MATERIALS
AND
METHODS
Biological materials:

Seeds collection:

For the present study, two cultivars (Nitya-33 and Pioneer - 30V92) of maize (Zea mays L.) seeds were selected and procured from local market of Anand (Fig. 14 N), Gujarat. These seeds were used either in vitro or field experimentation.

Collection of Fungal, Bacterial and Chemical elicitor:

For this study, Two fungal [Aspergillus flavus (MTCC 4613) and Aspergillus parasiticus (MTCC 411)] and two bacterial [Bacillus subtilis (ATCC 6051) and Pseudomonas aeruginosa (ATCC 25668)] strains were selected. Both the fungal strains were obtained from Junagadh Agriculture University, Gujarat and maintained on potato dextrose agar (Hi Media, India, pH 5.6) medium at 25±2°C. Both the bacterial strains were collected from Microbiology lab of Department of Biosciences, Sardar Patel University, Vallabh Vidyanagar, Gujarat and maintained on nutrient agar (Hi Media, India, pH 7.4) medium at 37°C. Headline (Pyraclostrobin 20% WG) (Fig. 14 M) was purchased from local market and used as an elicitor.

Morphological and Microscopic study of fungal and bacterial strains:

For the morphological and microscopic studies, seven days old cultures of Aspergillus flavus and A. parasiticus maintained on potato dextrose agar were used (Fig: 14 A and D). Small fragment of each Aspergillus flavus and A. parasiticus fungal mat was placed on glass slide and the hyphae were teased using a needle and stained with 2-3 drops of lactophenol cotton blue. The glass cover slip placed over it gently. The preparation was observed under light microscope (Carl Zeiss, Germany) and photomicrographs were taken with CCD camera attached to it.

Three days culture of Bacillus subtilis and Pseudomonas aeruginosa from the nutrient agar was used for morphological and microscopic studies. Microscopic study of both the bacteria was done by Gram’s staining. A single colony of each B. subtilis and P. aeruginosa was taken from the nutrient agar plates with the help of wire loop and smeared over the glass slide. The smear was fixed by gentle heating and then stained with crystal violet, gram’s iodine and safranin. The slides were observed under light microscope (Carl Zeiss, Germany) and photomicrographs were taken with CCD camera attached to it.
**Preparation and selection of elicitors:**

Fungal culture filtrate (FCF) of both fungi *Aspergillus flavus* and *A. parasiticus* were prepared by inoculating 8mm agar plugs of each fungus, individually in potato dextrose broth (Hi-media, India, pH 5.1) and incubated at 25±2°C for twenty eight days. The unspent medium of individual fungus was autoclaved and filtered by using muslin cloth and separated the fungal culture filtrate (FCF) and the fungal mat. The protein present in FCF was estimated upto 28 days at weekly interval for further use. The harvested fungal mat of *A. flavus* and *A. parasiticus* was washed with sterile distilled water and measured the fresh weight at weekly interval. The mat was crushed in 0.1M phosphate buffer (pH 7.4) and sterilized by autoclaving and then measured mycelial cell wall fraction (MCW) protein up to twenty eight days at weekly interval.

Cell free culture filtrate (CCF) of *Bacillus subtilis* and *Pseudomonas aeruginosa* was prepared by inoculating 8mm agar plugs of each bacterial culture in 100ml nutrient broth (Hi Media, India, pH 7.4) and incubated at 30°C for seven days. The unspent culture medium was filtered with Whatman filter paper no.1 and then sterilized by autoclaving. The protein level in CCF was measured for seven days at daily interval.

The total protein concentration of both fungal FCF and MCW and both bacterial CCF was measured by Folin-Lowry’s method (Lowry et al., 1951). For estimation of protein 200µl of each sample (FCF, MCW and CCF), 800µl of distilled water and 5ml of alkaline copper reagent were mixed together and incubated for 10 minutes at room temperature. 500µl of Folin-Ciocalteu reagent was added and incubated for 30 minutes in dark. The intensity of blue color was measured at 660nm using UV-visible spectrophotometer. Bovine serum albumin was used as an internal standard. The elicitors were selected based on higher total protein concentration of FCF and CCF for further experimentation.

**Standardization of concentration and elicitation time of elicitor for treatment**

Twenty-one-days old FCF and MCW of *A. flavus* and *A. parasiticus* (AFFCF, APFCF, AFMCW and APMCW) and 24hrs old CCF of *Bacillus subtilis* (BSCCF) and *Pseudomonas aeruginosa* (PACC) were selected as elicitor’s treatment on the basis of higher protein concentrations. The Headline (Pyraclostrobin 20% WG) as an elicitor was also used at different concentrations (W/V) for treatments. Maize seeds were washed with distilled water and kept in petriplates containing filter papers.
moistened with different concentrations (0.05%, 0.1%, 0.5%, 1%, 2%, 5%, 10%, 15% and 20%) of the selected elicitors separately and distilled water used as control. The seed samples were collected at different time intervals i.e., 0, 3, 6, 12, 24, 48, 72, 96, 120, 144 and 168 hrs of treatment with fungal elicitors as well as Headline and 0, 0.5, 1, 2, 4, 6, 12, 24, 48, 72, 96 with bacterial elicitors. The treated seeds were rinsed with distilled water and measured the Phenylalanine ammonia lyase (PAL) activity following the method described by Aguilar et al., (2000) and peroxidase (POX) activity following the method given by Thimmaiah, (2006) (see detailed method in subsequent pages). The concentration and time for treatment of elicitor was determined based on highest PAL and POX activities.

**Treatments with elicitors:**

To check the effect of various elicitors, the following experiments were conducted and their efficacy for induction of defense related enzymes were checked.

- **Treatment 1:** Elicitor treated seeds grown in *in vitro* condition.
- **Treatment 2:** Elicitor treated seeds grown on soil infested with *A. parasiticus* and *A. flavus*.
- **Treatment 3:** Untreated seeds sown in field and foliar application of various elicitors.

**Treatment 1: *In vitro* Treatment**

Murashige Skoog's (MS medium, Table 3) basal medium (Murashige and Skoog, 1962) was supplemented with ten percent concentrations (v/v) of various elicitors (AFFCF, APFCF, AFMCW, APMCW, BSCCF and PACCF) for the experiment. The pH of the medium was adjusted to 5.7-5.8 with 0.1 N NaOH or 0.1 N HCl prior to adding agar-agar 0.8% (w/v). MS basal medium without elicitor was used as a control. All the media were sterilized in an autoclave at 15 psi (121°C) for 15 minutes. Healthy maize seeds (Nitya-33 and Pioneer-30V92) were selected, washed with distilled water and germinated on autoclaved wet filter paper. Next day, the seeds were sterilized with 1% mercuric chloride for 2-3 minutes and washed 3-4 times with sterile distilled water and aseptically inoculated the seeds on to above mentioned media containing various elicitors. The bottles were incubated in growth room at 25±2°C temperature, 60-70% relative humidity and 16:8hr photoperiod of 35 μEm²S⁻¹ irradiance level provided by cool white fluorescent light during the whole experiment. Enzyme activities such as phenylalanine ammonia lyase (PAL),
peroxidase (POX) and β-1, 3 glucanase activities and also the total protein and phenols were (detailed method in subsequent pages) estimated in seven days old in vitro grown seedlings at 24 hour interval for one week.

<table>
<thead>
<tr>
<th>Name of stock</th>
<th>Constituent</th>
<th>Concentration of constituents (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>NH₄NO₃</td>
<td>1650.0</td>
</tr>
<tr>
<td>B</td>
<td>KNO₃</td>
<td>1900.0</td>
</tr>
<tr>
<td>C</td>
<td>kH₂PO₄</td>
<td>170.0</td>
</tr>
<tr>
<td></td>
<td>H₃BO₃</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>Na₂MoO₄</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>KI</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>CoCl₂·6H₂O</td>
<td>0.025</td>
</tr>
<tr>
<td>D</td>
<td>CaCl₂·2H₂O</td>
<td>440.0</td>
</tr>
<tr>
<td>E</td>
<td>MgSO₄·7H₂O</td>
<td>370.0</td>
</tr>
<tr>
<td></td>
<td>MnSO₄·4H₂O</td>
<td>22.3</td>
</tr>
<tr>
<td></td>
<td>ZnSO₄·7H₂O</td>
<td>8.6</td>
</tr>
<tr>
<td></td>
<td>CuSO₄·5H₂O</td>
<td>0.025</td>
</tr>
<tr>
<td>F</td>
<td>FeSO₄·7H₂O</td>
<td>27.8</td>
</tr>
<tr>
<td></td>
<td>Na₂EDTA</td>
<td>37.3</td>
</tr>
<tr>
<td>Vitamins</td>
<td>Nicotinic Acid</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Pyridoxine HCl</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Thiamine</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Glycine</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Sucrose: 30 g/l, Myo-Inositol: 100 mg/l, Agar-Agar: 8 g/l, pH: 5.7- 5.8

Treatment 2: Soil infestation assay

Fungal spore inoculum for both the fungi was prepared by using autoclaved wheat seeds. Wheat seeds were washed with water and soaked overnight and mashed with Sumeet mixer grinder and autoclaved at 15 psi for 15 minutes. After cooling fungal spores of *A. flavus* and *A. parasiticus* were inoculated on crushed wheat seeds and incubated for 48 hr at room temperature. The soil infestation was done by mixing the 40gm of wheat seeds with fungal spores with 1kg soil containing...
Materials & Methods

1:1 ratio of soil and manure. The mixture was filled in polybags of 15 cm diameter and 20 cm height and the soil was moistened with water before sowing seeds. Maize seeds of Nitya-33 and Pioneer-30V92 cultivars were treated with selected fungal and bacterial elicitors for 24 hrs and 30 mins respectively. The treated seeds were washed with distilled water and sown in infested soil (Kishore et al., 2005). Bioassays for PAL, POX and β-1, 3 glucanase activities and also estimated the total protein and phenols (detailed method in subsequent pages) by collecting the samples from 21 day old seedling leaves.

Treatment 3: Foliar application of Elicitors in field grown plants

Field experiments were conducted in experimental plots selected in farmer’s field at Lambhavel and Kanajari village, District Anand, Gujarat, India (Table 4) during the years 2010-2012. Untreated seeds of Pioneer-30V92 were sown in field and 15 m² plot area was chosen for each treatment in randomized block design. Twenty day old plants at V₆-V₉ leaf stage were selected and the plants were foliar sprayed with selected concentration of fungal culture filtrates and pyraclostrobin (Headline) (details see Table 4) by using a hand-operated atomizer along with control (untreated). The samples were collected after one hour of treatment which was considered as zero day and subsequently at regular intervals and estimated different enzymes.

Rabi season of 2011 and Kharif season of 2012 plants were given two foliar spray treatments with selected elicitors to check their effect at different stages of growth and development. The first foliar spray was given at V₆-V₉ stage (20 DAS) and the second foliar spray was given to the fifty day old plants at R₁-R₂ stage (50 DAS) with 10% concentration of AFFCF and APFCF and 0.1% Headline along with untreated control plants. The leaf samples from treated plants with elicitors were collected from 0-7 days after treatment (DAT) and at 10 days interval up to 70 DAT for estimation of various biochemical assays (PAL, POX, β-1,3-glucanase, NR, NiR, catalase, polyphenol oxidase, chitinase, glutathione reductase, salicylic acid, ethylene, lignin content, total protein, total phenol, total carbohydrate and total chlorophyll content) were carried out.
Table 4: Foliar application of selected elicitors on maize plants growing in field at different localities of Anand District, Gujarat during the period 2010-2012.

<table>
<thead>
<tr>
<th>Year</th>
<th>Season</th>
<th>Cultivar Grown</th>
<th>Location</th>
<th>Stage of plant for treatment</th>
<th>Selected Biochemical Parameters</th>
<th>Treatments</th>
<th>Concentration (V/V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2010</td>
<td>Kharif</td>
<td></td>
<td>Lambhavel village (Dist. Anand)</td>
<td>V6-V9 stage (20DAS)</td>
<td>PAL, POX, β-1, 3 glucanase, NR, NIR, Total protein, Total phenol, total carbohydrate and total chlorophyll content</td>
<td>10% AFFCF, APFCF and 0.1% Headline (pyraclostrobin 20% WG)</td>
<td></td>
</tr>
<tr>
<td>2011</td>
<td>Kharif</td>
<td>Pioneer-30V92</td>
<td>Kanajari village (Dist. Anand)</td>
<td>V6-V9 stage (20DAS) &amp; R1-R2 stage (50DAS)</td>
<td>PAL, POX, β-1, 3 glucanase, NR, NIR, Total protein, Total phenol, total carbohydrate, total chlorophyll content, catalase, PPO and lignin content</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2012</td>
<td>Kharif</td>
<td></td>
<td></td>
<td></td>
<td>PAL, POX, β-1, 3 glucanase, NR, NIR, Total protein, Total phenol, total carbohydrate, total chlorophyll and glutathione reductase</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Preparation of enzyme extract:

Different biochemical parameters were analyzed for all the experiments described above. One gm fresh leaf samples (fully developed third or fourth leaf from the top) were collected from the treated and control plants. The sample collected one hour after application of elicitors was considered as zero day for the estimation of various enzymes. The leaf tissues were homogenized in 0.1M phosphate buffer (pH 7); 0.1g polyvinyl pyrolidone and 10μl β-mercaptoethanol and then centrifuged at 10,000 rpm for 10 minutes. The supernatant was collected and used as an enzyme extract and estimated various enzyme activities at different time interval in all the three treatments mentioned above.

Phenylalanine ammonia lyase activity (PAL):
The PAL enzyme activity was estimated following the method of Aguilar et al. (2000).

Reagents: 150 mM Tris HCl buffer (pH 8.5) with 50mM L-Phenylalanine.

Method: The reaction mixture consisted of 2ml of PAL reagent (150mM Tris-Hcl buffer (pH 8.5) containing 50mM L-phenylalanine), 900μl distilled water and 100 μl of enzyme extract, which was then incubated at 30°C and absorbance was taken at 280nm in the interval of 30 seconds by using UV-spectrophotometer. One unit of enzyme activity was calculated as the amount of enzyme that formed 1mg cinnamic acid/hr.

Peroxidase activity (POX):
The peroxidase enzyme activity was measured by following the method described by Thimmaiah (2006).

Reagents: 0.01M potassium phosphate buffer (pH 6.0), freshly prepared 0.01M O-Dianisidine in methanol and 0.02M H₂O₂ (0.227 ml of 30% H₂O₂ was diluted to 100 ml with distilled water) and 2N H₂SO₄.

Method: The reaction mixture consisted of 200μl of enzyme extract, 1ml 0.1M potassium phosphate buffer (pH 6.0), 500μl of 20mM H₂O₂, 1ml of 0.01M O-dianisidine and incubated at 30°C for 5 minutes. The enzyme reaction was stopped by adding 1ml of 2N H₂SO₄. The change in absorbance was measured at 436 nm up to 3 min. at 15 seconds interval by using spectrophotometer. One unit (U) of enzyme defines as the change in the absorbance 0.1/unit (OD/min) under specific assay condition.
β -1, 3 Glucanase activity:
The estimation of β-1, 3 glucanase activity was done following the method of Pan et al. (1991).

Reagents: 0.5% laminarin, DNSA (Dinitrosalicyclic Acid), Enzyme extract.

Method: The reaction mixture consisted of 200µl of 0.5% laminarin and 200µl of the enzyme extract in the tubes and incubated at 37°C for one hour. The reaction was stopped by adding 200µl of Dinitrosalicyclic acid. The tubes were kept in boiling water bath for 5 minutes. 3.2ml of distilled water was added to all tubes after cooling. The absorbance was measured at 500nm by using spectrophotometer. The specific activity of β-1, 3 glucanase was expressed as 1µg glucose (released /min/gm of fresh tissue) equivalent to 4mg protein.

Estimation of total protein:
The total protein construction was estimated by using the method described by Lowry et al. (1951).

Reagents:
Solution A: 2% Sodium Carbonate in 0.1N NaOH
Solution B: 0.5% Copper Sulphate in 1% Sodium Potassium tartrate.
Solution C: Alkaline Copper Sulphate Solution (50ml of solution A was mixed with 1ml of solution B just prior to use).
Folin-Ciocalteu reagent: The commercial Folin-Ciocalteu reagent (2N) was mixed with an equal volume of distilled water.

Method: Total protein concentration either in elicitor or plant extract was checked by Folin-Lowry’s method (Lowry et al., 1951). For estimation of total protein 200µl of plant extract, 800µl of distilled water and 5ml of alkaline copper reagent were mixed together and then incubated for 10 minutes at room temperature. 500µl of Folin-Ciocalteu reagent was added and then incubated for 30 minutes in dark. The intensity of blue color was measured at 660nm using spectrophotometer. Bovine serum albumin was used as an internal standard.

Protein profiling by Sodium Dodecyl Sulphate-Polyacrylamide gel electrophoresis (SDS PAGE): The protein profiling was done by SDS-PAGE analysis by using the method of Laemmli et al. (1970).

I. Preparation of stock reagents
Materials & Methods

A. Acrylamide/Bisacrylamide:

Acrylamide 146.0 gm
N, N, Methylene-Bisacrylamide 4.0 gm

The volume was made up to 500ml with distilled water and filtered the solution and stored in dark at 4°C.

B. 1.5M Tris-HCl, pH 8.8 (For separating gel):

Tris base 54.54 gm
Distilled water 200 ml

pH was adjusted to 8.8 and then the volume was made up to 500 ml by adding distilled water.

C. 0.5M Tris-HCl, pH 6.8 (For stacking gel):

6 gm Tris base was dissolved in 60ml distilled water and pH was adjusted to 6.8 with 0.1N HCl and made the volume up to 100 ml with distilled water and stored at 4°C.

D. 10% Sodium dodecyl sulphate (SDS):

10 gm SDS was dissolved in 60ml water by gentle stirring and made the volume up to 100ml.

E. 10% Ammonium per sulphate (APS):

100mg APS was dissolved in 1ml distilled water.

F. Sample buffer:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>3.0 ml</td>
</tr>
<tr>
<td>0.2M Tris-HCl, pH 6.8</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>20% Glycerol (v/v)</td>
<td>1.6 ml</td>
</tr>
<tr>
<td>0.05% Bromophenol Blue (in D/W) (w/v)</td>
<td>0.4 ml</td>
</tr>
<tr>
<td>10mM β- mercaptoethanol</td>
<td>0.4 ml</td>
</tr>
<tr>
<td>10% Sodium Dodecyl sulphate (w/v)</td>
<td>1.6 ml</td>
</tr>
</tbody>
</table>

G. Electrode buffer pH 8.3:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>22.5 gm</td>
</tr>
<tr>
<td>SDS</td>
<td>1.5 gm</td>
</tr>
<tr>
<td>Tris Base</td>
<td>5.91 gm</td>
</tr>
</tbody>
</table>

Distilled water was added to make up the volume to 1.5 liters.

Induction of SAR in maize by using different elicitors
II. Gel preparation for SDS PAGE:

SDS PAGE (10%) gel preparation

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Separating gel</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15 ml</td>
<td>5 ml</td>
</tr>
<tr>
<td>Water</td>
<td>5.9 ml</td>
<td>3.4 ml</td>
</tr>
<tr>
<td>30% Acrylamide/Bisacrylamide</td>
<td>5.0 ml</td>
<td>0.83 ml</td>
</tr>
<tr>
<td>1.5M Tris-HCl (pH 8.8)</td>
<td>3.8 ml</td>
<td>-</td>
</tr>
<tr>
<td>1.0M Tris-HCl (pH 6.8)</td>
<td>-</td>
<td>0.63 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.15 ml</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>1.0M Ammonium persulphate</td>
<td>0.15 ml</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.006 ml</td>
<td>0.005 ml</td>
</tr>
</tbody>
</table>

III. Gel staining and de-staining:

- Gel staining solution:
  40:10: 50 Methanol: Acetic acid: Distilled water.
  0.1% Coomassie Brilliant Blue (R-250)

- Gel de-staining solution:
  40:10: 50 Methanol: Acetic acid: Distilled water.

Estimation of Total Phenol:
The total phenol was estimated by following the method given by Sumere et al. (1965).

Reagents: 80% ethanol, Folin-Ciocalteu reagent (FCR), 20% Na₂CO₃

Method:
Enzyme extraction: 200mg of fresh leaves were crushed in 80% methanol and incubated for 24 hrs at 4°C and then centrifuged at 10,000 rpm for 10 mins. The supernatant was collected and used for estimation of total phenol.

Enzyme assay: 200µl of extract was mixed with 2.5ml distilled water and 500µl of Folin-Ciocalteu reagent (1N). The mixture was incubated for 3mins at room temperature and then added 2ml of 20% Na₂CO₃. The mixture was kept in boiling water bath for 1min. The change in color was measured at 650nm by spectrophotometer. Total phenol concentration was expressed as mg phenol in terms of catechol per gram of fresh tissue.
Phenol profiling by High Pressure Thin Layer Chromatography (HPTLC):

The method described by Jayaraman (1981) was followed for phenol profiling using HPTLC. The sample having highest total phenol content from each treated and control plants was selected for phenol profiling. 10µl phenolic extract along with standard were separately spotted as bands by semi automatic applicator (Linomat 5, CAMAG, Switzerland) on 10×10 cm sq pre-coated aluminum sheets with silica gel G_60 F_254 (Merck, Germany) for HPTLC. Benzene: Ethyl acetate: formic acid (3:6:1) was used as solvent system for visualization of phenolic bands at 254nm and 366nm by using TLC Scanner 3, CAMAG Switzerland. The plates were photographed at 254nm and 366nm with the help of CAMAG Reprostar, Switzerland. The Rf Values were analyzed by using the software Win-cat. 1% FeCl₃ was used as spraying reagent for confirmation of phenolic bands. Rf values and number of bands were recorded for induced phenols. The HPTLC analysis was done separately for various standard phenols and scored the Rf values, which were used as standard Rf values for respective phenols.

Nitrate reductase activity (NR):
The method described by Thimmiah (2006) was followed to estimate the Nitrate reductase activity.

Reagents: 0.1M phosphate buffer (pH 7.5), grinding medium (0.1 M Tris, 0.01M cysteine and 0.0003M EDTA at a pH of between 7.3 & 7.8), 0.1M KNO₃, 2mM NADH, 1% sulphanilamide in 1.5N HCl (w/v), 0.02% N-(1-naphthyl) ethylene diamine HCl (NNED), 0.01M Standard potassium nitrite solution.

Method:
Preparation of enzyme extract: Fresh leaves were collected from treated and untreated plants as described above and immersed quickly in cold (2°C) deionized water; blot dried, weighed and cut into small pieces. Leaf tissues (1 g) were ground with mortar and pestle by using 3ml grinding medium and centrifuged at 20000 rpm for 2 min in grinding medium (2°C). The supernatant was collected and filtered through Whatman filter paper no.1 and stored at 3-5°C and the assay was completed within 2-3 hrs of sample preparation.

Enzyme Assay: The reaction mixture consisted of 1 ml of phosphate buffer, 0.2 ml KNO₃, 0.5 ml NADH, 0.1 ml deionised water and 0.2 ml of enzyme extract. Blank was set with distilled water. The experimental tubes were incubated at 27°C for 15 mins. The reaction was stopped by adding 1 ml of sulphanilamide followed by 1 ml...
NNED reagent and incubated for 5 mins. The absorbance was measured at 540 nm by using spectrophotometer. The activity of nitrate reductase was calculated and expressed as μmoles KNO$_2$ formed/h/g fresh weight.

**Estimation of nitrite reductase activity (NiR):**
The nitrite reductase enzyme activity method developed by Miflin (1967) was followed.

**Reagents:** 0.2M potassium phosphate buffer (pH 7.0), 5mM sodium nitrite, 1.5mM benzyl viologen, dithionite reagent.

**Method:**

**Extraction:** Fresh leaves (3 g) were crushed in two volumes of 20mM potassium phosphate buffer (pH 7.5) containing 5mM cysteine HCl and 0.05mM EDTA and centrifuged at 5000 rpm. The supernatant was collected and used for the enzyme assay.

**Enzyme assay:** The reaction mixture consist of 0.2 ml phosphate buffer, 0.1 ml sodium nitrite, 0.1 ml benzyl viologen and 0.2 ml enzyme extract and the volume was made up to 0.8 ml with distilled water at 30°C. The reaction was initiated by adding 0.2 ml of the dithionite reagent and incubated for 10 mins. The reaction was stopped by vigorously shaking the mixture until the dithionite was completely oxidized and disappearance of dye color. Blank was set with all above reaction mixture except enzyme extract. The amount of nitrite reductase was calculated and estimated as μmoles KNO$_2$ formed/h/g fresh weight.

**Estimation of Carbohydrates:**
The estimation of reducing and non reducing sugars was done by following the method described by Sadasivam and Manickam (1992).

**a) Estimation of Reducing Sugars by Di-nitro salicylic acid (DNS) method**

**Reagents:**
DNS reagent, 40% Rochelle salt solution (sodium potassium tartarate solution), Glucose Standard

**Method:** Fresh leaves (1 g) were collected and homogenized in hot 80% methanol twice (5 ml each time). The supernatant was collected and evaporated in boiling water bath for approximately 3 hrs. The reaction mixture consisted of 1 ml of alcohol-free extract and made up to 3 ml with distilled water and then 3 ml of DNS was added and
mixed thoroughly. The reaction mixture was kept in boiling water bath for 5 mins. After color has developed, 1 ml of 40% Rochelle salt solution was added and mixed. The experimental tubes were cooled under running tap water and the absorbance was measured at 510 nm using spectrophotometer. The amount of reducing sugar was calculated in the sample using a standard graph prepared from standard glucose solution (1 mg/ml).

b) Estimation of non-reducing sugars

**Reagents:** 1 N H$_2$SO$_4$, 1 N NaOH, Methyl red indicator

**Method:** The steps for sample preparation were followed as described for estimation of reducing sugars. The reaction mixture consisted of 1 ml of extract and 1 N H$_2$SO$_4$ which was hydrolyzed by heating at 49°C for 30 mins. The tubes were cooled and added 1 drop of methyl red indicator. The contents were neutralized by adding 1 N NaOH drop by drop. Appropriate reagent blank was maintained. The total reducing sugars formed was estimated as described above under reducing sugars. The total carbohydrate content was calculated by adding the reducing sugars with non-reducing sugars.

**Estimation of chlorophyll content:**

The chlorophyll content was estimated by following the method described by Sadasivam and Manickam (1992).

**Reagents:** 80% acetone (Pre-chilled).

**Method:** One gm of fresh leaf tissue was homogenized in mortar and pestle with 20 ml of prechilled 80% acetone and centrifuged at 10,000 rpm for 10 mins. The supernatant was taken in volumetric flask. The residue was ground again in 80% acetone and centrifuged at 10000 rpm for 10 mins. This procedure was repeated until residue turned colorless. The total volume was made up to 100 ml with 80% acetone and measured the absorbance at 645 nm, 652 nm & 663 nm against the solvent (80% acetone) as blank by using spectrophotometer.

The total chlorophyll content of intact leaf of field grown maize plants were measured by using SPAD-502 chlorophyll meter (Konica Minolta sensing INC. Japan).
Materials & Methods

Estimation of Catalase activity:
The Catalase activity was estimated following the method described by Thimmaiah (2006).

**Reagent:** 0.1M Potassium phosphate buffer (pH 7.0), freshly prepared 0.005M H₂O₂, 0.7N H₂SO₄, 0.01N KMnO₄.

**Method:** The reaction mixture consisted of 1.5ml of phosphate buffer, 1ml of H₂O₂ and 0.2ml of enzyme extract which was incubated at 20°C for 1min. After 1min, reaction was stopped by adding 5ml of 0.7N H₂SO₄. This reaction mixture was titrated against 0.01N KMnO₄ to find out the residual H₂O₂ until a faint purple color persists for at least 15sec. Blank was prepared by adding the enzyme extract to an acidified solution of reaction mixture at zero time. One unit of Catalase is defined as the amount of enzyme which breaks down 1μmole of H₂O₂ under the assay conditions.

Estimation of Polyphenol oxidase (PPO) activity:
The method described by Thimmaiah (2006) was followed to estimate the polyphenol oxidase activity.

**Reagent:** 0.2 M potassium phosphate buffer (pH 7.2), 0.05 M Catechol

**Method:** The reaction mixture contains 4.5 ml of 0.2 M potassium phosphate buffer and 1 ml of 0.05 M catechol. The mixture was incubated at 30°C and 0.1 ml enzyme extract was added to start the reaction. Change in OD was recorded at 30 sec interval up to 3 min at 410 nm by using spectrophotometer. Enzyme activity was expressed as units per min and the specific activity as units/min/gm of fresh weight.

Estimation of Chitinase Activity:
The method described by Monreal and Reese (1969) was used to estimate the Chitinase activity.

**Reagent:** 0.05 M citrate phosphate buffer (pH 6.6), colloidal chitin (Sigma Co.) and dinitro salicylic acid (DNS).

**Method:**
**Extraction:** Fresh maize leaves tissue (0.5 g) was homogenized in citrate phosphate buffer under ice-cold condition. The homogenates were centrifuged at 10,000 rpm for 30 min at 4°C, and the supernatant was stored at 4°C until use.
Enzyme assay: The reaction mixture consisted of 1ml of colloidal chitin, 3 ml of 0.05M citrate phosphate buffer (pH 6.6) and 1ml enzyme extract mixed thoroughly. The tubes were then incubated in a water bath at 37°C for 1hr. 1ml of the reaction mixture was added to 1ml of DNS solution and boiled for 5min which is used as blank. After cooling the solutions the reducing sugar was determined at 540 nm by using spectrophotometer.

Estimation of Glutathione reductase activity:
The estimation of glutathione reductase activity was done following the method of Rao et al. (1996)
Reagent: Extraction buffer (100 mM Potassium phosphate buffer (pH 7.6) and 1 mM EDTA), 10 mM dithiothreitol (DTT), 0.5 mM glutathione substrate (GSSG), 0.2 mM Nicotinamide adenine dinucleotide phosphate, tetrasodium salt (NADPH) and poly vinyl pyrrolidone (PVP).

Method:
Extraction: The enzyme extract was prepared by homogenizing 0.5 g of fresh leaf tissue with 1.5 ml of extraction buffer and 1% (w/v) PVP under ice-cold condition. The homogenates were centrifuged at 10,000 rpm for 30 min at 4° C. The supernatant fraction was mixed with 10 mM dithiothreitol (DTT) and used for the assay.

Enzyme assay:
The reaction mixture (3 ml) containing 1.5ml of extraction buffer (100 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA), 0.1ml of 0.5 mM GSSG, 0.25ml of 0.2 mM NADPH and 1% (w/v) BSA. The mixture was thoroughly mixed and maintained the constant OD before adding the 0.1 ml enzyme extract and immediately mixed by inversion and recorded decrease in OD for 5mins by using thermostat spectrophotometer A<sub>340nm</sub>. One unit of enzyme will reduce 1μmole of oxidized glutathione per minute at 25°C.

Estimation of Salicylic acid:
The salicylic acid was measured following the method of Yalpani et al. (1991).
Reagents: Hexane, HPLC grade methanol, 0.02 M phosphate buffer (pH 5.5)

Method:
Extraction: Fresh leaf tissue (1g) was chopped and placed in 10ml hexane and stored overnight at 4°C.
Enzyme assay: The hexane extract was filtered by using 0.22μm cellulose acetate filters (Sartorious, Germany). HPLC analysis was carried out by using C-18 ZORBAX ODS column (4.6 mm ID X 250 mm, 5 μm particle size) with the solvent system consisting of equal volume of methanol: phosphate buffer (pH 5.5, 0.02M), with flow rate 1 ml/min at 35°C to quantify the salicylic acid levels. Samples were monitored at 315 nm UV detector built-in HPLC instrument.

Estimation of Ethylene by using Gas-chromatography: The ethylene content was estimated by using the method described by Thimmaiah (2006).

Materials: Glass bottles (250ml), air tight syringes, Ethylene gas (standard), Gas Chromatography instrument (GC).

Method: Fresh leaf sample was trimmed into small pieces, weighed and placed in 250ml airtight glass bottle (specially designed for the estimation) and placed in light for 24hr. Pure ethylene was injected into empty bottle of same volume and incubated for 1h at 20°C which is used as standard. The ethylene content in the gas phase of the bottle was determined from the samples withdrawn with hypodermic syringe and injected into a GLC auto system XL (Perkin Elmer) gas chromatograph fitted with a flame ionization detector and 1.8 m × 2 meter column packed with 60-80 microns mesh silica gel. The oven temperature at 50°C and detector temperature at 150°C was maintained. Ethylene identification was based on the retention time compared with pure ethylene standard and peak height was measured. The quantity of ethylene produced is expressed as μl ethylene/hr/kg material.

Estimation of Lignin: The method described by Thimmaiah (2006) was used to estimate the lignin content.

Reagents: Diethyl ether, 0.1 M sodium phosphate buffer (pH 7.0), 0.1M and 0.5M NaOH, 2N HCL.

Method: 100mg of oven dried leaf was homogenized with diethyl ether and centrifuged for 10 min at 9200 rpm using Sigma refrigerated centrifuge (Laborzentrifugen GmbH, Osterode, Germany). The pellet was washed thoroughly and centrifuged for two times. The pellet was dissolved in 2ml of NaOH for 16hrs and then added 4.5ml of 2N HCl (pH 7.8). The mixture was centrifuged at 2500 rpm for 5 min. 0.8 ml of supernatant was taken in equal volume of 0.1M Potassium phosphate buffer and measured at 245nm and another 0.8 ml sample was dissolved in equal
Materials & Methods

volume of 0.1N NaOH and measured at 350 nm using UV-spectrophotometer. The difference between $A_{245}$ and $A_{350}$ was considered as the concentration of lignin in sample and amount of lignin was expressed as $A_{350}$/sample.

The entire above mentioned enzyme assays were performed in triplicates and the readings were recorded. The fold count of various enzyme activities were calculated as a measure of enzyme activity in treated plants divided by enzyme activity in untreated control plants at different time interval.

Histological studies of leaf:

The field grown maize plants untreated and treated with APFCF, AFFCF and Headline were considered for the study to see the structural details of leaf with respect to lignin deposition. Fully developed leaf samples were collected at different time intervals (0, 7, 10, 20, 40 and 60 DAT) and stored in Formaldehyde: Acetic acid: 70% Alcohol (FAA) in the ratio of 5:5:90 ml. A portion of lamina along with the midrib was cut from the maize leaf and free hand transverse sections of midrib were taken. The sections were stained with phloroglucinol for 30 seconds followed by 70% alcohol for 30 seconds and then immediately observed the sections under microscope (Carl Zeiss, Germany) and the photomicrographs were taken with charge coupled device (CCD) camera attached to it.

Harvesting:

The mature cobs were harvested from treated and untreated plants after 120 DAS. At the time of harvesting different parameters were checked i.e. height of plants (meter), fresh weight of cob (gm), Length and width of cob (cm) by using measuring tap. The cobs were sun dried for a week and then measured dry weight of cob (gm), No. of seeds per cob and seed weight (gm) was also recorded.

Purification and characterization of Elicitors:

The total protein of the crude fungal culture filtrate (FCF) is precipitated by using different concentrations (10%, 20% ...100%) of ammonium sulfate (Thimmaiah, 2006). The protein precipitated samples were then centrifuged and the pellet was collected and then dissolved in 5ml phosphate buffer. The concentration of protein after AMS precipitation was estimated and separated in SDS PAGE. The proteins precipitated by 80% ammonium sulphate giving highest protein concentration of both AFFCF and APFCF were kept for dialysis overnight in phosphate buffer at pH 7 to
Materials & Methods
remove excess salt. This sample was taken and fractionated by using Sephadex G-50 gel filtration column (GFC) of 30 cm length and 1 cm width and run with the phosphate buffer. Fifteen fractions of 2 ml each were collected with a flow rate of 1 ml/min. The fractions were monitored at 280 nm. A total of five fractions corresponding to absorption peak were selected for the biochemical assays in both Aspergillus flavus fungal culture filtrate (AFCF) and A. parasiticus fungal culture filtrate (APFCF). The protein concentration was estimated from the collected fractions separately to identify which fraction contains the elicitor protein.

The fractions containing the proteins were sprayed on seven day old maize plants (Pioneer-30V92) grown in plastic trays in lab condition and estimated the Phenylalanine ammonia lyase (PAL) and peroxidase (POX) enzymes after 1st day of treatment (DAT). The fraction containing elicitor protein was also tested for the hypersensitive response (HR). One drop of the column purified fraction having the elicitor protein and one drop of phosphate buffer as a control were placed over the upper leaf surface of plants growing in lab condition and HR response was observed after 1st DAT. On the basis of higher PAL and POX enzyme activity and HR response the fraction having elicitor protein was determined. The selected fraction of both AFCF and APFCF were run in SDS-PAGE using 12% polyacrylamide separating gel with 5% stacking gel to determine the protein profile of both the fungal FCF along with crude FCF. Purified protein fractions of both the fungi were loaded on separating gel with 1.5 M Tris-HCl (pH-8.8) and stacking gel with 0.5 M Tris-HCl (pH-6.8). 10% SDS and 10% ammonium persulphate also incorporated in gels. Small-slab gel electrophoresis system (Bangalore Genei) was used. The electrophoresis buffer composed of 0.6 M Tris HCl buffer pH6.8, 0.4 M Glycine (pH 8.3) and 0.006 M SDS. Samples were heated at 100°C for 1 minute in 1X sample buffer (0.5 M Tris HCl buffer (pH6.8), 1% SDS, 0.5% Bromophenol blue, 0.5% Mercaptoethanol and 1% Glycerol) to denature protein. 80 µl sample protein + 20 µl 1X sample buffer was added in each well. Standard protein marker (14.3-94kD) (Bangalore Genei) 1 mg/ml was prepared with the sample buffer and was loaded to compare and determine the approximate protein sizes. The gel was run at 100 volt AC current for 3-4 hrs. After the gel was completely run, the gel was stained with Periodic acid and Schiff’s reagent (PAS) (Moller and Poulsen, 1995) to detect the presence of glycoprotein. The gels were photographed with canon power soft G-2 (Japan) at 4 mega pixel camera. Molecular weight of the polypeptides and protein banding pattern was determined

Induction of SAR in maize by using different elicitors
Materials & Methods

using the standard protein marker (14-97 kDa). Molecular weight and expression of proteins was analyzed by using UN-SCANE-IT software (Silk Scientific Inc, USA).

Seed germination assay of harvested seeds (F1):

Harvested Maize seeds (F1) were collected from control and treated plants (10%AFFCF, 10%APFCF and 0.1% Headline) of Pioneer-30V92 cultivar and percentage of germination and vigor index (VI) was checked. This experiment was conducted by germinating the seeds on petriplates containing moistened filter paper in lab condition after treatments as follows: 1. The F1 harvested seeds collected from elicitor treated AFFCF, APFCF, Headline and control plants were treated with D/W. 2. The harvested seeds (F1) from control plants treated again with different elicitors. 3. The F1 elicitor treated seeds were treated again with respective elicitor. The emergence of radical was considered as germination. The percentage of germination and vigor index of seeds was recorded. Germinated seeds were collected for seven days at different time intervals i.e., 0-7 day of treatment and rinsed with distilled water and Phenylalanine ammonia lyase (PAL), Peroxidase (POX) and β-1, 3-glucanase activities were measured following the methods described earlier. The percentage of germination (%), seedling length (cm) and Vigor index was recorded after 7 days of planting on filter paper (Dezfuli et al., 2008).

The vigor index was calculated as per formula given below.

Vigor index (VI) = [seedling length (cm) × germination Percentage].

Percentage of survival of F1 harvested seeds in fungal infested soil:

This experiment was carried out to check survival rate and vigor index of F1 harvested Pioneer-30V92maize cultivar seeds treated again with standardized concentration of elicitor (AFFCF, APFCF and Headline) mentioned earlier. The seeds were germinated in A. flavus and A. parasiticus infested soils after elicitor treatment. Germination of seeds was counted in 24 hour intervals and continued until no further germination occurred (7th day) to score the percentage of survival. Final survival percentage (%) and Vigor index was recorded after 7 days of planting the treated seeds in infested soil. Leaf samples of 20 days old plants growing in infested soils were collected at different time intervals from (0, 1, 2, 3.... 7 days) and induction of Phenylalanine ammonia lyase (PAL), Peroxidase (POX) and β-1, 3-glucanase activities was also measured by using the methods described earlier.
Statistical Analysis

The experiments were run in triplicate and the mean values (±SD) were expressed. All enzyme activities were expressed in terms of units (U)/g fresh wt of leaf (GFW). Significance (P≤0.05) was calculated using Student’s ‘t’ test using statistical package for the social sciences (SPSS-Version 15) software.