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THE PROTEOMIC ANALYSIS OF RESISTANCE TO *SCLEROTINIA SCLEROTIORUM* FUNGUS IN SUNFLOWER SEEDLING STAGE

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ABSTRACT
Sclerotinia sclerotiorum is one of the most important soil-borne fungi which infect wide variety of plants such as sunflower. Study on resistance sources at the molecular level can have a significant role in the identification of resistant plants in the early stages. Considering the importance of *Sclerotinia* in sunflower, the current study was carried out to identify resistance sources at protein levels of two sensitive (CMS Farokh) and resistant (Tub-3234) sunflower lines. For this purpose, proteins were extracted from cotyledon leaves 12 h after infection and analyzed by two-dimensional electrophoresis. Due to the relative expression of proteins, six protein spot (SOD, CAT, AOS, LSP, LOX and ICL) were identified through Maldi-TOF-TOF or mass spectrophotometry. The results indicated that, relative expression of LOX and ICL proteins increased in sensitive line, while relative expression of enzymes such as SOD, CAT, AOS and LSP increased in resistant line. Consider the obtained results; resistance sources in sunflower are highly related to antioxidant system and programmed cell death is justifiable. On the other hand, susceptibility to *Sclerotinia sclerotiorum* might be due to a switch from the TCA to glyoxylate pathway which describes more carbohydrates production for fungi.

KEYWORDS: Glyoxylate, Maldi-TOF-TOF, Protein spot, *Sclerotinia sclerotiorum*, TCA pathway.

INTRODUCTION

The production of oilseeds is increasing worldwide due to the growing demand of oil as the main source of edible and industrial oil. The sunflower (*Helianthus annus* L.) is one of the most important oil crops globally and its oil is among the highest quality vegetable oils on the market. In addition to provide edible and industrial oil, sunflower meal can be also used to provide minerals, vitamins and antioxidants (Nasim and Bano, 2012). The fungal pathogen *Sclerotinia sclerotiorum* (Lib.) is a common and widespread soil borne fungal pathogen to more than 400 plant species. It was first described in 1837, and recognized as a sunflower pathogen in 1861 (Lu, 2003). It is encountered in all sunflower-growing regions of the world and attack several plant parts including stem, root, leaf and head. Under favorable climatic conditions the fungus causes important yield reductions, especially in the case of stem (stalk rot) or capitulum (head rot) infection. As chemical control is not practical, genetic control through the development of resistant lines appears to be the best crop protection strategy (Parts *et al.*, 2003).

In the recent years, proteomic analyses are widely used in understanding the plant responses to biotic and abiotic stress (Cánovas *et al.*, 2004; Rossignol *et al.*, 2006). Proteomics, which is known as the large-scale study of proteins, particularly their structures and functions is a complement to genomics techniques such as micro-arrays, providing information about gene expression and function. Proteomic analyses, combined with transcriptomics, have been used as a tool for identifying proteins involved in plant defense responses to different pathogens. Identifying these proteins is crucial, not only for understanding the plants defense system, but also for testing interaction between plants and pathogens (Mehta *et al.*, 2008). Plants have complex defense mechanisms, including stimulation of antioxidant enzymes which respond to pathogen attack quickly. A protein spot as peroxidase (POX) antioxidant enzyme in relation with *S. sclerotiorum* was observed in canola (*Brassica napus* L.) proteome (Yang *et al.*, 2010). This enzyme plays many important roles in plants cells for instance scavenging reactive oxygen species by removing H_2O_2, lignin biosynthesis and degradation in cell wall, hormone signaling, chemical oxidation and defense against pathogens. There are many reports suggesting that POX synthesis is stimulated due to pathogen attack (Fossdal *et al.*, 2001). In other words, increase in antioxidant enzymes, especially peroxidases in plant cells is known as a common sign of pathogens attack. Other antioxidant enzymes such as superoxide dismutases (SOD) are also synthesized under unfavorable conditions of pathogens attack (Subramanian *et al.*, 2005). The role of SOD in relation to *S. sclerotiorum* has been well documented by SOD enzymatic assays in canola. According to Yang et al. (2010), SOD activity in canola significantly decreased 24 h after inoculating with *S. sclerotiorum* followed by considerable increase was observed after 36-48 h. In

addition, the effect of glyoxylate on plants resistance has already been studied and the results provided by these studies indicated that glyoxylate (oxalic acid), especially oxalic acid *I* would increase three times 48 h after inoculating with *S. sclerotiorum*. Detoxification of methyl glyoxylate (MG) is mostly carried out by glyoxylate *I* and *II*. Methyl glyoxylate as by-product of *TPI* or as result of MG synthase can causes changes in protein structure and increases protein degradation as well as deactivates antioxidant enzyme defense system. Although *lase* oxalic acid system in response to abiotic stress is extensively studied, a potential role for oxalic acid *I* has been proposed in corn (*Zea mays* L.) against *Aspergillus flavus* (Chen *et al.,* 2004), suggesting that oxalic acid probably plays an important role in plant defense systems. However, the application of oxalic acid in engineering resistance to diseases is not proven. It has been reported that glyoxylate transcription increases 36-48 h after inoculating of canola plants with *S. sclerotinia*. These results have been confirmed by proteomics examinations (Yang *et al.,* 2010).

Peroxidase (POX), allene oxide synthase (AOS), superoxide dismutase (SOD), isocitrate lyase (ICL), lipoxygenase (LOX) and leaf senescence factors are identified proteins involved in resistance to *Sclerotinia* genus in sunflower (Zaefizadeh *et al.,* 2013). Sunflower resistance to necrotrophic fungi would obtain through the activation of related pathogen nuclease (NRP1), sensitive reaction 203j (HSR) and plant defense factor (PDF) genes (Radwan *et al.,* 2005). Study on biotrophic oomycete *Plasmopara halstedii* in sunflower indicated that Ha-PDF transcription increased in resistant hypocotyls 6 days after disease inducing and then reached to the maximum on ninth days (Radwan *et al.,* 2005). They have also reported that sunflower resistance to *Plasmopara halstedii* depends on chain activities of systemic-acquired resistance (SAR) and hypersensitive reaction (HR) and direct relation between them. In other words, the resistance of sunflower to *P. halstedii* is associated with an HR which fails to halt the parasite. By contrast, this HR triggers a SAR which takes places in the upper part of the hypocotyls and eventually leads to the arrest of parasite growth (Radwan *et al.,* 2005).

Kazemi-Pour et al. (2004) with proteomic analysis of bacterial plant pathogens obtained a comprehensive profile of proteins secreted by *Erwinia chrysanthemi*. By analysis of mutants, Western blotting, and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF-TOF), they could identify 55 spots representing 25 unique proteins. In uninduced conditions, they identified spots corresponding to the cellulase Cel5, the proteases PrtA, PrtB, and PrtC, the flagellin FliC, and some intracellular proteins whose presence probably resulted from spontaneous cell lysis (Kazemi-pour *et al.,* 2004).

During comprehensive analysis of the extracellular proteins from (*Xanthomonas campestris* pv. Campestris), 87 different proteins have been identified which most of them were characterized as destructive enzymes, possibly involved in infecting sensitive hosts (Watt *et al.,* 2005).

Pantoea stewartii subsp. *stewartii* (Pnss), the causal agent of Stewart's bacterial wilt and leaf blight of maize and sweet corn, is one of the quarantine pathogens in many countries and regions. Comparative proteomics and genetic analysis of *Pantoea stewartii* Led to the identification of genetic markers to distinguish the virulent and avirulent subspecies of *Pantoea stewartii* (Wu *et al.,* 2007). In addition, study on *Magnaporthe grisea*, also known as rice blast fungus, has led to the identification of a number of proteins linked to formation of appressorium (Kim *et al.,* 2004). Moreover, proteins linked to formation of appressorium in *P. infestans*, an oomycete that causes the serious potato disease known as late blight or potato blight, have identified (Grenville-Briggs *et al.,* 2005). During proteome anaylsis of Blumeria graminis f.sp, proteome map of conidiospores were identified (Noir *et al.,* 2009). Using a combination of two-dimensional polyacrylamide gel electrophoresis and matrix-assisted laser desorption ionizationtime-of-flight mass spectrometry, they have identified the proteins in 180 spots, which probably represent at least 123 distinct fungal gene products (Noir *et al.,* 2009).

Recently, extracellular proteins of pathogenic and non-pathogenic isolates of *Pyrenophora triticirepentis*, that causes tan spot of wheat, were compared and qualitative and quantitative differences among produced proteins were spotted (Cao *et al.,* 2009). Study on proteome of wheat black rust, caused by *Puccinia triticina*, showed that there are several known and unknown proteins which are more abundant after pathogen attack. Similar results were obtained from a proteomics experiment aimed to study wheat responses to F*usarium graminearum* (Zhou *et al.,* 2006).
Despite numerous reports on proteomic analysis of plant pathogens, poly-genetic features of resistance to *Sclerotina*, which causes natural resistance to *S. sclerotiorum*, through hybridization in sunflower cultivars, have not been reported

so far. *Sclerotina* resistant sunflower lines are limited in the world; however their desirable resistance is suitable for using in hybridization programs. Therefore, study on resistance mechanisms, especially molecular resistance in resistant and sensitive cultivars, could assist the crop breeders to release resistant lines. In addition, since the outcome of the gene expression is often a protein or enzyme, so study on proteins (proteomics) of sensitive and resistant lines could direct genetic engineers to new techniques.

As mentioned above, the current study was aimed to evaluate resistance mechanisms using comparative proteomics and transcriptomics molecular techniques in sunflower lines infected with *Sclerotinia sclerotiorum*. Furthermore, identifying the molecular mechanisms occurring during the first stage of infection, especially in TCA cycle was the second aim of the study.

MATERIAL AND METHODS

- Plant material and protein extraction

In the current study, in order to proteome analysis of glyoxylate cycles in relation to resistance source to *Sclerotinia sclerotiorum*, two sensitive and resistant sunflower lines (CMS Farokh and TUB -5-3234, respectively) were selected among four lines during a pre-experiment. The lines were provided from Seed and Plant Improvement Institute and National Institute of Genetic Engineering and Biotechnology.

- Protein assay

The protein was extracted from sunflower leaves according to Wang and coworkers method (2003 and 2006). In addition, protein absorbance was measured using Bradford (1976) techniques. The protein content of the crude extract was determined using bovine serum albumin (BSA) as a standard, according to the method of Bradford (1976). Five ml of Bradford solution was added to 10 µl crude extract and absorbance recorded at 595 nm (λ max =595 nm) for estimate of total protein content. The protein concentration was calculated from a BSA standard curve.

- Gel preparation, sample loading and electrophoresis

The agarose gel (0.5 % dissolved in deionized distilled water) was poured into outer edge of electrophoresis chamber to seal the chmaber. Then lower gel buffer included Tris-Hcl 5 M, SDS 0.4 g, APS 0.3 g, TEMED 0.012 ml and 12 ml acrylamid stock was poured into the chmaber. After 20-25 min to ensure the gel is solid, the upper gel included Tris-Hcl 5 M, SDS 0.4 gr, APS 0.06 gr, TEMED 0.006 ml and 1 ml acrylamid stock was placed on the lower gel. The comb was placed on the upper gel so that teeth were 1.5 cm away from lower gel surface. In order to prepare samples, 30 µl protein extract was mixed into 30 µl sample buffer and cooled on ice after heating for 3 min using benmary method. Afterwards, 10 µl of sample was loaded into the holes. In order to determine molecular weight of proteins, the appropriate ladder was loaded into the first hole. The device was set on 6 mA and stable voltage of 70-80 V. The device was set of when the bands reached to the end of the gel. Staining was performed using coomassie blue R-250 and the decolorization was done to get clear protein bands.

- Two-dimensional electrophoresis and identification of proteins

In order to set two-dimensional electrophoresis, 18 cm immobilized pH gradient (IPG) with pH 3-10 was used as first dimension (Xu *et al.*, 2013). To start the two-dimensional electrophoresis, rehydration of dried gels was performed based on 1000 µl rehydration buffer (urea 8.5 mM, CHAPS 2%, DTT 40 mM, ampholyte 0.1% (pH 3-10) and bromophenol blue 0.001%) for each gel strip for 16 at 25° C. Electrofocusing was conducted on gels using Gel Healthcare device (Ettan IPGphar 3 Amersham PROTEAN IEF). After rehydration of the gels moist WEEK papers were placed between electrode and gel and then the device was set for focusing at 20° C. At the end of focusing stage, the gels were shacked for 15 min in the first (urea 6 M, SDS 2%, Tris HCL 0.375 M pH 8.8, glycerol 20% and DTT 130 mM) and the second (urea 6 M, SDS 2%, Tris HCL 0.375 M pH 8.8, glycerol 20% and iodoacetamide 2.5%) equalization buffer. Subsequently the gels were washed in a graduated cylinder containing electrophoresis buffer. The lower and upper gels were 12 and 5% respectively. The strip gels were placed on the upper gel. Agarose solution (agarose 5%, Tri-base 25 mM, glycine 192 mM and SDS 0.1%) was used for covering strips and then gels were put into the electrophoresis tank. The device was turned on adjusted on 80 V after adding electrophoresis buffer tank. Mass spectrophotometry (MALDI TOF/TOF) was used for analyzing protein spots. Protein sequencing and identifying was carried out using Protein Probe and Peptid Sequencing programs related to BioLynx software.

RESULT AND DISCUSSION

- Identification of differential protein spots on two-dimensional electrophoresis analysis

According to the obtained results, 146 protein spots were identified in sensitive and resistant lines at germination stage. The differential study indicated that there are six protein spots differing from other spots (Figure 1 and 2). In addition, mass analysis demonstrated that these protein spots represent ICL, LOX, LSP, SOD, CAT and AOS (Table 1).

Table 1- The most abundant proteins in sunflower inoculated with Sclerotinia sclerotiorum

Spot	Protein description	S_{core}	P_{value}-E_{value}	Found in	Identification
LCL	Iso citrate lyase	151	≤0.006	*Cucumis sativus*	Glyoxysomal
LOX	Lipo oxygenase	124	2.1×10^{8}	-	B-Oxidation degradation
LSP	Leafseneceus protein	95	0.00002	-	
SOD	Super oxid desmotase	124	3.7×10^{9}	-	Natrual plant Antioxidant
CAT	Catalase	224	1.3×10^{-5}	*Arabidopsis*	Catalase
AOS	Alenoxide synthase	187	0.00001	*Zea maiz*	-

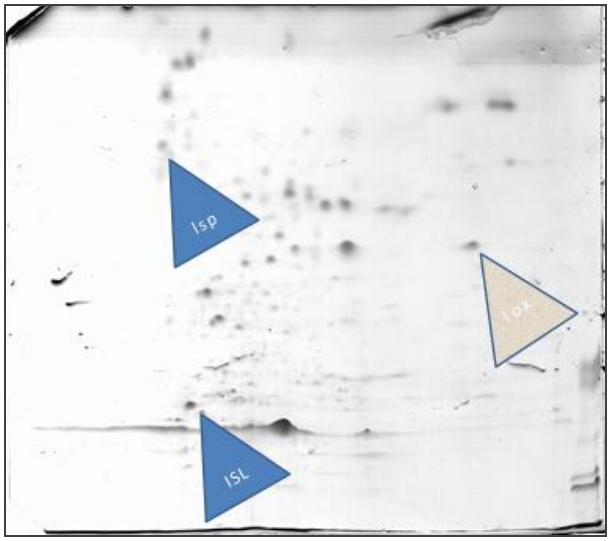

Figure 1. Gel of Two-dimensional electrophoresis for LOX, ICL and LSP protein

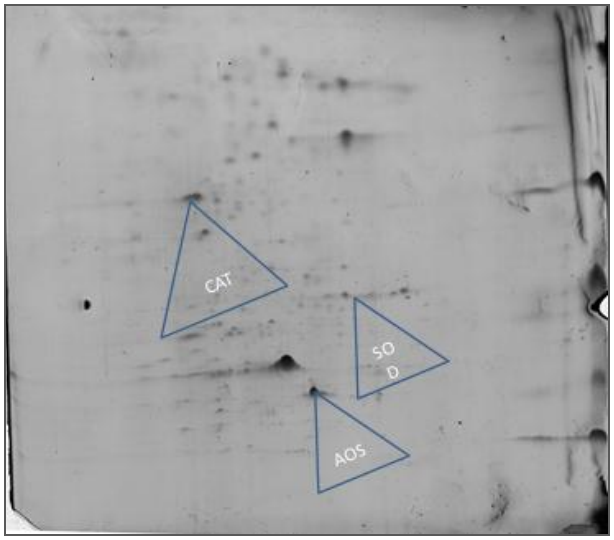

Figure 2. Gel of Two-dimensional electrophoresis for SOD, CAT and AOS protein

- Isocitrate lyase protein (ICL)

According to the results, the first spot was isocitrate lyase which was identified by Maldi Tof Tof (Figure 3). In sensitive line, ICL increased compared with fungi extract treatment during the first two hours. After this time ICL increased significantly until 24 h (Figure 3), which is indicative of stimulation through fungus elicitor during infection. ICL expression increased with increasing fungus extract application which represents more penetration of fungus extract into the host cells. The lack of significant difference between 12 and 24 h represents saturation of cells with fungi extract after 12 h. In resistant line, there was no significant difference between control and fungi extract treatments in terms of ICL expression during the first two h. It appears that during the first two h, fungi extract penetration into cells and ICL expression is considerably low. However, over the time expression levels increased, indicating fungi extract penetration into the cells. In other words, stimulation of the expression of the ICL, whether by stimulating fat cell wall or by reducing the amount of glucose in the resistant line, was not occurred during the early stages, indicating cellular resistance against fungi elicitor penetration. By contrast in sensitive line, penetration increased during the early hours of treatment. In general, the results indicate that the protein spot is less expressed in

resistant line than sensitive line. In addition, it can be concluded that this enzyme has up-regulate trend in sensitive line (Table 2). This might be due to this fact that, in TCA cycle, isocitrate lyase is converted to isocitrate dehyrogenase a-ketoglutarate, which is natural pathway to produce NADH and succinyl COA and can be converted to glyoxylate by isocitrate lyase and malate directly. The second pathway occurs during fungi penetration into the plant cells.

Therefore, ICL enzyme can be one of the most important factors in plants resistance to fungus. Furthermore, this pathway plays a key role in changing and converting and destroying lipids and also in lipids beta-oxidation cycle (Wang *et al.,* 2003) to convert it to acetyl coenzyme A. During fungal infections, fungus' elicitor affects glyoxylate cycle through two different mechanisms. First, through glucose consumption that leads to isocitrate lyase synthesis. Second, during the first stage of spore penetration by fungi mycelium and its penetration into plants cell wall, MAPK (Thines *et al.,* 2000) and cell membrane lipids are stimulated by stimulating LOX (Wang *et al.,* 2003) which lead to cell wall swelling and finally penetration into the cell.

Table 2. T-Test analysis for the relative expression of protein spots

Protein	ICL		LSP		CAT		SOD		AOS		LOX	
	RE*	P_{value}	RE	P_{value}	RE	P_{value}	RE	P_{value}	RE	P_{value}	RE	P_{value}
Susceptible cultivar (CMS Farokh)	Up	<0.05	Down	<0.05	Down	<0.05	Down	<0.05	Up	<0.05	Up	<0.05
Resistant cultivar (Tub-3234)	Down		Up		Up		Up		Down		Down	
*** Relative expression**												

The TCA cycle provides an opportunity to consume produced acetyl coenzyme A during lipids decomposition and provides this substance for carbohydrates synthesis. Sensitivity in plants is due to this fact that during the action of this enzyme, TCA cycle is not normal and the cycle use this enzyme to supply required carbohydrates. Due to pathogen resistance, the CMS Farokh line shows sensitivity indicating fungi elicitor stimulation during infection. Increase in ICL expression on account of increase in treating time, represents more fungi extract penetration into the plants cells.

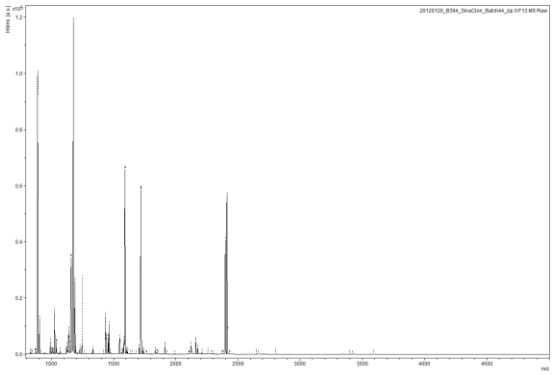

Figure 3. Mass analysis of ICL enzyme

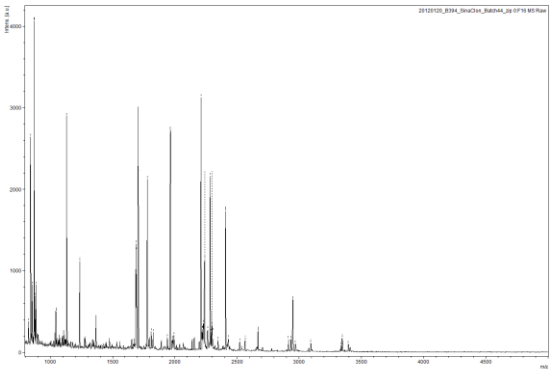

Figure 4. Mass analysis of LOX enzyme

- Lipoxygenase protein (LOX)

Obtained results from mass analysis indicated that the second spot was lipoxygenase protein (LOX) (Figure 4). According to table 2, lipoxygenase expression in sensitive line was higher than resistant line. Lipoxygenase enzyme is a dioxygenase enzyme which is able to add two oxygen atoms to substrate molecule which is usually along with hydro

peroxide. The main substrates for this enzyme are fatty acids isomers such as linoleic acid, linolenic acid and arachidonic acid. Therefore, the demand for substrate would increase with increasing expression of this enzyme. In sensitive lines, increase in expression is parallel with increase in fatty acid consumption while in resistant lines there is no need to fatty acid consumption at high levels. Under this condition, when cell wall is resistant, fungi extract penetration is low. In addition, oil content in resistant lines will increase.

- Leaf senescence protein (LSP)

Obtained results from mass analysis indicated that the third spot was leaf senescence protein (LSP) (Figure 5). According to the results shown in table 2, the expression of this enzyme reduced in sensitive line while the expression increased in resistant sunflower line. Therefore, it can be concluded that this enzyme has down-regulate trend. The LSP enzymes as regulators of leaf growth and development have important effects on other organs as well as the regulation of gene expression.

A number of transcription factors involved in leaf senescence have been identified. LSP enzyme transcription factor is related to genes associated with aging and developmental processes in plants in response to stress. Many of changes, observed in leaves aging process are appeared in plants phenotype and caused by hormones signaling. The process of aging is a process that requires energy and the required energy is supplied from the breakdown of intracellular lipids. In the current study, reduction in expression of this enzyme, especially in sensitive line, led to delay in ageing process and saved more energy, while in resistant line, although the expression was higher, the energy was saved due to being resistant. Therefore, plants are able to use intracellular lipids to gain more energy. It should be mentioned that LSP enzyme is located in downstream of genes coding defense signals, so increase in gene expression in resistant line lead to more defense by plant against pathogen attack. By contrast, in sensitive line, the expression of this enzyme is low and lower signals are transmitted, therefore ageing occurs quickly.

- Allene oxide synthase protein (AOS)

Obtained results from mass analysis indicated that the fourth spot was allene oxide synthase protein (AOS) (Figure 6). Comparison between sensitive and resistant lines showed that expression of this protein in sensitive line is lower than resistant line. Since the study was aimed to increase resistance in sensitive line, therefore, it can be stated that this enzyme had down-regulation in the sensitive line (Table 2). This enzyme as jasmonate plays an important role in preventing ageing and leaf abscission. It should be noted that this enzyme as a jasmonate hormone is the most important stress resistance hormone. The Jasmonate rapidly accumulates in all tissues hormone after mechanical injuries or wounds (Lu *et al.*, 2012). Therefore, it can be concluded that allene oxide synthase enzyme have low expression in sensitive line, while its expression in resistant line is much higher.

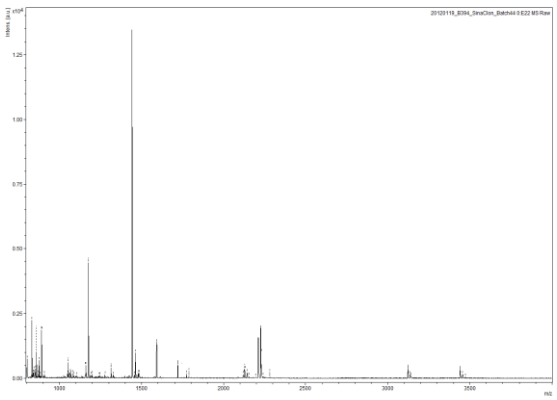

Figure 5. Mass analysis of LSP enzyme

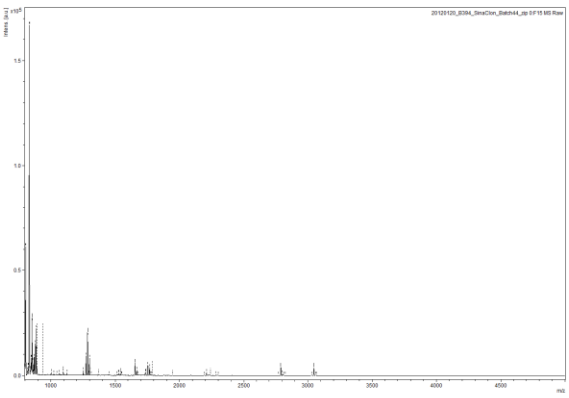

Figure 6. Mass analysis of AOS enzyme

- Superoxide dismutase protein (SOD)

Obtained results from mass analysis indicated that the fifth spot was superoxide dismutase protein (SOD) (Figure 7). This enzyme had low expression in sensitive line while in resistant line the expression was higher. In other words, superoxide dismutase has down-regulate trend. Superoxide dismutase is known as strong antioxidant conserving cells

against reactive oxygen species. Lack of this enzyme causes serious oxidative injuries. Therefore, when the expression of this enzyme is suppressed, especially in sensitive lines, then cellular conservation would decrease and sensitive line have weak defense against reactive oxygen species. By contrast, increase in expression of this enzyme enhances resistance to unfavorable conditions (Raychaudhuri and Deng, 2000).

- Catalase protein (CAT)

Obtained results from mass analysis indicated that the sixth spot was catalase protein (CAT) (Figure 8). The results indicated that in sensitive line the expression of this enzyme decreased so this enzyme has also down-regulate trend. Based on the results, the expression of this enzyme in resistant line was more than sensitive line so the resistant line is up-regulate line. Catalase plays an important role in physiology and virulence of pathogens. These enzymes not only detoxifies hydrogen peroxide, but is known as an early marker or indicator in controlling environmental pollution for instance in detecting bacteria in foods. In other words, catalase is one of the enzymes that protect cells against hydrogen oxide. Since oxidation properties of catalase against pathogens are considered a useful agent, therefore, up-regulation of this enzyme in resistant lines is useful. Lower expression in sensitive line compared with resistant line decreases resistant against oxidative factors and then reduces resistance in sensitive line.

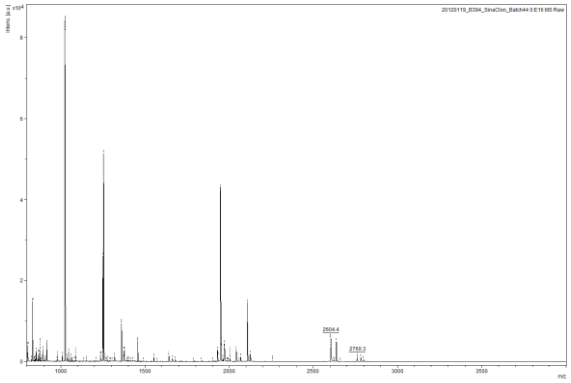

Figure 7. Mass analysis of LSP enzyme

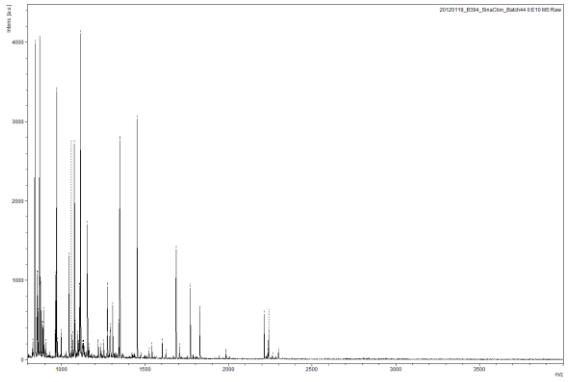

Figure 8. Mass analysis of AOS enzyme

CONCLUSION

As mentioned earlier, the protein spots were identified using Maldi Tof Tof process. In general, the results indicated that relative expression of LOX and ICL in sensitive line significantly increased, while significant increase in relative expression of LSP, CAT, SOD and AOS was observed in resistant line. Therefore it can be concluded that LSP, CAT, SOD and AOS proteins cause resistance against *Sclerotinia sclerotiorum*, especially in resistant line. This is due to normal TCA cycle in resistant line, whereas in sensitive line, TCA cycle does not take place properly. In other words, in the sensitive line, pathogen affect TCA cycle and plant is involved in supplying carbohydrate for pathogens. Moreover, plant uses glyoxylate cycle to supply more sugar, while in the resistant lines, there is no need to supply carbohydrates by TCA cycle and glyoxylate cycle remains inactive. Generally, Tub-3234 is known as resistant line, because pathogens stimulated LSP, CAT, SOD and AOS expression as up-regulate trend. On the other hand, LOX and ICL proteins showed down-regulate trend. Therefore, in resistant line, fungi extract penetration had no effect on plant and conservation was as barrier and not immunity. In the sensitive line, the plant was susceptible against fungi extract penetration due to down-regulate trends of AOS, SOD and CAT. The LOX and ICL proteins showed up-regulate trends in sensitive line, suggesting a switch from TCA cycle to glyoxylate pathways which in turns represents more sugar production. In plants, during infection and defense responses in addition to change in carbohydrate metabolism (Sutton *et al.*, 2007) reduction in photosynthesis (Bonfig *et al.*, 2006) monolignols (Chen *et al.*, 2000) and tannin (Miranda *et al.*, 2007) accumulation, respiration (glycolysis, TCA cycle and electron transport chain) changes to supply energy for resistance mechanisms (Bolton, 2009). For example, increase in respiration in infected barley led to reduction in seed yield. It shows that in incompatible infections, defense mechanisms need more energy (Smedegaard-Petersen and Stolen, 1981). It has been reported that in compatible or incompatible interactions between plant and pathogen, expression of genes involved in respiration increase. There is a correlation between gene expression and respiration rate during defense systems in plants. For example, phosphofructokinase expression in infected wheat would increase

(Bolton *et al.,* 2008). In potato, infection increased glyceraldehydes 3-phosphate dehydrogenase (GAPDH) activity (Laxalt *et al.,* 1996). Laxalt and co-workers have reported that GAPDH which is part of defense responses would be activated with delay and direct early metabolism towards production of required metabolites. Expression of genes coding both cytosolic subunit of GAPDH increases during compatible and incompatible infections. Most of TCA enzymes increase during infection suggesting that TCA is related to defense responses. Expression of genes, coding protein complex compounds increase due to fungi infection. In addition, expression of genes coding NADH dehydrogenase and oxidase (AOX1a, AOX1d) would increase during infection. Alternative respiration plays an important role in defense response in plants (Clifton *et al.,* 2006; Van Aken *et al.,* 2009). Alternative respiration in oxidative respiration does not produce ATP. However, when oxidative respiration is saturated, glycolysis and TCA would increase; therefore alternative respiration plays a critical role in the production of carbon structure (Clifton *et al.,* 2006). Alternative respiration correlates with senescence and programmed cell death due to infection with fungus or chemicals. During defense responses, alternative respiration reduces reactive oxygen species throughout programmed cell death. In general, change in genes could change up-regulating of some antioxidant proteins and result in resistance in plants. Generally, the obtained results indicated that in Tub-3234 line as a resistant line, pathogen penetration and elicitors stimulated up-regulating of LSP, CAT, SOD and AOS proteins.

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