Proteome analysis of organically grown yardlong bean [*Vigna unguiculata* sub sp. *sesquipedalis* (L.) Verdcourt]

By

DEEPTHI K. KUMAR

(2013-11-104)

THESIS

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VELLANIKKARA, THRISSUR – 680656

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DECLARATION

I, hereby declare that the thesis entitled **"Proteome analysis of organically grown yardlong bean [*Vigna unguiculata* sub sp. *sesquipedalis* (L.) Verdcourt]"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me any degree, diploma, fellowship or other similar title of any other University or Society.

Vellanikkara
Date: 25-01-16

Deepthi K. Kumar
(2013-11-104)

CERTIFICATE

Certified that the thesis entitled **"Proteome analysis of organically grown yardlong bean [*Vigna unguiculata* sub sp. *sesquipedalis* (L.) Verdcourt]"** is a bonafide record of research work done independently by **Ms. Deepthi K. Kumar (2013-11-104)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship or fellowship to her.

Vellanikkara
Date: 25.01.2016

Dr. Deepu Mathew
Major advisor, Advisory Committee
Assistant Professor
Centre for Plant Biotechnology and Molecular Biology
College of Horticulture

CERTIFICATE

We, the undersigned members of the advisory committee of **Ms. Deepthi K. Kumar (2013-11-104)**, a candidate for the degree of Master of Science in Agriculture with major field in Plant Biotechnology, agree that the thesis entitled **"Proteome analysis of organically grown yardlong bean [*Vigna unguiculata* sub sp. *sesquipedalis* (L.) Verdcourt]"** may be submitted by Ms. Deepthi K. Kumar, in partial fulfillment of the requirement for the degree.

25/1/16

Dr. Deepu Mathew
(Major Advisor)
Assistant Professor
Centre for Plant Biotechnology
and Molecular Biology
College of Horticulture

25/01/2016

Dr. P. A. Valsala
(Member, advisory committee)
Professor and Head
Centre for Plant Biotechnology
and Molecular Biology
College of Horticulture

25/01/2016

Dr. Abida P.S.
(Member, advisory committee)
Associate Professor
Regional Agricultural Research Station
Pattambi

Dr. George Thomas
(Member, advisory committee)
Professor Dept. of Agronomy
College of Horticulture

External Examiner

Dr. Arivalagan M
Scientist
Central Plantation Crop Research Institute
Kasargod

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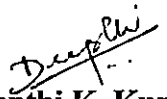

Deepthi K. Kumar

Dedicated to my Parents

and

Teachers

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ABBREVIATIONS

^0C	: Degree Celsius
cm	: Centimeter
DAFO	: Days After Flower Opening
EC	: Electrical Conductivity
g	: Gram
IPG	: Immobilized pH gradient
kDa	: kilo Dalton
M	: Molar
MAS	: Months After Sowing
MALDI-TOF	: Matrix Assisted Laser Desorption Ionisation- Time of Flight
mg	: Milligram
min	: Minute
ml	: Millilitre
mM	: Millimolar
MS	: Mass spectrometry
µg	: Microgram
µl	: Microlitre

OD : Optical Density

pH : Hydrogen ion concentrartion

% : Per cent

SDS : Sodium Dodecyl sulphate

Sec : Second

2DE : Two Dimensional Electrophoresis

UV : Ultra violet

V : Volts

Vhr : Volt-hour

Introduction

1. INTRODUCTION

Mizoram made history by becoming the first state in India to legislate for turning its entire agricultural produce organic. It passed the Mizoram Organic Farming Act, 2004 on July 12, 2004. Even though Sikkim resolved to rid agriculture of chemical fertilizers and pesticides in 2003, the Organic Mission of Sikkim aims to convert Sikkim into a totally organic state by 2016. The state of Kerala follows their footsteps. The Government of Kerala has initiated efforts to promote organic farming in the state and to convert it into fully organic state by 2016. Kasaragod is already declared as an organic district. This trend of conversion to organic farming is mainly due to the concern over environmental pollution and health risks. The continuous use of chemical inputs such as fertilizers and pesticides is diminishing and polluting the natural resources with a significant impact on environmental and agricultural sustainability. The urgent need for agricultural sustainability has been highlighted in by many eminent (Dale *et al.*, 2013). Organic systems those minimize the environmental effects on cropping environment and maintain soil health and fertility (Mehdizadeh *et al.*, 2013), can represent a possible solution to produce food with safer inputs.

Another reason for this trend is the increasing consumer demands for organic products. The term organic is gaining more and more popularity these days. In 2013, the global market for organic products was estimated to be worth 72 billion USD (FiBL, 2015). The United States is the leading market with 24.3 billion euros, followed by Germany (7.6 euros) and France (4.4 billion euros), China stood fourth with 2.4 billion euros. Organic farming has grown rapidly on a global scale. Two million organic producers were reported in 2013 by FiBL, with India having highest number of producers (650,000) followed by Uganda (189,610) and Mexico (169,703). This rapid growth may be traced to increased consumer confidence in organic foods as well as to concern about possible health risks and environmental impacts of conventional food production methods. Surveys indicate that consumers purchase organic foods because of a perception

that organic foods are healthier, one of the surveys reported that the main reasons consumers purchased organic foods were for the avoidance of pesticides (70 %), for freshness (68 %), and for health and nutritional benefits (67 %). Consumers believe that organic foods are safer, and therefore healthier, and have greater nutritional and sensory qualities than conventional foods (Chen, 2007; Magkos *et al*, 2006; Zekeli *et al.*, 2014). Even though the world carries so much of perceptions regarding the quality of organic foods, the scientific backing is missing for most of these assumptions.

Proteins are vital parts of living organisms, as they are the main components of the physiological metabolic pathways of cells. The entire set of proteins, produced or modified by an organism or system is proteome. This varies with time and distinct requirements, or stresses, that a cell or organism undergoes. An organism's genome is more or less constant, whereas the proteome differs from cell to cell and from time to time. Hence a comparative proteome analysis of organic and inorganic products will help us to understand the difference between the products and can effectively conclude the ongoing debate with scientific backing.

Pole type vegetable cowpea (*Vigna unguiculata* sub sp. *sesquipedalis*) commonly known as yardlong bean was selected for this study as it is an important vegetable crop in Kerala, next to bittergourd in coverage and preference. Proteome analysis of the embryogenic cell suspensions of cowpea were analysed and 550 proteins were resolved, among which 128 were isolated for trypsin digestion (Nogueira *et al.*, 2007). Sixty seven different proteins in cowpea involved in different biological processes like metabolism, hormone response, cell growth-division, transport, cytoskeleton composition, protein synthesis and processing, regulation and signal transduction, disease defence and stress response were already been identified. These data helps to provide background information about the plant.

The work titled "Proteome analysis of organically grown yardlong bean [*Vigna unguiculata* sub sp. *sesquipedalis* (L.) Verdcourt]" was taken up with the objective to compare the organically and conventionally grown yardlong bean through 2-dimensional proteome analysis. Thus this work will reveal the major proteins which are differentially expressed in the pods under both farming situations, contributing towards the peculiar qualities, furnishing a scientific background for the pod qualities.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1. YARDLONG BEAN (*Vigna unguiculata* subsp. *sesquipedalis*) AS AN IMPORTANT VEGETABLE CROP

Yardlong bean belongs to the genus *Vigna,* which is an important legume species (Pasquet, 2001; Wests and Francois, 1983). It is believed to have been selected in South-East Asia and developed for their long tender pods, from vegetable types of *Vigna unguiculata* introduced there from India (Steele and Mehra, 1980). Yardlong bean, also known as string bean, long-podded cowpea, chinese long bean, pea-bean, bora, asparagus bean, sitao, bodi bean, and snake bean, is widely cultivated and used as food ingredient in Southeast Asia (Rachie, 1985; Fery, 2002; Chen, 2007). Yardlong beans are cultivated for their strikingly long drooping pods (30-90 cm) which are used as vegetable. Besides the tender pods, the young leaves and seeds are also edible and consumed. They are referred to as 'poor man's meat' in Philippines. Yardlong bean is commercially important in parts of Indonesia, Thailand, Philippines, Taiwan and China, as a minor vegetable throughout its range of distribution. It was estimated that, area under yardlong bean in China alone exceeds 250,000 ha annually (Rubatzky and Yamaguchi, 1997), while in Thailand it is grown on about 18,560-20,160 ha annually (Sarutayophat *et al.*, 2007). Yardlong bean is one of the favourite vegetables in Thailand too, especially in the Central part. It is estimated that this bean is consumed at least once a week for each household in Thailand (Khansupa *et al.*, 2008).

Yardlong beans are mainly a warm-season crop and capable of surviving extreme humidity and heat. They can be planted in a wide range of climatic conditions but are very sensitive to cold. Although cowpeas originated in Africa, this vegetable variant has been introduced there only in recent times. It is now grown as a minor garden crop in many sub-tropical countries of Africa and America. It is popular in these countries because of its beautiful, large violet flowers and the long drooping pods.

In India, yardlong bean is a less known under-utilized vegetable grown in the peninsular region particularly Kerala, Tamil Nadu, coastal Andhra Pradesh and Odisha. It is also cultivated to some extent in West Bengal, Assam and the North Eastern Hill (NEH) region. It is a highly self-pollinating, vigorous climbing annual, growing up to a height of three to four meters. Dwarf and tall, climbing varieties exist. Depending upon the cultivar type, it bears blue to violet flowers after about 6-10 weeks of seedling. Pods appear after about another two to four days following flowering. These fleshy, pendulous pods are usually harvested while they are immature and eaten as green vegetables. It produces very long, slender and succulent pods that may be white, light green, dark green, brownish red or purple. Nutritionally, the tender green pods are rich in crude protein (3.5-5 perc ent), besides being a good source of vitamin A (941 IU) and C (13 mg), iron (2.5 mg), calcium (80 mg), phosphorus (74 mg) and dietary fibre (2 g), making it an excellent vegetable (Singh *et al.,* 2001). In Kerala's climate, yardlong beans may be cultivated throughout the year and significant research on this crop has been undertaken by the Kerala Agricultural University. Several high yielding varieties such as Lola, KMV-1, Mallika, Sharika, Vellayani and Vyjayanthi have been released for the long pods ranging from 40-60 cm. Even so, the yardlong bean is still a relatively minor legume crop, but its importance both as a vegetable and as a legume cannot be over-emphasized. In the 21st century with threats of global warming and climate change, yardlong beans forms an important component in the multiple cropping systems and fixing the atmospheric nitrogen, increasing phosphorus availability and revitalizing the degraded soils.

2.2 ORGANIC FARMING

Main principle to organic crop production is the design of a system that enhances the prevention of diseases and weeds and self-supplies nitrogen through the use of N-fixing crops and cover crops (Askegaard *et al.,* 1999). Crop diversification can deliver many simultaneous agronomic benefits such as reduced weed pressure (Bond and Grundy, 2001), reduced pest incidence which is an ecological benefits (Finckh and Wolfe, 1998) and maintaining or enhancing

production efficiency. Maintaining good growing conditions and avoiding stress enhances the natural tolerance of plants to plant competitors (Tamis and van den Brink, 1999). Stimulating biodiversity in and above the soil, by amendments, good soil management, and rotation allows for the control of most soilborne pests and diseases (Altieri, 1995). Finally, many foliar pests and diseases are effectively controlled by spatial crop diversification, including mixed cropping and variety mixtures (Finckh *et al.*, 2000), in combination with disease-resistance genotypes.

Major challenges faced by organic crop production are weed control and nutrient management (Barberi, 2002). Most of the farmers fail to apply recommended dose of manure, which leads to lower yield as compared to conventional system.

2.3 COMPARATIVE QUALITY PARAMETERS OF PRODUCTS FROM ORGANIC AND CONVENTIONAL FARMING SYSTEMS.

Organic farming is gaining recognition as the system of farming that goes in hand with sustainability, an overarching principle that will drive agricultural activities in the years to come (Knight and Newman, 2013). The demand for organic products is also constantly increasing since they are perceived by consumers as healthier and safer for the environment. Although these properties have not been unequivocally proven (Brandt and Mølgaard, 2001; Smith-Spangler *et al.*, 2012), variable accumulation of nutritional metabolites in organic cultivation has been documented. Zörb *et al.* (2006) have reported no significant differences for the levels of 44 secondary metabolites measured in wheat grain from organic and conventional farming. In line with these results, Röhlig and Engel (2010) have found that differences in the metabolic profile of maize kernels with respect to the two cultivation systems are mostly associated to the genetic component and environmental factors rather than the farming system itself. The levels of flavonoids and phenolic acids, two groups of secondary plant metabolites with potential health benefits, were not differentially affected in onions and carrots under conventional and organic agriculture (Søltoft *et al.*, 2010). For the

accumulation of these molecules, cultivar and environmental determinants, like climate and soil, seem to be more important than cultivation related factors such as type of fertilization and methods of weed control (Roose *et al.*, 2010).

Cardoso *et al.* (2011) compared the concentration of vitamin C (ascorbic acid, AA, and dehydroascorbic acid, DHA) and carotenoids (lycopene and β-carotene) between persimmon, acerola and strawberry produced by organic and conventional farming. Vitamin C and carotenoids were analysed by high-performance liquid chromatography, they observed no evidence of the nutritional superiority of the organically grown fruits.

Zuchowski *et al.* (2011) reported, that the concentrations of ferulic and p-coumaric acids have been found to be relatively higher in organic as compared to conventionally grown wheat. An increase in flavonoids and phenolic acids has been reported in organic tomato (Migliori *et al.*, 2012; Hallmann, 2012). Straus *et al.* (2012) have also demonstrated that the reduced nitrogen availability associated to organic farming may enhance the concentration of secondary metabolites, including free radical scavengers with high antioxidant activity.

A study by Lv *et al.* (2012) investigated the phenolic acid compositions and antioxidant and anti-inflammatory activities of commercial organic and conventional peppermints (*Mentha piperita* L.) and cinnamons (*Cinnamomum verum*). They reported that organic peppermint contained greater level of insoluble bound and total caffeic acid, and soluble free p-coumaric acid than the conventional counterparts whereas the conventional peppermints had greater amounts of total and insoluble syringic and ferulic acids. The organic and conventional cinnamons had no difference in their total phenolic contents or radical scavenging capacities under the same experimental conditions, though the organic cinnamon contained greater amount of insoluble bound catechin, syringic acid, and soluble free ferulic acid. Interestingly, the organic peppermints had stronger DPPH radical scavenging and anti-inflammatory activities than their conventional counterparts, but the conventional cinnamons generally had greater anti-inflammatory properties than the organic ones.

Studies conducted by Lombardo *et al.* (2012) and Bartovar *et al.* (2013) suggest different cultivars interact differently to organic cultivation. Early potato cultivars grown organically produced higher nutritional value tubers due to higher total phenolic and lower nitrate content, the tubers contained lower amount of soluble sugars and higher level of dry matter (Lambardo *et al.*, 2012). Bartovar *et al.* (2013) reported that late maturing cultivar Bionta exhibited high instability in tubers yield, total protein and patatin protein characteristics when produced under organic farming system, while the early maturing cultivar Karin reacted on the conditions of organic farming by an increase of the patatin content.

Maggio *et al.* (2013) tried to assess the effect of cultivation variables that may interact with farming systems and ultimately affect the final product quality. They compared the response to conventional vs. organic farming of cauliflower, endive and zucchini and demonstrated that the overall quality of organic products depends on many interacting variables. In cauliflower, the cultivar effect overwhelms other quality determinants with respect to *antioxidant* activity and nitrate accumulation. In endive, the liposoluble antioxidant activity increases under organic cultivation only in the absence of mulching. They also concluded that, organic farming promotes the accumulation of K in zucchini grown on clay but not on sandy soil.

According to the study conducted by Nunes-Damaceno *et al.* (2013), conventionally grown kiwis were larger and heavier than the others; they also had the greater soluble solids, glucose and fructose contents and were sensorially sweeter and juicier than those grown by integrated farming methods. Conventionally grown kiwis were judged to taste marginally better than the others by the consumers.

López *et al.* (2013) reported that in general, conventional management led to larger, firmer and thicker peppers than observed in organic fruits, with a similar greenish colour but lower colour intensity. They observed some exceptions which were accounted to the fact that the effect of cropping system on most fruit quality parameters depended on the harvesting time and/or cultivar. Conventional peppers

showed higher concentrations of N and P than organic fruits and also, only in the case of Almuden, a higher NO^{3-} concentration.

Lu *et al.* (2014) used High Performance Liquid Chromatographic (HPLC) and Flow-Injection Mass Spectrometric (FIMS) fingerprinting techniques to differentiate organic and conventional sweet basil leaf samples. This study suggested that the organic basil sample contained greater concentrations of almost all the major compounds than its conventional counterpart on a per same botanical weight basis.

Mazzoncini *et al.* (2015) while comparing organic and conventionally grown wheat observed that organic wheat yielded less than conventional wheat, mainly due to the nitrogen shortage, and its bread-making quality was lower, the cultivation system did not affect the total amounts of phenolics and phenolic acids.

Campiglia *et al.* (2015) reported that the durum grain yield was on average 15 per cent lower in organic compared to conventional, although the yield gap between the cropping systems varied from −5 to −32 per cent across the years. The air temperature influenced the grain yield more in organic than in conventional, while high rainfall during the grain filling stage produced a higher grain yield in conventional than organic due to a different weed infestation. A severe water stress period starting from stem elongation determined a poor grain yield and low protein concentration, while high temperatures and water stress throughout the grain filling period resulted in a poor yield, yet high protein content in both cropping systems. Conventional wheat generally showed a higher level of vitreousness and gluten quality, while protein and gluten content were higher in conventional compared to organic when a regular rainfall distribution occurred throughout the wheat reproductive period.

In 2015, Pongpresert and Srilaong compared conventional and organic yardlong bean. Their study suggested that the organically cultivated yardlong bean stored at 4 °C demonstrated lower weight loss and swelling of pod compared

with the pod from conventional produce. Organic produce showed higher total ascorbic acid and total chlorophyll content then conventional produce. It may be due that conventional produce have protein compound higher than that organic produce. It caused low pigment synthesis and low vitamin (Worthington, 2001).

From these reviews one can come to the conclusion that cropping system alone doesn't determine the quality of the produce. Some characters are better expressed in organically cultivated crops and some are better expressed in conventionally grown crops. There are too many variables involved in the cropping system to essentially pinpoint the effects of organic or inorganic fertilizers. Hence we planned an experiment with least no of variables to better understand the effects of organic and conventional cultivation practices. Since proteins are the keys in character expression, to gain insights into the molecular effects of organic farming we used proteome analysis.

2.4. PROTEOME PROFILING IN QUALITY ASSESMENT

All multicellular organisms are constituted with different types of cells. Even though each of the different type of cells in an organism carries the same genome they differ in their structure and function. This is accounted to differential gene expression i.e. the variations occurring among the cells of the same individual are due to the differential protein present in them. So, even if we were able to completely sequence the genome of an organism, we still will not be able to truly understand the biology of that organism. Therefore, there is a need for efficient approach for determining protein expression in different tissue and under different conditions, for identifying modification of protein in response to different stimuli and for characterizing proteins, which will be critical for understanding biological process in the post-genome era. In such a scenario proteomics comes in handy. Proteomics is the study of gene products, which enables the observation of the products of gene expression that have a physiological effect on the plant. The main advantage of using a proteomics approach is that proteomics allows the observation of post-transcriptional changes

to gene products that would not be identified in the transcriptome analysis. Proteome analysis refers to the systematic identification and quantification of the complete complement of proteins of a biological system at a specific point in time. It is an essential component of the emerging system biology approach that seeks to comprehensively describe biological systems (Giorgianni, 2003). Proteome analysis of the embryogenic cell suspensions of cowpea were analysed and 550 proteins were resolved, among which 128 were isolated for trypsin digestion (Nogueira *et al.*, 2007). The reviews on methods and technology for the identification and quantification of proteins in biological samples are discussed below.

2.4.1 Solubilization of Protein

The extraction of protein from a cell lysate is a critical step for establishing a stable and reproducible proteome data. Extraction of proteins for 2Dimensional Electrophoresis (2DE) from plant material is more challenging than extraction from animal tissue or microorganisms, mainly due to presence of cell wall and large vacuoles. The techniques developed to extract proteins from plant material are based primarily on precipitation of proteins from crude plant extracts and subsequent suspension of precipitated proteins in buffers used for electrophoretic separation (Mechin *et al.*, 2007)

2.4.2 Separation of Proteins

Separation of proteins is the first step of any proteome analysis. Basic technologies involved in protein separation include one dimensional electrophoresis, two dimensional electrophoresis and chromatography.

2.4.2.1 *One-dimensional Gel Electrophoresis*

SDS PAGE is used to resolve protein mixture according to its molecular mass. 1-DE is a simple and basic methodology that can resolve protein mixture with molecular weight ranging from 10-300 kDa (Schagger and Jagow, 1987).

Singh *et al.* (1991) used this method to separate out LMW subunits of glutenin. Limited resolving power is the fundamental drawback involved with this method. To overcome this concern whenever a complex protein mixture is encountered, then two dimensional gel electrophoresis is preferred (Graves and Timothy, 2002).

2.4.2.2 *Two-dimensional Gel Electrophoresis*

2DE has been a mainstay of biochemistry and has made important contributions to proteomic analyses. Two dimensional gel electrophoresis is preferred for the large scale purification of protein. Proteins are separated according to their net charge (pI) in first dimension and then according to their molecular weight in second dimension (O'Farrell, 1975). The separation in second dimension is carried out in a polyacrylamide matrix in a sodium dodecylsulfate (SDS) background. Acrylamide (10-20 per cent) is commonly used in the gel followed by visualization by various detection methods. Many protocols were exploited for protein staining, wherein Coomassie Brilliant Blue and silver staining have been extensively accepted. Initially it was very tedious and irreproducible technique but with the introduction of immobilized pH gradient gels the practical technical problems were resolved (Gorg *et al.*, 2000). It is a well-accepted protein separation technique, but there are a few restrictions to this technology (Newsholme *et al.*, 2000). Large hydrophobic proteins cannot penetrate the gel, hence limiting the identification of less abundant proteins when a total cell lysate is analysed, as rich proteins take a lead in the gel. Also post translational modifications of proteins significantly affects the observed molecular weight and isoelectric point (pI) of the protein, which can offer advantage in comparing protein expression of two samples both qualitatively and quantitatively.

2.4.3 Detection of Proteins Separated by 2-D Gel Electrophoresis

A number of methods have been developed to visualize proteins which were separated by gel electrophoresis. Coomassie blue and silver staining are the

most accepted methods of choice because of their ease of use and sensitivity. Some of the other methods developed for the detection of gel separated spots are the use of fluorescence staining (e.g LAVAPurple, Cy dyes and Sypro dyes), radiolabeling and immunodetection (Gauci *et al.*, 2011). These methods provide a superior sensitivity and a broad dynamic range as compared to the standard methods (Gevaert and Vandekerckhove, 2000). There is a chance of the spot to shift on the pH gradient and molecular weight scale depending on post-translational modifications, sample composition etc. Now a days 2-DE gel images are evaluated and analyzed using specialized software packages eg. Melanic package from Swiss Institute of Bioinformatics, Phoretix 2DE software from Phoretix, Gellab II from scanalytics etc. which can store all of the relevant information on all of the spots of a 2-DE gel in a database (Rabillouda and Lelong, 2011)

2.4.4 Isolation of Protein from the Gel

The information contained in a gel is not complete hence the additional information has to be extracted via blot digestion of proteins and In-gel digestion of proteins. In blot digestion of proteins (Electroblotting/ Electrotransfer), after separation of proteins by 1-DE/2-DE, proteins are separated from the gel by the technique called electro blotting, which was specifically designed for this purpose. The proteins are transferred from gel to a binding membrane (nitrocellulose membrane) under the influence of electric field. The protein trapped in the gel migrates and encounters nitrocellulose membrane to which it gets attached. The blot can be visualized by staining. Wilm *et al.* (1996) introduced In-gel digestion of proteins, which is the method of choice for generation of peptides from proteins separated on the gel. The protein spot of interest is digested in gel with trypsin after excising it from the gel followed by extraction to obtain the peptides.

2.4.2Protein Identification and Characterization

2.4.2.1 *Edman degradation*

Edman in 1949 developed a technique for the N-terminal sequencing of proteins. In this technique protein are chemically degraded (phenyl isothiocyanate) into individual amino-acid in a cycle-dependant manner from the N-termini of the proteins, typically upto 20 amino-acids (Graves and Timothy, 2002). It is a very tedious and extensively laborious approach to sequence a protein. Now a days protein identification by using N-terminal amino acid sequencing became obsolete due to availability of protein and DNA database such as Uniprot, DDBJ, EMBL, GenBank etc.

2.4.2.2 *Mass Spectrometry (MS)*

Mass spectrometers are instruments that are used to produce and separate ions according to their mass-to charge ratio (m/z). MS techniques have significantly advanced the proteomics-based research in recent years (Aebersold, and Mann, 2003). The idea of Mass spectrometry was established nearly a century ago and but it still is widely applied as an analytical technique (Borman *et al.*, 2003). MS is very sensitive and it is used to analyse the molecular weight (MW) or structural information such as peptide masses or amino acid sequences of a compound in a short period of time (Feny *et al.*, 1998). Earlier, MS was found to be a tedious and slow process and it required large volume of samples. But recently, the idea of using MS for identification of proteins has evolved with the improvement in instrumentation (Aitken, 2005). MS instrumentation separates and detects the ions in gas phase and therefore, prior to any separation by MS, molecules should be ionized and converted into gaseous state using different techniques. Development of two new ionization techniques Electrospray ionization (ESI) (Fenn *et al.*, 1989) and matrix-assisted laser desorption/ionization (MALDI) (Karas and Hillenkamp, 1988) in the late 1980s simplified the

acquisition of mass spectra with minuscule quantities of peptides and proteins thus making it possible to apply MS to the analysis of proteins. The introduction of Matrix Assisted Laser Desorption Ionization Time of flight (MALDI-TOF) and Electron Spray Ionization Tandem Mass Spectrometry (ESI tandem MS) has revolutionized the field of mass spectrometry. 2-DE coupled with MS is now widely used for protein analysis. The protein spots from 2- D gels are excised and subjected to in-gel digestion followed by identification of resulting peptide fragments by MS (Matt *et al.*, 2007). For identification of 2-D gel spots MALDI-TOF MS is commonly used. MS has three essential parts namely, the ion source that will produce ions from the sample, the mass analyzer to resolve ions based on mass/charge ratio (m/z) and the detector to detect the ions resolved by the mass analyzer. For separation electric or magnetic fields are generated within the instrument. Ion separation is necessarily done under vacuum to avoid the collision between accelerated ions and air molecules. The analyser and the detector also must be under vacuum, although ion production can also be done at atmospheric pressure depending on the type of ionization source. By using MS protein structural information like peptide mass, amino-acid sequence can be obtained. The analyser is the part of the MS where separation of ions in gas phase takes place. Mass spectrometers known as tandem mass spectrometers may have two or more mass analysers coupled together. Analysers like TOF, quadruples and ion traps (ITs) are used either alone or in tandem in proteomics applications. In ESI, ions are formed at atmospheric pressure, while ions in MALDI they may be generated either at atmospheric pressure (Laiko *et al.*, 2000) or under vacuum conditions, although the best performance is obtained when working at low pressure. The technique of MALDI-TOF MS is discussed as under.

2.4.2.2.1 Matrix Assisted Laser-Desorption-Ionization Time-of-Flight/Mass Spectrometry (MALDI-TOF MS)

In the field of MS, MALDI-TOF allowed rapid and accurate process to measure mass of analytes (James *et al.*, 1993; Harvey, 1996). MALDI is used to change the peptide from a solid phase to the gaseous phase. It is a competitive

process where the ionization of an analyte may be inhibited considerably by the existence of others (Laiko *et al.*, 2000). In MALDI, protein or peptide sample mix is allowed to combine with the matrix molecule in solution and small amounts of the mixture are deposited on a surface and allowed to dry. Here a matrix compound that is capable of absorbing ultraviolet (UV) light is employed (Karas and Hillenkamp, 1988). In this method of ionization, sample is incorporated into matrix molecules and then subjected to irradiation by a laser beam (Hillenkamp and Karas, 1990). Matrix is typically consists of small energy absorbing molecules such as 2, 5 dihydroxybenzoic acid or α-cyano-4 hydroxycinnamic acid and sinapinic. Matrices like ice can also be used for peptides and proteins (Hutchens and Yip, 1993). The analytes along with matrix are mixed in the appropriate solvent and spotted on a sample probe that can measure dozens to hundreds of samples. The solvent is allowed to evaporate resulting in crystal formation. The plate is placed in MS at high vacuum and a laser beam of a specific wavelength is targeted onto a limited area of the spot. The matrix molecules absorb photons from the beam and become excited. The excess energy is then transferred to the peptides in the sample, which are then ejected into gas phase (Hillenkamp *et al.*, 1991; Hunt *et al.*, 2005). Each peptide molecule takes up single proton hence peptide ions are singly charged (Graves and Timothy, 2002). A high voltage (+20 V to +30 V) is applied on MALDI plate to induce positively charged peptides to accelerate toward the orifice of the flight tube. TOF (Time-of-flight) necessarily comprises of a flight tube in high vacuum. It is simplest mass analyser which measure mass/charge ratio of an ion by determining the time required for it to traverse the length of flight tube (Graves and Timothy, 2012). The vacuum in the flight tube is such that the chance of molecule collision with each other or with the flight tube is very low. As the peptides generated in MALDI are equally charged, they traverse flight tube with different velocities, which are inversely proportional to their masses and hit the detector at different time intervals (Rodwell and Barnes, 2000). Lighter ions land at the detector faster than the higher ones separating the ions of different masses. To improve the resolution, reflectron a piece of hardware is placed in the path of ions (Kaufmann

et al., 1996). Principle application of MALDI-TOF-MS is peptide mass fingerprinting and large scale proteomics work. The technique is applicable not only for peptides and proteins, but for other bio-molecules like polysaccharides, lipids and polynucleotides (Mann and Talbo, 1996). It is a user friendly, fast, highly sensitive and accurate technique.

2.4.3. Database Utilization

All the mass spectra obtained by mass spectrometer are written to a file or loaded into a database and are then further analyzed, using the bioinformatics tools (Eng *et al.*, 1994; Quadroni and James, 1999). The goal of analysis of proteins/peptides by database searching is to identify a large number of proteins in a short span of time. Three types of database searching are given below

2.4.3.1 *Peptide Mass Fingerprinting Database Search*

Peptide mass fingerprinting is a type of protein identification method where the analysis and database search is fully automated (Mann *et al.*, 1993; Mann and Wilm, 1994). In this method, mass of each peptide obtained from the proteolytic digestion of an unknown protein is matched against the predicted mass of the peptide from the theoretical digestion of proteins obtained from the *in silico* digestion at the same enzyme cleavage sites of all protein amino acid sequences in a database (Pappin *et al.*, 1993; Jensen *et al.*, 1997). If they overlap, protein identification can be done. The proteins in the database are positioned depending upon their peptide masses matching their sequence within a given mass error tolerance. Programs used for protein identification are PepSea (Mann *et al.*, 1993), MS-Fit (Clauser *et al.*, 1999), PeptIdent/MultiIdent (Wilkins *et al.*, 1999) and ProFound (Zhang and Chait, 2000). The biggest advantage of database searching is rapidity and that it can be fully automated. However there are a few limitations also associated with this technique. First is the ambiguity in protein identification i.e. a peptide with ten amino acid will carry same mass by simple reorganization of its constitutive amino acids (Mirza and Olivier, 2008). Secondly it is effective for analysis of protein from organism whose genome is small and

completely sequenced (Qin *et al.,* 1997). Another important factor is mass accuracy i.e. if an unknown protein is extensively modified by post-translational modifications, the peptides produced from the proteins will not match with the unmodified proteins in database.

2.4.3.*2 Peptide Amino Acid Sequencing Database Search*

This is the most specific type of database searching for protein identification. By determining the amino acid sequence of a peptide, the information about protein from which it is originated can be obtained by search database. One such method based on this theory is peptide mass tag searching where peptide mass together with a short sequence produced by partial interpretation of the spectrum is used for database searching (Deutsh *et al.*, 2008). For protein identification, peptide mass tag searching is more specific and can be used for protein mixtures in contrast to peptide mass fingerprinting (Neubauer *et al.,* 1997). The major disadvantage of this procedure is that the process is not easily automated hence becomes time consuming.

2.4.3.3 *De novo* Sequencing

De novo peptide sequencing is independent of any information present in databases. When protein identification remains intangible with other methods, *de novo* peptide sequencing is the only option to acquire information about the analyte. The sequence of a peptide can be reconstituted from its fragmentation spectrum (Papayannopoulos, 1995).

2.4.4 Applications of Proteome Profiling

Proteome analysis gives us insight into the protein level scenario, which along with the genomic level information provides us better understanding any biological system. The applications of proteome profiling are listed below:

2.4.4.1 *To Study the Resistance in Host*

Castillejo *et al.* (2004) carried out an experiment to study the response of pea plant to *Orobanche crenata*, which is a parasitic plant that threatens legume production. The protein profile of healthy and infected *P. sativum* root tissue were analysed by two-dimensional electrophoresis. Approximately 500 individual protein spots were detected on silver stained gels. Out of these 22 different protein spots differentiated control, non-infected, Messire and Ps 624 accessions. Some of them were identified by MALDI-TOF mass spectrometry and database searching as cysteine proteinase, β-1,3-glucanase, endochitinase, profucosidase, and ABA-responsive protein. Hence proving that these molecules are involved the interaction between host plant and parasite.

Gokulakannan and Niehaus (2010) studied the changes in protein composition during interaction of *Medicago truncatula* cell suspension culture with a pathogen-derived yeast elicitor (YE) and suppressor using *Sinorhizobium meliloti* LPS using 2D-PAGE analysis. They identified proteins involved in defense, such as l-ascorbate peroxidase, specifically targeted proteins to the cell wall during defense, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and proteins that play an important role during growth and development.

Proteome profiling can be used to studying biochemical aspects underlying the interaction between host and the pathogen. Moura *et al.* (2014) planed an experiment on this basis. They studied the proteomics changes during the incompatible interaction between cowpea and *Colletotrichum gloeosporioide.* The analyses of two-dimensional gel electrophoresis patterns and protein identification indicated that the *C. gloeosporioides* infection-dependent cowpea leaf proteome changed in association with metabolism, photosynthesis ,response to stress, oxidative burst and scavenging, defense signaling, and pathogenesis-related proteins. It was also observed that the *C. gloeosporioides* responsive proteins interaction network in cowpea revealed the interconnected modulation of

key cellular processes involving particularly antioxidants proteins, photosynthetic apparatus forming proteins and proteins of the energetic metabolism that interact with each other suggesting that their expression changes are also important for resistance of cowpea to *C. gloeosporioides*.

2.4.4.2 *To Study the Molecular Basis of Changes Occurring in an Organism:*

A comparative proteome analysis at various developmental stages using 2DE approach provides differentially expressed spots, these when analysed can provide information about the proteins which are differentially expressed at different developmental stages, which in turn provides us opportunity to study the molecular basis of different stages of development.

Schiltz *et al.* (2004) followed a proteomic approach to analyze protein changes during nitrogen mobilization (N mobilization) from leaves to filling seeds in pea. First, proteome reference maps were established for mature leaves and stems. They displayed around 190 Coomassie Blue-stained spots with pIs from 4 to 7. A total of 130 spots were identified by mass spectrometry as corresponding to 80 different proteins implicated in a variety of cellular functions. Although the leaf proteome map contained more abundant spots, corresponding to proteins involved in energy/carbon metabolism, than the stem map, their comparison revealed a highly similar protein profile. Second, the leaf proteome map was used to analyze quantitative variations in leaf proteins during N mobilization. Forty per cent of the spots showed significant changes in their relative abundance in the total protein extract. The results confirmed the importance of RuBisCo as a source of mobilizable nitrogen, and suggested that in pea leaves the rate of degradation of RuBisCo may vary throughout N mobilization. Correlated with the loss of RuBisCo was an increase in relative abundance of chloroplastic protease regulatory subunits. Concomitantly, the relative abundance of some proteins related to the photosynthetic apparatus (RuBisCo activase, Rubisco-binding proteins) and of several chaperones increased. A role for these proteins in the maintenance of a RuBisCo activation state and in the PSII repairduring the intense

proteolytic activity within the chloroplasts was proposed. Finally, two 14-3-3-like proteins, with a potential regulatory role, displayed differential expression patterns during the massive remobilization of nitrogen.

Zhua *et al.* (2013) tried to investigate the molecular mechanism underlying the process of pod swelling of groundnut. A comparative proteome analysis between developing aerial and subterranean pods at various developmental stages was performed using 2-DE approach and significantly differentially expressed spots were selected to further identification by MALDI-TOF–TOF MS. They reported enzymes in lignin synthesis and ubiquitin proteasome system and several photosynthesis and oxidative stress proteins might function as potential candidate proteins and play critical roles to regulate pods swelling and development.

2.4.4.3 *To Assess Food Quality and Safety*

Initially aimed at the identification of proteins expressed by a genome, proteomics now involves a study of their structure, localization, modification, interactions and functions, that is largely taking advantage by progress in mass spectrometry (i.e. Q-TOF mass spectrometer for MS/MS based protein identification) and in robotics-based technology. A new challenge for proteomics has recently been recognized pointing out differences in food proteomes relevant for nutrition (Kvasnicka, 2003).

Zarkadas *et al.* (2007 b) determined the protein quality of 11 null and 2 tofu soybean genotypes from their total protein content, their amino acid composition, and their glycinin and b-conglycinin contents. They reported that two-dimensional gel electrophoretic (2-DE) reference maps, using narrow range immobilized pH gradient (IPG) strips, revealed unique differences in the proteome, and subunit expression of glycinin and b-conglycinin, among the null genotypes, which can be correlated with their protein quality. They suggested that 2-DE along with liquid chromatography on-line with electrospray LCQ DecaXP tandem quadrupole time-of-flight mass spectrometry (LC/MS/MS) will enable accurate evaluation of protein quality in soybeans, based on their protein

digestibility-corrected amino acid score, assessment of the genetic variability of soybean genotypes, and serve as very effective tools for assisting plant breeders in their selection of high quality soybean varieties.

Nasi *et al.* (2009) reviewed the main aspects of current and perspective applications of mass spectrometry and proteomic technologies to the structural characterisation of legumes are presented, with focus on issues related to detection, identification, and quantification of phytohemagglutinins relevant for their biochemical, immunological and toxicological aspects. As earlier methods to determine the lectin levels in foods were based on immunoenzymatic or toxicity tests, which are largely aspecific and proteomic methodologies has allowed to start development and validation of sensitive and specific assays for detecting trace amounts of harmful lectins in either raw or processed foods.

2.4.4.4 *To Establish Proteome Reference Map*

The existence of a reference map provides a starting point for ongoing functional genomics studies associated with biotic or abiotic stresses and for studies on natural product biosynthesis. It can also be used for assessing the quality of the agricultural products.

As a first step to study stress physiology of soybean, Xu *et al.* (2006) have separated and identified soybean leaf proteins from normal plants to establish a proteomic reference map for soybean leaves, using 2-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE) and Mass Spectrometry (MS). Tryptic digests of 260 spots were subjected to Peptide Mass Fingerprinting (PMF) by MALDI-TOF MS. Fifty-three of these protein spots were identified by searching NCBInr and SwissProt databases using the Mascot search engine. Sixty-seven spots that were not identified by MALDI-TOF-MS analysis were analyzed with LC-MS/MS, and 66 of these spots were identified by searching against the NCBInr, SwissProt and Expressed Sequence Tag (EST) databases. They have identified a total of 71 unique proteins.

Gokulakannan and Niehaus (2010) have established the proteome reference map of *M. truncatula* cell wall proteins using a combination of two-dimensional gel electrophoresis (2D-PAGE) and/or liquid chromatography–tandem mass spectrometry (LC–MS/MS).

Zarkadas *et al.* (2007 a) assessed 14 commercial soybean cultivars using two-dimensional gel electrophoretic separations and they have establishment high-resolution proteome reference maps, enabling polypeptide chain identification and calculation of the ratio of the constituent glycinin and b-conglycinin storage proteins of soybean.

2.4.5 Proteome Analysis as a Tool to Study Variation in Conventional and Organic Produce

To gain insights into the molecular effects of organic farming, Nawrockia *et al.*, (2011) used proteome analysis to analyse cabbage and carrot grown under three schemes: one conventional and two organic schemes. The patterns of 2DE separated proteins obtained were very similar for each of the vegetables across the three cropping systems. They detected significant quantitative differences in only 58 of 1300 proteins in cabbage and in 68 of 1800 proteins in carrot roots. The result was obtained based on a low average standard deviation of the protein-spot intensity among the three replicate samples from the field. This allowed them to identify even moderate changes in protein expression of only 30 per cent due to different treatments of the crops in the field. Low variability indicated that the field variation was low, which is a pre-requisite for being able to measure treatment effects, and also that the sampling and extraction protocols they developed kept sampling error at a low level. They reported that proteins of the glycolytic pathway and Krebs cycle as well as several proteins related to amino acid and protein metabolism were overexpressed in organically farmed cabbage, proteins related to detoxification processes were overexpressed in conventionally grown cabbage, proteins involved in metabolism of carbohydrates, polypeptides and secondary metabolites were affected by different cropping regimes in carrots.

They also observed that proteomes of conventionally grown vegetables varied from organically grown vegetables to a larger extent than the two organic cropping schemes varied from each other. With these conclusions one could infer that proteomics platform is suitable and useful for systematic studies of the effects of organic and conventional farming techniques on plant metabolism.

Similar work was done by Tétard-Jones *et al.* (2012) on wheat. To understand the molecular effects of organic cropping system compared to conventional cropping systems on plant utilisation of nutrients, they used proteomics to analyse winter wheat (*Triticum aestivum*). They aimed to investigate the effects of contrasting fertility management and crop protection regimes in organic and conventional cropping systems on the wheat flag leaf proteome and the association between the proteome and physiological traits. They extracted the flag leaf protein by TCA/acetone precipitation and they analysed the protein by 2DE and MALDI-TOF MS. A total of 219 spots were matched across gel images and analysed by ANOVA for the effects of fertilisation and crop protection regimes on protein expression. Contrasting fertilisation regimes resulted in significant differential expression of 111 protein spots. Five of these protein spots also changed in response to crop protection regimes, but with a lower level of significance. The expression of some of these 111 proteins spots were also affected by an interaction between fertilisation and crop protection regimes: 15 protein spots at the $p < 0.05$ level, and a further two proteins at the $p < 0.01$ level. The 111 protein spots differentially expressed in response to fertilisation treatment were selected for protein identification by Peptide Mass Fingerprinting (PMF). Twenty-six proteins were identified that were significantly ($p < 0.01$) up-regulated in wheat grown under compost compared to mineral fertilisation, eleven of these proteins were identified as the large subunit of RuBisCO. Thirty-one proteins were significantly up-regulated in flag leaves of wheat grown under mineral fertilisation when compared to compost-fertilised flag leaves. They concluded that, the most significant changes to the proteome were

due to the contrasting fertilisation regimes rather than the contrasting crop protection regimes.

To gain a better understanding of the factors affecting nutrient use efficiency (yield per unit fertilizer input) from organic fertilizers Tétard-Jones *et al.* conducted a study in 2013, wherein they showed that, previous crop management (organic *vs.* conventional fertilization and crop protection regimes), organic fertilizer type and rate (composted cattle manure *vs.* composted chicken manure pellets) and watering regimes (optimized and restricted) significantly affected leaf chlorophyll content, potato tuber N-concentration, proteome and yield. Protein inference by gel matching indicated several functional groups significantly affected by previous crop management and organic fertilizer type and rate, including stress/defense response, glycolysis and protein destination and storage. These results indicate genomic pathways controlling crop responses (nutrient use efficiency and yield) according to contrasting types.

2.5 ORGANOLEPTIC ANALYSIS

Comparative study of any food item should include affective sensory evaluation, to evaluate the acceptability by the consumer. Organoleptic properties are the aspects of food or other substances that an individual experiences via the senses including taste, sight, smell, and touch. Sensory analysis is a scientific discipline that applies principles of experimental design and statistical analysis to the use of human senses (sight, smell, taste, touch and hearing) for the purposes of evaluating consumer products. The discipline requires panels of human assessors, on whom the products are tested, and recording the responses made by them. By applying statistical techniques to the results it is possible to make inferences and insights about the products under test (Heintz and Kader, 1983). Sensory analysis can be classified into three types i.e effective testing (dealing with objective facts about products), affective testing (dealing with subjective facts such as preferences), perception (the biochemical and psychological aspects of sensation) (Peryam and Girardot, 1952). Here we have focussed on affective testing, which

is also known as *consumer testing*, this type of testing is concerned with obtaining *subjective* data, or how well products are likely to be accepted. Usually a panels of untrained personnel are recruited for this type of testing, although smaller focus groups can be utilised to gain insights into products. The range of testing can vary from simple comparative testing, structured questioning regarding the magnitude of acceptance of individual characteristics. Consumer testing also known as 'hedonic testing' involves having potential consumers of a product evaluate various products and a small number of items on a ballot (Peryam and Pilgrim, 1957). Most large consumer goods companies have departments dedicated to sensory analysis. There are many studies in which organoleptic tests were included. Odedeji and Oyeleke (2011) conducted organoleptic test of whole and dehulled cowpea seeds. Lombardo *et al.* (2012) conducted organoleptic tests to analyse the sensory characteristics of "early" potato cultivars under organic and conventional cultivation systems using a 5-point hedonic scale. Ojiako and Kayode (2014) conducted organoleptic tests to study the consumer acceptability of cowpea seeds treated with natural and synthetic insecticides against bruchid infestation, using five point hedonic scale.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study on proteome analysis of organically grown yardlong bean [*Vigna unguiculata* sub sp. *sesquipedalis* (L.) Verdcourt] was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Kerala Agricultural University during the period of 2013-2015. The objective of the study was to compare the quality parameters of the organically and inorganically grown cowpea, using the precise 2DE proteome analysis. The materials used and methodologies adopted are discussed in this chapter.

3.1 INITIAL SOIL NUTRIENT ANALYSIS

To derive a precise picture of the contribution of the types of nutrient supplies on the proteome, it was essential to get a clear picture of the initial nutrient status of the soil. From both the plots 10 random sites were selected to from where the soil was taken. The soil from all the 10 sites were mixed thoroughly and left to shade dry. The properly dried soil, with all the stones and other debris removed were used for further analysis. The soil organic carbon was determined based on the Walkley-Black chromic acid wet oxidation method (Walkley and Black, 1999). Available phosphorus was estimated using Bray's No. 1 reagent (Bray and Kurtz, 1947). Flame photometer was used to assess the potassium in the soil (Jackson, 1958). Calcium and magnesium were determined using Atomic Absorption Spectrometer. Available sulphur was assessed by Massoumi and Cornfield method (Massoumi and Cornfield, 1963) and the micronutrient were assessed using spectrometric methods (Sims and Johnson, 1991).

3.2 EXPERIMENTAL DETAILS

The yardlong bean variety Sharika, which is a trailing type released by Kerala Agricultural University, was selected for the study as it is one of the popular varieties and has wider consumer acceptability in Kerala. Yardlong bean variety Sharika was cultivated under organic and inorganic nourishments schemes during October - January 2014-15; rainy season was purposefully avoided to overcome

the loss of nutrients due to leaching. The land was thoroughly ploughed and weeds and stubbles were removed. Soil from the plots was analysed before the crop was sown. Two plots were included in the study, each with an area 40 m^2 and three rows of pits, each row having five pits. Since 'Sharika' is a trailing variety, it was sown in pits (at the rate of 3 plants/ pit) at 2 m x 2 m spacing. The crop was grown in 2 cents area. For nourishment in organic plot 8 kg/per pit cowdung was incorporated into the soil before sowing. In the inorganic plot Nitrogen, Phosphorous and Potassium were supplemented through commercial fertilizers (Factamphos 20:20:15 N P S and MoP 60 K), to provide 20:20:10 N P K/ha, as recommended by Kerala Agricultural University (2011), with slight modification to suit the pole type cultivar. Periodic manual weeding was done; none of the herbicides were applied.

During the cropping period, pea aphid (*Aphis craccivora*), American Serpentine leaf miner (*Liriomyza trifolii*), pod borers, and leaf folder were observed in the field. Since the infestation was not severe, no control measures were taken. In the later stages of growth the crop was infected by powdery mildew, no preventive measures were taken as application of fungicides may again lead to variations in the experiment.

Pods were harvested about two months after direct seeding or sowing days. Pods were collected at two stages for proteome profiling i.e. immature stage (vegetable purpose), when the seeds are not filled completely, pod snaps on bending and mature stage, when the seeds are completely filled. Immature pods at two stages of crop growth (at 2 month after sowing and 4 month after sowing) were analysed for its nutrient status.

3.3 LABORATORY CHEMICALS, EQUIPMENT AND MACHINERY

The chemicals utilized in this study were procured from Merck India Ltd., HIMEDIA and Bangalore Genei Ltd. All the plastic wares were obtained from Tarson India Ltd., and borosilicate glass wares were used.

For autoclaving autoclaves were used at 121 °C and 15 psi. High precision electronic balance (Shimazu), pH meter (EuTech Instruments PC 510), micropipettes (Eppendorf), Icematic (F100 compact) and high speed refrigerated centrifuge (KUBOTA 6500) were used for protein extraction. Extracted protein samples were stored at -80 °C and reagents were stored at -20 °C (SANYO medical freezer). Some chemicals were stored at 4-0 °C in refrigerator. Isoelectric focusing was carried out in PROTEAN® i12™ IEF system. (BioRad), second dimensional separation was carried out in PROTEAN® (BioRad) circulatory cooler (Analab) was connected to the electrophoresis unit to prevent preheating of the samples. Staining was aided by the rocker shaker Rocker 25 (Labnet).The gels were visualized and converted into digital image data using the GS-900™ calibrated densitometer (BioRad). Softwares used for the analysis of the digital image data were Quantity one and PDQuest.

3.4 SAMPLE COLLECTION:

Pods were randomly picked from both organic and inorganic yardlong bean plots which are separated by a trench. Pods were randomly selected at two stages i.e. immature pods (10 days after flower opening) and mature pods (15 days after flower opening). Following are the samples which were subjected to further analysis:

C1: Inorganically cultivated immature pods

C2: Organically cultivated immature pods

C3: Inorganically cultivated mature pods

C4: Organically cultivated mature pods

3.5 ORGANOLEPTIC TEST

Two hundred grams (200 g) each of the samples were washed and cooked in clean pots over a gas cooker for 10 min, same amount of salt was added to both the samples and the samples were presented to the panellists at the same temperature. To eliminate subconscious bias in the panellists, cryptic labelling

was employed. Samples were labelled as A and B. The cooked pods were served to a panel of twenty two judges in a tasting room devoid of environmental interferences Questionnaires were drawn to determine the parameters being sought after, that is, colour, taste, flavour, stringiness and general appeal. A nine-point hedonic scale method with scores ranging from 1 to 9 where 1 is dislike extremely and 9 like extremely (Peryam and Pilgrim, 1957) was used to rate the cooked pods.

3.6 PROTEIN EXTRACTION FROM PODS

Five gram of pod was ground into fine powder using autoclaved and prechilled mortar and pestle in liquid nitrogen. This fine powder was then added to an Oakridge tube containing 10 ml TCA extraction buffer (A) (Annexure II) (pre-cooled at -20 °C). These tubes were then kept in medical freezer at -20 °C for one hour for incubation. These were then centrifuged at 12000 rpm for 15 min in a precooled centrifuge (precooled at 4 °C). Supernatant was discarded and 10ml wash buffer was added and kept for one hour incubation at -20°C. Tubes were again centrifuged at 12,000 rpm for 15 min at 4 °C. Supernatant was discarded and washing was carried out for 2 more times (Natarajan *et al.*, 2005). The pellet was lyophilized and stored at -80°C.

3.7 PROTEIN SOLUBILISATION

Fifteen microgram of lyophilized sample protein was suspended in 250 µl of lysis buffer (Annexure II) and incubated for one hour at 37 °C with intermittent vortexing at 10 min interval. Contents were then centrifuged at 10000 rpm for 15 min at room temperature. The clear supernatant was transferred to eppendorf tubes and stored at -80 °C.

3.8 PROTEIN QUANTIFICATION

Protein quantification was carried out using Genei™ protein estimation kit (Lowry *et al.*, 1951). BSA was used as standards. One millilitre of distilled water

was added to 5 mg of BSA, from which 0.1 ml was taken and mixed with 0.9 ml distilled water to make the concentration to 0.5 mg/ml. Complex forming reagent was prepared by mixing solution I and solution II in the ratio 1:100. BSA standards were prepared with following concentrations: 0.05 µg/µl, 0.1 µg/µl, 0.2 µg/µl, 0.3 µg/µl and 0.4 µg/µl. Two millilitres of complex forming reagent was added to 200 µl of BSA standards and protein samples (1 µl protein sample and made upto 200 µl with water) were mixed well and kept at room temperature for 10 min, 0.2 ml of solution III was then added to each of the tubes and was kept for 30 min incubation. The optical density was measured in spectro-photometer at 660 nm.

3.9 PROTEIN PROFILING

3.9.1 SDS-PAGE (One Dimensional Electrophoresis)

3.9.1.1 Sample Treatment

Protein samples (10 µl) from the different treatments were mixed well with 2.5 µl of sample buffer (Annexure IV) in a microfuge tube.

3.9.1.2 Standardization of SDS-PAGE Protocol

SDS PAGE (Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis) analysis was done by the standardized protocol (Laemmli, 1970) with minimum modifications by adjusting the APS and TEMED concentrations.

3.9.1.3 SDS-PAGE Analysis of Proteins Extracted from Mature and Immature Pods from Organic and Inorganic Plots

The plates were assembled in a gel casting apparatus. Separating gel mixture was prepared and poured into the plates (reagents and gel mixture chemistry were provided in Annexure IV). A layer of water of about one cm was poured over the gel mix and allowed to solidify for 45 min with the presence of light and in the absence of air. After solidification, the layer of water was removed

by tilting, dried with tissue paper. Stacking gel mixture of 4 per cent was then prepared and poured above. Appropriate comb was placed at the top immediately and allowed to solidify for 30 min.

After complete polymerization, the plates were separated from the casting apparatus and fixed in the gel running gasket vertically. The dummy plate was fixed on the other side and setup was tightened before placing inside the buffer tank containing 1X tank buffer. The combs were then removed carefully and the wells were washed thoroughly to remove the unbound acrylamide.

Treated samples containing around 220 µg proteins from different treatments were loaded into the wells. The molecular marker of 6 kD to 180 kD was then loaded and electrophoresis was carried out at constant voltage of 80 V until the samples travel through the stacking gel. Then the voltage was adjusted to 100 V until the samples reach the bottom of the separating gel. To prevent the sample from degradation due to heating of the buffer while running, ice packs were kept on both sides of the buffer tank for the small unit. The large unit was connected to a cooling water circulator unit (Analab).

When the tracking dye reaches the bottom of the plates, the power was switched off and chords were removed. The gel running gasket containing the plates was taken out and lock was relaxed. The dummy plate was taken first followed by the glass plate assembly. The two plates were then separated carefully using the tool provided by with the setup and the fragile gel sticking to one of the plates was removed patiently with the help of water squeeze without breaking the gel.

3.9.1.4 Coomassie Brilliant Blue Staining

The gel was transferred to staining solution. After two hours the gel was immersed into destaining solution with uniform shaking for one and half hours and the process was repeated at least twice until the background of the gel became colorless. The gel was then stored in distilled water.

3.9.1.5 Documentation

Documentation was carried out using GS-900™ calibrated densitometer, which helps in visualization and conversion of gel into digital image.

3.9.2 Two Dimensional Gel Electrophoresis of Proteins Extracted from Mature and Immature Pods from Organic and Inorganic Plots

3.9.2.1 IPG strip rehydration

The IPG strips were taken out from the freezer and kept outside at room temperature. The solubilized protein was mixed with appropriate amount of rehydration buffer (Annexure III) to make up the concentration suitable for focussing, volume of rehydration buffer and protein concentrations needed for different strips sizes are given in the Table 3.1. Rehydration tray was placed at a level work bench. The mixture of solubilized protein and rehydration buffer was then pipetted as a line along the back edge of channel of the rehydration tray. Care was taken not to insert any bubbles. Coversheet of the IPG strips were peeled off and the strips were placed gel side down onto the sample in the rehydration tray. Precaution was taken not to trap any bubbles beneath the strips, and then 2 to 3 ml of mineral oil was added on top of the strips to prevent evaporation during rehydration step. The rehydration tray was covered using plastic lid and the tray was left on a level bench overnight (11 to 16 hours).

3.9.2.2 First Dimensional Focussing

Clean and dry PROTEAN IEF focusing tray with the same size as that of the IPG strips were placed on the lab bench. Paper wicks were dipped in distilled water and placed on both ends of the channels covering the wire electrodes using forceps. The lid was removed from the rehydration tray, the strips were taken out and the mineral oil was drained out from the strips using tissue paper. The IPG strips were then transferred to focussing tray (gel side down configuration). Mineral oil was added over the strips as already detailed and focussing tray was

placed in the PROTEAN IEF cell. A three step protocol was programmed in the PROTEAN IEF cell, details of which are given in Table. 3.2; default temperature was setup to be 20 °C and maximum current of 50 μA/strip. The electrophoresis run was initiated by pressing the START button.

3.9.2.3 Second Dimensional Focussing

After completion of first dimensional focussing the strips were taken out and the oil was drained out. Equilibration buffer I (Annexure III) was added to the channels of fresh rehydration tray and strips were placed on these channels. This setup was then kept in shaker for 10 min. At the end of 10 min the setup taken from the shaker and the buffer was decanted. Equilibration buffer II (Annexure III) was added to each of the strips and once again the setup was placed on the shaker for 10 min. During the incubation of 10 minutes overlay agarose was melt with a help of microwave oven. After the incubation, the buffer was decanted.

The plates were washed well with tap water and wiped with distilled water followed by ethanol and acetone. The plates were then assembled in a gel casting apparatus. Separating gel mixture was prepared and poured into the plates. The strips were taken out of the tray and dipped in 1X TGS (Tris-Glycine-SDS) buffer. Using forceps the strip was inserted into plates containing the solidified gel in such a way that the strip touches the gel. Care was taken not to introduce any bubbles. Over the strip, melted overlay agarose was poured using a pipette. The plates having the gel were then mounted over the gel box. The tank was then filled with 1X TGS buffer and electrophoresis was initiated. A constant current of 16 mA was provided for first minutes, later it was increased to 20 mA (for bigger unit, for small unit 80 V of constant voltage was provide) which was regulated using the power pack for electrophoresis. To prevent the sample from degradation due to heating of the buffer while running, ice packs were kept on both sides of the buffer tank for the small unit. The larger unit was connected to a cooling water circulator unit (Analab).

When the tracking dye reaches the bottom of the plates, the power was switched off and chords were removed. The gel running gasket containing the plates was taken out and lock was relaxed. The dummy plate was taken first followed by the glass plate assembly. The two plates were then separated carefully using the tool provided by with the setup and the fragile gel sticking to one of the plates was removed patiently with the help of water squeeze without breaking the gel.

3.9.2.4 Coomassie brilliant blue staining

The gel was transferred to staining solution. After two hours the gel was immersed into destaining solution with uniform shaking for one and half hours and the process was repeated at least twice until the background of the gel became colorless. The gel was then stored in distilled water.

3.9.2.5 Documentation

Documentation was carried out using GS-900™ calibrated densitometer, which helps in visualization and conversion of gel into digital image.

3.9.2.6 Peptide Mass Fingerprinting by MALDI-TOF/MS and In-Silico Analysis

The differentially expressed spots were identified, cut from the gel and send for peptide sequencing by Matrix Assisted Lazer Desorption/ Ionization-Time of Flight Mass Spectrometry (MALDI-ToF/MS). The spots from gels were eluted and digested with trypsin into peptides before embedding into a matrix made of aromatic compounds. A laser beam ionized the matrix along with the peptides, evaporated them and made to travel along a tube. The time of flight of each peptide is directly proportional to the molecular mass of the peptides, the data was fed to the computer and the peptide mass fingerprint was generated. This data so obtained were analysed with the online bioinformatics too, MASCOT/MS peptide search engine for the characterization of proteins.

Table 3.1: Protein content and volume of rehydration buffer needed for different sized strips

Strip size	Protein content (µg)	Volume of rehydration buffer (in µl)
7 cm	300	125
17 cm	900	300

Table 3.2: Programme set for isoelectric focusing

Strip size	Step 1	Step 2	Step 3
7 cm	250 V for 30 min	600 V for 30 min	1000 V till 15000 Vhr
17 cm	250 V for 30 min	600 V for 30 min	1000 V till 45000 Vhr

RESULTS

4. RESULTS

The results of the study entitled "Proteome analysis of organically grown yardlong bean [*Vigna unguiculata* sub sp. *sesquipedalis* (L.) Verdcourt]" undertaken during the period from 2013-15 at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara are presented in this chapter. The objective of the study was to compare the quality parameters of organically and inorganically grown cowpea, using precise 2D proteome analysis.

4.1 SOIL TEST ANALYSIS

The soil from the two plots were collected and sent for soil testing at Radio Tracer Laboratory, College of Horticulture, Kerala Agricultural University, for nutrient analysis. The results of the soil test data is provided in the Table 4.1. The initial soil nutrient status of the plots suggests that the soil possess similar nutrient levels.

4.2 NUTRIENT ANALYSIS OF FRESHLY HARVESTED PODS

The freshly harvested pods from both the plots were collected (250 g from each plot) and analysed at Radio Tracer Laboratory, College of Horticulture, Kerala Agricultural University. The result is given the Table 4.2 and Table 4.3. In the first harvest lot levels of P, N, K, B and Mg were almost the same. Variation was observed in levels of Ca, S, Fe, and Zn. Organic produce showed higher concentration of Cu (9.7 ppm), whereas higher concentrations of Ca (0.67 per cent), S (0.68 per cent), Fe (100.6 ppm), Zn (61.10 ppm) and Mn (117.30 ppm) were observed in inorganic produce. A huge variation of 86 ppm was observed in the levels of Mn in produce between inorganic plot and organic plot.

In the last harvest lot, levels of N, P, K, B, Cu, S and Mg were found to be almost same for both the plots. Unlike first harvest, higher concentrations of Ca (0.54 per cent) were found in organic produce of last harvest. Higher

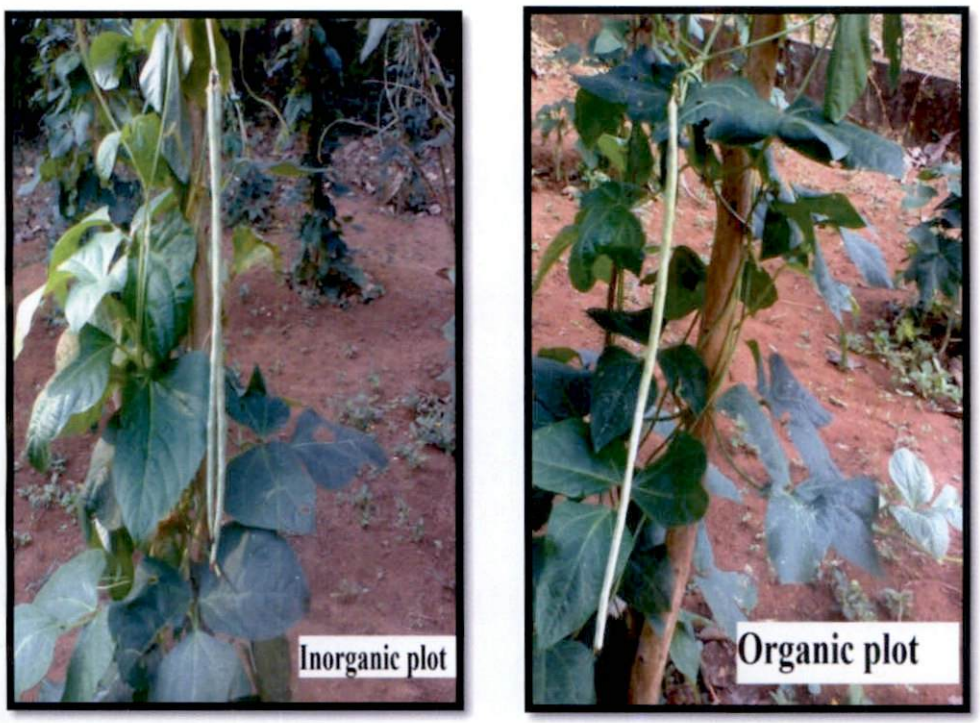


Plate 4.1: The plants grown organically and inorganically

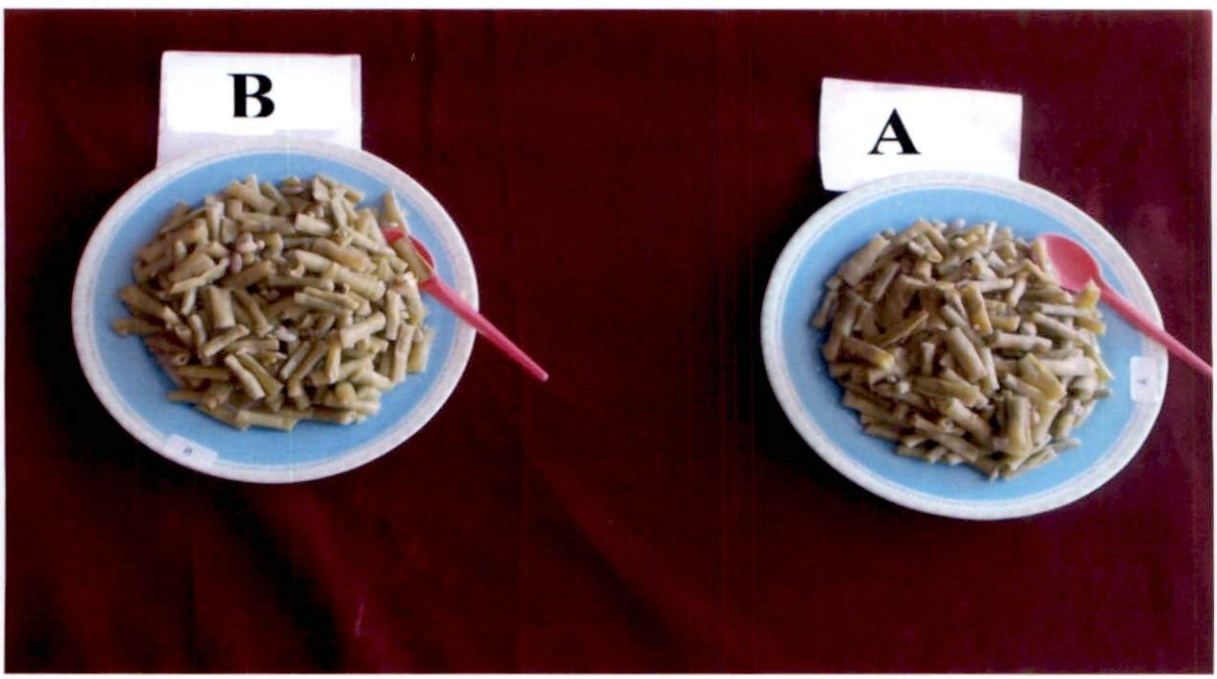


Plate 4.2: Samples prepared for organoleptic analysis; A - organically cultivated produce; B - inorganically cultivated produce.

concentrations of Fe (436.8 ppm), Zn (59.20 ppm) and Mn (161.40 ppm) were observed in inorganic produce. A huge difference of 101ppm and 53.9 ppm were observed in the concentrations of Mn and Fe respectively.

4.3 ANALYSIS OF YIELD AND YIELD PARAMETERS

For vegetable purpose immature pods of yardlong bean is usually harvestable in about two months after direct seeding. Pods at half the diameter of a pencil, before the seeds have filled inside and when they still snap on bending are used for vegetable purpose. The pods were harvested on alternate days and the yields were recorded as and when the pods were harvested. Organic plot (40 m^2), has yielded 12.1 kg, with an average of 327 g/plant whereas the plot under inorganic scheme (40 m^2), has yielded 10.35 kg, with an average of 345.3 g/plant.

4.4 ORGANOLEPTIC ANALYSIS

To eliminate subconscious bias in the panellists, cryptic labelling was employed. Samples were labelled A and B. The cooked pods were served to a panel of twenty one judges in a tasting room devoid of environmental interferences. The hedonic scale (Annexure V) and the score card (Annexure V) were also given to them. Their scores were then analysed through Mann-Whitney Test. The results of this test shows that, there is no significant difference between organically and inorganically grown pods in colour, taste, flavour, stringiness and general appeal. These results are presented in the Table 4.4.

4.5 EXTRACTION AND QUANTIFICATION OF TOTAL PROTEIN

Total protein was extracted from the pods by TCA/Acetone precipitation, which was then solubilized in lysis buffer (Annexure II). This sample was then used for protein quantification by Lowry's method (Lowry *et al.*, 1951). The concentrations of the protein in the samples were derived from the standard curves. The protein concentrations from immature pods cultivated organically and inorganically were found be 22.70 µg/µl and 22.68 µg/µl, respectively and that of

Table 4.1: Nutrient status of soil prior to the start of the experiment

Parameters	Organic plot		Inorganic plot	
	Quantity	Remarks	Quantity	Remarks
pH	5.1	Strongly acidic	5.1	Strongly acidic
Electrical Conductivity (dS/m)	0.03	Normal	0.03	Normal
Organic Carbon (%)	1.95	High	1.95	High
Total Nitrogen (%)	0.168	High	0.168	High
Available Phosphorus (kg/ha)	19.41	Medium	22.09	Medium
Available Potassium (kg/ha)	129.92	Medium	116.48	Medium
Available Calcium (mg/kg)	541.50	Sufficient	546.50	Sufficient
Available Magnesium (mg/kg)	93.0	Deficient	111.25	Deficient
Available Sulphur (mg/kg)	12.50	Sufficient	8.42	Sufficient
Micronutrients				
Copper (mg/kg)	4.13	Sufficient	3.09	Sufficient
Iron (mg/kg)	8.25	Sufficient	6.00	Sufficient
Zinc (mg/kg)	0.86	Deficient	1.19	Sufficient
Manganese (mg/kg)	15.22	Sufficient	15.10	Sufficient
Boron (mg/kg)	0.31	Deficient	0.34	Deficient

Table 4.2 Nutrient status of the pods harvested from organic and inorganic plots (first harvest)

Nutrient	Organically grown	Inorganically grown
Nitrogen (%)	3.92	4.85
Phosphorous (%)	0.24	0.26
Potassium (%)	0.93	0.98
Calcium (%)	0.44	0.67
Magnesium (%)	0.32	0.34
Sulphur (%)	0.57	0.68
Copper (ppm)	9.7	6.6
Iron (ppm)	83.70	100.60
Zinc (ppm)	40.00	61.10
Manganese (ppm)	31.30	117.30
Boron (ppm)	7.22	6.11

Table 4.3 Nutrient status of the pods harvested from organic and inorganic plots (last harvest)

Nutrient	Organically grown	Inorganically grown
Nitrogen (%)	4.80	4.86
Phosphorous (%)	0.43	0.37
Potassium (%)	1.02	1.02
Calcium (%)	0.54	0.18
Magnesium (%)	0.45	0.44
Sulphur (%)	0.78	0.81
Copper (ppm)	31.10	29.00
Iron (ppm)	382.9	436.8
Zinc (ppm)	47.20	59.20
Manganese (ppm)	60.40	161.40
Boron (ppm)	25.64	32.82

Table 4.4 Organoleptic qualities of pods harvested from organic and inorganic plots analysed through Mann-Whitney test

Quality parameters		Mean Rank	Mann-Whitney U	Z	Significance
Colour	Organically grown pods	22.34	238.50	-0.089	NS
	Inorganically grown pods	22.66			
Taste	Organically grown pods	19.36	173.00	-1.676	NS
	Inorganically grown pods	25.64			
Flavour	Organically grown pods	18.98	164.50	-1.876	NS
	Inorganically grown pods	26.02			
Stringiness	Organically grown pods	20.91	207.00	-0.843	NS
	Inorganically grown pods	24.09			
General appeal	Organically grown pods	19.84	183.50	-1.434	NS
	Inorganically grown pods	25.16			

No. of observation for each sample = 22

Table 4.5 Amount of protein recovered from organically and inorganically cultivated immature and mature yardlong bean pods

Sample		Protein per cent
Organically cultivated pods	Immature	1.58 ± 0.13
	Mature	1.79 ± 0.12
Inorganically cultivated pods	Immature	1.59 ± 0.18
	Mature	1.76 ± 0.13

mature pods cultivated organically and were found to be 22.86 µg/µl and 22.72 µg/µl respectively and the recoverable protein percentage from samples is presented in Table 4.5.

4.6 SDS-PAGE ANALYSIS OF PROTEIN EXTRACTED FROM MATURE AND IMMATURE PODS FROM ORGANIC AND INORGANIC PLOT

Laemmli (1970) protocol for the SDS-PAGE analysis was further appropriated to the laboratory conditioned by increasing the APS concentration from 0.5 per cent to 0.8 per cent, to promote the polymerization of the SDS-PAGE gel. The concentrations of Tris in separating and stacking buffer were slightly increased from 1.5 M to 1.87 M and from 0.5 M to 0.6 M, respectively (Shiny, 2013).

The SDS-PAGE analysis of isolated protein has shown distinct expression of bands for mature and immature pods, but differential bands were not observed between the organically and inorganically grown cowpea. A total of 11 bands were observed in all the four lanes (Plate 4.3). Differential bands were observed between mature and immature pods but no differential bands were identified between organic and inorganic samples, hence the samples were subjected to 2DE.

4.7 TWO DIMENSIONAL GEL ELECTROPHORESIS OF PROTEIN EXTRACTED FROM MATURE AND IMMATURE PODS FROM ORGANIC AND INORGANIC PLOT

The first dimension IEF was performed using 7cm and 17 cm linear IPG strips with pH range of 3.0-10.0. For the second dimension, the IPG strips were incubated with rehydration buffer I and rehydration buffer II for 15 minutes each and subsequently placed onto 12 per cent polyacrylamide gels prepared as described by Laemmli (1970). The 2D-PAGE gels were visualized by staining with Colloidal Coomassie Blue G-250 as described by Newsholme (2000). The 2DE gels obtained were separated into two sets, i.e., mature and immature, each

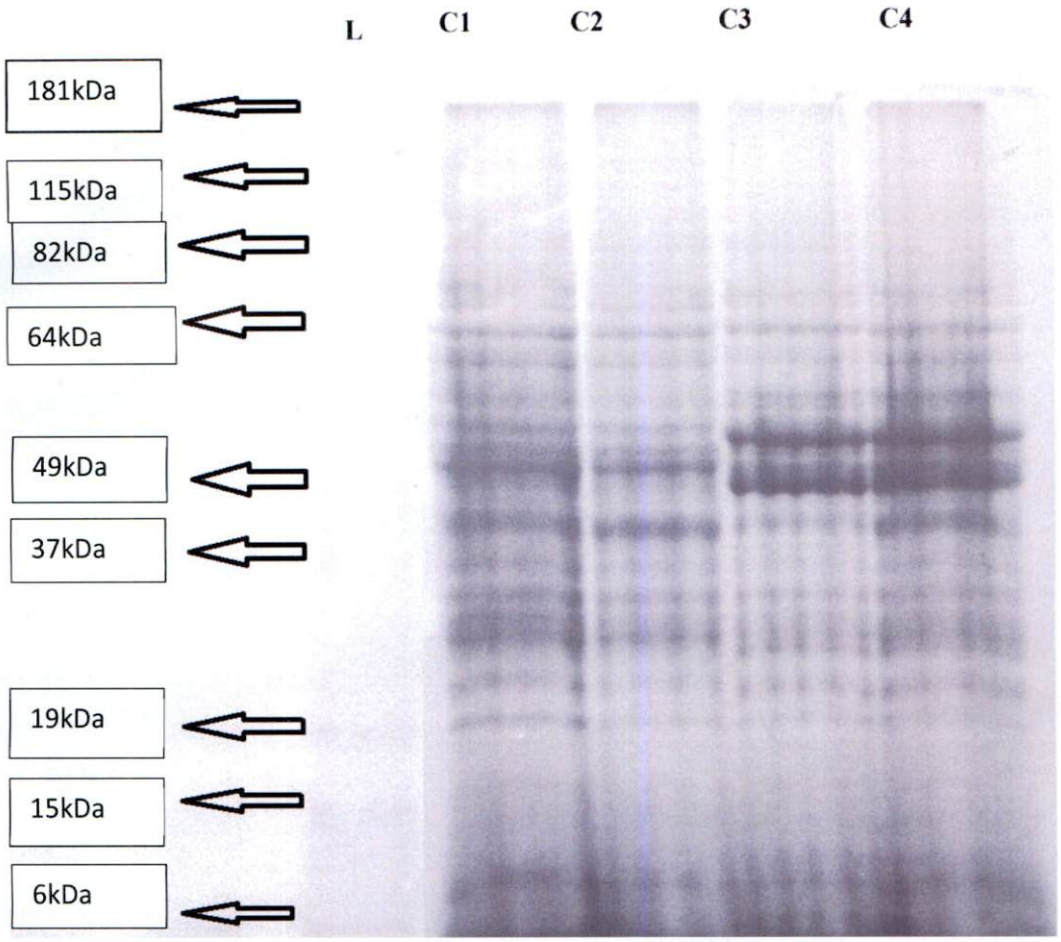


Plate 4.3. Protein profile of all the four samples using SDS-PAGE

L: Protein ladder (Invitrogen Benchmark TM prestained protein ladder)

C1: Protein from inorganically cultivated immature pods

C2: Protein from organically cultivated immature pods

C3: Protein from inorganically cultivated mature pods

C4: Protein from organically cultivated mature pods

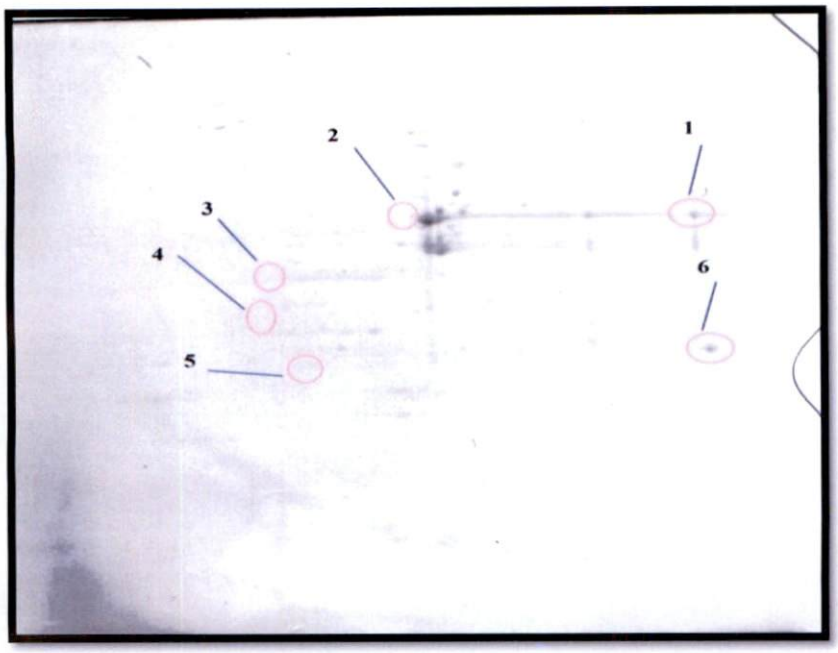


Plate 4.4. Protein profile of protein from organically cultivated mature pods through 2DE

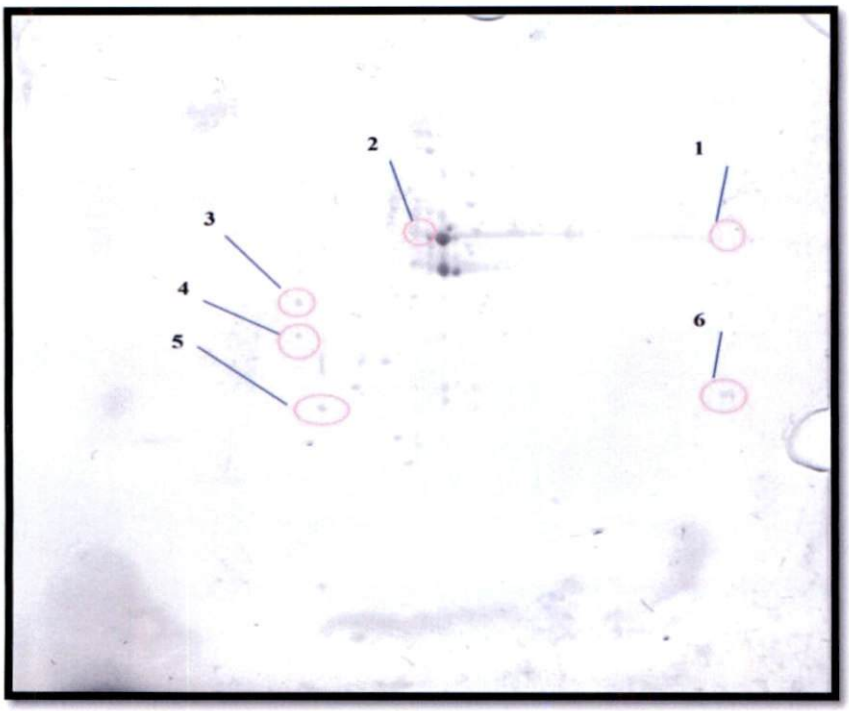


Plate 4.5. Protein profile of protein from inorganically cultivated mature pods through 2DE

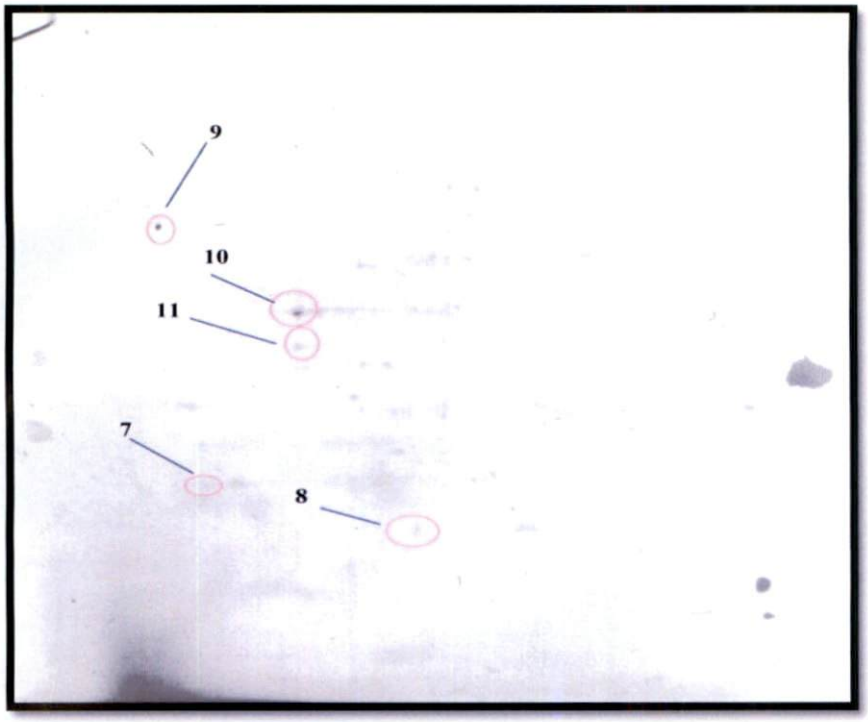


Plate 4.6. Protein profile of protein from organically cultivated immature pods through 2DE

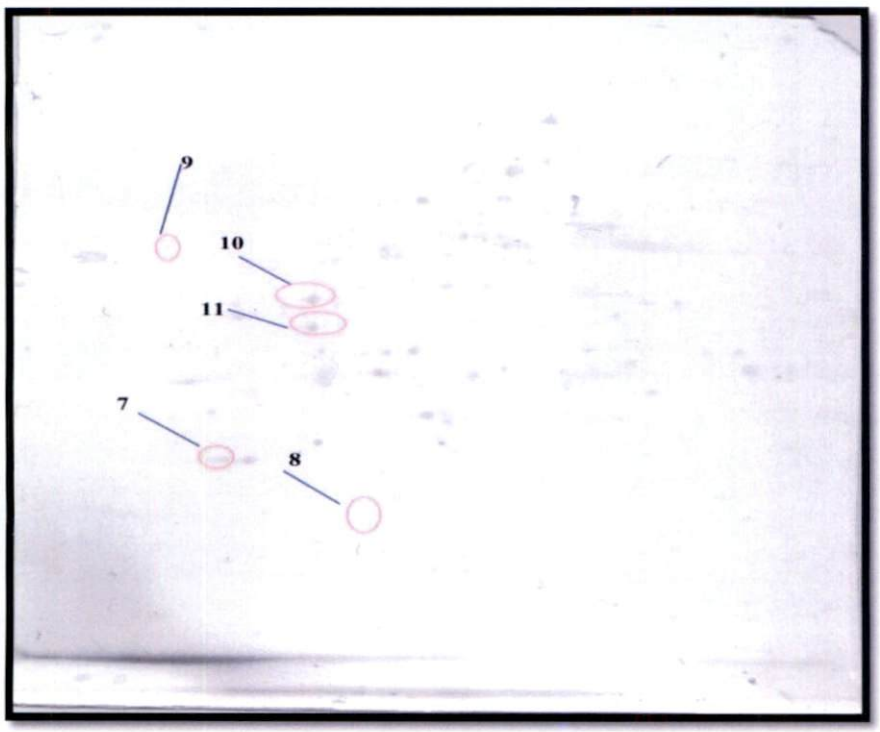


Plate 4.7. Protein profile of protein from inorganically cultivated immature pods through 2DE

set had two gels one of organically and another of inorganically cultivated pods. The gels were analysed set wise; the differential spots between organic and inorganically grown pods were cut out from the gel for further analysis. Gel of immature pods grown organically (Plate 4.6) contained 31 spots and that from immature pods grown inorganically (Plate 4.7) had 23 spots. 33 spots were observed in gel of mature pods grown organically (Plate 4.4) and 26 spots were present in gel of mature pods grown inorganically (Plate 4.5). From set1 containing gels from mature pods six spots and from set 2 five spots, a total of 11 spots were identified, which were carried for further analysis.

4.8 PEPTIDE MASS FINGERPRINTING BY MALDI-TOF/MS AND *IN-SILICO* ANALYSIS

After comparing the 2DE gel pictures of organic and inorganic samples, 8 differential spots, 2 upregulated and 1 down regulated spots were identified and cut from the gel (details about the selected spots are given in Table 4.6) and sent for peptide mass fingerprinting by MALDI-ToF/MS (Outsourced from Sandor sequencing, Hyderabad) for the identification of the protein. The protein from the spots were eluted from the gel, digested with trypsin and analysed by MALDI-ToF mass spectrometry (Stults, 1995). Mass spectrometric analysis of intact digest mixture from a spot thus provided a set of peptide molecular masses with the corresponding peaks separately for the proteins from all the 11 spots. The mass spectrometry data having the peak values of peptide mass fingerprint of the 11 spots were analysed with Mascot Server software from Matrix Science which is basically utilized for the identification, characterization and quantification of proteins using mass spectrometry data. As most of the protein showed similarity with hypothetical and uncharacterized protein, the protein showing maximum similarity was then used as query for Blastp and Smart BLAST to get the closely related proteins. Details of peptide mass fingerprint and in-silico analysis for each of the spots is given n Table 4.6.

Table 4.6 The excised protein spots and their expression in immature and mature yardlong bean pods from organic and inorganic plots

Protein spot ID	Immature pods		Mature pods	
	Inorganic C1	Organic C2	Inorganic C3	Organic C4
Spot 1	–	–	–	+
Spot 2	–	–	+	–
Spot 3	–	–	+	–
Spot 4	–	–	+	–
Spot 5	–	–	+	–
Spot 6	–	–	+	++
Spot 7	+	–	–	–
Spot 8	–	+	–	–
Spot 9	–	+	–	–
Spot 10	+	++	–	–
Spot 11	++	+	–	–

(-) –no expression; (+)- expression

Peptide mass spectra and other details such as m/z (mass to charge ratio), S/N (signal to noise ratio), quality factor, resolution, intensity, area of each of the masses identified using MALDI-ToF/MS of spot 1 extracted from organically cultivated mature pods is depicted in Fig. 4.1. Sixty three masses were identified with S/N greater than 5. These were then utilized in database searching using MASCOT server software. Protein from spot1 was represented by a mixture containing uncharacterized protein LOC104108377 isoform X1 [*Nicotiana tomentosiformis*] and hypothetical protein POPTR_0013s02260g [*Populus trichocarpa*]. This mixture had a total score of 107 and it had 24 matches. Uncharacterized protein LOC104108377 isoform X1 [*Nicotiana tomentosiformis*] and hypothetical protein POPTR_0013s02260g [*Populus trichocarpa*] were then used as query for BLASTp and SmartBLAST (Fig. 4.2 to Fig. 4.6) Uncharacterized protein LOC104108377 isoform X1 [*Nicotiana tomentosiformis*] showed maximum similarity with Uncharacterized protein LOC104108377 isoform X2 [*Nicotiana tomentosiformis*] and hypothetical protein POPTR_0013s02260g [*Populus trichocarpa*] showed maximum similarity with F-box protein At1g55000-like [*Populus euphratica*].

Peptide mass spectra and details of each of the masses identified using MALDI-ToF/MS of spot 2 extracted from inorganically cultivated mature pods is depicted in Fig 4.7. The number of masses identified were 129, which were used in MASCOT analysis and the protein showed maximum similarity with hypothetical protein OsJ_17213 [*Oryza sativa* Japonica Group], with a total score of 93 and 19 matches. BLASTp and SmartBLAST of hypothetical protein OsJ_17213 [*Oryza sativa* Japonica Group] showed maximum similarity with hypothetical protein OsI_18563 [*Oryza sativa* Indica Group] (Fig. 4.8 to Fig. 4.10).

Peptide mass spectra and details of each of the masses identified using MALDI-ToF/MS of spot 3 extracted from inorganically cultivated mature pods is depicted in Fig 4.11. The number of masses identified was 54. MASCOT search results suggested that the protein is represented by hypothetical protein

LR48_Vigan07g225300 [*Vigna angularis*], with a total score of 145 and 17 matches. BLASTp and SmartBLAST of hypothetical protein LR48_Vigan07g225300 [*Vigna angularis*] suggested that this hypothetical protein is similar to hypothetical protein PHAVU_001G067300g [*Phaseolus vugaris*] and alcohol dehydrogenase1 [*Glycine soja*] (Fig. 4.12 to Fig. 4.14).

Peptide mass spectra and details of each of the masses identified using MALDI-ToF/MS of spot 4 extracted from inorganically cultivated mature pods is depicted in Fig 4.15. The number of masses identified was 97. MASCOT search results suggested that the protein is represented by hypothetical protein LR48_Vigan07g183600 [*Vigna angularis*] with a total score of 161 and 19 matches. BLASTp and SmartBLAST of hypothetical protein LR48_Vigan07g183600 [*Vigna angularis*], showed that it is similar to Fructose-bisphosphate aldolase, cytoplasmic isozyme (*Glycine soja*) (Fig. 4.16 to Fig. 4.18).

Peptide mass spectra and details of each of the masses identified using MALDI-ToF/MS of spot 5 extracted from inorganically cultivated mature pods is depicted in Fig. 4.19. The number of masses identified was 73. MASCOT search results suggested that the protein is represented by hypothetical protein LR48_Vigan02g005500 [*Vigna angularis*] with a total score of 140 and 13 matches. BLASTp and SmartBLAST of hypothetical protein LR48_Vigan02g005500 [*Vigna angularis*] suggests it is similar to that hypothetical protein LR48_Vigan02g006400 [*Vigna angularis*], chain A, crystal structure of Hemopexin Fold protein Cp4 from cowpea and mungbean seed albumin (Fig. 4.20 to Fig. 4.22).

Peptide mass spectra and details of each of the masses identified using MALDI-ToF/MS of spot 6 present in both organically and inorganically cultivated mature pods is depicted in Fig. 4.23. The number of masses identified was 177. MASCOT search results suggested that the protein is represented by a mixture containing aldehyde dehydrogenase family 2 member C4-like [*Musa*

acuminata subsp. *malaccensis*], hypothetical protein SOVF_071130 [Spinacia oleracea] and flocculation protein FLO11-like [*Brassica oleracea* var. *oleracea*] with a total score of 108 and 78 matches. BLASTp and SmartBLAST of hypothetical protein SOVF_071130 [*Spinacia oleracea*] suggests it is similar to root phototropism protein 2 [*Beta vulgaris* sub sp. *vulgaris*] (Fig. 4.24).

Peptide mass spectra and details of each of the masses identified using MALDI-ToF/MS of spot 7 present in inorganically cultivated immature pods is depicted in Fig. 4.25. The numbers of masses identified were 152. MASCOT search results suggested that the protein is represented by a mixture containing protein PLASTID MOVEMENT IMPAIRED 2-like [*Camelina sativa*] and trans-resveratrol di-O-methyltransferase-like [*Cucumis sativus*] with a total score of 106 and 39 matches (Fig. 4.26).

Peptide mass spectra and details of each of the masses identified using MALDI-ToF/MS of spot 8 present in organically cultivated immature pods is depicted in Fig. 4.27. The number of masses identified was 122. MASCOT search results suggested that the protein is represented by hypothetical protein EUTSA_v10007083mg [*Eutrema salsugineum*], with a total score of 87 and 22 matches. BLASTp and SmartBLAST of hypothetical protein EUTSA_v10007083mg [*Eutrema salsugineum*] suggests it is similar to uncharacterized protein (Fig. 4.28 to Fig. 4.30).

Peptide mass spectra and details of each of the masses identified using MALDI-ToF/MS of spot 9 present in organically cultivated immature pods is depicted in Fig. 4.31. The number of masses identified was 135. MASCOT search results suggested that the protein is represented by hypothetical protein EUTSA_v10007083mg [*Eutrema salsugineum*], with a total score of 92 and 35 matches. BLASTp and SmartBLAST of hypothetical protein EUTSA_v10007083mg [*Eutrema salsugineum*] suggests it is similar to uncharacterized protein (Fig. 4.32 to Fig. 4.34).

Peptide mass spectra and details of each of the masses identified using MALDI-ToF/MS of spot 10 present in both organically and inorganically cultivated immature pods is depicted in Fig. 4.35. The number of masses identified was 42. MASCOT search results suggested that the protein is represented by hypothetical protein LR48_Vigan07g225300 [*Vigna angularis*], with a total score of 222 and 20 matches (Fig. 4.36). This also suggests that spot 3 and spot 10 are represented by the same protein.

Peptide mass spectra and details of each of the masses identified using MALDI-ToF/MS of spot 11 present in both organically and inorganically cultivated immature pods is depicted in Fig. 4.37. The number of masses identified was 32. MASCOT search results suggested that the protein is represented by hypothetical proteinLR48_Vigan07g183600 [*Vigna angularis*], with a total score of 117 and 10 matches (Fig. 4. 52 to Fig. 4. 54). This also suggests that spot 4 and spot 11 are represented by the same protein. The results from Mascot search has been summarised in Table 4.7.

Table 4.7 Summarized Mascot analysis results of protein spots expressed in immature and mature yardlong bean pods from organic and inorganic plots

Protein spot ID	Mascot analysis result: Protein accession	Details of the protein
Spot1 (C3)	gi|697123397	PREDICTED: uncharacterized protein LOC104108377 isoform X1 [*Nicotiana tomentosiformis*]
	gi|566198963	hypothetical protein POPTR_0013s02260g [*Populus trichocarpa*]
Spot 2 (C4)	gi|222630290	hypothetical protein OsJ_17213 [*Oryza sativa* Japonica Group]
Spot 3 (C4)	gi|920705324	hypothetical protein LR48_Vigan07g225300 [*Vigna angularis*]
Spot 4 (C4)	gi|920704907	hypothetical protein LR48_Vigan07g183600 [*Vigna angularis*]
Spot 5 (C4)	gi|920690680	hypothetical protein LR48_Vigan02g005500 [*Vigna angularis*]
Spot 6 (C3 and C4)	gi|695031782	PREDICTED: aldehyde dehydrogenase family 2 member C4-like [*Musa acuminata* subsp. *malaccensis*]

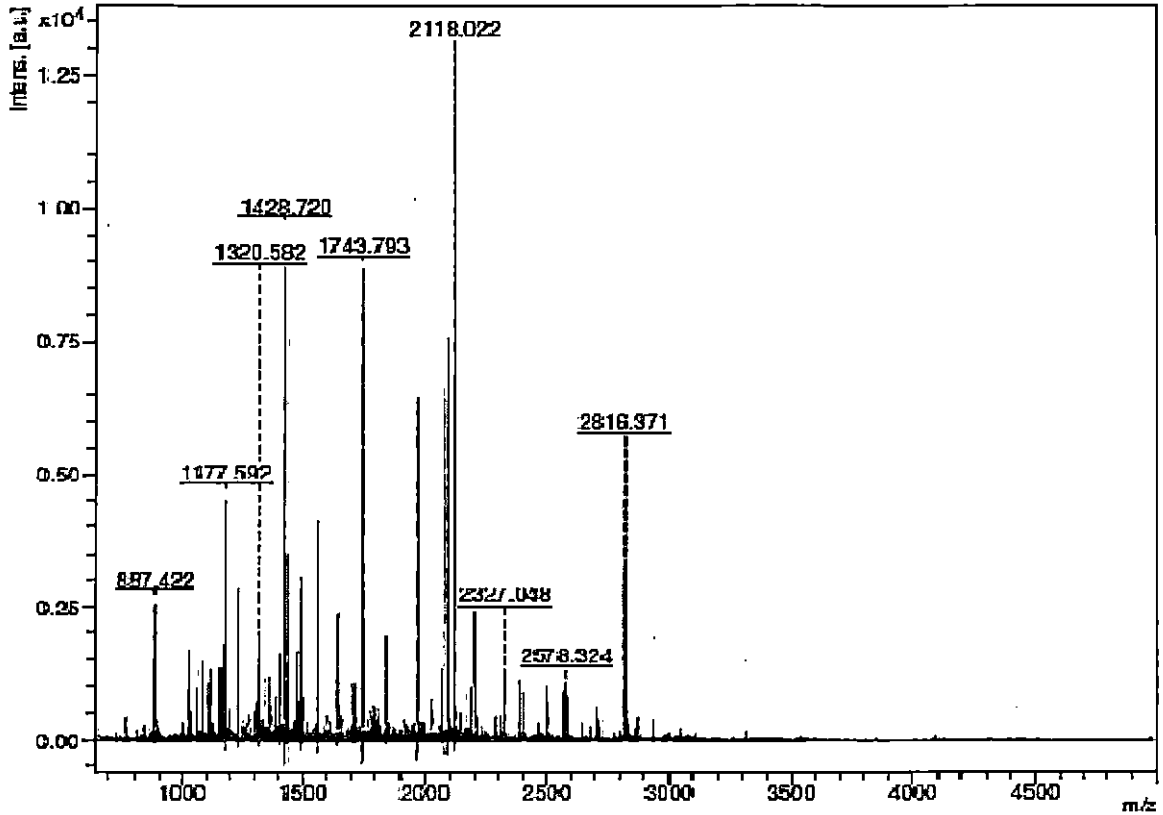
C1 and C2 - immature pods cultivated inorganically and organically; C3 and C4 - mature pods cultivated inorganically and organically

Table 4.7 Summarized Mascot analysis results of protein spots expressed in the pods in immature and mature yardlong bean pods from organic and inorganic plots (contd.)

Spot ID	Mascot analysis result: Protein accesion	Details of the protein
Spot 6 (C3 and C4)	gi|922481517	PREDICTED: flocculation protein FLO11-like [*Brassica oleracea* var. *oleracea*]
Spot 7 (C2)	gi|727509054	PREDICTED: protein PLASTID MOVEMENT IMPAIRED 2-like [*Camelina sativa]*
	gi|778726730	PREDICTED: trans-resveratrol di-O-methyltransferase-like [*Cucumis sativus*]
Spot 8 (C1)	gi|567149521	hypothetical protein EUTSA_v10007083mg [*Eutrema salsugineum*]
Spot 9 (C1)	gi|567149521	hypothetical protein EUTSA_v10007083mg [*Eutrema salsugineum*]
Spot 10 (C1 and C2)	gi|920705324	hypothetical protein LR48_Vigan07g225300 [*Vigna angularis*]
Spot 11 (C1 and C2)	gi|920704907	hypothetical protein LR48_Vigan07g183600 [*Vigna angularis*]

C1 and C2 - immature pods cultivated inorganically and organically; C3 and C4 - mature pods cultivated inorganically and organically

Fig. 4.1: Peptide mass fingerprint of the spot 1 protein extracted from mature organically cultivated pods, obtained by MALDI-ToF Mass spectrometry



m/z	S/N	Quality Fac.	Res.	Intens.	Area
769.496	9.6	2098	6026	445.05	93
887.422	54.8	12040	6940	2742.70	659
1028.598	32.7	8805	7803	1792.66	483
1037.518	9.2	935	7963	514.24	137
1064.598	17.2	2822	7239	902.20	300
1082.586	25.3	8983	7631	1467.79	437
1107.538	18.2	5310	7848	1072.11	324
1109.468	7.0	486	6776	416.06	142
1119.601	20.0	7877	6822	1253.80	430
1160.567	22.8	14175	7571	1373.51	456
1165.586	7.1	727	5510	445.33	208
1168.653	29.9	9438	8504	1828.47	558
1173.655	7.8	610	7942	492.49	157
1177.592	75.6	19784	8103	4748.45	1540
1179.594	28.6	6530	7905	1810.52	595
1193.609	9.8	329	7476	621.71	216
1254.668	46.6	15679	8023	3038.07	1072
1257.687	7.4	1788	8453	483.80	171
1302.681	7.7	860	7252	516.11	220
1307.663	8.8	374	8763	585.80	209
1313.680	11.7	1543	8793	785.41	283
1320.582	51.4	18186	8157	2028.91	823
1357.703	17.3	8210	8533	1220.66	474
1365.647	7.8	1295	6545	556.91	278
1390.691	12.1	228	8862	870.38	337
1406.729	25.8	3324	8780	1812.25	733
1428.720	154.3	39167	8563	9782.57	4131
1454.765	19.1	2478	9120	1405.33	593
1462.732	23.6	14980	9028	1716.21	734
1481.740	23.0	10889	8469	1682.34	760
1493.726	41.2	28791	9082	3038.87	1313
1503.681	10.4	2506	7691	761.36	388
1556.823	29.1	36028	9201	4424.65	2031
1638.844	54.4	12310	9590	2633.61	1300
1707.749	13.7	7790	8739	1038.24	609
1743.793	118.3	51200	8550	9041.40	5573
1779.905	6.4	472	9363	487.06	281
1791.723	6.5	1117	7525	492.24	358

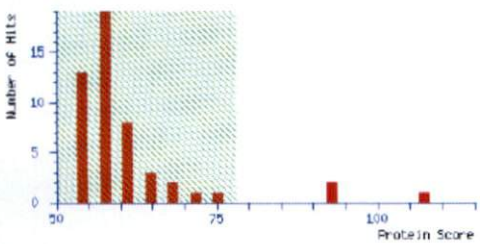
Fig. 4.2. *In-silico* analysis of spot 1 protein using Mascot Server software

MATRIX SCIENCE Mascot Search Results

```
User            : joy
Email           : joyprashant88@gmail.com
Search title    :
Database        : NCBInr 20151012 (72776944 sequences; 26510890717 residues)
Taxonomy        : Viridiplantae (Green Plants) (3263908 sequences)
Timestamp       : 15 Oct 2015 at 06:06:56 GMT
Top Score       : 107 for Mixture 1, gi|697123397 + gi|566198963
```

Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 78 are significant (p<0.05).



Index

	Accession	Mass	Score	Description
1.	Mixture 1		107	gi|697123397 + gi|566198963
2.	gi|697123397	60002	94	PREDICTED: uncharacterized protein LOC104108377 isoform X1 [Nicotiana tomentosiformis]
3.	gi|697123399	59874	93	PREDICTED: uncharacterized protein LOC104108377 isoform X2 [Nicotiana tomentosiformis]
4.	gi|302824717	159574	74	hypothetical protein SELMODRAFT_187668 [Selaginella moellendorffii]
5.	gi|566198963	37901	70	hypothetical protein POPTR_0013s02260g [Populus trichocarpa]
6.	gi|920716792	59511	67	hypothetical protein LR48_Vigan10g277200 [Vigna angularis]
7.	gi|727585205	60081	67	PREDICTED: protein disulfide isomerase-like 1-6 [Camelina sativa]
8.	gi|568845352	113485	65	PREDICTED: uncharacterized protein LOC102625808 isoform X1 [Citrus sinensis]
9.	gi|567894064	113573	65	hypothetical protein CICLE_v10018685mg [Citrus clementina]
10.	gi|641857511	113545	65	hypothetical protein CISIN_1g0019191mg [Citrus sinensis]
11.	gi|356534442	141343	63	PREDICTED: uncharacterized protein LOC100805723 isoform X1 [Glycine max]
12.	gi|302794426	159620	61	hypothetical protein SELMODRAFT_177444 [Selaginella moellendorffii]
13.	gi|727525408	35854	61	PREDICTED: probable 3-hydroxyisobutyrate dehydrogenase-like 1, mitochondrial [Camelina sativa]
14.	gi|920688956	11775	61	hypothetical protein LR48_Vigan01g249500 [Vigna angularis]
15.	gi|475588046	107959	60	Lipoxygenase 2.2, chloroplastic [Aegilops tauschii]
16.	gi|595814820	143827	60	hypothetical protein PRUPE_ppa000331mg [Prunus persica]
17.	gi|734420388	114870	60	C2 and GRAM domain-containing protein [Glycine soja]
18.	gi|567143603	45190	59	hypothetical protein EUTSA_v10005699mg, partial [Eutrema salsugineum]
19.	gi|474388044	57349	59	Zinc finger CCCH domain-containing protein 4 [Triticum urartu]
20.	gi|161138112	79550	58	cryptochrome 1a [Triticum aestivum]

Fig. 4.3. *In-silico* analysis of gi|697123397 protein using BLASTp

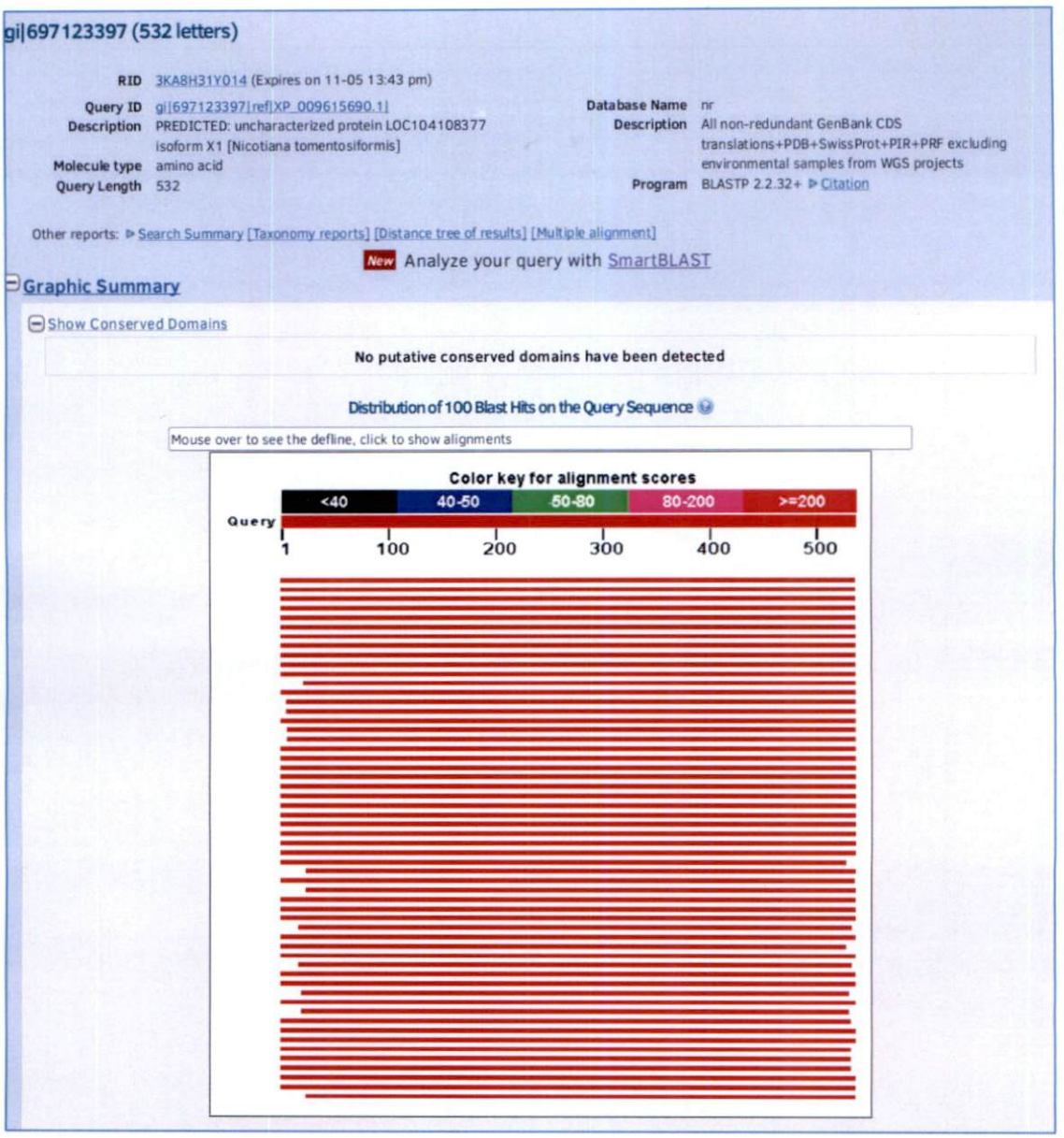


Fig. 4.4. *In-silico* analysis of gi|697123397 protein using smart BLAST

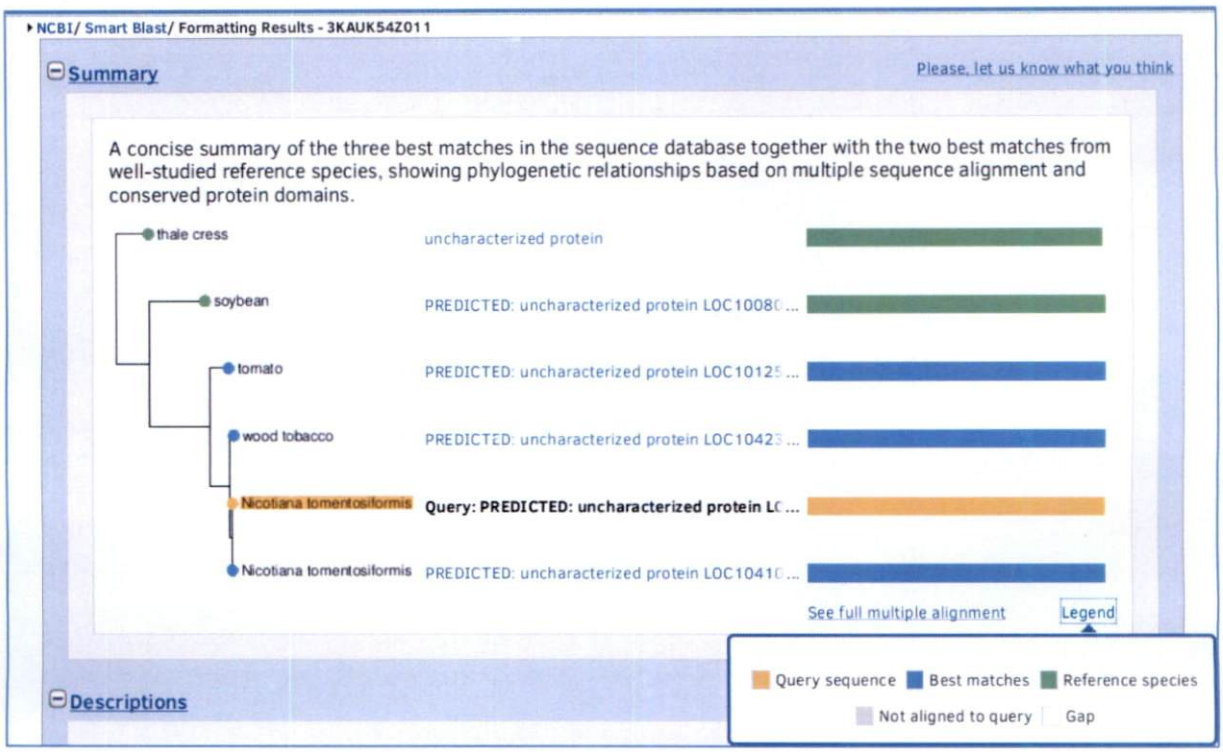


Fig. 4.5. *In-silico* analysis of gi|566198963 protein using BLASTp

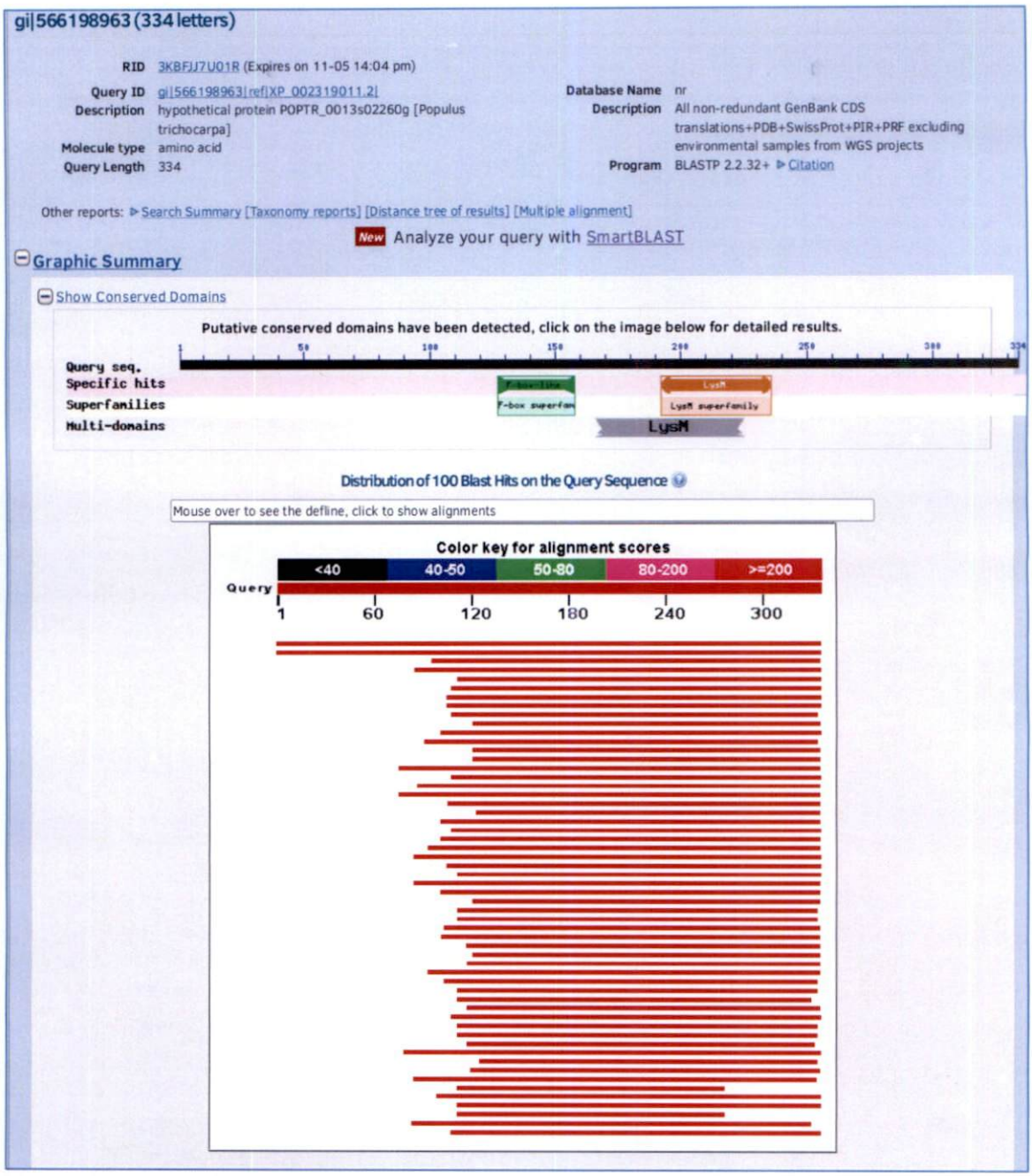


Fig. 4.6. *In-silico* analysis of gi|566198963 protein using smart BLAST

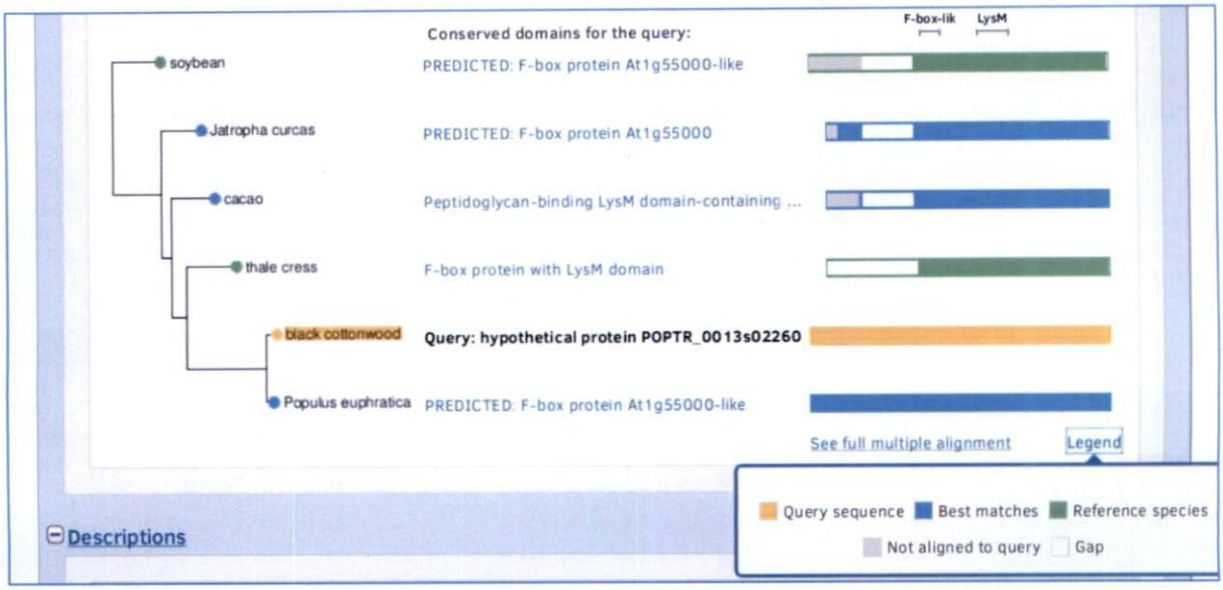
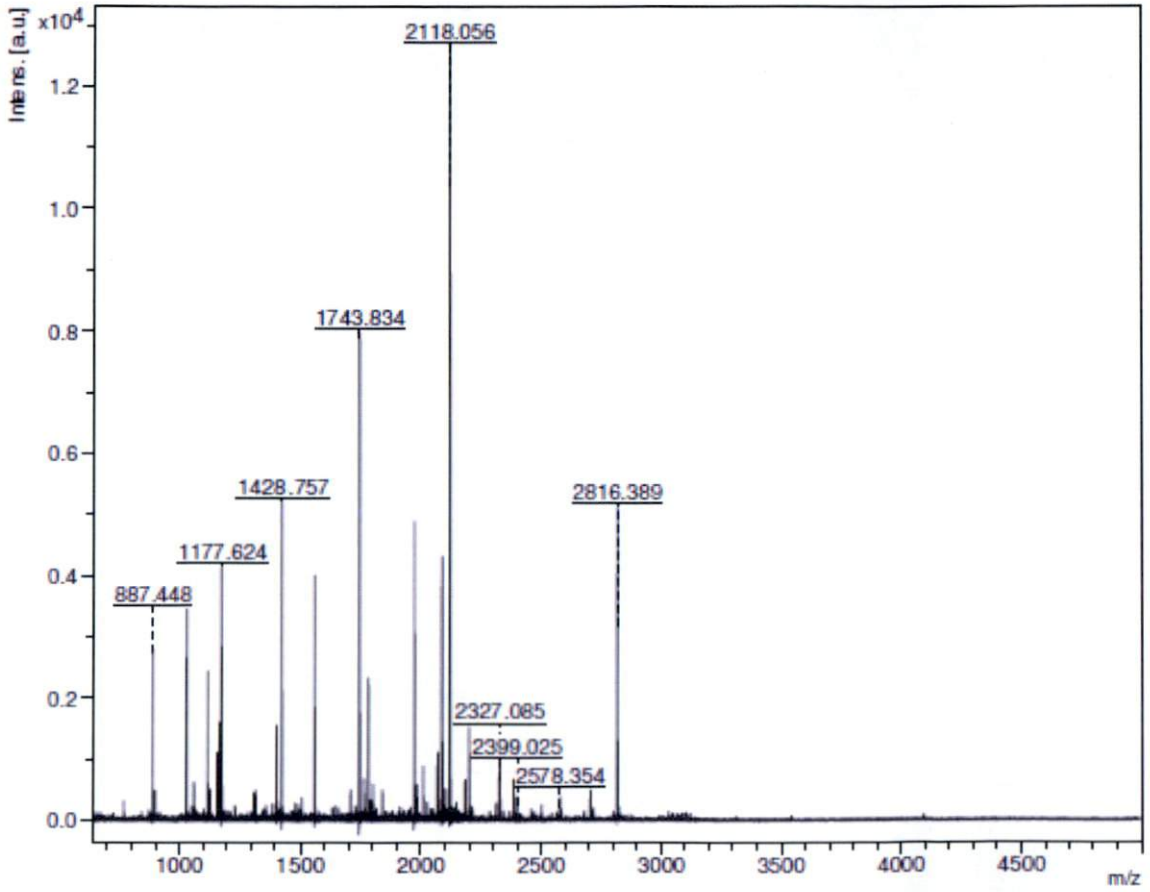


Fig. 4.7: Peptide mass fingerprint of the spot 2 protein extracted from mature inorganically cultivated pods, obtained by MALDI-ToF Mass spectrometry



m/z	S/N	Quality Fac.	Res.	Intens.	Area
768.518	7.3	704	3666	258.80	91
887.448	74.3	13739	4411	2726.81	999
899.458	11.7	1454	4456	428.66	159
1028.642	91.6	63088	6131	3426.25	1148
1061.612	19.0	981	6757	732.08	237
1119.660	61.9	33738	6237	2447.23	919
1131.658	11.6	4268	6115	455.75	175
1160.603	25.5	6403	5645	1016.89	448
1168.703	40.3	20982	6716	1628.94	619
1177.624	102.7	39128	6247	4156.52	1694
1313.718	10.1	2279	7463	422.10	170
1320.618	10.1	1107	6163	424.24	211
1406.760	36.9	7873	7096	1622.07	793
1428.757	118.4	29586	6894	5218.28	2700
1503.683	6.8	1507	6651	305.54	179
1556.856	87.5	19025	7052	3973.44	2343
1707.793	9.4	2237	8069	443.85	278
1743.834	168.0	54674	7401	7896.19	5626
1762.915	13.0	502	6554	609.79	495
1779.940	43.8	7917	7646	2069.85	1472
1800.860	10.1	1756	6500	475.97	407
1838.918	8.9	2497	6854	416.90	354
1970.918	100.5	26466	7993	4336.93	3456
1982.928	11.6	2383	7933	496.01	403
2009.063	17.3	3141	7697	728.33	617
2068.877	22.3	505	6003	900.93	1017
2085.876	90.6	38704	7705	3622.43	3236
2102.018	7.7	894	3207	300.52	638
2118.056	265.7	41014	7594	10365.47	9576
2183.080	11.7	1194	4311	430.38	725
2198.984	34.3	26983	8320	1251.39	1104
2312.183	6.4	1138	6729	206.12	266
2327.085	20.5	3003	7365	655.07	702
2383.964	14.8	2460	7250	451.83	511
2399.025	7.9	1653	6776	239.04	290
2578.354	7.5	555	6020	190.73	287
2705.137	11.7	1152	7331	263.12	351
2816.389	155.0	9902	8485	3166.31	3935

Fig. 4.8. *In-silico* analysis of spot 2 protein using Mascot Server software

MATRIX SCIENCE Mascot Search Results

```
User            : joy
Email           : joyprashant88@gmail.com
Search title    :
Database        : NCBInr 20151012 (72776944 sequences; 26510890717 residues)
Taxonomy        : Viridiplantae (Green Plants) (3263908 sequences)
Timestamp       : 15 Oct 2015 at 06:58:38 GMT
Top Score       : 93 for gi|222630290, hypothetical protein OsJ_17213 [Oryza sativa Japonica Group]
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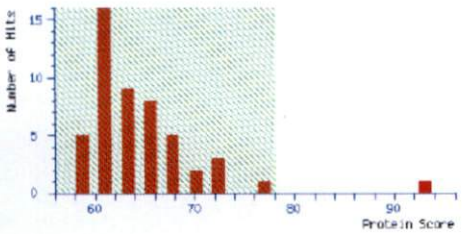
Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 78 are significant (p<0.05).



Index

	Accession	Mass	Score	Description
1.	gi|222630290	37171	93	hypothetical protein OsJ_17213 [Oryza sativa Japonica Group]
2.	gi|674866684	41894	76	BnaCnng53400D [Brassica napus]
3.	gi|697123397	60002	73	PREDICTED: uncharacterized protein LOC104108377 isoform X1 [Nicotiana tomentosiformis]
4.	gi|697123399	59874	72	PREDICTED: uncharacterized protein LOC104108377 isoform X2 [Nicotiana tomentosiformis]
5.	gi|697151580	230203	71	PREDICTED: uncharacterized protein LOC104120049 isoform X2 [Nicotiana tomentosiformis]
6.	gi|697151578	230472	71	PREDICTED: uncharacterized protein LOC104120049 isoform X1 [Nicotiana tomentosiformis]
7.	gi|356520996	55587	70	PREDICTED: glycerol-3-phosphate 2-O-acyltransferase 6-like [Glycine max]
8.	gi|527185015	32618	69	hypothetical protein M569_16181, partial [Genlisea aurea]
9.	gi|743875705	26783	68	PREDICTED: uncharacterized protein LOC105132954 isoform X2 [Populus euphratica]
10.	gi|743875700	26911	68	PREDICTED: uncharacterized protein LOC105132954 isoform X1 [Populus euphratica]
11.	gi|303283950	62325	68	predicted protein [Micromonas pusilla CCMP1545]

Fig. 4.9. *In-silico* analysis of gi|222630290 protein using BLASTp

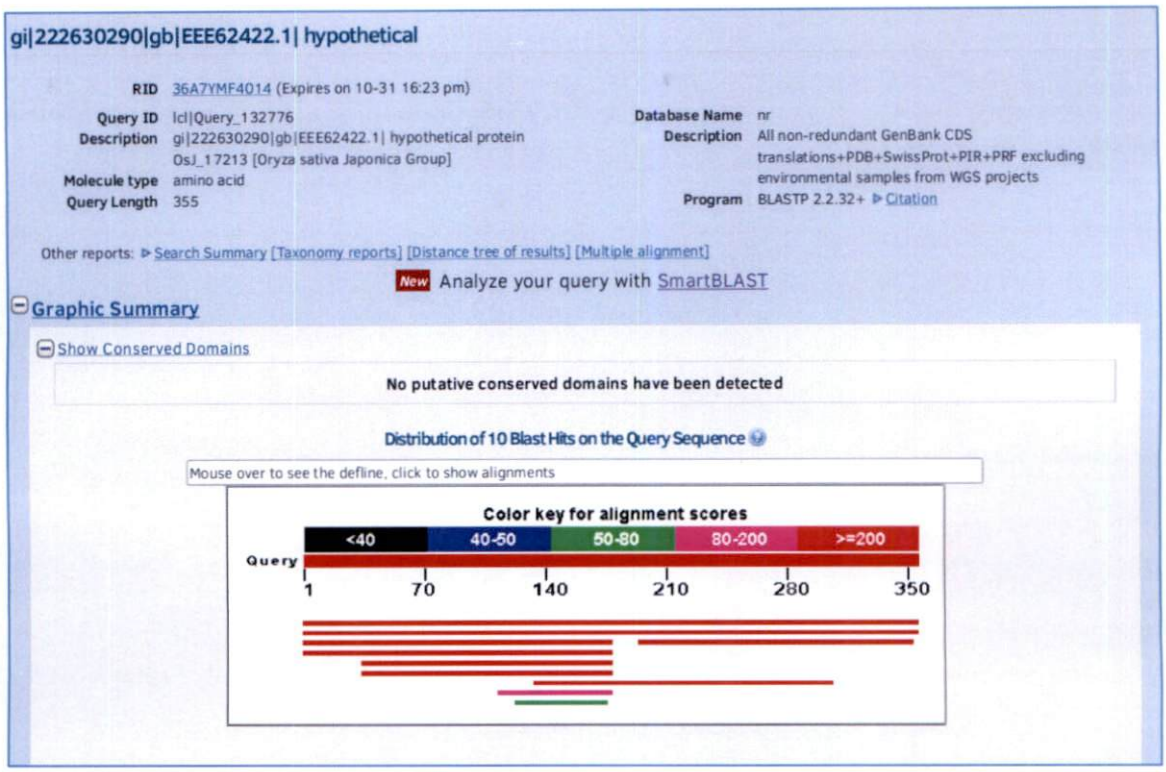


Fig. 4.10. *In-silico* analysis of gi|222630290 protein using smart BLAST

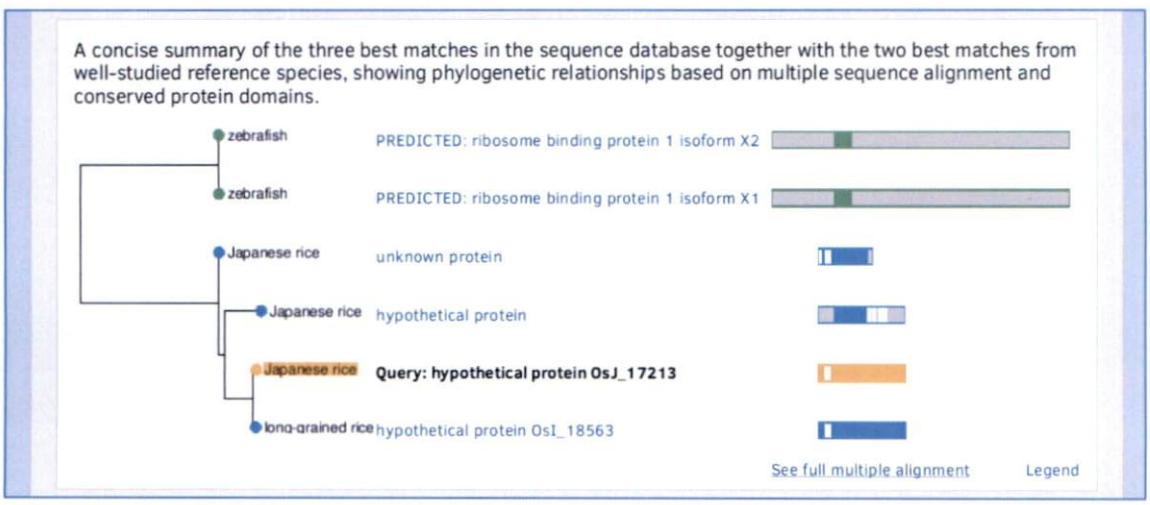
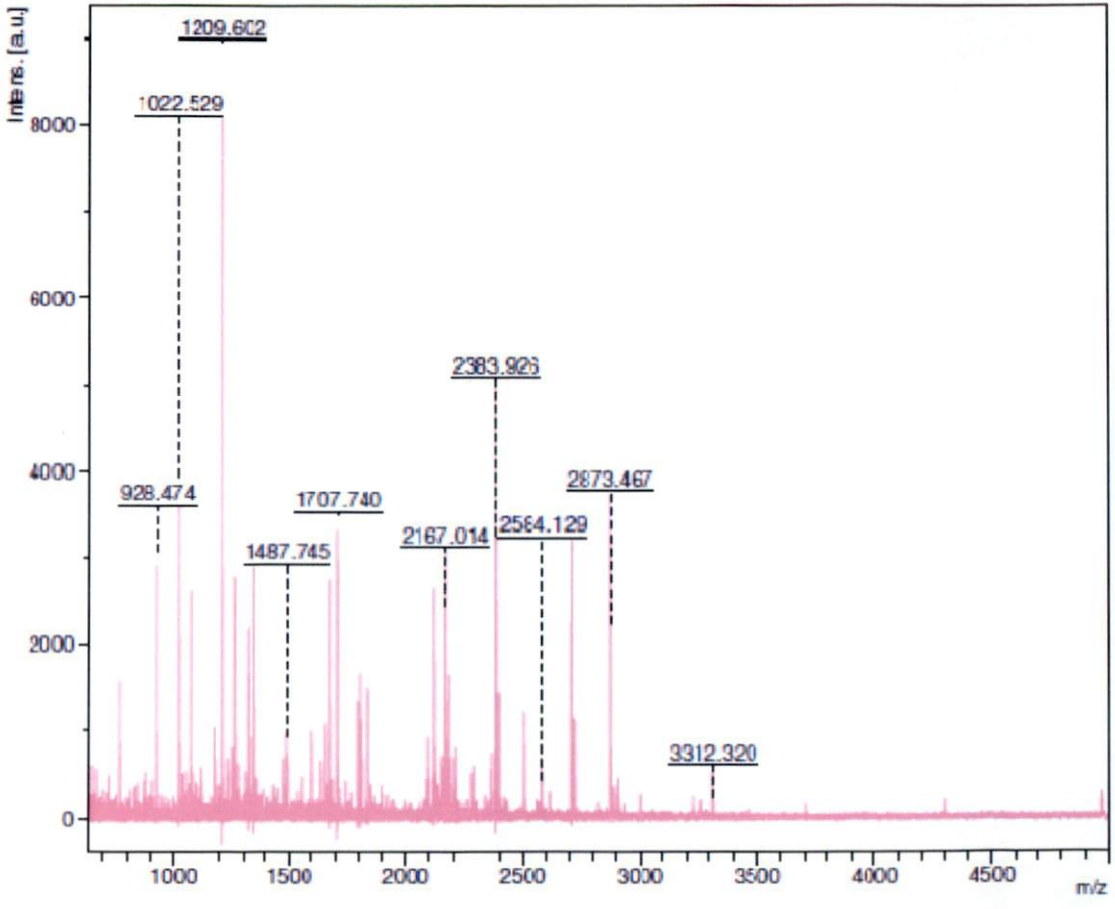


Fig. 4.11: Peptide mass fingerprint of the spot 3 protein extracted from mature inorganically cultivated pods, obtained by MALDI-ToF Mass spectrometry



m/z	S/N	Quality Fac.	Res.	Intens.	Area
653.296	7.9	342	4908	562.36	122
724.463	6.4	1004	5607	455.52	97
768.496	23.6	3848	6419	1711.48	359
882.544	6.9	918	6206	510.55	131
928.474	41.4	13743	7223	3058.49	753
1022.529	49.2	15390	7271	3760.35	1067
1087.538	6.4	1770	7705	494.91	137
1072.622	34.4	14397	7324	2649.77	808
1079.549	6.2	221	7595	475.29	138
1179.586	13.6	8762	7924	1039.30	340
1209.602	116.9	20935	8154	8951.22	3003
1234.646	8.2	353	6812	636.13	259
1251.651	11.0	2137	7709	839.54	313
1263.705	37.4	16426	7725	2867.79	1092
1265.580	10.4	240	0344	815.35	221
1278.682	7.6	610	7971	587.29	215
1300.569	26.9	4880	7626	2076.53	830
1336.705	10.5	893	6688	811.87	382
1349.631	42.4	8564	8821	3282.29	1228
1475.742	9.6	880	8476	746.74	335
1487.745	11.9	502	8155	914.80	428
1493.717	10.1	4341	9635	777.57	322
1600.813	12.8	6102	8401	959.33	505
1638.826	7.4	1277	7309	542.30	340
1657.765	13.8	3435	8409	1023.49	568
1675.907	39.4	22633	0312	2885.71	1395
1707.740	47.3	27557	0401	3481.35	1734
1791.690	16.7	5299	8622	1188.35	758
1800.796	20.8	5061	7249	1466.36	1127
1803.969	12.7	2487	5736	897.31	873
1813.851	15.9	12277	8982	1113.77	698
1838.874	21.1	32062	0090	1485.51	876
2096.910	11.9	1596	7842	694.40	603
2120.998	36.6	17454	8770	2099.86	1677
2125.316	9.8	3117	2953	540.21	1278
2152.973	9.3	879	7019	514.75	513
2160.996	7.4	260	7738	409.48	373
2167.014	44.6	28236	8784	2441.31	1999

Fig. 4.12 *In-silico* analysis of spot 3 protein using Mascot Server software

MATRIX SCIENCE Mascot Search Results

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User            : joy
Email           : joyprashant88@gmail.com
Search title    :
Database        : NCBInr 20151012 (72776944 sequences; 26510890717 residues)
Taxonomy        : Viridiplantae (Green Plants) (3263908 sequences)
Timestamp       : 15 Oct 2015 at 07:15:20 GMT
Top Score       : 145 for gi|920705324, hypothetical protein LR48_Vigan07g225300 [Vigna angularis]
```
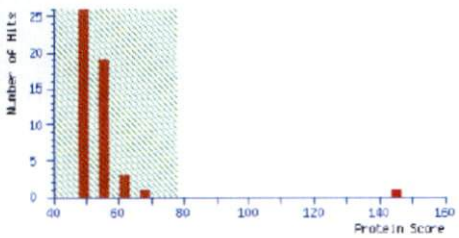

Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 78 are significant (p<0.05).



Index

	Accession	Mass	Score	Description
1.	gi|920705324	42000	145	hypothetical protein LR48_Vigan07g225300 [Vigna angularis]
2.	gi|661881841	17894	68	unnamed protein product [Coffea canephora]
3.	gi|222636454	24281	61	hypothetical protein OsJ_23136 [Oryza sativa Japonica Group]
4.	gi|115470635	13983	60	Os07g0154800 [Oryza sativa Japonica Group]
5.	gi|728813851	37540	59	putative ycf37 [Gossypium arboreum]
6.	gi|218190901	15786	58	hypothetical protein OsI_07527 [Oryza sativa Indica Group]
7.	gi|703100947	32637	58	hypothetical protein L484_019495 [Morus notabilis]
8.	gi|674868656	10862	58	BnaC08g46790D [Brassica napus]
9.	gi|727459664	67688	57	PREDICTED: uncharacterized protein LOC104787743 isoform X1 [Camelina sativa]
10.	gi|218199107	21540	57	hypothetical protein OsI_24941 [Oryza sativa Indica Group]
11.	gi|670400834	29432	56	PREDICTED: uncharacterized protein LOC103655249 [Zea mays]
12.	gi|727459666	67560	56	PREDICTED: HORMA domain-containing protein 1-like isoform X2 [Camelina sativa]
13.	gi|190613786	29490	56	NADH dehydrogenase subunit F [Oxyphyllum ulicinum]
14.	gi|671697703	17900	56	S-phase kinase-associated protein 1-like protein 5 [Malus hupehensis]
15.	gi|731357715	18810	55	PREDICTED: SKP1-like protein 1B [Beta vulgaris subsp. vulgaris]
16.	gi|731400474	153906	55	PREDICTED: centromere-associated protein E isoform X2 [Vitis vinifera]
17.	gi|253750642	161364	55	phytochrome 3 [Coniogramme intermedia var. glabra]
18.	gi|169647577	17730	54	SKP1-like protein 3 [Petunia x hybrida]
19.	gi|82470779	17814	54	Skp1-like protein 1 [Petunia integrifolia subsp. inflata]
20.	gi|82470781	17674	54	Skp1-like protein 2 [Petunia integrifolia subsp. inflata]

Fig. 4.13. *In-silico* analysis of gi|920705324 protein using BLASTp

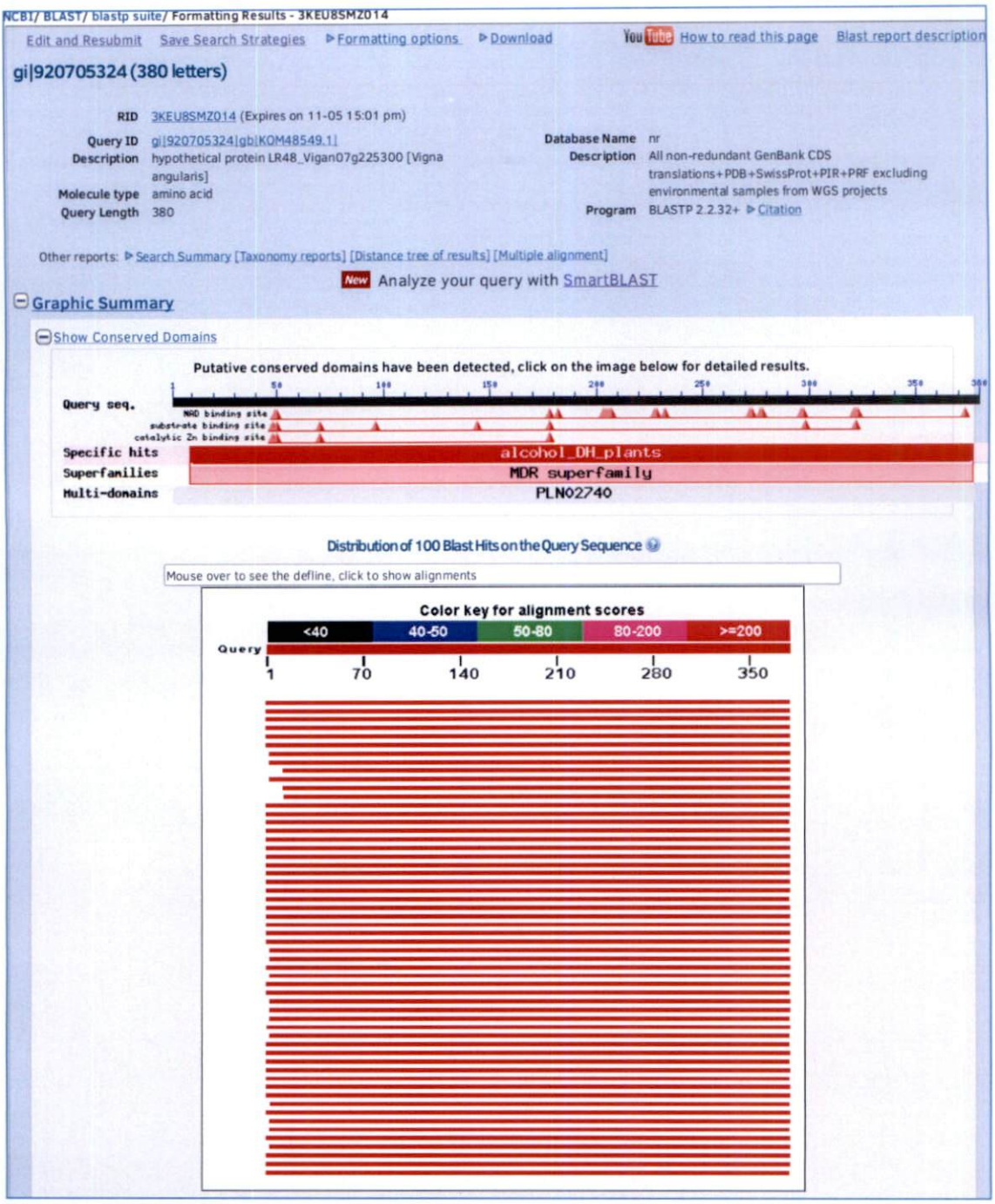


Fig. 4.14. *In-silico* analysis of gi|920705324 protein using smart BLAST

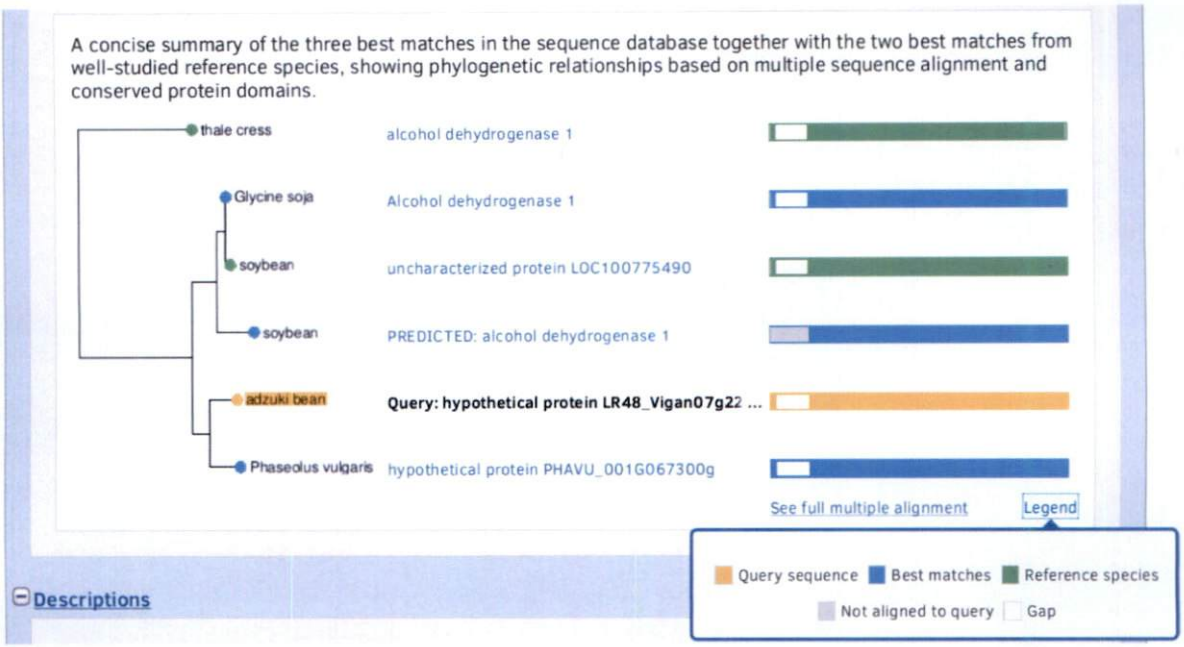
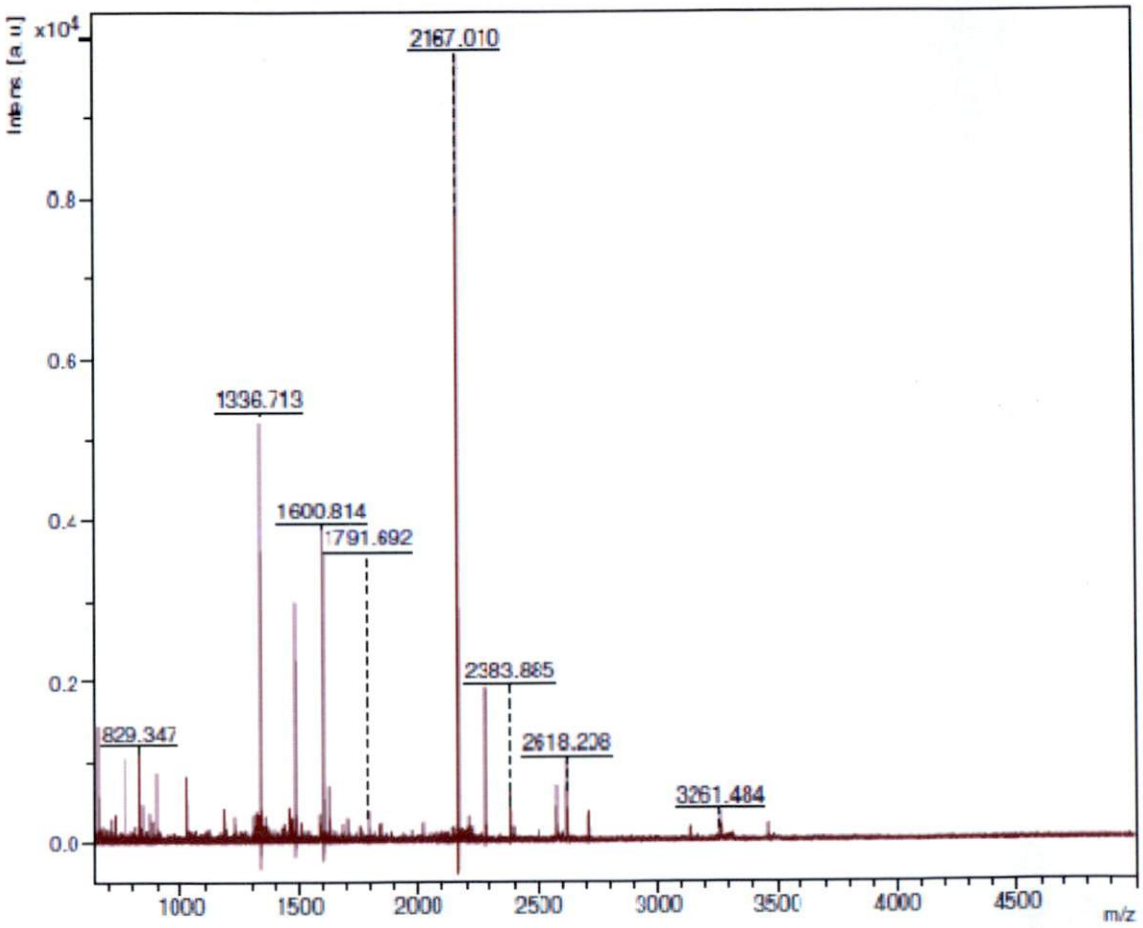


Fig. 4.15: Peptide mass fingerprint of the spot 4 protein extracted from mature inorganically cultivated pods, obtained by MALDI-ToF Mass spectrometry



m/z	S/N	Quality Fac.	Res.	Intens.	Area
653.304	31.5	3669	4263	1390.85	339
707.346	6.2	1404	5228	270.16	62
724.463	7.6	1232	5816	326.59	69
768.503	24.1	5987	5908	1038.00	236
829.347	25.3	12483	5260	1095.86	307
842.473	10.3	1531	5976	446.29	110
870.509	7.3	1053	5219	316.74	94
899.506	19.0	3286	5697	817.28	235
1027.617	19.6	9628	7014	857.20	250
1179.594	9.1	2577	7258	393.30	136
1231.624	7.7	203	6639	336.83	138
1308.657	7.0	1487	8086	304.40	115
1320.584	7.5	564	5599	322.77	176
1325.687	7.8	431	7619	336.36	136
1336.713	121.7	40097	8306	5297.63	2050
1357.693	7.9	1180	8211	339.87	135
1464.804	8.6	328	6370	368.24	213
1475.741	6.8	1498	7859	294.56	140
1488.786	66.1	41158	8664	2851.51	1282
1600.814	92.4	28626	7822	3886.43	2194
1622.864	17.0	13180	9110	720.65	360
1707.745	6.1	2589	7775	264.77	172
1791.692	7.0	837	7513	291.55	212
2167.010	237.2	28264	8737	7774.89	6361
2276.053	43.9	7967	8316	1345.30	1235
2383.885	15.5	1112	8269	443.57	434
2578.288	15.7	1537	8442	400.29	432
2618.208	24.9	2426	8407	621.62	689
2705.084	10.2	1386	8504	227.15	262
3261.484	9.8	845	7054	154.03	293

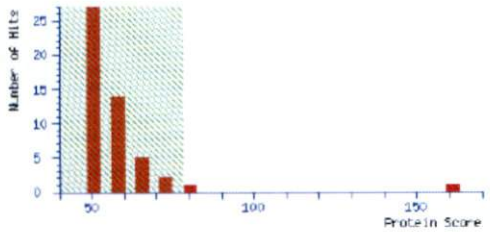
Fig. 4.16. *In-silico* analysis of spot 4 protein using Mascot Server software

MATRIX SCIENCE Mascot Search Results

```
User            : joy
Email           : joyprashant88@gmail.com
Search title    :
Database        : NCBInr 20151012 (72776944 sequences; 26510890717 residues)
Taxonomy        : Viridiplantae (Green Plants) (3263908 sequences)
Timestamp       : 15 Oct 2015 at 07:32:49 GMT
Top Score       : 161 for gi|920704907, hypothetical protein LR48_Vigan07g183600 [Vigna angularis]
```

Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 78 are significant (p<0.05).



Index

	Accession	Mass	Score	Description
1.	gi|920704907	38613	161	hypothetical protein LR48_Vigan07g183600 [Vigna angularis]
2.	gi|593488987	38805	79	hypothetical protein PHAVU_008G189200g [Phaseolus vulgaris]
3.	gi|727447202	38903	72	PREDICTED: fructose-bisphosphate aldolase, cytoplasmic isozyme [Camelina sativa]
4.	gi|629117480	36795	71	hypothetical protein EUGRSUZ_C03549 [Eucalyptus grandis]
5.	gi|555433275	39113	68	fructose-bisphosphate aldolase [Phaseolus vulgaris]
6.	gi|734432148	38483	68	Fructose-bisphosphate aldolase, cytoplasmic isozyme [Glycine soja]
7.	gi|303271723	68634	66	predicted protein [Micromonas pusilla CCMP1545]
8.	gi|823128452	74958	64	PREDICTED: U-box domain-containing protein 3-like isoform X2 [Gossypium raimondii]
9.	gi|363543273	86628	62	RGH3 splicing factor [Zea mays]
10.	gi|657989481	38633	61	PREDICTED: LOW QUALITY PROTEIN: fructose-bisphosphate aldolase, cytoplasmic isozyme-like [Mal
11.	gi|255635475	29508	61	unknown [Glycine max]
12.	gi|823128448	87179	60	PREDICTED: U-box domain-containing protein 3-like isoform X1 [Gossypium raimondii]
13.	gi|922355208	39356	59	12-oxophytodienoate reductase-like protein [Medicago truncatula]
14.	gi|747049456	73876	59	PREDICTED: probable leucine-rich repeat receptor-like protein kinase At1g68400 [Sesamum indic
15.	gi|920711504	24498	58	hypothetical protein LR48_Vigan09g141200 [Vigna angularis]
16.	gi|525313856	38599	58	fructose-bisphosphate aldolase, cytoplasmic isozyme [Cicer arietinum]
17.	gi|590717960	66519	58	GH3 family protein [Theobroma cacao]
18.	gi|357502073	10636	58	transmembrane protein, putative [Medicago truncatula]
19.	gi|703079577	106569	56	Kinesin-related protein 11 [Morus notabilis]
20.	gi|571554660	36385	56	PREDICTED: uncharacterized protein LOC100776711 isoform X4 [Glycine max]

Fig. 4.17. *In-silico* analysis of gi|920704907 protein using BLASTp

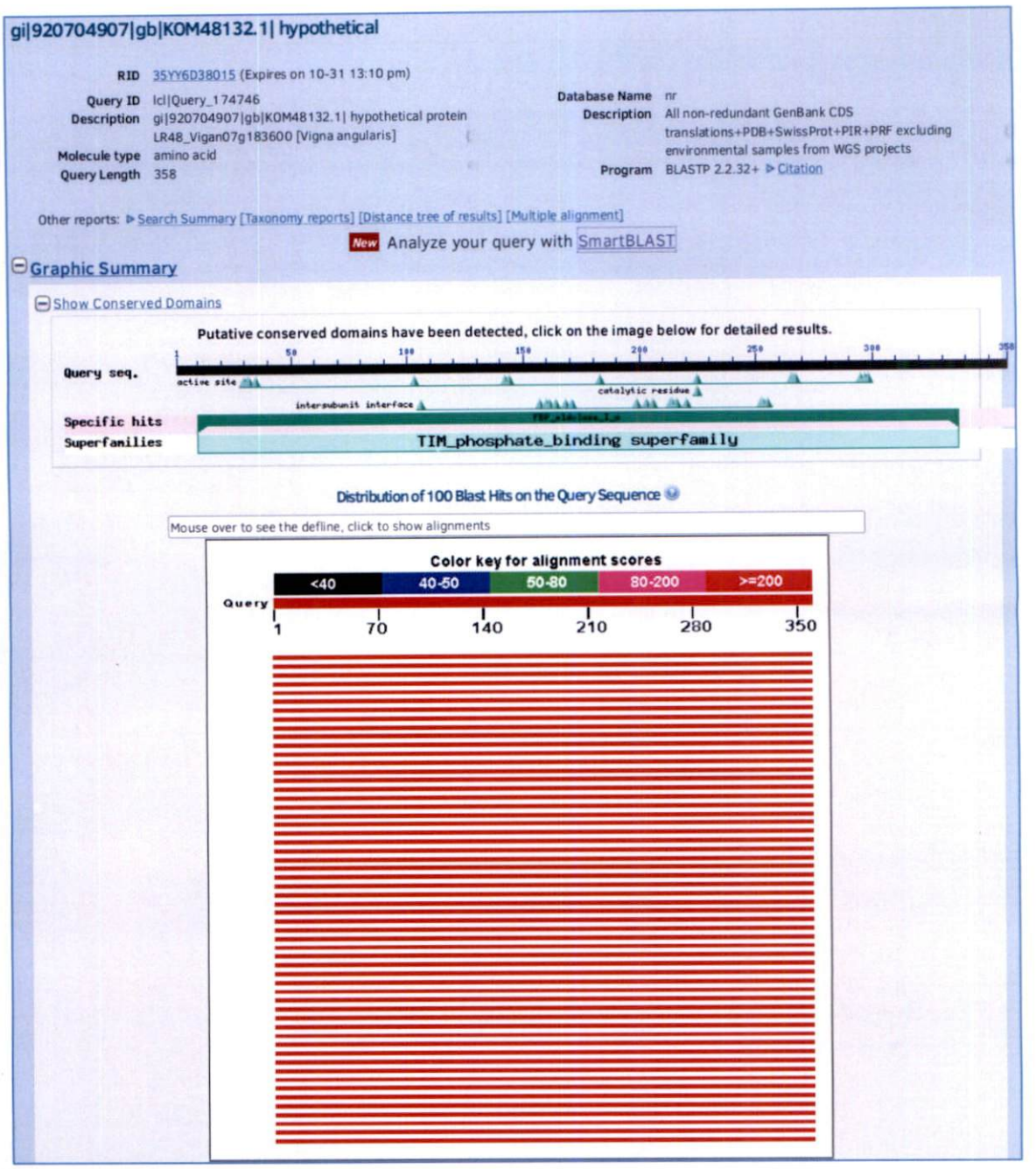


Fig. 4.18. *In-silico* analysis of gi|920704907 protein using smart BLAST

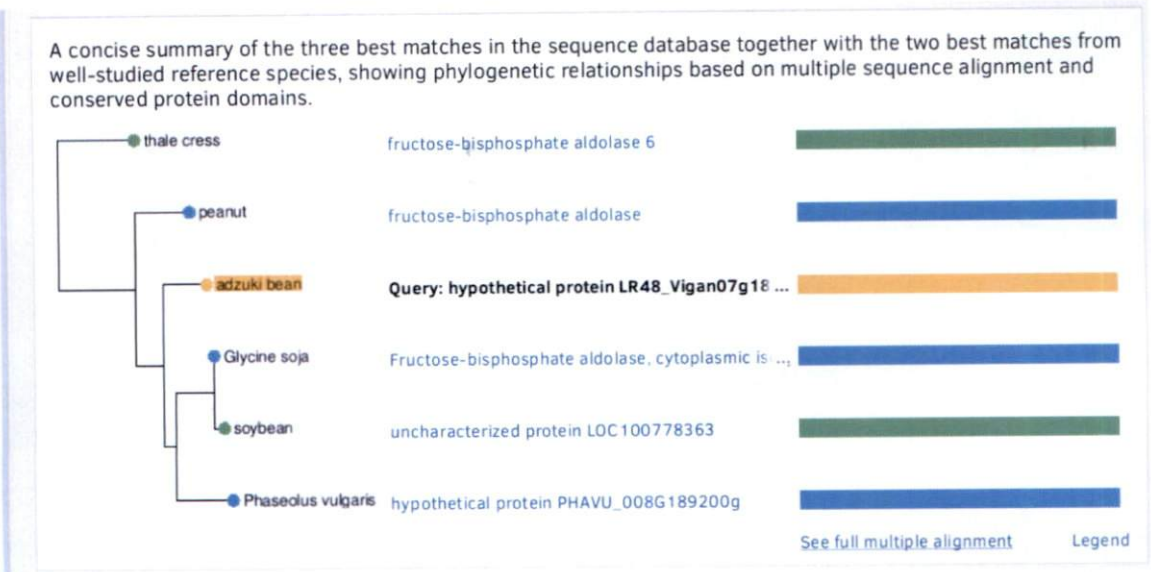
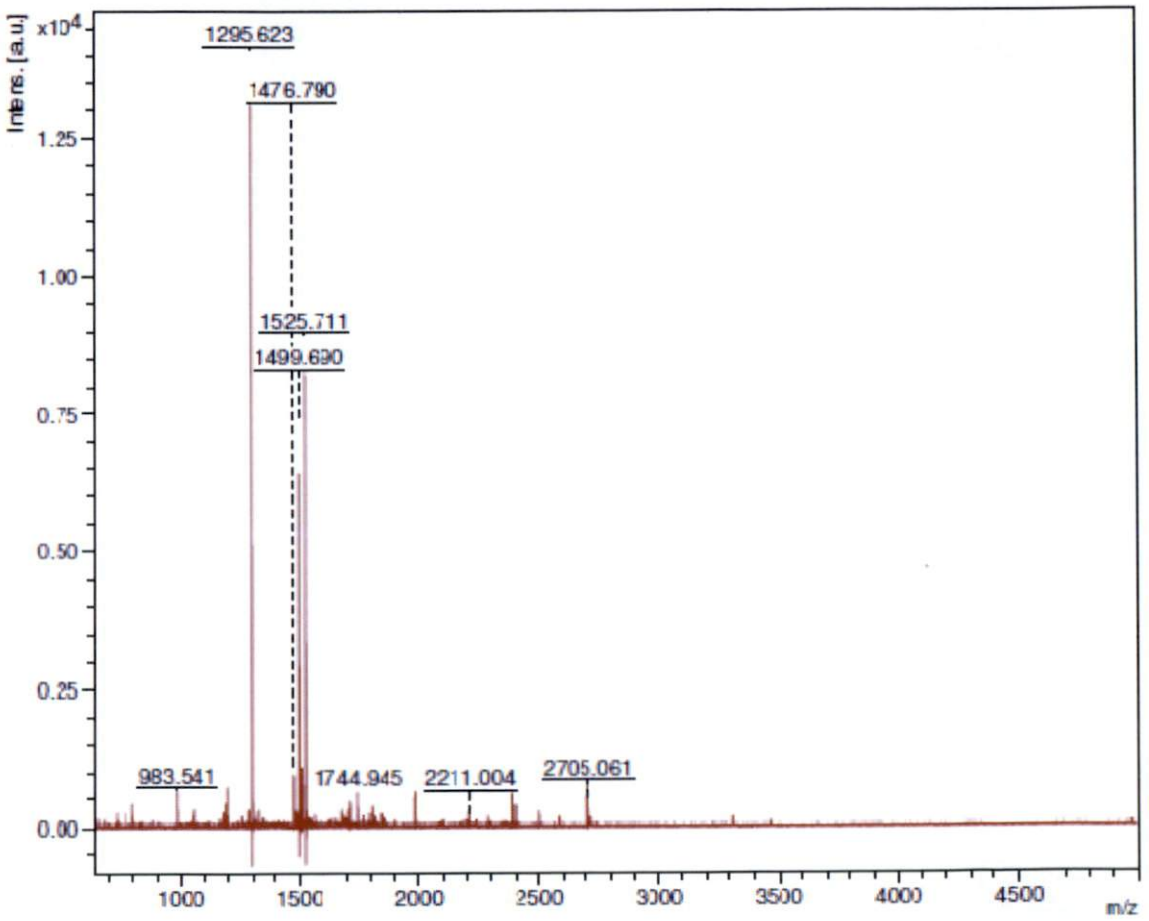


Fig. 4.19: Peptide mass fingerprint of the spot 5 protein extracted from mature inorganically cultivated pods, obtained by MALDI-ToF Mass spectrometry



m/z	S/N	Qualit y Fac.	Res.	Intens.	Area
729.404	8.1	1281	4667	236.90	61
768.498	8.9	1685	6035	261.59	58
799.439	13.6	2651	4951	398.14	111
983.541	17.6	5115	6875	652.55	179
1059.569	8.3	2018	7757	318.22	89
1188.647	8.4	1973	7671	410.85	137
1195.563	14.5	2526	7273	710.11	251
1295.623	287.2	21412	7136	14092.46	5933
1476.790	21.4	9319	9217	1068.32	451
1499.690	149.8	19359	9788	7429.51	3057
1511.688	21.6	4686	8299	1080.87	508
1525.711	180.7	30325	9332	8919.59	3944
1714.804	10.2	2140	8453	446.45	266
1744.945	15.4	9832	10620	671.63	342
1804.875	7.1	2094	7792	309.19	222
1985.019	20.6	18065	10370	588.14	370
2211.004	7.6	364	8269	185.15	163
2383.874	20.3	2423	9243	427.86	378
2398.935	12.2	1899	10013	255.22	211
2501.148	9.6	1651	7970	184.48	202
2705.061	28.9	3490	10650	490.67	452

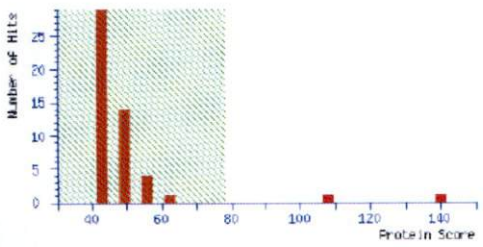
Fig. 4.20 *In-silico* analysis of spot 5 protein using Mascot Server software

MATRIX SCIENCE Mascot Search Results

```
User            : joy
Email           : joyprashant88@gmail.com
Search title    :
Database        : NCBInr 20151012 (72776944 sequences; 26510890717 residues)
Taxonomy        : Viridiplantae (Green Plants) (3263908 sequences)
Timestamp       : 14 Oct 2015 at 11:06:03 GMT
Top Score       : 140 for gi|920690680, hypothetical protein LR48_Vigan02g005500 [Vigna angularis]
```

Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 78 are significant (p<0.05).



Index

	Accession	Mass	Score	Description
1.	gi|920690680	25652	140	hypothetical protein LR48_Vigan02g005500 [Vigna angularis]
2.	gi|920690689	25460	109	hypothetical protein LR48_Vigan02g006400 [Vigna angularis]
3.	gi|657945095	114226	62	PREDICTED: uncharacterized protein LOC103441207 [Malus domestica]
4.	gi|475578002	10099	54	hypothetical protein F775_15854 [Aegilops tauschii]
5.	gi|659091473	24489	54	PREDICTED: uncharacterized protein LOC103489264 [Cucumis melo]
6.	gi|703100570	105597	53	Eukaryotic translation initiation factor 3 subunit C [Morus notabilis]
7.	gi|923655049	30939	53	PREDICTED: uncharacterized protein LOC106350417 [Brassica napus]
8.	gi|125571351	55765	50	hypothetical protein OsJ_02787 [Oryza sativa Japonica Group]

Fig. 4.21. *In-silico* analysis of gi|920690680 protein using BLASTp

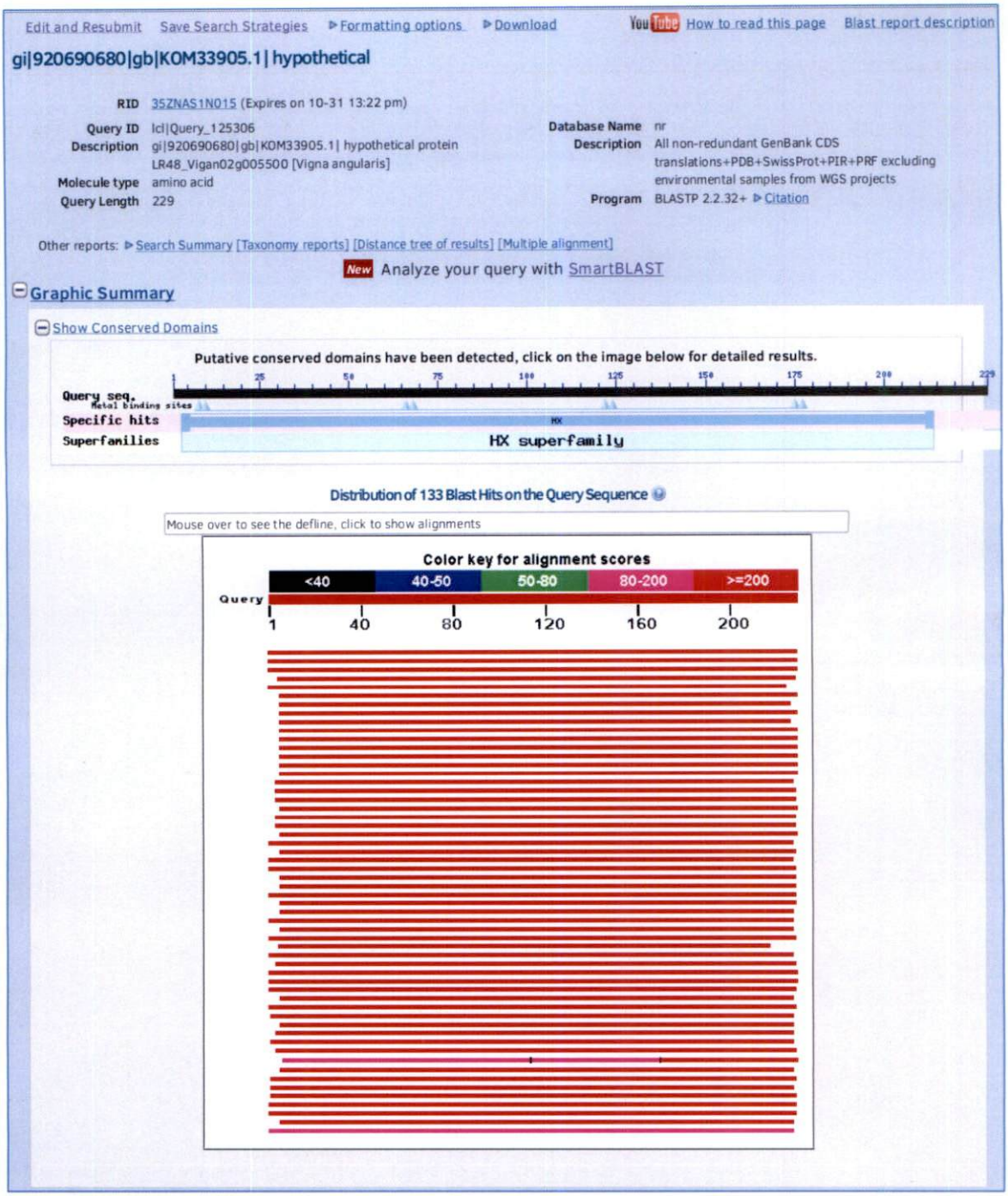


Fig. 4.22. *In-silico* analysis of gi|920690680 protein using smart BLAST

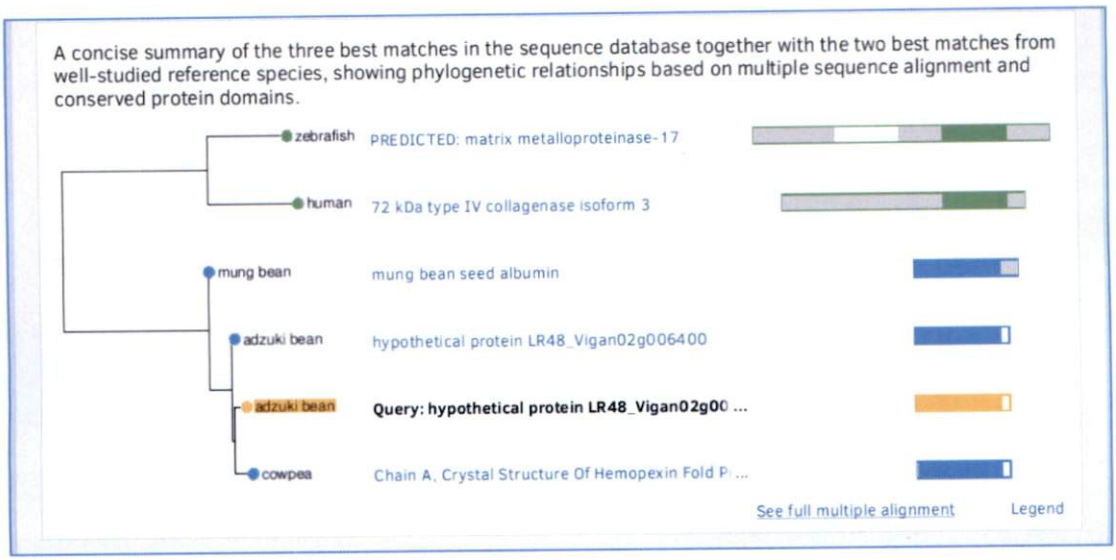
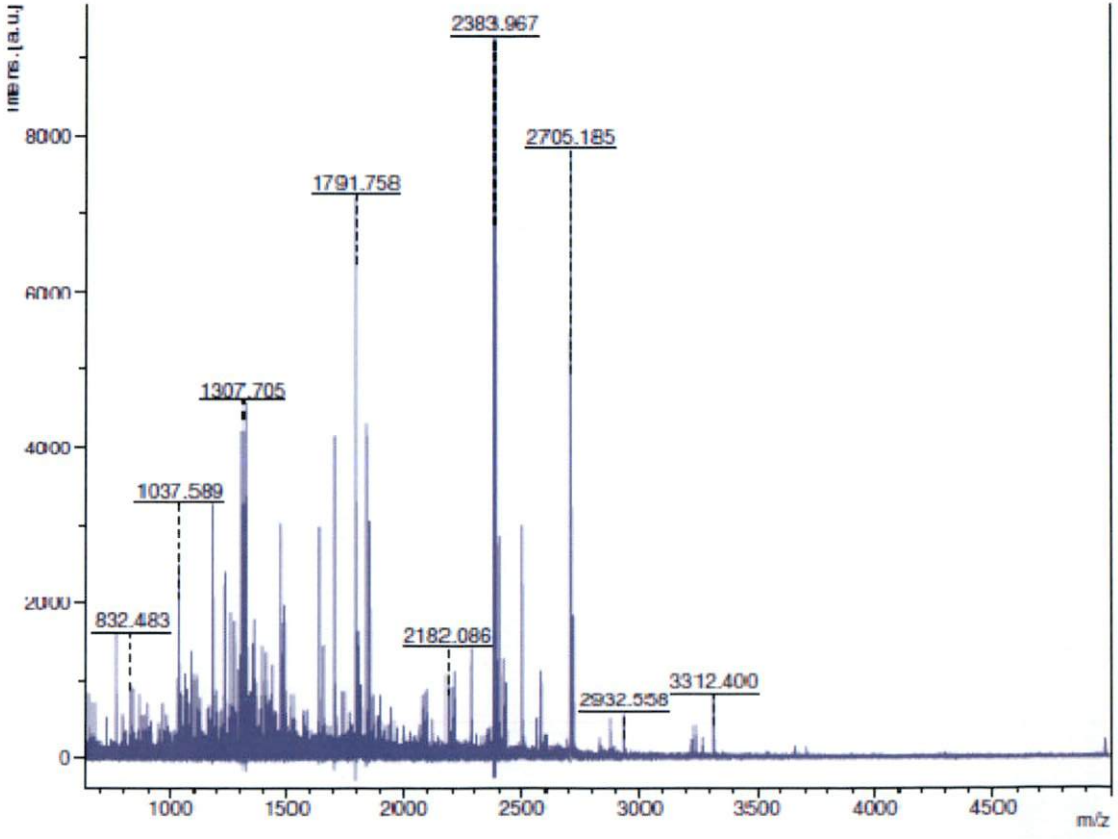


Fig. 4.23: Peptide mass fingerprint of the spot 6 protein extracted from mature organically cultivated pods, obtained by MALDI-ToF Mass spectrometry



m/z	S/N	Quality Fac.	Res.	Intens.	Area
653.319	8.7	1190	3822	782.40	213
672.362	6.5	589	3242	589.62	197
768.530	16.0	3073	4491	1528.14	439
832.483	9.0	5108	3247	887.09	249
842.491	8.0	1220	4332	795.19	272
870.541	7.0	1380	4266	691.65	253
1033.550	8.3	2853	6036	939.24	319
1037.589	18.1	6413	5953	2032.39	700
1045.592	6.2	388	5188	703.77	278
1060.599	6.4	1573	6924	737.49	231
1064.615	10.1	254	5649	1164.66	446
1074.556	6.3	1404	5945	728.52	268
1094.606	11.2	625	5085	1309.16	576
1107.575	8.4	388	5431	985.11	416
1111.599	7.6	1954	5675	892.30	364
1179.626	24.5	8283	6135	2982.05	1233
1194.643	6.0	757	5510	739.12	346
1234.678	14.4	303	4390	1788.40	1090
1235.541	14.1	329	8974	1752.08	558
1259.709	15.6	1624	6189	1705.88	784
1277.733	14.7	2043	7521	1847.42	725
1295.673	8.3	2285	9043	1050.34	358
1302.730	9.0	292	6619	1147.05	512
1307.705	34.3	3994	7053	4364.07	1888
1309.697	7.4	372	8191	942.03	343
1320.620	33.8	16905	6626	4317.55	2018
1323.715	7.7	979	6380	986.12	476
1350.717	10.1	1838	6849	1317.39	614
1357.731	14.3	715	7321	1850.29	793
1365.692	6.0	1098	7024	781.82	364
1393.766	8.5	1425	6457	1109.54	581
1410.756	8.7	1984	7405	1137.35	535
1438.745	7.8	749	6029	1015.96	602
1475.785	23.2	11466	7266	3034.50	1583
1487.786	12.0	4363	7282	1572.60	832
1493.766	14.4	6186	7410	1871.22	973
1638.881	23.6	10783	7906	2994.32	1749
1657.827	10.2	2086	6806	1285.49	889
1707.792	32.7	16866	7472	4042.64	2710

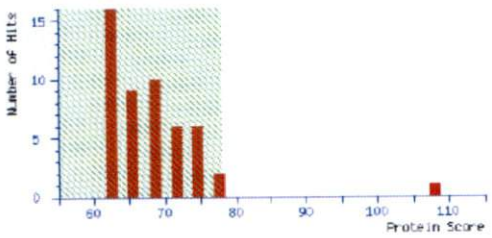
Fig. 4.24. *In-silico* analysis of spot 6 protein using Mascot Server software

MATRIX SCIENCE Mascot Search Results

```
User           : joy
Email          : joyprashant88@gmail.com
Search title   :
Database       : NCBInr 20151012 (72776944 sequences; 26510890717 residues)
Taxonomy       : Viridiplantae (Green Plants) (3263908 sequences)
Timestamp      : 14 Oct 2015 at 11:23:49 GMT
Top Score      : 108 for Mixture 1, gi|695031782 + gi|902219732 + gi|922481517
```

Mascot Score Histogram

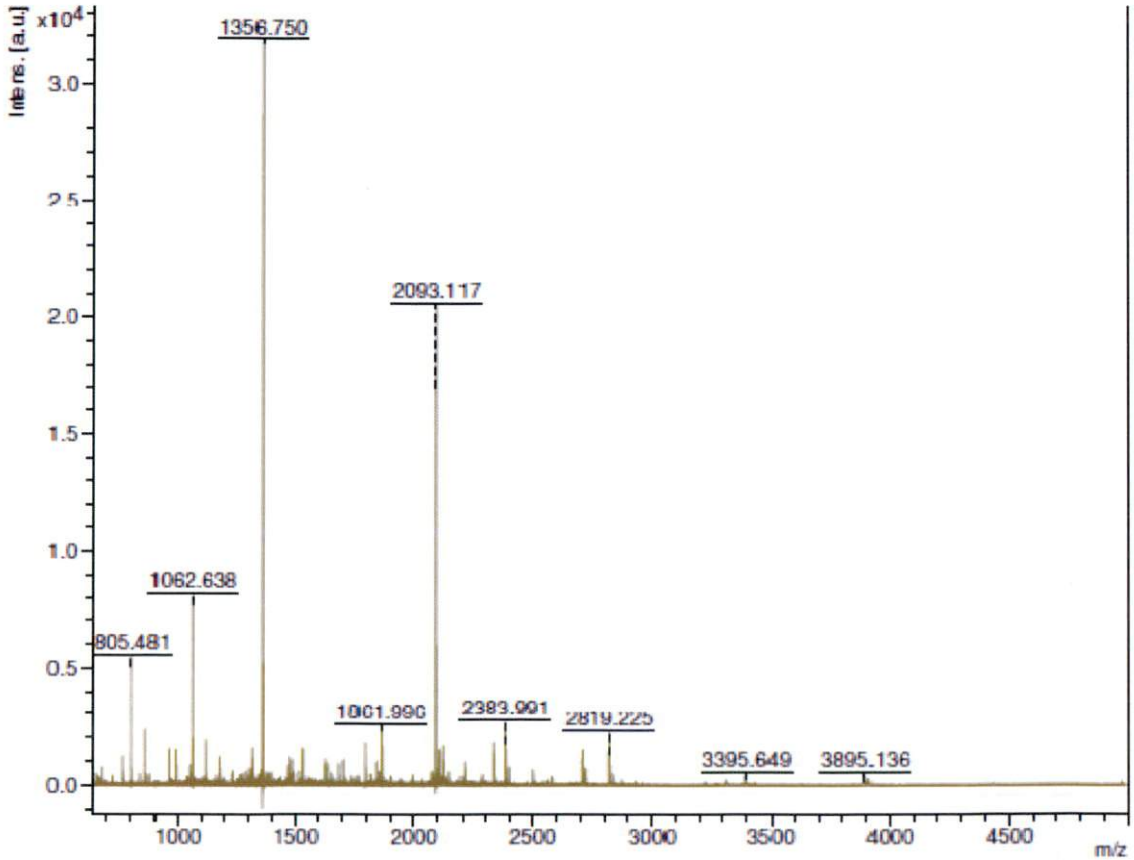
Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 78 are significant (p<0.05).



Index

	Accession	Mass	Score	Description
1.	Mixture 1		108	gi|695031782 + gi|902219732 + gi|922481517
2.	gi|695031782	55065	79	PREDICTED: aldehyde dehydrogenase family 2 member C4-like [Musa acuminata subsp. malaccensi
3.	gi|567203838	93078	78	hypothetical protein EUTSA_v10020078mg [Eutrema salsugineum]
4.	gi|698459331	166883	75	PREDICTED: uncharacterized ATP-dependent helicase C29A10.10c [Nicotiana sylvestris]
5.	gi|836026642	102311	75	PREDICTED: probable DNA gyrase subunit A, chloroplastic/mitochondrial [Setaria italica]
6.	gi|50313187	25543	74	dual-specificity phosphatase protein [Oryza sativa]
7.	gi|115467010	25640	74	Os06g0208700 [Oryza sativa Japonica Group]
8.	gi|552833814	65705	74	expressed protein [Chlorella variabilis]
9.	gi|21672054	204658	73	Putative retroelement [Oryza sativa Japonica Group]
10.	gi|720017265	90707	73	PREDICTED: golgin candidate 4 [Nelumbo nucifera]
11.	gi|743767765	84585	72	PREDICTED: VIN3-like protein 2 isoform X1 [Elaeis guineensis]
12.	gi|923555941	121341	72	PREDICTED: disease resistance protein RML1A-like [Brassica napus]
13.	gi|308800972	679428	71	modular polyketide synthase (ISS) [Ostreococcus tauri]
14.	gi|693501321	795862	71	Polyketide synthase/Fatty acid synthase, KR [Ostreococcus tauri]
15.	gi|473880406	69854	71	T-complex protein 1 subunit theta [Triticum urartu]
16.	gi|727476880	67026	70	PREDICTED: root phototropism protein 2-like [Camelina sativa]
17.	gi|727456851	67081	70	PREDICTED: root phototropism protein 2-like [Camelina sativa]
18.	gi|727593633	66950	70	PREDICTED: root phototropism protein 2 isoform X1 [Camelina sativa]
19.	gi|475499789	61337	69	Phospholipase C 2 [Aegilops tauschii]
20.	gi|702338219	26569	68	PREDICTED: uncharacterized protein LOC104443973 [Eucalyptus grandis]

Fig 4.25: Peptide mass fingerprint of the spot 7 protein extracted from immature inorganically cultivated pods, obtained by MALDI-ToF Mass spectrometry



m/z	S/N	Quality Fac.	Res.	Intens.	Area
653.321	8.1	718	3988	469.69	124
679.278	11.7	851	3299	671.48	223
767.356	8.0	1101	4819	479.69	131
768.324	20.3	2103	4566	1217.61	360
805.481	83.3	7291	4277	5035.27	1639
842.499	6.3	594	3722	383.16	152
863.489	36.2	12346	4533	2220.90	764
887.579	6.3	447	3156	384.89	193
961.618	23.4	6167	4971	1453.84	535
991.497	21.9	4899	4941	1376.16	532
1051.755	13.0	3611	6598	843.67	276
1062.638	119.5	13784	5178	7754.13	3194
1119.604	26.1	14748	3433	1709.81	740
1179.643	15.5	3757	5655	1033.89	463
1234.671	7.2	166	4127	479.99	316
1277.741	7.0	1241	7966	471.50	175
1290.769	7.6	1525	5226	510.81	288
1307.708	10.9	333	6854	735.30	326
1312.732	8.3	729	5900	560.98	289
1320.636	19.3	5549	5819	1298.49	694
1350.732	7.9	943	5877	529.67	287
1526.720	469.5	25085	5402	31671.29	19124
1467.816	9.3	533	4166	624.73	557
1475.798	12.4	372	4107	833.78	756
1487.798	14.1	4807	6432	946.16	565
1493.777	6.9	1225	6046	466.64	295
1515.798	7.5	1205	5893	495.13	331
1531.797	22.6	9333	6892	1494.95	886
1628.889	14.1	1017	6840	930.10	622
1638.903	11.9	3603	6890	792.24	533
1687.995	11.9	4336	7222	785.76	529
1707.813	14.5	7084	6875	952.50	695
1791.773	24.7	16151	7185	1573.39	1217
1838.957	13.0	6180	6868	815.89	694
1851.950	7.3	974	6610	455.03	402
1861.996	36.1	28118	7531	2239.53	1798
2076.145	7.1	157	4072	361.04	593
2093.117	337.6	65068	7177	16957.14	16383
2098.014	8.4	859	4041	403.06	738

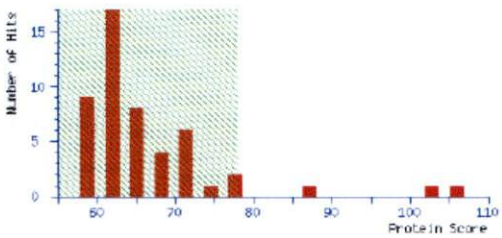
Fig. 4.26. *In-silico* analysis of spot 7 protein using Mascot Server software

MATRIX SCIENCE Mascot Search Results

```
User            : joy
Email           : joyprashant88@gmail.com
Search title    :
Database        : NCBInr 20151012 (72776944 sequences; 26510890717 residues)
Taxonomy        : Viridiplantae (Green Plants) (3263908 sequences)
Timestamp       : 14 Oct 2015 at 11:48:07 GMT
Top Score       : 106 for Mixture 1, gi|727509054 + gi|778726730
```

Mascot Score Histogram

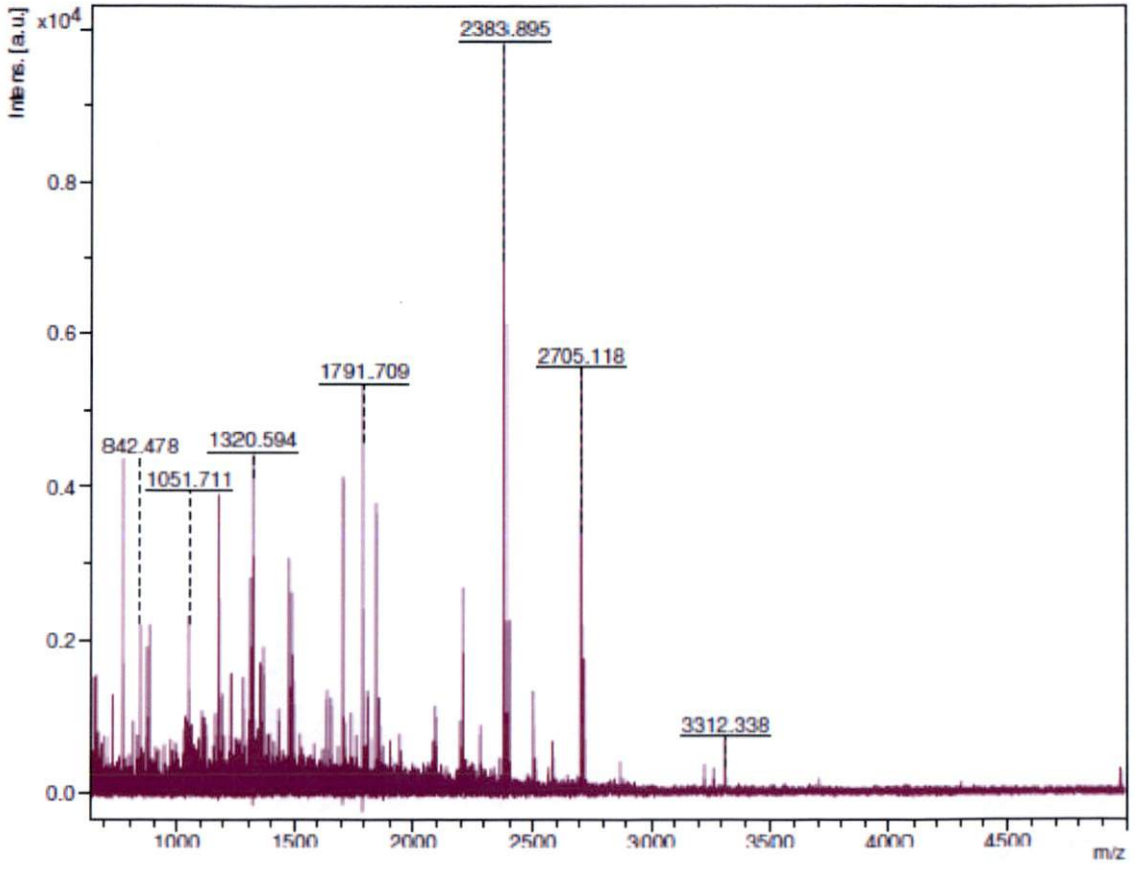
Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 78 are significant (p<0.05).



Index

	Accession	Mass	Score	Description
1.	Mixture 1		106	gi|727509054 + gi|778726730
2.	Mixture 2		102	gi|727509054 + gi|760438885
3.	gi|727509054	32969	88	PREDICTED: protein PLASTID MOVEMENT IMPAIRED 2-like [Camelina sativa]
4.	gi|778726730	42810	79	PREDICTED: trans-resveratrol di-O-methyltransferase-like [Cucumis sativus]
5.	gi|685358302	69442	78	PREDICTED: LOW QUALITY PROTEIN: microtubule-associated protein 70-2-like [Brassica rapa]
6.	gi|802760569	74842	74	PREDICTED: ankyrin-2-like [Jatropha curcas]
7.	gi|923711473	69360	72	PREDICTED: microtubule-associated protein 70-2-like [Brassica napus]
8.	gi|641846238	113891	71	hypothetical protein CISIN_1g0004212mg, partial [Citrus sinensis]
9.	gi|747046872	73844	71	PREDICTED: uncharacterized protein LOC105179636 isoform X4 [Sesamum indicum]
10.	gi|923698504	162605	71	PREDICTED: protein NETWORKED 1B-like [Brassica napus]
11.	gi|747046870	73902	71	PREDICTED: uncharacterized protein LOC105179636 isoform X3 [Sesamum indicum]
12.	gi|747046864	73973	71	PREDICTED: uncharacterized protein LOC105179636 isoform X1 [Sesamum indicum]
13.	gi|674935934	132961	69	BnaC08g12560D [Brassica napus]
14.	gi|760438885	51390	69	IMP-specific 5'-nucleotidase 1 [Auxenochlorella protothecoides]
15.	gi|923873934	151081	67	PREDICTED: putative WEB family protein At1g65010, chloroplastic [Brassica napus]
16.	gi|297742628	26987	67	unnamed protein product [Vitis vinifera]
17.	gi|475505056	106312	66	Pentatricopeptide repeat-containing protein [Aegilops tauschii]
18.	gi|922359871	7640	66	40S ribosomal S10-like protein, putative [Medicago truncatula]
19.	gi|685261967	181919	66	PREDICTED: myosin-11 [Brassica rapa]
20.	gi|922552631	150890	65	PREDICTED: putative WEB family protein At1g65010, chloroplastic isoform X1 [Brassica oler

Fig. 4.27: Peptide mass fingerprint of the spot 8 protein extracted from immature organically cultivated pods, obtained by MALDI-ToF Mass spectrometry



m/z	S/N	Quality Fac.	Res.	Intens.	Area
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724.463	8.5	1430	5078	1210.45	285
768.500	30.2	8653	5773	4375.75	1020
842.478	14.5	3586	6378	2188.33	529
870.514	11.7	2453	5260	1768.58	520
882.547	14.2	3090	5881	2160.37	588
1051.711	13.3	5189	6677	2194.66	704
1179.605	23.6	12220	7982	3998.78	1311
1193.620	6.8	167	6516	1161.49	463
1234.658	9.3	144	5655	1578.43	760
1277.708	8.4	2895	7959	1433.56	537
1307.678	16.1	628	9078	2781.61	965
1320.594	23.8	8133	6910	4105.02	1841
1350.692	10.6	1526	8581	1846.99	708
1365.651	10.1	2303	7392	1770.71	787
1475.758	18.4	15142	8272	3139.82	1462
1487.752	14.3	5883	7089	2430.60	1314
1493.735	9.0	1955	7747	1523.03	810
1638.844	7.2	867	7908	1168.24	676
1707.754	25.7	20735	7760	3988.38	2579
1791.709	31.4	24363	7807	4601.36	3292
1838.899	25.1	53115	8789	3580.73	2378
1851.897	7.4	2025	7567	1040.04	812
2211.051	22.2	24797	8285	2045.46	1805
2286.067	6.0	1409	5377	500.72	710
2383.895	94.7	16635	8029	6958.37	7078
2398.983	21.2	4943	7537	1531.01	1680
2501.166	13.1	3106	7000	832.88	1062
2584.154	6.5	1231	6648	379.48	521
2705.118	62.4	8496	8094	3583.24	4123
2717.047	18.0	1514	6986	923.12	1304
3312.338	9.9	1827	7461	319.78	605

Fig. 4.28. *In-silico* analysis of spot 8 protein using Mascot Server software

MATRIX SCIENCE Mascot Search Results

```
User            : joy
Email           : joyprashant88@gmail.com
Search title    :
Database        : NCBInr 20151012 (72776944 sequences; 26510890717 residues)
Taxonomy        : Viridiplantae (Green Plants) (3263908 sequences)
Timestamp       : 14 Oct 2015 at 11:58:27 GMT
Top Score       : 87 for gi|567149521, hypothetical protein EUTSA_v10007083mg [Eutrema salsugineum]
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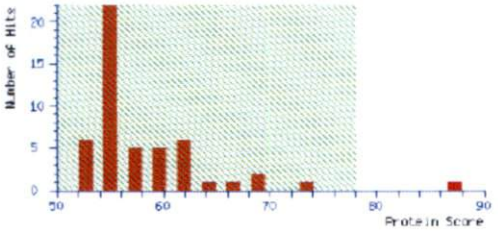
Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 78 are significant (p<0.05).



Index

	Accession	Mass	Score	Description
1.	gi|567149521	71755	87	hypothetical protein EUTSA_v10007083mg [Eutrema salsugineum]
2.	gi|612391130	43079	73	exodeoxyribonuclease III [Bathycoccus prasinos]
3.	gi|674868656	10862	70	BnaC08g46790D [Brassica napus]
4.	gi|414589545	31529	69	TPA: hypothetical protein ZEAMMB73_157272 [Zea mays]
5.	gi|720017265	90707	68	PREDICTED: golgin candidate 4 [Nelumbo nucifera]
6.	gi|242070679	113028	64	hypothetical protein SORBIDRAFT_05g008250 [Sorghum bicolor]
7.	gi|645235306	113580	63	PREDICTED: U-box domain-containing protein 43-like [Prunus mume]
8.	gi|645232316	153921	62	PREDICTED: DNA repair protein RAD50 [Prunus mume]
9.	gi|297791059	134206	62	hypothetical protein ARALYDRAFT_494346 [Arabidopsis lyrata subsp. lyrata]
10.	gi|661898513	78519	62	unnamed protein product [Coffea canephora]
11.	gi|566159719	32894	62	hypothetical protein POPTR_0002s24180g [Populus trichocarpa]
12.	gi|21554345	22634	61	ribosomal protein L17-like protein [Arabidopsis thaliana]
13.	gi|719983464	98916	60	PREDICTED: uncharacterized protein LOC104592923 isoform X1 [Nelumbo nucifera]
14.	gi|719983467	98788	60	PREDICTED: uncharacterized protein LOC104592923 isoform X2 [Nelumbo nucifera]
15.	gi|242070683	106453	60	hypothetical protein SORBIDRAFT_05g008270 [Sorghum bicolor]
16.	gi|694410883	85482	59	PREDICTED: uncharacterized protein LOC103926731 isoform X4 [Pyrus x bretschneideri]
17.	gi|39545916	39438	59	TAF15b [Arabidopsis thaliana]
18.	gi|694410878	89970	58	PREDICTED: uncharacterized protein LOC103926731 isoform X2 [Pyrus x bretschneideri]
19.	gi|727619914	35355	56	PREDICTED: myelin transcription factor 1-like protein [Camelina sativa]
20.	gi|743797275	22802	56	PREDICTED: ras-related protein RIC1-like [Elaeis guineensis]

Fig. 4.29 *In-silico* analysis of gi|567149521 protein using BLASTp

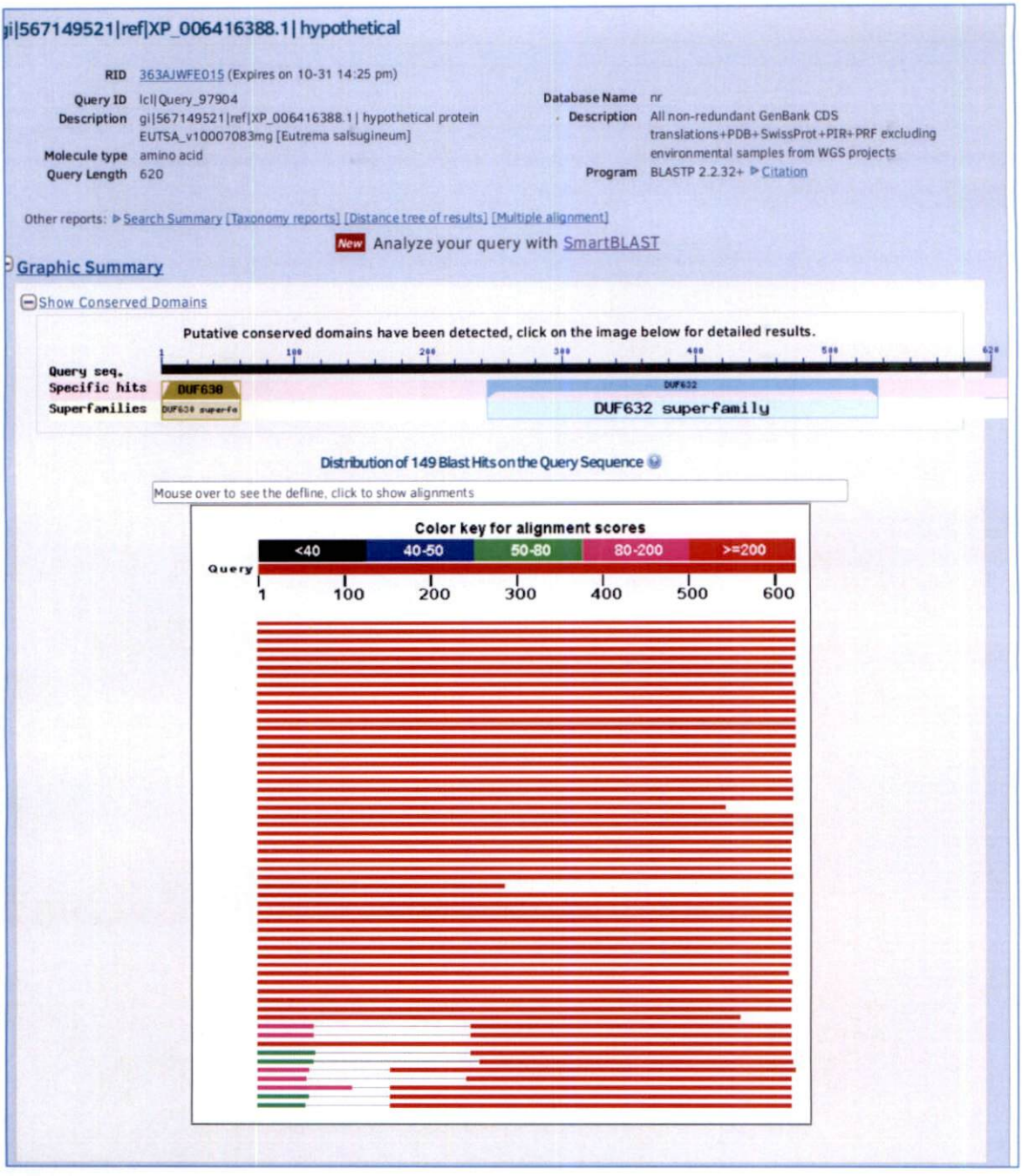


Fig. 4.30. *In-silico* analysis of gi|567149521 protein using smartBLASTp

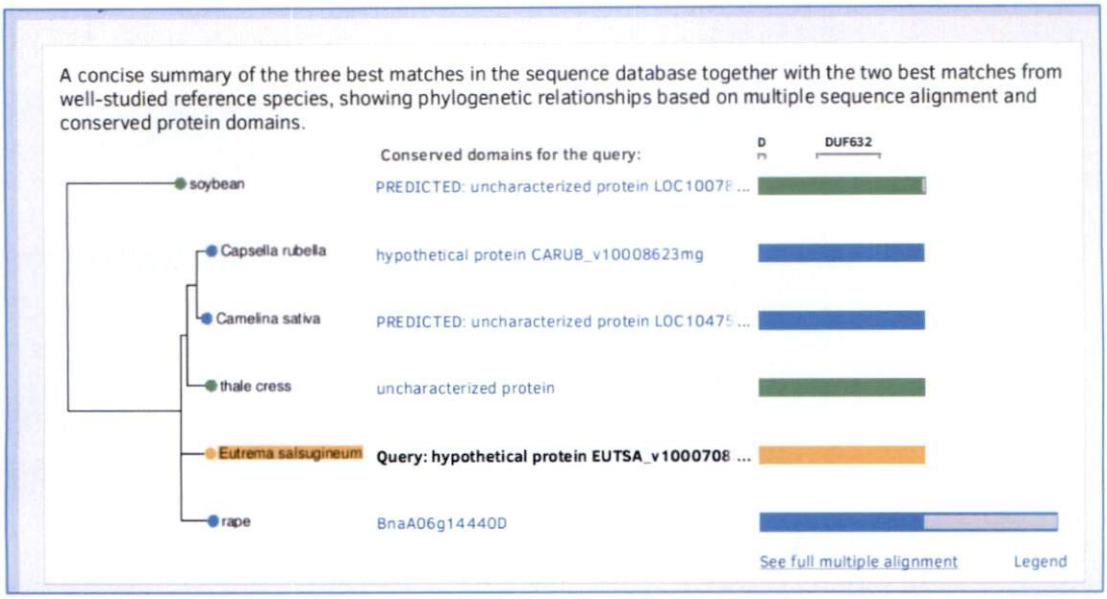
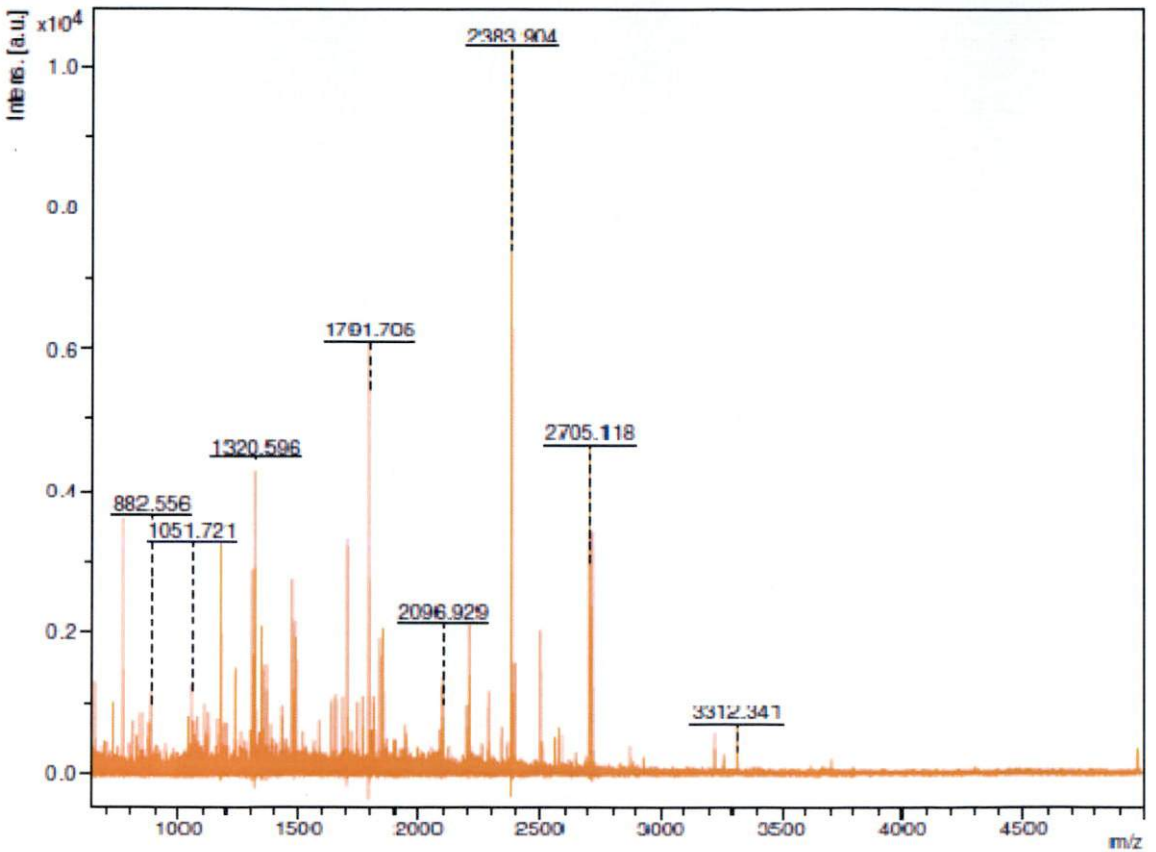


Fig. 4.31: Peptide mass fingerprint of the spot 9 protein extracted from immature organically cultivated pods, obtained by MALDI-ToF Mass spectrometry



m/z	S/N	Quality Fac.	Res.	Intens.	Area
655.308	12.6	1461	5555	1255.14	252
724.470	9.4	1958	5495	940.11	209
768.512	36.5	7569	6250	3733.72	806
812.537	6.7	1320	5569	686.18	176
842.477	7.9	1218	5478	820.53	228
870.516	6.3	1649	5868	649.20	176
881.244	8.7	699	5456	896.30	263
882.556	9.5	786	4900	983.60	319
1037.540	6.4	1313	7398	705.31	200
1051.721	10.7	2449	7351	1171.80	343
1107.546	9.1	233	7571	1009.50	313
1125.544	7.1	409	5765	784.92	319
1179.607	28.7	13140	7592	3239.31	1100
1234.632	10.9	871	3628	1239.07	604
1235.522	9.3	429	9079	1063.03	333
1307.679	28.1	3470	9203	3257.30	1109
1320.596	37.9	8276	8801	4410.73	1601
1350.690	17.2	7305	8945	2029.64	753
1365.645	11.9	1286	7589	1392.90	597
1434.751	6.7	1662	7602	785.64	362
1475.764	23.5	23373	8509	2765.34	1252
1487.752	19.0	13151	8947	2224.30	967
1493.779	15.9	9079	8847	1860.08	818
1638.843	9.2	3453	8925	1052.13	555
1657.772	8.5	1385	8231	964.33	547
1687.940	7.4	1268	6975	833.07	579
1707.759	30.8	35552	9692	3468.27	1807
1765.730	8.0	1495	7175	882.56	654
1791.705	30.2	19460	8379	3429.89	3303
1813.863	6.8	637	5732	735.72	720
1858.899	15.8	13993	9609	1696.37	1040
1851.902	16.7	5371	7692	1762.25	1348
2091.852	11.1	1104	8058	928.95	783
2096.929	11.5	1843	8201	962.20	801
2198.921	10.3	3020	8552	767.75	658
2211.049	22.8	18912	8737	1677.76	1419
2286.061	11.9	2598	7483	794.31	811
2342.049	7.0	1077	8119	442.48	432
2383.904	121.7	22139	9056	7374.23	6665

Fig. 4.32. *In-silico* analysis of spot 9 protein using Mascot Server software

MATRIX SCIENCE Mascot Search Results

```
User            : joy
Email           : joyprashant88@gmail.com
Search title    :
Database        : NCBInr 20151012 (72776944 sequences; 26510890717 residues)
Taxonomy        : Viridiplantae (Green Plants) (3263908 sequences)
Timestamp       : 14 Oct 2015 at 12:15:22 GMT
Top Score       : 92 for gi|567149521, hypothetical protein EUTSA_v10007083mg [Eutrema salsugineum]
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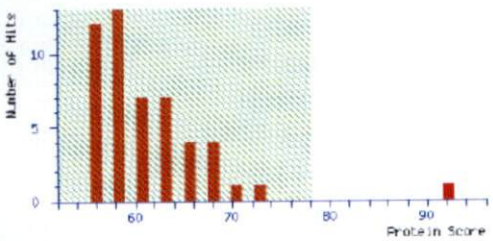
Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 78 are significant (p<0.05).



Index

	Accession	Mass	Score	Description
1.	gi|567149521	71755	92	hypothetical protein EUTSA_v10007083mg [Eutrema salsugineum]
2.	gi|902219732	66315	73	hypothetical protein SOVF_071130 [Spinacia oleracea]
3.	gi|764644063	88817	70	PREDICTED: TMV resistance protein N-like isoform X1 [Fragaria vesca subsp. vesca]
4.	gi|778701651	82459	69	PREDICTED: probable serine/threonine-protein kinase At1g09600 isoform X1 [Cucumis sativus]
5.	gi|778701658	81886	69	PREDICTED: probable serine/threonine-protein kinase At1g09600 isoform X2 [Cucumis sativus]
6.	gi|802760818	68493	67	PREDICTED: uncharacterized protein LOC105647950 isoform X1 [Jatropha curcas]
7.	gi|698534620	35791	67	PREDICTED: serine/arginine-rich splicing factor RS41-like isoform X2 [Nicotiana sylvestris]
8.	gi|674906807	84025	66	BnaA06g12610D [Brassica napus]

Fig. 4.33. *In-silico* analysis of gi|567149521 protein using BLASTp

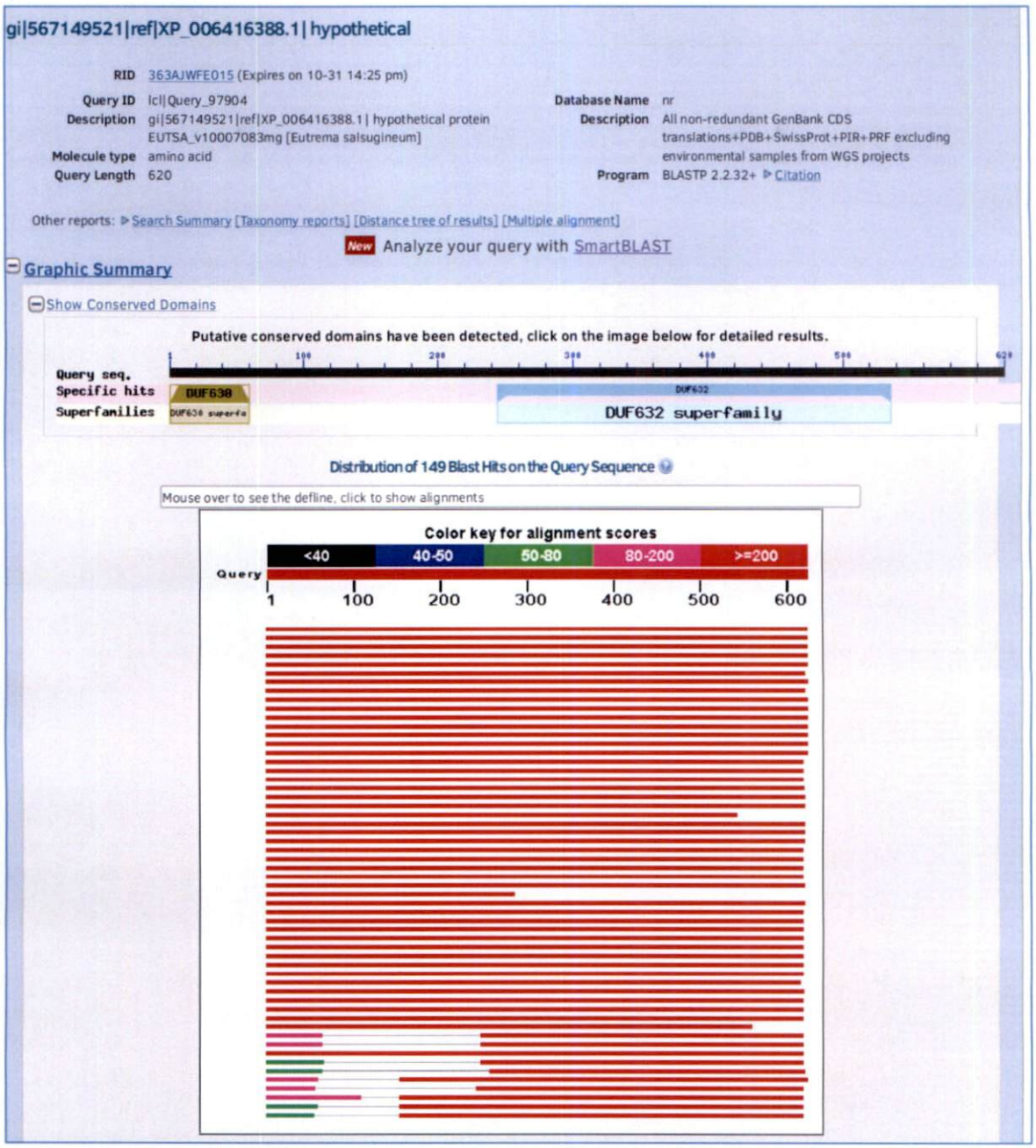


Fig. 4.34. *In-silico* analysis of gi|567149521 protein using smartBLAST

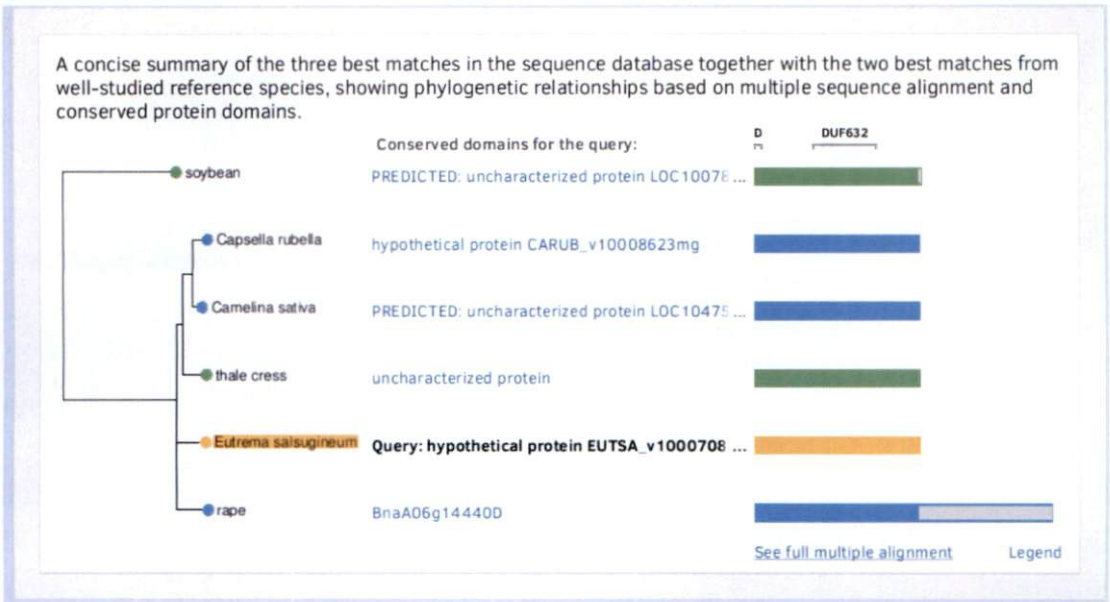
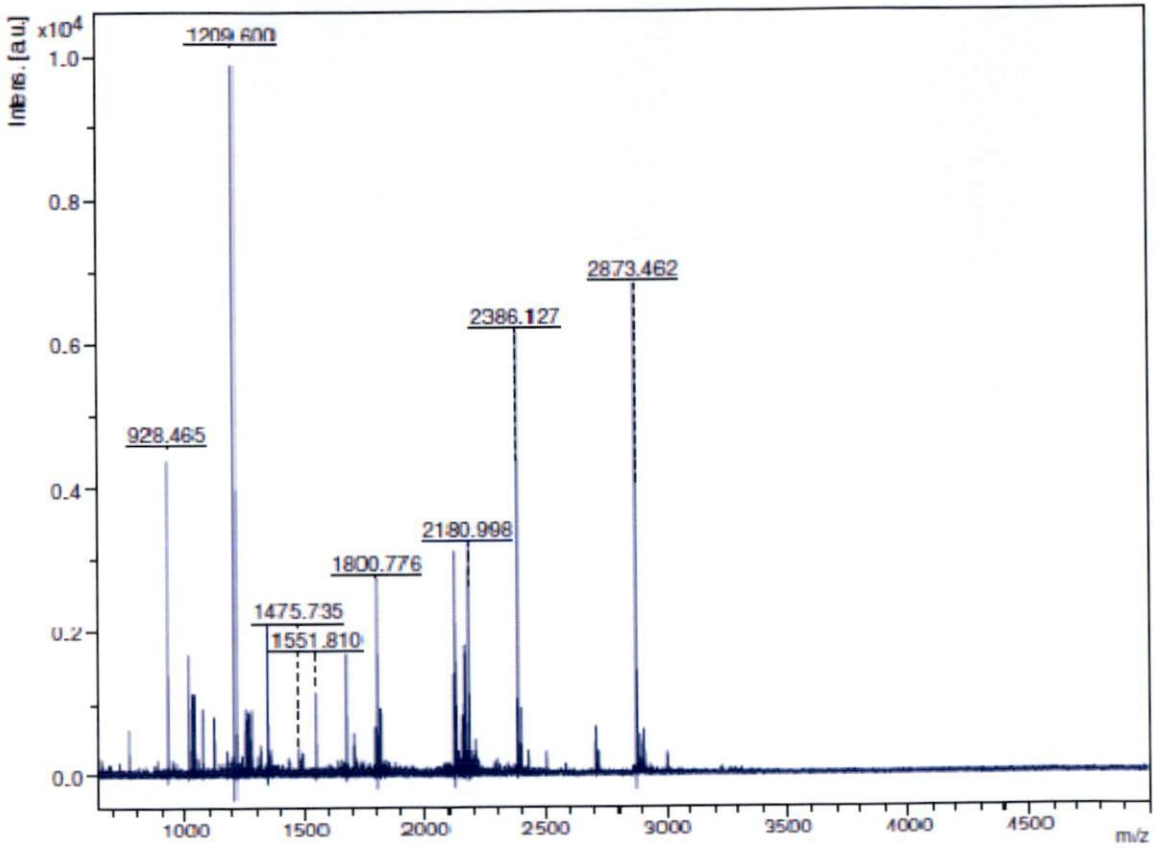


Fig. 4.35: Peptide mass fingerprint of the spot 10 protein extracted from immature organically cultivated pods, obtained by MALDI-ToF Mass spectrometry



m/z	S/N	Quality Fac.	Res.	Intens.	Area
768.487	15.6	7635	5737	646.58	149
928.465	102.8	41158	6431	4541.81	1242
1022.518	39.8	16131	7370	1776.80	498
1036.536	26.5	7845	7318	1192.21	344
1072.618	20.1	2863	7596	899.98	260
1173.560	18.0	5035	7485	819.81	267
1179.576	6.6	1006	7326	309.87	107
1209.600	217.3	48482	7256	10108.33	3738
1211.531	32.0	2999	7777	1488.64	524
1251.647	20.0	7739	7729	938.50	350
1263.705	13.7	297	6727	642.71	272
1265.586	17.4	888	5716	817.29	412
1277.706	9.5	1074	7052	445.67	180
1278.690	12.0	2674	7818	566.36	214
1320.568	7.9	867	7088	373.81	163
1349.615	45.1	11145	7781	2140.66	880
1364.634	7.4	294	8575	350.15	134
1475.735	8.3	1798	8535	401.81	181
1551.810	24.8	13761	9400	1227.64	552
1675.899	35.8	31868	9474	1736.25	896
1708.750	11.0	820	9783	534.90	283
1791.692	11.9	2589	7557	576.51	423
1800.776	56.5	12933	8579	2736.21	1789
1804.951	6.1	471	4784	294.06	338
1814.800	18.1	5232	8433	878.05	504
2117.011	25.2	376	6371	1006.80	1087
2120.982	57.0	4284	7586	2262.76	2063
2125.308	31.5	6613	4253	1226.83	2072
2152.970	15.2	1732	8811	584.05	468
2161.994	20.9	653	8552	799.65	664
2165.002	38.8	4044	7732	1330.46	1226
2180.998	69.1	25144	8274	2612.70	2289
2211.016	8.6	1048	9089	316.09	243
2383.851	17.2	82	8551	557.06	528
2386.127	134.4	19090	8811	4353.27	4027
2400.130	21.3	2417	7940	681.90	700
2705.075	16.1	2329	9395	387.80	405
2717.007	6.9	637	6439	164.11	251
2873.462	194.2	26219	9584	4041.22	4606

Fig. 4.36. *In-silico* analysis of spot 10 protein using Mascot Server software

MATRIX SCIENCE Mascot Search Results

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User            : joy
Email           : joyprashant88@gmail.com
Search title    :
Database        : NCBInr 20151012 (72776944 sequences; 26510890717 residues)
Taxonomy        : Viridiplantae (Green Plants) (3263908 sequences)
Timestamp       : 14 Oct 2015 at 12:32:10 GMT
Top Score       : 222 for gi|920705324, hypothetical protein LR48_Vigan07g225300 [Vigna angularis]
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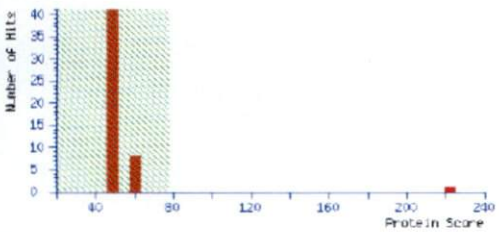
Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 78 are significant (p<0.05).



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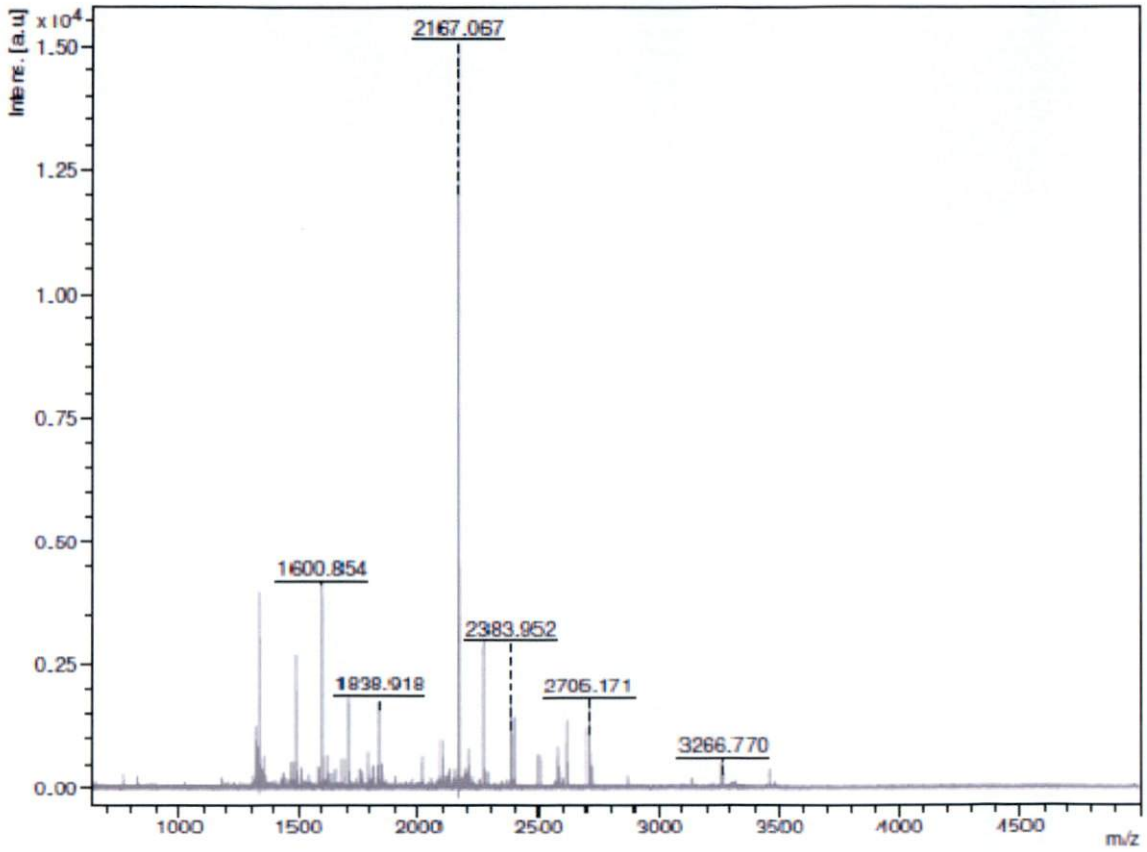
	Accession	Mass	Score	Description
1.	gi|920705324	42000	222	hypothetical protein LR48_Vigan07g225300 [Vigna angularis]
2.	gi|303284531	51102	64	predicted protein [Micromonas pusilla CCMP1545]
3.	gi|670434667	17549	61	PREDICTED: sorting nexin 1-like [Zea mays]
4.	gi|567213109	54683	60	hypothetical protein EUTSA_v10016603mg [Eutrema salsugineum]
5.	gi|902157173	112624	57	hypothetical protein SOVF_184220 [Spinacia oleracea]
6.	gi|629118219	18700	57	hypothetical protein EUGRSUZ_C04263 [Eucalyptus grandis]
7.	gi|222636454	24281	56	hypothetical protein OsJ_23136 [Oryza sativa Japonica Group]
8.	gi|731351608	99546	56	PREDICTED: uncharacterized protein LOC104901272 [Beta vulgaris subsp. vulgaris]
9.	gi|45558475	108510	55	minichromosomal maintenance factor [Triticum aestivum]
10.	gi|695028891	11819	54	PREDICTED: uncharacterized protein LOC103985751 [Musa acuminata subsp. malaccensis]
11.	gi|657960434	58125	54	PREDICTED: uncharacterized protein LOC103435179 [Malus domestica]

Fig. 4.37: Peptide mass fingerprint of the spot 11 protein extracted from immature organically cultivated pods, obtained by MALDI-ToF Mass spectrometry



m/z	S/N	Quality Fac.	Res.	Intens.	Area
768.485	7.6	739	5774	338.16	77
1320.597	21.5	3028	6742	1178.30	537
1325.713	14.5	2132	6884	795.65	356
1336.736	71.0	11687	6960	3020.50	1782
1357.789	10.2	1366	6028	561.64	300
1475.771	8.1	2075	6830	469.86	257
1488.817	42.7	16789	7126	2479.49	1339
1510.813	7.3	761	9243	429.99	189
1600.854	69.4	35020	7543	4148.79	2444
1622.893	9.1	1122	5978	540.99	405
1687.971	7.2	1380	6799	434.06	310
1707.787	29.5	34968	8572	1787.14	1057
1791.736	9.9	2263	6928	598.33	476
1813.892	6.0	1036	6439	361.57	315
1838.918	26.4	16186	7816	1581.34	1179
1851.922	7.0	1340	7473	419.88	332
2018.013	8.3	1114	6329	447.00	457
2096.969	14.1	1761	6592	710.03	736
2167.067	252.0	58877	7779	13071.64	11098
2211.071	10.4	1382	6854	478.96	511
2276.087	48.7	32281	7683	2137.33	2131
2383.952	29.5	8363	7079	1178.20	1368
2399.027	23.9	11496	7216	948.27	1088
2501.214	12.8	5425	6288	463.77	650
2578.369	15.0	2062	6917	511.26	675
2584.221	7.9	1466	6219	266.66	389
2618.291	25.7	12732	7536	842.64	1056
2705.171	25.7	20513	8176	1099.08	1302
2717.104	8.7	1607	6568	264.84	402
3261.586	10.4	1013	5689	217.94	513
3266.770	12.7	3459	7700	266.77	484
3460.764	9.6	2443	7641	180.18	353

Fig. 4.38. *In-silico* analysis of spot 11 protein using Mascot Server software

MATRIX SCIENCE Mascot Search Results

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User            : joy
Email           : joyprashant88@gmail.com
Search title    :
Database        : NCBInr 20151012 (72776944 sequences; 26510890717 residues)
Taxonomy        : Viridiplantae (Green Plants) (3263908 sequences)
Timestamp       : 14 Oct 2015 at 12:33:25 GMT
Top Score       : 117 for gi|920704907, hypothetical protein LR48_Vigan07g183600 [Vigna angularis]
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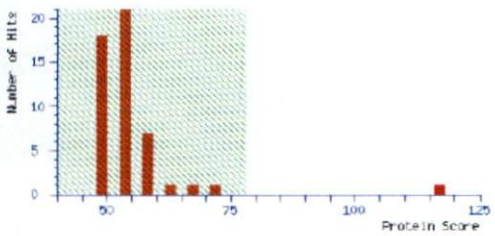
Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 78 are significant (p<0.05).



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	Accession	Mass	Score	Description
1.	gi|920704907	38613	117	hypothetical protein LR48_Vigan07g183600 [Vigna angularis]
2.	gi|727447202	38903	72	PREDICTED: fructose-bisphosphate aldolase, cytoplasmic isozyme [Camelina sativa]
3.	gi|593686734	22738	66	hypothetical protein PHAVU_007G123800g [Phaseolus vulgaris]
4.	gi|593792358	65846	65	hypothetical protein PHAVU_002G219100g [Phaseolus vulgaris]
5.	gi|475600336	167571	59	hypothetical protein F775_03017 [Aegilops tauschii]
6.	gi|147794423	145854	58	hypothetical protein VITISV_010581 [Vitis vinifera]
7.	gi|590636929	41937	57	NAD(P)-binding Rossmann-fold superfamily protein isoform 1 [Theobroma cacao]
8.	gi|590636932	42036	57	NAD(P)-binding Rossmann-fold superfamily protein isoform 2 [Theobroma cacao]
9.	gi|226528108	14934	57	glutaredoxin 2 [Zea mays]
10.	gi|388504930	13319	56	unknown [Lotus japonicus]
11.	gi|641847731	58474	56	hypothetical protein CISIN_1g007251mg [Citrus sinensis]
12.	gi|590701886	100472	55	RB1-inducible coiled-coil protein 1, putative isoform 2 [Theobroma cacao]
13.	gi|629120134	50435	55	hypothetical protein EUGRSUZ_B01453 [Eucalyptus grandis]
14.	gi|835997423	19543	55	PREDICTED: uncharacterized protein LOC101759530 isoform X3 [Setaria italica]
15.	gi|514733755	45851	55	PREDICTED: protein trichome birefringence-like 13 [Setaria italica]
16.	gi|567918650	62289	54	hypothetical protein CICLE_v10007707mg [Citrus clementina]
17.	gi|698464416	79225	53	PREDICTED: putative U-box domain-containing protein 50 isoform X2 [Nicotiana sylvestris]
18.	gi|747098791	85600	53	PREDICTED: ATP-dependent RNA helicase-like protein DB10 [Sesamum indicum]
19.	gi|30697362	40940	53	AAR2 protein family [Arabidopsis thaliana]
20.	gi|474072501	13086	52	hypothetical protein TRIUR3_29973 [Triticum urartu]

DISCUSSION

5. DISCUSSION

Organic farming is receiving increasing attention from consumers, citizen groups, the media, market players, and governments. Although the growth in popularity of organic foods has not met the expectations that its promoters anticipated during the 1970s, the organic sector is now firmly established in the majority of developed countries and provides the basis of a creative tension with the evolution of the inorganic agri-food system. Hence, this research work was done with the objective to compare the quality difference between organic and inorganically grown yardlongbean to get a view about the quality difference in produce cultivated organically and inorganically. The results obtained in the present study "Proteome analysis of organically grown yardlong bean [*Vigna unguiculata* sub sp. *sesquipedalis* (L.) Verdcourt]" are discussed in this chapter based on the earlier reports and best possible interpretations.

5.1 SOIL TEST ANALYSIS

Initial soil analysis data suggests that the nutrient status of soils from the plots were almost the same, it was varying only in Zn content. Since the plots were side by side, the nutrient status was expected to be similar.

5.2 NUTRIENT ANALYSIS OF FRESHLY HARVESTED PODS

When the pods from these plots were analysed at two stages of crop growth, larger variation were seen in Ca, Cu, Fe, Zn, Mn, level, with a huge difference of 86 ppm in Mn level. Pods from inorganic plot showed better accumulation of Mn than organically grown pods. A general trend was observed in case of micronutrients, the level of micronutrients were found to be higher in pods from last harvest compared to the pods of first harvest, except in the case of Zn. Maggio *et al.* (2013) reported that organic farming promotes the accumulation of K in zucchini. But our study had shown that accumulation of K is similar in organic as well as the inorganic produce. López *et al.* (2013) documented that Inorganic peppers showed higher concentrations of N and P than organic fruits.

Nutrient analysis of the pods from both the plots showed that the level of N in organic pods of first harvest was lower than from inorganic pods of first harvest, but the pods from last harvest showed that the level of N was similar in both organic and inorganic pods, this may be due to the fact that the N from cowdung takes time to get available to the plants whereas N from fertilizers are readily available to the plants.

5.3 ANALYSIS OF YIELD AND YIELD PARAMETERS

While working with wheat, Mazzoncini *et al.* (2015) and Campiglia *et al.* (2015) have suggested that organic wheat yielded less than inorganic wheat. Our study shows lesser yield/plot under inorganic. This is due to the fact that crop stand in inorganic plot was less compared to organic plot; the average yield was more in inorganic plot. Higher average yield in inorganic field may be due the fact that soil enrichment under organic farming is a time taking process and this could lead to the initial decline in the crop yield (Stanhill, 1990; Watson *et al.*, 2002; de Ponti *et al.*, 2012).

5.4 ORGANOLEPTIC ANALYSIS

Several studies have reported that consumers were surprised (given the prevailing notion that organically grown produce is better-tasting) to find that the product they had actually identified as better-tasting had been grown inorganically (Barrios and Costell, 2004). Nunes-Damaceno *et al.* (2013), in their comparative study documented that 43.8 per cent of consumers preferred inorganically grown kiwis, 26.8 per cent and 29.4 per cent opted for organically and IFS (Integrated Farming System) grown kiwis, respectively. The higher flavour and overall acceptability ratings given by consumers to inorganically grown kiwis in their study are accordingly probably explained by their greater soluble solids, fructose and glucose contents, while their greater overall acceptability may also be related to the greater juiciness and lesser fibrosity perceived by the sensory analysis panel. The organoleptic tests in our study shows that the panellists gave better score for inorganically cultivated pods. But when these scores were analysed

using Mann-Whitney Test, it concluded that differences in scores in all of the five characters were non-significant. Hence, we can say that the there was no significant difference in the acceptability of consumers to organic and inorganically grown produce, which goes against the common notion that organic food are better tasting than inorganically produced food.

5.5 PROTEIN PROFILING BY SDS-PAGE

The SDS-PAGE profile of the proteins extracted from the four samples i.e, immature pods from organic and inorganic plots and mature pods from organic and inorganic plots, were compared. In all the four samples, 11 bands were observed. Differential bands were identified between mature and immature pods this was due accumulation of protein during seed development. Hill and Breidenbach (1974) had also observed difference in the protein profiles of seed extracts at various stages of seed development. No differential bands were observed while comparing the bands of pods grown in organic and inorganic systems. This result inferred to the fact that there was no protein with different charge to mass ratio which were expressed differentially in organic and inorganically grown cowpea, but this result does not eliminate the possibility of difference in the protein profile among organic and inorganically cultivated pods. Hence the samples were further analysed by 2DE.

5.6 PROTEIN PROFILING BY 2DE

For broader range, IPG strips of pH 3-10 of 17 cm were utilized in 2DE. The 2DE gels obtained were separated into two sets, i.e., mature pod stage and immature pod stage, each set had two gels one of organically and another of inorganically cultivated pods. The gels were analysed set wise; the differential spots between organic and inorganically grown pods were cut out from the gel for further analysis.

5.7 *IN SILICO* ANALYSIS OF THE EXCISED SPOTS

Analysis of the peptide mass fingerprint of all the eleven spots in Mascot Server Software compared the data with all the protein sequences in NCBI database.

The spot 1 which was present in the gel from organically cultivated mature pods and absent in inorganically cultivated pods, was found to be represented by a mixture of PREDICTED: uncharacterized protein LOC104108377 isoform X1 [*Nicotiana tomentosiformis*] and hypothetical protein POPTR_0013s02260g [*Populus trichocarpa*]. Hypothetical proteins are created by gene prediction softwares during genome analysis. When the bioinformatics tool used for gene identification finds a large open reading frame without a characterized homologue in the protein database, it returns "hypothetical protein" as an annotation remark. Uncharacterized proteins are those whose functions are not yet determined. Since the mixture consisted of hypothetical and uncharacterized protein, this result was not able to shed light into the basic function of the protein present in spot 1. Hence protein blast was carried out to identify protein which shows similarity to the uncharacterized protein LOC104108377 isoform X1 [*Nicotiana tomentosiformis*] and hypothetical protein POPTR_0013s02260g [*Populus trichocarpa*]. BLASTp of uncharacterized protein LOC104108377 isoform X1 [*Nicotiana tomentosiformis*] was not helpful as it showed similarity to other uncharacterized protein. Whereas, hypothetical protein POPTR_0013s02260g [*Populus trichocarpa*] was found to be similar to F-box protein At1g55000-like [*Populus euphratica*]. F-box protein At1g55000-like is a component of SCF (ASK-cullin-F-box) E3 ubiquitin ligase complexes, which may mediate the ubiquitination and subsequent proteasomal degradation of target proteins (Uniprot, 2015a).

Spot 2, present in the gel of mature inorganically cultivated pod, was found to be represented by hypothetical protein OsJ_17213 [*Oryza sativa* Japonica Group]. BLASTp of this protein showed that it is similar to hypothetical

protein OsI_18563 [*Oryza sativa* Indica Group]. These results were not useful in determining the protein present in spot 2.

Spot 3, present in gel of inorganically grown mature pods and spot 10 present in immature pods both from organic and inorganic plots but is upregulated in organically grown pods , was found to be represented by hypothetical protein LR48_Vigan07g225300 [*Vigna angularis*], which in turn was found similar to hypothetical protein PHAVU_001G067300g [*Phaseolus vugaris*] and alcohol dehydrogenase1 [*Glycine soja*]. The ADH enzyme is essential for anaerobic metabolism. In higher plants, alcohol fermentation is essential for survival under some environmental stress situations. The *Adh* gene is induced strongly under low oxygen conditions, and the Arabidopsis *ADHl* gene is also induced by low temperature and osmotic stress (Dolferus *et al.* 1997). Pathuri *et al.* (2011) studied the function of alcohol dehydrogenase (ADH) in the interaction of barley with the parasitic fungus *Blumeria graminis*. They found out that ADH knock-down/inhibition resulted in reduced fungal success hence they concluded that ADH activity supports biotrophy by maintaining glycolytic metabolism in pathogen-stressed barley.

Spot 4, present in the mature pods cultivated inorganically, and spot 11 which is present in both the immature pods but upregulated in inorganically cultivated ones, has been represented by hypothetical protein LR48_Vigan07g183600 [*Vigna angularis*], which showed similarity towards Fructose-bisphosphate aldolase, cytoplasmic isozyme (*Glycine soja*). Fructose-bisphosphate aldolase (EC 4.1.2.13), often just aldolase, is an enzyme catalyzing a reversible reaction that splits the aldol, fructose 1,6-bisphosphate, into the triose phosphates dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (G3P). Aldolase can also produce DHAP from other (3S, 4R)-ketose 1-phosphates such as fructose 1-phosphate and sedoheptulose 1, 7-bisphosphate. Gluconeogenesis and the Calvin cycle, which are anabolic pathways, use the reverse reaction. Glycolysis, a catabolic pathway, uses the forward reaction. Aldolase has also been implicated in many "moonlighting" or non-catalytic

functions, based upon its binding affinity for multiple other proteins including F-actin, α-tubulin, light chain dynein, WASP, Band 3 anion exchanger, phospholipase D (PLD2), glucose transporter GLUT4, inositol trisphosphate, V-ATPase and ARNO (a guanine nucleotide exchange factor of ARF6). These associations are thought to be predominantly involved in cellular structure, however, involvement in endocytosis, parasite invasion, cytoskeleton rearrangement, cell motility, membrane protein trafficking and recycling, signal transduction and tissue compartmentalization have been explored (Ahn, *et al.*, 1994; Rangarajan *et al.*, 2010; Merkulova *et al.*, 2011).

Spot 5, present in the inorganically cultivated mature pods, was found to be represented by hypothetical protein LR48_Vigan02g005500 [*Vigna angularis*], which has shown similarity to hypothetical protein LR48_Vigan02g006400 [*Vigna angularis*], chain A, crystal structure of Hemopexin Fold protein Cp4 from cowpea and mungbean seed albumin [*Vigna radiata* var. *radiata*]. Vigeolas *et al.* (2008) reported that albumin protein in pea, PA2, play an important role in regulating polyamine metabolism, which has important function in development, metabolism, and stress responses in plants. Gaur *et al.* (2010), also suggested that the interactions of heme and spermine with LS-24 (hemopexin fold protein) bear physiological implications. While binding of spermine to LS-24 can be linked with polyamine biosynthesis that of heme correlates with oxidative stress. Mutually exclusive binding of heme and spermine in different oligomeric states suggest a role for LS-24 in sensing oxidative. Role of polyamines in the physiology of the plant, if any, is still uncertain but their titre is very responsive to external conditions, such as light, temperature, and various chemical and physical stress agents. Application of exogenous polyamines to plants or plant parts can produce visible effects such as the prevention of senescence in excised leaves and the formation of embryoids or floral primordia in certain otherwise vegetative tissue cultures (Galston and Sawhney, 1990). Fujihara and Harada (1989), reported that rapidly growing root nodule bacteria of the genus *Rhizobium*

produce large quantities of aminobutyl homospermidine, a tetraamine not found in slowly growing strains.

Spot 6, present in both the mature pods but upregulated in organically cultivated pods, was found to be represented by a mixture containing PREDICTED: aldehyde dehydrogenase family 2 member C4-like [*Musa acuminata* sub sp. *malaccensis*], hypothetical protein SOVF_071130 [*Spinacia oleracea*] and PREDICTED: flocculation protein FLO11-like [*Brassica oleracea* var. *oleracea*]. Aldehyde dehydrogenase family 2 member C4-like is involved in ferulic acid and sinapic acid biosynthesis by oxidation of conyferylaldehyde and sinapaldehyde, respectively (Uniprot, 2015b). Ferulic acid (FA), a ubiquitous natural phenolic phytochemical present in seeds, leaves, both in its free form and covalently conjugated to the plant cell wall polysaccharides, glycoproteins, polyamines, lignin and hydroxy fatty acids. FA plays a vital role in providing the rigidity to the cell wall and formation of other important organic compounds like coniferyl alcohol, vanillin, sinapic, diferulic acid and curcumin. FA exhibits wide variety of biological activities such as antioxidant, anti-inflammatory, antimicrobial, anti-allergic, hepatoprotective, anti-carcinogenic, antithrombotic, increase sperm viability, antiviral and vasodilatory actions, metal chelation, modulation of enzyme activity, activation of transcriptional factors, gene expression and signal transduction (Kumar and Pruthi, 2014). Sinapic acid is an orally bioavailable phytochemical, extensively found in spices, citrus and berry fruits, vegetables, cereals, and oilseed crops and is known to exhibit antioxidant, anti-inflammatory, anticancer, anti-mutagenic, anti-glycemic, neuroprotective, and antibacterial activities. The literature reveals that sinapic acid is a bioactive phenolic acid and has the potential to attenuate various chemically induced toxicities (Chen, 2015).

Spot 7, present in the inorganically cultivated pods, had shown to be represented by a mixture containing protein PLASTID MOVEMENT IMPAIRED 2-like [*Camelina sativa*] and trans-resveratrol di-O-methyltransferase-like [*Cucumis sativus*]. Protein PLASTID MOVEMENT IMPAIRED 2-like is

required for the chloroplast avoidance response under high intensity blue light. This avoidance response consists in the relocation of chloroplasts on the anticlinal side of exposed cells. Acts in association with WEB1 to maintain the velocity of chloroplast photorelocation movement via cp-actin filaments regulation (Uniprot, 2015c). Trans-resveratrol di-O-methyltransferase-like catalyzes the biosynthesis of pterostilbene from resveratrol. Pterostilbene has both antifungal and pharmacological properties (Uniprot, 2015d). Pterostilbene (trans-3,5-dimethoxy-4-hydroxystilbene) is a natural dietary compound and the primary antioxidant component of blueberries. It has higher bioavailability in comparison to other stilbene compounds, which may enhance its dietary benefit and possibly contribute to a valuable clinical effect. Multiple studies have demonstrated the antioxidant activity of pterostilbene in both *in vitro* and *in vivo* models illustrating both preventative and therapeutic benefits. The antioxidant activity of pterostilbene has been implicated in anti-carcinogenesis, modulation of neurological disease, anti-inflammation, attenuation of vascular disease, and amelioration of diabetes (McCormak and McFadden, 2013).

Spot 8 and spot 9, present in the organically cultivated pods were represented by hypothetical protein EUTSA_v10007083mg [*Eutrema salsugineum*]. BLASTp of this protein has shown that protein EUTSA_v10007083mg shows similarity with hypothetical protein. Hence the function of these proteins cannot be assessed. Availability of the identified proteins from the 11 spots is depicted in Table 5.1.

Table 5.1 Differential expression/ upregulation/ downregulation of proteins in yardlong bean pods

Sl.no	Protein identified	Availability			
		Immature pods		Mature pods	
		Inorganic C1	Organic C2	Inorganic C3	Organic C4
1	F-box protein At1g55000-like [*Populus euphratica*]	-	-	-	+
2	Alcohol dehydrogenase1 [*Glycine soja*]	+	++	+	-
3	Fructose-bisphosphate aldolase, cytoplasmic isozyme (*Glycine soja*).	++	+	+	-
4	Chain A, crystal structure of Hemopexin Fold protein Cp4 from cowpea	-	-	+	-
5	Mungbean seed albumin [*Vigna radiata* var. *radiata*]	-	-	+	-
6	Aldehyde dehydrogenase family 2 member C4-like [*Musa acuminata* subsp. malaccensis]	-	-	+	+
7	Protein PLASTID MOVEMENT IMPAIRED 2-like [*Camelina sativa*]	+	-	-	-
8	Trans-resveratrol di-O-methyltransferase-like [*Cucumis sativus*]	+	-	-	-

(-) –no expression; (+)- expression and (++)- over expression

SUMMARY

6. SUMMARY

The study entitled "Proteome analysis of organically grown yardlong bean [*Vigna unguiculata* sub sp. *sesquipedalis* (L.) Verdcourt]" was conducted at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, during the period of 2013-2015. The objective of the study was to compare organically and inorganically grown yardlong bean in terms of protein expression through 2DE proteome analysis.

The yardlong bean variety Sharika was selected for the study. Soil from the plots was analysed before the crop was sown. Two plots were included in the study, each with an area 40 m^2 and three rows of pits, each row having five pits. Since Sharika is a trailing variety, it was sown in pits (at the rate of 3 plants / pit) at 2 m x 2 m spacing. For nourishment in organic plot 8 kg/per pit cowdung was incorporated into the soil before sowing. In the inorganic plot Nitrogen, Phosphorous and Potassium were supplemented through commercial fertilizers (Factamphos 20:20:15 NPS and MoP 60 K), to provide 20:20:10 N P K / ha, as recommended by Kerala Agricultural University (2011), with slight modification to suit the pole type cultivar. Immature pods at two stages of crop growth (at 2 MAS and 4 MAS) were analysed for its nutrient status. Pods were collected at two stages for proteome profiling i.e. immature stage (vegetable purpose), and mature stage.

Organoleptic properties of immature pods from organic and inorganic plots were evaluated by 22 panelists using a 9-point hedonic scale. This organoleptic analysis suggested no significant difference between the organic and the inorganic produce.

Molecular work was initiated with protein extraction from the organic and inorganic pods at two stages of maturity (10 DAFO and 15 DAFO). The extracted protein was quantified using Lowry's method. The quantified sample was then analysed through SDS-PAGE. No differential band was observed when the proteome profiles of organic and inorganic produce were compared. Hence the

samples were further analysed through 2DE. Eight differential and two upregulated and a downregulated spot were identified while comparing proteome profiles of organic and inorganic produce. These spots were then excised from the gel and analysed by MALDI-ToF/MS through outsourcing. The peptide fingerprint data thus generated was used as query in Mascot analysis. Mascot search results revealed that two of the spots represent characterized protein and nine of them represented either hypothetical or uncharacterized proteins. Protein from those nine spots was analysed through BLASTp and SmartBLAST, which suggested that proteins from 6 of them shows similarity with characterized proteins and proteins from rest of the three spots shows similarity with hypothetical and uncharacterized proteins.

Spot 6 consisted of a mixture of PREDICTED: aldehyde dehydrogenase family 2 member C4-like, hypothetical protein SOVF_071130 [*Spinacia oleracea*] and PREDICTED: flocculation protein FLO11-like [*Brassica oleracea* var. *oleracea*]. Spot 7 was represented by a mixture containing protein PLASTID MOVEMENT IMPAIRED 2-like [*Camelina sativa*] and trans-resveratrol di-O-methyltransferase-like [*Cucumis sativus*].

Thus, this study shows that the numerous proteins contributing to quality aspects in cowpea are differentially expressed under both the systems of crop nourishment and thus a conclusive decision on the enhancement of nutritional quality of the pods based on the method of crop nourishment cannot be drawn.

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ANNEXURE

ANNEXURE – I

Details of laboratory equipment items used for the study

Microwave oven	: Samsung
Autoclave	: Kdt, YS-18L
Icematic	: SIMAG
Micropipettes	: Eppendorf
Balance	: Shimadzu, AX200
pH meter, PC 510	: EuTech Instruments
Medical freezer	: SANYO
Refrigerator	: Godrej, LG, Samsung
High speed refrigerated centrifuge	: Kubota , Model 6500
Minispin	: Eppendorf, MiniSpin®
Cylindrical water bath	: Rotex
Vertical electrophoresis system	: BIO-RAD, Mini-PROTEAN®
Power PAC	: BIO-RAD, Power PAC 300 and 1000
Circulating cooling system	: Annalab, Model-A11-RCB10
Rocker 25	: Labnet
2Dimensional Electrophoresis unit	: BIO-RAD, PROTEAN IEF cell
Spectrophotometer	: SHIMADZU, UV-1800

ANNEXURE-II

Chemicals used in protein extraction:

A) TCA extraction solution

10 per cent TCA: 10 g

0.07 per cent DTT: 0.07 g

Made upto 100ml with acetone

B) Sample washing buffer

0.07 per cent DTT: 0.07 g

Made upto 100ml with aceone

Stored in glassbottle at -20 °C

C) Lysis buffer:

9M Urea: 5.4 g

CHAPS 4 per cent: 0.4 g

DTT 1 per cent: 0.1 g

pH 3-10 ampholytes: 250 µl

35 mM Tris base: 0.0424 g

Lysis buffer was made up to 10 ml with milliQ water and filtered through 0.2 µm pore size membrane. Small aliquots were made and stored at -80 °C. Sample buffer once thawed cannot be refrozen for further use.

ANNEXURE-III

Chemicals used in one dimensional focussing

A) Rehydration buffer:

Urea: 12 g

CHAPS: 0.5 g

Bromophenol blue: few grains

Double distilled water: 25 ml

Ampholyte pH 3-10

DTT: 0.1 g

B) Equilibration buffer I:

6M Urea: 3.6 g

30 per cent w/v Glycerol: 3 ml

2 per cent w/v SDS: 0.2 g

1per cent w/v DTT: 0.1 g

1.5 mM Tris HCl buffer pH 8.8: 0.200 ml

C) Equilibration buffer II:

6M Urea: 3.6g

30 per cent w/v Glycerol: 3ml

2 per cent w/v SDS: 0.2g

Iodoacetamide: 0.25g

1.5 mM Tris HCl buffer pH 8.8: 0.200 ml

ANNEXURE-IV

Chemicals for SDS-PAGE analysis

Acrylamide-bisacrylamide stock

29.2 g of acrylamide and 0.8 g of N N Bismethlene acrylamide was dissolved in 80 ml of distilled water and made up the 100 ml. The solution was then filtered and stored at 4 °C in dark upto 30 days.

Separating gel buffer (pH 8.8)

22.7 g of Tris base was dissolved in 80 ml of distilled water, the pH was adjusted to 8.8 with 1 N HCl and the volume was made up to 100 ml with distilled water. The solution was stored at 4 °C.

Stacking gel buffer (pH 6.8)

7.26 g of 0.6 M Tris base was dissolved in distilled water and the volume was made upto 100 ml with distilled water. The solution was stored at 4 °C.

10 per cent Sodium Dodecyl Sulphate (SDS)

1 g of SDS was dissolved in distilled water and the volume was made up to 10 ml with distilled water. The solution was stored at room temperature.

10 per cent Ammonium Persulphate (APS)

0.1 g of APS was dissolved in 1 ml of distilled water to obtain 10% of APS.

Separating Gel (12%)

- Acrylamide solution (30 per cent): 19.79 ml
- Distilled water: 16.8 ml
- Tris (1.5 M, pH 8.8): 12.5 ml

- 10 per cent SDS: 0.5 ml
- 10 per cent APS: 0.25 ml
- TEMED: 0.025 ml

Stacking Gel (4%)

- Acrylamide solution (30 per cent): 1.34 ml
- Distilled water: 6.150 ml
- Tris (1.5M, pH 6.8): 2.5 ml
- 10 per cent SDS: 0.05 ml
- 10 per cent APS: 0.080 ml
- TEMED: 0.016 ml

Sample buffer (10 ml)

- SDS (10 per cent): 4 ml
- Tris HCl (pH 6.8): 2.5 ml
- β mercaptoethanol: 0.2 ml
- Glycerol: 2 ml
- Bromophenol blue: 0.2 g

Tank buffer (1000ml, 10X)

- Tris Base: 12.2 g
- Glycine: 57.6 g
- SDS: 0.5 g

  All the components were mixed and made upto 1L. The buffer can be stored at 4 °C and warmed to 37 °C before use. The same buffer can be used 2-3 times for running the gel.

Solutions for Coomassie staining

Staining solution

- Coomassie brilliant blue R250 dye : 0.1 g
- Methanol : 40 ml
- Acetic acid : 10 ml
- Distilled water: 50 ml
- The dye was first dissolved in methanol and all the other components were added. Every time fresh preparation of the dye solution was prepared.

Destaining solution

- Methanol : 40 ml
- Acetic acid : 10 ml
- Distilled water : 50 ml

ANNEXURE-V

Scale and score card used in organoleptic analysis

9 point hedonic scale

Like extremely	**9**
Like very much	**8**
Like moderately	**7**
Like slightly	**6**
Neither like nor dislike	**5**
Dislike slightly	**4**
Dislike moderately	**3**
Dislike very much	**2**
Dislike extremely	**1**

Score card of organoleptic evaluation

Sl no.	Characteristics	Sample A	Sample B
1.	Colour		
2.	Taste		
3.	Flavour		
4.	Stringiness		
5.	General appeal		

PROTEOME ANALYSIS OF ORGANICALLY GROWN YARDLONG BEAN [*VIGNA UNGUICULATA* SUB SP. *SESQUIPEDALIS* (L.) VERDCOURT]

By

DEEPTHI K. KUMAR

(2013-11-104)

ABSTRACT OF THESIS

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Kerala Agricultural University, Thrissur

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COLLEGE OF HORTICULTURE

VELLANIKKARA, THRISSUR – 680656

KERALA, INDIA

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Abstract

The term 'organic' is gaining more and more popularity these days. This rapid growth may be traced to increased consumer confidence in organic foods as well as to the concern about the possible health risks and environmental impacts of inorganic food production methods. Even though the world carries so much of perceptions regarding the quality of organic foods, the scientific backing is missing for most of these assumptions. The study entitled 'Proteome analysis of organically grown yardlong bean [*Vigna unguiculata* sub sp. *sesquipedalis* (L.) Verdcourt]' aimed at comparing organically and inorganically grown yardlong beans in terms of protein and amino acid expression through 2-dimensional proteome analysis (2DE).

Seeds of yardlong bean 'Sharika' were sown, in plots each of 40 m^2 area. The nutrient status of organic and inorganic plot was assessed prior to the study. At two stages of crop growth i.e. at two months after sowing and four months after sowing, immature pods were analysed for their nutrient status. Among the pods of two plots, prominent variation was observed in the levels of iron and manganese. In organoleptic analysis, 22 judges have evaluated the organic and inorganic produce with a 9 point hedonic scale. Mann-Whitney test of these data revealed that there is no significant difference between the organic and inorganic produce.

Freshly harvested pods were used for the extraction of protein using TCA/acetone buffer. This powdered sample was then solubilized in lysis buffer, which was used for further analysis. Protein concentration was estimated using Lowry's method. The samples with good quantity of protein for all the four category .i.e, immature pods from inorganic and organic plot (C1 and C2) and mature pods from inorganic and organic plot (C3 and C4), were subjected to SDS-PAGE. Since there was no differential bands observed between pods from inorganic and organic plot, the samples were subjected to 2DE. Eight differential and two upregulated spots and one downregulated spot were identified while

comparing the gels of inorganic and organic samples. These spots were then excised and characterized using MALDI-ToF/MS through outsourcing. Data from MALDI-ToF/MS was used as query for mascot search. Mascot search of some spots yielded only hypothetical and uncharacterized proteins, so these proteins were used as query for BLASTp and SmartBLAST analyses. Mascot results have suggests that a protein, aldehyde dehydrogenase family 2 member C4-like, which is involved in ferulic acid and sinapic acid biosynthesis, is present in the mature pods from both the plots but is upregulated in organic produce. Another protein trans-resveratrol di-O-methyltransferase-like is present in immature pods from inorganic plot, which catalyses the biosynthesis of pterostilbene. Pterostilbene has antifungal, antioxidant and pharmacological properties.

This study concludes that there is no significant difference in the organoleptic properties between organic and inorganic produce and similarly there is no much difference in the protein profile except that the inorganic produce consisted of two proteins that are involved in biosynthesis of antioxidants, whereas, in the organic produce, only one protein was identified to be involved in the biosynthesis of antioxidants.