKPf 5*) which was evident from the pre-activation of antioxidative enzymes and defense related proteins in treated plants.

Based on the present findings, a hypothesis has been proposed (Fig 4.6.1 and 4.6.2). to explain the basis of innate and induced stem tolerance in groundnut.

- The fungus *S. rolfsii* produces oxalic acid, a non-host specific toxin that synergistically promotes the infection by assisting the cell wall degrading enzymes, especially Polygalacturonase(PG) produced by the pathogen. The higher expression of Polygalacturonase Inhibitory Proteins (PGIPs) in tolerant as compared to the susceptible genotypes provide tolerance by preventing the activity of PG.
- The stronger antioxidant machinery along with the ability of tolerant genotypes to induce higher and stable expression PR proteins at early stage of stem rot infection possibly could be the reason of tolerance observed in tolerant genotypes.
- Use of *Pseudomonas aeruginosa* enhances stem rot tolerance in groundnut by promoting overall plant growth and imparting Induce Systemic Resistance (ISR).

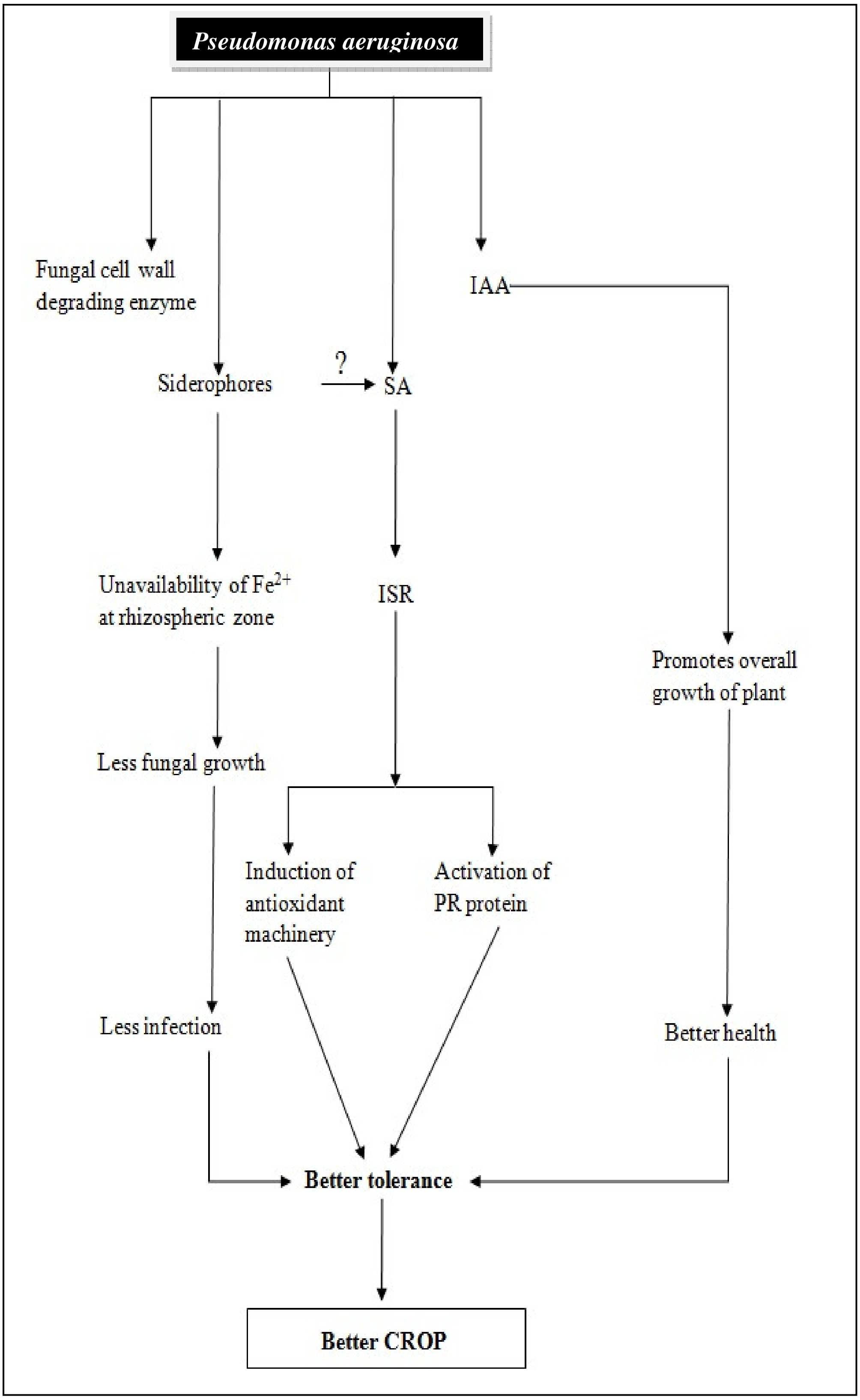

Fig 4.6.1: A hypothetical depiction of the basis of *Pseudomonas* induced stem rot tolerance in groundnut

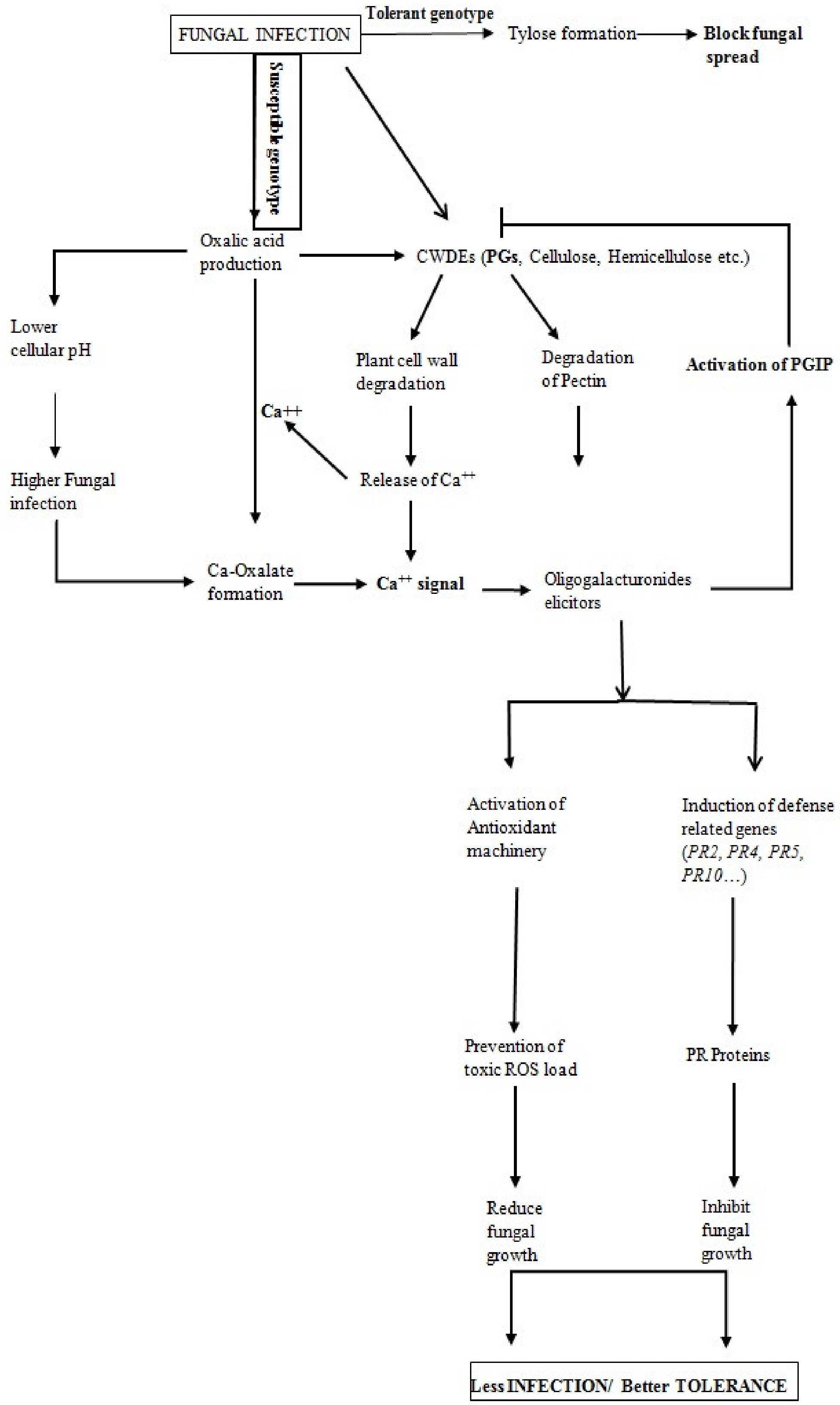

Fig. 4.6.2: A hypothetical depiction of the basis of stem rots tolerance in groundnut

CHAPTER–V

SUMMARY AND CONCLUSIONS

The study was conducted to understand the mechanism of tolerance to stem rot causing pathogen, *S. rolfsii* in groundnut through biochemical, molecular and histological studies. The main aim of the experiment was to decipher the basis of innate immunity or susceptibility of genotypes towards the pathogen. It was also to validate the biocontrol mechanism of fluorescent Pseudomonas species and basis of the induced stem rot tolerance on their application in groundnut genotypes. The study was conducted with following objectives such as, (1) To screen groundnut genotypes for stem rot tolerance under high disease pressure in sick plot, (2) To identify potent antagonistic *Pseudomonas fluorescens* isolates against *Sclerotium rolfsii* from groundnut growing areas of Gujarat, (3) To investigate the histo-pathological basis of *Sclerotium rolfsii* infection in groundnut, (4) To study the induced biochemical changes during host-pathogen and biocontrol agent interactions, (5) To study the differential expression of defense related gene(s) in tolerant and susceptible genotypes. The results of the experiments conducted in the study are summarised as below.

EXERIMENT I:

Evaluations of groundnut genotypes for stem rot tolerance in sick plot

Twenty-two groundnut genotypes were screened for stem rot tolerance in sick plot and the per cent disease incidence in terms of mortality of plants were recorded at harvest. Among the 22 genotypes, only four such as CS19 (tolerant), GG16 (moderately tolerant), GG20 (susceptible) and TG37A (highly susceptible), were selected based on their varying degree of the disease tolerance for further study. The per cent disease incidence was in the range of 19 to 79% corresponding to the genotypes CS19 and TG37A whereas, the genotypes GG16 and TG37A showed about 23% and 63% disease incidence respectively.

EXPERIMENT II:

Isolation and in-vitro antagonism study of antagonistic fluorescent pseudomonad isolates towards *S. rolfsii*

Eleven isolates of fluorescent pseudomonads were isolated from soil of groundnut rhizosphere from 10 different groundnut growing locations of *Saurasthra* region of Gujarat. Molecular identifications of those isolates were made by 16S sequencing, and subsequently the partial 16S sequences were submitted to the NCBI database. Isolates were differing at their species level but all belonged to the large and heterogeneous group of fluorescent Pseudomonads. The isolate *SKPf 5,* identified to be a *Pseudomonas aeruginosa*, was selected as the best antagonist among all. The isolate produced significantly higher amount of lytic enzymes such as chitinases, ß-1,3 glucanase and protease; antifungal metabolites like siderophores, salicylic acid and IAA and also was capable of inhibiting the activity of fungal polygalacturonase and cellulase *in vitro* as compared to other isolates.

EXPERIMENT III:

Histo-pathological basis of S. *rolfsii* infection in groundnut

Two major findings of this experiment could able to differentiate the tolerant and susceptible genotypes. The tolerant genotype CS19 formed tyloses as a distinct response of tolerance to the pathogen attack whereas accumulation of Ca-oxalate crystals like structures in the infected stems of the most susceptible genotype TG37A corresponded to the higher attack of *S. rolfsii* at 5 dpi. Neither of the above two structures were observed in genotypes GG16 and GG20 corresponding to moderately tolerant and susceptible to stem rot of groundnut.

EXPERIMENT IV:

Induced biochemical changes during host pathogen interaction

Infection of *S. rolfsii* induced antioxidative enzymes such as Superoxide Dismutase (SOD), Catalase (CAT), Ascorbate Peroxidase (APX) and Glutathione Reductase (GR) in all genotypes. There was a variable response of genotypes to the intensity and time of induction, but in general the induction was rapid, early and stronger for tolerant genotypes as compared to that of susceptible ones. The activity of those antioxidative enzymes gradually increased from 1 dpi and reached to the peak at 5 dpi; and then steadily reduced towards the later stage of infection. Application of *Pseudomonas aeruginosa* (*SKPf 5*) as seed-priming and root inoculants resulted comparatively higher induction of antioxidative enzyme activity in all genotypes compared to their respective control plants. Induction of the enzymes was higher in *SKPf 5*-treated genotypes when infected with the fungus as compared to the respective non-treated genotypes.

All genotypes resulted a higher induction of Pathogenesis related (PR) proteins such as Peroxidase (POX), Chitinases and ß-1, 3-glucanases as a common response to the fungal infection but there existed a varied genotypic responses. Tolerant genotypes resulted higher and rapid induction of PR proteins at the early stage of infection which gradually increased with the disease intensity and reached the maxima at 5 dpi which followed by a steady reduction in activity towards 10 dpi. Application of *SKPf 5* also induced higher expression of PR proteins in healthy plants. Exposure of the bio-primed plants to the fungal infection, reported lesser induction of PR proteins as compared to the untreated infected plants. It can be summarized that as a common response to fungal infection, all genotypes resulted higher induction of antioxidative enzymes and PR proteins. Possibly as a result of induced systemic resistance by the biocontrol agent, *SKPf 5* treated plants showed lesser incidence of disease and therefore a relatively lesser induction of the above defensive proteins.

As a result of infection, the ascorbic acid content in all genotypes reduced however, the reduction was comparatively higher in susceptible than tolerant genotypes. Plants treated with *SKPf 5* were healthier possibly with pre-induced antioxidative machinery, managed to maintain the ascorbic acid content to a desired amount upon the fungal infection. On the contrary, infected stem tissues accumulated higher amount of oxalic acid as compared to the healthy tissues of the groundnut genotypes. The oxalic acid started accumulating rapidly in the susceptible genotypes even at 1 dpi, whereas it was delayed for the tolerant genotype CS19. With the increasing susceptibility to the pathogen from 1 dpi to 10 dpi, the oxalic acid accumulated gradually at higher rate. Application of *SKPf 5* significantly reduced the accumulation in infected plants, which was correlated with lesser disease incidence.

Fungal infection in both tolerant and susceptible genotypes altered the protein expression and the number of stress induced proteins was more in tolerant genotypes as compared to the susceptible. The number of stress induced proteins was highest in genotype GG16, followed by the genotypes such as CS19, TG37A and GG20 respectively. Some proteins expressed during controlled conditions were either non expressed or less expressed under stress conditions or the *vice-versa*. As the protein expression is modulated by a number of factors, hence giving a conclusive remark seems to be difficult.

EXPERIMENT V:

Differential expression of defense related gene(s) in tolerant and susceptible groundnut genotypes

Groundnut genotypes showed differential expression pattern of PR protein genes such as *PR2*, *PR4*, *PR5*, and *PR10* as a response to *S. rolfsii* at different stages of fungal infection. In general, tolerant genotypes had an instant and stronger expression of PR protein genes while susceptible ones reported comparatively either weaker or late induction. Tolerant genotype CS19 had an early induction of *PR2* (1.74 fold) at 1dpi, and at the same point of time moderately tolerant genotype GG16 reported relatively less induction (1.58 fold). In contrast to the tolerant genotypes, TG37A did not have induction of *PR2* at any point of time. Similarly for *PR4* expression, CS19 had late (5 dpi), but quite stronger induction (~20 folds) compared to all other genotypes and the expression remained stable even at the later stage of infection (10 dpi). The moderately tolerant genotype (GG16), showed an early induction of *PR4* expression at 1 dpi (about 7 fold), which was substantially decreased to 3.21 fold at 5 dpi and to basal level at 10 dpi. In contrary to the tolerant genotypes, a weaker or no induction was observed for susceptible genotypes. The genotype CS19 reported the maximum induction of the *PR5* gene (15 fold) followed by for genotype GG16 (11.83 fold) at 1 dpi. Although TG37A is susceptible to stem rot, but upon fungal infection at 1 dpi, resulted about 5 fold increased *PR5* expression. However, the genotype GG20 could not able to show the expression of *PR5* at any stage of infection.

As response to the *S. rolfsii* infection, the induction of *PR10* (gene for a ribonuclease like protein) was reported for both susceptible and tolerant genotypes. Both the genotypes CS19 and GG16 had quicker, stronger and stable expression of *PR10* as compared to delayed and weaker induction for genotype GG20, and remained almost at basal level up to 5 dpi. On the contrary, the susceptible genotype TG37A, showed an early induction at 1 dpi (about 5 fold) which increased up to 7 fold at 5 dpi and dropped down to basal level at 10 dpi.

A partial putative PGIP protein encoding gene (*pgip*) was reported for the first time in groundnut based on the sequence similarity to many PGIP genes reported in both related and unrelated crops. The DNA sequence was submitted to the NCBI database (Accession no. *KP844637*). Tolerant genotypes reported higher expression of *pgip* as compared to susceptible genotypes as a response to *S. rolfsii* infection suggested its possible role in stem rot stress tolerance in groundnut. However, the susceptible genotype TG37A did not have any induction of *pgip* throughout the period of infection whereas CS19 marked as high as 5.56 fold higher expression as compared to the respective control at 10 dpi. Genotype GG20, had an early induction (2.6 fold) of *pgip* at 1dpi, and then after the expression reduced to basal level at the following stages. The genotype GG16 showed an early induction of 3.30 fold at 5 dpi which rose up to about 5 fold.

CONCLUSIONS

The sole aim of the present study was to decipher the biochemical, molecular and histopathological basis of innate and fluorescent Pseudomonas induced stem rot tolerance in groundnut. Four groundnut genotypes were screened out based on their varying degree of tolerance to the stem rot pathogen, *S. rolfsii*. A Pseudomonas isolate (*SKPf 5*) identified as *Pseudomonas aeruginosa* through 16S sequencing was selected out of eleven isolates to use as a treatment based on its superior *in vitro* antagonistic potential. Biochemical analysis reported genotypic and stage specific differential expression of antioxidative enzymes, PR proteins, and other stress related metabolites accumulation in *S. rolfsii* infected plants. Bio-priming of groundnut seeds before sowing and subsequent rhizosperic inoculation of *SKPf 5* showed plant growth enhancing effect and hence resulted in establishing a stronger pre-induce antioxidant and defence machinery. The biocontrol agent supposed to activate the Induce Systemic Resistance (ISR) in the treated plants. Electron Microscopy study depicted the presence of tyloses only in genotype CS19 and accumulation of Ca- oxalate in genotype (TG37A), represented distinct responses of the tolerant and susceptible genotypes respectively to fungal infection. Differential gene expression analysis showed comparatively a stronger, earlier and stable expression of pathogenesis related proteins in tolerant genotypes as compared to the susceptible ones. A putative partial gene sequence for Polygalacturonase Inhibitory Protein (PGIP) was reported for the first time in groundnut. The relatively higher induction of the gene in tolerant compared to the susceptible genotypes as a response to *S. rolfsii* infection, suggested its possible role in imparting stem rot tolerance in groundnut.

FUTURE PERSPECTIVES

Our study has generated some basic information but a lot more are needed to completely understand the beneath mechanism of *S. rolfsii* infection in crop plants. Large number of Plant Growth Promoting rhizobacteria (PGPR) associated with groundnut rhizosphere needs be screened to identify superior fungal antagonistic biocontrol agents. Besides this, a stringent screening of the groundnut germplasm is required to identify resistant groundnut genotypes for stem rot tolerance which can be used as breeding materials. As reported here, a partial gene of PGIP in groundnut has been identified. Hence in future attempts should be made to isolate and functionally validate the complete PGIP gene (*pgip*) with its upstream and downstream regulatory sequences to completely understand its role in stem rot tolerance. It is quite possible that there may exist a *pgip* gene family in groundnut, hence a more detailed investigation is required to identify and characterise each members of the gene family, which can be used for future molecular breeding programmes to develop groundnut genotypes with better tolerance to stem rot.

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APPENDIX I

NCBI SUBMISSION OF *SKPf1*

Locus	KR422298 898 bp DNA linear BCT 07-MAY-2015
Definition	***Pseudomonas putida*** strain ***SKPf1*** 16S ribosomal RNA gene, partial sequence
Accession	KR422298
Version	KR422298
Source	***Pseudomonas putida***
Organism	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas.
Reference	(bases 1 to 898)
Authors	Bishi,S.K., Vakharia,D.N. and Golakiya,B.A.
Title	*In-vitro* characterization of fluorescent pseudomonad isolates from groundnut rhizosphere against stem rot pathogen (*Sclerotium rolfsii*)
Journal	Unpublished
Journal	Submitted (02-MAY-2015) Biochemistry, Junagadh Agricultural University, Directorate of Groundnut Research, Ivnagar Road, Pb.05, Junagadh, Gujarat 362001, India
Comment	Sequences were screened for chimeras by the submitter using DECIPHER. #Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##
Features Source	Location/Qualifiers 1..898, /organism="Pseudomonas putida"/ mol _type="genomicDNA" /strain="SKPf1"/ isolation_ source="stem rot-infected peanut rhizosphere" /db_xref="taxon:303 "/country= "India" /collection_date="20-Aug-2013"rRNA <1..>898 /product="16S ribosomal RNA"
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NCBI SUBMISSION OF *SKPf2*

Locus	KR422299 1386 bp DNA linear BCT 07-MAY-2015
Definition	***Pseudomonas monteilii*** strain ***SKPf2*** 16S ribosomal RNA gene, partial sequence.
Accession	KR422299
Version	KR422299
Source	***Pseudomonas monteilii***
Organism	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas.
Reference	(bases 1 to 1386)
Authors	Bishi,S.K., Vakharia,D.N. and Golakiya,B.A.
Title	Direct Submission
Journal	Submitted (02-MAY-2015) Biochemistry, Junagadh Agricultural University, Directorate of Groundnut Research, Ivnagar road, PB.05, Junagadh, Gujarat 362001, India
Journal	unpublished
Comment	Sequences were screened for chimeras by the submitter using DECIPHER ##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##
Features Source	Location/Qualifiers 1..1386 /organism="Pseudomonas monteilii" /mol_ type=" genomic DNA" /strain="SKPf2" / isolation_source="stem rot-infected peanut rhizosphere"/db_xref="taxon:76759"/country="India" /collection_date="20-Aug-2013" rRNA <1..>1386 /product="16S ribosomal RNA"
Origin	AGTCGAGCGGATGACGGGAGCTTGCTCCTTGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGC CTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGA CCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAATGGCTCACCAAG GCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTAC GGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAA GGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGT TACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAAT CGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTTGGATGTGAAAGCCCCGGGCTCAACCT GGGAACTGCATCCAAAACTGGCGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAA ATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCG AAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTT GGAATCCTTGAGATTTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGT TAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGA AGAACCTTACCAGGCCTTGACATGCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCTGACA CAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCC TTGTCCTTAGTTACCAGCACGTTATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGT GGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAGAG GGTTGCCAAGCCGCGAGGTGGAGCTAATCTCACAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCG ACTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTGT ACACACCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAGCTAGTCTAACC

NCBI SUBMISSION OF *SKPf3*

Locus	KP859615 731 bp DNA linear BCT 23-APR-2015
Definition	*Pseudomonas monteilii* strain ***SKPf3*** 16S ribosomal RNA gene, partial sequence.
Accession	KP859615
Version	KP859615
Source	***Pseudomonas monteilii***
Organism	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas.
Reference	(bases 1 to 731
Authors	Bishi,S.K., Vakharia,D.N. and Patel,K.G.
Title	*In-vitro* characterization of fluorescent pseudomonad isolates from groundnut rhizosphere against stem rot pathogen (*Sclerotium rolfsii)*
Journal	Unpublished
Journal	Submitted (24-FEB-2015) Biochemistry, Junagadh Agricultural University, Directorate of Groundnut Research, Ivnagar road, PB.05, Junagadh, Gujarat 362001, India
Comment	Sequences were screened for chimeras by the submitter using DECIPHER. ##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##
Features Source	Location/Qualifiers 1..731 /organism="Pseudomonas monteilii"/mol _type="genomic DNA" /strain="SKPf3" / isolation_ source ="stem rot infected peanut rhizosphere" /db_xref="taxon:76759" /country=" India"/ collection _date="28-Aug-2013" rRNA <1..>73 /product="16S ribosomal RNA"
Origin	TCGGCGTGCACCGACCACTAGCCATGCAGTCGAGCGGATGACGGGAGCTTGCTCCTTGATTCAGCGGCGG TAGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGC ATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGC TAGTTGGTGGGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTG GAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTG ATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATGGTAAAGCACTTTAAGTTGGGAGGAAGGGCAG TAAGTTAATACCTTGCTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGC GGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGT TGGATGTGAAAGCCCCGGGGCTCAACCTGGGGAACTGCATCCAAAAACTGGGCAAGCTAGAGTACGGTAG AGGGTGGTGGAATTTTCCCTGTGTAGCGGTGAAATGCGTAGGATATGAGGAAGGAACACCAGTGGCCGAA AGGCGACCACCTGTACTGATACTGACCCTTG

NCBI SUBMISSION OF *SKPf4*

Locus	KR 492887 1320 bp DNA linear BCT 07-MAY-2015
Definition	***Pseudomonas putida*** strain ***SKPf4*** 16S ribosomal RNA gene, partial sequence
Accession	KR492887
Version	KR492887
Source	***Pseudomonas putida***
Organism	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas.
Reference	(bases 1 to 1320)
Authors	Bishi,S.K., Vakharia,D.N. and Golakiya,B.A.
Title	*In-vitro* characterization of fluorescent pseudomonad isolates from groundnut rhizosphere against stem rot pathogen (*Sclerotium rolfsii*)
Journal	Unpublished
Journal	Submitted (05-MAY-2015) Biochemistry, Junagadh Agricultural University, Directorate of Groundnut Research, Ivnagar road, PB.05, Junagadh, Gujarat 362001, India
Comment	Sequences were screened for chimeras by the submitter using DECIPHER ##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##
Features Source	Location/Qualifiers 1..1320 /organism="Pseudomonas putida" /mol _type="genomic DNA" /strain=" SKPf4"/isolation_source="stem rot-infected peanut rhizosphere"/db_xref="taxon:303" /country="India" /collection _date="21-Aug-2013" rRNA <1..>1320 /product="16S ribosomal RNA"
Origin	CTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCATACGTCCTACGGGAGAAAGCAG GGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAATGGCTCAC CAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTC CTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGA AGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTG ACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGT TAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAAGCCCCGGGCTCA ACCTGGGAACTGCATCCAAAACTGGCAAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGG TGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGG TGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCC GTTGGAATCCTTGAGATTTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAA GGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTTGTTTAATTCGAAGCAACG CGAAGAACCTTACCAGGCCTTGACATGCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCTG ACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAA CCCTTGTCCTTAGTTACCAGCACGTTATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAA GGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACA GAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCTCACAAAACCGATCGTAGTCCGGATCGCAGTCTGCAAC TCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCT TGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAGCTAGTCTAACCT

NCBI SUBMISSION OF *SKPf5*

Locus	KP859614 728 bp DNA linear BCT 23-APR-2015
Definition	***Pseudomonas aeruginosa*** strain ***SKPf5*** 16S ribosomal RNA gene, partial sequence.
Accession	KP859614
Version	KP859614
Source	***Pseudomonas aeruginosa***
Organism	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas.
Reference	(bases 1 to 728)
Authors	Bishi,S.K. and Vakharia,D.N.
Title	*In-vitro* characterization of fluorescent pseudomonad isolates from groundnut rhizosphere against stem rot pathogen (*Sclerotium rolfsii*)
Journal	Unpublished
Journal	Submitted (24-FEB-2015) Biochemistry, Junagadh Agricultural University, Directorate of Groundnut Research, Ivnagar road, PB.05, Junagadh, Gujarat 362001, India.
Comment	Sequences were screened for chimeras by the submitter using DECIPHER ##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##
Features Source	Location/Qualifiers 1..728 /organism="Pseudomonas aeruginosa" /mol _type="genomic DNA" /strain="SKPf5" /isolation_source="stem rot infected peanut rhizosphere" /db_xref="taxon:287" /country="India" /collection _date="20-Aug-2013" rRNA<1..>728/product="16S ribosomal RNA”
Origin	GCGGTGGTTCAGCTGATATAGCCATGACAAGTCGAGCGGATGAAGGGAGCTTGCTCCTGGATTC AGCGGCGGTAGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTCCGGAAACG GGCGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGA TGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTG GTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCA GTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCT TCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACG TTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAG CGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGCAAGTTGGATGTGAAATCC CCGGGCTCAACCTGGGGAACTGCATCCAAAACTACTGAGCTAGAGTACGGTAGAGGGTGGTGGA ATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACC TGGACTGATACTGACACTGAGGTG

NCBI SUBMISSION OF *SKPf6*

Locus	KP859616 731 bp DNA linear BCT 23-APR-2015
Definition	***Pseudomonas monteilii*** strain ***SKPf6*** 16S ribosomal RNA gene, partial sequence.
Accession	KP859616
Version	KP859616
Source	***Pseudomonas monteilii***
Organism	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas.
Reference	(bases 1 to 731)
Authors	Bishi,S.K. and Vakharia,D.N.
Title	*In-vitro* characterization of fluorescent pseudomonad isolates from groundnut rhizosphere against stem rot pathogen (*Sclerotium rolfsi*i)
Journal	Unpublished
Journal	Submitted (24-FEB-2015) Biochemistry, Junagadh Agricultural University, Directorate of Groundnut Research, Ivnagar road, PB.05, Junagadh, Gujarat 362001, India
Comment	Sequences were screened for chimeras by the submitter using DECIPHER. ##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##
Features Source	Location/Qualifiers 1..731 /organism="Pseudomonas monteilii" /mol _type ="genomic DNA" /strain="SKPf6"/isolation_source="stem rot infected peanut rhizosphere" /db_xref="taxon: 76759" /country ="India " /collection_date="24-Aug-2013" rRNA <1..>731/product="16S ribosomal RNA"
Origin	TCGGCGTGCACCGACCACTAGCCATGCAGTCGAGCGGATGACGGGAGCTTGCTCCTTGATTCAGCGGCGG TAGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGC ATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGC TAGTTGGTGGGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTG GAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTG ATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATGGTAAAGCACTTTAAGTTGGGAGGAAGGGCAG TAAGTTAATACCTTGCTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGC GGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGT TGGATGTGAAAGCCCCGGGGCTCAACCTGGGGAACTGCATCCAAAAACTGGGCAAGCTAGAGTACGGTAG AGGGTGGTGGAATTTTCCCTGTGTAGCGGTGAAATGCGTAGGATATGAGGAAGGAACACCAGTGGCCGAA AGGCGACCACCTGTACTGATACTGACCCTTG

NCBI SUBMISSION OF *SKPf7*

Locus	KP859617 741 bp DNA linear BCT 23-APR-2015
Definition	Pseudomonas plecoglossicida strain SKPf7 16S ribosomal RNA gene, partial sequence.
Accession	KP859617
Version	KP859617
Source	Pseudomonas plecoglossicida
Organism	Pseudomonas plecoglossicida Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas.
Reference	(bases 1 to 741)
Authors	Bishi,S.K., Golakiya,B.A. and Vakharia,D.N.
Title	*In-vitro* characterization of fluorescent pseudomonad isolates from groundnut rhizosphere against stem rot pathogen (*Sclerotium rolfsii*)
Journal	Unpublished
Journal	Submitted (24-FEB-2015) Biochemistry, Junagadh Agricultural University, Directorate of Groundnut Research, Ivnagar road, PB.05,Junagadh, Gujarat 362001, India
Comment	Sequences were screened for chimeras by the submitter using DECIPHER. ##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##
Features Source	Location/Qualifiers 1..741/organism="Pseudomonas plecoglossicida" /mol _type="genomic DNA" /strain="SKPf7" /isolation_source="stem rot infected peanut rhizosphere"/db_xref="taxon:70775"/country ="India" /collection _date="29-Aug-2013" rRNA <1..>741 /product="16S ribosomal RNA"
Origin	TGGTAAGTGCAGTGTCCATCGCCATGCAAGTCGAGCGGATGACGGGAGCTTGCTCCTTGATTCAGCGGCG GTATGGGTGAGATAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATAC CGCATACGTCCTACGGGGGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATT AGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACA CTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGC CTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGG CAGTAAGTTAATACCTTTTCTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAG CCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGT TAAGTTGGGATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGGCGAGCTAAAGTACCG GTAGAGGGTGGTGGAATTTTCCTGTGTACCGGTGAAATGCGTTAGATAGAGGGAAAGGAACACCAGGTG CTGATGGGCGACCCCCCTGGAACTGGAATACTGACAATTGA

NCBI SUBMISSION OF *SKPf8*

Locus	KP859617 741 bp DNA linear BCT 23-APR-2015
Definition	Pseudomonas putida strain SKPf8 16S ribosomal RNA gene, partial sequence.
Accession	KR422300
Version	KR422300
Source	Pseudomonas putida
Organism	Pseudomonas putida Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas.
Reference	(bases 1 to 1383)
Authors	Bishi,S.K., Vakharia,D.N. and Golakiya,B.A.
Title	*In-vitro* characterization of fluorescent pseudomonad isolates from groundnut rhizosphere against stem rot pathogen (*Sclerotium rolfsii*)
Journal	Unpublished
Journal	Submitted (02-MAY-2015) Biochemistry, Junagadh Agricultural University, Directorate of Groundnut Research, Ivnagar road, PB.05, Junagadh, Gujarat 362001, India
Comment	Sequences were screened for chimeras by the submitter using DECIPHER. ##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##
Features Source	Location/Qualifiers 1..1383 /organism="Pseudomonas putida" /mol_typ e="genomic DNA /strain="SKPf8 /isolation_source="stem rot-infected peanut rhizosphere"/db_xref="taxon:303"/country="India" /collection _date="20-Aug-2013" rRNA <1..>1383/product="16S ribosomal RNA"
Origin	CGAGCGGATGACGGGAGCTTGCTCCTTGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTG GTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCT TCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAATGGCTCACCAAGGCG ACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGG AGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGT CTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTAC CGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGG AATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAAGCCCCGGGCTCAACCTGGG AACTGCATCCAAAACTGGCAAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATG CGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAA GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGAA TCCTTGAGATTTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAA ACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTTGTTTAATTCGAAGCAACGCGAAGAA CCTTACCAGGCCTTGACATGCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCTGACACAGG TGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGT CCTTAGTTACCAGCACGTTATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGG ATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTT GCCAAGCCGCGAGGTGGAGCTAATCTCACAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTG CGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTGTACAC ACCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAGCTAGTCTAACCT

NCBI SUBMISSION OF *SKPf9*

Locus	KR492888 715 bp DNA linear BCT 07-MAY-2015
Definition	***Pseudomonas plecoglossicida*** strain ***SKPf9*** 16S ribosomal RNA gene, partial sequence.
Accession	KR492888
Version	KR492888
Source	*Pseudomonas plecoglossicida*
Organism	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas.
Reference	(bases 1 to 715)
Authors	Bishi,S.K., Vakharia,D.N. and Golakiya,B.A.
Title	*In-vitro* characterization of fluorescent pseudomonad isolates from groundnut rhizosphere against stem rot pathogen (*Sclerotium rolfsii*)
Journal	Unpublished
Journal	Submitted (05-MAY-2015) Biochemistry, Junagadh Agricultural University, Directorate of Groundnut Research, Ivnagar road, PB.05, Junagadh, Gujarat 362001, India
Comment	Sequences were screened for chimeras by the submitter using DECIPHER. ##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END## 1..715
Features Source	Location/Qualifiers /organism="Pseudomonas plecoglossicida" /mol _type="genomic DNA"/strain="SKPf9" /isolation_source="stem rot-infected peanut rhizosphere"/db_xref="taxon:70775" /country="India" /collection _date="28-Aug-2013" rRNA <1..>71/product="16S ribosomal RNA"
Origin	TGGTAAGTGCAGTGTCCATCGCCATGCAAGTCGAGCGGATGACGGGAGCTTGCTCCTTGATTCA GCGGCGGTATGGGTGAGATAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAG GAACGCTAATACCGCATACGTCCTACGGGGGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAG ATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACT GGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGC AGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTC TTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTTTCTGTTTTGA CGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCA AGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTTGGGATGTGAA AGCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGGCGAGCTAAAGTACCGGTAGAGGGTG GTGGGAATTTTCCTGTGTACCGGTGAAATGCGTTAGATAGAGGGAAAGGAACACCAGGTGCTGA TGGGCGACCCC

NCBI SUBMISSION OF *SKPf10*

Locus	KR422301 1391 bp DNA linear BCT 07-MAY-2015
Definition	***Pseudomonas putida*** strain ***SKPf10*** 16S ribosomal RNA gene, partial sequence.
Accession	KR422301
Version	KR422301
Source	***Pseudomonas putida***
Organism	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas.
Reference	(bases 1 to 1391)
Authors	Bishi,S.K., Vakharia,D.N. and Golakiya,B.A.
Title	In-vitro characterization of fluorescent pseudomonad isolates from groundnut rhizosphere against stem rot pathogen (*Sclerotium rolfsii*)
Journal	Unpublished
Journal	Submitted (02-MAY-2015) Biochemistry, Junagadh Agricultural University, Directorate of Groundnut Research, Ivnagar road, PB.05, Junagadh, Gujarat 362001, India
Comment	Sequences were screened for chimeras by the submitter using DECIPHER. ##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##
Features Source	Location/Qualifiers 1..1391 /organism="Pseudomonas putida"/mol_ type ="genomic DNA"/strain="SKPf10" /isolation_source="stem rot-infected peanut rhizosphere"/db_xref="taxon:303"/country="India /collection_ date="20-Aug-2013" rRNA <1..>1391 /product="16S ribosomal RNA"
Origin	CGAGCGGATGACGGGAGCTTGCTCCTTGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTG GTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCT TCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAATGGCTCACCAAGGCG ACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGG AGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGT CTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTAC CGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGG AATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAAGCCCCGGGCTCAACCTGGG AACTGCATCCAAAACTGGCAAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATG CGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAA GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGAA TCCTTGAGATTTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAA ACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAA CCTTACCAGGCCTTGACATGCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCTGACACAGG TGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGT CCTTAGTTACCAGCACGTTATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGG ATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTT GCCAAGCCGCGAGGTGGAGCTAATCTCACAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTG CGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTGTACAC ACCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAGCTAGTCTAACCTTCGGAGGA

NCBI SUBMISSION OF *SKPf11*

Locus	KR492889 1260 bp DNA linear BCT 07-MAY-2015
Definition	***Pseudomonas putida*** strain *SKPf11* 16S ribosomal RNA gene, partial sequence.
Accession	KR492889
Version	KR492889
Source	***Pseudomonas putida***
Organism	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas.
Reference	(bases 1 to 1260)
Authors	Bishi,S.K., Vakharia,D.N. and Golakiya,B.A.
Title	*In-vitro* characterization of fluorescent pseudomonad isolates from groundnut rhizosphere against stem rot pathogen (*Sclerotium rolfsii*)
Journal	Unpublished
Journal	Submitted (05-MAY-2015) Biochemistry, Junagadh Agricultural University, Directorate of Groundnut Research, Ivnagar road, PB.05, Junagadh, Gujarat 362001, India
Comment	Sequences were screened for chimeras by the submitter using DECIPHER. ##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##
Features Source	Location/Qualifiers source 1..1260 /organism="Pseudomonas putida"/mol_type="genomic DNA" /strain="SKPf11" /isolation_ source ="stem rot-infected peanut rhizosphere " /db_ xref ="taxon :303" /country ="India"collection_date=" 23-Aug-2013" rRNA <1..>1260 /product="16S ribosomal RNA"
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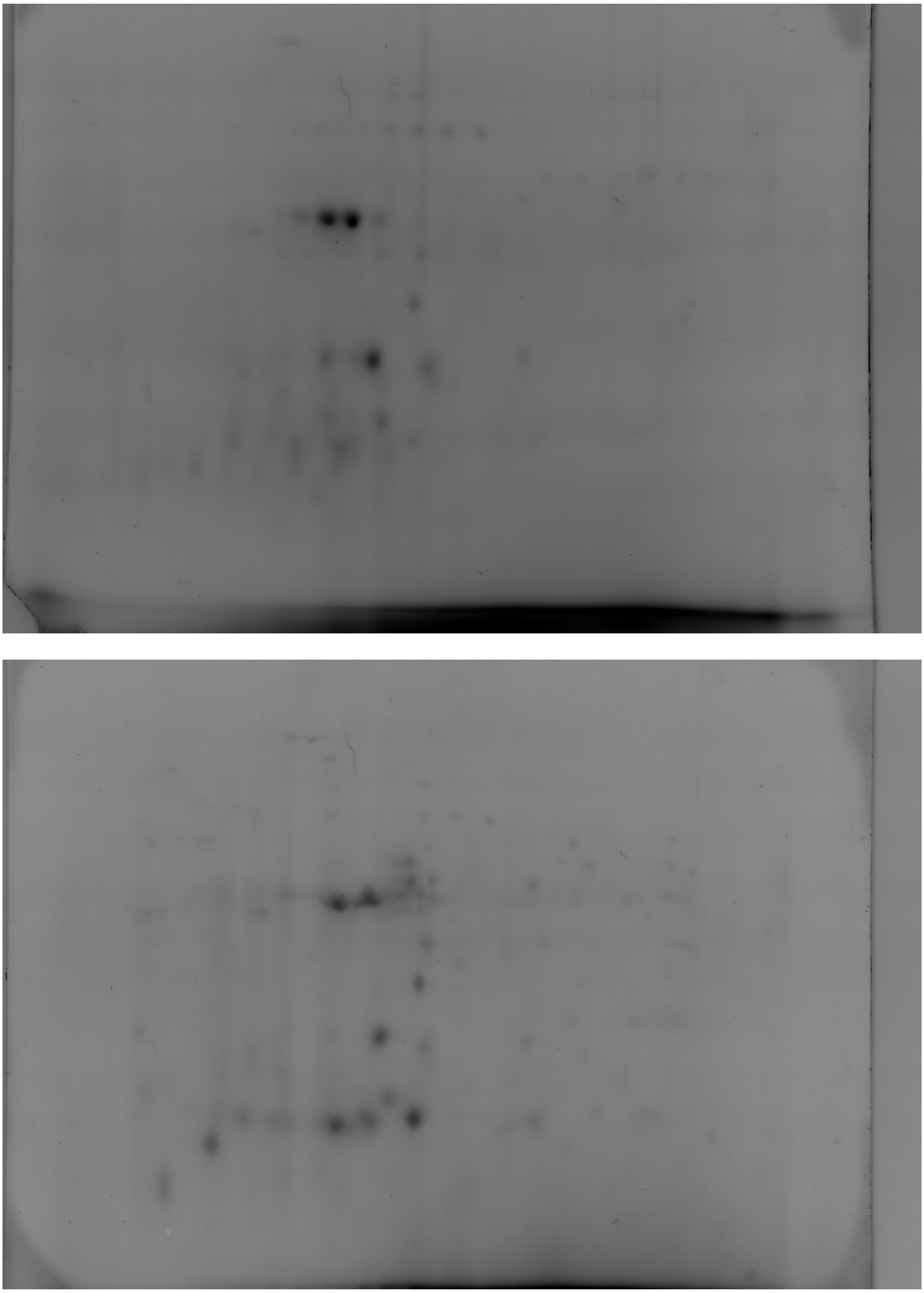
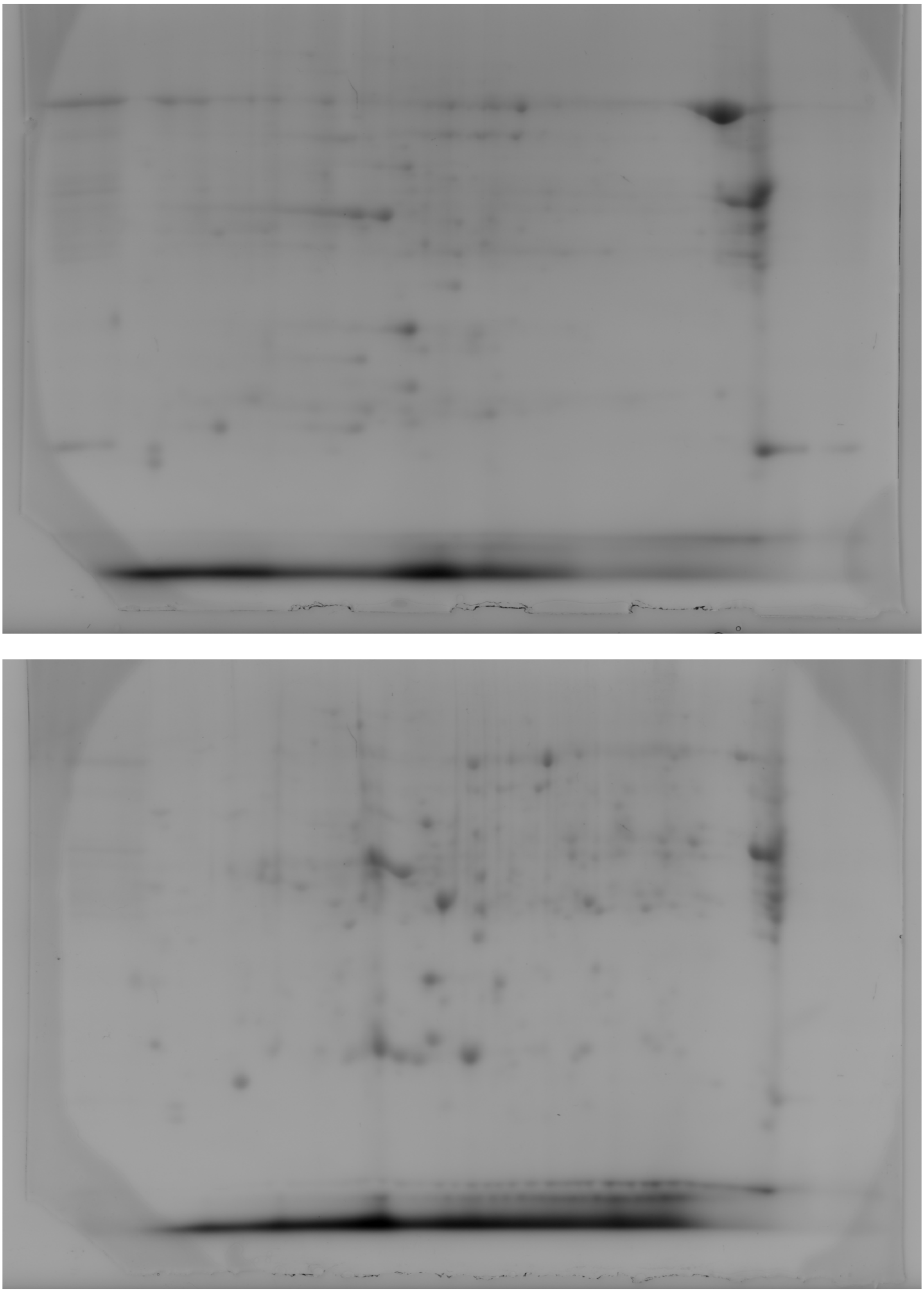
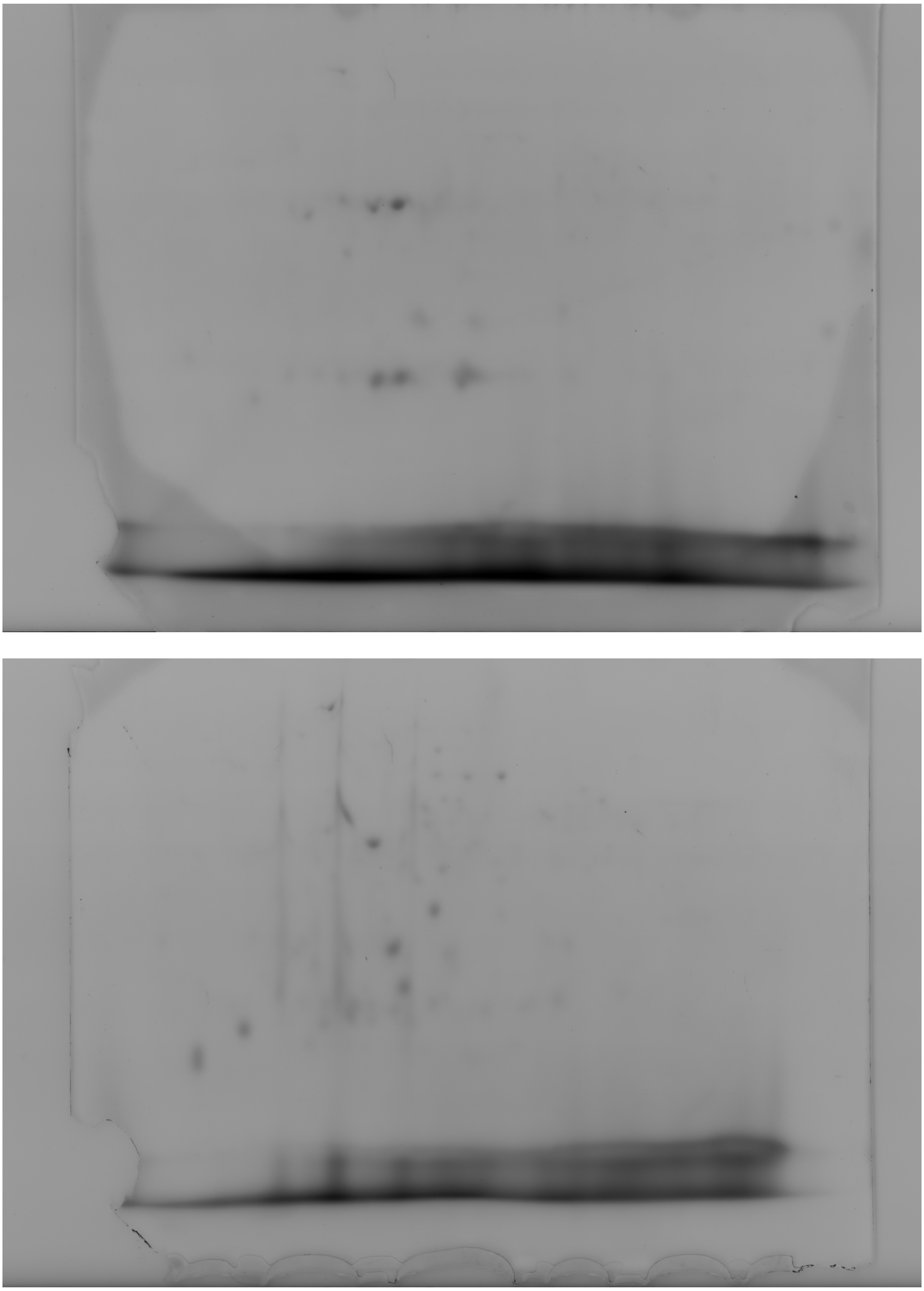
APPENDIX IIa



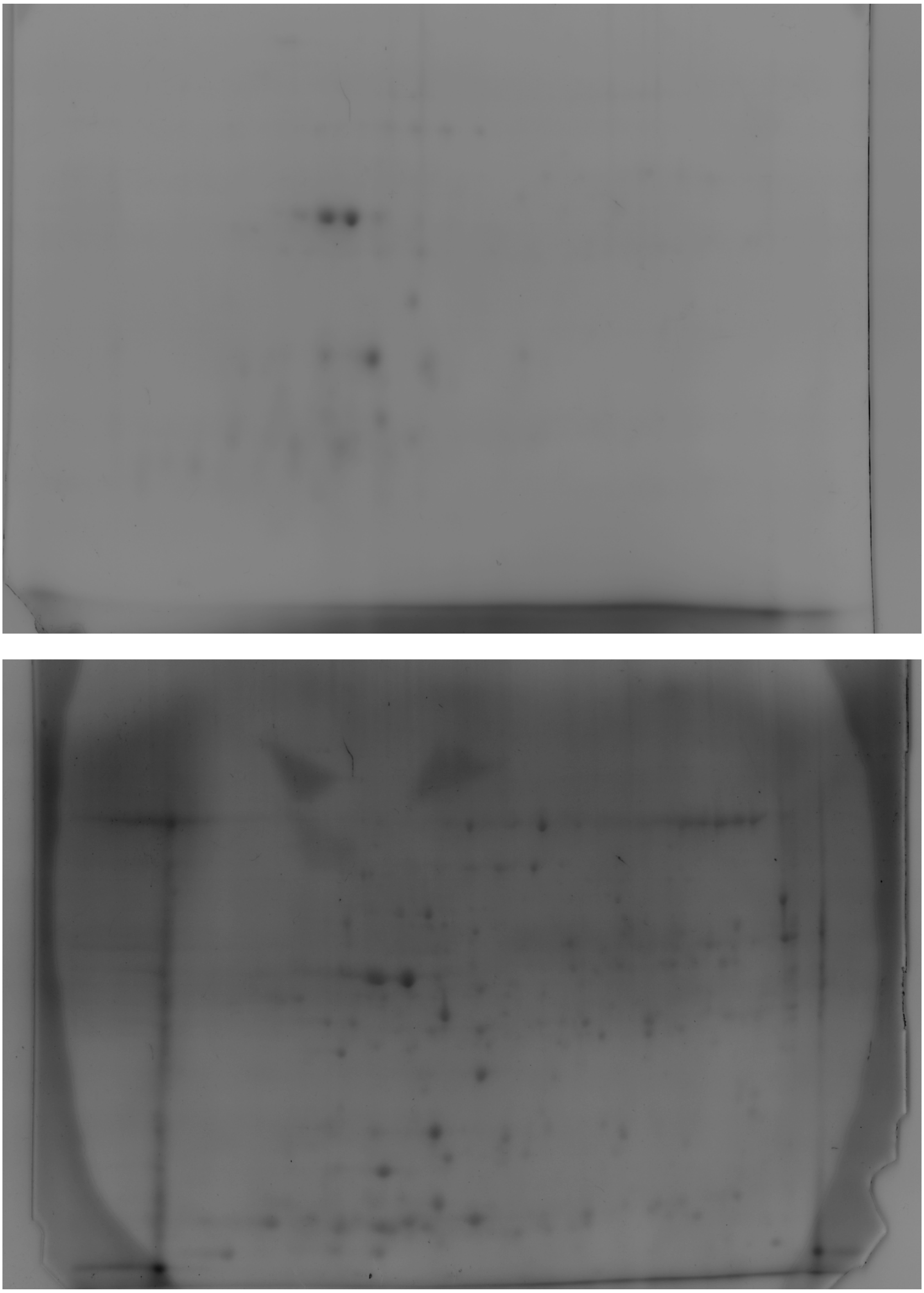
Leaf proteome of genotype CS19 expressed under control (a) and infected with *S. rolfsii* (b) conditions



Leaf proteome of genotype GG16 expressed under control (a) and infected with *S. rolfsii* (b) conditions



Leaf proteome of genotype GG20 expressed under control (a) and infected with *S. rolfsii* (b) conditions



Leaf proteome of genotype TG37A expressed under control (a) and infected with *S. rolfsii* (b) conditions

APPENDIX IIb

SPOT TABLE FOR CS19, Relative volume (%) of spots							
Spot match ID	**Control CS19**	**Stress CS19**	**Fold change under stress**	**Spot match ID**	**Control CS19**	**Stress CS19**	**Fold change under stress**
0	0.646	0.374	0.580	34	1.013	0.272	0.268
1	0.170	0.092	0.538	35	0.097	0.142	1.465
2	0.153	0.377	2.473	36	0.225	0.416	1.845
3	0.147	0.782	5.310	37	0.258	0.534	2.069
4	0.118	0.235	1.990	38	0.118	0.495	4.183
5	0.405	0.993	2.454	39	0.142	0.123	0.865
6	0.130	0.411	3.161	40	0.338	0.199	0.588
7	0.478	0.868	1.814	41	0.344	0.349	1.013
8	0.156	0.653	4.197	42	0.115	0.137	1.191
9	0.019	0.194	10.142	43	0.121	0.174	1.442
10	0.337	0.156	0.461	44	0.083	0.290	3.484
11	0.183	0.148	0.808	45	0.147	0.271	1.837
12	0.249	0.508	2.043	46	0.818	0.395	0.482
13	0.264	0.327	1.240	47	0.275	0.816	2.972
14	0.210	0.267	1.274	48	0.193	0.409	2.121
15	0.492	0.337	0.686	49	0.411	1.569	3.818
16	0.585	0.860	1.470	50	0.143	0.456	3.180
17	0.626	0.561	0.896	51	0.478	0.737	1.540
18	0.376	0.129	0.343	52	1.372	0.312	0.227
19	0.269	1.002	3.731	53	0.500	0.192	0.385
20	0.343	0.097	0.282	54	1.333	0.627	0.470
21	0.251	0.937	3.729	55	0.096	0.138	1.437
22	0.582	1.101	1.890	56	0.420	0.325	0.773
23	0.285	0.686	2.410	57	0.233	0.239	1.026
24	0.591	0.136	0.230	58	0.296	0.141	0.477
25	0.615	0.371	0.604	59	0.520	0.356	0.684
26	0.148	0.341	2.301	60	0.432	0.150	0.347
27	0.070	0.606	8.651	61	0.357	0.331	0.925
28	0.277	0.120	0.433	62	0.116	0.359	3.102
29	1.015	0.496	0.488	63	0.154	0.219	1.427
30	0.938	0.223	0.238	64	0.206	0.326	1.585
31	1.128	0.367	0.325	65	0.063	0.168	2.661
32	0.737	0.735	0.997	66	0.044	0.449	10.303
33	0.157	0.708	4.498	67	0.196	0.193	0.986
68	0.252	0.114	0.454	70	0.059	0.438	7.379
69	0.052	0.470	9.057	71	0.112	0.162	1.448

SPOT TABLE FOR GG16, Relative volume (%) of spots

Spot match ID	Control GG16	Stress GG16	Fold change under stress	Spot match ID	Control GG16	Stress GG16	Fold change under stress
0	1.009	1.694	1.679	36	0.122	0.143	1.174
1	2.652	1.139	0.429	37	0.217	0.343	1.582
2	1.355	0.174	0.128	38	0.089	0.101	1.131
3	0.719	0.831	1.156	39	0.271	0.118	0.437
4	0.511	0.524	1.027	40	0.484	0.463	0.958
5	0.341	0.440	1.291	41	0.789	0.556	0.704
6	0.312	0.272	0.870	42	0.166	0.326	1.966
7	1.158	0.469	0.405	43	0.388	0.122	0.315
8	0.829	0.555	0.670	44	0.169	0.260	1.539
9	0.041	0.127	3.120	45	0.633	0.550	0.869
10	0.457	0.274	0.601	46	0.140	0.055	0.397
11	0.117	0.148	1.263	47	0.300	0.259	0.863
12	0.601	0.555	0.924	48	0.663	0.517	0.780
13	0.045	0.095	2.119	49	0.389	0.290	0.746
14	0.323	0.152	0.470	50	0.258	0.582	2.256
15	0.227	0.178	0.784	51	0.388	0.060	0.154
16	1.203	0.194	0.161	52	0.236	0.267	1.132
17	0.136	0.200	1.472	53	0.128	0.081	0.636
18	1.294	0.597	0.461	54	0.142	0.539	3.786
19	0.143	0.137	0.957	55	1.655	2.340	1.414
20	0.631	0.220	0.349	56	0.651	0.479	0.736
21	0.204	0.175	0.861	57	0.593	1.158	1.951
22	0.400	0.596	1.491	58	0.255	0.488	1.912
23	0.307	0.396	1.289	59	0.413	0.280	0.678
24	0.069	0.507	7.386	60	0.307	0.477	1.556
25	0.092	0.251	2.714	61	0.335	0.646	1.930
26	0.520	0.165	0.317	62	0.456	1.291	2.829
27	0.206	0.852	4.141	63	0.062	0.209	3.358
28	0.181	0.490	2.715	64	0.051	0.316	6.147
29	0.409	0.072	0.176	65	0.103	0.065	0.630
30	0.430	0.421	0.980	66	0.201	0.379	1.881
31	0.850	1.096	1.289	67	1.517	0.858	0.565
32	0.087	0.179	2.047	68	0.241	0.464	1.923
33	0.365	0.434	1.190	69	0.166	0.133	0.803
34	0.144	1.285	8.931	70	0.088	0.310	3.529
35	0.117	0.099	0.846	71	0.442	0.153	0.345
72	0.658	1.004	1.526	102	0.139	0.256	1.837
73	0.723	1.884	2.604	103	0.385	0.222	0.576

74	0.152	0.141	0.924	104	0.294	0.327	1.110
75	0.131	0.235	1.803	105	0.464	0.207	0.446
76	0.268	0.562	2.097	106	0.188	0.088	0.470
77	0.137	0.242	1.770	107	0.169	0.099	0.586
78	0.368	0.172	0.469	108	0.359	0.265	0.738
79	0.295	0.178	0.601	109	0.455	0.823	1.808
80	0.113	0.149	1.320	110	3.726	0.602	0.162
81	0.173	0.093	0.537	111	1.196	0.953	0.797
82	0.042	0.113	2.679	112	0.489	0.540	1.106
83	0.251	0.513	2.047	113	0.144	0.984	6.847
84	0.377	0.238	0.632	114	0.409	0.419	1.024
85	0.300	0.406	1.352	115	0.249	0.285	1.143
86	3.857	3.900	1.011	116	0.458	0.193	0.420
87	0.058	0.670	11.594	117	0.298	0.413	1.387
88	0.062	0.477	7.720	118	0.487	0.173	0.354
89	0.216	0.222	1.028	119	0.316	0.177	0.562
90	0.055	0.259	4.720	120	0.145	0.239	1.653
91	0.405	0.281	0.694	121	0.039	0.145	3.753
92	0.314	0.385	1.227	122	0.293	0.571	1.948
93	0.167	0.484	2.894	123	0.103	0.499	4.839
94	0.067	0.290	4.298	124	0.088	0.246	2.788
95	0.261	0.150	0.575	125	0.080	0.129	1.620
96	0.099	0.127	1.278	126	0.093	0.221	2.381
97	0.039	0.146	3.714	127	0.059	0.299	5.062
98	0.829	0.295	0.356	128	0.516	0.907	1.759
99	0.075	0.231	3.076	129	0.126	0.338	2.678
100	0.195	0.411	2.104	130	0.039	0.458	11.828
101	0.158	0.205	1.295	131	0.018	0.391	21.226

SPOT TABLE FOR GG20, Relative volume (%) of spots							
Spot match ID	**Control GG20**	**Stress GG20**	**Fold change under stress**	**Spot match ID**	**Control GG20**	**Stress GG20**	**Fold change under stress**
0	0.312	3.635	11.63	23	1.692	0.182	0.11
1	1.056	2.993	2.83	24	0.090	0.585	6.51
2	0.680	7.156	10.53	25	0.320	0.378	1.18
3	2.569	1.400	0.55	26	0.148	0.505	3.41
4	0.264	0.124	0.47	27	0.434	0.316	0.73
5	0.895	0.193	0.22	28	0.272	0.885	3.26
6	0.255	0.260	1.02	29	0.415	0.273	0.66
7	1.335	0.505	0.38	30	0.202	0.302	1.49
8	0.250	0.187	0.75	31	0.615	0.191	0.31

9	0.169	0.091	0.54	32	0.513	0.920	1.79
10	0.919	1.049	1.14	33	0.315	0.630	2.00
11	0.360	0.932	2.59	34	0.185	0.568	3.08
12	0.419	0.446	1.06	35	0.130	0.463	3.56
13	0.515	0.414	0.80	36	0.079	0.372	4.71
14	0.838	0.413	0.49	37	0.180	0.096	0.53
15	6.637	0.563	0.08	38	0.101	0.050	0.49
16	0.317	0.273	0.86	39	0.616	0.289	0.47
17	0.287	0.548	1.91	40	0.445	0.374	0.84
18	0.515	0.154	0.30	41	0.102	0.091	0.89
19	1.405	0.543	0.39	42	0.300	0.365	1.22
20	0.650	0.488	0.75	43	0.441	0.151	0.34
21	0.330	0.223	0.68	44	0.250	0.289	1.16
22	0.215	0.144	0.67				

SPOT TABLE FOR TG37A, Relative volume (%) of spots

Spot match ID	**Control TG37A**	**Stress TG37A**	**Fold change under stress**	**Spot match ID**	**Control TG37A**	**Stress TG37A**	**Fold change under stress**
0	0.032	0.272	8.47	33	0.302	0.277	0.92
1	0.309	0.206	0.67	34	0.054	0.163	2.99
2	0.290	0.379	1.31	35	0.360	0.241	0.67
3	0.617	0.348	0.56	36	0.099	0.153	1.55
4	0.241	0.221	0.92	37	0.076	0.221	2.92
5	0.142	0.080	0.57	38	0.153	0.332	2.18
6	0.519	0.471	0.91	39	0.421	0.184	0.44
7	0.512	0.195	0.38	40	0.108	0.173	1.60
8	0.296	0.247	0.83	41	0.160	0.179	1.12
9	0.098	0.046	0.47	42	0.080	0.272	3.40
10	0.304	0.222	0.73	43	0.127	0.067	0.53
11	0.345	0.207	0.60	44	0.425	0.280	0.66
12	0.420	0.180	0.43	45	0.298	0.687	2.31
13	0.173	0.140	0.81	46	0.590	0.100	0.17
14	0.656	0.263	0.40	47	0.150	0.069	0.46
15	0.309	0.043	0.14	48	0.667	0.182	0.27
16	0.605	0.255	0.42	49	0.468	0.182	0.39
17	0.864	0.111	0.13	50	0.060	0.635	10.68
18	0.113	0.630	5.55	51	0.805	0.121	0.15
19	0.251	0.044	0.17	52	0.130	0.652	5.03
20	0.123	0.070	0.57	53	0.418	0.137	0.33
21	0.791	0.311	0.39	54	0.509	0.136	0.27
22	0.096	0.333	3.47	55	1.358	0.818	0.60
23	1.160	0.361	0.31	56	0.537	0.558	1.04
24	0.749	0.146	0.20	57	0.057	0.090	1.58

25	0.855	0.335	0.39	58	0.199	0.298	1.50
26	0.944	0.226	0.24	59	0.165	0.258	1.57
27	0.254	0.170	0.67	60	0.074	0.091	1.24
28	0.171	0.430	2.51	61	0.192	0.290	1.51
29	0.206	0.151	0.73	62	0.253	0.111	0.44
30	0.232	0.377	1.63	63	0.167	0.044	0.27
31	0.297	0.525	1.77	64	0.476	0.322	0.68
32	0.097	0.163	1.68	65	0.451	0.081	0.18
66	0.259	0.117	0.45	81	0.486	0.036	0.07
67	1.260	0.089	0.07	82	0.489	0.250	0.51
68	0.076	0.065	0.86	83	0.267	0.113	0.42
69	0.210	0.137	0.65	84	0.283	0.806	2.85
70	0.266	0.116	0.44	85	0.207	0.324	1.56
71	0.273	0.100	0.37	86	0.041	0.583	14.12
72	0.367	0.105	0.29	87	0.224	1.092	4.87
73	0.144	0.117	0.81	88	0.147	0.130	0.89
74	0.143	0.526	3.67	89	0.173	0.235	1.36
75	0.244	0.199	0.82	90	0.165	0.142	0.86
76	0.180	1.136	6.30	91	0.271	0.642	2.37
77	0.121	0.202	1.66	92	0.123	1.121	9.12
78	0.284	0.591	2.08	93	0.298	0.204	0.68
79	0.120	0.246	2.05	94	0.051	4.485	88.72
80	0.147	0.533	3.63				

APPENDIX III

Arachis hypogaea cultivar GG20 polygalacturonase inhibitory protein (PGIP) mRNA, partial cds

GenBank: KP844637.1

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LOCUS       KP844637                 611 bp    mRNA    linear   PLN 28-APR-2015
DEFINITION  Arachis hypogaea cultivar GG20 polygalacturonase inhibitory protein
            (PGIP) mRNA, partial cds.
ACCESSION   KP844637
VERSION     KP844637.1  GI:814556877
KEYWORDS    .
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REFERENCE   1  (bases 1 to 611)
  AUTHORS   Bishi,S.K. and Vakharia,D.N.
  TITLE     Biochemical and molecular basis of innate and Pseudomonas
            fluorescens induced stem rot tolerance in groundnut (Arachis
            hypogeae L.)
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 611)
  AUTHORS   Bishi,S.K. and Vakharia,D.N.
  TITLE     Direct Submission
  JOURNAL   Submitted (23-FEB-2015) Biochemistry, Directorate of Groundnut
            Research, Previously NRCG, Ivnagar Road, Pb.05, Junagadh, Gujarat
            362001, India
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      181 CCCTCCATCG CCACCCTCCC TTACCTTAAC ACTATCCAAT TGGGCCCCGC CAACTTCACC
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      421 CTCCAATCCC TCGACTTACA GTCTAACAGC CTCAGCGGAT CGATCCCGGA CATATTCGAC
      481 TCCTTCCCAA ACTTCTCCTT CCTGTGGCTA AGCTCGAACC AGCTCTCCGG GAAGATACCA
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Polygalacturonase inhibitory protein, partial [*Arachis hypogaea*]

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GenBank: AKE32094.1
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DEFINITION  polygalacturonase inhibitory protein, partial [Arachis hypogaea].
ACCESSION   AKE32094
VERSION     AKE32094.1  GI:814556878
DBSOURCE    accession KP844637.1
KEYWORDS    .
SOURCE      Arachis hypogaea (peanut)
  ORGANISM  Arachis hypogaea
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            Spermatophyta; Magnoliophyta; eudicotyledons; Gunneridae;
            Pentapetalae; rosids; fabids; Fabales; Fabaceae; Papilionoideae;
            Dalbergieae; Arachis.
REFERENCE   1  (residues 1 to 203)
  AUTHORS   Bishi,S.K. and Vakharia,D.N.
  TITLE     Biochemical and molecular basis of innate and Pseudomonas
            fluorescens induced stem rot tolerance in groundnut (Arachis
            hypogeae L.)
  JOURNAL   Unpublished
REFERENCE   2  (residues 1 to 203)
  AUTHORS   Bishi,S.K. and Vakharia,D.N.
  TITLE     Direct Submission
  JOURNAL   Submitted (23-FEB-2015) Biochemistry, Directorate of Groundnut
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     Region          165..187
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ORIGIN
        1 ALLQIKKDLN NPTALSSWKT TADCCRHWKG VSCDRTKTHR VNFLNLDNID MKPGHPLSIP
       61 PSIATLPYLN TIQLGPANFT GPIPRSLLNL TNLKDLSITS TLLSGTIPEF LSQITTLTDL
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      181 KSMGNINVTY LYLDNNELEG DAS
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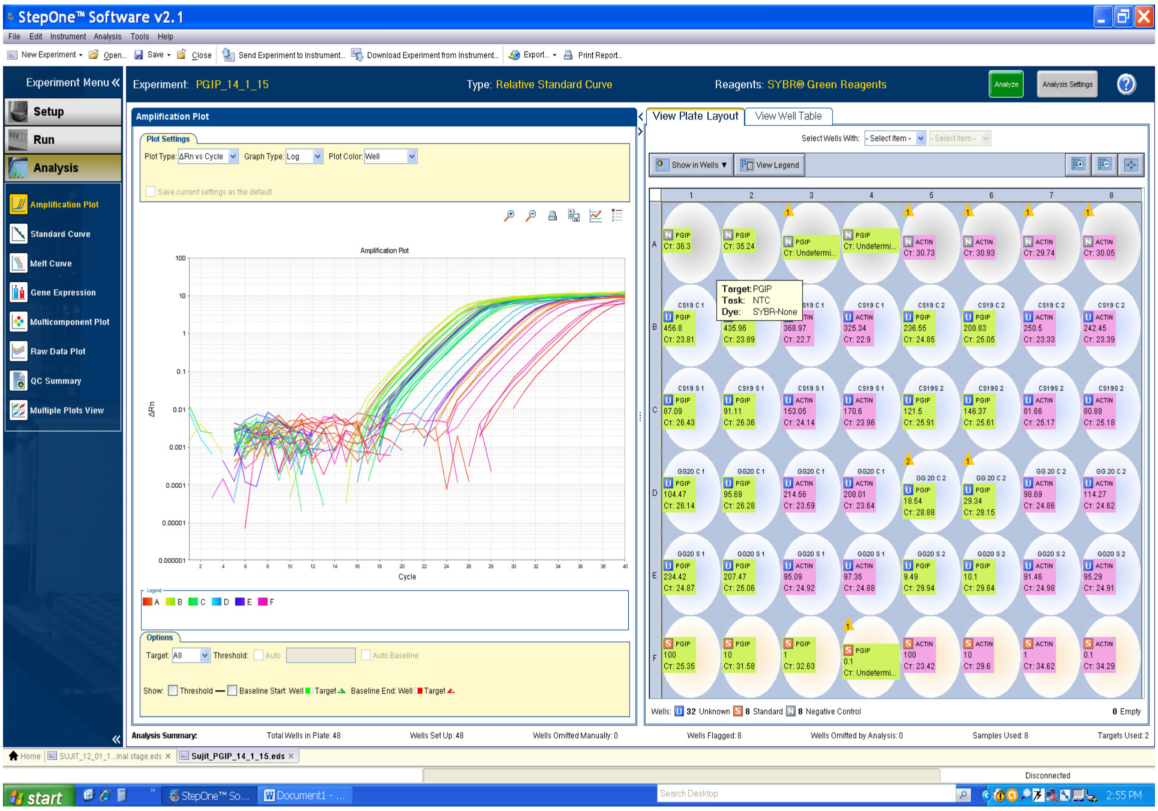


Experiment: PGIP_14_1_15

Type: Relative Standard Curve

Reagents: SYBR® Green Reagents

Plot Settings

Plot Type: ΔRn vs Cycle

Graph Type: Log

Plot Color: Well

Options

Target: All

Threshold: ☐ Auto

☐ Auto Baseline

Show: ☐ Threshold ☐ Baseline Start: Well Target Baseline End: Well Target

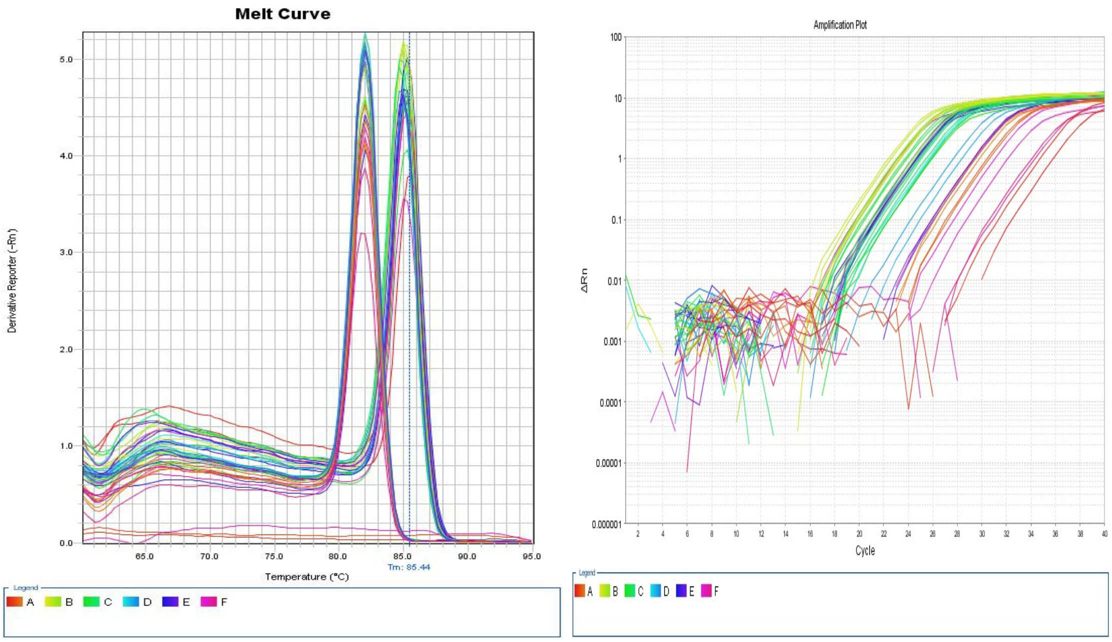
Select Wells With: - Select Item - - Select Item -

	1	2	3	4	5	6	7	8
A	N PGIP CT: 36.3	N PGIP CT: 35.24	1 N PGIP CT: Undetermi...	N PGIP CT: Undetermi...	1 N ACTIN CT: 30.73	1 N ACTIN CT: 30.93	1 N ACTIN CT: 29.74	1 N ACTIN CT: 30.05
B	CS19 C 1 U PGIP 456.8 CT: 23.81	435.96 CT: 23.89	S19 C 1 TIN 368.97 CT: 22.7	CS19 C 1 U ACTIN 325.34 CT: 22.9	CS19 C 2 U PGIP 236.55 CT: 24.85	CS19 C 2 U PGIP 208.83 CT: 25.05	CS19 C 2 U ACTIN 250.5 CT: 23.33	CS19 C 2 U ACTIN 242.45 CT: 23.39
C	CS19 S 1 U PGIP 87.09 CT: 26.43	CS19 S 1 U PGIP 91.11 CT: 26.36	CS19 S 1 U ACTIN 153.05 CT: 24.14	CS19 S 1 U ACTIN 170.6 CT: 23.96	CS19S 2 U PGIP 121.5 CT: 25.91	CS19S 2 U PGIP 146.37 CT: 25.61	CS19S 2 U ACTIN 81.66 CT: 25.17	CS19S 2 U ACTIN 80.88 CT: 25.18
D	GG20 C 1 U PGIP 104.47 CT: 26.14	GG20 C 1 U PGIP 95.69 CT: 26.28	GG20 C 1 U ACTIN 214.56 CT: 23.59	GG20 C 1 U ACTIN 208.01 CT: 23.64	2 GG 20 C 2 U PGIP 18.54 CT: 28.88	1 GG 20 C 2 U PGIP 29.34 CT: 28.15	GG 20 C 2 U ACTIN 98.69 CT: 24.86	GG 20 C 2 U ACTIN 114.27 CT: 24.62
E	GG20 S 1 U PGIP 234.42 CT: 24.87	GG20 S 1 U PGIP 207.47 CT: 25.06	GG20 S 1 U ACTIN 95.09 CT: 24.92	GG20 S 1 U ACTIN 97.35 CT: 24.88	GG20 S 2 U PGIP 9.49 CT: 29.94	GG20 S 2 U PGIP 10.1 CT: 29.84	GG20 S 2 U ACTIN 91.46 CT: 24.98	GG20 S 2 U ACTIN 95.29 CT: 24.91
F	S PGIP 100 CT: 25.35	S PGIP 10 CT: 31.58	S PGIP 1 CT: 32.63	1 S PGIP 0.1 CT: Undetermi...	S ACTIN 100 CT: 23.42	S ACTIN 10 CT: 29.6	S ACTIN 1 CT: 34.62	S ACTIN 0.1 CT: 34.29

Target: PGIP
Task: NTC
Dye: SYBR-None

Wells: U 32 Unknown S 8 Standard N 8 Negative Control 0 Empty

Analysis Summary: Total Wells in Plate: 48 Wells Set Up: 48 Wells Omitted Manually: 0 Wells Flagged: 8 Wells Omitted by Analysis: 0 Samples Used: 8 Targets Used: 2



Representative snaps of results showing differential gene expression study using real time PCR