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Research Article

ALTERATIONS IN BARLEY PROTEOME UPON FUNGAL INFECTION AND TRICYCLAZOLE TREATMENT

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Abstract: The barley proteome was investigated upon fungal infection and subsequent treatment by tricyclazole (TCZ), which is known to have applications in spot blotch disease management in barley. Significantly enhanced chlorophyll content was recorded in TCZ treated plants. The disease severity was significantly reduced after TCZ application in pathogen inoculated plants by reducing the appressoria formation at infection site in barley leaves. Two-dimensional gel electrophoresis (2-DE) revealed the expression profile of proteins from (I) control plants (healthy barley leaves; application with sterile water), (II) plants after foliar application of TCZ (100 µg/ml), (III) plants inoculated with *B. sorokiniana* and (IV) plants treated with TCZ (72 h after *B. sorokiniana* inoculation). A set of 33 proteins expressed differentially after TCZ treatment. Out of this 19 had known functions, while others were unknown or hypothetical proteins. These differentially expressed proteins were related to redox-activity and gene expression, electron transfer, cell division and chromosome partitioning, cell envelop biogenesis, energy metabolism and conversion, respiration and pathogenesis related functions in the barley plants. The study provides a platform and documents the proteins that might be involved in disease management in barley following TCZ application. It is expected that the study will provide boost in understanding proteome regulation upon fungal infection and subsequent anti-fungal treatment and will attract researchers for further validation leading to better pest management.

Keywords: Barley; ***Bipolaris sorokiniana;*** Proteomics; Mass Spectrometry; Tricyclazole

Note: Coloured Figures available on Journal Website in "Archives" Section

Introduction

Barley (*Hordeum vulgare* L.) is an important crop that represents fourth most abundant cereal in terms of both area and tonnage (Blake *et al.*, 2011). A significant yield reduction of barley is reported due to spot blotch disease caused by the fungus *B. sorokiniana* in South Asian countries (Duveiller *et al.*, 1998; Saari *et al.*, 1998). *Bipolaris sorokiniana* synthesizes 1,8- dihydroxynaphthalene (DHN) melanin *via* pentaketide pathway and promotes the development of aerial mycelia and conidia (Kumar *et al.*, 2014). A melanin biosynthesis inhibitor TCZ ($C_9H_7N_3S$) [5-methyl-1, 2, 4,-triazolo (3,4,-b) (1,3) benzothiazole] is commonly used to control the disease (Chida *et al.*, 1987). Aggressiveness of *B. sorokiniana* is regulated by the presence or absence

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of melanin which plays a crucial role during pathogen infection (Wheeler and Bell, 1988; Chand *et al.,* 2014). Exposure of TCZ reduces the melanin content thereby depicting altered morphology, virulence and enzyme activities of the pathogen (Kumar *et al.,* 2014). Plant stress response is a dynamic process in several phases that can be distinguished by unique proteome composition (Larcher, 2003).

Proteins are directly involved in spot blotch disease and play a vital role in many structural and functional components of the cell. Therefore, the proteomic study ideally provides information about changing protein profile in various tissues (Finnie and Svensson, 2009; Al-Daoude *et al.,* 2013a). Generally, abiotic and biotic stresses causes alterations in protein network that includes signaling, energy metabolism (glycolysis, Krebs cycle, ATP biosynthesis, photosynthesis), storage proteins, protein metabolism, proteins involved in protein folding and chaperone activities, other protective proteins (LEA, PR proteins), ROS scavenging enzymes as well as proteins affecting regulation of plant growth and development (Kosová *et al.,* 2014) for plant stress tolerance. Since proteins are directly involved in plant stress response so it is important to study the changes in protein profile under various stress condition.

Most of the proteomic studies in plant stress responses (abiotic and biotic) are comparison of proteome composition in stressed plants *vs.* control ones, and also in differentially-tolerant genotypes exposed to stress. Comparison of proteome responses in barley has been attempted in this investigation to gain insight into the proteins involved in host-pathogen interactions. Present work aims to study the proteins involved in plant defence mechanism upon TCZ treatment through proteomic studies using two-dimensional polyacrylamide gel electrophoresis (2-DE PAGE), coupled with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS/MS). The study will be help to add to our understanding about the altered protein expression in barley and *B. sorokiniana* interactions under TCZ influences.

Methodology

Material and experimental setup

The seeds of susceptible barley genotype *cv.* RD-2508 were raised in pots (60 × 40× 8 cm) in greenhouse at 22 ± 1°C (day) and 17 ± 1°C (night) temperatures with a day length of 12 h and a relative humidity (RH) of 80-90% and arranged in a complete randomized block design in three replicates. Forty days old plants were inoculated with highly virulent strain of *B. sorokiniana* WPB-24 (MTCC-11881). Experiments were carried out in four different treatments: **(I) control plants (healthy barley leaves;** application **with** sterile water**), (II) plants after foliar** application of TCZ (100 µg/ml), (III) plants inoculated with ***B. sorokiniana*** **and (IV) plants** treated with TCZ (72 h after ***B. sorokiniana*** inoculation).

Disease parameters and chlorophyll levels in barley leaves upon B. sorokiniana infection

Forty days old barley plants were inoculated by spraying 25 ml of conidial suspension (10^4 conidia/ml) of *B. sorokiniana* by a hand-held sprayer (Zadoc *et al.,* 1974; Joshi and Chand, 2002; Eisa *et al.,* 2013). Number of lesions/leaf, lesion size (cm^{-2}), percent (%) disease severity and chlorophyll level (SPAD value) were observed seven days after *B. sorokiniana* inoculation. Disease severity was scored in double digit scale (DD, 00-99) as described by Saari and Prescott (1975).

Chlorophyll content in the leaves was measured as SPAD values using chlorophyll meter (SPAD 502, Minolta, Japan). Five measurements were recorded for SPAD value corresponding to leaf chlorophyll level in leaves starting from tip to base (Rosyara *et al.,* 2007 and 2010).

Histopathological study

Barley leaf samples were collected after 72 h of *B. sorokiniana* spore inoculation. Leaves were cleared with mixture of absolute ethanol/glacial acetic acid (vol/vol; 1:1) and fixed in lactoglycerol (lactic acid/glycerol/water, vol/vol/vol; 1:1:1) (Sillero and Rubiales, 2002). Microscopic observations were performed for spore germination in barley leaves. However, seven-days after inoculation the leaves were also studied for spore formation and their position on the leaf surface under light microscope. Suitable photographs were taken under a combination of eye piece and objective (12.5 × 25) using the Nikon Eclipse E200MV R microscope (Nikon Corporation, Tokyo, Japan).

Protein extraction and quantification

Total soluble protein in barley leaves were quantified from all the four treatments. Barley

leaves (1.0 g) were ground to fine powder using liquid nitrogen and protein extraction were performed in 12 ml Mg/NP-40 buffer containing 0.5 M Tris-HCl (pH 8.3), 2% v/v NP-40, 20 mM $MgCl_2$, 2% v/v β-mercaptoethanol, 1 mM phenyl methyl sulfonyl fluoride (PMSF) and 1% w/v polyvinyl pyrrolidone (PVP) (Kim *et al.,* 2001). Protein pellets were suspended in lysis buffer (7 M urea, 2 M thiourea, 4% Triton X 100, 1% CHAPS, 65 mM dithiothreitol (DTT) and protein content were measured using bovine serum albumin (BSA) (Sigma) as standard (Bradford, 1976).

2-DE and spot analysis

Protein (250 µg/ml) from all of the experimental sets were rehydrated for one-dimensional separation on 13 cm Immobiline Dry Strip (pH 3"11) (GE Healthcare Bio-Sciences AB, Sweden) in rehydration solution for 16 h at room temperature (RT) and subjected to isoelectric focusing (Zhou *et al.,* 2013). Strips were equilibrated in equilibration buffer **I** [6 M urea, 2% SDS, 75 mM Tris-HCl (pH 8.8), 30% glycerol,100 mg DTT and 0.002% bromophenol blue (BPB)] and **II** [6 M urea, 2% SDS, 75 mM Tris-HCl (pH 8.8), 30% glycerol, 0.002% BPB and 100 mg iodoacetamide] for 15 min each. Subsequently, strips were placed onto 12% SDS polyacrylamide gels and sealed with 0.5% agrose and protein were separated at constant current of 30 mA. The protein spots were visualised by Coomassie Brilliant Blue G-250 (CBB G-250) staining. The images were captured under bright light using gel documentation system (Bio-Rad, model-Universal hood II, USA). The expression or density of protein spots were analysed by PD Quest software (Basic-7.2.0; Bio-rad, CA, USA) and **spots showing differential proteins expression under different treatments were selected** for further analysis.

Mass spectrometric analyses

Targeted protein spots were manually excised from 2D-gels and placed in protein low binding Eppendorf tubes for protein digestion using trypsin (Shevchenko *et al.,* 1996). MALDI-TOF-MS/MS analysis was performed according to Person *et al.* (2006), using a time-of-flight mass analyzer (ABI Microflex MALDI/TOF mass spectrometer, USA).

Database search

Peptide masses (mass list) generated from the peptide mass fingerprint (PMFs) were searched at NCBI (*National Center for Biotechnology Information*) using MASCOT search engine (MASCOT 2.1.03, Matrix Science, UK) for protein identification at significant level *P* dH 0.05 (Perkins *et al.,* 1999).

Statistical analysis

The statistical significance (*P* dH 0.05) of the results were analyzed using statistical analysis software (SAS; version 9.2; SAS Institute Inc., Cary, NC 2010) with one-way ANOVA followed by Duncan's multiple range test (DMRT). Data analysis for the histograms of **(a)** number of lesions/leaf, **(b)** lesion size (per cm^2 of leaf area), **(c)** % disease severity and **(d)** chlorophyll level (SPAD Value) of barley leaves at 7 days after spore inoculation were performed by Sigma Plot Software (version 10.0).

Figure 1: Phenotypic response of TCZ and spot blotch symptoms caused by *Bipolaris sorokiniana* at 7days after spore inoculation on 40 days old barley plants for (I) control plants (healthy barley leaves; application with sterile water), (II) plants after foliar application of TCZ (100 µg/ml), (III) plants inoculated with *B. sorokiniana* and (IV) plants treated with TCZ (72 h after *B. sorokiniana* inoculation) for spot blotch disease management.

Results and Discussion

B. sorokiniana is initially a hemibiotrophic pathogenic fungus that later turns necrotrophic causing spot blotch disease (Aggarwal *et al.,* 2008). However, barley faces challenges upon infection by *B. sorokiniana* and has to cope with the resulting metabolic changes. An understanding of the physiological process that occurs in plants upon fungal pathogen interaction may potentially lead to new and more effective disease management strategies (Kumar *et al.,* 2016). It is known that TCZ application leads to the interruption in virulence by pathogenic fungi *B. sorokiniana* (Kumar et al., 2014). The response in barley upon TCZ application is likely to include changes in pathogen infection ability, physiological and proteome regulation. Recent studies on wheat have reported the roles of sub-cellular proteomes such as chloroplast (Kamal *et al.,* 2012) and mitochondrial (Jacoby *et al.,* 2010 and 2013) proteomes as well as post translational modifications (PTMs) such as phosphoproteomics (Yang *et al.,* 2013; Zhang *et al.,* 2014) when exposed to stress.

Physiological changes in barley seedlings upon pathogen infection and exposure to TCZ

Figure 1 shows the effect of TCZ on the spot blotch severity and physical appearance of leaves. A significant effect of TCZ was recorded upon the pathogen infection such as appearance of number of lesions/leaf, lesion size (cm^{-2}), % disease severity and chlorophyll level (SPAD value) upon *B. sorokiniana* infections (Figure 2 a, b, c, d). No lesions (symptoms) were observed in the **control healthy barley leaves** (Figure 2a, I) and leaves treated with TCZ (Figure 2a, II). However, 269 lesions were observed in *B. sorokiniana* inoculated barley leaves (Figure 2a, III). Following TCZ application (72 h after ***B. sorokiniana*** inoculation), the lesions were reduced by 2.59 fold (Figure 2a, IV). Similarly, *B. sorokiniana* inoculated barley leaves showed 0.93 cm^2–lesion size and 9.21 % disease severity (Figure 2b, III and c, III). However, the lesion size and % disease severity reduced by 9.89 and 1.36 folds after application of TCZ on barley infected leaves (Figure 2b, IV and c, IV). Joshi *et al.* (2007) suggested that changes in leaf chlorophyll (SPAD value) are associated with the disease. In this study chlorophyll level (SPAD value) was enhanced by 1.2 fold in TCZ treated barley leaves compared to non-treated ones (Figure 2d, I and II). The SPAD value however, decreased by 1.73 fold in *B. sorokiniana* infected barley leaves (Figure 2d, III). TCZ applications to *B. sorokiniana* infected barley leaves led to a significant 1.37 fold recovery in chlorophyll levels (Figure 2 d, IV). Maintenance of chlorophyll in pathogen inoculated and TCZ treated plants is due to the effect of TCZ (Rosyara *et al.,* 2010).

Histopathological study of infection process upon foliar application of TCZ

Figure 3 shows the microscopic view of conidia on conidiophores at the surface of barley leaves. As expected, the control and TCZ treated barley leaves did not support any conidia formation (Figure 3 I, II). Upon infection, a large number of hyphae, conidia and conidiophors were observed on barley leaf surface (Figure 3 III), which significantly reduced upon foliar application of TCZ (72 h after ***B. sorokiniana*** inoculation) (Figure 3 IV). Inhibition in conidia germination on the surface of barley leaves suggested a significant effect of TCZ in the disease management. An earlier study reported that inhibition in conidial germination may be one of the mechanisms for resistance in barley cultivars that is characterized as penetration prevention rather than as a slow rate of mycelial growth (Lehnackers *et al.,* 1990; Xi *et al.,* 2000; Kumar *et al.,* 2016).

Differential abundance pattern of proteins in response to *B. sorokiniana* infection and TCZ application in barley by MALDI-TOF-MS/MS

Present work is a pilot study wherein protein expressions have been investigated to understand the (i) changes in barley plants upon *B. sorokiniana* infection and (ii) TCZ exposure on *B. sorokiniana* infected and non infected barley plants used in disease management. Earlier reports on proteome analysis in barley revealed several proteins to be common in host and pathogen during host-pathogen interaction (Al-Daoude *et al.,* 2013b; Bhadauria *et al.,* 2010; Xu *et al.,* 2007). In extension of this study the regulation in protein expression was performed for the disease management using TCZ. Herein, two dimensional gel electrophoresis (2-DE) studies revealed 746, 577, 686 and 860 protein spots by ignoring faint spots from proteomes of **(I) control plants (healthy barley leaves;** application **with** sterile water**), (II) plants after foliar** application of TCZ (100 µg/ml), (III) plants inoculated with ***B. sorokiniana*** **and (IV) plants** treated with TCZ (72 h after ***B. sorokiniana*** inoculation) (Figure 4A I - IV).

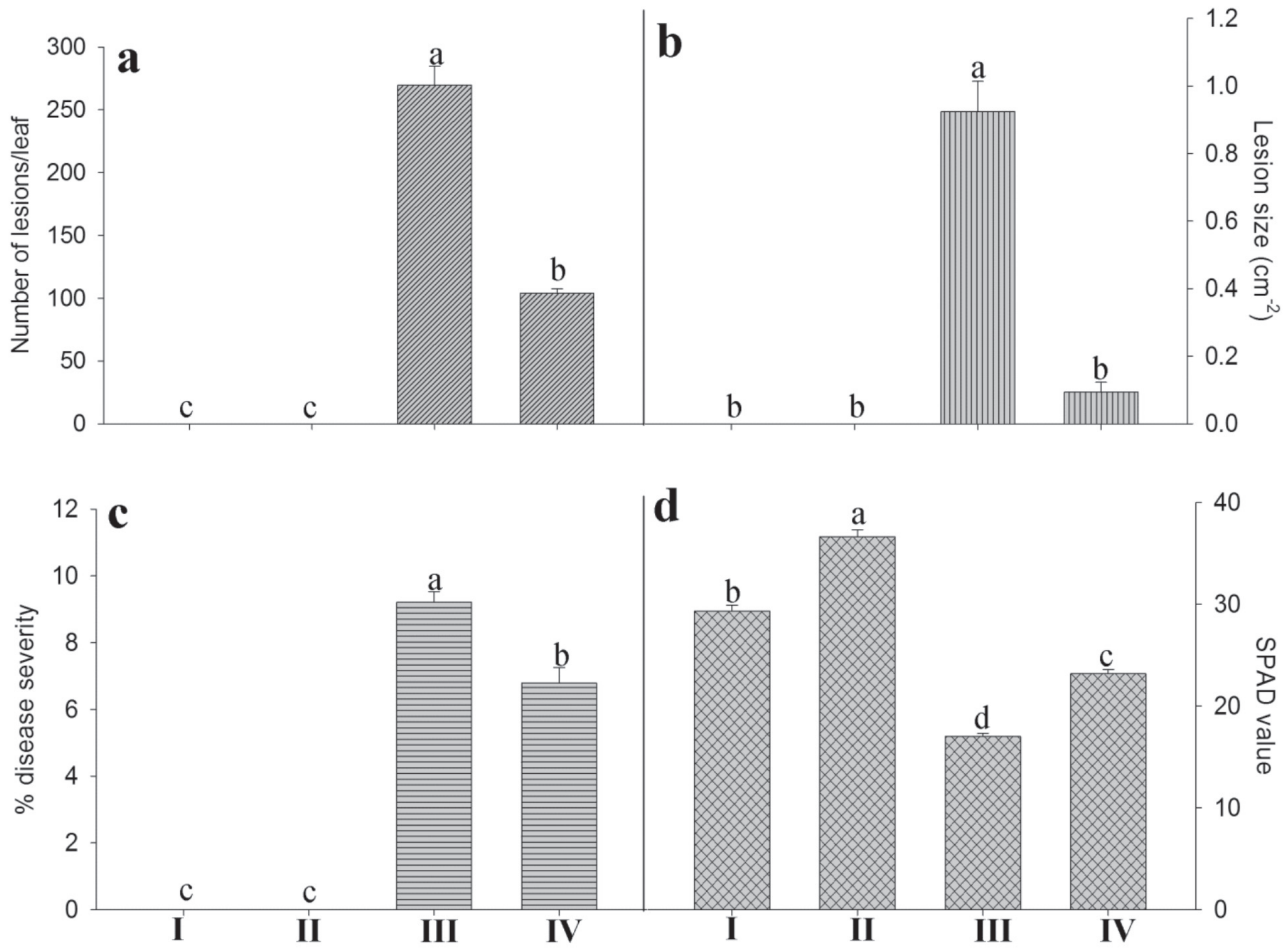

Figure 2: The response of TCZ on: (a) number of lesions/leaf, (b) lesion size (per cm^2 of leaf area), (c) % disease severity and (d) chlorophyll level (SPAD Value) on barley leaves at 7 days after spore inoculation on four different treatments; (I) control plants (healthy barley leaves; application with sterile water), (II) plants after foliar application of TCZ (100 µg/ml), (III) plants inoculated with *B. sorokiniana* and (IV) plants treated with TCZ (72 h after *B. sorokiniana* inoculation) for spot blotch disease management.

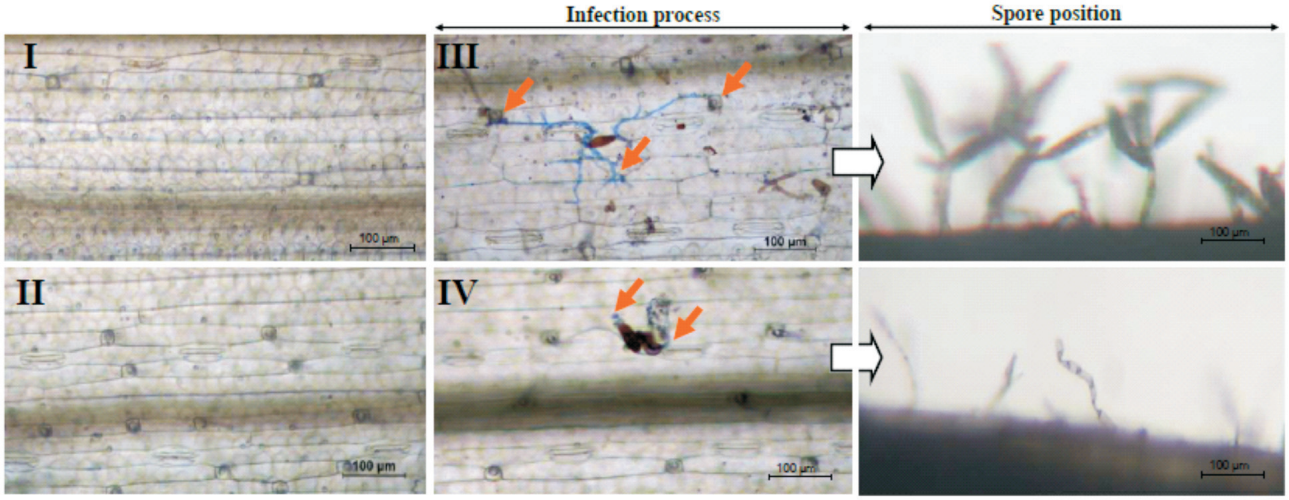

Figure 3: Histopathological study and spore position from: (I) control plants (healthy barley leaves; application with sterile water), (II) plants after foliar application of TCZ (100 µg/ml), (III) plants inoculated with *B. sorokiniana* and (IV) plants treated with TCZ (72 h after *B. sorokiniana* inoculation). The barley plants were inoculated by 1×10^4 conidia ml^{-1} from 10 days old cultures. Arrows indicate appressoria formation, Bar = 100 µm.

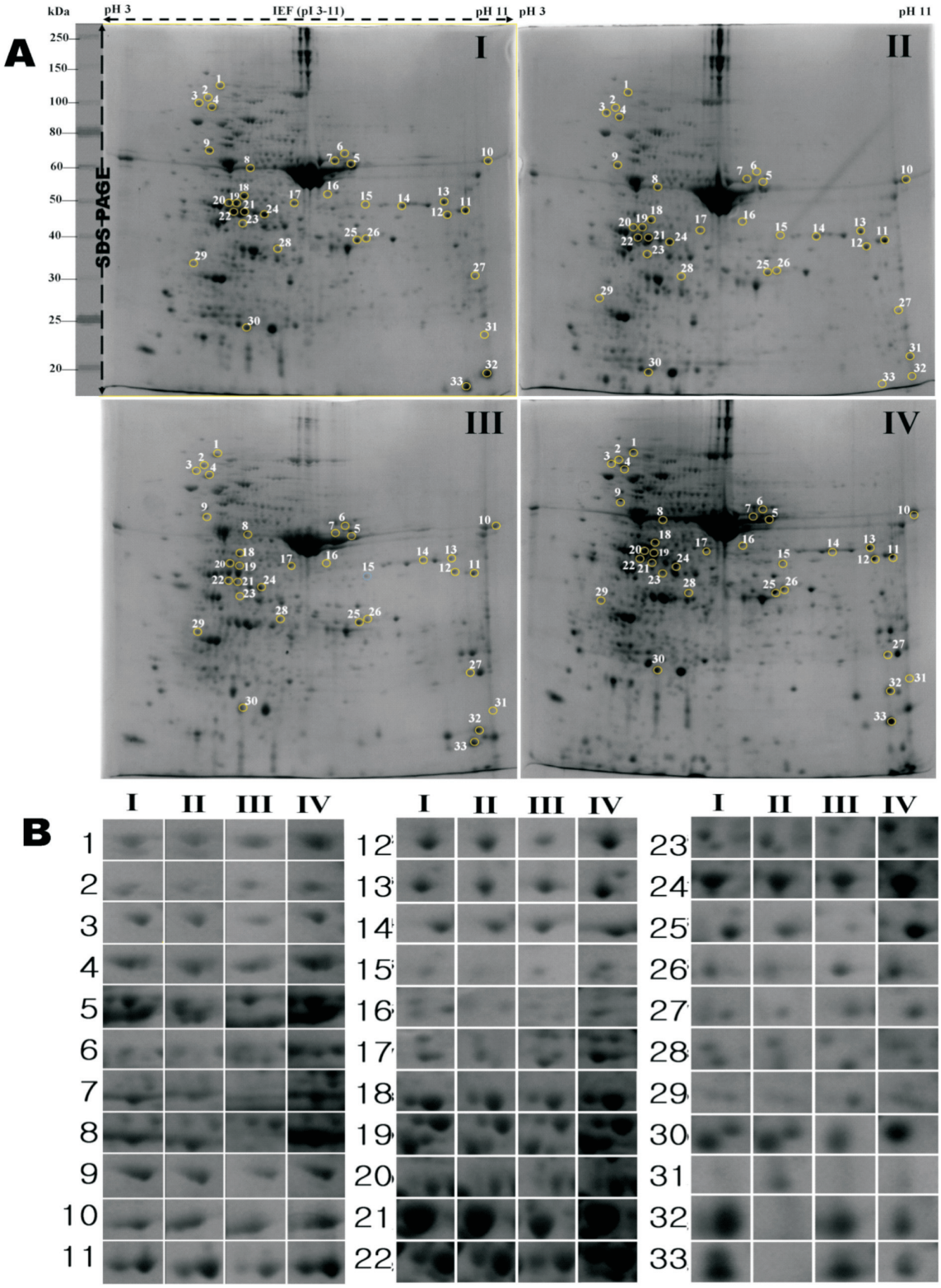

Figure 4: (A) The 2-DE images of crude protein extracted from: (I) control plants (healthy barley leaves; application with sterile water), (II) plants after foliar application of TCZ (100 µg/ml), (III) plants inoculated with *B. sorokiniana* and (IV) plants treated with TCZ (72 h after *B. sorokiniana* inoculation). The numbered circles on 2-DE gel image indicate the protein expression, which are further identified by MALDI-TOF MS/MS analysis. The molecular mass marker (Sigma) and *pI* are indicated on the left side and above the gels respectively. (B) Snapshots of expression pattern of selected protein spots from 2-DE gels. Details are given in Table 1. Number represents the spots no. on 2DE gel image.

Close-up view of 33 protein spots were constantly observed as differential alterations in the proteome profile of ***B. sorokiniana*** inoculated and non-inoculated barley plants treated with foliar application of TCZ for disease management (Figure 4B I - IV). MASCOT searches and *de novo* sequence analysis of the MS data *via* NCBI- n and EST database search revealed that 24 spots matched plant proteome and eight proteins spots matched fungal proteins that were implicated in metabolic functions, structural integrity and pathogenesis. Surprisingly one protein spot matched with P-450 super-family protein from *Drosophila.* The identified spots represented proteins that have known functions such as c**ell division, redox activity, electron transfer, cell envelop biogenesis of outer membrane, energy production and conversion, respiration and pathogenesis, respectively** (Table 1)**. In this regard the findings echo** a mini review that has already been published regarding proteomic studies in temperate cereal crops wheat and barley under abiotic and biotic stresses (Kosová *et al.,* 2014).

The proteins involved in oxidative stress response such as ascorbate peroxidase and jasmonic acid were identified. However, PR proteins such as â- glucanases, chitinase and *thaumatin-like proteins* and amino acids, nitrogen metabolism related proteins (such as cysteine synthase, glutamate dehydrogenase and tryptophan synthase) play a vital role in signalling pathway. In accordance a significant up-regulation of ATPase-protein in *B. sorokiniana* infected barley leaves treated with TCZ was observed as spot 1 which is identified as a protein similar to that from *Arabidopsis lyrata* subsp. Lyrata (gi|297850656) hypothetical protein ARALYDRAFT_889705.

Constitutive expression of defence elements could be a basis for resistance pattern as well as a pathogen induced gene expression. TCZ increases the resistance under stress in plants through enhanced level of both non-enzymatic and enzymatic antioxidant potentials (Kishorekumar *et al.,* 2008). Significant over production of reactive oxygen species (ROS) in plants leading to oxidative stress is a common response to most of the stresses (Shah *et al.,* 2013). Additionally, expression levels of proteins involved in sucrose metabolism and PR proteins such as chitinase were differentially affected upon infection. In accordance the upregulation of transcription/translation regulator was observed herein as spot 2 in TCZ treatment for disease management. A similar protein had antioxidant activity in *Melampsora larici-populina* as hypothetical protein MELLADRAFT_104640; gi|328859839 (Table 1).

The protein from spot 3 was a manganese (Mn) catalase protein similar to the one from *Nostoc sp.* PCC 7120, hypothetical protein alr3090 (gi|17230582). Protein in spot 4 resembled proteins with unknown function from *Glycine max* and associated with the formation of nucleosome core. Protein in spot 5 was identified as a fungalysin (secreted protease) known to degrade extra cellular proteins and peptides for nutrition. This protein was significantly higher in *B. sorokiniana* infected barley than TCZ treated plants. Spot 6 represented a RNA polymerase sigma factor SigJ found in *Bacillus sp.* FJAT-13831 (gi|397746830) containing a DNA gyrase subunit B having an important role in topoisomarase II activity significantly reduced under ***B. sorokiniana*** **infection and recovered by the TCZ** (Table 1). Spot 9 was found to contain a protein that represents a transcriptional regulator protein from *Gemella sanguinis* and consists of a domain DUF1027 from super family/cl09961 with unknown function significantly down regulated under ***B. sorokiniana*** **infection**. Protein in spot 10 was identified as hypothetical protein - AsmA_2 protein in *Sphingomonas melonis* that encodes for outer membrane protein AsmA-like C-terminal region involved in control and invasion of pathogenic *E. coli* and outer membrane biogenesis proteins. Surprisingly, the presence of AsmA-like protein involved in control and invasion of pathogenic *E. coli* was found significantly down-regulated upon *B. sorokiniana* infection and TCZ exposure in this study and this protein may serve an important role in maintenance of the outer cell membrane. However, in *B. sorokiniana* infected plants, it might be significantly involved in the control and invasion of pathogenic ability under TCZ application.

Protein in spot 15 was identified as a PAS/PAC sensor protein bearing proline-specific peptidase like activity. Elongation factors Tu and Ts identified in spot 18 from *Triticum urartu* was significantly down-regulated under *B. sorokiniana* infection and TCZ exposure in this work may be a consequence of enhanced GTP binding and protein synthesis resulting from induced cellular injuries which were predicted to be localized to the mitochondrion and cytoplasm, respectively. This indicated that the metabolic processes related to protein synthesis in

Table 1: **Proteins identified by peptide mass fingerprinting analysis using MASCOT search of barley (*cv.* RD-2508) treated with tricyclazole (100 µgml^{-1}) upon infection with pathogen *Bipolaris sorokiniana*. The functions of each proteins identified using domain search tool at National Center for Biotechnology Information (NCBI), USA**

Major Functions (I – VII)	Spot No.	Description	Organism	NCBI Accession No.	No. and name of conserved domains	Score	Sequence Coverage (%)	Exp. Size (Da)	pI	Residues	Spot density regulation (at cursor point) # Healthy plant (Control)	Plant + Pathogen (P)	Plant + TCZ	Plant + P +TCZ	LSD*
(I) Cell division and chromosome partitioning or chromosome segregation															
	1	Hypothetical protein ARALYDRAFT_889705	*Arabidopsis lyrata* subsp. lyrata	gi|297850656	**3/** NT-C2/ pfam10358; Smc/ COG1196 (2)	68	11	231020	5.00	2000	2232.7± 226.4^{ab}	1965.7 ±112.2^{b}	2006.3 ±11.7^{ab}	2484.3 ±43.1^{a}	506.28
	4	Unknown	*Glycine max*	gi|255639235	**1/** H4 super family/ cl00074	70	35	12704	4.73	113	2448.7± 66.2^{b}	2262.0± 54.2^{c}	1694.3 ± 25.4^{d}	2454.3± 68.1^{a}	67.93
(II) Redox activity and regulation of gene expression															
	2	Hypothetical protein MELLADRAFT_104640	*Melampsora larici-populina* 98AG31	gi|328859839	**1/** JGIDBMellp1_104640	56	18	38225	4.79	339	2173.0 ±21.1^{a}	1714.3 ±42.8^{b}	1708.3 ±56.1^{b}	2106.3 ±5.2^{a}	132.07
(III) Electron transfer															
	3	Hypothetical protein alr3090	*Nostoc sp.* PCC 7120	gi|17230582	**2/** Mn_catalase/cd01051; Ferritin/ pfam00210	88	13	25.65	5.2	230	2448.7 ±66.2^{a}	2262.0 ±54.2^{b}	1694.3 ±25.4^{c}	2454.3 ±68.1^{a}	154.5
(IV) Cell envelop biogenesis of outer membrane															
	5	Hypothetical protein PNA2_1097	*Pyrococcus sp.* NA2	gi|332158738	**1/** GluZincin super family/cl14813	52	27	44.39	6.1	386	3368.7 ±10.3^{a}	2525.0 ±28.2^{b}	2190.0 ±49.5^{c}	3376.3 ±21.6^{a}	67.07
	6	RNA polymerase sigma factor SigJ	*Bacillus sp.* FJAT-13831	gi|515716933	**1/** DNA gyrase Subunit B	46	30	33750	6.97	296	2970.0 ±38.2^{a}	1989.3 ±55.4^{c}	2436.3 ±45.7^{b}	2924.0 ±19.3^{a}	160.73
	9	Transcriptional regulator	*Gemella sanguinis*	gi|493408549	**1/** DUF1027 super family/cl09961	40	45	11134	5.09	95	2731.3 ±28.8^{ab}	2595.7 ±15.4^{b}	2212.7 ±25.3^{c}	2793.7 ±105.2^{a}	193.1
	10	Hypothetical protein	*Sphingomonas melonis*	gi|516602237	**2/** AsmA_2/ pfam13502; AsmA/COG2982	51	20	75303	10.31	705	2740.3 ±34.9^{a}	2400.7 ±31.5^{ab}	2269.3 ±35.9^{c}	2645.7 ±117.0^{ab}	260.18
	15	Putative PAS/ PAC sensor protein	*Sphingomonas sp.* PAMC 26605	gi|497871628	**1/** PRK03592	59	21	30455	8.65	295	2024.7 ±32.6^{a}	1577.3 ±33.6^{b}	1476.0 ±59.8^{b}	2091.0 ±51.0^{a}	149.56
	18	Elongation factor Tu, chloroplastic	*Triticum urartu*	gi|474198705	**3/** EFTU_II/ cd03697; EFTU_III/cd03707; Ras_like_GTPase super family/cl17170	544	27	45795	4.61	414	3463.7 ±62.8^{a}	2668.7 ±300.4^{b}	3041.3 ±72.9^{ab}	3486.3 ±37.4^{a}	573.06
	27	Chitinase	*Hordeum vulgare* subsp. vulgare	gi|563487	**1/** chitinase_glyco_hydro_19/ cd00325	675	40	27377	8.74	256	1891.7 ±51.5^{b}	1394.0 ±33.8^{c}	3305.3 ±30.8^{a}	3515.7 ±105.4^{a}	224.62
	28	Ferredoxin-NADP (H) oxidoreductase	*Triticum aestivum*	gi|20302471	**2/** CYPOR_like_FNR/ cd06208; PLN03115/ PLN03115	174	7	39181	8.29	353	2671.3 ±55.1^{b}	2211.7 ±36.7^{c}	2840.0 ±32.1^{b}	3045.3 ±69.4^{a}	200.21
	29	Predicted protein	*Hordeum vulgare* subsp. vulgare	gi|326500992	**1/** PAP_fibrillin/ pfam04755	295	22	33254	5.85	313	2332.7 ±9.2^{b}	1835.0 ±49.1^{c}	2570.7 ±19.4^{a}	2523.3 ±37.5^{a}	128.63
	30	RecName: Full=Triosephosphate isomerase, cytosolic; Short = TIM; Short=Triose-phosphate isomerase	*Hordeum vulgare*	gi|2507469	**1/** TIM/ cd00311	72	11	26948	5.39	253	3112.7 ±39.1^{a}	2826.0 ±21.0^{b}	2645.3 ±49.9^{c}	3166.3 ±27.7^{a}	139.05

contd. table 1

Spot No.	Description	Organism	NCBI Accession No.	No. and name of conserved domains	Score	Sequence Coverage (%)	Exp. Size (Da)	pI	Residues	Spot density regulation (at cursor point) # Healthy plant (Control)	Plant + Pathogen (P)	Plant + TCZ	Plant + P +TCZ	LSD*
(V) Energy production and conversion														
7	NADPH-dependent glutamate synthase beta chain-like oxidoreductase	*Thermoplasmatales archaeon* SCGC AB-540-F20	gi|495879621	**11/** Fer4_20/ pfam14691; NAD_binding_8/ pfam13450 **(2);** HCP_like super family/cl19102 **(2);** Pyr_redox/ pfam00070; Pyr_redox super family/cl15766; HdrA/COG1148 **(3);** ltD/PRK12810	68	11	166.1	6.2	1469	3578.0 ±75.0^{a}	2851.3 ±40.0^{b}	2657.3± 195.4^{b}	3607.7 ±91.0^{a}	412.38
11	Predicted protein	*Hordeum vulgare* subsp. vulgare	gi|326527219	**2/**alpha_hydroxyacid_oxid_FMN/ cd02809; PLN02493/PLN02493	346	21	40360	8.99	370	2660.0 ±53.4^{a}	2360.3 ±37.3^{b}	1671.0± 7.8^{c}	2730.7 ±45.7^{a}	150.26
12	Similar to Glycolate oxidase Os07g0152900	*Oryza sativa* Japonica Group	gi|115470621	**2/** alpha_hydroxyacid_oxid_FMN/ cd02809; PLN02493/ PLN02493	337	19	40276	8.50	369	2809.0 ±46.1^{a}	2650.7 ±130.8^{a}	2021.3± 27.0^{b}	2797.0 ±35.3^{a}	240.43
13	Predicted protein	*Hordeum vulgare* subsp. vulgare	gi|326490678	**1/** AGAT_like/ cd06451	662	31	44126	8.16	401	2878.3 ±9.4^{a}	2749.7 ±41.7^{b}	1851.3± 32.7^{a}	2861.7 ±27.4^{ab}	112.1
14	Unknown (shadow)	*Drosophila melanogaster*	gi|24646122	**1/** p450 super family/ cl12078	56	21	60220	9.08	520	2598.3 ±11.0^{ab}	2432.0 ±64.0^{bc}	2413.3± 28.3^{c}	2768.7 ±52.2^{a}	172.27
17	Predicted protein	*Hordeum vulgare subsp. vulgare*	gi|326493350	**1/** PLN00103/ PLN00103	406	22	46151	5.99	409	3101.7 ±41.3^{a}	1669.3 ±695.7^{b}	2796.3± 15.3^{ab}	3219.7 ±36.5^{a}	1207.5
20	Predicted: phosphoglycerate kinase, chloroplastic-like	*Oryza brachyantha*	gi|573944490	**1/**Phosphoglycerate_kinase/ cd00318	127	9	42509	5.14	405	3232.3 ±27.2^{a}	2914.0 ±46.9^{b}	2631.7± 46.0^{c}	3312.0 ±10.7^{a}	91.152
24	Glyceraldehyde-3-phosphate dehydrogenase B, chloroplastic	*Triticum urartu*	gi|473912215	**4/** Gp_dh_C/ pfam02800; Gp_dh_N/pfam00044; CP12 super family/ cl14670; PLN02237/ PLN02237	247	13	47337	6.03	444	3443.7 ±23.5^{a}	3204.0 ±48.4^{b}	3201.3± 55.5^{b}	3611.0 ±89.0^{a}	227.91
25	RecName: Full = Glyceraldehyde-3-phosphate dehydrogenase 2, cytosolic	*Hordeum vulgare*	gi|120668	**3/** Gp_dh_C/ pfam02800; Gp_dh_N/ pfam00044; GAPDH-I/ TIGR01534	191	11	33443	6.20	305	3181.7 ±13.2^{a}	2918.0 ±32.9^{b}	2785.7± 74.4^{b}	3272.3 ±28.4^{a}	148.96
26	Chloroplast RNA binding	*Theobroma cacao*	gi|590644896	**3/** SDR_a1/ cd05265; Epimerase_Csub super family/ cl15760; PLN00016/ PLN00016	115	10	42717	8.62	379	2383.3 ±41.7^{a}	2091.0 ±37.3^{b}	2169.7± 77.6^{b}	2511.3 ±38.9^{a}	201.59

contd. table 1

Spot No.	Description	Organism	NCBI Accession No.	No. and name of conserved domains	Score	Sequence Coverage (%)	Exp. Size (Da)	pI	Residues	Spot density regulation (at cursor point) # Healthy plant (Control)	Plant + Pathogen (P)	Plant + TCZ	Plant + P +TCZ	LSD*
31	RecName: Full=Photosystem I reaction center subunit II, chloroplastic; AltName: Full= Photosystem I 20 kDa subunit; Short=PSI-D; Flags: Precursor	*Hordeum vulgare*	gi|548603	**1**/ PLN00041/ PLN00041		11	21976	9.81	205	1883.0 ±20.1^{a}	2081.0 ±51.4^{b}	1579.0 ±30.4^{c}	1617.0 ±67.7^{c}	138.68
(VI) Respiration														
8	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast)	*Zexmenia serrata*	gi|38146650	**2**/ RuBisCO_large_I/ cd08212; rbcL/ CHL00040	121	23	52.43	6.2	468	3436.7 ±23.9^{a}	2940.0 ±32.1^{b}	2572.0 ±5.5^{c}	3516.0 ±67.5^{a}	156.06
16	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	*Flaveria ramosissima*	gi|321273313	**2**/ RuBisCO_large_I/ cd08212; rbcL/CHL00040	196	31	54368	6.00	485	2216.3 ±19.1^{b}	1878.7 ±21.4^{c}	1728.7 ±20.9^{d}	2317.3 ±30.7^{a}	85.425
19	plastid 3-phosphoglycerate kinase, partial	*Triticum aestivum*	gi|383512798	**1**/Phosphoglycerate_kinase super family/cl00198	485	31	31426	4.79	298	3459.3 ±16.9^{ab}	3357.0 ±35.1^{b}	3064.0 ±25.7^{c}	3521.3 ±67.3^{a}	155.65
21	Ribulose-1,5-bisphosphate carboxylase/oxygenase activase 2	*Gossypium hirsutum*	gi|12620883	**1**/ PLN00020/ PLN00020	223	9	48609	5.06	435	3741.7 ±31.4^{a}	3684.3 ±2.3^{a}	3504.3 ±8.7^{b}	3796.3 ±47.9^{a}	115.27
22	Ribulose-bisphosphate carboxylase activase (EC 6.3.4.-) A long form precursor - barley (fragment)	*Hordeum vulgare* subsp. vulgare	gi|100614	**1**/ PLN00020/ PLN00020	280	8	47496	5.64	426	3589.7 ±69.3^{a}	3548.7 ±63.9^{ab}	3335.0 ±29.7^{b}	3763.3 ±66.9^{a}	219.05
23	Ribulose bisphosphate carboxylase/oxygenase activase 1, chloroplast precursor, putative	*Ricinus communis*	gi|255584538	**1**/ PLN00020/ PLN00020	136	5	52453	5.42	474	2426.0 ±69.2^{b}	2419.3 ±108.3^{b}	1797.3 ±20.3^{c}	2930.3 ±30.8^{a}	210.75
(VII) Pathogenesis related proteins														
32	PR-1a pathogenesis related protein (Hv-1a)	*Hordeum vulgare* subsp. vulgare	gi|401831	**1**/ SCP_PR-1_like/ cd05381	73	20	17771	8.19	164	3281.3 ±19.2^{a}	1207.3 ±14.7^{c}	3092.0 ±48.5^{b}	3132.0 ±18.1^{b}	89.967
33	Pathogenesis-related protein PRB1-2; Flags: Precursor	*Hordeum vulgare* subsp. vulgare	gi|548588	**1**/ SCP_PR-1_like/ cd05381	100	13	18010	9.08	164	3171.0 ±26.9^{a}	1297.7 ±15.4^{d}	2880.7 ±1.8^{b}	2504.3 ±16.6^{c}	41.644

Represents relative abundance of protein spots of barley plant leaves following different experimental sets. Values are mean±SE. Different alphabets show significantly different values (P<0.05, DMRT).

* Represents Least Significant Difference.

barley might be different in response due to oxidative stress in TCZ exposure or *B. sorokiniana* infection; however this needs further study. The functional cycles of these elongation factors depend on GTP binding and its hydrolysis. During the synthesis of proteins, the GTP translation factors regulate initiation, elongation, termination and release in translation, while the Era-like GTPases regulate sporulation, cell division and DNA replication also. Chitinase protein is separated to cause direct neutralization of the pathogen. Chitinase from *Hordeum vulgare* subsp. vulgare (gi|563487) with a domain chitinase_glyco_hydro_19 (cd00325) identified as spot 27 and must play an important role in the hydrolysis of eta-1, 4-N-acetyl-D-glucosamine linkages in chitin polymers which are mostly found in the fungi. A significant down regulation of the protein was found in plant infected by *B. sorokiniana*, however it was up-regulated under TCZ exposure. Protein in spot 28 was significantly down regulated in *B. sorokiniana* infected plants and up-regulated under TCZ exposure. It was identified as ferredoxin-NADPH oxidoreductase protein of *Triticum aestivum* (gi|20302471), related to the NADPH cytochrome P450 reductases (CYPOR), which catalyzes the reversible electron transfer between NADP(H) and electron carrier proteins, such as ferredoxin and flavodoxin. Protein in spot 29, identified as down-regulated upon *B. sorokiniana* infection and up-regulated upon TCZ exposure is a predicted protein (gi|326500992) from *Hordeum vulgare* having a domain PAP_fibrillin (pfam04755), a specific region found in plastid lipid-associated and putative fibrillin proteins. The protein in spot 30 is characterized as triosephosphate isomerase, a glycolytic enzyme responsible for catalysis of the dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate significantly reduced under ***B. sorokiniana*** **infection and recovered by the TCZ application for disease management** (Table 1).

Proteins from spot 7 and 11 play a significant role in energy production and conversion. Post-translation modification such as phosphorylation of proteins plays an important role in mediating plant response to pathogen. Up regulation of these proteins support the high energy requirements of the plant cell for repair upon pathogen attack (Ghazvini *et al.*, 2008). Protein of spot 12, 13, 14, 17, 20, 24 and 25 were largely identified as proteins involved in plant metabolic pathways such as Krebs cycle (Table 1). Proteins involved in photosynthesis and carbon metabolism, energy production, and signal transduction were identified in barley leaves following infection. The nature of the constitutively up regulated proteins in the host pathogen interactions (proteins involved in photosynthetic carbohydrate metabolism and TCA cycle) may lead to enhanced overall plant health, which may help them better tolerate to the pathogen challenge. Protein in spot 26 was characterized as a chloroplast-RNA binding protein similar to that from *Theobroma cacao*, whereas, protein in spot 31 was a chloroplastic photosystem I reaction centre subunit II protein from *Hordeum vulgare* involved in photosynthesis. Several antioxidant enzymes including dehydroascorbate reductase and *peroxiredoxin* along with proteins involved in photosynthetic and nitrogen metabolism were found to be up regulated in the barley leaves suggesting that post-translation modification. This perhaps could be the reason for an altered chlorophyll levels upon infection as reflected by the altered SPAD values in this work.

A decreases in the level of small sub unit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) following fungal infection in barley leaves suggest an altered carbon assimilation within the host plant as a consequence of infection. Proteins in spots 8, 16, 19, 21, 22 and 23 are similar to RuBisCO protein with photosynthesis related functions that play a crucial role in the Calvin reductive pentose phosphate pathway in plants for efficient CO_2 and healthy photosynthetic apparatus in barley (Table 1). Recent studies on rice blast lesion revealed a significant increase in the expression of pathogenesis-related class 5 and 10 proteins, as well as an increase in the expression of oxidative- stress related proteins as catalase, APX and SOD (Jung *et al.,* 2006).

Proteins in spot 32 and 33 were identified as pathogenesis related proteins (PRs)- PR-1a (Hv-1a) and PRB1-2; Flags: Precursor from *Hordeum vulgare* which may play an important role in plant defence against pathogen invasion. There is a possibility of a cumulative effect of different proteins involved in several mechanisms responsible for the observed tolerance to this pathogen.

The host cells have formation and accumulation of stress combating compounds like ethylene, salicylic acid, proteins and other stress specific bio molecules under various stresses including

pathogen attack (Singh *et al.,* 2014). An increasing level of receptors like protein kinases, â 1-3, glucanase thaumatin like protein, probenazole inducible protein and rice PR protein is suggested to play a possible role in mediating tolerance to the pathogen (Kim *et al.,* 2004). The presence of two pathogenesis related proteins namely- PR-1a (Hv-1a) and PRB1-2, were up-regulated upon infection, and can be well accounted for the fact that the PR-proteins accumulate after infection. Such proteins are reported to have antifungal activity or have implications in cell wall loosening (Bryngelsson *et al.,* 1994). The plant pathogen infections in this study suggested an involvement of antioxidant, photosynthesis and nitrogen metabolism related enzyme protein whereas protein related to photosynthesis were down-regulated (Zhou *et al.,* 2006). In summary, almost all of the plant cell organelles were predicted to undertake challenges towards *B. sorokiniana* infection or application of TCZ on barley leaves, and the biological processes involved in growth and development of barley were possibly bi-functional. This study will provide better understanding about the protein regulation in host defence mechanisms during *B. sorokiniana* infection and TCZ exposure for spot blotch disease management. To further strengthen this study, the isolation of anti-fungal toxic proteins may be useful for an integrated management of spot-blotch disease in barley.

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Conflict of Interest

All the authors have contributed equally to this work and there is no conflict of interest.

Abbreviations

2-DE: Two**-dimensional gel electrophoresis; BSA:** bovine serum albumin; **CBB:** coomassie brilliant blue; **DD:** double digit scale; **DMRT:** duncan's multiple range test; **DTT:** dithiothreitol; **MALDI-TOF MS/MS:** matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; **NCBI:** national center for biotechnology information; **PAGE:** polyacrylamide gel electrophoresis; **PMFs:** peptide mass fingerprint; **PMSF:** phenyl methyl sulfonyl fluoride; **PR:** pathogenesis related proteins; **RH:** relative humidity; **RT:** room temperature; **SAS:** statistical analysis software; **SPAD:** soil plant analysis development; **TCZ:** tricyclazole.

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