MATERIALS
&
METHODS
CHAPTER II: MATERIALS AND METHODS

1. Chemicals:

Protein molecular weight marker-high range was purchased from Genei, Bangalore, India. Bovine serum albumin (BSA) was purchased from S.D. Fine-Chemical Limited, Mumbai, India. Antisera to *A. thaliana* NPR1 protein was kindly gifted by Dr. Fobert Pierre, Plant Biotechnology Department, National research council, Canada. Nitrocellulose, Horse reddish POX labeled secondary goat anti rabbit antibodies and Tetramethyl benzidine (TMB)-H2O2 were procured from Genei, Bangalore, India. All other chemicals and solvents were obtained from Qualigens, Merck, Rankem, Himedia or Loba Chem Companies, India.

2. Plant material and preparation of Fungal culture filtrate:

All the four different varieties of potato plants namely Badshah, Bahar, Pukhraj and Lauvkar were gifted by Dr. N.H. Patel 'Potato Research Station', Deesa, North Gujarat. Potato plants of all four varieties were grown and maintained during the month of November to January in the departmental backyard as per the local farming practices for potato. 7-8 plugs of 5 day old mycelium of *P. infestans* was inoculated and grown in liquid media (composition Gm/l: 30 glucose; 7 peptone; 1 yeast extract; 0.01 thiamine HCl; 0.4 KH2PO4; 0.26 K2HPO4; 0.1 MgSO4.7H2O; 1 CaCl2.2H2O; 1mg FeSO4.7H2O; 0.004 ZnSO4.7H2O; 0.05 CuSO4; 0.04 Na2MoO4; 0.045 MnCl2) under static condition at 25°C in dark for 21 days and then filtered using 4 layers of cheesecloth. This Fungal culture filtrate (FCF) was used for treatment to plants. *F. oxysporum* and *A. solani* were grown for five days on the Potato dextrose agar (PDA) plates and 7-8 plugs of 5 day old mycelium was
transferred to the Potato dextrose broth (PDB) and the broth was inoculated at 28°C for 5 Days and then culture was filtered to obtain FCF.

3. Application of the Fungal culture filtrate to the plants:

The 100 µl of FCF was spreaded over the dorsal surface of the leaves of the 40 days old plants in the field or on detached leaves placed in the sterile petriplate having wet filter paper.

4. Total protein extraction for enzyme assays:

1 gm of the leaves of each variety was homogenized in 0.1 M potassium phosphate buffer, pH 7.0, containing 0.1 mM Phenyl methyl sulfonyl fluoride (PMSF) using a pre-chilled mortar and pestle. 1% Polyvinyl pyrrolidone (PVP) was added during the homogenization process and the volume was made up to 10 ml. The sample was centrifuged at 8000 revolutions per minute (RPM) for 15 min. Supernatant was used as the extract for the enzyme assays and protein estimation by Lowry et al, 1951. This extract was also used to study the 10% SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis) profile of the PR proteins.

5. Measurement of Total Chlorophyll:

Chlorophyll measurement was done as described in Thiammaihi, 1998. One gm of leaf was crushed in mortar and pestle. Then 20 ml of 80 % acetone was added. Centrifuged at 5000 RPM for five minutes and supernatant was transferred to a 100 ml volumetric flask and then the residue was grinded again with 20 ml of 80% acetone. The supernatant was then transferred to the volumetric flask. This
process was repeated till the residue was colorless. Final volume was constituted to 100 ml with 80% acetone. Absorbance of the solution was read at 645, 663 and 652 nm using 80% acetone as a blank.

6. Measurement of Cell death:

After treatment of the FCF, leaves from infected plants were taken at the indicated times and then cut in to equal size using the 5 millimeter borer. Then these leaf discs were soaked in 10 ml of 0.25% Evans blue. Leaves were then washed briefly in 10 ml water and de-stained in 96% ethanol for 10 min. Optical density (OD) of de-staining solution was measured at 660 nm (Liu et al, 2008).

7. Measurement of Ion leakage:

Ion leakage measurement was done as described by Khayyat et al, 2009. Ion leakage was determined by measuring the conductivity, in milli volt (mV) of the solution using the pH meter. Leaves treated with the FCF of pathogen were taken. 5 millimeter circular leaf disc segment were cut out at random from each leaf, washed three times with distilled water in order to remove surface contaminants and then placed individually in 100 ml conical flasks sealed with the aluminum foil vials containing 10 ml of milliq water. The vials were shaken moderately for 15 min and then incubated at RT (room temperature) (25°C) for 24 hours. Electrical conductivity of this bathing solution was measured by measuring the voltage (mV) after incubation using the pH meter. The conductivity of the milliq water was taken as the reference.
8. Measurement of Lipid peroxidation:

Lipid peroxidation measurement was done by measuring the MDA content. MDA measurement was followed the method described by Reezi et al, 2009. Plant leaves (0.2 gm) were homogenized in 1.5 ml, 5% (w/v) trichloroacetic acid (TCA) using a mortar and pestle. The homogenates were centrifuged at 13000 g for 20 min. A reaction mixture of the supernatant (0.5 ml) and 1 ml TCA 20% (w/v) and thiobarbituric acid (0.5%) was incubated at 95°C in a water bath for 25 min, and then cooled immediately before centrifugation. Absorbance of the supernatants was determined at 450, 532 and 600 nm, respectively. Calculation of MDA was done based on the following formula: \( C (\mu m/l) = 6.45(A_{532} - A_{600}) - 0.56(A_{450}) \).

9. Measurement of FRAP value:

Antioxidant power was measured by using the Standard operating procedure (S.O.P) adapted from Benzie and Strain, 1996. Antioxidant power can be presented as ferric reducing ability. The assay measured the formation of a colored ferrous: 2, 4, 6-tripyridyl-s-triazine (Fe\(^{+2}\) -TPTZ) complex from ferric ion at low pH. One gm of leaves were cut into small pieces and mashed with a cool mortar and pestle using and 9 ml cool 0.1 M phosphate buffer (pH 7.6, containing 0.1 mM Ethylenediamine tetraacetic acid (EDTA)). This mixture was filtered through a filter paper and centrifuged at 15000 RPM for 10 min. The supernatant was used for the measurements. Sample containing 3 ml of freshly prepared FRAP solution (0.3 M acetate buffer (pH 3.6)) containing 10 mM 2, 4, 6- TPTZ and 40 mM FeCl\(_3\).6H\(_2\)O and 100 μl of extract (as prepared for phenol determination)
was incubated at 37°C for 4 min. The absorbance was measured at 593 nm (UV/Visible Spectrophotometer, Halios). An intense blue color is formed when the ferric-tripyridyltriazine (Fe³⁺-TPTZ) complex is reduced to the ferrous (Fe²⁺-TPTZ). A standard solution of Fe²⁺ (FeSO₄·7H₂O) in the range of 100-1000 µmol/l in milliq water was prepared. The absorbance change was converted into a FRAP (Ferric reducing ability of Plasma) value, by relating the change of absorbance at 593 nm of the test sample to that of the standard solution of Fe²⁺ and results were expressed as µ mol Fe²⁺ g⁻¹ fw.

10. Measurement of Catalase activity:

Catalase activity was measured as described by Beers and Sizer, 1952. In 3 ml of reaction mixture, 3.0 ml of 50 mM Potassium Phosphate buffer (pH 7.0) was added in blank, 2.9 ml of 0.036 % H₂O₂ (OD between 0.520 - 0.550 at A₂₄₀ nm) in test was added. Then A₂₄₀ was recorded till constant. 100 µl of enzyme solution was added to the test and time required for the decrease of OD from 0.45 to 0.40 was recorded. One unit of enzyme decomposed 1.0 µmole of H₂O₂ per minute at pH 7.0 at 25°C, as observed at 240 nm.

11. Measurement of Superoxide dismutase activity:

The assay mixture contained 1.2 ml of potassium pyrophosphate buffer pH 7.0, 0.1 ml of Phenazine methosulfate (PMS), 0.3 ml of Nitro blue tetrazolium (NBT), 0.2 ml of the enzyme preparation and water in a total volume of 2.8 ml. The reaction was initiated by the addition of 0.2 ml of NADH (Reduced Nicotinamide adenine dinucleotide). The mixture was incubated at 30°C for 90 seconds and arrested by the addition of 1.0 ml of glacial acetic acid. The reaction mixture was
then shaken with 4.0 ml of n-butanol, allowed to stand for 10 minutes and centrifuged. The intensity of the chromogen in the butanol layer was measured at 560 nm in a spectrophotometer. One unit of enzyme activity is defined as the amount of enzyme that gave 50% inhibition of NBT reduction in one minute.

12. Measurement of NADPH oxidase activity:

NADPH oxidase (NOD) activity was done as described by Reusch and Burger, 1974. 3 ml of reaction mixture contained 1.90 ml deionised water, 51 mM potassium phosphate buffer pH 7.0 (0.60 ml), 0.07 mM β-NADPH (0.30 ml), 0.1 mM Flavin adenine dinucleotide (FAD). The reaction mixture was mixed well and equilibrated by inversion at 30°C. OD A_340/minute was taken till it became constant. Then 100 µl of enzyme extract in test and 100 µl of potassium phosphate buffer in blank was added. Again reaction mixture was mixed by inversion and decrease in A_340 nm was recorded. Then A_340/minute was obtained. One unit of NOD was defined as the enzyme that will oxidize 1.0 µmole of β-NADPH per minute at pH 7.0 at 30°C.

13. Measurement of Polyphenol oxidase activity:

PPO activity was measured as described by Dawson and Magee, 1955. In a 3 ml reaction mix, the final concentrations contained 2.60 ml 50 mM potassium phosphate buffer (pH 6.5), 0.10 ml 5 mM L-3,4-dihydroxyphenylalanine, 0.100 ml of 2.1 mM L-ascorbic acid, 0.100 ml of 0.065 mM EDTA. Immediately mixed by inversion and recorded A_265 nm till it became constant. Then 100 µl of enzyme extract was added in test and decrease in A_265 nm for approximately 5 min was recorded. The change in OD at A_265 nm/min for test and blank were obtained.
1.0% (w/v) BSA. Mixed by inversion and equilibrated to 25°C. Monitor the $A_{340}$ nm until constant, using a suitably thermostated spectrophotometer. Then 0.10 ml of enzyme solution was added in test and 0.1 ml of BSA was added in blank. Immediately mixed by inversion and the decrease in the $A_{340}$ nm for approximately 5 min was recorded. The change in OD at $A_{340}$ nm / min using the test and blank was obtained. One unit of GR reduced 1.0 µmol of oxidized glutathione per min at pH 7.6 at 25°C.

17. Measurement of Reduced glutathione content:

GSH from the extract was determined by the method of Ellman's assay. The reaction mixture contained 2.0 ml of 0.1 M Potassium phosphate buffer, 0.20 ml of extract, and 0.04 ml of Ellmann's reagent. Incubated at RT for 15 min. The absorbance was read at 412 nm. The level of GSH was expressed as µmoles/mg.

18. Measurement of Total phenol:

The leaves were grounded in to mortar and pestle in to 80 % ethanol (1mg/ml) for the extraction of total phenol. Then the homogenate was centrifuged at 10000 RPM for 20 min and the residue was reextracted five times with 80 % ethanol, centrifuged and supernatant pooled. Then it was evaporated to dryness, and then dissolved in to 5 ml of distilled water. 0.2-2 ml of supernatant was then taken in to test tube and then made up to 3 ml with distilled water. Then it was added with 0.5 ml of Folin ciocalteau reagent (FCR). After 3 min, 2 ml of 20 % $Na_2CO_3$ was added, mixed thoroughly, then tubes were kept in boiling water bath for one min, cooled and absorbance was read at 650 nm. Standard curve was
prepared using different concentrations of Catechol (Catechol std 100-600 μg/ml).

19. Extraction and quantitation of Salicylic acid:

1 gm of FCF treated *S. tuberosum* leaves were sliced using scissor and kept overnight in 5 ml of ice-cold methanol. Then the extract was constituted to 500 μl. 20 μl of sample from this was loaded on to the HPLC and run in 0.01 M potassium phosphate buffer pH 5.5 and methanol (50:50 v/v) was used as mobile phase. SA was separated in Zorbex C18 column at flow rate of 1 ml/ min and detected by UV detector at 315 nm. 20 μl of 1 mg/ ml of SA (Ramkem) in methanol was loaded as the standard. SA was identified from the retention time and quantified from the peak area in comparison with the standard.

20. Extraction and quantitation of Jasmonic acid using HPTLC:

JA was extracted from the 5 gm of potato leaf samples collected at different time intervals after spraying with the FCF of different pathogens. Then this extract was kept overnight in 5 ml of 70 % methanol. This was collected in eppendorf tube and the JA was extracted in 500 μl of ethyl acetate. Aluminum- backed silica gels 60 F 254 TLC foils (20 X10 cm) of 0.25 mm thickness (Merck, Germany) were run with methanol as mobile phase and then dried in oven at 120°C for 20 min before sample loading. Sample loading was performed using Linomat V applicator (CAMAG, Germany) using 100 μl syringe (Hamilton). Then 20 μl of this sample was loaded on to the TLC plate and ran in the 20 X 10 cm twin trough chamber using Iso propanol : ammonia : water (10:1:1 v/v) mobile phase. TLC chamber (Camag) was already preconditioned with the mobile phase for 20 min.
before start of the run. 50 μg of the JA was loaded as the internal standard in each plate. After development, the plate was air dried for 10 min and peak area of the JA band in the sample and standard were quantified at 295 nm using CAMAG TLC Scanner 3 with deuterium source. The JA was quantified using the peak area generated using win CATS software version 1.2.2

21. Measurement of Lipoxygenase activity in plant leaves:

Substrate preparation:

The substrate was prepared as described by Anthon and Barett (2001). 25 mM stock solution was prepared by adding 155 μl (140 mg) of linoleic acid and 257 μl (280 mg) of tween 20 to 5 ml of water. The mixture was emulsified by drawing back and forth in a Pasteur pipette and then clarified by adding 0.6 ml of 1 N NaOH. After dilution to the final volume of 20 ml using water, the solution was divided in to 1 ml aliquots in air tight tubes which were stored at 0°C in a freezer.

Lipoxygenase assay:

LOX activity was measured from the plant material using conjugated diene method as described by Surrey (1964). This method makes use of the fact that the hydroperoxy lipid product of reaction contains a conjugated diene which absorbs strongly at 234 nm. Reaction rates thus can be determined by measuring the product formation through the change in the absorption at this wavelength. The reaction was carried out at 25°C in a mixture of 200 μl of the respective enzyme extract and 2.784 ml of Potassium Phosphate buffer (pH 6.0) and 15 μl of substrate (linoleic acid) in presence of O2. After 1 minute this 1 ml sample was
transferred in to each of three reaction tubes containing 2 ml of absolute alcohol. This would have stopped the reaction and the product was allowed to come in to the alcoholic layer. To each tube 7 ml of 60 % ethanol was added to make total volume of 10 ml. All the reactions were performed in triplicates for more accuracy in the interpretation and statistical analysis. Optical densities of the clear alcoholic solutions were read at 234 nm against a suitable control by a HALIOS UV spectrophotometer. The use of this method with crude enzyme preparation is however limited by the presence of other UV absorbing material in the assay. Therefore the control system was prepared using the same volumes of substrate and enzyme as those used in the test samples. The enzyme was added to 2 ml of absolute alcohol. After mixing the tube was allowed to stand for a few minutes. 60 % alcohol was then added followed by substrates. The spectral blank was prepared without adding the enzyme and it would compensate by adding the potassium phosphate buffer (10 mM, pH 6.0). One unit of LOX activity was calculated as the enzyme that will produce an optical density of 0.001 at 234 nm in 1 minute in a total volume of 10 ml of 60% ethanol solution.

22. Measurement of Phenylalanine ammonialyase activity:

Measurement of PAL activity was done as described by Hodgins et al, 1987. Phenyl alanine is converted to transcinnamic acid and ammonia. This decrease in the L-Phenyl alanine is measured at 270 nm and hence the activity of the PAL is measured. 2 ml of 3 mM L-Phenylalanine solution and 0.9 ml of deionized water was added in all the test and the blank tubes. The tubes were mixed by inversion and equilibrated at 30°C. Then 0.1ml of enzyme solution was added in the test and 0.1 ml 150 mM Tris HCL Buffer, pH 8.5 was added in the blank. The tubes
were immediately mixed by inversion and increase in A$_{270nm}$ for approximately 5 min was recorded. $\Delta$A$_{270nm}$/min were obtained for both the test and the blank. One unit of PAL deaminated 1.0 µmol of L-Phenylalamine to transcinnamic acid.

23. S and G lignin staining:

Detection of S lignin was performed using Maule’s staining. Leaf/stem were rinsed with water, treated with 1% (w/v) KMnO$_4$ for 15 min, rinsed with water twice and then with 2% (w/v) HCl for 5 min. Then washed and treated with 2N NH$_4$Cl. S lignin gives purple red to brown coloration.

24. Metabolome profiling:

Six days after treatment of the FCF of all three pathogens, 1 gram of the plant material was made in to final slices and then was kept overnight in 80 % Methanol. Extract was then made up to equal volume (500 µl) with same solvent and 20 µl of it was loaded on to TLC plates. The plates were given prewash of 10 min with methanol. This and other plates were run in twin trough chamber (CAMAG, Switzerland) previously saturated at least for 30 min with 30 ml of solvent system Butanol : Acetic acid : Water [4: 1: 5 (v/v)] for 20 min. It was allowed to dry for 10 min at RT and different metabolites separated bands were then scanned at 400 nm - 700 nm using Scanner-3 (CAMAG, Switzerland). Differential bands were then identified and their $\lambda_{\text{max}}$ was determined using Wincats planner chromatography software (CAMAG, Switzerland).
25. Measurement of Oxalic acid from Fungal culture filtrate and Plants:

Oxalic acid was measured as described by Hodginson, 1970. To 1 gm of the leaf powder or 10 ml FCF, 10 ml 1 N sulfuric acid was added. The solution was carefully shaken for 10 min and then filtered using Whatman No.1 filter paper. The filtrate was then collected and titrated against 0.01 N KMnO4 solution. (1.0 ml of 0.01N KMnO4 is equivalent to 0.45 mg of anhydrous oxalic acid)

26. Histochemical assay for the detection of H₂O₂:

The detection of H₂O₂ in leaf sample was done using the DAB (3.3'-Diamino benzidine) staining method. Leaves were detached and placed in 1mg/ml DAB-HCl, pH 3.8, DAB generates a reddish-brown DAB polymer that can be detected at the site of the H₂O₂ formation. After staining, leaves were cleared in 96% boiling ethanol and observed with a microscope (Veronese et al, 2006).

27. Lignin staining:

Phloroglucinol (2.0 gm) in 80 ml of 20% ethanol solution was prepared. Solution was then filtered. A drop of phloroglucinol was applied over section of leaf/stem and this was then covered with a drop of 12 N HCl for 1 min and then covered with cover slip observed under microscope.

28. Measurement of β-1,3 Glucanase activity:

32 μL of 4 % laminarin and 32 μL of the enzyme extract were added in all the experimental tubes. In the control tubes only 32 μL of 4 % laminarin was added. The tubes were incubated at 40°C for 10 min in water bath. The reaction was
stopped by adding 187 μL of DNSA (3,5-Dinitrosalicylic acid). 32 μl of enzyme extract was added in all the control tubes after words. The tubes were then kept in boiling water bath for 5 min. 2 ml of distilled water was added in all of the tubes after cooling. The absorbance was measured at 550 nm.

29. Measurement of Chitinase activity:

Measurement of Chitinase activity was done as described by Monreal and Reese, 1969. 0.1% w/v solution of NAG (N-acetyl glucose amine) was prepared in deionised water. 100-500 μg of this NAG was used to plot the standard curve. The test [2.0 ml of 1.25% (w/v) Chitin Suspension and 0.5 ml of enzyme] and Blank [2.0 ml 1.25% (w/v) Chitin Suspension and 0.5 ml deionised water] were incubated for two hours at 25°C and then kept in boiling water bath for 5 min, cooled to RT and Centrifuged the suspension. 1.0 ml of this test supernatant, 2.0 ml deionised water and 1.5 ml of DNSA reagent were taken for test and 2.0 ml blank supernatant, 1.0 ml deionised water and 1.50 ml of DNSA reagent for the blank. All the containers were placed in a boiling water bath for 5 min, cooled to room temperature and the OD was recorded at A540 nm. One unit of the enzyme was defined as the enzyme that liberated 1.0 mg of NAG from chitin per hour at pH 6.0 at 25°C in a two step reaction.

30. Extraction of NPR1 protein from the plants:

1 gm of 40 day old potato (S. tuberosum) leaves were collected and treated with 7 day old FCF of P. infestans for 2 hours and then subjected to protein extraction. Leaves were homogenized in pestle and mortar with 1 ml of various solvents. Various solvents that were used for extraction were - 1) Chilled water, 2) Salt
solution with Tris buffer, 50 mM Tris HCl (pH 7.5), 150 mM NaCl, 10 mM MgCl₂, 10 % glycerol and 1.0 mM PMSF, 3) 0.1 M Tris buffer (pH 7) and 4) Phosphate buffer, pH 7. 1% PVP was added during the grinding in all extraction processes. Then centrifugation was done at 15000 RPM for 30 min at 4°C. The pellet was discarded and the supernatant was taken. Total protein from this supernatant was estimated using method of Lowry et al, 1951. The supernatant was further used for the protein separation by Sodium Dodecyl Sulfate Polyacrylamide gel electrophoresis (SDS-PAGE) for NPR₁ analysis. The solvent capable of extracting higher number of proteins were used for further studies. Leaves of S. tuberosum treated with FCF of P. infestans, F. oxysporum and A. solani as shown above along with control leaves were subjected to total protein extraction using salt solution with Tris buffer (pH 7.5) and SDS-PAGE analysis.

31. SDS-PAGE and Native PAGE analysis:

SDS and Native PAGE were done as described by Laemmli, 1970. Proteins extracted after treatment of the FCF of the all three pathogens were subjected to 10% SDS PAGE analysis. About 400 µg of the total protein was loaded in the wells along with the protein molecular weight marker and the gel was run at 90 volt for initial 2 hours in 5% stacking gel and then at 110 volt for 4 hours in 10% resolving gel till complete development of gel. The gel was then removed and stained using Comassie brilliant blue R-250 (CBB R-250) prepared in Methanol: acetic acid: water (425:75:500) for 15 hours and destained with Methanol: acetic acid: water (425:75:500) for 1.5 hours.
32. Western blot analysis:

Western blotting using antibodies against *A. thaliana* NPR1 was carried out to confirm the identity of one of the induced band of 65.9 kD found in SDS PAGE analysis. Briefly, the gel was electro transferred on to the nitrocellulose membrane at 35 volt at 4°C for 15 hours. The membrane was blocked by immersing in 3% BSA at RT for two hours. The membrane was then probed with the polyclonal rabbit antibodies against *A. thaliana* NPR1 protein at 1:833 dilution for 50 min and then washed by immersing in 1X Phosphate buffered saline (PBS) (pH 7.2). The membrane was then incubated in 10 ml goat horse radish peroxidase conjugated goat anti-rabbit antibody at 1:1000 dilution with gentle agitation. The blot was then developed by immersing in 10 ml of TMB-\(H_2O_2\) solution with gentle shaking for 30 min.

33. Generation of the calibration curve for the determination of the protein concentration:

200 ng to 50 \(\mu\)g of the standard protein, Bovine serum albumin (BSA) was loaded in the gel and the calibration curve was drawn based on generation of peak area using Alpha DigiDOCT™ RT Image processing and image analysis software (USA). Limit of detection (LOD) and limit of quantification (LOQ) were determined. Regression analysis was measured to check the linearity.

34. Determination of the inter gel variation:

2.5 - 30 \(\mu\)g of the BSA protein was ran in three different gels and the calibration curve was drawn from the peak area generated. Standard deviation (SD) and
Coefficient of variation (CV) were calculated for the same concentration of the protein between three different gels.

35. Determination of the intra gel variation:

3 μg and 7 μg BSA were loaded in triplicate and 2, 4, 6, 8 and 10 μg of the BSA protein as standard in the same gel to generate the calibration curve to estimate the difference in the protein loaded on gel and protein calculated from the gel. CV was calculated for the protein loaded and protein estimated.

36. Estimation of the protein of interest using Alphadigidoc TM RT:

400 μg protein samples extracted from FCF 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 (charged coupled device) Digital camera of Alpha DigiDoc™ RT System. The images were then processed with the Alpha DigiDoc™ RT software. A calibration curve was drawn and the concentration of the band of interest (65.9 kD) from samples was determined using the same software.

37. In gel analysis of induced PR proteins:

400 μg protein samples extracted from FCF treated and control plant leaves were loaded on the to the SDS PAGE gels and scanned using Alpha DigiDoc™ RT software. The bands with increased intensity as well as differentially induced bands were studied for comparison of the induction of the PR proteins.