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Proteome level changes in the root of *Brassica alboglabra* induced by alachlor herbicide

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Chinese kale (*Brassica alboglabra*) is a famous and extensively grown vegetable in Southeast Asia. Despite its nutritional values, pesticides are heavily applied to it. In this study, changes in protein expression due to alachlor treatment on *B. alboglabra* were investigated by using the 2-dimensional PAGE. Differential protein expressions were determined by using Image Master Software with volume (%) ≥2 fold as significant. Ten spots of interest have been identified by LC/MS/MS showing significant increases in differential protein expression between *B. alboglabra* roots treated with alachlor as compared to the untreated group which include HSC-cognate binding proteins, adenosylmethionine synthetase and beta-tubulin involved in defence mechanism in plants. Little is known about the function of other proteins identified which include knox-like proteins and hypothetical protein. Further investigations on plant proteomics would provide more information on the effects of different types of pesticides.

Key words: *Brassica alboglabra*, pesticides, proteomics, two-dimensional electrophoresis.

INTRODUCTION

Brassica are cruciferous vegetables belonging to the Brassicacea family which include Chinese kale, cabbage, cauliflower, broccoli and Brussel sprouts. Brassica are widely grown and consumed worldwide due to their taste and high nutritional values. Moreover, studies have been extensively carried out on Brassica with regards to their antioxidant and anti-carcinogenic properties (Podsedek, 2007; Sameca et al., 2011). Despite these, the contamination of pesticide residues in vegetables remains a global concern due to the increased usage of pesticides by farmers (Bankar et al., 2012).

According to the US Environmental Protection Agency (EPA) (Environmental Protection Agency, 2012), a pesticide is defined as any substance or mixture of substances used to prevent, destroy, repel or mitigate any pests. Pesticides include insecticides, herbicides, weedicides, rodenticides and others according to the target pest. In Thailand, herbicides represent the biggest import of pesticides which accounted for 32,971 tons (74.2%), followed by insecticides (12.2%) and others

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Abbreviations: 2 DE, Two-dimensional electrophoresis; **PAGE,** polyacrylamide gel electrophoresis; **LC/MS,** liquid chromatography mass spectrometry; **HPLC,** high performance liquid chromatography.

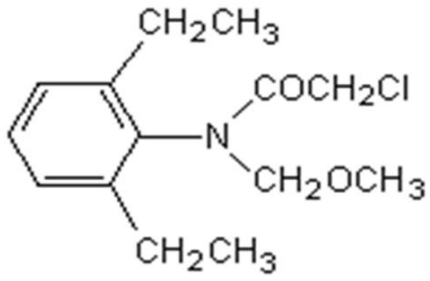


Figure 1. Structure of alachlor.

(13.6%). The most frequently imported herbicides include glyphosate, atrazine and alachlor (Department of Agriculture, Thailand, 2004).

Chloroacetanilide herbicides such as alachlor are important in the agriculture sector. In the United States, alachlor has been used for more than 20 years as an emergent herbicide (Zhao et al., 2006). The chloroacetanilide pesticides act as seedling growth inhibitor in weeds. It inhibits elongase of the root and geranylgeranyl pyrophosphate (GGPP) cyclisation enzymes which are parts of the gibberelin pathway. It also interferes with the plants' ability to produce protein (Rao, 2000).

Proteomics serve as a modern approach to ascertain the protein alterations due to stress or the response of tolerance in plants to stress environment. The studies on abiotic stress proteomics revealed the major results in different types of plants such as rice (Ahsan et al., 2008; Chen et al., 2011; Nam et al., 2012), tomato (Zhou et al., 2009; Manaa et al., 2011), grapevine (Giribaldi et al., 2010; Marsh et al., 2010; Yang et al., 2011) and pea (Dumont et al., 2011; Barilli et al., 2012). The abiotic stresses include salt stress, drought stress, desiccation tolerance, cold stress, heat stress, mineral toxicity, mineral deficiency, nitrogen exposure, hypoxia, ozone exposure, sulphur dioxide exposure and pesticide exposure (Salekdeh and Komatsu, 2007).

There are several studies carried out on plant proteomics with regards to the effects of pesticides. Interestingly, different classes of pesticides may also induce differential protein expression due to their different modes of action. Proteomic study was done in grapevine (*Vitis vinifera L*.) exposed to the herbicide 'flumioaxin', the protein alterations in the photosynthesis-related protein, enzymatic antioxidant system and also enzymes in photorespiratory pathway were observed (Castro et al., 2005). In another study, the herbicide 'glyphosate' demonstrated the induction of oxidative stress enzymes such as glutathione-S-transferase and superoxide dismutase and also decrease in the photosynthetic enzymes such as ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in rice leaves (Ahsan et al., 2008).

Studies on Brassica families are rapidly expanding with regards to proteomics and metabolomics along with other well-established plant species such as *Arabidopsis thaliana* or rice (Kehr and Buhtz, 2011). Most Brassicas serve as food and feed sources and have moreover become valuable due to their great importance as bio-energy crops (Franzaring et al., 2008). Studies on the effects of environmental stresses on Brassica by using 2-DE have been documented such as root responses to infection (Hansen et al., 1994; Ito et al., 1996) and root responses to cadmium treatment (Alvarez et al., 2009).

In this study, the effect of alachlor (2-chloro-2',6'-diethyl-N-methoxymethylacetanilide) (Figure 1) on *Brassica alboglabra* roots was investigated. Alachlor, which is a type of chloroacetanilide herbicide, has been widely used worldwide to control annual grasses and broadleaves weeds (EPA, 2012). The herbicide is known to be quickly eliminated by plants upon uptake; however, the effects on the Brassica proteomes have not yet been determined.

Proteomic studies in plants in relation to pesticides are of interest due to their great importance in the agricultural sector. Further investigations in this field would provide more evidence and understanding of the effects of herbicides on plant proteomes which would contribute to advancement in plant biotechnology.

MATERIALS AND METHODS

The seeds of *B. alboglabra* were obtained from a local seed company, with the information of 80% germination and 98% purity. On day 1, about 15 to 20 seeds of *B. alboglabra* were grown in a pot of 8 cm in diameter filled with decomposed soil in the open environment under the direct sunlight at Chulaborn Research Institute compound. The plants were concurrently supplied with organic and chemical fertilizers. The plants were harvested on the seventh week in the morning prior to extraction to minimize the possibility of affecting the protein level. The plants parts used were cut leaves, stems and roots and weighed before subjection to protein extraction. The roots were washed thoroughly to avoid any contamination from soil which might affect the protein separation.

Herbicide application

The commercially available alachlor were applied by pouring the solution on the soil once/week starting from day 1. The plants were divided into control, half dose, recommended dose and maximum dose of alachlor. The used concentration for half dose, recommended dose and maximum dose poured in the pot for each condition were 200, 400 and 500 µL/m^2 of alachlor. The plants were harvested at the seventh week of growth for analysis of proteins and detection of herbicide residues.

Alachlor analysis

The 300 µL of methanol was added to 0.3 g of samples. The mixture was mixed with a glass rod followed by sonication for 10 min in water bath followed by vortex mixed for 15 min. The mixture was then centrifuged at 2000 rpm for 5 min at 4°C. The supernatant was subjected to HPLC. Pesticides analyses were conducted by using HPLC. Extract was analyzed using an 1100 Series HPLC (Hewlett Packard) equipped with DAD detector (signal at 280 and 230 nm). A Phenomenax Selectosil C18 100 A column 250 x 4.6 mm were used. The mobile phase was 80% acetonitrile/20% distilled water. Injection of 2 µL at flow rate of 0.7 ml/min at pressure of 54 bars was

used. Alachlor residues were detected at 220 nm.

Protein extraction by using TCA/acetone precipitation

0.1 g of samples was ground in liquid nitrogen and resuspended in 1 mL solution of 10% TCA and 0.07% DTT in cold acetone. The samples were incubated at 20°C for 1 h followed by centrifugation at 14 000 rpm for 10 min. The supernatant was discarded, and the pellet was resuspended in 1 mL of 0.07% DTT in cold acetone incubated and centrifuged as previously described, twice. The pellet was vacuum-dried for 30 min and resuspended in 500 µL of rehydration buffer consisting of 8 M urea, 2% CHAPS, 40 mM DTT, 0.2% ampholytes 3-10, and 2 mM tributylphosphine (TBP). The samples were then vigorously vortex for 5 min and incubated at 4°C overnight. The next day, the samples were centrifuged as previously described. The supernatant was collected for protein determination.

Two-dimensional electrophoresis

The two-dimensional electrophoresis was performed using Immobiline^{TM} DryStrip pH 3-10 Non-Linear, 7 cm (GE Healthcare). The 125 µL containing 150 µg of protein and rehydration buffer was loaded and kept at room temperature overnight. The first IEF was conducted at 200 Vh, and 55 µA/strip. Prior to the second dimension SDS-PAGE gels, the strips were soaked with equilibration buffer 1 (0.5 M Tris-HCl, pH 6.8, 6 M urea, 30% glycerol, 1% SDS and 2.5% DTT) with gentle agitation at room temperature for 10 min followed by soaking in equilibration buffer 2 (0.5 M Tris-HCl, pH 6.8, 6 M urea, 30% glycerol, 1% SDS and 2.5% Iodoacetamide). The IPG strips were embedded within molten agarose directly on top of a 1 mm 12.5% SDS-PAGE gel. The SDS-PAGE was performed at 200 V and 44 mA for 3 h.

Gel staining and image analysis

The gels were stained with the staining solution Commassie Blue, destaining with 40% methanol and 5% acetic acid for 2 h and 10% of methanol and 5% of acetic acid for 1 h. The gels were scanned by an Amersham Biosciences image scanner. Image was analysed with the Image Master 2D Platinum 6.0 by the GE Healthcare.

Liquid chromatography-mass spectrometry (LC/MS/MS)

2 to 3 spots were excised, pooled and underwent the steps of destaining with 0.1 M NH_4HCO_3/50% acetonitrile, reduction of disulphide bonds with 0.1 M NH_4HCO_3/10 mM DTT/1 mM EDTA, alkylation with 100 mM iodoacetamide/0.1 M NH_4HCO_3 and digestion with 0.01 µg/µL trypsin. The LC/MS/MS analyses were carried out using a capillary LC system (Waters, US) coupled to a Q-TOF mass spectrometer (Micromass, Manchester, UK) equipped with a Z-spray ion-source working in the nanoelectrospray mode. Glu-fibrinopeptide was used to calibrate the instrument in MS/MS mode. The tryptic peptides were concentrated and desalted on a 75 µm ID x 150 mm C18 PepMap column (LC Packings, Amsterdam, Netherlands). Eluents A and B were 0.1% formic acid in 97% water, 3% acetonitrile and 0.1% formic acid in 97% acetonitrile, respectively. 6 µl sample was injected into the nanoLC system, and separation was performed using the following gradient: 0 min 7% B, 35 min 50% B, 45 min 80% B, 49 min 80% B, 50 min 7% B, 60 min 7% B. The database search was performed with ProteinLynx screening SWISS-PROT and NCBI. The Mascot search toll available on the Matrix Science site screening NCInr was used for some protein which was not available in previous database.

Statistical analysis

Significant changes are referred to as the ratio of the volume (%) that is more than two-folds by using Image Master Software. The p-values were calculated by using Student t-test for comparing treated and control group. The two-tailed P value less than or equal to 0.05 was considered to be statistically significant.

RESULTS

Detection of alachlor residues by HPLC

Detection of alachlor residues has been carried out on the seventh week roots of *B. alboglabra* by using HPLC as shown in Figure 2. The roots were divided into control, half dose recommended dose and maximum dose groups. Standard alachlor was detected at 5.887 min. Alachlor residues were not detected in the control root, but present in the half-dose, recommended dose and the maximum dose roots. The concentrations of alachlor residues increased based on the areas under the curve, shown in the half dose, the recommended dose and maximum dose.

Effects of herbicide (alachlor) on *B. alboglabra* root using proteomics

The 2-DE was carried out to determine the differential protein expression between the control and the treated *B. alboglabra* with half dose, recommended dose and the maximum dose. Protein functions were obtained from UniProt database (www.uniprot.org) as shown in Table 1. The protein spots from the untreated roots were compared with the treatment groups of half dose, recommended dose and maximum dose. The protein spots were enlarged as shown in Table 2 to compare the patterns of treated and the control *B. alboglabra* roots. There were significant differences in the levels of proteins in the alachlor treated group with the untreated group (Figure 3). Seven spots were found to be significantly increased which included endoplasmic reticulum HSC70-cognate binding protein precursor, knox-like protein, beta-tubulin, alpha methylacyl-CoA racemase putative, adenosylmethionine synthethase, MLP like protein 328 and hypothetical protein MICPUN_55958 and three spots were significantly decreased which included knox-like protein, RNA polymerase II second largest subunit and MLP like protein 328.

DISCUSSION

The mechanisms of action of any pesticide are intended to kill or mitigate the target pests; however not many studies have reported the unintentional effects on non-target plants. In this study, we investigated alachlor-induced proteome patterns on the roots of *B. alboglabra* by using 2-DE techniques to gain more understanding of herbicide effects on the non-target species. Plant samples

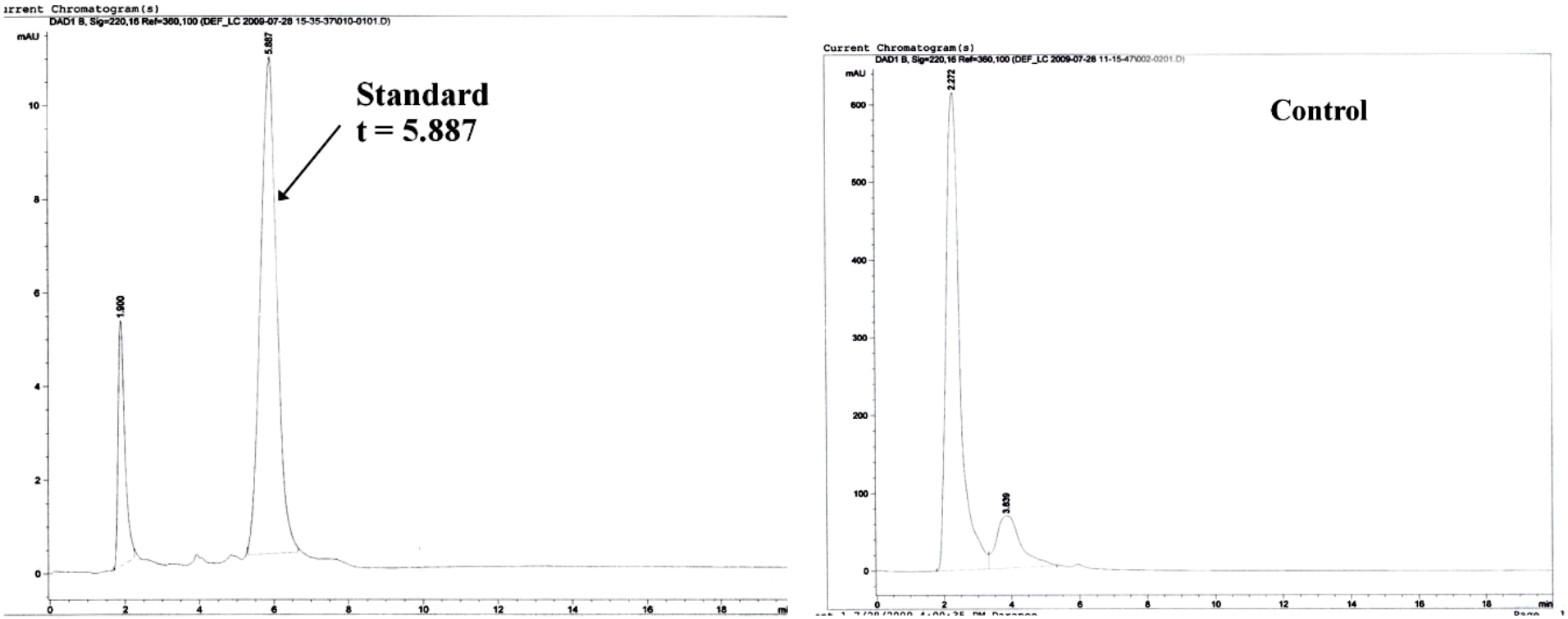
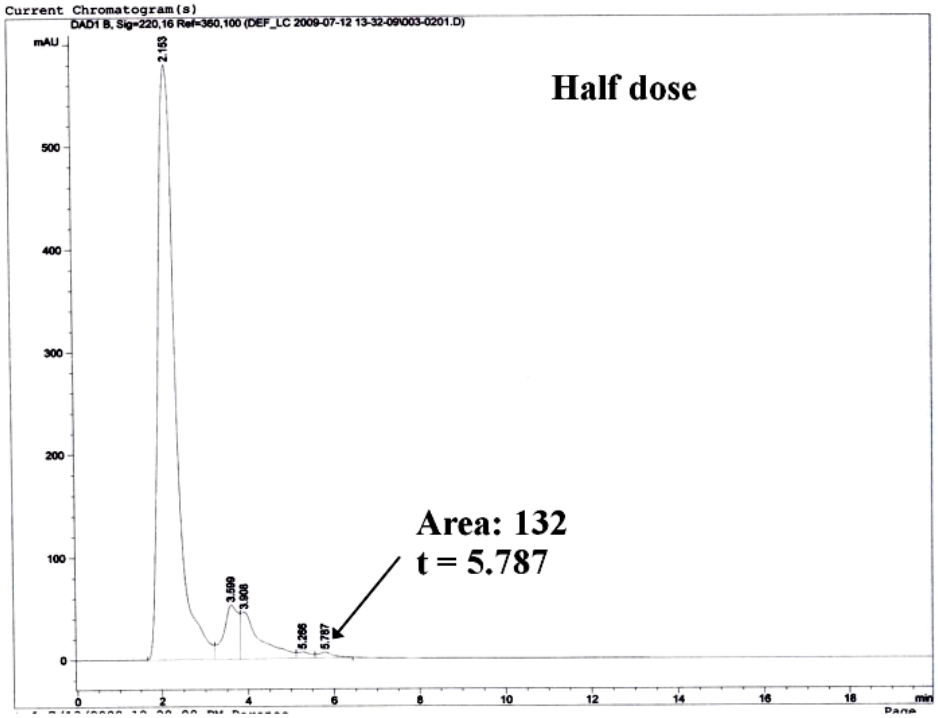


Figure 2. Alachlor residues in *B. alboglabra* detected by HPLC. Standard alachlor was detected at approximately 5.887 min. Alachlor residues were not present in untreated roots (A) but present in the *B. alboglabra* roots of half dose (B), recommended dose (C) and maximum dose (D) roots.

samples had been treated with alachlor at three various concentrations of half-dose, recommended dose and the maximum dose directly to the soil. We found that the proteins mainly involved in the defence mechanism of plants showed significant increase in differential protein expression between *B. alboglabra* roots treated with alachlor as compared to the untreated group.

By using HPLC, alachlor residues were detected in all treatment groups of half-dose, recommended dose and the maximum dose roots as depicted in Figure 2. There were also increases in concentrations of alachlor residues from the half dose, recommended dose to maximum dose of the roots. This finding is in line with those of Szovati et al. (2007), who observed that chloroacetanilide pesticides have the tendency to accumulate in the root treated with herbicides as compared to other plant segments.

Other than glyphosate, which has been known to inhibit the shikimic acid pathway, thus leading to deficiency in protein synthetic precursor, alachlor has been also reported to act on plants by interfering with protein synthesis and root elongation process (Warmund, 1985; Department of Botany and Plant Pathology Purdue University, updated 2008). From the 2-DE of *B. alboglabra* roots, 5 spots were found to be significantly increased by the volume (%) ≥2 fold which included endoplasmic reticulum HSC70-cognate binding protein precursor (Spot 1), beta-tubulin (Spot 9), alpha methylacyl-CoA racemase putative (Spot 14), adenosylmethionine synthethase (Spot 15) and hypothetical protein MICPUN_55958 (Spot 49). Two spots were significantly decreased which included RNA polymerase II second largest subunit



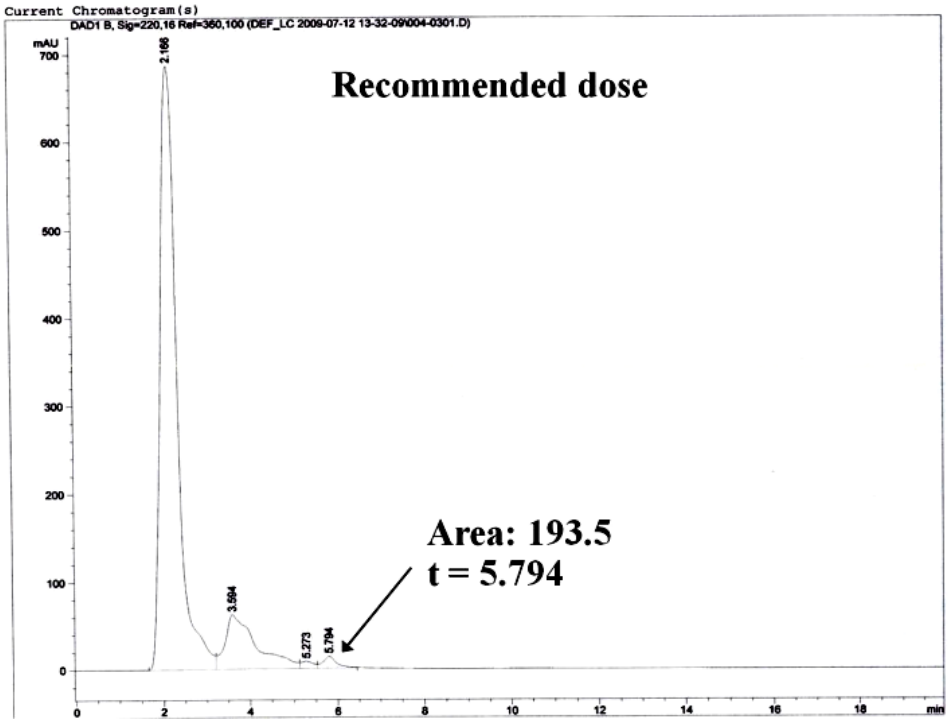


Figure 2. Contd.

Table 1. Proteins classified according to the main functions obtained from UniProt (www.uniprot.org).

Spot number	Accession number	Protein description	Theoretical MW/pI	Experimental MW/pI	Protein coverage	Function
1	gi|2642238	endoplasmic reticulum HSC70-cognate binding protein precursor (Glycine max)	73.59/5.15	77.7/5.06	7	Stress response
2	gi|255084752	knox-like protein (Micromonas sp. RCC299)	51.18/6.99	75.65/6.00	2	Transcription
3	gi|255084752	knox-like protein (Micromonas sp. RCC299)	51.18/6.99	73.7/6.35	2	Transcription
8	gi|255084752	knox-like protein (Micromonas sp. RCC299)	51.18/6.99	60.25/4.6	2	Transcription

Table 1. Contd.

9	gi|166640	beta-tubulin	49.88/4.79	53.35/4.8	13	Microtubule-based movement
14	gi|255618086	alpha methylacyl-CoA racemase	19.86/4.77	45.85/5.28	6	Metabolic process
15	gi|1709002	S-adenosylmethionine synthetase;	43.14/5.53	45.85/5.78	6	Stress response
17	gi|46277289	RNA polymerase II second largest subunit (Camellia japonica)	66.75/7.72	41.75/5.87	3	Transcription
45	ML328_ARATH	MLP like protein 328	17.50/5.32	17.5/6.1	7	Defense
49	gi|255072969	hypothetical protein MICPUN_55958 (Micromonas sp. RCC299)	23.87/4.67	19.8/6.75	3	Unidentified

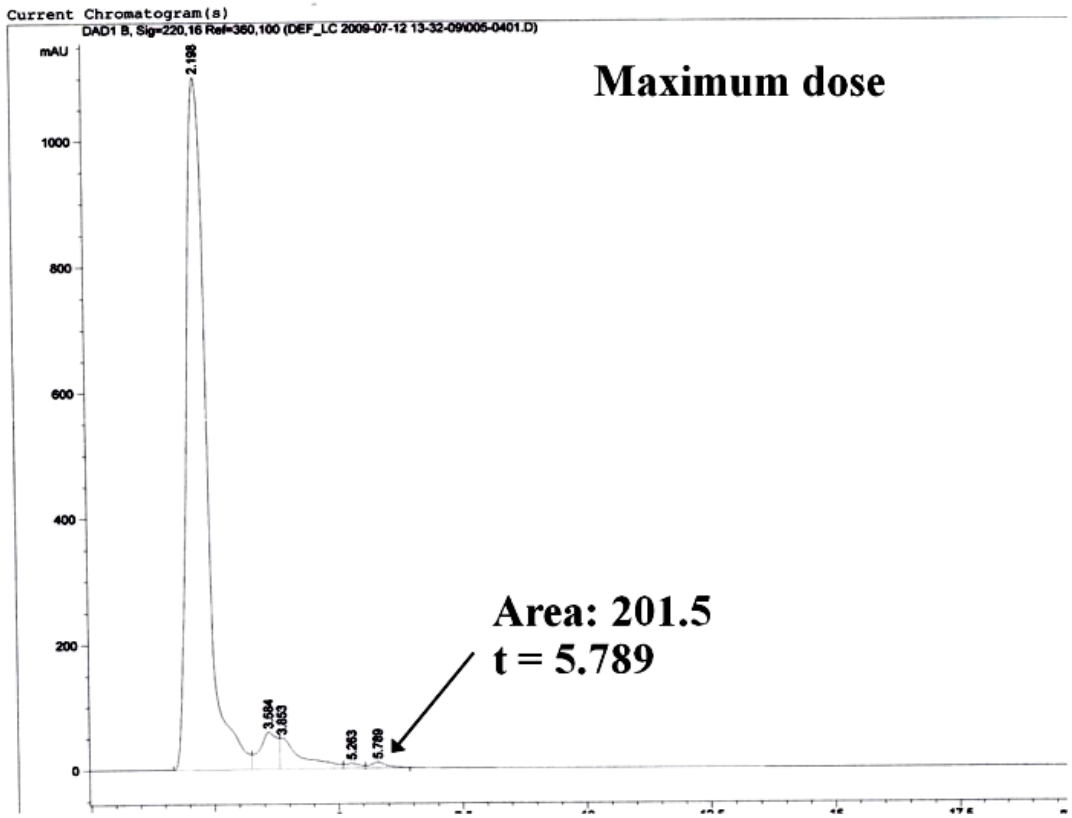
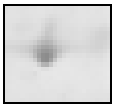
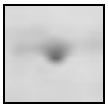
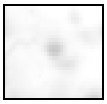
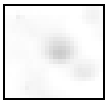
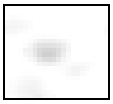
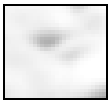
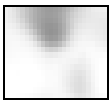
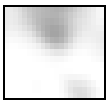
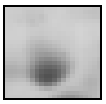
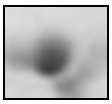
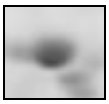
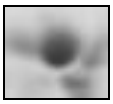
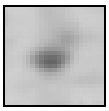
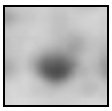
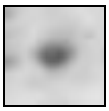
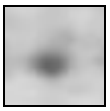
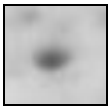
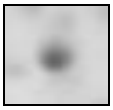
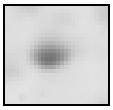
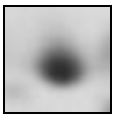
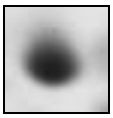
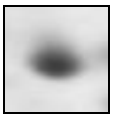
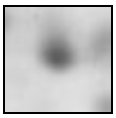
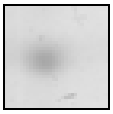
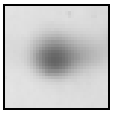
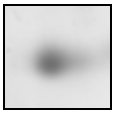
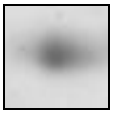


Figure 2. Contd.

Table 2. Spots of interest identified by LC/MS/MS, showing differential protein expression in roots of *B. alboglabra* treated with alachlor as compared to the control group.

Spot	Protein description	Control	Half	Recommended	Maximum	p-value
1	Endoplasmic reticulum HSC70-cognate binding protein precursor	 1	 +2.0*	 +2.8*	 +1.6	0.0183*
2	knox-like protein (*Micromonas* sp. RCC299)	 1	 +1.2	 -3.2*	 -2.1*	0.3097
3	knox-like protein (*Micromonas* sp. RCC299)	 1	 +1.6	 +1.9	 +2.6*	0.0068*
8	knox-like protein (*Micromonas* sp. RCC299)	 1	 +1.0	 +4.5*	 +3.6*	0.2722
9	beta-tubulin	 1	 +1.6	 +1.2	 +2.8 *	0.0748
14	Alpha methylacyl-CoA racemase, putative	 1	 +1.9	 +1.9	 +2.5*	0.0572
15	Adenosylmethionine synthetase	 1	 +3.9*	 +2.7*	 +2.4*	0.0142*
17	RNA polymerase II second largest subunit (*Camellia japonica*)	 1	 -1.2	 -1.3	 -2.8*	0.4437
45	MLP like protein 328	 1	 +1.7	 -1.0	 -2.1*	0.650
49	Hypothetical protein MICPUN_55958 (*Micromonas* sp. RCC299)	 1	 +2.5*	 +2.5*	 +2.8*	0.0009*

Significant changes marked as * are referred to as: the ratio of the % Volume ≥2 fold. (-) and (+) refers to decrease and increase of fold change, respectively. The two-tailed P value is significant at ≤0.05 with 95% CI.

(Spot 17) and MLP like protein 328 (Spot 45) (Figure 3).

Two proteins involved in stress responses: endoplasmmic reticulum HSC70-cognate binding protein precursor (Spot 1) and adenosylmethionine synthethase (Spot 15) were significantly increased by two-fold. HSC-cognate protein precursor functions as a transport protein and molecular chaperone with plays a role in protein folding, assembly and transport. Moreover, it has been found to

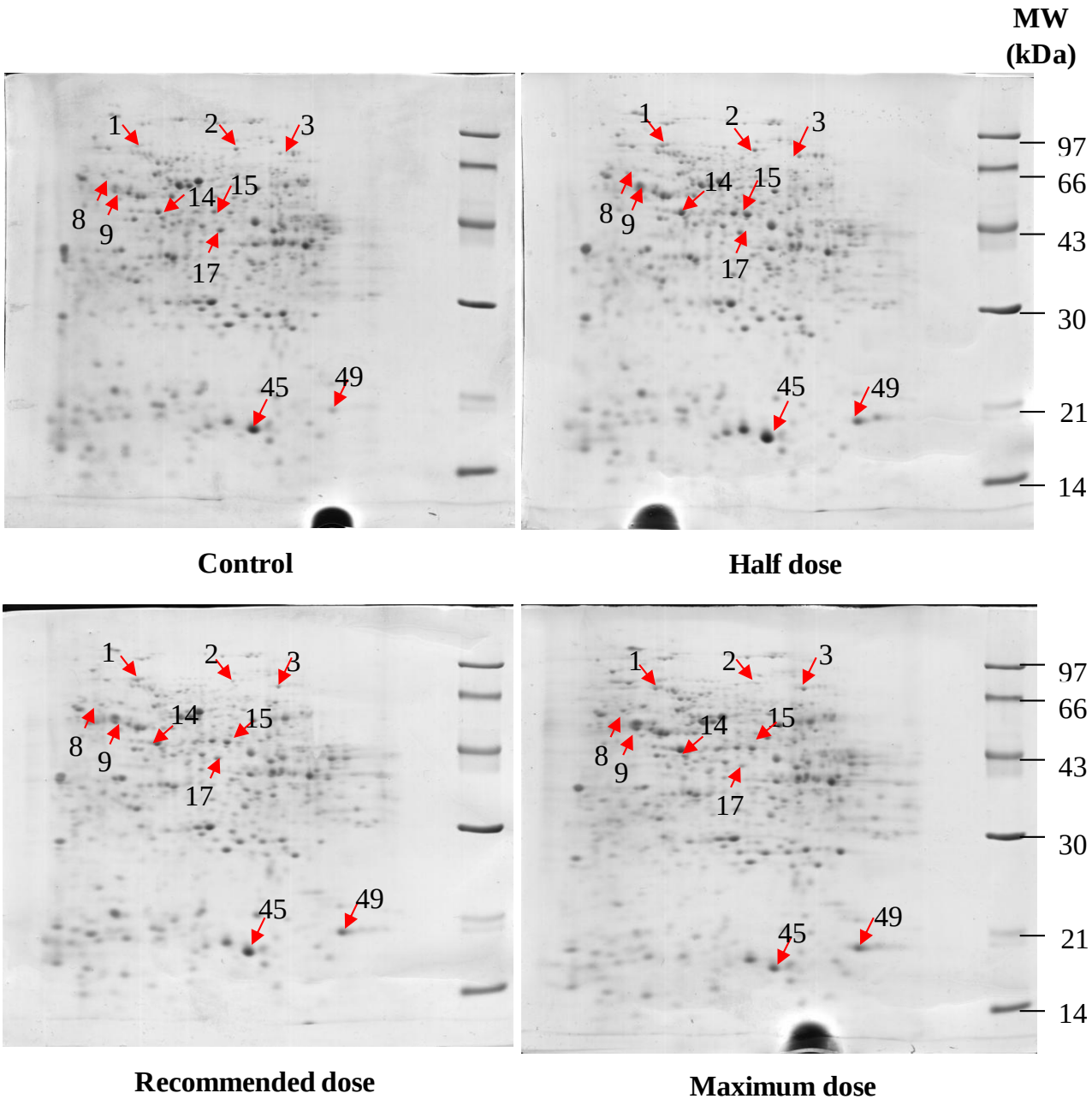


Figure 3. Two-dimensional PAGE performed on the roots of untreated (A), half dose (B), recommended dose (C) and maximum dose (D) with commercial alachlor herbicides. The arrows and numbers represent significant differential protein expression in the treated groups (half dose, recommended dose and maximum dose) as compared to the control.

play a pivotal role in protecting plants from environmental and genetic stresses (Castro et al., 2005; Aoki et al., 2002). S-adenosylmethionine synthethase (Spot 15) was known to be increased upon stresses in response to salt stress, cadmium and iron. A study by Esparto et al. (1994) showed that NaCl stress causes the accumulation of S-adenosylmethionine synthethase in the roots of tomato seedlings. Therefore, the identification of those proteins involved in the stress response induced by alachlor suggested similar effects as shown by other abiotic stressors and on other plant species such as *Oryza* sp. (Jin-Ju et al., 2011).

Protein involved in the organization of microtubules was found to be increased which indicates the response to developmental and environmental stressors (Oakley et al., 2007). Microtubules are important in maintaining the cell structure and play a role in transportation especially during development or cell repair. In this study, we found that beta-tubulin (Spot 9) was increased in the treatment group. Our finding is supported by another study which

was carried out in other plant species, *Arabidopsis* sp, whereby beta-tubulin was induced upon stress stressors (Saito et al., 2003).

In contrast, another protein involved in defence mechanism, MLP-like 328 (major latex protein) (Spot 45), was found to be decreased. MLP-like proteins were found to have significant increases in expression due to oxidative stress in Arabidopsis; however, not much is known about this protein in other plant species (Stanley et al., 2005).

Other than proteins involved in stress response, we identified other proteins involved in transcription processes; RNA polymerase II and knox-like protein. We found that, RNA polymerase was decreased in the treatment plants. Alachlor has been known to inhibit protein and RNA biosynthesis (Rao, 2000). Meanwhile, knox-like proteins increased significantly in the treatment group. These proteins act as regulators in transcription in developmental processes in plants such as shoot apical meristem development (Hay and Tsiantis, 2010) and root development (Truernit and Haseloff, 2007). However, the knowledge on these proteins is still limited with regards to environmental stressors and the interaction with pesticide should be extensively discussed.

Even though alachlor is intended to affect the target plants (monocotyledons), some proteins in *B. alboglabra* have also been altered. Similar to other environmental stressors studied thus far, alachlor induced the expression of proteins involved in the stress response in experimental plants. However, different stress proteins were altered depending on the parts of the plant studied which also indicate the specificity of the proteins. The stress response is suggested to be a way of adaptation by the plants for their sustainable growth. Different classes of herbicides may also induce differential protein expression due to their different modes of action. Further investigations in this field would provide better understanding and advancement on the effects of different types of environmental stressors on plant proteomics.

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