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Research Article

Differential Proteomics Study on Se-enriched *Pleurotus ostreatus*

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Abstract: It is well known that Selenium plays important roles in human health. In this study, Selenium-enriched *Pleurotus ostreatus* (*P.ostreatus*) was cultivated by adding selenoarginine to substrate. Selenium content in mushrooms was measured by Atomic Fluorescence Spectrophotometer (AFS). We demonstrated that that selenium concentration in samples increased significantly and it had a significant positive relationship with selenium concentration in the cultivation material. Finally, we identified the total proteins extracted from ordinary and Se-enriched *P.ostreatus* by SDS-PAGE and 2-DE. We found there had no significant changes in protein content. Two-dimensional electrophoresis combining with mass spectrometry technology was applied to identify proteins that are differentially expressed in mushroom grown in the substrates with 44.26 mg/kg of Se. From 2D maps, 51 differentially expressed proteins were detected, 17 of those were up-expressed and 34 of those were down-expressed. 8 up-expressed proteins were analyzed by MALDI-TOF-MS/MS: 3 proteins were identified, one was hypothetical protein (gi|270056453) and the other two proteins are respectively NAD-dependent formate dehydrogenase (gi|164564766) and Aldo keto reductase (gi|628847367). These two proteins are mainly involved in substance and energy metabolism in the cell.

Keywords: Selenium-enriched *pleurotus ostreatus*, selenoarginine, two-dimensional electrophoresis

INTRODUCTION

P.ostreatus is one of the most appreciated mushrooms due to its delicious taste and high quantities of proteins, carbohydrates, minerals (calcium, phosphorus, iron) and vitamins (thiamin, riboflavin and niacin) (Baysal *et al*., 2003; Shah *et al*., 2004). *P.ostreatus* with nutritional and medicinal properties has significant value as an edible fungus which is commercially used world-wide. Since research has tended to focus on the dietary value of *P.ostreatus*, there is relatively little information pertaining to their antioxidant properties and their possible use to treat oxidative stress. The genetic analysis of *P.ostreatus* has gained considerable interest (Moussa, 2009), however, the need for an easy and efficient transformation system became obviously to allow functional genomics in *P.ostreatus*. Selenium (Se) is essential to human diets and it is in low concentration in the soil and consequently in food. Selenium has several physiological functions for enhancing immune system function, anti-aging and reducing cancer risk while transforming to organic selenium, especially combining with protein or polysaccharide (Finley, 2006). It has been a research hotspot in the fields of foodstuff science and medicine pharmacy to develop the edible and medicinal Se-enriched products in recent years. *P.ostreatus* is a good carrier for enriching selenium, however, it is a question that whether the protein combined with selenium from selenium accumulation selenium accumulation substrate has changed compared to control (Schmidt *et al*., 2003). In this research Se-enriched *P.ostreatus* were systematically studied for its different protein spots. The aim of this study was to evaluate the protein expression of Se-enriched *P.ostreatus*.

MATERIALS AND METHODS

Materials: The *P.ostreatus* was provided by Shanxi Zituan of ecological agriculture LTD, CHINA, SHANXI (Shanxi, China). The vegetative mycelium was maintained at 25°C on potato dextrose agar covered with autoclaved cellophane membrane in a Petri dish, for producing fruiting bodies and germinated spores. After the cotton seed shells mixed with bran, lime and other accessories stacking fermentation and sterilizating at 100°C for 12 h, when temperature was reduced to 30°C, mushroom species were accessed in aseptic conditions, around 30 days later, the mushroom species developed. This selenium acid arginine was synthesis by our laboratory. Selenium content is 26% (Liu *et al*., 2007).

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Se determination in se-enriched *P.ostreatus*: For fortied Se, various selenoarginine concentrations (11.07; 22.13; 44.26 mg/kg) was added to ordinary mushroom cultivation (da Silva *et al*., 2012). The inoculated packs were incubated at 25°C for 15 days. Fungi were placed at 20°C; stored at -20°C for 24 h and freeze-dryed for 48 h. After crushing 200 mesh sieve, sample were saved into the sample bag.

The test Samples were digested with 10 mL of mixture of HNO_3 and $HClO_4$ (v/v = 9:1) at 150°C for 2 h, after cooling, adding to 5 mL 6 mol/L HCl, then the samples was diluted with ultrapure water to 50 mL, then 10 mL of the solution was transferred to a reaction vessel, with 2 mL of 6 mol/L HCl and 1 mL of KBH_4 added. The control with the same procedure was used to provide a blank value. A titrisol standard solution of 4-20 μg/L of Se (Merck) was used to prepare the reference analytical solutions. The determination of Se in the samples was performed by an atomic fluorescence spectrophotometry 830 (Beijing cygnet instrument company, Beijing, China).

Protein extraction and determination of the selenium: 5.0 g sample adding 100 mL 0.1 mol/L NaOH solution 100 mL, 60°C water bath, stirring and extraction for 4 h, then 4000 r/min centrifugal 10 min, the precipitation added 50 mL 0.1 mol/L NaOH solution, repeat extracted once, combined the supernatant. To adjust the pH to 3.5 with acetic acid, 4°C overnight, 4000 r/min centrifuged 20 min, supernatant was precipitated 50°C vacuum drying, the determination of the protein content of selenium (Zhao *et al*., 2004).

Two-dimensional gel electrophoresis (2-DE): For 2-DE (Tran *et al*., 2015), briefly, a total of 300 μg proteins extracted from *P.ostreatus* by TCA method was loaded onto the IPG strips (Immobiline DryStrip pH 3-10, 7 cm, GE Healthcare UK Ltd., Buckinghamshire, UK). First, the IPG strips were rehydrated overnight with rehydration buffer (7 mol/L urea, 2 mol/L Thiourea, 4% CHAPS, 40 mmol/L DTT, 2% IPG buffer 3-10 and trace amount of bromophenol blue) and focused using an Ettan IPGphor III (GE Healthcare UK Ltd., Buckinghamshire, UK) as follows step: step and hold at 300 V for 4 h, gradient to 1000 V for 30 min, gradient to 5000 V for 90 min, step and hold at 4000 and hold at 300 V for 1 h, gradient to 1000 V for 30 min, gradient to 5000 V for 30 min, step and hold at 2500 V-h for 30 min. Prior to PAGE, the IPG strips strips were incubated in equilibration buffer (6 mol/L urea, 75 mmol/L Tris-HCl, pH 8.8, 29.3% glycerol, 2% SDS, 0.002% bromophenol blue) with 10 mg/mL DTT for 15 min, followed in equilibration buffer with 25 mg/mL iodoacetamide for 15 min. The second dimension separation was run on 12% SDS-PAGE gels at 10 mA per gel for 15 min, 20 mA per gel until the bromophenol blue reached the bottom of the gels. After electrophoresis, gels were fixed and stained with Coomassie Brilliant Blue (CBB) R-250 dye. Finally, the gel was destained with destaining solution and imaged using GelDoc XR imaging (Bio-Rad, USA). Gel images were analyzed using PDQuest 2D Analysis Software (Bio-Rad, USA).

Sample preparation for MS analysis: The gel piece was destained with 100 μL of destaining solution (100 mmol/L NH_4HCO_3, 30% ACN) for 10 min and washed with 500 μL of distilled water for 15 min. The above destaining-steps were repeated twice. Then the gel piece was dehydrated with 100% ACN for 10 min. The gel pieces was covered with 8 μL trypsin solution (25 mmol/L NH_4HCO_3, containing 12.5 μg/mL) overnight at 37°C.

MALDI-TOF/TOF mass spectrometry and database searching: Prior to MALDI-TOF/TOF analysis, a volume of 1 μL of each sample adding 0.8 μL matrix was applied onto a MALDI plate and was allowed to air dry at room temperature. The sample was analyzed by ultrafleXtreme MALDI-TOF/TOF (Bruker Daltonics, Inc., Billerica, MA, USA). To identify the proteins, massspectra (excluding trypsin autolytic peptides and other known background ions) were searched against the Mascot search engine (Matrix Science Limited), run on an in-house server using in NCBInr database and SWISS-PORT database retrieval. The search was performed using trypsin digestion allowing 0 or 1 missed cleavage, Carbamidomethyl (C) as a fixed modification, variable modifications of oxidation and carbamidomethyl, maximal mass tolerance of 0.1 Da, precursor tolerance of 100 ppm and Other Fungi.

Statistical analysis: The data were analyzed using one-way analysis of variance. Triplicate samples were used to generate each data point. Statistical significance was set at $p \leq 0.05$.

RESULTS AND DISCUSSION

Se in *P. ostreatus*: *P.ostreatus* were able to absorb and accumulate Se when Selenoarginine was added in mushrooms cultivation. The influence of Selenoarginine on *P.ostreatus* was shown in Fig. 1. Selenoarginine on *P.ostreatus* has great influence on the formation of physical properties. Firstly, the change of the mushroom type is very large. Secondly, Selenoarginine can contribute to the development of stipe, at the same time inhibit the grows of pileus.

The selenium concentration on each part of *P.ostreatus* are shown in Fig. 2. The selenium content of ordinary *P.ostreatus* entity was only 0.34 μg/g, while Se-enriched *P.ostreatus* entity of A1, A2 and A3 group were 11.07 μg/g and 22.13 μg/g, 44.26 μg/g, respectively. When selenoarginine concentrations in the mushrooms cultivation is lower than 44.26 mg/kg, selenium content in *P.ostreatus* is increases with the

Fig. 1: Effect of selenoarginine on phenotype of fruiting body of *P.ostreatus*. Selenoarginine concentrations was added to ordinary mushroom cultivation. A1: 11.07 mg/kg; A2: 22.13 mg/kg; A3: 44.26 mg/kg

Table 1: A list of protein spots identified by mass spectrometry analysis

Spot No.	Protein name	Accession no.	pI
5	Hypothetical protein	gi|270056453	7.96
6	NAD-dependent formate dehydrogenase	gi|164564766	6.28
8	*Aldo keto reductase*	gi|628847367	5.81
Spot No.	Taxonomy	Coverage	Score
5	*P. ostreatus*	31%	319
6	Ceriporiopsis subver	10%	104
8	Aldo keto reductase	4%	102

linear as the selenoarginine increases in the cultivation (R = 0.9967), selenium can improve about 220 times compared to the control.

Selenoarginine affect the distribution of *P.ostreatus* fruiting bodies protein: Proteins of ordinary *P.ostreatus* fruiting bodies and Se-enriched *P.ostreatus* fruiting bodies were analyzed by SDS-PAGE electrophoresis. As shown in Fig. 3, *P.ostreatus* fruiting bodies of proteins widely distributed in the molecular weight of 10 KD-120 KD. This suggests that there is no new protein band appear and no protein band disappear just content of protein slightly different. This shows that the selenium will not affect the distribution of fruiting body protein and change the protein synthesis metabolic pathways.

Differentially expressed proteins in *P.ostreatus* and Se-enriched *P.ostreatus* fruiting body: Total protein of Se-enriched *P.ostreatus* fruiting bodies were extracted by TCA precipitation method, using gel imager to capture images. The result was shown in Fig. 4. Ordinary and Se-enriched *P.ostreatus* fruiting body of 2D-PAGE spectra is similar, but there are large differences in the expression of proteins. Analyzed by PDQuest 8.0.1 software, two-dimensional gel electrophoresis proteomic analysis picked out 51 protein spots differentially expressed respectively, including a significant increase of 17 protein spots and 34 proteins significantly reducing. No. 1 to 9 was No. 1 significantly increase expression of the protein spots, No. 10 to 18 is significantly reduce. No. 1 to 8 proteins was analysised and identificated using matrix-assisted laser desorption ionization time of flight mass spectrometry (MADI-TOF-MS/MS). The result showed that eight proteins have mass spectrum signal, to search peptide mass fingerprinting of these proteins in the MASCOT database, three proteins were initially identified, the mass spectrum is shown in Fig. 5, the identification results is shown in Table 1. From Table 1, successfully identified proteins, only one is from mushroom species but it is a hypothetical protein of

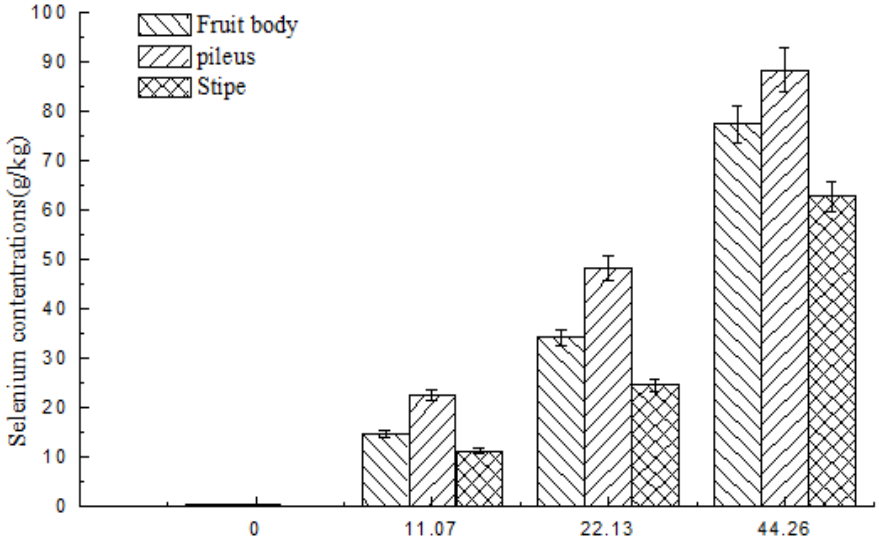


Fig. 2: Effect of the combining ability of *P.ostreatus* with Se

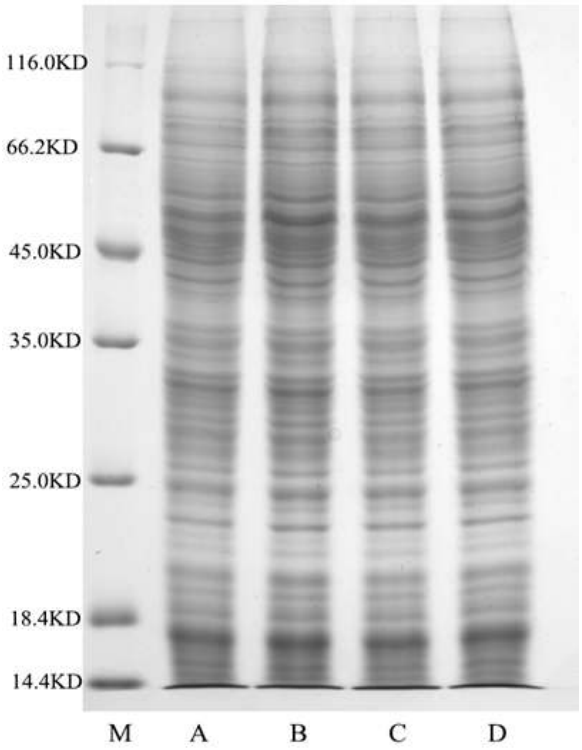


Fig. 3: SDS-PAGE analysis of the proteins in fruiting bodies of selenium-enriched *P.ostreatus*. M: Standard protein maker, A: ordinary *P.ostreatus* protein; B: 11.07 mg/kg; C: 22.13 mg/kg; D: 44.26 mg/kg

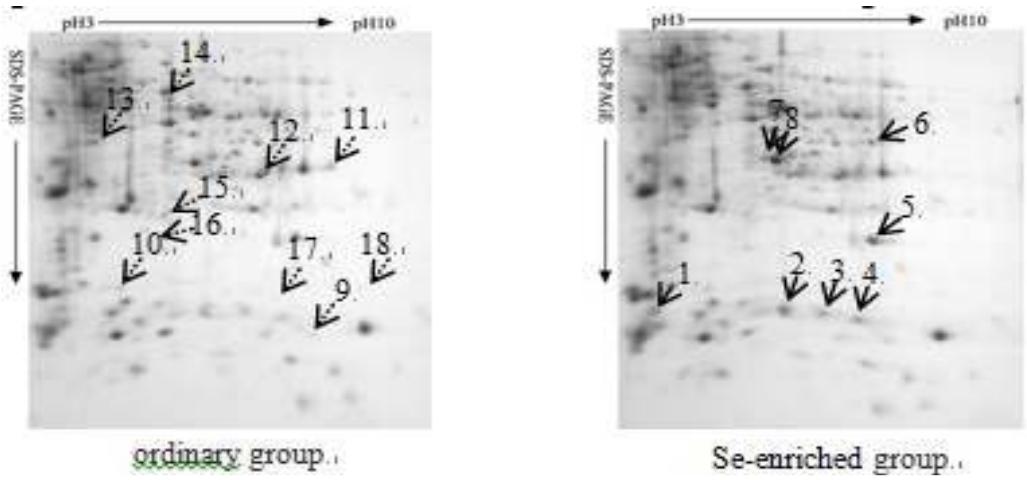


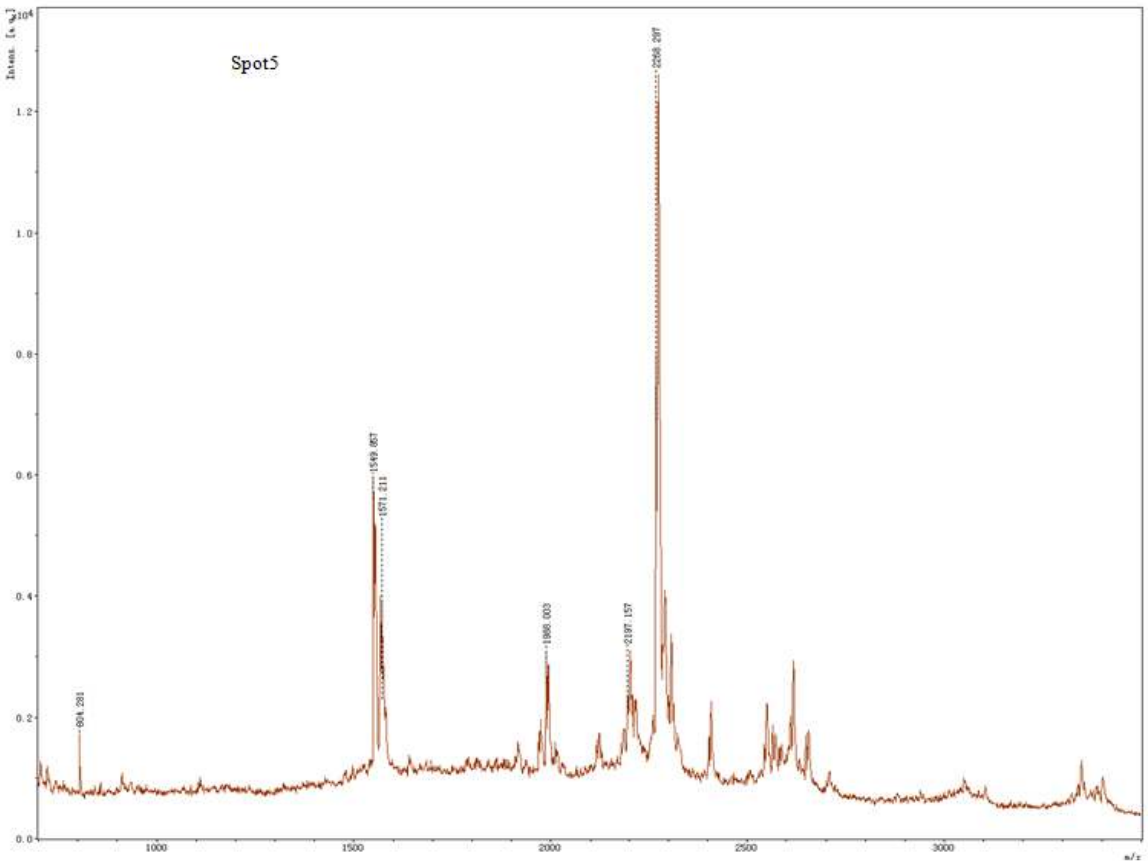
Fig. 4: 2-DE maps of *P.ostreatus*

unknown function (gi|270056453), the other two proteins are intended formate dehydrogenase from insects (gi|164564766) and pink wax species of the genus Alternaria leather AKR strains of the genus (gi|628847367).

CONCLUSION

The cultivation of *P.ostreatus* enriched with selenoarginine was feasible, not only promoting Se absorption but also elevating biological efficiency. These results indicate the great potential of selenoarginine in the production of Se-enriched *P.ostreatus* biotransformation Se. Se present in the *P.ostreatus* has been shown to be bioavailable because it can cross the intestinal barrier and be inserted in peptides.

NAD-dependent formate and Aldo Keto Reductase (AKR) are mainly involved in substance and energy metabolism in the cell. NAD (P)+-dependent dehydrogenases comprise a substantial and diverse group of proteins differing in structure and function.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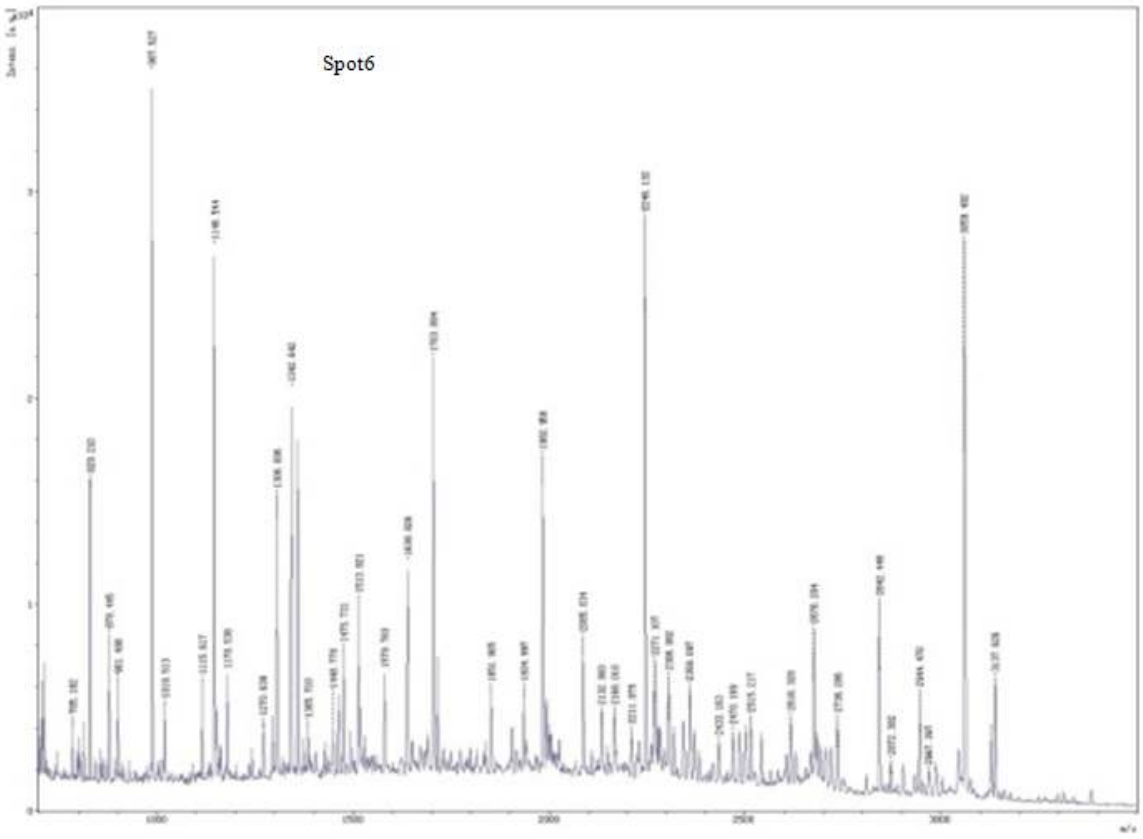
Spot6

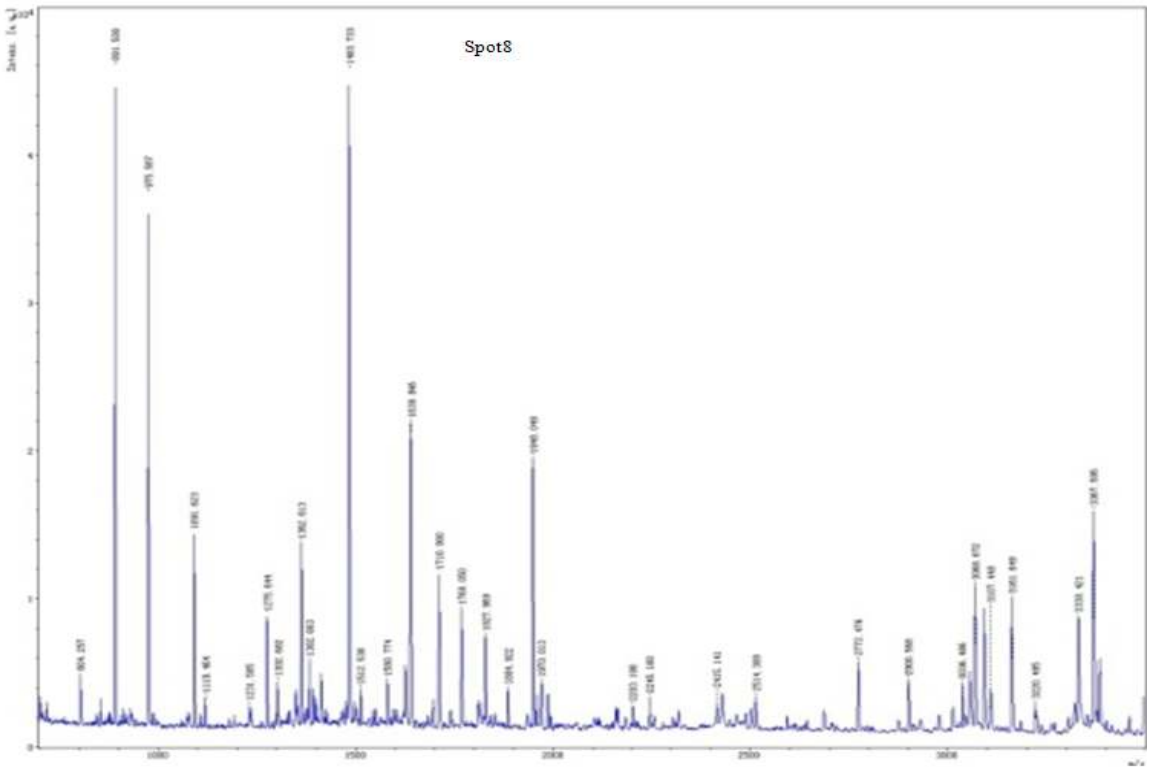

Fig. 5: Identification of differential expression proteins by MADI-TOF-MS/MS

They catalyse a number of key metabolic steps and belong to one of the most extensively studied protein families, it play a important role when plant responses to hypoxia or anoxia. AKR protein superfamily contains >190 members that fall into 16 families and are found in all phyla. AKRs exist in nearly all phyla, they are mainly monomeric soluble proteins, NAD(P)(H) dependent oxidoreductases.

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