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Effect of Popcorn Disease Infected Leaves on Silkworm Performance and Differential Proteome Analysis of Mulberry Popcorn Disease

Pei-feng Yin[1,2], Xiu-xiu Li[1], Qi-wei Zeng[3], Cheng-chen Shen[1], Le Gao[1] and Jian-zhang Ton[2,*]

[1]*School of Architecture, Soochow University, Suzhou 215123, Jiangsu, China*
[2]*School of Bio- and Food Engineering, Chuzhou University, Chuzhou 239000, Anhui, China*
[3]*State Key Laboratory of Silkworm Genome Biology, Southwest University, Chongqing 400715, China*

ABSTRACT

Popcorn disease, a fungal infection is reportedly known to affect the production of mulberry, however, there is no known research data available regarding the effects of popcorn infected leaves on silkworm performance. One group of silkworms was fed infected mulberry leaves and the second silkworms group was given control mulberry leaves that were infection free. Results showed that infected leaves exhibited limited toxicity to the silkworm; however, the cocoons yield found in infected group was deeply affected. Compared with control group, the biggest body weight, cocoon weigh, cocoon shell weight, pupal weight in infected group was 9.3%, 8.8%, 11.4% and 10.2%, respectively, lower than that of control group. Although the groups obtain a similar group oviposition, group good oviposition and group oviposition rate (P>0.05), the single oviposition, single good oviposition and single oviposition rate were significantly reduced by infected leaves. Furthermore, to elucidate the molecular resistance mechanism of fruit mulberry "Da10" *i.e.,* popcorn disease, Two-dimensional electrophoresis (2-DE), matrix-assisted laser desorption/ ionisation time-of-flight tandem mass spectrometry (MALDI-TOF-TOF MS) and bioinformatics technique were used for characterize the differential expressed proteins. Almost 78 patho-stress responsive proteins which expression level more than 1.5-fold were identified, where 50 proteins were up-regulated and 28 proteins were down-regulated; The identified proteins were categorized into 16 classes, which are mainly including energy metabolism, gene expression regulation, oxidation-reduction reaction, cellular component and stress responses, and the stress proteins including Mn-superoxide dismutase and thaumatin-like protein. The results means that mulberry young fruits can regulate the expression levels of multiple proteins to reply popcorn disease and these pathogenesis-related proteins provide valuable information to further study the pathogenesis of popcorn disease and disease-resistant molecular breeding in mulberry.

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Authors' Contribution
PY conceived and designed the study. PY, XL and CS performed the experiments. LG prepared and collected mulberry leaves and QZ provided the silkworms. PY wrote the paper and JT reviewed and edited it.

Key words
Mulberry sorosis, Popcorn disease, Patho-stress, Comparative proteomics, Mass spectrometry analysis.

INTRODUCTION

The history of Sericulture in China is more than 5000 years (Zhang *et al*., 2010 #2482), and China owns the biggest silk production in the world, which product 300000 metric tons of silkworm cocoons in 2007 (Sun *et al*., 2012 #2484). Regarding the mulberry leaf is the only food to silkworm, the safety of mulberry leaf is the safeguard of silkworm, however, plenty factors would affect the quantity of mulberry leaf. The pesticide is no doubt the biggest problem of mulberry leaf. Exposure of silkworm larvae to pesticides may cause acute effects on survival of the insects (Zhao *et al*., 2004), or sub-lethal effects on silk production and quality (Sun *et al*., 2012 #2484). However, limited research focus on the effect of mulberry disease on the silkworm (Gençoğlan *et al*., 2016).

On the other side, the mulberry Popcorn disease invades in the mulberry inflorescence during flowering. This disease poses serious threats to mulberry fruit production and thus, often associated with economic losses. In order to breed disease-resistant varieties to reduce the losses linked with this disease, the understanding of mulberry response mechanism to sclerotinia at molecular level become more important. Pathogen shows variedly threats to plants. With co-evolution, different plants organs build-up different respond to invading pathogens. With the

* Corresponding authors: JianzhangTon@outlook.com
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infecting, the vulnerable organs in host activate the defense system, which including enzymes activities changing and synthesize resistance-related proteins, as resistance gene (R gene), allergic reactions, signal transduction pathways and pathogenesis-related proteins (PR) to conduct biological defense (Liz *et al*., 2011; Gu *et al*., 2015).

Plenty of PRs have been recognized as the key role in the plant resistance, such as glutathione S-transferase (GST) (Overby *et al*., 2015), plant cell wall-degrading enzymes (PCWDEs) (Kubicek *et al*., 2014), chitinase and chitinase-like proteins (CHI) (Marcato *et al*., 2016), catalase (CAT) (Iannone *et al*., 2015), peroxidase (POD) (Kwon *et al*. 2015), superoxide dismutase (SOD) (Jing *et al*., 2015) and polyphenol oxidase (PPO) (Chi *et al*., 2014), *etc*. A better understanding of the pathogens induced proteins will undoubtedly contribute to its resistance mechanisms research.

Sclerotinia is dangerous in agriculture, as Arabidopsis, Tomato, kidney bean and rape are susceptible to the disease (Zhou *et al*., 2014; Zhao *et al*., 2015). In addition, this fungal diseases (usually named as popcorn disease) limited the cultivation of mulberry, which invaded in the mulberry inflorescence during flowering, thus caused a terrible taste and commercial loss of the product (Xue *et al*., 2014).

It is clear that, a better understanding of molecular mechanism of the mulberry response to Popcorn disease will prevent the invasion of the disease efficient. In previous proteins studies, 2-DE was utilized for mulberry study, the responsive proteins of mulberry dwarf disease was identified in mulberry leaves (Borges *et al*., 2015). Chen *et al*. (2013) optimized the methods of two-dimensional electrophoresis system of mulberry fruits, and Niu *et al*. (2013) separation and identification of stage-specific proteins in pistillate flowers of mulberry. A wide variety of disease resistance genes of mulberry have been isolated, including NBS, MaPGIP1, phenylalanine ammonia-lyase gene (Wang *et al*., 2015; Zhang, 2009). Some pathogen response proteins, such as NUDIX/mut, SOD, F-box, heat shock protein were reported also (Xianling *et al*., 2009).

Since limited research concern the effect of popcorn disease to the silkworm and the mechanism of the Popcorn disease infecting the mulberry. The objectives of the present research were therefore focused upon the performance of clerotium disease infected mulberry leaves on silkworm development, and the molecular mechanism of mulberry response to popcorn disease. 2-DE and mass spectrometry technology was utilized to distinguish the protein expression in the mulberry, thus revealed the disease-resistant mechanism and confirmed the resistance gene of the disease.

MATERIALS AND METHODS

Silkworms

Newly hatched silkworms species "Liang-Guang No. 2" were gathered during the year May 1st, 2015, and feed with mulberry leaves which were collected from agricultural garden of Soochow University, China. 10 days later, the silkworm was feed different mulberry leaves, and terminated at 22d.

Mulberry leaves

The pathogenic strains were popcorn disease . Leaves were selected from diseased plants which total sorosis were infected by disease last more than two years and whole plant showed a marked decline, the healthy leaves comes from the mulberry that were not infected by disease in the same environmental conditions with the same annual.

Mulberry

The healthy and diseased mulberry fruits were enclosed by transparent paper bags. After 72 h inoculation, the collected samples were rinsed for dust and dirt and sipped up surface water. The final samples were putted into 2 mL EP tubes and stored in -80°C liquid nitrogen, respectively. Mass spectrometric analysis of protein was carried on in Proteome analysis Center, Shanghai Institutes for Biological Sciences.

Silkworm seed production

Hatching silkworms, 1-3 instar young silkworms were feed on control and infected leaves according to the method (Greis and Petkov, 2000). The silkworms fed by infection group and control group leaves were supported by enough mulberry leaves, 3 times/d at same temperature and humidity. During the study, the effect of vitality, silkworm cocoon quality and quantity, silkworm seed quality and quantity were evaluated.

Protein extraction and two-dimensional gel electrophoresis

The frozen mulberry fruit were ground to fine powder in liquid nitrogen (Wang *et al*., 2008). The improved phenol extraction method was utilized for total proteins extraction, all of the reagent were precooled (-20°C). TCA/cetone (100 % TCA10 mL, cetone 90 mL, β-mercaptoethanol 70μL) was added to frozen tissue powder (1 g), and the sample was vortexed for 5 min, then stored at -20°C temperature for 30 min, after centrifugation (15,000 ×g at 4°C for 5 min), the upper phase was discarded. 0.1 M cold ammonium acetate buffer solution (ammonium acetate 0.77 g, 80% acetone 100 mL) was added to the precipitate collected, which was washed with 80% acetone two times. The proteins obtained were purified by SDS and phenol

phase (1:1), and the middle phase phenol was collected after a centrifugation, the precipitated was washed two times in ammonium acetate buffer solution at -20°C for 8-10 h, then dissolved in lysis buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.5% (v/v) IPG buffer, and 1% (w/v) DTT g).

The concentration samples were detected by the Bio-Rad protein assay reagent. The qualified protein samples (800 µg) was analyzed by 2-DE. First carried isoelectric focusing on pH 3-10 IPG strip, total protein was loaded onto immobilised pH gradient (IPG) strip and rehydrated for 10-12 h. Following the procedure: 30v 12h, 500v 1 h, 1000v 1h, 8000v 8h, 500v 4h. After the strips were subjected to isoelectric focussing, the IPG strip was equilibrated in solution (6 mol/L urea, 2 mol/L thiourea, 2% CHAPS, 0.5% IPG buffer containing 1% DTT w/v for 15 min, then incubated in 4% iodoacetamide w/v for 15 min on Ettan IPGphor Isoelectric Focusing System (GE Amersham). Then IPG strip was carried the second dimension electrophoresis in SDS-PAGE gel (containing 12% polyacrylamide) for three repeats on Hofer SE 600 (GE Amersham) to separate the proteins (Parkhey *et al*., 2015; Shen *et al*., 2003).

The sample obtained was stained by silver staining for 2-DE images analysis and by CBB-R250 for mass spectrometry analysis. After decolorization, scaned by gel-specific transparency scanner (UMax Powerlook 2110XL), then analyzed by image Master 5.0 (GE Healthcare), replicates were considered to calculate volume% of all protein spots. The differential protein spots in the gel by 1.5-fold or more were selected for mass spectrometry analysis (class report ratio >=1.5).

Mass spectrometry analysis

Protein spots were decolorizated by a solution to transparent (200-400 Ml, 100 mmol/L NH_4HCO_3 /30% ACN), silver staining: 30-50µL 30mmol/L $K_3Fe(CN)_6$: 100mmol/L $Na_2S_2O_3$ =1:1 (v:v). The samples were swollen at room temperature for 15 min and digested by Trypsin (Promega) at 37°C for 12 h. After digestion, the samples obtained was ultrasound with 100 µL 60% ACN/0.1%TFA, desalted by Ziptip (millipore).

After a redissolving in 2 µL 20% acetonitrile, the peptides were eluted onto the target plate with natural drying, added supersaturated CHCA (solvent: 50%ACN0.1%TFA) for nitrogen blasting.

The samples were identified by MALDI-TOF/TOF MS (5800 MALDI-TOF/TOF/, AB SCIEX), Test method: laser source: 355 nm Nd:YAG, accelerating voltage: 2kV, positive ions; Automatic acquisition data, sample target: 384 opti-TOF123mm x 81 mmssabsciex, matrix: CHCA, MS: 800–4000Da, selected parent ion(SNR>50) to MS/MS analysis. Two-way and gel electrophoresis system special scanner Image scanner (GE Healthcare).

The result was retrieved by NCBI database under taxonomy of Rosales and Viridiplantae with Mascot 2.2 software. Type of search were MS+MS/MS. Enzyme: Trypsin; Fixed modifications: Carbamidomethyl (C); Dynamical modifications: Oxidation (M); Mass value: Monoisotopic; Protein Mass: unrestricted; Peptide Mass Tolerance: ± 100 ppm; Fragment Mass Tolerance: ± 0.4 Da; Peptide Charge State: 1+; Max Missed Cleavages: 1. Protein score and protein CI% score over 60 and 95 respectively were successfully identified proteins, which function were annotated by online software available at http://www.geneontology.org/.

RESULTS

Effect of different leaves to the vitality of silkworm

Table I describes the vitality of silkworms (%). According to the results, the vitality of silkworms found similar in both groups, suggesting limited effects of the infected leaves over silkworms' performances.

Table I.- The effect of different leaves to the vitality of silkworm (%).

	Infection group	Control groups	P value
HR of 3-instar silkworm	100.00±0	100.00±0	>0.05
HR of 4-instar silkworm	100.00±0	100.00±0	>0.05
HR of 5-instar silkworm	100.00±0	100.00±0	>0.05
Cocooning rate	100.00±0	100.00±0	>0.05
Dead silkworm cocoon rate	1.31±2.02	1.17±3.11	>0.05
Late pupa death rate	4.35±2.39	4.12±3.19	>0.05
3 instar pupation rate	97.32±2.61	96.68±3.04	>0.05
3 instar moth rate	92.21±3.33	93.16±5.42	>0.05

HR, healthy rate.

Effect of different leaves to the cocoon quality and quantity

Results regarding various parameters of the silkworms fed on infected and control leaves are presented (Table II). Controlled groups have more body weight, cocoon weight, Pupal weight ($P \leq 0.01$) versus infected group. There was no statistical difference found in cocoon shell rate (%) in both groups ($P \leq 0.05$).

Effect of different leaves to the silkworm seed quality and quantity

Silkworm seed quality and quantity is presented in

Table III. According to the results, Spawning moth rate (%) was significantly higher in infected groups than in control group. Furthermore, group oviposition egg per 15 moths remained similar (P≥0.05) in both groups. Similar trends were found in case of group good oviposition (eggs/15 moths) and group oviposition rate (%), respectively. On the other hand, single oviposition (eggs/moth), single good oviposition (eggs/moth) and single oviposition rate (%), respectively, were significantly different (P≤0.01) in control and infected groups. In general, control groups were found to be superior in these parameters compared with the infected groups.

Table II.- The effect of different leaves to the cocoon quality and quantity.

	Infection group	Control group	P value
Body weight of silkworm (g)	3.52±0.09	3.88±0.11	<0.01
Cocoon weigh (g)	1.46±0.06	1.60±0.04	<0.01
Cocoon shell weight (g)	0.31±0.15	0.35±0.01	<0.01
Cocoon shell rate (%)	21.33±1.01	21.37±0.39	<0.05
Pupal weight (g)	1.14±0.03	1.27±0.02	<0.01

Table III.- The effect of different leaves to the silkworm seed quality and quantity.

	Infection group	Control groups	P value
Spawning moth rate (%)	97.13±3.21	94.36±2.37	<0.01
Group oviposition (eggs/15 moths)	9 220±344	9 425±631	>0.05
Group good oviposition (eggs/15 moths)	9 174±357	9 424±631	>0.05
Group oviposition rate (%)	99.69±0.01	99.21±1.33	>0.05
Single oviposition (eggs/moth)	627±26	654±17	<0.01
Single good oviposition (eggs/moth)	625±25	653±17	<0.01
Single oviposition rate (%)	99.72±0.03	99.87±0.06	<0.01

Reactions of mulberry fruits toward the popcorn disease

As shown in Figure 1, after a 72 h inoculation, the control and inoculated fruits exhibited an infection appearance. However, the inoculated fruits of Da-Shi were redder and bigger than control sample. In comparison to the healthy mulberry, which mature fruits color were purple, the disease fruit were white or gray, and followed by a significantly enlarged perianth. Because of the mulberry were composed of obviously swelled small fruit, look like popcorn, so the disease also called popcorn disease.

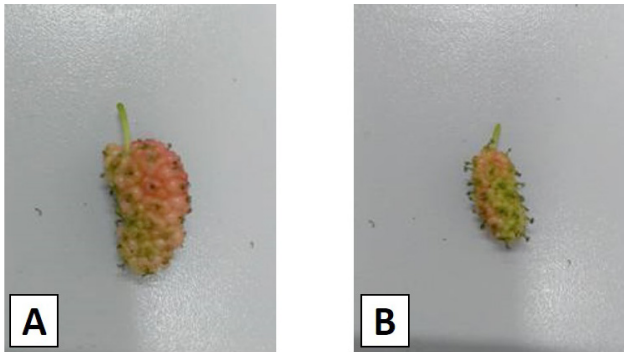

Fig. 1. Morphological comparison of the control (A) and inoculated fruits (B).

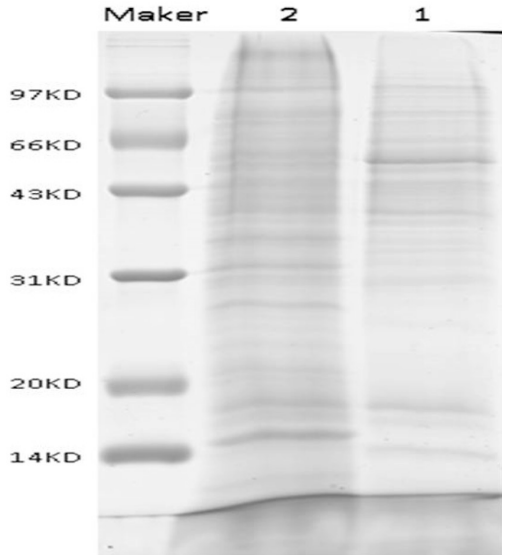

Fig. 2. SDS-PAGE of mulberry proteins of two samples. Maker, protein molecular weight markers; loading amount, 10 ug; coomassie brilliant blue G250 dying.

Comparative analysis of protein in the two samples

The protein expressions of pathogen in normal and inoculated samples were 5.607 μg/μL and 2.455 μg/μL, respectively. On the other hand, as shown in Figure 2, the protein components of samples were difference based upon SDS-PAGE result. The protein expression of pathogen samples was enhanced; whose molecular weight increased from 20kD to 31KD, which means, the pathogen invasion caused a protein up or down-regulated expression in mulberry.

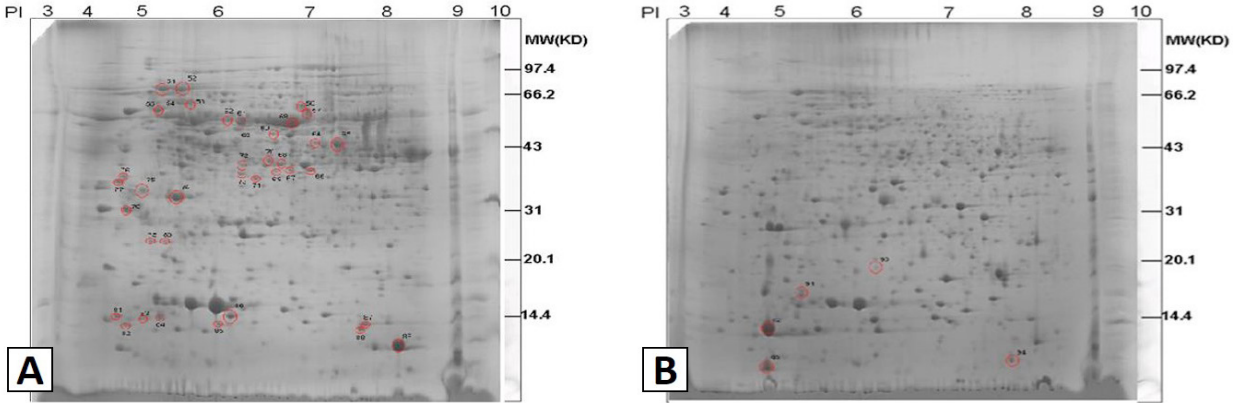

Fig. 3. 2-DE specific expression protein spots: A, inoculated group; B, control group.

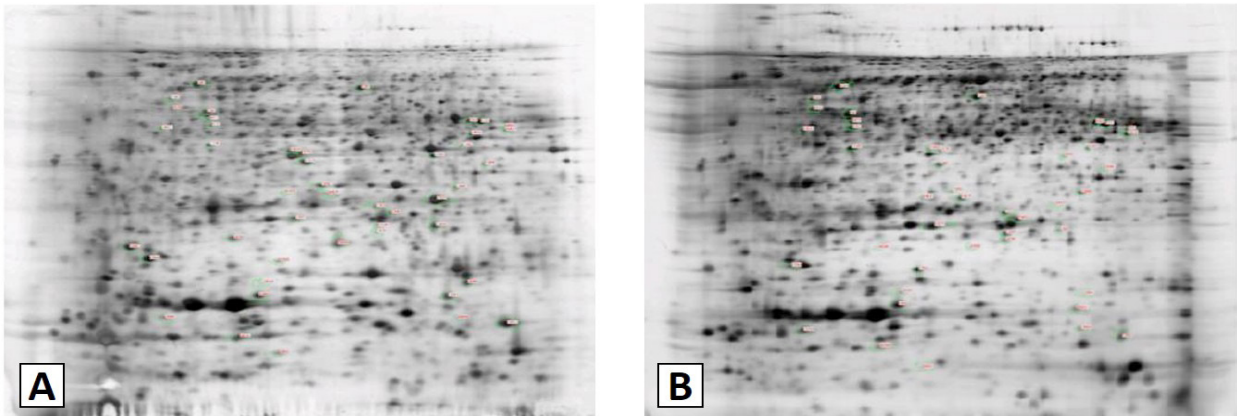

Fig. 4. Analysis of 2-DE images of fruit proteins from mulberry after popcorn disease infection: A, inoculated group; B, control group.

2-DE analysis of proteins from inoculated and normal mulberry

The 2-DE images were analyzed by software of Image Master 2 dimensional platinum. As shown in Figures 3, 4 and 5, after a 72 h inoculation, 822, 688, 994 and 1547, 1998, 1384 protein spots were separated in samples. Although minor differences are seen among the samples, it is clear that, the expressed proteins were dissimilar according 2-DE results. Inoculated mulberry exhibited a 50% higher 45 protein expression compared with the control group, followed with 18 and 27 proteins were up and down regulation, respectively.

Protein identification and functional categorisation

Form the 2-DE result, 45 proteins were identified successfully, where 13 and 27 proteins were regard as up and down expressed proteins. 44 specific proteins were selected as the aim proteins, however, just 1 specific protein can be identified in the normal mulberry sample and 37 specific proteins were identified in inoculated sample. The 2-DE result means that, there were 50 up-regulated and 28 down-regulated proteins in inoculated mulberry sample. 5 proteins were identified from Viridiplantae database; the others were highly matched with proteins from Rosales database.

These known proteins could be categorised into 16 classes (Fig. 6 and Table IV), which including photosynthesis proteins (24.62%), protein synthesis (10.77%), ATP synthesis (9.23%), ROS scavenging (9.23%), amino acid metabolism (7.69%), carbohydrate metabolism (4.62%), ruit ripening (4.62%), defense and stress related proteins (1.54%) etc. Up-regulated proteins mainly related to gluconeogenesis related proteins, ATP synthesis related proteins, transport related proteins, fruit ripening proteins, and stress related proteins. Others mainly are belonging to down expressed proteins. Among these classes, the category related to resistance was defense response, ROS scavenging proteins. Some proteins classified into metabolism also played important role in resist to pathogen, such as lipoxygenase, cytochrome oxidase, heat shock, S-adenosyl-L-methionine synthetase.

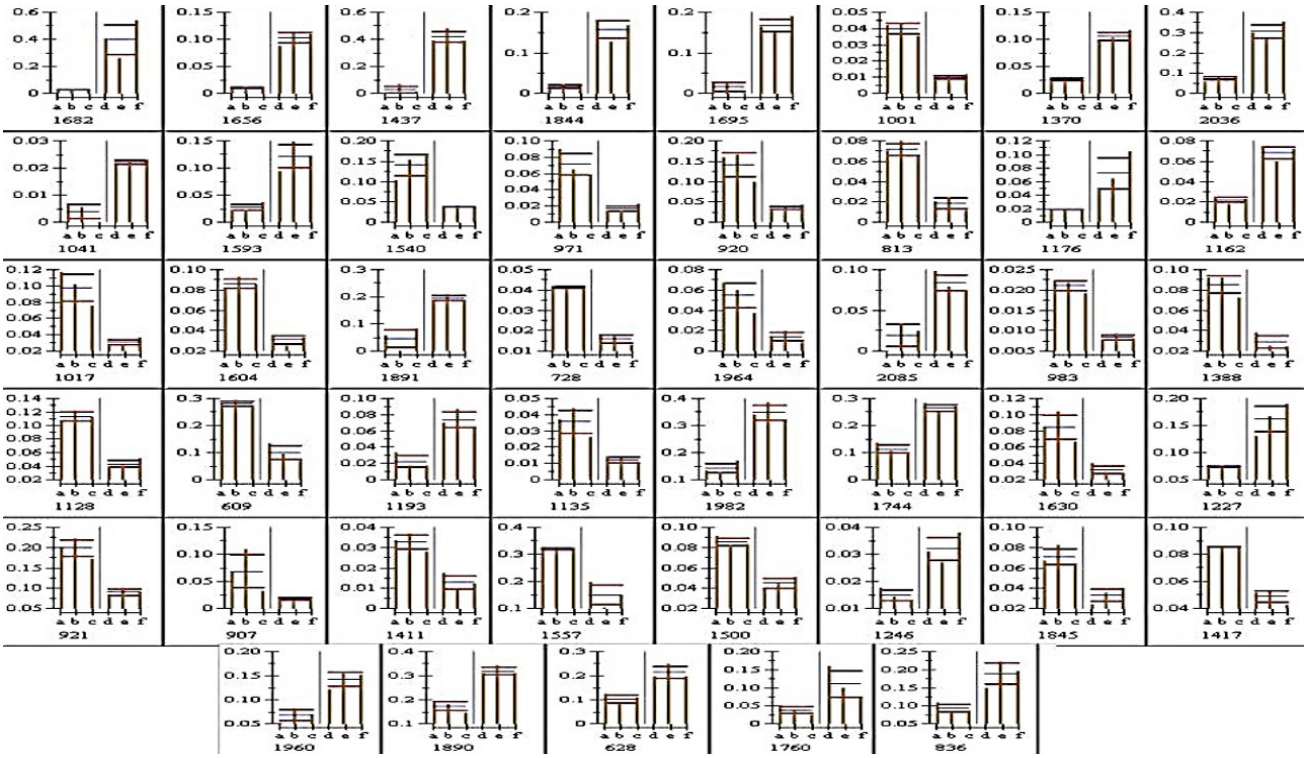

Fig. 5. Differential protein classes and group histograms.

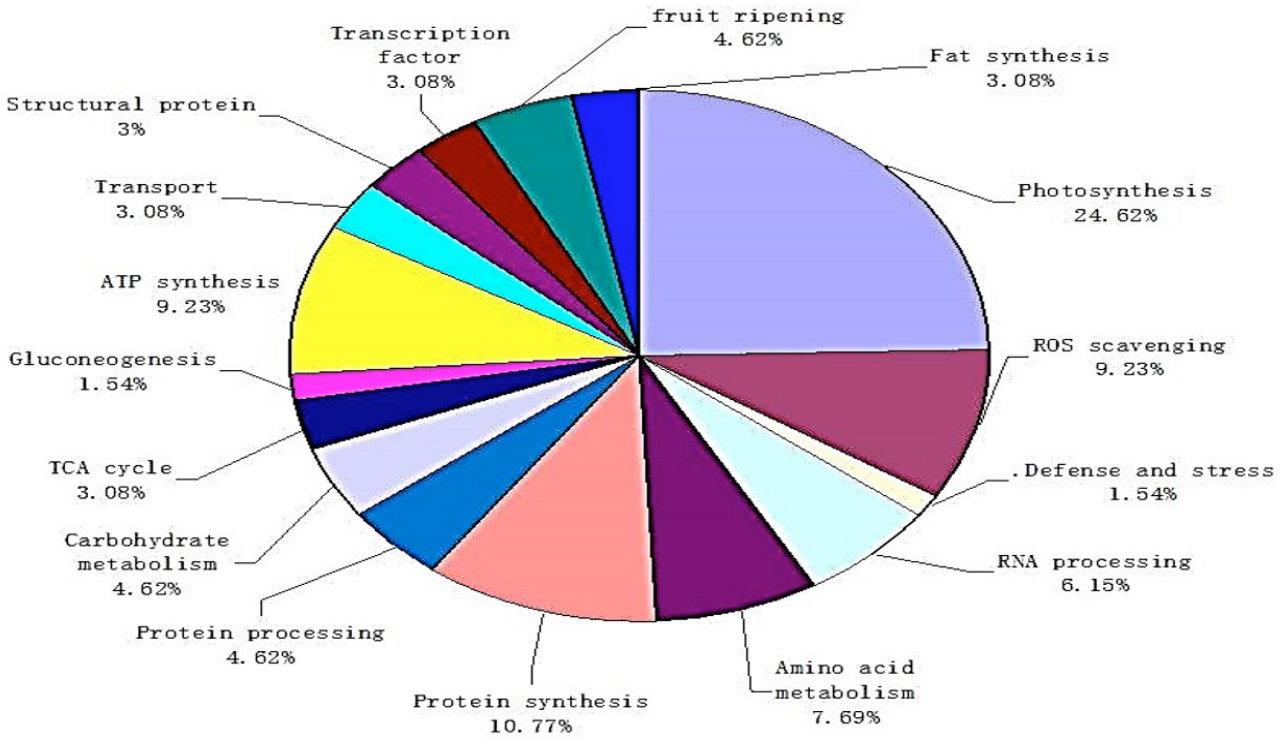

Fig. 6. Functional categorization of the identified proteins.

Table IV.- Differentially expressed proteins of mulberry fruits related popcorn disease.

Group ID	DEQ	Protein name	Accession No.	Protein			
				MW	PI	Score	Score C.I. %
Photosynthesis proteins							
1540/K11	-2.89309	ribulose-phosphate 3-epimerase,chloroplastic-like	gi|470126730	30055	8.92	165	100
813/K14	-2.72266	chloroplast ribulose-1,5-bisphosphate carboxylase/ oxygenase activase	gi|119855475	27345.5	4.76	675	100
983/K23	-2.18539	ribulose bisphosphate carboxylase oxygenase	gi|9968281	52163.2	6	122	100
907/L11	-1.77796	ribulose bisphosphate carboxylase/oxygenase activase 2	gi|567774771	48327.3	6.28	526	100
1417/L17	-1.58823	gamma carbonic anhydrase 1,mitochondrial-like	gi|470111253	29633.4	6.1	271	100
1960/L18	-1.57676	ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit	gi|114804273	53090.7	6	247	100
1246/L15	1.62532	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast)	gi|379062327	51017.6	6.04	79	99.904
1001/K6	-3.30513	chloroplast sedoheptulose-1,7-bisphosphatase	gi|118175929	42824.9	6.06	356	100
1500/L14	-1.64788	soluble inorganic pyrophosphatase-like	gi|470102884	24340.2	5.3	134	100
1411/L12	-1.77162	soluble inorganic pyrophosphatase-like isoform 2	gi|470133818	24675.4	5.51	138	100
286/L14	-	ribulose bisphosphate carboxylase	gi|19581	20718.4	9.04	81	99.932
255/K7	+	ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit	gi|114804273	53090.7	6	871	100
254/K6	+	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	gi|533040	52030.2	6	707	100
285/L13	+	ribulose bisphosphate carboxylase small chain, partial	gi|527193675	19481.8	9.01	83	99.465
270/K22	+	chloroplast photosynthetic water oxidation complex 33kDa subunit precursor	gi|152143640	28477.5	5.48	788	100
Defense and stress related proteins							
275/L3	+	thaumatin-like protein-like	gi|470137923	25581.8	8.32	75	99.724
ROS scavenging							
1604/K18	-2.31303	chloroplast Mn-superoxide dismutase 1B-d	gi|383386107	26083.6	8.57	96	99.998
1760/L21	-1.52408	phospholipid hydroperoxide glutathione peroxidase 6, mitochondrial-like	gi|470125537	25568.1	9.31	132	100
1630/L8	-1.84426	methionine sulfoxide reductase	gi|372864100	21874.7	6.3	362	100
276/L4	+	2-Cys peroxiredoxin BAS1-like,chloroplastic-like	gi|470135402	28882.9	6.9	199	100
262/K14	+	NADP-dependent alkenal double bond reductase P2-like	gi|470101429	41644.7	5.3	82	99.945
265/K17	+	thioredoxin reductase 2-like	gi|470102522	35769.1	5.84	192	100
268/K20	+	coproporphyrinogen-III oxidase, chloroplastic-like	gi|470137537	43834.6	6.15	287	100
RNA processing							
1845/L16	-1.59956	regulator of ribonuclease-like protein 3-like	gi|470117445	26953.7	8.59	94	99.997
1891/K19	2.31014	Bifunctional DNA-directed RNA polymerase subunit beta-beta' OS=Wolbachia pipientis wMel GN=rpoBC PE=3 SV=1		319977	6.02	78	99.123
250/K2	+	poly(rC)-binding protein 1-like	gi|470132392	57626	4.8	169	100
283/L11	+	glycine-rich RNA-binding protein	gi|34851124	17373.9	7.82	101	100

Group ID	DEQ	Protein name	Accession No.	Protein			
				MW	PI	Score	Score C.I. %
Amino acid metabolism							
1890/L19	-1.56931	S-adenosyl-L-methionine synthetase	gi|13540318	43564.9	5.5	121	100
628/L20	-1.55981	S-adenosylmethionine synthase 3-like isoform	gi|470122103	43396.9	5.5	474	100
1162/K16	2.47934	cysteine synthase-like	gi|470132276	34541	5.49	76	99.818
267/K19	+	cysteine synthase-like	gi|470126275	34381.1	6.06	291	100
256/K8	+	S-adenosylmethionine synthase 3-like isoform 5	gi|470122107	41474.7	5.08	499	100
Protein synthesis							
1557/L13	-1.67692	20 kDa chaperonin, chloroplastic-like	gi|470102438	26123.1	9.03	152	100
728/K20	-2.2365	26S protease regulatory subunit 6A homolog A-like	gi|470133049	47833.6	5.02	627	100
971/K12	-2.79719	Probable protein disulfide-isomerase A6 OS=Medicago sativa PE=1 SV=1		40808.8	5.44	79	99.287
1128/L1	-2.15889	60S acidic ribosomal protein P0-1-like	gi|470143935	33989.8	5.32	89	99.989
1593/K10	2.96497	Translation initiation factor IF-2 OS=Leuconostoc citreum (strain KM20) GN=infB PE=3 SV=1	gi|189028330	91089.2	9.18	83	99.71
278/L6	+	60S acidic ribosomal protein P2B-like	gi|470134336	11448	4.56	215	100
259/K11	+	polyadenylate-binding protein RBP45-like isoform 1	gi|470116402	46768.3	5.68	123	100
Protein processing							
248/J24	+	HSP70	gi|325961435	71560.3	5.17	744	100
247/J23	+	stromal 70 kDa heat shock-related protein, chloroplastic-like	gi|470129443	74291.6	5.13	631	100
249/K1	+	heat shock 70 kDa protein-like	gi|470101369	71857.6	5.17	220	100
8. Carbohydrate metabolism related protein							
920/K13	-2.78185	glyeraldehyde 3-phosphate dehydrogenase	gi|353259703	36539.1	7.68	234	100
1135/L4	-1.9928	2-dehydro-3-deoxyphosphooctonate aldolase 1-like	gi|470141667	32073.8	6.61	210	100
258/K10	+	enolase-like	gi|470134388	48251.6	5.76	365	100
TCA cycle							
1017/K17	-2.34452	succinyl-CoA ligase [ADP-forming] subunit alpha-1, mitochondrial-like	gi|470126989	35348.5	9.06	204	100
264/K16	+	NAD-dependent malate dehydrogenase	gi|307707110	34811.8	5.92	292	100
1041/K9	3.12876	malate dehydrogenase, mitochondrial-like	gi|470115832	35885.9	8.46	80	99.919
ATP synthesis related proteins							
1744/L6	1.95422	Deoxyuridine 5'-triphosphate nucleotidohydrolase OS=Arabidopsis thaliana GN=DUT PE=1 SV=1	gi|470143935	17603.2	5.34	106	100
2036/K8	3.22111	ATPase 10, plasma membrane-type-like	gi|470135969	105284.6	5.34	63	96.01
1193/L3	2.08659	ATP synthase subunit beta,mitochondrial-like	gi|470126069	60119.4	6.01	99	100
609/L2	-2.14693	ATP synthase subunit beta,mitochondrial-like	gi|470126069	60119.4	6.01	854	100
1982/L5	1.9678	Nucleoside diphosphate kinase OS=Emericella nidulans (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) GN=swoH PE=3 SV=1	gi|81652940	16954.8	7.82	248	100
257/K9	+	ATP synthase beta subunit	gi|114804272	53815.1	5.46	985	100
Transport related proteins							
1370/K7	3.28649	protein SEC13 homolog	gi|470133041	33072.1	5.27	183	100
282/L10	+	Lea protein precursor	gi|351727923	49483.7	7.08	93	99.95

Group ID	DEQ	Protein name	Accession No.	Protein			
				MW	PI	Score	Score C.I. %
Structural protein							
836/L22	-1.51375	actin, partial	gi|355329944	40369.4	5.67	651	100
260/K12	+	putative SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 3-like 2-like	gi|470137919	114165.2	6.55	78	99.862
Transcription factor							
274/L2	+	nascent polypeptide-associated complex subunit alpha-like protein-like	gi|470141512	21912.8	4.3	123	100
251/K3	+	retrotransposon protein, putative, Ty3-gypsy subclass	gi|77556709	150106.6	8.74	74	96.213
Fruit ripening proteins							
252/K4	+	cytochrome c oxidase subunit 6b-1-like	gi|470127428	20119.4	4.33	137	100
273/L1	+	lipoxygenase homology domain-containing protein 1-like	gi|470103585	19632.5	4.52	76	99.781
280/L8	+	lipoxygenase homology domain-containing protein 1-like	gi|470103585	19632.5	4.52	76	99.781
Fat synthesis related protein							
263/K15	+	enoyl-[acyl-carrier-protein] reductase [NADH], chloroplastic-like	gi|470118022	41515.4	9.1	104	100
266/K18	+	1-acyl-sn-glycerol-3-phosphate acyltransferase 2-like	gi|470140148	44110.1	8.02	66	98
Hypothetical protein							
921/L10	-1.79273	hypothetical protein PRUPE_ppa007714mg	gi|462397790	38763.1	6.69	204	100
1388/K24	-2.16414	hypothetical protein PRUPE_ppa010444mg	gi|462398011	27217.2	7.74	194	100
964/K21	-2.22111	hypothetical protein PRUPE_ppa019177mg, partial	gi|462417841	10550.6	5.29	136	100
1682/K1	8.77246	hypothetical protein PRUPE_ppa010901mg	gi|462406268	25660.8	9.25	75	99.748
1227/L9	1.8271	hypothetical protein PRUPE_ppa006270mg	gi|462419522	45577.6	8.48	63	95.822
1176/K15	2.49684	uncharacterized protein LOC101313290	gi|470116624	27439.8	5.79	128	100
281/L9	+	hypothetical protein PRUPE_ppa000105mg [Prunus persica]	gi|462422423	201978.7	4.7	64	97.042
271/K23	+	hypothetical protein PRUPE_ppa010342mg	gi|462396799	27547.6	6.77	69	98.926
277/L5	+	hypothetical protein PRUPE_ppa013496mg	gi|462408108	11875.7	4.47	73	99.61
272/K24	+	hypothetical protein PRUPE_ppa011150mg	gi|462414826	24255.7	4.39	305	100
284/L12	+	hypothetical protein PRUPE_ppa019177mg, partial	gi|462417841	10550.6	5.29	313	100
269/K21	+	uncharacterized protein LOC101313290	gi|470116624	27439.8	5.79	238	100
289/L17	+	unnamed protein product	gi|257734680	61157.9	6.19	66	97.756
290/L18	+	hypothetical protein PRUPE_ppa001809mg	gi|462402783	84394.4	8.91	75	99.754

+, proteins specific expression in inoculated sample; -, proteins specific expression in normal sample; DEQ, differential expression quantity.

DISCUSSION

Effect of popcorn disease infection on silkworm development

According to the result obtained, it is clear that, the infected leaves exhibited limited toxicity to the silkworm, which including general toxicity, developmental toxicity, neurotoxicity, organ and reproductive toxicity. The present study feed the silkworm with infected leaves form 3-instar to 5-instar, and found no clear or at all vomiting, apastia and twist and other acute poisoning symptoms during the study period. As shown in Table I, the healthy rates of 3rd, 4th and 5th instar silkworms were 100% and the cocooning rate was 100%, also. The result obtained means that, the Popcorn disease infection cause bare effect to the vitality of silkworm.

Regarding the growth and development of infected group, the silkworm finished a whole generation which including eclosion, copulation, oviposition, and the group exhibited a high uniformity of moulting silkworm and matured silkworm. Although the matured time of infected group was 1 day later compared with the control group, the silkworm in the group were mature in same day, which means, the infected leaves were developmental non-toxic to the silkworm.

As shown in Table III, compared with the control group, the silkworm feed with infected leaves exhibited a normal copulation, oviposition ability, and which is similar as control group, it means, the there was no reproduction toxicity substance in the leaves. Moreover, infected leaves exhibited limited toxicity to the silkworm; the cocoon yields found in infected group was deeply affected. Compared with control group, the biggest body weight, cocoon weigh, cocoon shell weight, pupal weight in infected group was 9.3%, 8.8%, 11.4% and 10.2%, respectively, were lower than that of control group. As shown in Table III, although the groups obtain a similar group oviposition, group good oviposition and group oviposition rate ($P>0.05$), the single oviposition, single good oviposition and single oviposition rate were significantly reduced by infected leaves. This indicated that due the infection, there was not enough nutrition. When the mulberry branches are infected, the moisture and nutrients in the leaves are reduced by the use of the bacteria.

Metabolism and energy production related proteins

As described above, 24.62% of aim proteins were photosynthesis related proteins. After an invasion by pathogen, the mulberry could induce 16 differentially expressed photosynthetic proteins, which means a dynamic influence of the pathogen on the host photosynthetic machinery. The up- and down-regulation of RuBisCO may be related to the complicated defense response to the pathogen.

In the present study, photosynthesis related protein Rubisco activase, gamma carbonic anhydrase, were down-regulated, where the Rubisco activase was the regulatory enzyme of Rubisco, and the gamma carbonic anhydrase (CA), a zinc metal enzyme, was the Key enzymes in carbondioxide concentrating mechanism. On the contrary, ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit was up-regulated. The down-regulated was the result of chloroplast degradation, as the up-regulation photosynthesis proteins came from ribosome fragment (Galmés *et al*., 2014). The carbohydrate metabolism related proteins down regulated would break the metabolic balance, cause metabolic disorder and variety of symptoms in plants (He *et al*., 2014).

As the young fruit exhibited a larger and redder appearance compared with normal, which can be attributed to the up–regulation of other metabolism and energy production related proteins, including: fat synthesis, fruit ripening proteins, ATP synthesis related proteins, malate dehydrogenase. It is clear that, host metabolism acceleration need more energy and substrate (Karlsson *et al*., 2015; Chen *et al*., 2008).

Protein synthesis, protein processing and amino acid metabolism related proteins were dissimilar expressed in the groups. Heat shock 70 act as protective protein under biological and abiotic stress, is a kind molecular chaperone participate in the folding and unfolding, transportation and degradation of proteins. Disulfide-isomerase (PDI) is an abundant oxidoreductase enzyme in theendoplasmic reticulum (ER).

Defense-related proteins

Some plant disease resistance related proteins were identified in the samples: S-adenosylmethionine synthase (SAMS) is closely related to transmethylation, transaminopropyl and transsulfur physiological functions, participated in the synthesis of ethylene and polyamine. In genetically modified soybean, wild soybean SAMS gene can improve its ability to resist drought, salt tolerance and low temperature (Xiao *et al*., 2015; Chen *et al*., 2014). Cysteine synthase can promote the use of sulfur, further converted into antioxidant substances of glutathione, and it's up-regulated expression can enhance the disease resistance of plants (Romero *et al*., 2015).

The expression of Mn-superoxide dismutase (Mn-SOD) was decreased, as Mn-SOD was regard as an important anti-oxidant enzyme, which plays a key role in resisting various stress of plants. SOD could protect cell membrane damage from oxygen or other peroxide radicals (Wang *et al*., 2016; Kim *et al*., 2014). The result means that, the defensive system of young fruits had been destroyed, which caused a lower Mn-SOD expression.

In order to resist the pathogen attacks, the host plants produce a series PR proteins, which could improve the defensive capacity of plants. Thaumatin-like protein (PR5) was identified in the sample. It can combine and degradation the components beta 1, 3 glucanase of the cell walls of fungi results from its glucanase activity. It means PR5 may contribute to against the mulberry popcorn disease pathogen (Misra *et al*., 2016).

Heat shock proteins play a critical role when pathogen infecting host plant (Wang *et al*., 2004). Hsp70 take part in plant defence responses, especially pathogen recognition, and in this study, Hsp70 were up-expressed in host. This is assumed attributed to a resistance function of the protein.

The other proteins

During pathogen-mulberry interaction, some

physiologically regulated proteins were identified, such as transport, transcription factor and RNA processing related proteins. SEC13 protein was transport of proteins. NAC transcription factors play regulatory roles in diverse developmental processes and stress responses, which means, many pathways are involved resistant in host during the process.

CONCLUSIONS

The present study showed that infected leaves exhibited limited toxicity to the silkworm; however, the cocoon yields found in infected group was deeply affected. Compared with control group, the biggest body weight, cocoon weigh, cocoon shell weight, pupal weight in infected group was 9.3%, 8.8%, 11.4% and 10.2%, respectively, were lower than that of control group. As shown in Table III, although the groups obtain a similar group oviposition, group good oviposition and group oviposition rate ($P>0.05$), the single oviposition, single good oviposition and single oviposition rate were significantly reduced by infected leaves. Furthermore, to elucidate the molecular resistance mechanism of fruit mulberry "Da10" *i.e.,* Popcorn disease, Two-dimensional electrophoresis (2-DE), matrix-assisted laser desorption/ionisation time-of-flight tandem mass spectrometry (MALDI-TOF-TOF MS) and bioinformatics technique were used for characterize the differential expressed proteins. Almost 78 patho-stress responsive proteins which expression level more than 1.5-fold were identified, where 50 proteins were up-regulated and 28 proteins were down-regulated; The identified proteins were categorised into 16 classes, which are mainly including energy metabolism, gene expression regulation, oxidation-reduction reaction, cellular component and stress responses, and the stress proteins including Mn-superoxide dismutase and thaumatin-like protein. The results means that mulberry young fruits can regulate the expression levels of multiple proteins to reply popcorn disease and these pathogenesis-related proteins provide valuable information to further study the pathogenesis of popcorn disease and disease-resistant molecular breeding in mulberry.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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