4.1 Introduction

Among the various fungal diseases of groundnut, collar rot caused by *A. niger* has got economical importance as this fungus causes rot of both seed and seedling which drastically reduces the plant stand (Kishore et al., 2005). In moist soil, seeds may be attacked and killed due to rotting. Seeds removed from soil show black sooty cover. The infected areas of seedlings are covered with black fungal spores. Mature plants are also attacked. Symptoms include wilt of branches permanently and/or wilting of entire plant. The dead and dried branches are easily detached from the collar region. Infected pods reveal patches of black sooty spores (Gajera and Vakharia, 2010). About 25–50% of the crop loss is due to collar rot (Ghewande and Nandagopa, 1997). *A. niger* is a necrotrophic pathogen. Necrotrophic plant pathogens were initially considered to invade their hosts in a rather unsophisticated manner; however, now they are known to use subtle mechanisms to subdue host plants (Jan and Van, 2006). They usually possess all the enzymatic activities required to utilize the extracellular matrix of the plant cells as a nutrient source. Moreover, they often trigger nutrient leakage from the host cells and are able to live from dead tissues as well. Investigation of natural defense mechanism in plants in recent years has given new dimension to disease control. Instead of targeting the pathogen by using fungicides and pesticides, plant defense mechanism can be activated to control the diseases. Plants possess both preformed and inducible mechanisms to resist pathogen invasion. Whether or not a plant turns out to be susceptible or resistant is determined by the speed and magnitude with which these mechanisms are activated and expressed and by their effectiveness against individual pathogens with different modes of attack (Van Loon et al., 2006). For the betterment of crop productivity with demand of groundnut, various molecular studies carried out throughout the world. Molecular markers such as Restriction Fragment Length Polymorphism (RFLP), Random Amplification of Polymorphic DNA (RAPD), and Amplified Fragment Length Polymorphism (AFLP) techniques are widely used to analyze complex traits and identifying a Quantitative Trait Loci (QTLs) (Burrow and Blake 1998). However, little progress was made as these techniques have shown limited polymorphism in groundnut plants which exhibit narrow genetic base and shows low genetic variation (Stalker and Mozingo, 2001). Several up-regulated genes in response to drought and *Aspergillus* infestation were identified using the cDNA microarray (Luo et al., 2005). Several ESTs from
**A. hypogaea** are presently accessible in public databases, but the protein expression data remain extremely inadequate. Proteomic research involves investigation of synthesis, turnover and modification of many proteins in order to understand gene function and characteristics of various genotypes (Jorrn et al., 2006). However, very little information is available about the proteome changes to biotic stress in peanut (Drame et al., 2007). Proteomic analysis may, therefore, provide a powerful tool to address biochemical and physiological aspects of plant responses to biotic stresses (Jones et al., 2004).

In the present work, we have studied differential response of susceptible and tolerant varieties of groundnut seedlings to **A. niger** infection. The main objective was to investigate mechanism of infection and to devise a strategy to combat the infection. Most plants produce reactive oxygen species (ROS) in response to pathogen ingress at the site of infection. One of the first enzymes found to be active in response to pathogen is NADPH oxidase (Egan et al., 2007). The ROS produced causes hypersensitive reactions (HRs) by which plants try to physically remove its infected portion along with the pathogen. Such HR is for the defense purpose but can be a boon to necrotrophic pathogen. To study induction of ROS upon pathogen infection, NADPH Oxidase and Glutathione Reductase (GR) were analyzed in the tolerant and susceptible varieties. Differential protein profile, oil content and the presence of unsaturated fatty acids (FAs) in oils obtained from the seeds of tolerant and susceptible varieties were also studied to find the differences in response to **A. niger** infection. It was observed that **A. niger** induces ROS in **A. hypogaea** very rapidly. To protect the plants from the damage caused by ROS, zinc was applied as an antioxidant and change in response of plants was studied. Also, in India, about 50% of the groundnut soils show zinc deficiencies causing considerable yield losses (Singh, 2006). The micronutrient zinc has an essential role in physiological and metabolic processes in plants as a cofactor or structural element in 300 catalytic and noncatalytic proteins. The basis of resistance development by zinc application was evaluated by protein profiling, estimation of salicylic acid (SA) and differential lipid content observation of live plants. We have also carried out proteome research in groundnut seedling challenged with **A. niger** in three groundnut cultivars and identified resistant a novel protein in groundnut.
4.2 Materials and method

4.2.1 Plant material and seed germination
Three varieties of groundnut seeds (*Arachis hypogaea* L.), GG 11, GG 20 and GG 24 were obtained from the Junagadh Agricultural University, Junagadh, Gujarat, India. Seeds were surface-sterilized with 0.1% HgCl$_2$, rinsed carefully with sterile water and put for germination in sterilized Petri dishes with sterile filter paper damped either with distilled water (control) or spore suspension of *A. niger* or 15 ppm of ZnSO$_4$ solution. Similar seed germination was also carried out in pots containing sterile soil. Infection by spore suspension was always made in the seeds grown for 48 h. Zinc sulphate solution was applied on germinating seeds prior to 24 h of infection.

4.2.2 Isolation and inoculation of fungal culture
*A. niger* was isolated on Potato dextrose agar (PDA) from the infected groundnut plant obtained from Junagadh Agricultural University and it was confirmed by observing spore structure. It was maintained on PDA plate by sub culturing every 15 days. Inoculum was prepared by flooding sterile water on sporulated plate and spores were collected and counted using Neubauer’s chamber. A suspension containing $10^5$ spores/ml was prepared and used for infection.

4.2.3 Sample collection
Treated and untreated plant material was powdered using liquid nitrogen (LN$_2$) and stored at 4 °C till further analysis. Each treatment was replicated three times. For all the analysis, the samples were collected in triplicates. The experiments were repeated twice.

4.2.4. Disease severity
Disease incidence was calculated as follows. A total of 200 seeds of each variety were incubated with *A. niger* spore suspension, the infected seeds were counted and the percentage of disease incident was calculated.
4.2.5 Oil extraction and iodine value
The total oil content was extracted and estimated according to (Bligh and Dyer, 1959). The 5 g of tissue was extracted in chloroform: methanol (10: 20), filtered and re-extracted with chloroform. The chloroform layer was evaporated to dryness, weighed and the percent total oil was calculated. Iodine value of oil was determined by the method described previously (Horowitz, 1975).

4.2.6 Defense related enzymes, Zinc estimation and total antioxidant power
NADPH oxidase activity, glutathione reductase activity, zinc estimation and total antioxidant power were determined as described in chapter 2.

4.2.7 Extraction and analysis of Salicylic Acid
One gm germinating seeds of all the three varieties was powdered using LN₂. The powdered tissues were ground in 5 ml of pre-chilled methanol using pre-chilled mortar and pestle for SA extraction. The slurry was collected in a vial and kept overnight at 4 °C to let the complete extraction of SA. It was then brought to room temperature, filtered through 0.2 mm membrane and concentrated by keeping the vials open at room temperature for 30 min. SA was separated and estimated by high performance liquid chromatography (HPLC) as described by Noordin and Chung (2007) using Zorbax RP C18 column and mobile phase of a mixture of methanol (60%) and water (40%). The flowrate used was 0.8 ml/min and the detection was done at 254 nm. The injection volume used was 20 µl. Salicylic acid (1 mg/ml, Sigma) was used as a standard. The detection was carried out using UV detector at 254 nm. The average retention time of standard SA was found to be 0.9 min, the corresponding retention time and peak area of the samples were noted and SA concentration in the samples were calculated.

4.2.8 Protein extraction and electrophoresis
Total protein was extracted according to Roulin and Buchala (1995). Plant material was frozen in LN₂ and grounded to fine powder with a pre-chilled mortar and pestle and extracted by adding 100 mM Tris buffer (pH 7.2) containing 1 mM phenyl methane sulphynl fluoride (PMSF) and 1% polyvinyl-pyrrolidone (PVP). Extracted protein was quantified by method of Lowry et al. (1951). Total protein from all three
varieties were profiled using SDS/ Native PAGE (10% separating gel with 4% stacking gel) according to the method of Laemmli (1970), with the Mini-PROTEIN Dual Slab Cell System (BIO-RAD). Total proteins (120µg) from each sample were loaded onto SDS-PAGE gels. Low-range protein markers (Sigma) were used as molecular mass standard. The gels were electrophoresed (100 V), stained with 0.125% Coomassie blue R-250 in 40% methanol and 10% acetic acid.

4.2.9 Densitometric quantification of protein of interest
120 µg protein samples extracted from A. niger treated and control plant leaves were loaded along with 5, 10, 15 and 20 µg of BSA in the 10 % SDS gel. After staining with Coomassie brilliant blue, images were taken using the Olympus CAMEDIA-C-5060 RM (Japan) 5.1 mega pixel CCD Digital camera of Alpha DigiDocTM RT System. The images were then processed with the Alpha DigiDoc TM RT software. A calibration curve was drawn and the concentration of the band of interest (110kd) from samples was determined using the same software (Densnijs et al., 1990).

4.2.10 Purification of induced protein
The crude protein fraction of 42 hr post infection was precipitated out using different levels of ammonium sulphate saturation from 0% to 80%. The pellets were collected after each saturation interval by centrifugation at 12 000 x g for 20 min at 4°C, and dissolved in 0.01 M Tris HCL buffer pH 7.2, and checked by Native PAGE for maximum precipitation. From these, dissolved pellets were loaded onto Sephadex G-100 (1.5 cm × 10 cm) using 0.01 M sodium phosphate buffer, pH 7.2 as a mobile phase. Further purification was carried out by gel filtration using Sephadex G- 75 (1.5 cm × 15.0 cm). Peak fractions of G-75 gel filtration were collected and reloaded onto a Sephadex G-75 equilibrated with 0.01 M sodium phosphate buffer, pH 7.2. The column was washed with the same buffer. All the fractions obtained were subjected to UV spectrophotometry and Native–PAGE analysis. The fractions containing band of interest were identified and used for further purification and characterization. Purified protein bands of interest were cut from Native polyacrylamide gels and dissolved in tris HCL buffer and reloaded in SDS PAGE for checking subunits of purified peptide.

4.2.11 Protein digestion and sequencing
Protein bands of interest were cut from polyacrylamide gels and digested overnight using trypsin (Sigma) as described elsewhere (Shevchenko et al., 1996). The cleaved
peptides were eluted, concentrated by vacuum centrifugation and separated by RP nano-LC (LC1100 series, Agilent Technologies, Paolo Alto, California; column: Zorbax 300SB-C18, 3.5 m, 150mm×0.075 mm; eluate: 0.1% formic acid in 0–60% acetonitrile). The peptides were analyzed by on-line MS/MS (LC/MSD TRAP XCT mass spectrometer, Agilent Technologies) (Benndorf et al., 2006). Thereafter, a database search was conducted using the MS/MS ion search (MASCOT, http://www.matrixscience.com) against all plants entries of NCBI nr (GenBank; http://www.ncbi.nlm.nih.gov/index.html) with subsequent parameters: trypsin digestion, up to one missed cleavage site, fixed modifications: carbamidomethyl (C), variable modifications: oxidation (M), peptide total:±1.2 Da, MS/MS total: ±0.6 Da, peptide charge: +1, +2 and +3.

4.2.12 Bioassay of partially purified protein

Two bioassays were performed. One was to find out direct anti-fungal activity of partially purified protein against A. niger by the agar cup diffusion method (Murray et al., 2009). Potato dextrose agar plate was prepared as per the standard directions and two cups were made at the same distance from the center by punching agar surface with a sterile cork borer. The punched part of the agar media was removed by scooping. In the center of PDA plate 48 h old A. niger was inoculated. Each cup was added with 200 µL partially purified protein (20µg/mL) carefully. Plates were then incubated at 35 °C for 24–48 h to observe growth.

Another indigenous bioassay was performed to find out effect of this purified protein on control and infected peanut seed germination. Surface sterilized seeds of groundnut were put for germination for 24 hrs in two sterile petri plates having filter paper soaked with water. After 20 hrs, partially purified resistance protein was applied on germinating seeds in one of the plates. Both the plates were treated with the inoculums having 10^5 spore of A. niger after 24hrs and incubated at 35°C for 24 hrs.

4.2.13 2D PAGE analysis

Plant material was frozen in LN₂ and grounded to fine powder with a pre-chilled mortar and pestle and extracted by adding 100 mM Tris buffer (ph 7.2) containing 1mM phenyl methane sulphonyl fluoride (PMSF) and 1% polyvinyl-pyrrolidone (PVP). Extracted protein was quantified by method of Lowry et al. (1951). Total seed proteins (120 µg) were loaded into tube gels (8 M urea, 4% acrylamide, 2% Igepal CA-630, 0.5% ampholyte pH 3.0–10, 0.5% ampholyte pH 4–6, 1.5% ampholyte pH
6–8, 0.01% ammonium persulfate, and 0.1% TEMED), and overlaid with 20 μl sample overlay buffer (4 M urea, 0.25% ampholyte pH 3.0–10, 0.25% ampholyte pH 4–6, 0.75% ampholyte pH 6–8, 2.5% -mercaptoethanol, 1% Igepal CA-360, and 0.05% Bromophenol blue). Isoelectric focusing (IEF) was conducted by using Mini-Protean 2-D Electrophoresis Cell (BIO-RAD). The upper and lower chamber buffers were 100 mM NaOH and 10 mM H₃PO₄ respectively. IEF conditions were 200 V for 15 min, 300 V for 15 min, 400 V for 30 min, and 750 V for 6 h. The focused tube gels were equilibrated immediately for 30 min in 10 ml SDS equilibration buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 0.05% Bromophenol blue), or kept at -20°C until use. After equilibration, the tube gels were embedded in a 1% agarose solution at the top of the 2-D gel. The second dimension was run on 15% polyacrylamide-SDS gels in a Mini-Protean 3 Cell (BIORAD), 120 V for 90 min. The gels were stained with Coomassie Brilliant Blue R250 and all gels were scanned and the spot intensities were analyzed using the software Image Master-2D (BIO-RAD).
4.3 Results and discussion

4.3.1 Disease severity
Germinating seeds of three groundnut varieties GG 11, GG 20 and GG 24 infected with *A. niger* showed 76%, 56% and 70% of disease severity respectively, which means GG 20 was found more tolerant compared to other two varieties. Survival of about 50% of seeds of GG 20 variety in spite of exposure to *A. niger* in soil generated our interest in investigating natural resistance mechanism in this variety. When pre-treated with 15 ppm zinc the disease incident were found 8% for GG 24 and 15% for GG 11 and 100% for GG 20 (Table 4.3.1). Similar results were repeated thrice and an average of disease severity was calculated. Disease was found to be aggravated in zinc treated GG 20 variety (fig. 4.3.1), which led us to estimate zinc levels in germinated seedlings.

<table>
<thead>
<tr>
<th>Variety</th>
<th>Seeds grown in presence of spores (1x10^6/ml) of <em>A. niger</em></th>
<th>Seeds pre-treated with 15 ppm of Zinc were grown in presence of spores (1x10^6/ml) of <em>A. niger</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24hrs</td>
<td>40hrs</td>
</tr>
<tr>
<td>GG 11</td>
<td>Nil</td>
<td>61%</td>
</tr>
<tr>
<td>GG 20</td>
<td>Nil</td>
<td>41%</td>
</tr>
<tr>
<td>GG 24</td>
<td>nil</td>
<td>58%</td>
</tr>
</tbody>
</table>

Table: 4.3.1 Disease severity in groundnut varieties.
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Fig: 4.3.1 Effect of Zinc on *A. niger* infected seedlings of GG-20 variety

after 48 hrs.

1= Seeds of GG-20 variety germinated in water for 48 hrs (control), 2 = Seeds of GG-20 variety infected with *A. niger*, 3 = Seeds of GG-20 variety pretreated with 15 ppm of Zinc and then infected with *A. niger*.

4.3.2 Zinc concentration

It was found that in GG 20 variety the rise in zinc level after application of 15 ppm of zinc sulphate solution was very negligible (about 0.2 mg/g of tissue), whereas in GG 11 and GG 24 zinc level rose by 0.5 to 1.0 mg/g of tissue. The increase in zinc in these two varieties indicated their zinc deficiency where as GG 20 was not deficient as zinc level did not rise in this variety (Fig. 4.3.2). Differential response to exogenous application of zinc was observed which might be due to sensitivity of particular variety. Davis et al. (2008) reported that groundnut is more sensitive to zinc than other crop.
Fig: 4.3.2 Zinc content of control and experimental seedlings exogenously applied with 15PPM Zinc in all three varieties. Error bars indicate ± SE.

4.3.3 NADPH oxidase

NADPH oxidase activity was found to increase in germinating seeds of all the three varieties infected with *A. niger* (Fig. 4.3.3a, b, c), indicating increase in ROS. Its activity increased and reached to its peak very rapidly (4 hrs post infection) in GG 11 and GG 24 varieties but in GG 20 its activity increased gradually to its peak at 40hrs post infection. The gradual increase of NADPH Oxidase in GG 20 variety can be interpreted as lesser ROS development, delayed death of host tissue and more resistance compared to GG 11 and GG 24, which corroborated with the disease severity results. Controlling ROS is one of the important effect against necrotrophic pathogen (Moshe and Robert, 2006), which could be achieved by controlling activity of NADPH oxidase. That Zinc exerts inhibitory effect on NADPH Oxidase in animal cells has been observed by other workers (Chvapil, 1979; Hammermuller et al., 1987). Application of 15 ppm of zinc resulted in decrease in activity of NADPH oxidase in GG 11 and GG 24 varieties. However in GG 20 variety, application of zinc induced its activity almost four times than control, indicating very high ROS levels and reasoning why disease went aggravated after zinc treatment in this variety. In GG 20 variety, increase in NADPH Oxidase activity due to zinc treatment could be due to its toxicity. Chaney (1993) has reported that zinc toxicity increases in acidic soil. Citric acid production by *A. niger* could also be the reason for disease aggravation due to zinc treatment in this variety.
Fig: 4.3.3a NADPH Oxidase activity in A. hypogaeae (GG11, GG20 and GG24 varieties) seedlings germinated for various times.

Sample identity: 11c =GG 11 control, 11z =GG 11 pre-treated with zinc, 11a =GG 11 infected with A. niger, 11az =GG 11 pre-treated with zinc and infected with A. niger. Error bars indicate ± SE.

Fig: 4.3.3b NADPH Oxidase activity in A. hypogaeae GG20 seedlings germinated for various times.

Sample identity: 20c =GG 20 control, 20z =GG 20 pre-treated with zinc, 20a =GG 20 infected with A. niger, 20 az = GG 20 pre-treated with zinc and infected with A. niger. Error bars indicate ± SE.
Fig: 4.3.3c NADPH Oxidase activity in *A. hypogaeae* GG24 seedlings germinated for various times.

Sample identity: 24c = GG 24 control, 24z = GG 24 pre-treated with zinc, 24a = GG 24 infected with *A. niger*, 24az = GG 24 pre-treated with zinc and infected with *A. niger*. Error bars indicate ± SE.

4.3.4 Glutathione reductase

Glutathione reductase (GR) activity was also found to increase very rapidly in germinating seed of GG 11 and GG 24 varieties post infection with *A. niger*, where as its activity raised gradually in GG 20 variety (fig. 4.3.4a, b, c). Increased GR activity was found as per the expectations as GR is involved in reducing oxidized glutathione produced due to increase in ROS.

Application of zinc brought down the activity of GR to what was seen in control samples in all three varieties. This could be due to decrease in NADPH Oxidase activity by zinc resulting in decrease in ROS. Zinc may not be playing role in expression of GR. Expression of various enzymes affected by zinc has been reported and GR is not one of them (Cakmak, 2000; Martin et al., 2007). GR is one of the key enzymes in the active oxygen scavenging system. Number of reports where the GR activity was shown to increase in various plants under different types of stresses has been found (Rao and Reddy, 2008; García-Limones et al., 2009).
Fig: 4.3.4a Glutathione reductase activity in *A. hypogaeae* GG11 seedlings germinated for various times.

Sample identity: **11c** = GG 11 control, **11z** = GG 11 pre-treated with zinc, **11a** = GG 11 infected with *A. niger*, **11az** = GG 11 pre-treated with zinc and infected with *A. niger*. Error bars indicate ± SE.

Fig: 4.3.4b Glutathione reductase activity in *A. hypogaeae* GG20 seedlings germinated for various times.

Sample identity: **20c** = GG 20 control, **20z** = GG 20 pre-treated with zinc, **20a** = GG 20 infected with *A. niger*, **20az** = GG 20 pre-treated with zinc and infected with *A. niger*. Error bars indicate ± SE.
Fig: 4.3.4a Glutathione reductase activity in *A. hypogaeae* GG24 seedlings germinated for various times.

Sample identity: **24c** =GG 24 control, **24z** =GG 24 pre- treated with zinc, **24a** =GG 24 infected with *A. niger*, **24az** =GG 24 pre- treated with zinc and infected with *A. niger*. Error bars indicate ± SE.

4.3.5 Total Anti-Oxidative Power/FRAP

FRAP value was found to be decreasing due to infection in all the three varieties (fig. 4.3.5 a, b, c). The FRAP value represents total antioxidative power of cell at particular time and level of oxidative stress by means of available antioxidant (Szollosi and Szollosi 2002). Decrease in FRAP value means increase in ROS. FRAP analysis corroborated our results of NADPH Oxidase and GR. Upon zinc treatment, FRAP value was found to be replenished very soon in all the three varieties.
Fig: 4.3.5a FRAP assay, Total Anti-oxidative/ total reducing power in *A. hypogeae* (GG 11) seedlings germinated for various times. Sample identity: 11c = GG 11 control, 11z = GG 11 pre- treated with zinc, 11a = GG 11 infected with *A. niger*, 11az = GG 11 pre- treated with zinc and infected with *A. niger*. Error bars indicate ± SE.

Fig: 4.3.5b FRAP assay, Total Anti-oxidative/ total reducing power μM/gm of tissue in *A. hypogeae* GG20 seedlings germinated for various times. Sample identity: 20c = GG 20 control, 20z = GG 20 pre- treated with zinc, 20a = GG 20 infected with *A. niger*, 20az = GG 20 pre- treated with zinc and infected with *A. niger*. Error bars indicate ± SE.
Fig: 4.3.5c FRAP assay, Total Anti-oxidative/ total reducing power µM/gm of tissue in *A. hypogaeae* GG24 seedlings germinated for various times.

Sample identity: 24c = GG 24 control, 24z = GG 24 pre-treated with zinc, 24a = GG 24 infected with *A. niger*, 24az = GG 24 pre-treated with zinc and infected with *A. niger*. Error bars indicate ± SE.

4.3.6 Salicylic Acid

Increased level of SA was found in all three varieties post infection of *A. niger* (fig. 4.3.6 a, b, c) SA level was found to be continuously increasing up to 72 h in GG-11 variety but in GG 20 and GG 24 varieties it reached to its peak post 40 h of infection. SA is the major signal and indication of plant defense mechanism (Gary and Murray, 2007; Glazebrook 2005; Vasyukova and Ozeretkovskaya, 2007). SA level was found to reduce to normal levels in zinc treated samples in all the three varieties, indicating role of zinc in controlling signaling molecule also.
Fig: 4.3.6a Salicylic acid µM/gm of tissue in *A. hypogaeae* GG 11 seedlings germinated for various times.

Sample identity: **11c** = GG 11 control, **11z** = GG 11 pre-treated with zinc, **11a** = GG 11 infected with *A. niger*, **11az** = GG 11 pre-treated with zinc and infected with *A. niger*. Error bars indicate ± SE.

Fig: 4.3.6b Salicylic acid µM/gm of tissue in *A. hypogaeae* GG20 seedlings germinated for various times.

Sample identity: **20c** = GG 20 control, **20z** = GG 20 pre-treated with zinc, **20a** = GG 20 infected with *A. niger*, **20az** = GG 20 pre-treated with zinc and infected with *A. niger*. Error bars indicate ± SE.

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Fig: 4.3.6c Salicylic acid μM/gm of tissue in *A. hypogaeae* GG24 seedlings germinated for various times.

Sample identity: 24c =GG 24 control, 24z =GG 24 pre- treated with zinc, 24a =GG 24 infected with *A. niger*, 24az =GG 24 pre- treated with zinc and infected with *A. niger*. Error bars indicate ± SE.

4.3.7 Total Oil Content and iodine no.

The variable disease severity inspired us to analyze differential lipids and proteins in resistant and susceptible varieties. The total oil content was found to be higher in GG 11 and GG 24 varieties then GG 20 (Table- 4.3.2). Iodine no was found to be highest in GG 20 (tolerant variety). Level of unsaturated Fatty acids (FAs) was found to be decreasing after 48 hrs of *A. niger* infection in all three varieties (Fig. 4.3.7). Total oil content may not be related to resistance but highest iodine no found in GG 20 (which indicated that more unsaturated fatty acid present in GG 20 variety) may be related to resistance. Level of unsaturated Fatty acids (FAs) was found to be decreasing after 48 hrs of *A. niger* infection in all three varieties which could be due to use of these FAs for the synthesis of signaling molecules. Biosynthesis of Jasmonic acid and other signaling molecules from unsaturated FAs is reported in infection of necrotrophic pathogen (Kachroo and Kachroo, 2009). Application of zinc increased the Iodine number and brought to the level of control samples.
### Table-4.3.2: Total oil content and iodine value of seeds

<table>
<thead>
<tr>
<th>Variety</th>
<th>Oil content mg/gm of seeds</th>
<th>Iodine value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG 11</td>
<td>260 mg(±7)</td>
<td>62.18(±0.3)</td>
</tr>
<tr>
<td>GG 20</td>
<td>203 mg(±6)</td>
<td>74.87(±0.6)</td>
</tr>
<tr>
<td>GG 24</td>
<td>278mg(±9)</td>
<td>57.105(±0.5)</td>
</tr>
</tbody>
</table>

**Fig: 4.3.7 Total oil content and iodine value of 48 hrs old germinating seedling of groundnut post 48 hr infection with *A. niger.*

Sample identity: **11c** = GG 11 control, **11z** = GG 11 pre- treated with zinc, **11a** = GG 11 infected with *A. niger*, **11az** = GG 11 pre- treated with zinc and infected with *A. niger*, **20c** = GG 20 control, **20z** = GG 20 pre- treated with zinc, **20a** = GG 20 infected with *A. niger*, **20 az** = GG 20 pre- treated with zinc and infected with *A. niger*, **24c** = GG 24 control, **24z** = GG 24 pre- treated with zinc, **24a** = GG 24 infected with *A. niger*, **24az** = GG 24 pre- treated with zinc and infected with *A. niger*. Error bars indicate ± SE.
4.3.8 Proteome analysis

Native PAGE profiling of all three varieties post infection with *A. niger* was carried out which showed one induced protein band in GG20 variety (fig. 4.3.8). Molecular mass of induced protein was found to be 110 kD by PAGE as well as by molecular size exclusion chromatography. Purified protein (fig. 4.3.10) was subjected to SDS-PAGE for subunit confirmation, which showed that this protein contain three sub units of about 41 kDa, 39 kDa and 30 kDa (fig. 4.3.11).

![Figure 4.3.8 Native PAGE of showing induced protein](image)

Well no. M= molecular weight marker, 1 =GG 11 control, 2=GG 11 infected with *A. niger*, 3 =GG 20 control, 4=GG 20 infected with *A. niger*, 5 =GG 24 control, 6=GG 24 infected with *A. niger*.

4.3.9 Quantification of induced protein

Densitometric quantification of induced protein was carried out which showed continued induction of this protein from 24 to 72 hrs (fig. 4.3.9) and its concentration was found to be increased as the disease progressed. Maximum induction was found three fold; after 42 hrs of pathogen infection (Table 3.4.3)
<table>
<thead>
<tr>
<th>Lane</th>
<th>Area</th>
<th>Conc. (µ gm)</th>
<th>Rf</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 BSA</td>
<td>2549</td>
<td>20</td>
<td>0.5364</td>
</tr>
<tr>
<td>2 BSA</td>
<td>3171</td>
<td>25</td>
<td>0.5330</td>
</tr>
<tr>
<td>3 BSA</td>
<td>3466</td>
<td>30</td>
<td>0.5353</td>
</tr>
<tr>
<td>4 BSA</td>
<td>4055</td>
<td>35</td>
<td>0.5330</td>
</tr>
<tr>
<td>72a</td>
<td>4347</td>
<td>36.72</td>
<td>0.2917</td>
</tr>
<tr>
<td>48a</td>
<td>4391</td>
<td>38.72</td>
<td>0.2977</td>
</tr>
<tr>
<td>24a</td>
<td>2639</td>
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<td>0.2857</td>
</tr>
<tr>
<td>72c</td>
<td>2900</td>
<td>23.23</td>
<td>0.3057</td>
</tr>
<tr>
<td>48c</td>
<td>1290</td>
<td>6.51</td>
<td>0.2677</td>
</tr>
<tr>
<td>24c</td>
<td>1482</td>
<td>8.507</td>
<td>0.2797</td>
</tr>
</tbody>
</table>

Table 3.4.3 Densitometric analysis of induced protein in GG20 variety

Figure 4.3.9 Densitometric analysis of induced protein in GG20 variety.
4.3.10 Purification and sequencing of protein

Sequencing of all three peptide was carried out by LC–ESI–MS/MS. This sequence segment was searched for homologous sequences using MASCOT data base program in which 41 kDa peptide [MLVESEGR] shown maximum score 44 for Hypothetical protein (Q1T5F9_MEDTR) *Medicago truncatula*, 39 kDa peptide [AAFLNNDYTK] shown maximum score 96 for p27SJ (gi|57868106) *Hypericum perforatum*. As far as protein identity concern more than 43 MASCOT score would be identical for protein identification (Jouili et al., 2008) but in sequenced peptides were not found significant score for any reported protein sequences of groundnut or other plant defense related proteins. Even when the expected value parameters were increased, it yielded the same result. The searches were done against non redundant database. Further, no match to this sequence was found from a search on the Express Sequence Tag of groundnut, shows that the sequence may be unique. Praxedes et al., (2011) reported similarly 51 kDa novel anti fungal protein from seeds of *Sesbania virgata*. Mitra et al. (2008) reported similarly novel cadmium induced protein in wheat. Varietal differences of protein profiles in mature groundnut seeds were investigated and identified 20 proteins by Kottapalli et al. (2008). More than 250 groundnut leaves protein identified by Katam et al. (2010). Although many PR proteins reported by different author in plants, there were very less emphasis found on identification of defense related protein in groundnut in literature.

![Figure 4.3.10 Native PAGE of showing induced and purified protein](image)

**Figure 4.3.10 Native PAGE of showing induced and purified protein**

Well no. M= molecular weight marker, 1 =GG 11 control, 2=GG 11 infected with *A. niger*, 3 =GG 20 control, 4=GG 20 infected with *A. niger*, 5 =GG 24 control, 6=GG 24 infected with *A. niger*. 
Figure 4.3.11 SDS PAGE of Purified protein showing tri-peptide

Well no. **M** = molecular weight marker, **1** = Purified tripeptide

4.3.11 Bioassay of partially purified protein

Direct antifungal activity assay of partially purified protein revealed that this protein do not have any growth inhibiting activity on *A. niger*. However to our surprise this protein showed decrease infection severity of *A. niger* and increased rate of germination when applied on germinated seedling prior to 4 hrs of infection (fig. 4.3.12). Praxedes et al. (2011) reported similarly novel *A. niger* inhibiting protein from seeds of *Sesbania virgata*. Thus, we assume that this induced protein is a unique and novel protein which is not reported earlier in groundnut. The complete sequence of induced protein and its functional role against *A. niger* however, await future investigation.

Figure 4.3.12 Bioassay of partially purified protein
4.3.12 2D PAGE analysis of GG20 variety infected with *A. niger*

Proteome analysis of GG 20 variety post 40 hrs infected with *A. niger* carried out using 2D PAGE which shown 124 differentially induced protein in infected germinating seed than control (fig. 4.3.13). Similarly Kottapalli et al. (2008) carried out Proteomics analysis of mature seed of four peanut cultivars using two-dimensional gel electrophoresis.

**4.3.13 2D PAGE analysis of GG20 variety infected with *A. niger***

<table>
<thead>
<tr>
<th>Gel Image Name</th>
<th>Spot Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>2D fine spot G20 after 72 hrs ...infected</td>
<td>911</td>
</tr>
<tr>
<td>2D fine spot G20 after 72 hrs ...control</td>
<td>787</td>
</tr>
</tbody>
</table>

Software used: PDQUEST
4.4 Conclusion

Increase of NADPH Oxidase and GR activity and decrease in FRAP value due to \textit{A. niger} infection indicated that the mechanism of pathogenesis is by oxidative stress. This may be true for all necrotrophic pathogens’ infections to plants. Possible role of zinc in protecting \textit{A. hypogaeae} seedlings from damage by ROS has been studied. Zinc treatment could protect GG 11 and GG 24 varieties from disease probably due to its antioxidant properties; however GG 20 variety succumbed to death very soon. Disease aggravation in GG 20 variety due to zinc treatment could be due to its toxicity at higher concentration. Treatment of higher concentration of \textit{ZnSO}_4 solution caused similar toxicity in GG 11 and GG 24 varieties. So optimum concentration of zinc is essential for development of natural disease resistance in \textit{A. hypogaeae} against \textit{A. niger}. We report for the first time, a 110-kDa \textit{A. niger} induced protein. Using bioassay result we assumed that this protein have role in defense response against \textit{A. niger} in groundnut. This 110 kDa trimeric protein seems to be a new protein, as search for the occurrence of other such biotic stress proteins in ground nut plant do not show homology to this induced protein. The exact role and nature of this protein however, awaits further investigations. Proteome analysis using 2D PAGE showed more than 100 down regulated protein expressed due to \textit{A.niger} infestation.
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4.5 References


Horowitz W (1975) *Official Methods of Analysis of AOAC*:488


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