3.1. Introduction

Biotic or abiotic elicitors of resistance are eco-friendly compounds that could modulate the protein and metabolic machinery of plants to counteract stress conditions. In terms of cost of fitness, elicitor treatment ensures that energy required for activating the defense response of plants is activated only upon encountering stress condition (Conrath, 2009). This phenomenon of priming if observed among elicitors that can alleviate stress condition in plants by about at least more than 60% then, comprehending the mechanism by which they confer resistance to plants and can aid in sustainable agricultural practices. This could be achieved by obtaining a holistic view of plant protein and metabolic machinery during priming and its subsequent exposure to stress which can be studied by high-throughput approaches such as proteomics.

Proteomics in simple terms refers to study of large set of proteins in a given cell with relevance to their abundance level, sub-cellular localization, post-translational modification of proteins, their interaction with other proteins of particular or different metabolic pathways (X. Fang et al. 2015). Generally, research communities working on stress biology in plants apply comparative proteomic approach to comprehend the resistance mechanism of plants with an effort in developing a cultivar with resistant trait. However, it is important to note that there is scarcity in information on proteomic analysis on these aspects: (i) effect of elicitor treated plants exposed to biotic stress (ii) affect of elicitor treated plants of millet family or among monocots exposed to abiotic and biotic stress (iii) pearl millet, whose genome is not sequenced yet.

Considering these facts into account and to add onto the existing knowledge of pearl millet- *Sclerospora graminicola* interaction, the present investigation was carried out which deals with dynamics of elicitor primed pearl millet proteome during infection with the downy mildew pathogen. This effort was made for deciphering the resistance mechanism conferred by the elicitors onto the host to counteract the biotic stress. The present chapter deals with standardization of protocols for total protein extraction from pearl millet seedlings using SDS-PAGE approach and optimization of
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dimensions and other parameters to be considered for 2DE (two-dimensional gel electrophoresis).

The optimized parameters were then considered in the fractionation and detection of significantly and differentially accumulated proteins in pearl millet seedlings earlier primed with an abiotic elicitor- β-amino butyric acid or a biotic elicitor- Pseudomonas fluorescens and its subsequent encounter with downy mildew pathogen. This was achieved using two-dimensional gel electrophoresis, 2D-gel analysis software and univariate statistics.

3.2. Materials and methods

3.2.1. Plant material, pathogen and elicitors

Seeds of pearl millet cv.7042S exhibiting high susceptibility [downy mildew disease incidence (dmdi) of 85-95%] and cv.IP18296 with high resistance (0% dmdi) to the S. graminicola were used in the present study. The seeds were obtained from All India Coordinated Pearl Millet Improvement Project, Mandor, Jodhpur, India. The elicitor β-aminobutyric acid (BABA) was procured from Sigma Chemical Co, St. Louis, USA, while the isolate of P. fluorescens (ID No. UOM SAR14), was obtained from the culture collection of Department of Studies in Biotechnology, University of Mysore, Mysore, India. The inoculation experiments were carried out using a virulent pathotype of downy mildew biotrophic pathogen S. graminicola isolated from pearl millet cv.7042S and was maintained on the same cultivar under greenhouse conditions.

3.2.2. Pathogen inoculum preparation

The inoculum of S. graminicola was prepared as previously described (Safeeulla, 1976). Initially, leaves of pearl millet with profuse sporulation of S. graminicola on the abaxial side were collected in the evening from plants maintained under greenhouse conditions (25–30 °C, >95% 120 RH). These leaves were thoroughly washed under running tap water to remove the previous crop of sporangia which were then blot-dried, cut into small pieces, and kept in a moist chamber for
sporulation. The next morning, zoospores released from the fresh crop of sporangia were harvested in sterile distilled water which served as pathogen inoculum.

3.2.3. Elicitor treatment

Surface sterilization of pearl millet seeds of cv.7042S were carried out for 15 min with 0.1% (w/v) sodium hypochlorite solution, with subsequent washing in sterile distilled water for 2 min. Priming one set of seeds were with BABA, an abiotic inducer and the other set with *P. fluorescens* UOM SAR14, a biotic inducer was carried out as described by (Raj, Shetty, & Shetty, 2004; Shailasree et al., 2001) respectively. The seeds primed with these elicitors were germinated at 25± 2 °C in dark for two days on moist filter paper under aseptic conditions. The seeds treated with water served as control for elicitor treatment.

3.2.4. Challenge inoculation of plant material with pathogen

The two-day old seedlings from elicitor treated seeds were root-dip inoculated in *S. graminicola* zoospore suspension (4 × 10⁴ zoospores/mL) as described previously (Safeeulla, 1976). Seedlings immersed in sterile distilled water served as control. The elicitor primed seedlings were harvested at 24 h of post-inoculation (hpi) and snap frozen in liquid nitrogen before being stored at −80 °C for further studies. Thus six different treatment samples were generated: untreated-non-inoculated or control (UC), untreated-pathogen inoculated (UI), BABA treated-non-inoculated (BTN), BABA treated-pathogen inoculated (BTI), *P. fluorescens* UOM SAR14 treated-non-inoculated (PTN) sample and *P. fluorescens* UOM SAR14 treated-pathogen inoculated (PTI) samples. The experiment was repeated thrice to obtain three biological replicates for further proteomic analysis.

On the other hand, seedlings of resistant cv. IP18296 were harvested at 0, 6, 12 h.p.i. along with its corresponding control and were utilized during standardization of proteomic experiment. Among these samples, the early time point of post inoculation with the pathogen i.e. 6 h.p.i was considered for analysis of dynamics of pearl millet seedling proteome during early phase of pathogen infection.
3.2.5. Standardization of protein extraction, quantification and two-dimensional gel electrophoresis (2DE) procedure for fractionation of pearl millet seedling proteome

3.2.5.1. Standardization of protein extraction protocol

Initially, the protein extraction was performed using TCA-acetone based protocol. Due to unsatisfactory results obtained from this method, comparatively better phenol based method was adopted for standardizing the extraction procedure. The total proteins were extracted from the seedlings by modified method of (Hurkman & Tanaka, 1986).

- Pearl millet seedlings (1 g) were homogenized in a pre-chilled pestle & mortar with acid washed sand. Ground tissue (0.4 g) was suspended in a pre-weighed microfuge tube containing 800 µl of the extraction buffer [500 mM Tris-HCl, 50 mM EDTA, 700 mM Sucrose, 100 mM KCl, pH was adjusted to 8.0 with HCl. 1 mM PMSF & 2% β-Mercaptoethanol was added just before extraction. 0.1% PVP (optional)] and incubated by shaking for 10 min. on ice. This step was performed in three different sets and 8 replicates were maintained in each set. The mixture in each of the three different sets was processed further in three different ways.

  - (i) The first set was processed by adding equal volume of Tris-saturated phenol of pH 8.0.
  - (ii) The second set was processed by adding equal volume of Tris-saturated phenol, pH 8.0 along with SDS buffer [30% Sucrose, 2% SDS, 0.1M Tris-HCl (pH 8.0) and 5% β-Mercaptoethanol] in 1:1 ratio.
  - The solution in both set 1 and 2 were incubated on a shaker for 10 min. at room temperature.
  - (iii) While the third set was processed by centrifuging the above mixture at 12,000 rpm at 4°C for 25 min. The supernatant was collected and further divided into two subsets: subset 1 and subset 2. Subset 1 and subset 2 was processed in the same manner as mentioned in (i) and (ii) respectively.

- A fourth and fifth experimental set was also maintained where samples were processed in a manner similar to that of set 3 of subset 1.
For convenience, the 5 different experimental set ups are referred as Set 1, Set 2 and Set 3 (with its subsets 1 and 2, Set 4 and Set 5).

All the 5 experimental sets were further subjected to extraction process (independently) in the same manner, as mentioned below.

To separate the insoluble material for aqueous and organic phases, the samples were centrifuged for 10 min. at 10,000 rpm at 4 °C. The upper phenol phase is recovered carefully and poured into a new 2ml tube.

The phenol phase was then back extracted with 800 µl of the extraction buffer. The samples were shaken and vortexed for 3 min. Centrifugation for phase separation was repeated for 10 min. at 10,000 rpm at 4 °C.

The phenolic phase collected from 8 replicates of each set on top of the tube is carefully recovered and poured into new centrifuge tubes; 4 volume of precipitation solution (0.1 M ammonium acetate in cold methanol) was added. The tube was shaken by inverting and incubated overnight at -20 °C.

The proteins were pelleted by centrifugation for 15 min at 10,000 rpm at 4 °C.

After centrifugation, the pellet was washed thrice with ice cold 100% methanol. After each washing step, the samples were centrifuged 10 min. at 10,000 rpm at 4 °C. Finally, the protein pellet was air dried for 2 min.

The pellets obtained from experimental sets 1 to 3 were solubilised and diluted in 5X SDS-PAGE sample buffer (45% glycerol, 5% SDS, 20% β-mercaptoethanol, 35% of 0.5 M Tris-Cl pH 6.8 and a pinch of bromophenol blue) before boiling the samples for 5 min and loading 50 µg equivalent of total protein from the extract for SDS-PAGE.

The protein pellets from set 4 were solubilized in lysis buffer containing 9M urea, 10% Triton X-100 and 2% dithiothreitol (DTT).

In case of set 5, the protein pellets were solubilized in lysis buffer containing 7 M urea, 2 M thiourea, 2% of 3-[(3-Cholamidopropyl) dimethyl ammonio]-1-propanesulfonate (i.e. CHAPS), 0.5% ampholytes of pH 3–10 and 2% DTT. The solubilized protein pellet from set 4 and 5 were diluted using lysis buffer prior to running by SDS-PAGE.
3.2.5.2. Optimization of quantification procedure for loading equivalent amount of protein amount onto IPG strips

Initially bovine serum albumin working solution is prepared in water at a concentration of 1 mg/ml. Protein standards from this solution is then prepared with concentration range from 0-50 µg. 10 µl each of lysis buffer and 0.1N HCl is added to the reaction mixture followed by addition of 2 ml of Bradford reagent prior to quantification of protein standards at 595 nm. Quantification of protein extract (test sample) is determined using the BSA standard values (Ramagli & Rodriguez, 1985).

3.2.5.3. Optimization of dimensions for two-dimensional gel electrophoresis

Initially, standardization of protocol for the protein extract from pearl millet seedlings were performed on 3-10 pI range IPG strips of 7cm length prior to determining the coverage of pI range of the fractionated proteins on 2D gels. Based on the preliminary observation, narrow pI range IPG strips (4 to 8 pI or 5 to 8 pI range) as well as bigger dimension 17cm IPG strips were chosen for further experiments. The IEF program for the 7cm IPG strips consisted of 0-250V linear ramp for 15 min. followed by gradual increase to 4000V for 2 h and then 10000 Vh as per the manufacturer’s instruction (Ready Strip IPG, Bio-Rad). Both silver staining as well as Coomassie blue staining was adopted during the standardization.

3.2.6. Analysis of differentially expressed protein in elicitor primed pearl millet seedling during its interaction with downy mildew causing S. graminicola

3.2.6.1. Protein extraction and two-dimensional gel electrophoresis

Total protein was extracted from the seedlings obtained from each of the six treatment conditions (that includes elicitor treatment) by phenol based precipitation protocol as described by (Anup et al., 2015). The precipitated protein was solubilized in lysis buffer (7 M urea, 2 M thiourea, 2% CHAPS, 0.5% ampholytes pH 3–10 and 2% DTT) and protein estimation was performed by modified Bradford method with BSA as standard (Ramagli & Rodriguez, 1985). Solubilized proteins obtained from three biological replicate samples were stored at -80 °C for further experiments.
Four hundred microgram equivalent proteins were subjected to one-dimensional fractionation on IPG Ready strip (Bio-Rad) 17 cm, linear pH range of 5–8 by initially rehydrating the strip with the protein sample at 50 μA for 12 h. This was followed by iso-electric focusing of the proteins in IPG strips on PROTEAN IEF unit (BioRad) at 20 °C with increasing linear voltage: 1 h at 1000 V and 2.5 h at 10,000 V followed by constant 10,000 V maintained until 60,000 Vh is attained. Subsequently, the IPG strips were incubated initially for 15 min in 500 mM Tris–HCl buffer pH 8.8, 6 M urea, 2% SDS, 20% glycerol solution containing 2% DTT and then for 15 min in the same solution containing 2.5% iodoacetamide instead of 2% DTT. The one-dimensional fractionated proteins on the IPG strips were then subjected to second dimensional separation on 12% SDS-polyacrylamide gels electrophoresis which was carried out using Protean xi cell (Bio-Rad) at 15 °C for 30 min at 25 V and then at 50 V/gel for about 14 h, until the dye front reached the bottom of the gel. Visualization of protein spots on 2D gels were performed by colloidal Coomassie blue staining (X. Wang et al., 2012).

3.2.6.2. Image acquisition and statistical analysis of dataset

Eighteen gels (6 treatments × 3 independent biological replicates) were scanned using Gel Doc-XR (BioRad) densitometer and the gel images were analyzed with PD-Quest software (version 8.01). Spot detection tool of the 2D gel analysis software was employed in detecting and matching spots between gels of six different treatment samples and addition of spots missed by the software and deletion of artifacts identified as spots were performed manually. The analysis was re-evaluated by visual inspection, by considering the spots with significant fold-change (with two-fold-change in protein abundance in at least one of the treatments) among treatments which were consistently observed across biological replicates. All the six treatment samples along with their replicates were analyzed simultaneously. Total density in gel image as normalization factor of the software was used in determining normalized intensities of the protein spots (expressed in parts per million).

For further analysis, protein spots that met the following criteria were considered: the protein spots displaying reproducible treatment ratios differing at least 2-fold with statistical (Student t-test) significance \( p \leq 0.05 \) between treatments and...
which were consistently present or absent among the replicates. Normalized intensities of the protein spots that met the criteria were log transformed (log10) and subjecting to one-way ANOVA with Tukey's HSD test at a significance of \( p \leq 0.05 \).

3.3. Results and discussion

For a proteomic experiment, it is necessary to ensure the protein extraction procedure adopted extracts maximum amount of protein from a tissue under study, followed by their subsequent solubilization in a buffer that is compatible with fractionation techniques adopted. For instance, the proteins extracted for 2DE need to be solubilized using buffer containing chaotropes, detergents that are non-ionic, reducing agents and ampholytes and the sample with little or no salt content to enhance the efficiency of resolving power of IEF on IPG strips. The former parameter was achieved in our study by improvisation of the phenol method of protein extraction by Hurkman and Tanaka (Hurkman and Tanaka, 1986). The initial TCA-acetone based protein extraction procedure was unsatisfactory in providing better protein solubilization as well as resolution by SDS-PAGE approach which led to adoption of phenol-based protein extraction protocol. The main objective of standardization of protein extraction procedure is to ensure maximum extraction of the total proteins such that it is compatible with 2DE workflow.

3.3.1. Standardization of protein extraction procedure to be adopted for two-dimensional gel electrophoresis

The experimental sets 1 to 3 as detailed in material and method section were not efficient based on the observation of SDS-PAGE profile [Figure 3.1.(a) and (b)]. Though comparatively, there was an improvement in the resolution of bands for the experimental set 2 and 3. This could be due to evasion of the contaminating debris such as polysaccharides from the homogenized pearl millet seedlings sample which otherwise might block the pores of polyacrylamide gels and inturn cause undesirable resolution of protein bands during SDS-PAGE. Though this issue was sorted out by experimental set 3 [Figure 3.1.(c)], the final protein pellet obtained from the extraction is efficiently solubilized in lysis buffer containing chaotropes such as urea rather than using SDS-PAGE sample buffer. This is evident from Figure 3.1. (d) and
(e) where samples were processed as per experimental procedures of set 4 and set 5 respectively.

However, it is important to note that the resolution of protein bands on SDS-PAGE gels improved to a greater extent upon inclusion of additional chaotropic agent such as thiourea and comparatively better zwitterionic detergents such as CHAPS over Triton X-100 [Figure 3.1. (e)].

Inference of the standardization of protein extraction protocols that would be useful for further 2DE experiments:

- Supernatant portion of the homogenized tissue need to be considered for phenol phase extraction.
- Tris-saturated phenol yielded better result over its combination with SDS buffer used in the initial phase of extraction.
- Efficient solubilization of protein pellet in lysis buffer containing chaotropes rather than using 5X-SDS PAGE loading buffer.
- Lysis buffer containing additional chaotrope such as thiourea and superior quality zwitterionic detergent such as CHAPS enhanced the solubility of the extracted protein pellet.

It is important to note that after protein extraction protein enrichment or protein depletion is employed to narrow down the proteome analysis for a set of proteins (e.g. phosphoprotomics) and to remove the presence of any of the abundant proteins (e.g. RuBisCO) that would mask the detection of any low abundant proteins (Righetti & Boschetti, 2016). However, presence of RuBisCO which generally would appear as big blotch in the higher molecular mass region of 2D gels was not evident in our study. This could be attributed to the presence of low amount of RuBisCO in the germinating young seedling samples employed in the current study.
3.3.2. Optimization of quantification procedure for loading equivalent amount of protein amount onto IPG strips

Relative quantification of protein extract is a requisite for loading equivalent amount of proteins onto IPG strips to avoid bias in the quantification of protein abundance from 2D gels. The protein quantification protocol adopted need to be sensitive, compatible with buffers used in solubilizing the extracted protein and economical. With this consideration Bradford method seem to be more appropriate over other commonly used procedures such Lowry’s and Bicinchoninic methods.
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However, accuracy of protein estimation by the usual Bradford method was unfavorably influenced by the presence of alkaline chaotropes and detergents of the lysis buffer in which the sample is solubilized. To overcome this issue, the reaction mixture was acidified with 0.1 N HCl for both protein standards as well as that of test samples. In addition, to simulate the reaction mixture of test samples, incorporation of 10 µl of lysis buffer to each of the bovine serum albumin (BSA) protein standards is done.

An attempt was made in preparing the BSA standard itself in lysis buffer instead of water. But the erroneous reading of the standards with linear regression value par below 0.95 was obtained. Thus, the above strategy was adopted to prepare BSA protein standard as well as to quantify the protein mixture prior to loading of their equivalent amount onto IPG strips.

3.3.3. Optimization of dimensions for two-dimensional gel electrophoresis (2DE)

The initial standardization of 2DE was performed using 3-10 pI range IPG strips of 7cm length. From the figure 3.2., it was observed that most of the proteins were concentrated (focused) in the pI range between 4 to 8 pI. In addition, the protein spots seem to be overlapping which would cause complications in densitometric based protein quantification (by 2D gel analysis software) and during excision of the spots for mass spectrometric based protein identification.

This issue was overcome by narrowing down the pI range of IPG strips to 5 to 8 and running larger 2D gels of 20 cm x 17 cm. which seem to yield a better result as evident from figure 3.3. The type of staining procedure adopted seems to be crucial during subsequent phase of the proteomic experiments. For instance, initially, silver staining procedure was adopted for staining the 2D gels, which was more sensitive than CBB staining method. But due to lower linearity of dynamics during densitometric based protein abundance estimation and possibility of interfering silver ions during mass spectrometric analysis, the CBB-G250 based staining was adopted for further experiments.
Figure 3.2. Gel images of protein extract from 12 h control (water treated) and 12 h pathogen inoculated pearl millet seedlings. Protein extract from these samples were fractionated by 2DE on 3-10 pI range IPG strips and 12.5% SDS-polyacrylamide gels and the gels were silver stained.

Figure 3.3. Gel images of total protein extracted from 6 hrs. harvested control (water inoculated) and pathogen inoculated pearl millet seedlings. The first dimension isoelectric focusing of the total protein extract of 400µg equivalent was carried out on 18cm IPG strip (SERVA™) of pI range 5 to 8. The focussed proteins were then subjected to 12 % SDS-PAGE and then visualized by CBB-G250 staining solution.
3.3.4. Analysis of differentially accumulated proteins in pearl millet seedlings during early hours of post infection with downy mildew pathogen

After the initial standardization of protocols for protein extraction, quantification and 2DE, protein samples from 6 h.p.i. and its corresponding control was utilized in performing the 2DE experiments. The main objective behind this study was to determine the dynamics in pearl millet seedling proteome during initial phase of pathogen infection. Upon analysis of triplicate 2D gels derived from these samples about 200 protein spots were detected by the PD-Quest software, 35 protein spots were found to be differentially regulated of which 21 and 14 spots exhibited up-regulation and down-regulation at two-fold levels with significance at $p \leq 0.05$. These spots were subjected to mass spectrometric analysis as discussed in subsequent chapter.

3.3.5. Analysis of differentially accumulated proteins in elicitor primed pearl millet seedling during its interaction with *S. graminicola* causing downy mildew

As per our knowledge, the study is first to report on proteomic analysis of elicitor primed pearl millet seedlings and their interaction with downy mildew causing *Sclerospora graminicola*. The challenging aspect of the study was in terms of protein identification due to lack of sufficient protein database for pearl millet. The study was performed to shed light on the resistance mechanism of elicitor primed pearl millet seedlings which were challenge inoculated with downy mildew pathogen. The proteomic approach employed in the present study provides a picture on the dynamics of protein abundance which added onto the existing knowledge about the resistance mechanism of elicitor primed host against the downy mildew when compared to earlier histochemical and biochemical studies. As previous studies have shown that maximum differential response was observed in two-day old pearl millet seedlings with 24 h of post inoculation (hpi) with downy mildew pathogen, the particular time point was considered for sampling and analysis. Moreover, in-depth microscopic studies in the past have revealed that the pathogen makes its way into the host tissue
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within 24 h of inoculation of two-day old pearl millet seedlings by root dip method (Sharada, Shetty, & Shekar Shetty, 1995).

In the past field studies have shown that BABA and UOM SAR14 strain of *P. fluorescens* primed pearl millet seeds have reduced the disease incidence level by 74% and 75% respectively (Raj et al., 2004; Shailasree et al., 2001). To deduce the mode of action of these elicitors in conferring resistance to the host plant against downy mildew, they were considered in the present study. Though abundant reports on priming agents against downy mildew of pearl millet are available the mechanistic explanation of resistance induced by the elicitors in pearl millet is still unclear. Thus in our study, efforts were made to shed light on this regard using pearl millet–downy mildew host–pathosystem by proteomic analysis. The root-dip technique considered in our study is mainly due to pathogen’s ability to ingress the host via meristematic tissues (i.e. shoot tip or root tip) of pearl millet seedlings.

Preliminary experiments have revealed that majority of proteins from the extract of elicitor primed pearl millet seedlings fractionated by two-dimensional gel electrophoresis (2DE) were well resolved on IPG strips of pI range 5-8 pI (Figure 3.3). Thus, subsequent 2DE experiments were performed on 5-8 pI range IPG strips (Figure 3.4). It was observed that the proteins from the samples fractionated in this pI range were distributed in the molecular weight range of 18 kDa to 108 kDa. We assume that either due to stability of proteins in the pH of the extraction buffer considered or in the normal physiological condition that supported protein stability, most of the proteins were found in this pI range.

In order to nullify the bias that might have occurred during interpretation of 2D gel analysis three independent biological replicates were analyzed by 2DE experiments. Consistency was observed in terms of abundance pattern of protein spots from all the three replicate gels of each of the six treatments (Figure 3.6, protein spot intensities with standard error values).
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Figure 3.4. Representation of differentially accumulated proteins on master gel image generated by the PD-Quest software. About 63 significantly differentially accumulated proteins detected by the software (designated in the form of spot No.) upon analysis of 2D gels of UC, BTN, PTN, UI, BTI and PTI samples. These proteins were fractionated on IPG strips of pI range 5 to 8 and in the mass range of 14.4 kDa to 116 kDa of 12.5% polyacrylamide gels.
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Analysis of 2DE gels for the samples-UC, BTN, PTN, UI, BTI and PTI, stained in colloidal Coomassie blue (Figure 3.5.) by PD-Quest software (v8.01) resulted in detection of 570±39, 508±24, 536±38, 492±27, 533±35 and 543±44 protein spots in the samples respectively. Normalization factor considered for analysis of 2D gels was total density in gel image. This resulted in detection of 63 differentially accumulated protein spots with two-fold-change at p ≤ 0.05 (Student t-test) among at least one of the treatments. Representation of these protein spots by a synthetic master gel image created by the software is shown in Figure 3.4. The significant difference in the ratios of most drastically altered protein abundance among one of the six treatments with respect to their control are shown as p-values in Table 4.1. of subsequent chapter.

Figure 3.5. Representation of 2D-gel images for each of the six treatment samples of 24 h.p.i. proteomic experiment. The gels were stained in colloidal Coomassie blue G-250 solution.
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Figure 3.6. Line graph representation of the logarithmic abundance profile of the 63 differentially accumulated proteins. Significant differences among the samples were represented in the form of different alphabets on top of each standard error bar.
The significant changes in the dynamics of protein abundance of the 6 treatment samples was further confirmed by performing one-way ANOVA with Tukey's HSD at $p \leq 0.05$ (using SPSS tool v.8.0) on the normalized spot intensities of 63 significantly differentially accumulated protein spots (Figure 3.6). These protein spots were identified by mass spectrometric analysis as described in the subsequent chapter. The present chapter describes the standardization procedures adopted for total protein extraction from pearl millet seedlings, an appropriate protein estimation method and optimization of dimensions that is suitable for 2DE experiments. Based on the optimized protocols, fractionation by 2DE and detection of differentially accumulated proteins in 6 h.p.i. as well as elicitor primed pearl millet seedlings during downy mildew infection was performed with aid of 2D gel analysis software. Univariate statistical analysis by student t-test and one-way ANOVA at $p \leq 0.05$ using SPSS package (version 8) was performed.

The subsequent chapter deals with processing of these significant 2D gel protein spots and subjecting them to mass spectrometric analysis for identification. Multivariate statistics and bioinformatical analysis was further performed to further shed light on the resistance mechanism of pearl millet during downy mildew infection.