2.1 Introduction:

The tikka late leaf spot disease caused by *Cercosporidium personatum* results in extensive damage to the leaf area of groundnut (*Arachis hypogeae* L.) plants. When groundnut leaves are infected with *C. personatum*, brown spots spread rapidly and they eventually cause premature defoliation. The most economic and effective method of leaf spot control, however, would be to use an agronomically acceptable disease resistant variety. In the absence of resistance, copper and sulphur based fungicides which are used to control the leaf spot disease. The use of resistant cultivars is a more sustainable alternative method of control. However, groundnut plants with resistance to *C. personatum* have not yet been reported. Therefore, it is necessary to find approaches for obtaining groundnut plants resistant to this pathogen. Problems related to leaf spot diseases cause nearly complete defoliation and yield losses more than 50% (Shew et al., 1988). However, in groundnut plants, there are fewer reports on evaluation of biochemical responses for resistance trait against biotic stress. An improved understanding of resistance mechanisms in cultivated groundnut could provide insights into developing strategies to engineer durable and broad-spectrum resistance.

Fig.2.1 Disease cycle for late leaf spot caused by *C. personatum*

With the ever increasing speed of sequencing of genes, the emphasis of research is moving towards finding the function of genes. The focus was first on proteomics, as there is a logical link between the sequence of a gene and the amino
acid sequence of a protein. However, such a sequence does not give much information about the function of the protein. For most proteins the function is best reflected by the phenotype. The characterization of the phenotype thus has become an important objective, and here metabolomics comes into the picture. In recent years, more and more attention is being paid to the chemical characterization of the phenotype. Chemical characterization of macromolecules (e.g., proteomics and characterization of polysaccharides and lignins) or low molecular weight compounds (the metabolome) can be done. The biochemical defense mechanism may consist of the presence or absence of a particular chemical substance or a group of substances in a host plant which interferes with the growth and multiplication of the pathogen. The biochemical may be present before infection or may be produced by the interaction of the host and pathogen. Plants develop a complex variety of defense responses when infected by pathogens. The synthesis of new proteins that can have direct or indirect action on the course of pathogenesis is an ubiquitous response of monocot and dicot plants to pathogen attack (Lamb et al., 1989; Bowels, 1990). These induced proteins include cell wall proteins, enzymes involved in phenylpropanoids and flavonoid metabolism, toxic proteins (thionins), proteins with antimicrobial properties (enzyme inhibitors), oxidative enzymes, lytic enzymes and heterogenous group of proteins collectively known as pathogenesis related (PR) proteins. Early release of preformed phenolics and their later intensive production after stimulation of phenylpropanoid metabolism are a part of resistance reactions to disease in many plants (Peltonen, 1998). Phenylpropanoid pathway has received much more attention due to secondary metabolites such as lignin, phenolic acids and phytoalexins, which are responsible for adding mechanical rigidity and strength to cell walls and for providing barriers to infection by pathogen (Samia and Khallal, 2007). Phenylalanine ammonia lyase (PAL) is a key enzyme in the phenylpropanoid metabolism and plays a significant role in the regulation of phenol biosynthesis in plants as a response to pathogen infection (Wen et al., 2005). Phenylalanine ammonia lyase (PAL) catalyzes the deamination of L-phenylalanine to trans-cinnamic acid, which is the first step in the biosynthesis of large group of plant products such as lignin monomers and phytoalexins. Induction of phenylalanine ammonia lyase, peroxidase and the accumulation of phenolics occur in response to pathogen attack. PAL is the key enzyme in inducing the synthesis of salicylic acid (SA), which is responsible for inducing systemic resistance in many plants.
Polyphenol oxidase (PPO) accumulation takes place upon wounding in plants. PPO is induced via octadecanoic defense signal pathway which is usually associated with feeding by insects or similar physical trauma. It involves jasmonic acid as an intermediate signal and culminates in the production of proteins such as PPO and proteinase inhibitors (Schaller et al., 1995). Application of jasmonic acid results in induction of PPO and proteinase inhibitors resulting in decrease in the preference, performance and abundance of the pathogens. The activation of phenylpropanoid pathway in plants by environmental stimuli is one of the universal biochemical stress responses known. Phenolic compounds are common constituents of many plants. They include simple phenols, coumarins, most flavanoids and certain amino acids, prosthetic groups of some enzymes, plant pigments and complex derivatives such as lignins. Phenolic substances are known to participate in a number of biochemical processes, such as oxidation-reduction reactions and stimulation as well as inhibition of auxin activity. Phenolic compounds occur in a variety of simple and complex forms. Simple phenols such as cinnamic acid, coumarin, caffeic acid, protocatechoic acid, chlorogenic acid and quinic acid exhibit antimicrobial activities. Infection in certain diseases is characterized by increased synthesis of certain precursors of phenolic compounds and oxidation products of phenolics, such as quinines, which exhibit more toxicity to microorganisms than their reduced forms. In many instances, there is a positive correlation between the amount of phenolic content and degree of resistance to plant disease. Peroxidases, besides their main function as antioxidant enzyme, can also catalyze super oxide radical (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) formation by a complex reaction in which NADH is oxidized using trace amounts of H$_2$O$_2$ first produced by the non-enzymatic breakdown of NADH. Next, the NAD radical formed reduces O$_2$ to O$_2^-$, some of which dismutases to H$_2$O$_2$ and O$_2$ (Lamb and Dixon, 1997). Expressions of plant peroxidases have been implicated in a variety of defense-related processes including hypersensitive reaction, lignification, cross-linking of phenolics and glycoproteins, suberisation and phytoalexin production (Baysal et al., 2003). Peroxidase (PO) are involved in the regulation of plant cell elongation, phenol oxidation, polysaccharide cross-linking, IAA oxidation, oxidation of hydroxy cinnamyl alcohols into free radical intermediates and wound healing. These enzymes are involved in the polymerization of proteins and lignin or suberin, which acts as precursors in the plant cell wall, thus constructing a physical barrier that could prevent pathogen penetration through cell walls or movement through vessels.
Similarly catalase is also a part of plant defense system acting as radical scavenging enzyme. Efficient induction of hypersensitive cell death requires a balance between ROIs and NO production such that high levels of NO are ineffective in the absence of a correspondingly strong oxidative burst. The effects of NO depend on many factors including rates of production and diffusion, levels of ROIs, and the activities of ROI scavengers such as superoxide dismutase (SOD) and catalase (Massimo et al., 2000). The disposal of peroxide, which is toxic in high concentration, is done by catalase. Catalase is a four subunit heme protein, catalyzing the release of oxygen from hydrogen peroxide according to the equation –

$$2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$$

To this end, catalase is frequently used by cells to rapidly catalyze the decomposition of hydrogen peroxide into less reactive gaseous oxygen and water molecules. (Keen and Buzzell, 1991). Zinc and iron have definite role in plant growth and plant defense mechanism directly or indirectly. Role of zinc and iron beside their nutritional value, elaborated in oxidative and antioxidative mechanism while biotic and abiotic stress in plant by many researcher.

This study aimed to examine whether constitutive defense mechanisms are governing resistance in A. hypogaea plants against C. personatum. In the present study locally available Groundnut genotypes were selected based on its popularity especially in Saurashtra region of Gujarat. Present chapter describes the five varying genotypes of Groundnut namely GG 2, GG 5, GG 13, GG 20 and Desi, which were evaluated for total phenol, total antioxidative power, Catalase, Peroxidase, PAL, PPO, Glutathione reductase, NADPH Oxidase activities, concentration of Zinc and Iron. All the parameters were co-related with naturally occurring leaf spot disease incidents in all five varieties of groundnut.
2.2 Materials and Methods

2.2.1 Glass ware

All glassware were soaked in mild chromic acid solution for overnight, cleaned with lab wash, water and rinsed thoroughly with double distilled water.

2.2.2 Reagents

Chemicals used for the preparation of reagents were of analytical grade, obtained from standard companies (Sigma, Hi Media, Merck and Bangalore Genei).

2.2.3 Plant material

All the five different varieties of groundnut plants namely GG 2, GG 5, GG 13, GG 20 and Desi were obtained from National Research Center for groundnut, Junagadh, Gujarat. These were grown in pot and maintained in the departmental premises as per the local farming practices. Samples were collected randomly from all the varieties of healthy plants grown post 30 days. The experiments were repeated three times.

2.2.4 Morphological characteristic

Morphological characteristic were noted in all five varieties of groundnut (GG 2, GG 5, GG 13, GG 20 and Desi) at the different stages of growth up to harvest.

2.2.5 Isolation and identification of pathogens

2.2.5.1 Aspergillus niger

\textit{A. niger}, a causative agent of Collar rot disease, was isolated from infected groundnut plant obtained from Agricultural University Junagadh, Gujarat, India. Infected plant part was stamped on PDA plates and grown for 72 hrs. \textit{A. niger} was isolated from this plate by sub culturing and its identity was confirmed by studying spore morphology using a light microscope. It was maintained on PDA plate by sub culturing regularly every 15 days. Inoculum was prepared by flooding sterilized water on sporulated plate and spores were collected and counted using Neubauer chamber. A suspension containing $10^5$ spores /ml was prepared and used for infection of plants.

2.2.5.2 Cercospora sp.

\textit{Cercospora} was isolated by exposing vertically excised and surface sterilized infected groundnut leaf portion to white light illumination for 48 hrs and then stamping it on Peanut Oat-Meal agar plates and grown for 3 days. It was purified by
sub-culturing many times. The isolated fungi was grown for 8 days in the same medium and subjected to amplification and sequencing of Internal Transcribed Spacer (ITS) sequence of 18s rDNA.

**DNA extraction.**

Genomic DNA was extracted by a slightly modified method described by Crespo et al. (1997). About 1 to 2 mg of dry ice-frozen mycelia and diseased plant leaf material were ground to a fine powder in 100 ml of 2% cetyltrimethyl ammonium bromide (CTAB), using a mortar and pestle on dry ice. The ground material was suspended in 400 ml of CTAB with 1% b-mercaptoethanol and incubated at 60°C for 30 min. An equal volume of chloroform-isoamyl alcohol (24:1 v/v) was added, mixed, and centrifuged at 13,000 g for 10 min. The upper aqueous layer was collected. DNA was precipitated by the addition of 0.54 volume isopropanol and centrifuged for 5 min. The pellet was re suspended in 700 µl of 1 mM TE buffer and 20 units of ribonuclease, then incubated for 30 min at 37°C. Further purification was achieved by extraction with equal volumes of chloroform-isoamyl alcohol. DNA was finally precipitated by adding 2 volumes of 95% ethanol. The pellet was dried and re dissolved in 100 µl of 1mM TE buffer and stored at 20°C until used.

**PCR amplification.**

Two primers, ITS4 Reverse primer (TCCTCCGCTTATTGATATGC) and ITS1 Forward primer (TCCGTAGGTAACCTGCGG) were used (White et al., 1990). The 100 µl PCR reaction mixture contained 10 ml Taq PCR buffer, 2 ml of 2.5 mM dNTPs about 300 mM each of ITS4 and ITS1, 3 µg of genomic DNA, 25 units of Taq polymerase (Genpak Ltd.), 3 mM MgCl₂, and 55 µl of sterile distilled water. The mixture was gently vortexed and centrifuged briefly to collect the sample at the bottom, then overlaid with 100 ml of sterile mineral oil. The amplification parameters were standardized and PCR amplification products were detected by agarose gel electrophoresis; purified by GeNei PCR product purification Kit and sent for sequencing to Xcelris genomic Pvt. Ltd. Ahmedabad.

2.2.6 Leaf spot Diseases incidence

Disease assessment of naturally infected all five varieties were done manually on 100 day old plants. Total infected leaves from randomly selected 100 plants from each variety were calculated and % average disease incidence was measured.
2.2.7 Phenol

Leaf samples were homogenized in 10 ml of 80% methanol and agitated for 15 min at 70°C. To 1 ml of the extract 5 ml of distilled water and 5 ml of Folin-ciocalteau reagent were added and incubated at 25°C for 3 min. After that 1 ml of 20% sodium carbonate was added and mixed well. Then the tubes were placed in boiling water for 1 min and cooled. The absorbance was read at 750 nm and catechol was used as the standard. The total phenol content was expressed in mg of catechol/g of fresh tissue (Zieslin and Ben Zaken, 1993).

2.2.8 Phenylalanine ammonia lyase (PAL)

One gm of groundnut leaves was homogenized in 2 ml of ice cold 0.1 M phosphate buffer, pH 7.0 and centrifuged at 10000 rpm for 20 min at 48°C. The supernatant was used to assay the enzyme activity. PAL activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid (Dickerson et al., 1984). Sample extract of 0.4 ml was incubated with 0.5 ml of 0.1 M borate buffer, pH 8.8 and 1 ml of 12 mM L-phenylalanine and incubated for 1 h at 30°C. The reaction was initiated by the addition of L-phenylalanine and stopped with 0.5 ml of 2 N HCl. A blank was maintained by adding L-phenylalanine after the addition of 2 N HCl. The absorbance was read at 290 nm and the results were expressed as nmol transcinnamic acid formed/min/g of fresh tissue.

2.2.9 NADPH Oxidase

NADPH Oxidase activity was determined according to Cuevas et al. (2004). Plant material was frozen in LN₂ and grounded to fine powder with a pre-chilled mortar and pestle. Powdered samples (1gm) were homogenized in 5 ml HEPES-KOH buffer (pH 7.8) containing 250 mM sucrose and 0.1mM EDTA and the homogenate was centrifuged at 10,000 rpm for 15 min. The 200 µl supernatant of sample was added to the reaction buffer (50 mM HEPES-KOH pH 7.8, 100 µM EDTA and 1 µM KCN) in final volume of 1 ml. Samples were pre-incubated at 30°C for 1 min and the reaction was initiated by addition of 100 µM NADPH. NADPH oxidation rate was determined following the decrease in the absorbance at 340 nm for 5 min with UV-Visible spectrophotometer and calculated by the extinction coefficient of 6.2 mM⁻¹ cm⁻¹. Enzyme activity was expressed as nM/mg protein.
2.2.10 Polyphenol oxidase (PPO)

The reaction mixture consisted of 1.5 ml of 0.1 M sodium phosphate buffer pH 6.5 with 0.1 ml of enzyme extract. To this 0.2 ml of 0.01 M catechol was added to initiate the reaction. The change in absorbance was recorded at 495 nm and the results were expressed as change in absorbance/min/g of fresh tissue (Mayer et al., 1965).

2.2.11 Total Antioxidant power/ FRAP analysis

Ferric reducing antioxidant power (FRAP) analysis was carried out according to Szollosi and Szollosi (2002). One gm leaves of all five varieties were powdered using LN\textsubscript{2}. It was homogenized in 9 ml cool 0.1 M phosphate buffer (pH 7.6, containing 0.1 mM EDTA) in a pre-chilled mortar and pestle. This mixture was filtered through a filter paper and centrifuged at 15000 rpm for 10 min. The supernatant was used for the measurements. 1.5 ml of FRAP reagent and 5 ml of plant extract were mixed and absorbance was taken at 593 nm. FRAP reagent (25 ml acetate buffer 300 mM pH 3.6, 2.5 ml 10 mM 1,2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl, and 2.5 ml 20 mM FeCl\textsubscript{3} 6 H\textsubscript{2}O in distilled water) was used as a blank. Aqueous solution of known Fe (II) concentration was used for calibration (100-1000 µM).

2.2.12 Glutathione Reductase

GR activity was determined at 25°C according to Christophe et al. (1996). One gm of leaves of all five varieties were powdered using LN\textsubscript{2} and was homogenized using a cool mortar and pestle in 0.1 M phosphate buffer (pH 7.6, containing 0.1 mM PMSF) and centrifuged at 15000 rpm for 10 min. The supernatant was used for GR activity by analyzing the rate of NADPH oxidation at 340 nm. The assay mixture contained 0.5 mM NADPH, 10 mM oxidized glutathione, 3 mM MgCl\textsubscript{2} in 0.1 ml phosphate buffer (pH 7.3), and 100 µl enzyme extract in a total volume of 2 ml, GR activity was expressed as µM NADPH oxidized /mg protein/min.

2.2.13 Catalase

The enzyme activity was evaluated by estimating the residual H\textsubscript{2}O\textsubscript{2} in the reaction mixture with the help of KMnO\textsubscript{4} titrimetrically (Barber, 1980). The enzyme activity was expressed against reference reaction mixture containing enzyme extract in an acidified solution at zero time, using its extinction coefficient of 0.036 \( \mu \text{mol} \cdot \text{ml}^{-1} \cdot \text{l}^{-1} \) and expressed as µgm min\textsuperscript{-1} ml\textsuperscript{-1}.

---

Chapter 2

Biochemical evaluation of........ 81
2.2.14 Peroxidase (PO)

One gm of leaf sample was homogenized in 1 ml of 0.1 M phosphate buffer pH 7.0 in a pre-cooled pestle and mortar. The homogenate was centrifuged at 10000 rpm for 20 min at 48°C. The supernatant was used to assay activities of PO and PPO. The activity of peroxidase was determined as detailed by Hammerschmidt et al. (1982). The reaction mixtures viz. 1.5 ml of 0.05 M pyrogallol and 0.1 ml of enzyme extract were taken and added to the cuvette. The reaction was initiated by adding 0.5 ml of 1% H$_2$O$_2$. The change in absorbance was recorded at 420 nm at 30 s intervals for three minutes from zero seconds of incubation at room temperature. The results were expressed as change in absorbance/min/g of fresh tissue.

2.2.15 Zinc and Iron analysis

Determination of zinc and iron in all five varieties was carried out according to Laura et al. (2003). 1g of leaves were collected and mineralized by heating in muffle furnace at 825±25°C for 2h. Cooled remnant were digested by addition of 10 ml diluted HNO$_3$ (1:1). The resultant solution were diluted to 25 ml with doubled distilled water and analyzed. Zinc and Iron determinations were carried out by direct aspiration into an air acetylene flame atomic absorption spectrometer fit with zinc and iron hallow cathode lamp.
2.3 Results and Discussion

2.3.1 Morphological Features of all five varieties

Morphological features of all the varieties were noted (Table-2.3.1). The data showed that GG 2 and GG 5 share common morphological features where as GG 20 and Desi have similar features.

<table>
<thead>
<tr>
<th>Variety</th>
<th>GG-2</th>
<th>GG-5</th>
<th>GG-20</th>
<th>Desi</th>
<th>GG-13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Life time (days) cycle</td>
<td>110-115</td>
<td>110-115</td>
<td>125-130</td>
<td>130-135</td>
<td>120-125</td>
</tr>
<tr>
<td>Initiation of flowering time (days)</td>
<td>24-30</td>
<td>24-30</td>
<td>30-40</td>
<td>30-40</td>
<td>30-40</td>
</tr>
<tr>
<td>Seed</td>
<td>two-seeded pod</td>
<td>two-seeded pod</td>
<td>two-seeded pod</td>
<td>three-seeded pod</td>
<td>two-seeded pod</td>
</tr>
<tr>
<td>Colour</td>
<td>pinkish-brown</td>
<td>pinkish-brown</td>
<td>light-pink</td>
<td>dark-brown</td>
<td>light-pink</td>
</tr>
<tr>
<td>Shape</td>
<td>oval</td>
<td>oval</td>
<td>elongated</td>
<td>elongated</td>
<td>elongated</td>
</tr>
<tr>
<td>Size</td>
<td>small</td>
<td>small</td>
<td>big</td>
<td>big</td>
<td>big</td>
</tr>
<tr>
<td>Habit</td>
<td>bunch</td>
<td>bunch</td>
<td>runner</td>
<td>runner</td>
<td>semi-runner</td>
</tr>
</tbody>
</table>

Table-2.3.1 Morphological features of five groundnut varieties

2.3.2 Isolation of the two pathogens used in this study

Isolation of *Cercospora* sp.

*Cercospora* species was isolated by exposing vertically excised and surface sterilized infected groundnut leaf portion to illumination for 48 hrs and then stamping it on Peanut Oat-Meal agar plates and grown for 3 days. It was purified by sub-culturing many times. The isolated fungi was grown for 8 days in the same medium and subjected to amplification of about 550bp fragment of 18s ribosomal DNA and sequencing of Internal Transcribed Spacer (ITS) sequence of 18s ribosomal DNA. The following cycling parameters were standardized: initial denaturation at 94°C for 3 min, primer annealing at 56°C for 90 s. The 550 bp sequence alignment with database of fungi using BLAST showed only 90% similarity to *Fusarium*. The symptom analysis study in field however supported that this fungus could be *Cercospora*. Since, there could be doubt on identity of this fungus maintained in lab in Peanut Oat-
Meal Agar, to study defense responses infected plants were brought from the field every time.

**Fig. 2.3.2.1 Isolation of Cercospora sp.**

**Fig. 2.3.2.2 Amplification of 18s ribosomal DNA of isolated pathogen.**

1 and 2: PCR products, M: 100 bp ladder

Sequence obtained using ITS1 Forward primer

TTGGCWCGAGTGCTCTCCCAACCCCTGTGACATACCTATACGTTCCTCSG
YGGATCAGCCCGCCGCCAGAGAAGGACCGGCGCCGCCAGGACCC
CTAAACTTCTGTGTTTTAGTGGAACTTCTGAGTAAAAAAACAAATATATGC
AAAATTTCAACACGGATCTCTTGGTTTCTGGGATCTGGAAGAAGCGGACG
CAAAATTCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCAGAAT
CTTTGAACGCACATTTCGCCCCCAAGTATTCTGGCTGAGCACCTGCTTTGCG
AGCGTCATTTCACCCCTCAAGCTCAGCTTGTTGGGACTCGGCTGTAACC
CGCGTTCCCCAAATCGATTGGCGGKACGTGAGCTTCCATAGCGTAGTA
ATCATAACCTCGTTACTGGTAATCGTCGGCCACCGCAYTAAACCCCA
Sequence obtained using ITS4_Reverse primer

Isolation of *A. niger*

The pathogen isolated from samples showing Collar rot disease showed sporulation on PDA after 48 hrs. When observed under light microscope, the morphological characters and sporulation pattern confirmed the isolate as *A. niger*. Inoculum was prepared by flooding sterilized water on sporulated plate and spores were collected and counted using Neubauer chamber. A suspension containing $10^5$ spores /ml was prepared and used for infection.

*Fig. 2.3.2.3 Isolation of A. niger*
2.3.3 Disease incidence of late leaf spot on five groundnut cultivars

The modified 9-point scale for late leaf spot as given by Subbarao et al. (1990) was used for screening groundnut varieties (Table 2.3.3.1). The visual scores 1-9 and extent of leaf area damage (1-100%) are linearly related. The field disease scores are mainly based on the extent of leaf area damaged.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Description</th>
<th>Disease severity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No disease</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Lesions present largely on lower leaves; no defoliation</td>
<td>1-5</td>
</tr>
<tr>
<td>3</td>
<td>Lesions present largely on lower leaves, very few on middle leaves; defoliation of some leaflets evident on lower leaves</td>
<td>6-10</td>
</tr>
<tr>
<td>4</td>
<td>Lesions present on lower and middle leaves but severe on lower leaves; defoliation of some leaflets evident on lower leaves</td>
<td>11-20</td>
</tr>
<tr>
<td>5</td>
<td>Lesions present on lower and middle leaves, over 50% of defoliation of lower leaves</td>
<td>21-30</td>
</tr>
<tr>
<td>6</td>
<td>Severe lesions on lower and middle leaves; lesions present but less severe on top leaves; extensive defoliation of lower leaves; some defoliation of middle leaves</td>
<td>31-40</td>
</tr>
<tr>
<td>7</td>
<td>Lesions on all leaves but less severe on top leaves; defoliation of all lower and middle leaves</td>
<td>41-60</td>
</tr>
<tr>
<td>8</td>
<td>Defoliation of all lower and middle leaves; severe lesions on top leaves evident.</td>
<td>61-80</td>
</tr>
<tr>
<td>9</td>
<td>Almost all leaves defoliated, leaving bare stem; some leaflets my remain, but show severe leaf spot</td>
<td>81-100</td>
</tr>
</tbody>
</table>

Table 2.3.3.1 Modified 9-point scale used for screening groundnut genotypes for resistance to late leaf spot.
The highest disease incidence of the late leaf spot disease was observed in GG 5 variety of groundnut cultivar, followed by GG 20, GG 2 and Desi variety. The lowest disease incidence was observed in GG 13 variety (Table-2.3.2).

<table>
<thead>
<tr>
<th>Variety</th>
<th>% Disease incident</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG 2</td>
<td>65.33%</td>
</tr>
<tr>
<td>GG 5</td>
<td>79.29%</td>
</tr>
<tr>
<td>Desi</td>
<td>54.0%</td>
</tr>
<tr>
<td>GG 20</td>
<td>70.7%</td>
</tr>
<tr>
<td>GG13</td>
<td>30.66%</td>
</tr>
</tbody>
</table>

Table 2.3.3.2 Disease incidence

2.3.4 Total Phenols

Total phenol concentration was found to be higher in GG 5 and Desi, where as lower in GG 20, GG 2 and GG 13 varieties (Fig. 2.3.4). Phenolic compounds are the most important group implicated in both constitutive and induced resistance and a distinct correlation between the degree of plant resistance and phenolics present in plant tissue has been demonstrated. In the present study the level of constitutive phenols did not indicate distinct pattern in susceptible and resistant genotypes. Similar observations were recorded at pre-infection stage by Gogoi et al. (2001) in Karnal bunt susceptible wheat genotype WL-711 and in grey mildew susceptible cotton lines by Chakrabarty et al. (2002). Pollock and Drysdale (1976) reported low quantity of phenolic compounds in resistant tomato cultivars than the infected one. Brammachari and Kolte (1983) reported a decrease in total phenols in groundnut cultivars resistant towards *Cercospora*. Similar results have also been reported in muskmelon varieties resistant to powdery mildew (Jindal et al., 1979). The total phenol content was increased in susceptible genotypes of pearl millet without conferring any resistance. Such stimulated production of phenolics, following the invasion by infectious agents, is a physiological syndrome in host-pathogen interactions and is probably due to increase in peroxidase activity in present study (Fig. 2.3.7). Consistently, higher phenolic content has been reported in grape twigs infected with greening pathogens (Singh et al., 1982), in leaf spot infected turmeric leaves (Agarwal et al., 1982), and in susceptible muskmelon varieties infected with powdery mildew (Jindal et al., 1979).
2.3.5 Phenylalanine Ammonia Lyase Activity (PAL)

Phenylalanine Ammonia Lyase activity were found higher in GG 5 and Desi than other three varieties (Fig. 2.3.5). PAL is primary enzyme for synthesis of phenols in plant showing positive co-relation with total phenol content in groundnut. Induced PAL activity was marker for resistance in many plants but constitutive higher activity did not show relevance with resistance trait in groundnut. The constitutive level of PAL was higher in susceptible genotypes. Shiraishi et al. (1995) found that PAL activity increased regardless of the resistance or susceptibility of the barley cultivar to the powdery mildew fungus. Nagarathana et al. (1993) also reported higher PAL activity after 24 h post-inoculation of downy mildew pathogen in susceptible genotypes of pearl millet compared to resistant genotypes. In contrast, the PAL activity increased significantly in resistant genotypes (120.7-155.5%) of rapeseed after inoculation with Albugo candida compared to susceptible genotype Varuna (67%) (Jain et al., 2002). The higher activity of PAL in susceptible genotypes is in agreement with higher phenol content and POX activity in present study. This indicates that the response of PAL activity was not associated specifically with either the susceptibility or the resistance of the genotypes to Leaf spot pathogen. Thus, the augmentation of PAL activity can not reflect a response of either host that can be specifically attributed to the expression of susceptibility or resistance. In fact, it may be a general response of the tissue to infection, a phenomenon possibly similar to a
general non-host resistance response to stress (Fernandez and Heath, 1989). Such a hypothesis is supported by the observations of Cho and Smedegaard-Petersen (1986), who demonstrated that inoculation of whole barley leaves with either compatible or incompatible races of *Erysiphe graminis* f. sp. *hordei* resulted in an initial expression of induced resistance to subsequent inoculation even with a compatible race of the fungus. Thus, one might presume that the initial response is a general expression of resistance.

![Fig. 2.3.5 PAL activity in five groundnut varieties.](image)

### 2.3.6 NADPH oxidase Activity

Similar to Glutathione Reductase Activity, NADPH oxidase Activity was found higher in 45 day old plant than in 30 day old plant in all the varieties (Fig. 2.3.6). NADPH oxidase is known for its instant activity while any biotic or abiotic stress occurs in plant. Among all the varieties, GR activity found almost similar in 30 day old plant where as in 45 day old plant highest activity was found in GG 5 and GG 20 followed by GG 2 which shows maximum disease incident. NADPH oxidase activity positively correlates with least disease incident found in GG 13 and Desi. In most cases, induced production of $O_2^-$ is catalysed by NADPH-oxidizing enzyme systems localized in different cell compartments, such as cell walls (Gross et al., 1977) plasma membranes (Pinton et al., 1994) cytosol and microsomes.
2.3.7 Poly Phenol Oxidase Activity

The Poly Phenol Oxidase activity was found to be highest in Desi variety followed by GG 5, GG 20 and GG 2 (Fig. 2.3.7). The least PPO activity was observed in GG 13 variety. PPO activity negatively co-relates with disease incidence but positively co-relates with Total phenol contents and PAL activity in particular varieties. PPO is copper containing enzyme, which oxidises phenolics to highly toxic quinines and is involved in the terminal oxidation of diseased plant tissue, which was attributed for its role in disease resistance. In our study polyphenol oxidase activities have been recorded higher in susceptible groundnut varieties. These results showed the possible involvement of PPO in host resistance would not be its constitutive state but may be induced state.
Fig. 2.3.7 Poly Phenol Oxidase Activity in five groundnut varieties.

2.3.8 Total Anti-oxidative Power

Total Antioxidative power is a measure of antioxidant status of cell at particular time. Total Antioxidative power was found to be higher in GG 5 and Desi varieties. Among five groundnut varieties antioxidative power were found least and similar in GG 13 and GG 20 varieties (Fig. 2.3.8), which shows positive correlation with less disease incidence found in GG 13 but negatively correlation with GG 20 verity. Thus the data revealed that constitutive presence of enzymatic or non enzymatic antioxidative system was not responsible for resistant trait of particular varieties.
2.3.9 Glutathione Reductase Activity (GR)

Glutathione Reductase Activity was found higher in 45 day old plant than 30 day old plant (Fig. 2.3.9) in all the varieties which shows positive correlation with NADPH oxidase activity. The higher GR activity in 45 day old plant may be due to some abiotic stress at particular stage of growth which positively correlates with higher NADPH oxidase activity. Glutathione reductase (GR) is thought to be a bottleneck in the antioxidative cascade of plants, since it is present in lowest amounts compared to other enzymes of the defence system against free radical attack. GR activity showed least variation in 30 day old plants of all varieties where as in 45 day old plants highest activity was found in GG 2 followed by GG 5 and GG 20. GR activity positively correlates with least disease incidence found in GG13 and Desi.
2.3.10 Catalase Activity

The highest catalase activity was observed in both GG 2 and Desi variety of groundnut cultivars. The activity was found to be decreasing in order GG 5, GG 13, GG 20 (Fig. 2.3.10). The least activity was seen in GG 20 which was negatively correlated with higher disease incident found in particular genotype. Catalase is key enzyme of plant antioxidative system. Role of catalase in defense against biotic stress has been evaluated by many authors in plants (Mhamdi et al., 2010).

![Catalase Activity in five groundnut varieties.](image)

2.3.11 Peroxidase Activity

The highest peroxidase activity was observed in Desi variety followed by GG 13, GG 5 variety of groundnut cultivars (Fig. 2.3.11). The least and almost equal activity was observed in GG 20 and GG 2 variety. Peroxidase activity found to be similar in all the groundnut varieties revealed that constitutive level of expression of peroxidase have not significant role in resistant trait of groundnut but may be the magnitude of induction of the peroxidase. Rani and Yasur (2009) recorded increased activity in tolerant groundnut varieties post infection to *C. personatum*. Similar to our result they also found that basal level of Peroxidase was higher in susceptible genotype of groundnut which revealed that constitutive level of peroxidase not but its induction amplitude was important for resistance. Anjana et al. (2007) found constitutively higher Peroxidase activities in resistant than in susceptible genotypes. The relationship between high amounts of constitutive PO and plant resistance has been reported (Nawar and Kuti 2003).
2.3.12 Zinc and Iron

Zinc and iron are essential micronutrients for plants. Zinc concentration were found higher in 30 day old plants than in 45 days old plants. The activity of O$_2^-$-generating NADPH oxidases is greatly influenced by Zn deficiency in animal and plant cells. Zinc exerts a strong inhibitory effect on the generation of O$_2^-$ by NADPH oxidase. In Zn deficient animal cells, NADPH-dependent production of O$_2^-$ was much higher than in Zn sufficient cells, and this was considered as a major reason for Zn deficiency-induced membrane damage (Chvapil, 1979). Hammermuller et al. (1987) reported that Zn deficiency results in a two- to three-fold increase in NADPH-dependent H$_2$O$_2$ production in rat microsomes. In corroboration with Cakmak (2000), our result revealed that in 30 day old plants GR activity found to be lesser but with decreasing zinc concentration in 45 day old plants, GR activity was raised. Iron is known for generating reactive oxygen species in plant. Zinc deficiency results in a high accumulation of Fe in roots and shoots. Similar to our results, numerous report were found on an excessive accumulation of Fe in leaves of Zinc deficient plants in a nutrient solution experiment with nine plant species. Ambler et al. (1970) made similar observations on soybean plants growing in nutrition solution. They demonstrated a substantial decrease in Fe concentration in xylem exudate with increasing concentration of Zn from a deficient to an adequate level in nutrient solution. The higher content of Iron in 45 day old plant shows positive correlation with higher NADPH oxidase activity in all the varieties. Similarly Glinn et al., 1991 observed many enzymatic and non-enzymatic lipid peroxidation processes show a
high dependency on NADPH as a basic electron donor, particularly in the presence of redox active transition metals such as Iron.

**Fig. 2.3.12.1** Zinc concentration in five groundnut varieties

**Fig. 2.3.12.2** Iron concentration in five groundnut varieties
2.4 Conclusion

- A comparison of the disease incidence and constitutive levels of defense related enzymes among five varieties did not show relevance with resistance trait in groundnut.

- Constitutive basal Total phenol, PAL, PPO, Catalase, Peroxidase, Glutathione reductase, NADPH oxidase and total antioxidative power analyzed among five tolerant and susceptible groundnut varieties revealed that single parameters did not confirmed the resistance trait.

- Interestingly our observation suggest that Zinc may be useful to control infection by improving anti-oxidation system of plant and controlling iron mediated generation of free radical.
2.5 References


- Barber JM (1980) Catalase and peroxidase in primary bean leaves during development and senescence. Z. Pflanzenphysiol. 97:135-144


- Christophe B, Abdelilah B, Francoise C, Daniel C (1996) Changes in malondialdehyde content and in superoxide dismutase, catalase and glutathione
Chapter 2

reductase activities in sunflower seeds as related to deterioration during accelerated aging. Physiologia plantarum 97:104-110


Chapter 2


- Keen NT, Buzzell RI (1999) New Disease resistance genes in soyabean against pseudomonasd-syringae pv glycinea - evidence that one of them interact with a bacterial elicitor. Theor Appl Genet 81:133–138


- Moerschbacher BM, Wite U, Konigs D, Reisener HJ (1989) Changes in the level of enzyme activities involved in lignin biosynthesis during the temperature-sensitive resistant response of wheat (Sr 6) to stem rust (P 6). Plant Science 65:183-190


Wen PF, JY Chen, Kong WF, Pan QH, Wan SB, Huang WD (2005) Salicylic acid induced the expression of phenylalanine ammonia-lyase gene in grape berry Plant Science 169:928-934
