A methodological investigation of the proteins present in the roots of the resurrection plant, *Xerophyta viscosa*, for further proteomic analyses

Rizqah Kamies

Supervisor: Prof. J.M. Farrant
Co-supervisor: Dr. M.S. Rafudeen

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"*We are what we repeatedly do,*

Excellence then,

Is not an act,

But a habit"

Aristotle (384BC-322BC)

"An education is not how much you have committed to memory,

Or even how much you know,

It's being able to differentiate between what you do know

And what you do not"

Anatole France (1844-1924)

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Declaration

I know the meaning of plagiarism and declare that all work done in the document, save for which is properly acknowledged, is my own. This work has not been presented at any other university for examination or for any other purposes.

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List of abbreviations

1-DE	one- dimensional electrophoresis
2DE	two-dimensional electrophoresis
ABA	abscisic acid
ALDH	aldehyde dehydrogenase
ATP	adenosine triphosphate
BCA	bicinchoninic acid
BSA	bovine serum albumin
CAT	catalase
CBB	coomassie brilliant blue
CHAPS	((3-cholamido propyl)-dimethylammonio)-1-propane sulfonate
DTT	dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EF-Tu	elongation factor Tu
FBP	fructose-bisphosphate
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
GRP	glycine-rich RNA binding protein
GTP	guanosine triphosphate
HSP	heat shock polypeptide
IEF	isoelectric focusing
IPG	immobilised pH gradient
iTRAQ	isobaric tags for relative and absolute quantification
KCl	potassium chloride
LC-MS/MS	liquid chromatography-tandem mass spectrometry
MS	mass spectrometry
PAGE	polyacrylamide gel electrophoresis
p*I*	isoelectric points
PMSF	phenylmethylsulfonyl Fluoride
PVPP	polyvinylpolypyrrolidone
RFO	raffinose family oligosaccharide
RNA	ribonucleic acid
ROS	reactive oxygen species
RWC	relative water content

SAM	S-adeonsylmethionine
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOD	superoxide dismutase
TCA	trichloracetic acid
TIM	triosephosphate isomerise
tRNA	transfer ribonucleic acid
ULB	urea lysis buffer

Abstract

In order to conduct proteomic analysis on the hydrated root tissues of the resurrection plant, *Xerophyta viscosa* Baker, aeroponically grown plant roots were subjected to various proteomic techniques. Three protein extraction methods were investigated, of which one method, was the most suited in isolating total protein from the root tissues of *X. viscosa.* This method (Method C) was optimised using maize (*Zea mays*) roots as a positive control and testing material. Once optimised, the modified method of protein extraction was used to extract good quality, stable protein from the root tissues of *X. viscosa* under hydrated aeroponic conditions. The extracted proteins were subjected to two-dimensional electrophoresis (2-DE) and OFFGEL fractionation analysis in order to separate proteins according to their respective isoelectric points (p*I*). It was found when using these techniques, that OFFGEL fractionation analysis provided a reproducible and complete protein profile in comparison to 2-DE analysis.

Two OFFGEL fractions chosen from the separated protein profile were analysed using liquid chromatography tandem mass spectrometry (LC-MS/MS) for protein identification. For the two fractions analysed, it was found that 128 peptides out of 150 peptides identified (OFFGEL fraction 5) and 80 out of the 109 peptides identified (OFFGEL fraction 7) were above the protein identity threshold score. The identified proteins were then functionally classified according to their annotated biological processes, in which it was found that a number of proteins identified in the roots of *X.viscosa* were involved in metabolism. These functional categories include carbohydrate metabolism (representing 24 and 35% of the total proteins present in fractions 5 and 7, respectively), energy metabolism (4 and 5%), protein metabolism (15 and 26%), antioxidant mechanisms (22 and 9%), stress response (1 and 2%) and unknown proteins (18 and 5%). A number of proteins involved in other metabolic processes were also identified such as apoptosis (5 and 9%), protein transport and signalling (5 and 5%) as well as nucleasome assembly (1 and 2%).

Although a large number of the proteins identified were linked to metabolism and general day to day maintenance of the roots, a number of stress-related proteins were found to be constitutively present in the roots of *X. viscosa.* One antioxidant enzyme in particular, unique to desiccation tolerant material such as seeds and resurrection plants, 1-cys peroxiredoxin, was identified. Other antioxidant enzymes identified, known to offer protection during stress conditions include, class III peroxidases, the "housekeeping" antioxidants, superoxide

dismutase and catalase as well as proteins known to function in response to stress such as Hsp 70, oxidoreductase and aldehyde dehydrogenase.

This study provides a comprehensive overview of the methods used to isolate pure proteins from hydrated roots of *X. viscosa,* by using a physiologically tested aeroponic plant growth system. A series of optimisation procedures for the extraction of intact proteins and further proteomic analyses of proteins present in the hydrated root tissues of *X. viscosa* are presented and identified, some of which include those that are potentially constitutively involved in stress response.

1.) Introduction

1.1) Desiccation tolerance: the enigma that captivates us

The ability of plants to survive in times of low water availability is not common. Most flowering plants are sensitive to desiccation and usually cannot cope with drastic water loss, these plants usually die when their relative water content (RWC) drops to below 20-50% (Toldi *et al*., 2009). However, there are a small group of plants termed "resurrection plants" that are able to tolerate extreme water loss and revive from the air-dried state (Gaff, 1971). Resurrection plants have the ability to acquire desiccation tolerance when environmental conditions become harsh and rapid water loss occurs, enabling the loss of more than 95% of their cellular water (Farrant, 2007). These plants remain in the dry state for extended periods of time and yet are able to recover to full metabolic and physiological activity upon rehydration (Gaff, 1971; Farrant, 2000; Farrant *et al.*, 2007).

Xerophyta viscosa Baker is one such species and is able to survive drying to 5 % RWC while still resuming full physiological activity within 80 h of re-watering (Sherwin and Farrant, 1998; Ingle *et al*., 2007). This angiosperm resurrection plant is one of approximately 350 known angiosperms that have the innate ability to induce specialised protection mechanisms during dehydration to minimize the damage associated with subcellular water-loss (reviewed by Oliver *et al*., 1998; Vicre *et al*., 2004; Farrant, 2007; Moore *et al*., 2009). The stresses associated with water-deficit are many and these mechanisms used by resurrection plants to ameliorate such stresses are shown in Fig. 1 (adapted from Farrant *et al*., 2011). The stresses include mechanical stress associated with a loss of cell turgor (Vertucci and Farrant, 1995; Vicre *et al.*, 2004), and various metabolic stresses associated with excessive reactive oxygen species (ROS). These metabolic stresses are intensified by the concentration of the cytoplasmic constituents leading to ionic stress, loss of membrane integrity and protein denaturation as water is rapidly lost (Mundree *et al*., 2002). Protection mechanisms preventing damage associated with the stresses are multiple but interestingly there are many similarities between seeds and resurrection plants, with some that are unique to resurrection plants (Farrant *et al*., 2011; Fig. 1).

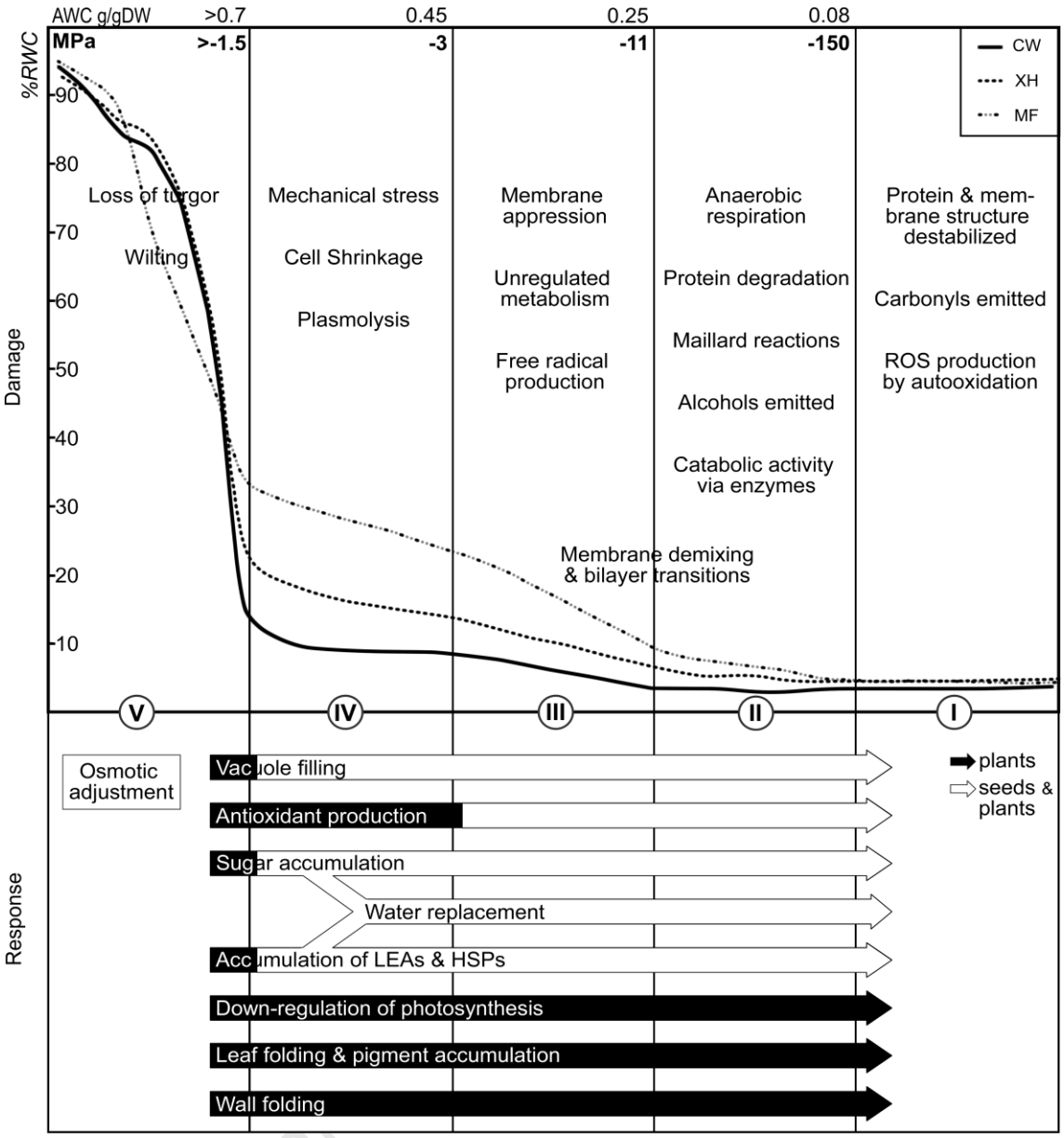

Fig. 1 Diagram depicting changes in RWC in seeds and the resurrection plants *Craterostigma wilmsii* (CW), *Xerophyta humilis* (XH) and *Myrothamnus flabellifolia* (MF) with the associated physical and metabolic effects reported to occur during desiccation. The appropriate protection mechanisms laid down in response to water-deficit are shown by white arrows for both seeds and plants and black for those present in resurrection plants only. The decline in water concentration (g H_2O gDW^{-1}) as a function of Relative Water Content (RWC) is shown for several resurrection plant species (Farrant, 2007) and is similar to the trend for seeds given in Berjak *et al*. (2007) (adapted from Farrant *et al*., 2011).

From the figure above (Fig. 1), some of the protection mechanisms laid down as a consequence of metabolic stress in response to water deficit in resurrection plants are the upregulation of antioxidants, the production and accumulation of non-reducing sugars and the accumulation stress-related or dehydrin proteins (Farrant, 2000; Scott, 2000; Vicre *et al.*,

2004; Illing *et al*., 2005; Farrant *et al*., 2007). Some of the physical changes that occur in resurrection plants are wall folding, leaf folding and pigment accumulation as well as the down regulation of photosynthesis (Farrant, 2007).

1.2) Resurrection plant research historically focused on leaf vegetative tissues

To date virtually all the research reported on resurrection plants have mostly been performed on leaf vegetative tissues (Sherwin and Farrant, 1998; Farrant, 2000; Mowla *et al*., 2002; Balsamo *et al*., 2005; Farrant *et al.*, 2007; Ingle *et al.*, 2007; Moore *et al*., 2007), while very little research has been previously reported on the root tissues of resurrection plants (Farrant, 2007). Reported studies to date have focused on sucrose accumulation during desiccation in the root and leaf tissues of the resurrection plant, *Craterostigma plantagineum* (Norwood *et al.*, 2000; 2003). The accumulation of sucrose during desiccation is thought to be linked to stabilising macromolecular interactions between membranes and proteins (Scott, 2000; Farrant, 2007), as well as helping in stabilising the subcellular milieu and aid in glass formation or vitrification of the cytoplasm during desiccation (Farrant, 2007; Farrant *et al*., 2007; 2011). Djilianov *et al*. (2005) were able to demonstrate successful *in vitro* propagation and regeneration of the endangered resurrection plant native to the Balkan region, *Haberlea rhodopensis*, and through the use of such studies, observed root and shoot development in a low nutrient tissue culture medium (Djilianov *et al*., 2005). It should be noted however, that both studies were conducted by using aseptic cultures only, either by germination from seed on (Murashige and Skoog, 1962) tissue culture medium (Djilianov *et al*., 2005), or by side-shoot cutting and growing on peat (Norwood *et al*., 2000). Although the root study was successful, these aseptic techniques were found to be laborious and time consuming, sometimes taking up to six or seven months to achieve conclusive results (Norwood *et al*., 2000; Djilianov *et al*., 2005).

1.3) Problems that deter the study of plant roots – the good, the bad and the ugly

The ability to conduct root study on plants in general is not widespread due to the complex and problematic nature of root tissues (Mehta *et al*., 2008), as well as the methodological difficulties associated with research on the root tissues (Waisel, 2001). The majority of plant roots are buried in soil and would need to be unearthed before any observation, treatment or analysis can be performed (Waisel, 2001). It is thus known that when conducting any observational, biochemical and physiological investigations on the root tissues of plants,

careful consideration needs to take place beforehand to avoid contamination by soil particles and damage when plants are uprooted from soil and rocky surfaces. With regards to conducting root study on resurrection plants, there is an added difficulty as specialised techniques would have to be employed due to plants often inhabiting ecological niches where water availability is low either in shallow soils or rocky outcrops (Porembski and Barthlott, 2000), and excavation would lead to breakage of finer roots or damage to larger ones (Waisel, 2001). Even if this is adhered, the soil particles adhering to the root tissues cannot always be thoroughly removed. One might be able to wash the excess soil particles off root tissues when observing plants in a hydrated state, but when conducting dehydration experiments, washing the root tissues off with water is not an option, as this would alter the root water content and thus it's biochemistry.

1.4) Aeroponic root study – the salvation?

In order to overcome the above mentioned problems, aeroponics can be used as an alternative method of cultivation. Aeroponic plant growth is the only method thus far that allows comprehensive studies of root systems without hampering the root tissues (Waisel, 2001). Not only has a wide range of plant species been shown to grow aeroponically, but most of the plants examined, displayed excellent growth under these conditions and were able to develop well-formed root systems (Waisel, 2001). In this optimised system, plants are grown in a nutrient rich air environment by regular electronic spraying of the root tissues with a plant nutrient solution, typically, Hoagland's solution (Hoagland and Arnon, 1950) (see Fig. 2 for *X. viscosa* growth in aeroponics system). Plant roots are free from soil particles or any other adhering material, allowing easy and direct access to the root tissues without inflicting unnecessary damage (Hayden *et al*., 2004). The root tissues are free from contaminating material caused by soil-borne microbes that might interfere with biochemical processes (Pagliarulo and Hayden, 2002; Pagliarulo *et al*., 2004). There is also an increase in airflow that occurs in the root tissues of plants growing aeroponically, which has previously demonstrated enhanced root growth (Zobel *et al*., 1975). In conjunction with all these positive points, controlled dehydration experiments of plants can be performed by reducing and then halting the rate of root spray. Once nutrient spraying ceases and plants are considered to be dry, the roots can be observed and analysed in the dry state. Such systems have been successfully used to study root tissues of many plant species including evergreen and

deciduous trees, ornamental plants, crop plants, xerophytes, hygrophytes and lithophytes (Waisel, 2001).

Fig. 2 Aeroponic plant growth system containing *X. viscosa* plants. (A) Well maintained *X. viscosa* leaves and (B) free hanging *X. viscosa* roots.

We have designed and optimised an aeroponic plant growth system to suit the growth and cultivation of the resurrection plant, *X. viscosa*, and have described the use of this physiologically tested system in Kamies *et al*. (2010). This system was used to investigate the roots response to desiccation in *X. viscosa*, by observing one of the key protection mechanisms induced by resurrection plants during desiccation, namely the upregulation of antioxidant activity (Farrant, 2000, 2007; Mowla *et al*., 2002; Moore *et al*., 2004; Kranner and Birtic, 2005; Illing *et al*., 2005; Moore *et al*., 2009; Farrant *et al*., 2011). This novel study provided us with the much needed insight to how the roots cope with desiccation with regard to antioxidant accumulation and by using this physiologically tested aeroponic plant growth system, presented an opportunity to investigate even further what occurs within the root tissues of *X. viscosa.*

1.5) Plant Root proteomics – "Rooteomics" thus far

Proteomics has been used in many regards to observe and analyse a wide range of protein profiles present in various organisms. The most common method of protein analysis involves two-dimensional electrophoresis (2-DE) analysis (O'Farrell, 1975), which essentially allows for the separation of protein samples based on their isoelectric points (p*I*), molecular mass

(Braun *et al.,* 2007), solubility and relative abundance (Gorg *et al*., 2004). Once protein spots have been successfully separated according to the above parameters, they are then excised from the 2-DE gels and identified using different types of mass spectrometry (MS) (Yates, 1998). The technique of using mass spectrometry to identify proteins has developed over the years into a more sophisticated tool of protein identification and has enhanced our abilities to compare proteomes of the same or different species, or proteomes from the same species but from different organs (comparative proteomics).

For investigations of plant tissues, many studies have been conducted using the above mentioned techniques, especially on the leaf (Bahrman *et al*., 2005; Albertin *et al*., 2009; Prinsi *et al.,* 2009; Jellouli *et al.,* 2010), and seed proteomes (Rajjou *et al.,* 2004; Hochholdinger *et al*., 2005; Hajduch *et al*., 2006; Miernyk and Hajduch, 2011). To date, little has been reported with regards to the root proteomes of plants due to the technical difficulties associated with root observation and analysis. Those that have been successfully performed and reported were able to analyse various forms of stress factors that affect the root tissues of plants, most of which were predominantly reported for crop plants such as maize, wheat, barley and rice. These factors would include heat stress (Nieto-Sotelo *et al*., 2002; Xu *et al*., 2008), salt stress (Yan *et al*., 2005; Witzel *et al.,* 2009; Manaa *et al.,* 2011), cold stress (Komatsu *et al*., 1999) and drought stress (Perez-Molphe-Balch *et al.,* 1996; Costa *et al*., 1998; Yoshimura *et al*., 2008).

For resurrection plants, there have been few proteomic studies mainly focusing on the leaf vegetative tissues, these would include proteomic studies performed on the leaves of the resurrection plant, *X. viscosa* and the desiccation-tolerant grass *Sporobolus stapfianus* in response to desiccation (Ingle *et al*., 2007; Oliver *et al*., 2011). Ingle *et al*. (2007) have reported on proteins that were upregulated in response to desiccation such as a few *de novo* synthesized proteins involved in antioxidant metabolism, PSII stabilizers, chaperonins and RNA-binding proteins as well as the down regulation of proteins involved in photosynthesis. For the desiccation-tolerant grass *S. stapfianus,* enzymes involved in glycolysis and the Calvin cycle were upregulated in response to desiccation, as well as the possible formation of a protein kinase-based signalling cascade and brassinosteroid involvement in the regulation of the cellular protection in response to desiccation (Oliver *et al*., 2011). When proteomic studies were targeted to the nucleus structure in the leaves of *X.viscosa* to identify possible nuclear

proteins involved in desiccation, eighteen proteins were found to be upregulated when plants were subjected to drying to 35% RWC. Of those proteins identified, four were involved in gene regulation, four were associated with translation, while others were linked to molecular chaperone type activities and energy metabolism (Abdalla *et al*., 2010) (For a further overview on proteomic studies performed on resurrection plants see Morse *et al.,* 2011). While there have been a few studies reported on proteomes of angiosperm resurrection plants, there have been no reports on the proteomes of root tissues in any resurrection plant (Farrant, 2007; Morse *et al*., 2011).

There have been a few reports on the physiological factors that affect the root tissues of plants such as the identification of differentially expressed proteins in response to high concentrations of nitrogen and nitrogen deficiency in the roots of wheat (*Triticum aestivum L.*) (Bahrman *et al*., 2005) and changes in proteins particularly induced by nitrate in the roots and leaves of maize (*Zea mays* L.) (Prinsi *et al*., 2009). Recently there have been some advances in the process of phytoremediation whereby plant roots were actively used to extract heavy metals or metalloids from contaminated soils (Requejo and Tena, 2005; Alvarez *et al*., 2009). These have been reported for maize (Requejo and Tena, 2005) and Indian mustard (*Brassica juncea* L.) roots (Alvarez *et al*., 2009) by observing changes in their root proteomic profiles in response to the toxic heavy metals, arsenic and cadmium respectively.

In the field of comparative proteomics, some researchers have compared the proteomes of different organs present in the same plant, such as the root, stem and leaf for functional and structural studies. It was found that when using 2-DE to observe the three organs of *Brassica napus* or oil seed rape (Albertin *et al*., 2009), that 43% of the observed protein spots functioned in "housekeeping" or were common in all three organs, while the green organs (stem and leaf) were more closely related with more than 80% common spots in comparison to the roots, who displayed protein spots that were specific to the root tissues only (approximately 10%) (Albertin *et al*., 2009). By observing these protein maps generated from the proteomes of the three organs, the authors were able to draw conclusions on which proteins showed root specific expression and would thus have a more specialised function, and which proteins in comparison in the leaf and stem proteomes that would function more in general housekeeping and maintenance (Albertin *et al*., 2009). Similarly comparative proteomics of root, stem and leaf was achieved in rice (Nozu *et al.,* 2006), where a contrasting

result was observed and only 12% of all observed spots were present in all tissues while more proteins were found to be tissue specific (21% for leaf, 14% for stem and 36% for root) (Nozu *et al*., 2006). Of those spots identified, proteins that function in photosynthesis and energy production were located in the leaf and stem tissues, while those functioning in cell defence and response to disease were located in the root tissues (Nozu *et al*., 2006). This would be a very sensible explanation when one takes into account all the naturally occurring environmental factors that each of these plants face. We can also deduce that conducting proteomics in a comparative manner provides insight as to how plant proteins interact between organs and how they are involved and linked with regard to signalling, metabolism, energy, cell defence and virulence to name a few (Nozu *et al*., 2006; Albertin *et al*., 2009; Mavlonov *et al*., 2009). This could potentially enable researchers to observe the whole plant response, not only to day to day maintanence, but also to proteins that are expressed as a result of biotic and abiotic stresses such as water-deficit (Perez-Molphe-Balch *et al*., 1996; Albertin *et al*., 2009), and changing environmental factors (Xu *et al*., 2008).

Although numerous studies have been performed on the proteome of root tissues of various other plants (see section 1.5), none has been reported on the roots of resurrection plants. As outlined above (section 1.3) the lack of such information is mainly due to the difficulties associated with root observation. In general, root studies are confounded by the fact that root tissues are particularly difficult to work with (Mehta *et al.*, 2008), mostly because of being a hard, fibrous tissue with low concentrations of intact proteins and high concentrations of contaminants such as salts, secondary metabolites and other interfering compounds (Wang *et al*., 2008). In addition, due to root tissues having a high fiber and lignin content, the process of rupturing cell walls for protein extraction and removing non-protein contaminants is complicated (Xu *et al.*, 2008). The root tissues are also highly vacuolated, that when ruptured, release high concentrations of proteases and secondary metabolites that interfere with protein extraction (Laing and Christellar, 2004; Wang *et al.*, 2008) and make subsequent analysis problematic. As a consequence of this, the main factors responsible for the limited data regarding root proteomics are both low protein concentrations and low root tissue amounts (Mehta *et al.*, 2008). In studies on resurrection plants, these are exacerbated by the fact that protein extractions are conducted on roots with various water contents and changing physical and physiological characteristics as the tissues are desiccated. Use of soil grown plants has an added complication of not being able to wash away associated contaminants and microbes as

it changes the RWC and in turn changes the nature of the subcellular milieu. Not only could the changes in plant root water content be affected but proteins from microbes present in soil could contaminate the proteome and transcriptome of the resurrection plant under study. Such constraints have been overcome by the use of aeroponics as outlined in section 1.4 above.

1.6) Objectives of this study

It was our initial aim to observe the total protein profile present in *X. viscosa* root tissue during normal hydrated conditions through the use of our physiologically tested and optimised aeroponic plant growth system. In order to analyse the proteins present in the roots tissues of *X. viscosa*, the first step was to determine a successful protocol for the extraction of proteins for further analysis of proteome content. In order for proteomic analysis to be successful, the protein extraction procedure is critically important and the ideal protein extraction method should be able to reproducibly capture the most wide-ranging collection of proteins and minimize proteolysis as well as contamination by non-proteinaceous compounds (Isaacson *et al.,* 2006). However, because all proteins are different with regards to molecular size, charge, hydrophobicity, post-translational modification, complexity and cellular distribution, no protein extraction protocol or solvent system can capture the entire proteome (Rose *et al.,* 2004). It was for this reason that we investigated three protein extraction protocols. As a consequence of limited *X. viscosa* root tissue availability growing aeroponically, we used maize roots as a positive control and as a testing material for the protein extraction methods investigated. Furthermore, all protein optimisation procedures were conducted on maize roots and once optimised, our second step was to analyse the root proteome changes in *X. viscosa* during desiccation.

Two-Dimensional Electrophoresis (2-DE) analysis was used in conjunction with OFFGEL analysis for subsequent analysis and separation of complex root protein samples from *X. viscosa* roots according to their isoelectric points (p*I*). Following successful pre-fractionation and separation techniques, the total proteins obtained in the hydrated state, were analysed using mass spectrometry, particularly Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) and the proteins detected were identified using various plant protein database searches. However, due to time constraints and technical challenges associated with *X. viscosa* root protein, studies on root proteins in the dry state were not conducted.

In this study we describe a series of methodological optimisations for the purpose of extracting intact proteins from the hydrated roots of *X. viscosa*, to be further used for large scale, platform proteomic analysis.

2.) Materials and Methods

2.1) Plant material

Seeds taken from plants of *Xerophyta viscosa* (Baker) collected in Cathedral Peak Nature Reserve (KwaZulu Natal Province, South Africa) were germinated in the dark on M.S (Murashige and Skoog, 1962) tissue culture medium under sterile conditions. The resultant seedlings were left to grow and mature before being clonally multiplied by transferring explants from the mother plant to cultivation media. These clonal plantlets were transferred to vermiculite and hardened under tissue culture room conditions of 16 h light, 132.5 µMol/m^2/sec; 8 h dark, 24-25^{o}C. Once hardened, the six plantlets from the same parental line were maintained in plant growth chambers with the set growth conditions of 14 h light, 155-165 µMol/m^2/sec; 10 h dark, 25^{o}C for some time before transferral into the aeroponics growth chamber.

Maize plant root tissue (*Zea Mays A188*) was kindly donated by Shakiera Sattar (Msc, UCT-pers.comm.) for use in optimisation of protocols for extraction and separation of root proteins. Plants from which the roots were harvested were approximately four months old and served as a positive control to test the efficacy of all the methods investigated.

The aeroponic plant growth chamber(s) (Fig. 3) consisted of a polyurethane black plastic box (0.4 m x 0.3 m and 0.4 m high) with tight fitting lids containing 15 foam lined plant holders. Only 5 plants were used per chamber. Within the chamber, roots were misted through 20 cm high rigid riders with attached 360^0 rotating micro-jet sprays. Each rigid rider was inserted into agricultural piping (diameter 10 mm) that was connected to a 45 watt submersible pump (Aqua H_2O submersible pump-APH 2500) with attached filter and was used to pump nutrient solution from a 25l reservoir at a flow rate of 2700 l.hr^{-1}. The mist was formed from Hoagland's solution (Hoagland and Arnon, 1950), recirculated from the nutrient solution reservoir and was renewed once a week. A 24 hour electrical timer (Major tech) was connected to the submersible pump and controlled the pumping of nutrient solution in the chamber at set time intervals.

2.2) Aeroponic growth conditions

Transfer of the *X. viscosa* plants to the aeroponics system was done as outlined in Kamies *et al*. (2010) and was achieved with minimal damage to the roots. Plant roots were sprayed with Hoagland nutrient solution at 07:00, 13:00 and 19:00, each day to maintain root hydration. The plants were then maintained and acclimated under these aeroponic growth conditions (14 h light, 155-165 µMol/m^2/sec; 10 h dark, 25^{o}C) for a period of 6-8 months before protein analysis commenced.

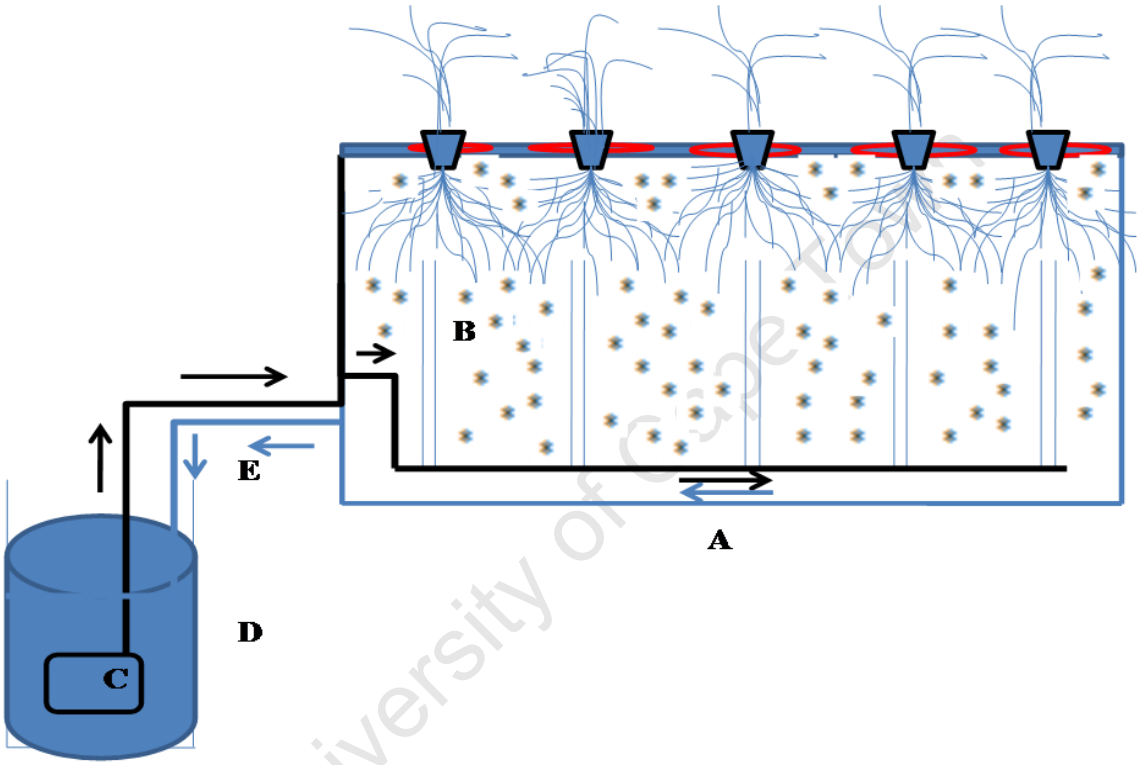

Fig. 3 Diagram depicting aeroponic plant growth system (A). Plant roots were sprayed with dilute Hoagland's nutrient solution through irrigation spouts (B), by electronic pumping of a submersible pump (C) situated in an adjacent nutrient reservoir (D). Nutrient solution was then re-circulated back to the reservoir through hosepipe outlet (E).

2.3) Protein Extraction Methods

All protein extraction methods investigated and subsequent analyses were performed on *X. viscosa* plant roots, while optimisation procedures were conducted on maize roots. These methods are detailed below.

2.3.1) Method A: (Phenol extraction, methanol/ammonium acetate precipitation)

This protocol was based on the work of Ingle and co-workers (2005) with a few modifications. Total protein was extracted by grinding 1 g root tissue to a powder with liquid nitrogen in a chilled mortar and pestle whilst gradually adding 1% (w/w) insoluble polyvinylpolypyrrolidone (PVPP). Prior to aliquoting ground tissue into 2 ml micro-centrifuge tubes, a protease inhibitor tablet (Roche Complete Mini) was added during the final stages of grinding. Root tissue aliquots were resuspended in 1 ml ice-cold extraction buffer (0.5 M Tris–HCl, pH 7.5, 10 mM EDTA, 1% (v/v) Triton X-100). The reducing reagent β-mercaptoethanol as well as the protease inhibitors, phenylmethylsulfonyl fluoride (PMSF) and benzamadine were added just before use at a final concentration of 2% (v/v) β-mercaptoethanol, 1 mM PMSF and 1 mM benzamadine (respectively). Root tissue was homogenised by vortexing for 5 minutes at room temperature followed by centrifugation at 14 000 g for 5 minutes at 4^{o}C. The supernatant was then collected and transferred to a new microcentrifuge tube and an equal volume of ice-cold Tris (0.5 M, pH 8.0)-saturated phenol was added and mixed by vortexing for 10 minutes at room temperature. Samples were then centrifuged at 14 000 g for 5 minutes at 4^{o}C to allow two-phase separation of protein-containing phenol and aqueous layers. Approximately 80% percent of upper (aqueous) layer was removed, discarded, and the protein-containing phenol layer re-extracted with an equivalent volume of extraction buffer, mixed by vortexing for 5 minutes and centrifuged at 14 000 g for 5 minutes. Once more, 80% of the top aqueous layer was removed and 5 volumes of ice-cold 0.1 M ammonium acetate in methanol were added to the protein-containing phenol layer. Samples were incubated at -20^{o}C for 16 hours to allow protein precipitation to occur. Proteins were then recovered by centrifugation at 14 000 g for 20 minutes at 4^{o}C. Protein pellets were washed once with cold 0.1 M ammonium acetate in methanol and centrifuged at 14 000 g for 5 minutes. The resultant pellets were washed twice with 80% (v/v) acetone. Protein pellets were then air-dried under a fume hood for 5 minutes before storage at -80^{o}C until further use.

2.3.2) Method B: (Tissue cleanup, phenol/ SDS extraction)

2.3.2.1) Preparation of dry tissue powder

This method was taken from Wang *et al*., (2003) with some modifications. Root tissue was ground (as described in 2.3.1) and the ground tissue was resuspended in 2 ml ice cold acetone. Tissue samples were mixed by vortexing for 1 minute followed by centrifugation at 10 000 g

for 3 minutes at 4^{o}C. The supernatant was discarded and the resultant tissue pellet was washed again in ice cold acetone in the same manner as before. All tissue pellets, after discarding the supernatant, were transferred to a clean, dry mortar and left to dry at room temperature under a fume hood for approximately 20 minutes. The dried root tissue was then further ground to a fine powder using acid-washed sand before being aliquoted into 2 ml microcentrifuge tubes. These fine tissue samples were then rinsed with cold 10% TCA in acetone 3 times until the supernatant was colourless (each time by vortexing for 1 minute and centrifuging for 3 minutes at 10 000 g). The resultant tissue samples were then washed twice with cold 10% TCA in H_2O, followed by two final washes with 80% acetone. The pellets were air dried at room temperature under a fume hood for approximately 1 hour. The dry tissue samples were either used immediately for protein extraction or stored at -80^{o}C till further use.

2.3.2.2) Protein extraction

Approximately 0.1 g of dry root tissue powder in a 2 ml microcentrifuge tube was re-suspended in 0.8 ml (0.5 M Tris, pH 8.0)-saturated phenol and 0.8 ml dense SDS extraction buffer (30% (w/v) sucrose, 2% (w/v) SDS, 0.1 M Tris-HCl, pH 8.0, 5% (v/v) β-mercaptoethanol). The mixture was vortexed for 5 minutes and separated by centrifugation at 10 000 g for 5 minutes. The upper phenolic phase was collected and transferred to a new 2 ml microcentrifuge tube while the supernatant (aqueous phase) and cell debris were discarded. The collected phenolic phase was then re-extracted with an equal volume of fresh extraction buffer mixed by vortexing for 5 minutes and separated again as before by centrifugation at 10 000 g for 5 minutes. The collected phenolic phase is then precipitated with 5 volumes of ice cold 0.1 M ammonium acetate in methanol for 30 minutes at -20^{o}C. Protein pellets were recovered by centrifugation at 14 000 g for 10 minutes at 4^{o}C and washed twice with cold ammonium acetate in methanol and twice with 80% acetone (each wash step was performed for 5 minutes at 10 000 g at 4^{o}C). Protein pellets were then dried under a fume hood at room temperature and stored at -80^{o}C or dissolved in a buffer of choice suitable to downstream application.

2.3.3) Method C: (Phenol extraction coupled with ammonium acetate precipitation)

This method was adapted from Isaacson *et al*. (2006) with some modifications. Total soluble root protein was extracted from 2 g of root tissue ground in liquid nitrogen to fine powder with the addition of 1% (w/w) insoluble PVPP. A protease inhibitor tablet (Roche Complete Mini) was added during the final stages of grinding. Ground root tissue was then aliquoted into 2 ml microcentrifuge tubes up to 0.1 ml mark and 1 ml ice-cold extraction buffer (0.7 M sucrose, 0.1 M KCl, 0.5 M Tris-HCl, pH 7.5, and 50 mM EDTA) together with 1 ml Tris (0.5 M, pH 8.0)-saturated phenol was then added. The reducing reagent β-mercaptoethanol and serine protease inhibitor PMSF were added to extraction buffer at a final concentration of 2% (v/v) and 1 mM respectively, just before use and the samples were well-mixed by vortexing for 15 minutes at 4^{o}C. These samples were then centrifuged at 14 000 g for 10 minutes at 4^{o}C to allow phase separation to occur. Once centrifugation was complete, the upper phenolic phase containing phenol soluble proteins was then carefully removed (without disturbing the white inter-phase) and transferred to a new microcentrifuge tube while the lower aqueous phase containing all cell debris and contaminants was discarded. An equal volume of fresh extraction buffer to that of collected phenolic phase was added and the mixture was vortexed again for 10 minutes at 4^{o}C. The samples were once again centrifuged at 14 000 g for 10 minutes to recover the phenolic phase and the process of re-extraction was repeated. To precipitate the proteins, 5 volumes (to that of the collected phenolic phase) of cold 0.1 M ammonium acetate in methanol was added and samples were incubated at -20^{o}C for 16 hours. Protein pellets were recovered by centrifugation at 14 000 g for 20 minutes at 4^{o}C and resulting supernatant was removed and discarded. The protein pellets were then firstly washed twice with 100% methanol at 4^{o}C, to remove phenol, ammonium acetate, lipids and pigments and centrifuged at 14000 g for 5 minutes at 4^{o}C. This was then followed by two washes with 100% acetone at 4^{o}C to remove traces of methanol and to allow rapid drying. Protein pellets were then air-dried under a fume hood for 5 minutes and stored at -80^{o}C till further use.

2.4) Protein solubilisation and quantification

Protein pellets from methods A and C were dissolved in a buffer of choice such as Urea Lysis Buffer (7 M urea, 2 M thiourea and 2% (w/v) 3-((3-cholamido propyl)-dimethylammonio)-1-propane sulfonate (CHAPS)), for use in 2-DE analysis or 2X SDS loading buffer (Laemmli buffer) (0.5 M Tris-HCl, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 0.1% (w/v) Bromophenol Blue) (Laemmli, 1970) for analysis by SDS-PAGE. Protein pellets from

method B were dissolved either in Laemmli buffer as described above or 2-DE rehydration solution (8 M urea, 4% (w/v) CHAPS, 2% (v/v) IPG Buffer (pH 3-10 or pH 4-9) (Sigma-Aldrich, USA), and 20 mM dithiothreitol), as described by Wang *et al*. (2003). Proteins from all three extraction methods were resuspended and solubilised by mild vortexing in either buffer for 2 hours at room temperature. The concentration of proteins solubilised in Urea Lysis Buffer (ULB) was spectrophotometrically quantified using a modified Bradford method according to Ndimba *et al* . (2003), using Bovine Serum Albumin (BSA) (Bio Basic, USA) as a standard, while proteins dissolved in Laemmli buffer were quantified according to the standard Bradford method also using BSA as a standard.

2.5) Protein separation – One-Dimensional Electrophoresis (1-DE) analysis

Separation of proteins in the first dimension by SDS-PAGE was performed at room temperature in the Mini-Protean Tetra Cell (BioRad) on 15% polyacrylamide SDS PAGE gels (30% (v/v) acrylamide, 0.375M Tris-HCl, pH 8.8, 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulfate and TEMED). The protein samples were electrophoresed at constant voltage of 150 V for 2 hours in SDS running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS). Once electrophoresis was complete the gels were carefully removed from glass plates and stained in Coomassie Brilliant Blue (CBB) R-250 staining solution (45% (v/v) methanol, 10% (v/v) acetic acid and 0.02% (w/v) Coomassie Brilliant Blue R-250) on a shaker at 37^{o}C for 1 hour. The gels were then destained in destain solution (40% (v/v) methanol and 10% (v/v) acetic acid) usually overnight until proteins bands and protein markers was clearly visible. The gels were then placed in 7% (v/v) acetic acid and later in distilled water (dH_2O) until the background blue colour was completely clear and the appearance of the protein bands enhanced.

2.6) Two-Dimensional Electrophoresis (2-DE) Analysis

Isoelectric focusing (IEF) was carried out on resuspended protein samples extracted from all three protein extraction methods using 7-cm immobilized pH gradient (IPG) strips (Bio-Rad, USA) with a pH range of 3-10 and 4-7 respectively. These IPG strips were rehydrated with 200–300 µg of dissolved protein in rehydration buffer containing the following at final concentrations of: 1% (v/v) carrier ampholytes (pH 3-10 or pH 4-9) (Sigma-Aldrich, USA), 2% (w/v) DTT in dH_2O containing 0.002% (w/v) bromophenol blue and ULB (7 M urea, 2 M thiourea, and 2% (w/v) CHAPS) in a total volume of 140 µl. Each 7-cm strip was left to

rehydrate in a rehydration/equilibration tray (Bio-Rad, UK) for about 1 hour at room temperature before being covered in 1 ml mineral oil (Sigma Aldrich, USA) and further left to rehydrate on bench top for 16 hours for complete absorption. Isoelectric focusing of proteins was carried out using a Protean IEF Cell (Bio-Rad, USA) with the voltage settings initially set at: stage 1, linear gradient up to 250 V in 20 minutes; stage 2, linear gradient up to 450 V in 2 hours and stage 3, rapid gradient up to 4000 V at 20 000 $V.h^{-1}$at 20^{o}C with a maximum current of 50 µA/strip. For optimisation purposes, the voltage settings were then later changed according to Requejo and Tena (2005), where the voltages were set at: stage 1, linear gradient up to 250 V in 20 minutes; stage 2, linear gradient up to 4000 V in 2 hours and stage 3, exponential gradient up to 4000 V at 10 000 $V.h^{-1}$ at 20^{o}C with a maximum current of 50 µA/strip. Following isoelectric focusing, the proteins in the strips were subjected to reduction of their disulphide bridges by incubation in equilibration buffer (6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) bromophenol blue) containing 2% (w/v) dithiothreitol (DTT), for 15 minutes and for an additional 15 minutes in the same equilibration buffer to which 4.5% (w/v) iodacetamide was added for alkylation of their cysteinyl residues. IPG strips were equilibrated at room temperature with mild agitation. Separation of proteins in the second dimension was achieved by SDS-PAGE as described above in section 2.5 where equilibrated strips were placed onto the 15% polyacrylamide gels and sealed with 1% (w/v) agarose. After protein electrophoresis at constant voltage of 160 V for 1.5 hours, the gels were stained in CBB R-250 staining solution for 2-4 hours on a shaker at room temperature followed by destaining in destain solution overnight. The gels were then left for a few hours in 7% (v/v) acetic acid for optimal visualisation.

2.7) Protein OFFGEL Fractionation analysis

For separation of protein samples according to their isoelectric points (p*I*), the 3100 OFFGEL fractionator (Agilent Technologies) with a 12-well setup was used. Protein pellets were resuspended in 140 µl 1.25X OFFGELL buffer (8 M urea, 2 M thiourea, 70 mM DTT) by vortexing for 1 hour. Prior to loading and electrofocussing, resuspended protein samples were pooled together and the following were added at final concentrations of: 6% (v/v) glycerol and 1% (v/v) IPG buffer, at pH 5-8 (Sigma-Aldrich, USA). The 11-cm immobilized pH gradient (IPG) strips (Bio-Rad, USA), with a linear pH 5-8 range, were left to rehydrate in 1.25X OFFGELL buffer for 15 minutes according to the Agilent 3100 Quick Start Guide. Following IPG strip rehydration, 150 µl of resolubilised protein sample in 1.25X OFFGEL

buffer was loaded into each of the 12 wells and electrofocussing of proteins was performed at 20^{o}C using the preset program OG24PR00, until a voltage of 20 kV.h^{-1} was reached. After focusing, the 12 fractions were transferred to pre-labelled microcentrifuge tubes and the fractionated proteins were precipitated with ice cold acetone and 50 mM Tris- HCl pH 8.8 for 1 hour at -20^{o}C, after which protein pellets were recovered by centrifugation at 14 000 g for 5 minutes at room temperature. These protein pellets were then resuspended in a bicinchoninc acid (BCA) protein assay compatible resuspension solution (50 mM Tris-HCl pH 6.8, 1 mM DTT and 5% (w/v) SDS) and quantified according to the Pierce BCA protein assay kit (Thermoscientific, USA) where BSA was used as a standard. The protein content of samples were read using the KC4 96-well microplate reader (Bio-Tek Instruments Inc., USA) and concentrations determined via a standard curve. The fractionated protein samples were then heated in Laemmli sample buffer for 5 minutes at 80^{o}C before being separated on 15% SDS-PAGE gels by electrophoresis at constant voltage of 150 V for 2 hours. The gels were then carefully removed and stained and destained as described above in section 2.5.

2.8) LC-MS/MS analysis: Identification of fractionated root protein samples

2.8.1) Root protein digests

After protein OFFGEL fractionation and precipitation of fractionated root protein samples into a pellet form, protein pellets were subjected to *in situ* enzymatic digestion and the digested peptides were analysed by LC-MS/MS. LC-MS/MS protein sequencing was conducted by the W. M. Keck biotechnology resource laboratory at Yale University using the following procedure. The dry protein pellets were rehydrated with 0.1 μg of modified trypsin per approximately 15 mm^{3} of protein pellet in 15 μl 10 mM ammonium bicarbonate. The samples were then left to digest for 16 hours at 37^{o}C.

2.8.2) LC-MS/MS procedure

LC-MS/MS was conducted using an LTQ Orbitrap that is equipped with a Waters nanoAcquity UPLC system, and used a Waters Symmetry® C18 180 μm x 20 mm trap column and a 1.7 μm, 75 μm x 250 mm nanoAcquity™ UPLC™ column (35^{o}C) for peptide separation. Trapping is done at 15 μl/min, 99% Buffer A (100% water, 0.1% formic acid) for 1 minute. Peptide separation was performed at 300 nl/min with Buffer A (100% water, 0.1% formic acid) and Buffer B (100% CH_3CN, 0.075% formic acid). A linear gradient (51 minutes) was run with 5% buffer B at initial conditions, 50% B at 50 minutes, and 85% B at 51 minutes. MS was acquired in the Orbitrap using 1 microscan, and a maximum injection

time of 900 ms followed by four data dependant MS/MS acquisitions of in the ion trap. An additional MS^3 fragment spectrum was obtained if any of the phosphopeptide neutral loss ions (-98.0, -49.0, -32.7, and 24.5 amu) were detected. The data were searched using Mascot Distiller and the Mascot search algorithm.

2.8.3) Database searches

Database searches were conducted by the W. M. Keck biotechnology resource laboratory at Yale University. All MS/MS spectra were searched using the Mascot algorithm (version 2.2.0) for un-interpreted MS/MS spectra after using the Mascot Distiller program to generate Mascot compatible files. The Mascot Distiller program combines sequential MS/MS scans from profile data that have the same precursor ion. A charge state of +2 and +3 are preferentially located with a signal to noise ratio of 1.2 or greater and a peak list is generated for database searching. Using the Mascot database search algorithm, the Keck Facility considered a protein identified when Mascot listed it as significant and more than 2 peptides matched the same protein. The database searched was typically the NCBInr that is chosen over genome specific databases since a match to the correct species had more significance in the larger databases and, for some incomplete genomes, a match may have been found based on homology to another species. The Mascot significance score match was based on a MOWSE score and relied on multiple matches to more than one peptide from the same protein. The protein score (or Mowse score) was set at -10x log (P), where P was the probability that the observed match was a random event. The expectation value was the number of matches that were expected to occur by chance alone ($P < 0.05$). The typical parameters used for database searching were partial methionine oxidation and carboxamidomethylated cysteine, a peptide tolerance of $\pm$20ppm, MS/MS fragment tolerance of $\pm$0.6 Da, and peptide charges of +2 or +3. Normal and decoy database searches were performed as well.

2.8.4) Protein classification

After root protein identification, proteins were functionally classified according to their annotated biological process using the PANTHER classification system (PANTHER: a library of protein families and subfamilies indexed by function, Thomas *et al*., 2003).

3.) Results and Discussion

3.1) Proteomic Analysis (Protein Extraction, 1-DE and 2-DE Analysis)

In order to perform high quality protein analysis on the proteins present in the roots tissues of *X. viscosa* during hydrated conditions, three protein extraction methods were investigated (designated Method A, B and C, respectively). All three methods were successful in isolating proteins from the root tissues as indicated by the production of comparable amounts of protein quantified by the Bradford (1976) method shown in Table 1. Furthermore, the quality of protein extracted using each method and subsequent protein analysis are discussed below.

Table 1. Relative concentrations of *X. viscosa* root protein using all three methods of extraction

Protein extraction method	Root weight (g)	Protein yield (µg/g)
Method A	1.2 ± 0.28	41.1 ±5.24
Method B	1.7 ± 0.37	40.1 ± 3.44
Method C	1.7 ± 0.28	45.1 ± 16.11

Protein yields shown are means of three replicates (n=3) for each protein extraction.

3.1.1) Method A

The first protein extraction method investigated was previously described by Ingle and co-workers (2005) for proteomic analysis of the hyperaccumulator plant *Alyssum lesbiacum*. One-dimensional electrophoresis (1-DE) separation of proteins using this method (Fig. 4A) showed relatively high-quality protein bands, with *ca* 20 proteins bands between the molecular masses of 100-17 kDa being evident, even though background protein streaking was present. We then further attempted to analyse the protein profile of hydrated *X.viscosa* root tissue by using two-dimensional electrophoresis (2-DE) analysis on the same protein extract (Fig. 4B). The result shows a poorly resolved total protein separation in the pH range of 4-7. A few indistinct protein spots were present in the molecular weight range of 100-17 kDa accompanied by prominent vertical streaking. Though distinct protein bands could be seen on the 1-DE result (Fig. 4A), the resolution and quality of the 2-DE result (Fig. 4B) was poor.

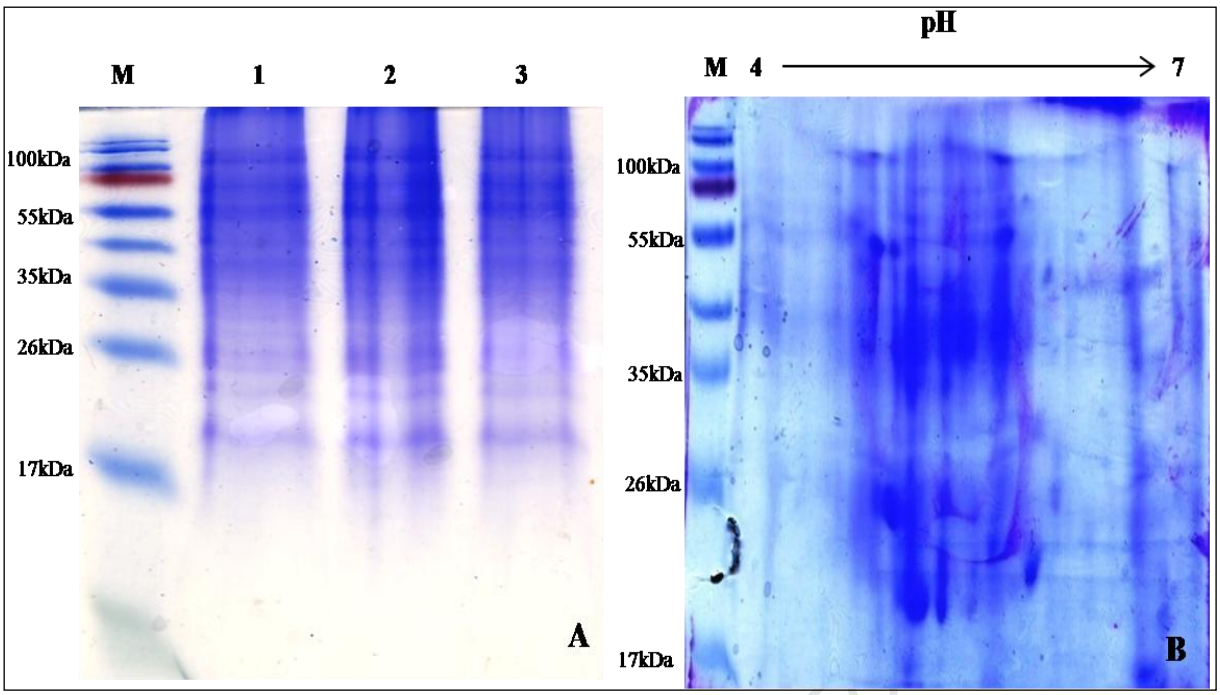

Fig. 4 (A) 15% SDS-PAGE representation of *X. viscosa* root total proteins extracted using Method A. Lane M: MW marker (Fermentas). The MW are given in kDa. Lanes 1, 2 and 3 depict *X. viscosa* root proteins mixed with 4XSDS loading buffer and loaded at an amount of 30 µg. (B) Two–dimensional electrophoresis of *X.viscosa* root protein extracted using Method A. *X. viscosa* root protein was loaded at an amount of 200 µg and separated across pH 4-7 using a 7-cm IPG strip on a 15% polyacrylamide gel. Protein bands and spots were visualised with Coomassie brilliant blue (CBB) stain.

It is commonly known that protein impurity is usually correlated with background streaking occurring on 1-DE and 2-DE protein gels (Schmidt *et al*., 2009). The incorporation of phenol during protein extraction is used to remove contaminants associated with protein isolation (Carpentier *et al*., 2005). However, from the 2-DE result (Fig. 4B), it is clear that impurities altering protein separation were still present in the protein samples. One of the main disadvantages of this particular protein extraction method is the ineffective removal of contaminants present in the supernatant. Only 80% of the upper aqueous layer is removed during centrifugation, while the other 20% is still present in the protein samples (including the white inter-phase). Although centrifugation of protein samples during the extraction procedure allows separation of the protein containing phenol (bottom) layer from the aqueous (upper) supernatant, it is difficult to do so without removing some of the protein-containing phenol layer as well. Thus the removal of supernatant in the protein extraction occurs with contamination during the two-phase separation of the phenol-aqueous layer, which then leads to precipitation of the contaminants with the protein pellet.

As mentioned previously, protein streaking on 2-DE gels are caused (amongst other factors) by the presence of interfering compounds such as secondary metabolites, which could have adverse effects on the protein extraction as well as separation on 2-DE gels (Gorg *et al*., 2004; Xu *et al*., 2008). These secondary metabolites, the most common being phenolics (Wang *et al*., 2008), are able to induce irreversible complexes with proteins that, subsequent to the oxidation of these phenolics by phenol-oxidases and peroxidases, cause streaking and the presence of artefactual spots on 2-DE gels (Vâlcu and Schlink, 2006). The presence of polysaccharides, pigments and lipids can also interfere with 2-DE analysis (Wang *et al*., 2008). Therefore the 2-DE result obtained in Fig. 4B is suggested to be an example of non-proteinaceous contamination in protein samples that caused streaky results.

We found that these contaminants present in the residual supernatant not only made resolubilisation in a resolubilising buffer difficult but also affected the downstream applications as well. The IEF runs were long, which usually resulted in incomplete focusing. During this time the risk of proteolysis or degradation could have increased due to the extended electrophoretic run as well as the possibility of protein migration out of the IPG strips. The other factors that could potentially have contributed to the poor 2-DE resolution are mostly linked to the conditions of protein extraction. While phenol was added in order to disrupt non-covalently bound protein complex, it is possible that the amount added or conditions under which it was added (after ground material has been extracted in cold extraction buffer, but at room temperature) did not completely facilitate breakdown of these complexes. Furthermore this process resulted in incomplete protein resolubilisation.

These problems might be solved by the establishment of a sucrose gradient in the extraction procedure, whereby sucrose is present in the aqueous buffer. Since sucrose is denser than phenol, the aqueous layer containing cell debris, PVPP, salts, nucleic acids and carbohydrates will form at the bottom layer during centrifugation, while the upper phenol layer will contain phenol-soluble proteins, lipids and pigments (Isaacson *et al.*, 2006). We therefore attempted another protein extraction method that incorporates phenol for protein purity and a sucrose gradient to allow better separation of samples as well as improve protein separation from the contaminants.

3.1.2) Method B

The next method that we used to extract proteins from the root tissues involved using both phenol and SDS, as described in Wang *et al*. (2003). Phenol addition to extraction procedures has been known to have a high cleanup capacity, as it is not only highly selective, but it is one of the strongest dissociating agents known to decrease molecular interactions between proteins and other compounds (Carpentier *et al*., 2005). The apparent advantage of this protein extraction method is that it was optimised and modelled to extract good quality proteins from tissues of the highly recalcitrant olive tree (*Olea europea* L.). Olive leaf tissue has a high concentration of interfering compounds and it would therefore be a good model for working with *X. viscosa* roots. Hurkman and Tanaka (1986) originally observed that a phenol protein extraction prevents proteolysis to the same extent as solubilisation in 4% SDS. Thus if these two reagents were combined in a phenol-SDS protein extraction it could potentially isolate proteins from *X. viscosa* root tissue that is protected from proteolysis (Hurkman and Tanaka, 1986), and relatively free from interfering compounds such as pigments, polyphenols, lipids , polysaccharides and nucleic acids (Wang *et al*., 2003).

Fig. 5 shows 1 and 2-DE PAGE separations of proteins using this method. 1-DE separation showed that there were prominent bands at 100 and 43 kDa (arrows), however it was evident that there was considerable protein degradation and few bands were found with molecular weights below 35 kDa. Although the inclusion of phenol in this protein extraction method initially might have offered some protection against proteolysis (Hurkman and Tanaka, 1986), the long waiting periods occurring during the preparation of dry tissue stage at room temperature (as explained in section 2.3.2.1 of Materials and Methods) provides the opportunity for proteolysis to occur even before the extraction of protein takes place. In this extraction protocol, root tissue is ground in liquid nitrogen, allowing plant cell walls to be ruptured and intracellular proteases to be released (Laing and Christellar, 2004). The ground matter is then subjected to numerous washes with TCA in either acetone or water, followed by washes with 80% acetone. Following these wash steps, an incubation step at room temperature occurs to facilitate the drying of ground plant matter which the authors estimate to be about 20 minutes, however we found this drying stage to take approximately 1 hour before the tissue was completely dry and protein extraction could take place.

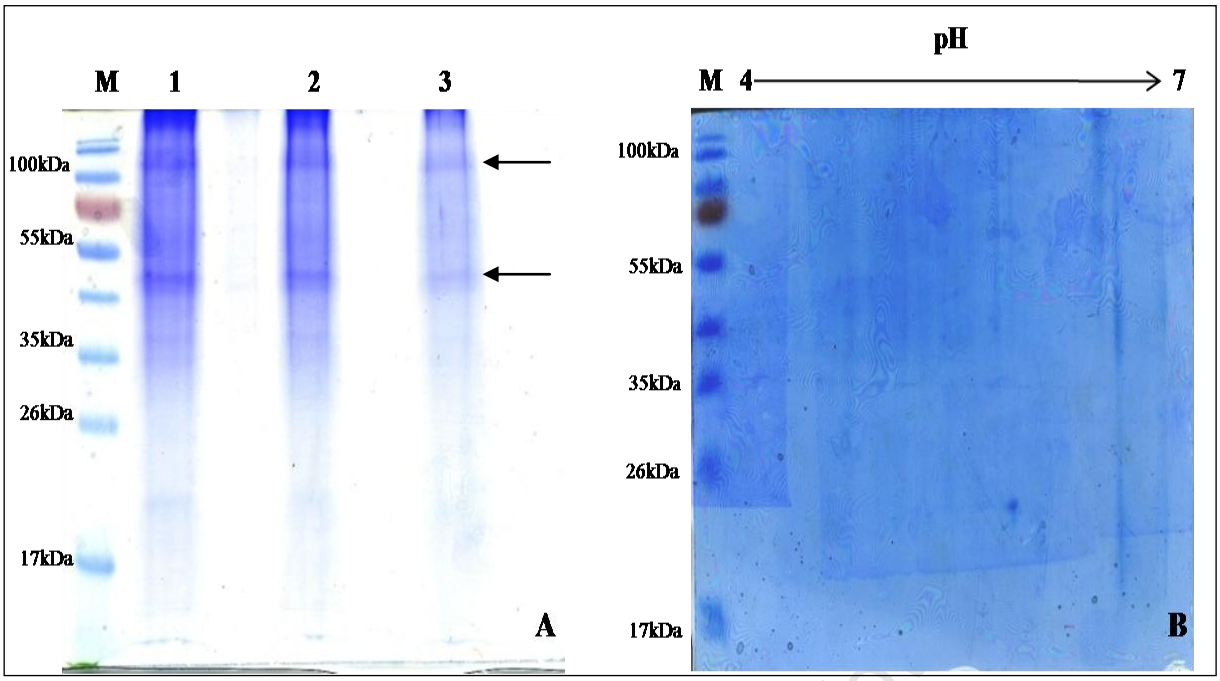

Fig. 5 (A) 15% SDS-PAGE representation of *X. viscosa* root total protein extracted using Method B. Lane M: MW marker (Fermentas). Their MW are given in kDa. Lanes 1, 2 and 3 depict *X. viscosa* root proteins dissolved in Laemmli buffer and loaded at an amount of 30 µg. (B) Two–dimensional electrophoresis of *X. viscosa* root proteins extracted using Method B. *X .viscosa* root proteins were loaded at an amount of 200 µg and separated across pH 4-7 using a 7-cm IPG strip on a 15% polyacrylamide gel. Protein bands and spots were visualised with Coomassie brilliant blue (CBB) stain.

It could be that in this time the intracellular proteases present become active at room temperature and are able to degrade the protein present, thereby explaining the loss of protein bands in Fig. 5A.

Wang *et al*. (2003) found that polyphenolic and other contaminants would co-purify with extracted proteins and lead to the formation of a brownish pellet upon protein extraction by homogenizing olive leaf in an aqueous buffer, followed by precipitation in organic solvents. A similar result was observed for *X. viscosa* root protein pellets in all our protein extraction methods investigated, indicating that polyphenol oxidation is widespread in the root tissues (our observations). Though this protein extraction method appears most suitable for the type of tissue we were working on, we still obtained a low yield of extracted proteins where most of the the proteins were degraded even when we included a protease inhibitor tablet (Roche) to the extraction buffer (data not shown).We therefore decided that given the ample opportunity for proteolysis to occur even before protein extraction takes place, that this method would not be the most suitable in isolating pure, stable protein from hydrated roots.

In 2-DE protein separations using this method (Fig. 5B), hardly any spots were present, except for 1 just below 26 kDa. The lack of protein spots is most probably due to low extraction yield or protein degradation occurring prior to the extraction as explained above. Method B attempts to reduce the presence of contaminants, however this protocol has its limitations when trying to preserve intact proteins, free from proteolysis (Fig. 5A and 5B). The authors of this paper were able to isolate pure proteins from the highly recalcitrant Olive leaf tissue, but with regards to isolating pure, stable proteins from the root tissues of *X. viscosa* in our case, the method was unsuccessful. The method is able to potentially generate high-quality protein samples for certain kinds of recalcitrant plant tissues and may need further optimisation. However, the authors do state that protein loss could occur due to the numerous cleanup steps and the long waiting periods in between them (Wang *et al.*, 2008). We found this to be the main cause of protein spot loss and nearly blank 2-DE gels.

3.1.3) Method C

The third protein extraction method that was attempted was originally developed by Hurkman and Tanaka (1986) and was later modified by Isaacson *et al.* (2006). This method incorporates an equal volume of both phenol and extraction buffer at the beginning of extraction just after ground tissue has been aliquoted into microcentrifuge tubes. The entire extraction procedure is then performed at 4^{o}C to minimise degradation of extracted proteins and a sucrose gradient is formed to efficiently separate the phenol (top) layer from the aqueous (bottom) layer, which allows better separation and a cleaner protein sample to be isolated. Due to phenol being included at the beginning stages of the protein extraction we observed that more contaminants and impurities were removed at the start of tissue homogenisation.

As seen from Fig. 6A, protein bands ranging from 100-35 kDa were present in lanes 1 to 4, while a loss of smaller molecular weight proteins less than 35 kDa occurred. Some streaking and smearing was still apparent across all four lanes in Fig. 6A, however the protein banding pattern and resolution seemed to be the same in all the samples loaded. The loss of protein bands in comparison to the result obtained using Method A can be explained by various reasons. Firstly, this method has an isolation bias for high molecular weight proteins due to the nature of the protein extraction method or secondly, the low molecular weight proteins may be lost during the extraction procedure due to the longer periods of vortexing as well as multiple re-extraction steps in fresh extraction buffer (Isaacson *et al*., 2006).

When 2-DE analysis was applied to the protein samples extracted using Method C (Fig. 6B), we found a few distinct protein spots at set molecular weights. The presence of background horizontal and vertical streaking was limited by the effective extraction procedure that gave good protein resolution. However, there seemed to be an extensive loss of protein spots, which could coincide to what was observed in Fig. 6A, with respect to the loss of low molecular weight proteins (Fig. 6B). There is a possibility that few proteins are indeed present in the roots of *X. viscosa* during fully hydrated conditions, explaining the low protein content. Furthermore the loss of proteins during analysis cannot always be avoided, due to common occurrences of incomplete protein precipitation and insufficient protein resolubilisation (Xu *et al.*, 2008).

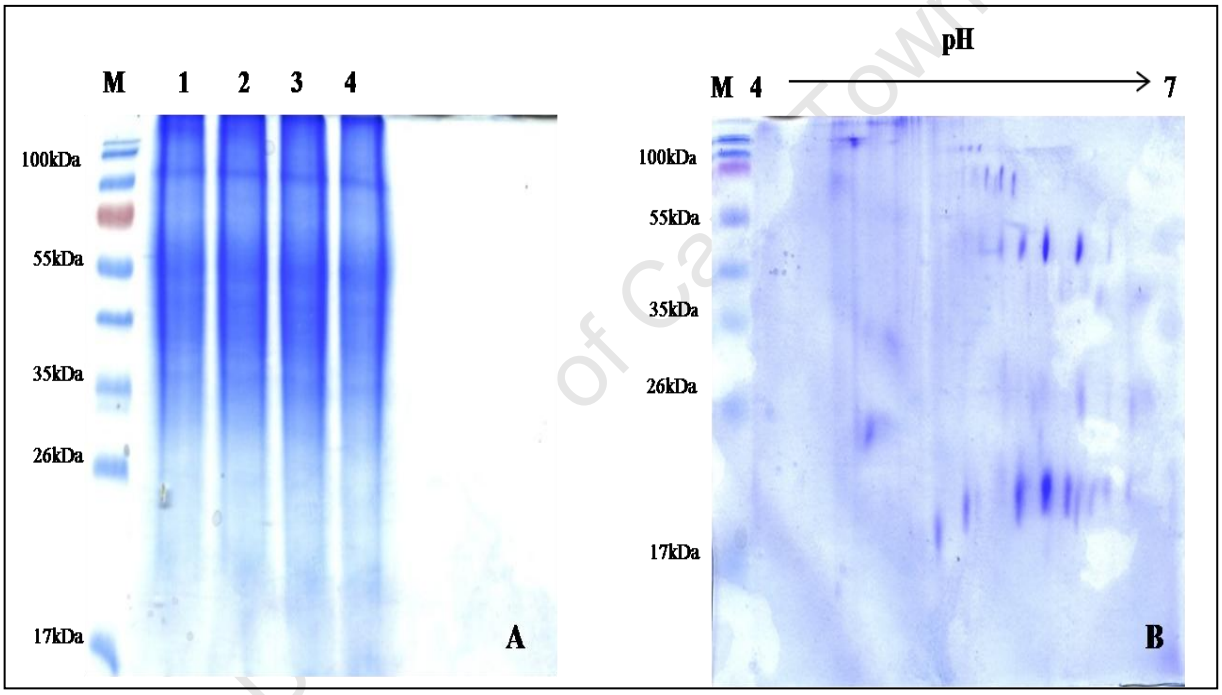

Fig. 6 (A) 15% SDS-PAGE representation of *X.viscosa* root total proteins extracted using Method C. Lane M: MW marker (Fermentas). Their MW are given in kDa. Lanes 1, 2, 3 and 4 depict *X. viscosa* root proteins mixed with 2XSDS loading buffer, heated for 5 minutes at 80^{o}C and loaded at an amount of 30 µg. (B) Two–dimensional electrophoresis of *X. viscosa* root proteins extracted using Method C. *X. viscosa* root proteins were loaded at an amount of 200 µg and separated across pH 4-7 using a 7-cm IPG strip on a 15% polyacrylamide gel. Protein bands and spots were visualised with Coomassie brilliant blue (CBB) stain.

Method C provides root proteins of a relatively good quality at both the 1 and 2-DE levels, given all the interfering substances naturally present in root tissues (Wang *et al.*, 2008). The reagents involved in the extraction are able to initiate an environment where proteins are able to freely move from the tissues into the phenol-capturing layer and sucrose is present to

facilitate a phase inversion (Carpentier *et al*., 2005). The high pH of the buffer can potentially cause the ionization of phenolic compounds and the neutralization of acidic compounds during disruption of the vacuoles (Hochstrasser *et al.,* 1988; Loomis and Battaile, 1966). All of this together with the fact that the entire extraction procedure is performed at 4^{o}C is able to slow down the rate of protein degradation (Isaacson *et al*., 2006). The presence of KCl is also able to facilitate the salting in of proteins by increasing protein solubility and EDTA is able to inhibit metalloproteases and polyphenol oxidases by chelating metal ions (Carpentier *et al.,* 2005). The addition of PVPP during the grinding process did not have any particular effect on the method (to our knowledge). However, Isaacson *et al.* (2006) found the addition of PVPP was beneficial to their extraction procedure when dealing with tissues high in phenolic compounds while Carpentier *et al.* (2005) did not find the addition of PVPP to improve their protocol. Nevertheless because PVPP is a strong H-acceptor, it could potentially function in absorbing polyphenols (Carpentier *et al.,* 2005). The main problem arising with most protein extractions involves the co-precipitation of proteins with contaminants forming a complex that is difficult to separate and hence remove (Wang *et al*., 2008). This effect generally results in downstream applications such as 2-DE analysis, difficult to achieve. However, even after optimisation procedures the final 2-DE result usually shows a loss of protein spots (Xu *et al.,* 2008).

Plant tissues normally contain low levels of proteins and high levels of proteases that potentially limit tissue disintegration and interfere with ensuing protein separation and identification (Isaacson *et al*., 2006). In general we observed that the proteins present in the root tissues of *X. viscosa*, are highly recalcitrant to extract, unstable and highly populated with contaminants. For proteomic analysis to be successful, the protein extraction procedure is critically important, thus the ideal protein extraction method should be able to reproducibly capture the most wide-ranging collection of proteins and minimize proteolysis as well as contamination by non-proteinaceous compounds (Isaacson *et al.,* 2006). However, because all proteins are different with regards to their molecular size, charge, hydrophobicity, post-translational modification, complexity and cellular distribution, no protein extraction protocol or solvent system can capture the entire proteome (Rose *et al.,* 2004). It was for this reason that we investigated three protein extraction protocols that all incorporate phenol at some stage during the extraction procedure. The inclusion of phenol during the extraction procedure is widely known to have the ability to isolate pure, soluble protein from recalcitrant tissues,

free from non-proteinaceous compounds (Saravanan and Rose, 2004; Carpentier *et al.*, 2005; Isaacson *et al*., 2006; Mehta *et al*., 2008; Xu *et al*., 2008), suitable for further proteomic analyses. In addition, previous studies have suggested that each extraction procedure needs to be optimised for different plant samples and tissues, as it is known that different types of plants and tissues contain differing amounts of protein and non-protein interfering compounds (Shaw and Riederer, 2003; Gorg *et al*., 2004; Carpentier *et al.*, 2005; Xu *et al*., 2008).

When performing 2-DE analysis on our protein samples we encountered many technical hurdles and challenges that required extensive optimization. We found that performing 2-DE analysis on proteins extracted using Method A yielded SDS-PAGE results with both horizontal and vertical streaking and even though we tried alternative methods to minimize the occurrences of interfering compounds, our protein samples still contained contaminants. Method C provided cleaner protein samples, while Method B on the other hand could not produce enough protein spots for analysis. Despite all our efforts in choosing an optimal protein extraction procedure that provides good-quality protein suitable for analysis, we also found IEF to be a problem. Long focusing runs would still occur even after we had removed salts and contaminating material. It was common for the IEF runs to exceed 24 hours, which provided ample opportunity for proteolysis to occur. It was clear that there was still high levels of contamination that resided within the protein complexes itself that resulted in interferences when analysis took place. This strongly suggested that *X.viscosa* root proteins were unpredictable in nature and intense care would be needed in further proteomic applications.

It should be noted that during our analysis of the root proteome of *X. viscosa*, we mainly investigated total protein isolated from hydrated roots; however when we attempted to isolate proteins from dehydrated root tissues using both Methods A and C, it was unsuccessful.

3.2) 1-DE and 2-DE Optimisation Procedures (Maize root tissue)

After the comparison of all three methods it was decided that Method C was the most suited for further optimisation. We wanted to determine if the poor protein resolution and streaking obtained could have been due to technical issues or whether *X. viscosa* root tissue is intractable in producing sufficient proteins. In addition, we decided to use maize tissue (*Zea mays*) as a positive control and conducted a number of technical optimisation procedures focusing primarily on the roots. This would help us determine if these technical issues were inherent to the plant species chosen or if there were certain technical errors. One of the other factors that contributed to our choice of tissue was that a great deal of research has been conducted on the maize proteome (Brouquisse *et al*., 2001; Mohamed, 2005; Requejo and Tena 2005; Zhu *et al*., 2006; Isaacson *et al*., 2006; Prinsi *et al*., 2009), and our method chosen (Method C) was also originally modelled to suit the extraction of maize root proteins (Isaacson *et al*., 2006). Maize was chosen not only because it was readily available for use but also because our supply of *X. viscosa* roots was limited.

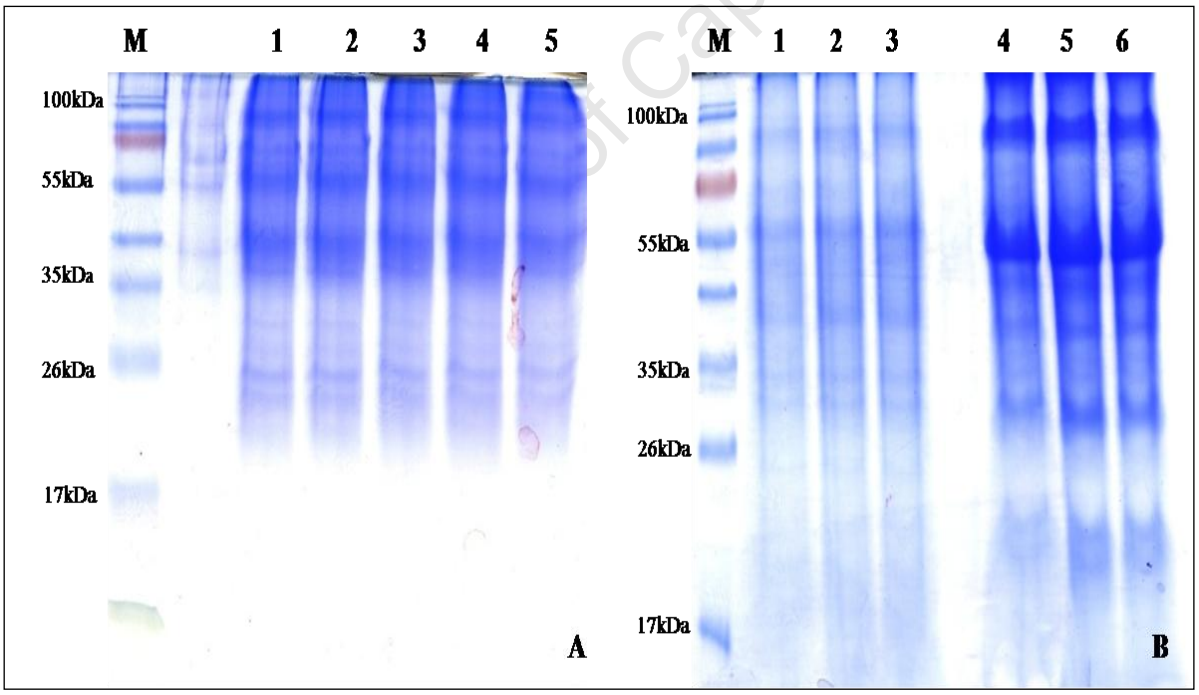

Fig. 7 (A) 15% SDS-PAGE representation of maize (*Zea mays*) root proteins. Lane M: MW marker (Fermentas). Their MW are given in kDa. Lanes 1-5 depict maize root proteins. (B) 15% SDS-PAGE of maize root proteins in comparison to maize leaf proteins. Lanes 1-3 maize root proteins and lanes 4-6 maize leaf proteins. All proteins were dissolved in 2X SDS loading buffer, heated at 80^{o}C for 5 minutes and loaded at an amount of 30 µg. Protein bands were visualised with Coomassie brilliant blue (CBB) stain.

In Fig. 7 the SDS-PAGE protein patterns of maize root tissue (A) and a combination of maize root and leaf tissue (B) were displayed. The protein banding patterns of both results (Fig. 7A and 7B) were distinct, well resolved and clearly visible at set molecular weights from approximately 96 kDa to 17 kDa. Protein extraction on maize roots using Method C seemed to be successful in isolating good quality proteins that were easily separated and visualised, with no occurrences of streaking or degradation. The results obtained were similar to what has been previously shown to be present in maize root and leaf tissue (Mohamed, 2005), with over 30 protein bands present. From the data above (Fig. 7), it was evident that these proteins were relatively free from proteolysis and the banding pattern was much better resolved in comparison to *X. viscosa* roots (see Fig. 4A, 5A and 6A).

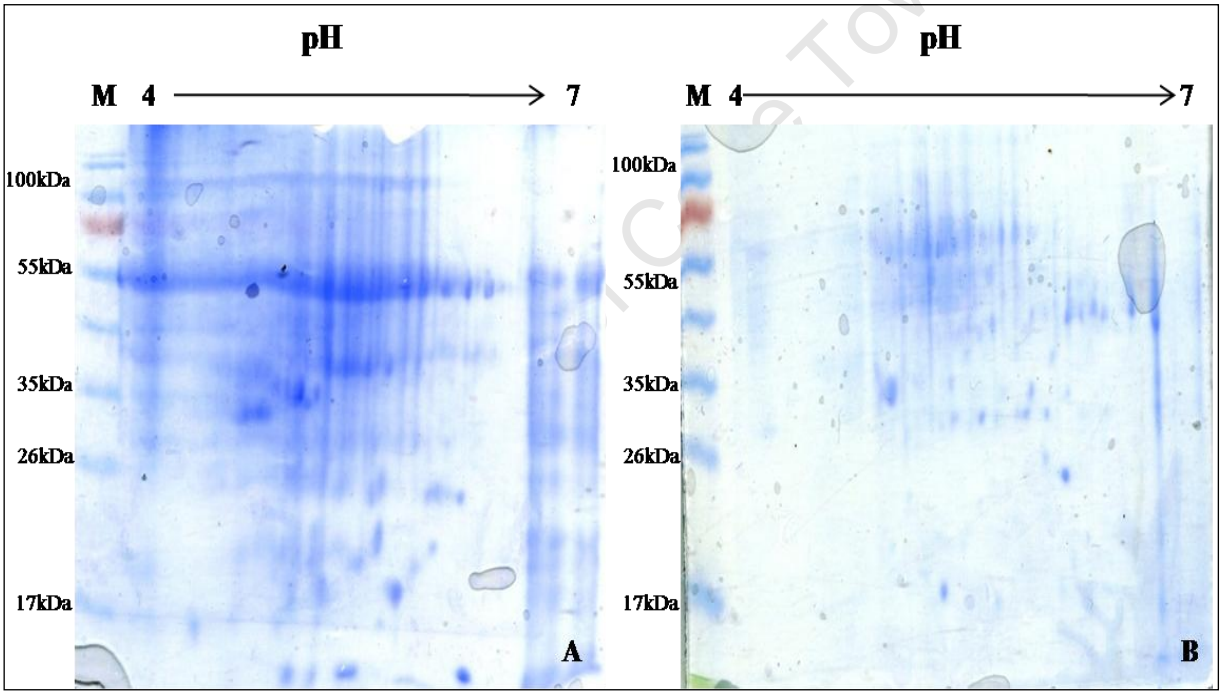

Fig. 8 Two-dimensional electrophoresis of maize leaf proteins (A) and root proteins (B); Lane M: MW marker (Fermentas), their MW are given in kDa. Maize protein was loaded at an amount of 200 µg and separated across pH 4-7 using a 7-cm IPG strip on a 15% polyacrylamide gel. Protein spots were visualised with Coomassie brilliant blue (CBB) stain.

When observing the 2-DE PAGE results (Fig. 8), we were able to visualize total protein spots for both maize leaf proteins (Fig. 8A) and maize root proteins (Fig. 8B). Although protein spots were present, there was still some horizontal and vertical streaking. This streaking effect could be attributed to many factors such as: loss of protein solubility at investigated p*I*, dust

contaminants, non-proteinaceous contamination, insufficient or excess reducing agent, protein overloading, sample solubility and incomplete focusing (Berkelman *et al*., 2000). In our specific protein samples, we found that sample solubility, incomplete focusing and non-proteinaceous contamination to be the main causes of poorly resolved 2-DE results, while dust contaminants, improper reducing agent and protein overloading to be negligible. Plant tissues usually have low protein content (Isaacson *et al*., 2006), while root tissues have even less protein due to having a high fiber and lignin content (Xu *et al.*, 2008). Thus the chances of high protein concentrations and hence overloading were slim. Dust contaminants were eliminated by filter-sterilising our reagents just before use and the reducing reagent, dithiothreitol (DTT) is well known in its ability to reduce disulphide bridges (Carpentier *et al*., 2005; Vâlcu and Schlink, 2006; Wang *et al.*, 2008).

We were able to remove further contaminants by employing the Wang *et al*. (2003) method during the extraction of maize proteins by the addition of acid-washed sand to the grinding stage in liquid nitrogen. This, combined with increased grinding times was able to increase tissue fineness which aids in more thoroughly removing contaminants when extraction buffer is added (Wang *et al*., 2003). We found that increased washes with 80% methanol and acetone subsequent to precipitation in methanol and ammonium acetate were able to produce a clean white protein pellet (free from polyphenolic oxidation). These washes facilitate the removal of residual ammonium acetate, phenol, lipids and pigments (Isaacson *et al*., 2006). The following resolubilisation step of protein pellets in a resolubilising buffer such as the widely used ULB (see Materials and Methods section 2.4) (Shaw and Riederer, 2003; Gorg *et al*., 2004) is critical to ensure that individual polypeptides are denatured and reduced to disrupt the intra- and intermolecular interactions while maintaining the charge properties of the proteins (Gorg *et al.*, 2004). We included DTT in the resolubilisation buffer at a concentration of 1% (w/v) that resulted in the samples being completely resolubilised after an hour of moderate vortexing. Sonication of protein samples has also been noted to increase resolubilisation of protein samples (Sarma *et al*., 2008), however we found this unnecessary once our samples were completely dissolved after 1 hour of moderate vortexing.

For IEF optimisation, we used the isoelectric focusing conditions used by Requejo and Tena (2005) (see Materials and Methods section 2.6). This decreased the time required for IEF focusing without affecting the 2-DE results and allowed an exponential gradient of 4000V at

10 000 $V.h^{-1}$ to be reached in under 8 hours. This was an improvement in comparison to *X. viscosa* proteins that would sometimes take up to 24 hours to reach focusing completion.

Though we optimised the 2-DE IEF conditions for maize root protein, we found that the results obtained on the 2-DE were still not sufficiently well-resolved (Fig. 8A and 8B). The amount of protein spots depicted in our results were less than expected (in comparison to the concentration of protein being loaded) and was not similar to what has been previously shown to be in maize roots, with respect to spot quantity (Brouquisse *et al*., 2001; Mohamed, 2005; Requejo and Tena 2005; Zhu *et al*., 2006; Isaacson *et al*., 2006; Prinsi *et al.,* 2009). The loss of protein resolution when observing our 2-DE results (Fig. 8A and 8B), may possibly be due to poor transfer of proteins to the second dimension (Shaw and Riederer, 2003). This has been shown to occur when resolubilisation of proteins in a resolubilising buffer such as ULB, with increased thiourea concentrations, results in SDS-protein binding inhibition or improved solubilisation of lipids, which affect protein spot resolution on 2-DE gels (Shaw and Riederer, 2003). Thiourea is usually used at a concentration of 2 M in conjunction with 7 M urea, as with our case, and has been generally used at these concentrations. However, there have been reports of decreased protein spot resolution with increased concentrations of thiourea (Musante *et al*., 1998; Shaw and Riederer, 2003). Nevertheless, we did not consider this to be a limiting factor in our further proteomic approaches, as it is well established that 2-DE analysis is difficult to automate, resulting in limited throughput and greater experimental variability (Rose *et al*., 2004), with every plant species and plant tissue analysed (Saravanan and Rose, 2004). One other factor that would have to be considered when observing 2-DE analysis results with regards to decreased protein spot number and resolution is the protein staining technique, in order to quantify and compare protein samples. Here again technical issues might arise and limit the quantification of proteins in a sample (Rose *et al.,* 2004) as the range of protein concentrations can often span numerous orders of magnitude from highly abundant to low abundant proteins (Rose *et al*., 2004).

It should also be re-iterated that sample preparation is of key importance when conducting 2-DE analysis and high quality protein samples are vital for good quality results as contaminants could drastically interfere with protein separation (Rose *et al.,* 2004). From our SDS-PAGE for maize leaf and root proteins (Fig. 7B), we observed no indication that our method chosen (Method C) for protein isolation was limiting in producing good quality proteins. We therefore concluded that 2-DE analysis has its own technical limitations

especially with regards to specific classes of proteins such as hydrophobic proteins and glycoproteins (Rose *et al.*, 2004; Braun *et al.*, 2007).

One other interesting result that we observed was the comparison of *X. viscosa* root proteins and maize proteins when both samples were analysed in the first dimension (Fig. 9). We conducted protein extractions on both maize root and leaf tissue, as well as on *X. viscosa* root tissue, under the same extraction conditions, using the same reagents and protein extraction method (Method C). These samples were stored at -80^{o}C for approximately two weeks before use. Upon subsequent SDS-PAGE analysis (Fig. 9), we were able to observe protein bands for both maize root and leaves in lanes 1-5 (Fig. 9A), but obvious protein degradation and smearing for *X.viscosa* root protein in lanes 6-7 (Fig. 9A).

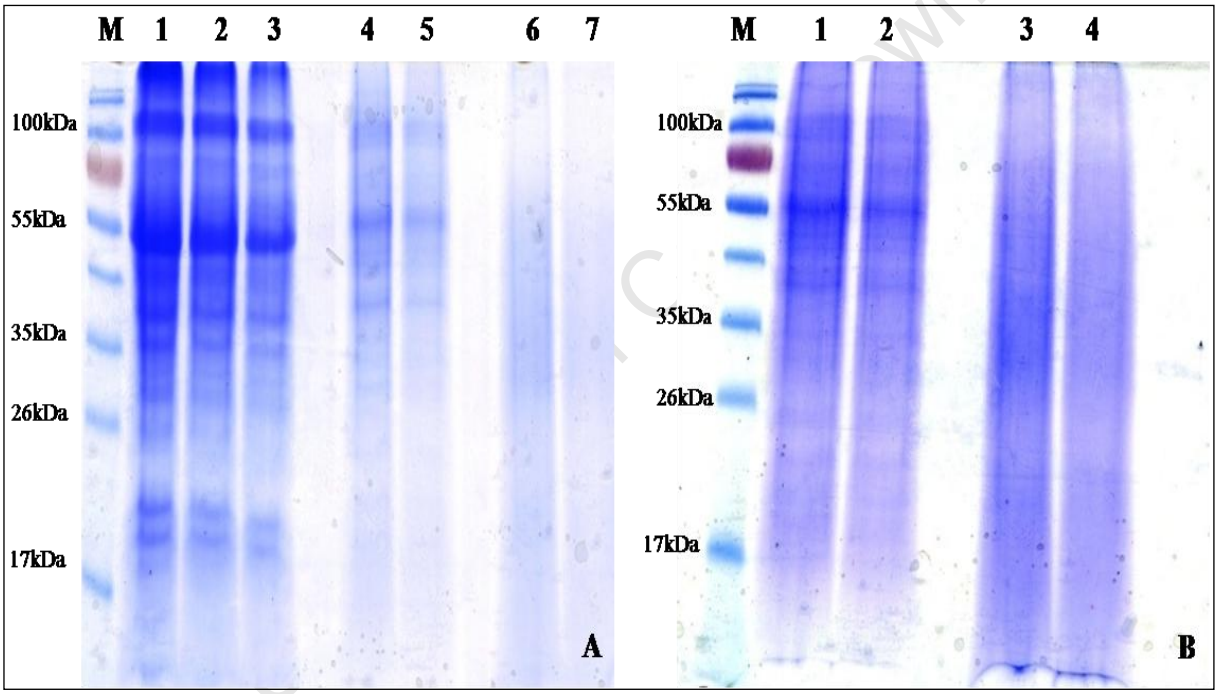

Fig. 9 SDS-PAGE gels of protein extracted from maize leaves and roots (A), maize and *X. viscosa* roots (B). Lane M: MW marker (Fermentas); MW are given in kDa. (A) Lanes 1-3 depict maize leaf proteins, lanes 4-5 maize root proteins and lanes 6-7 *X. viscosa* root proteins. (B) Lanes 1-2 maize root proteins and lanes 3-4 *X. viscosa* root proteins. All samples were dissolved in Laemmli buffer, heated at 80°C for 5 minutes and loaded onto 15% polyacrylamide gels. Protein bands were visualised with Coomassie brilliant blue (CBB) stain.

When focusing on the two root tissues only (Fig. 9B), we observed an even greater contrast between the two samples, lanes 1-4 (Fig. 9B). Maize roots have distinct bands in the molecular weight range of 100 kDa to 17 kDa in lanes 1 and 2 (in a similar molecular weight range to what was previously obtained (Fig.7B)), while lanes 3-4 for *X. viscosa* roots (Fig.

9B) have very few bands present, which were smeared, degraded protein. It would seem that maize root protein is able to produce a banding pattern (similar to Fig. 7A), while there is protein degradation in *X.viscosa* root samples, even when samples are stored at a very low temperature (-80^{o}C).

This result could potentially suggest the presence of an intracellular protease that is able to degrade proteins under what is normally considered to be stable conditions. Protein samples are usually frozen at very low temperatures, however it could also be speculated that these intracellular proteases are tightly bound to protein complexes and during incubation periods at low temperatures (< -20^{o}C), these proteases are activated and actively degrade protein. Schaffer and Fischer (1990), hypothesized the presence of a thiol protease present in tomato, that when induced by cold temperatures, was able to function in the degradation of damaged or denatured polypeptides that have been exposed to low temperatures. Our protein samples might not necessarily be damaged in this case, however they are denatured when resolubilisation takes place in a resolubilising buffer containing urea (Shaw and Riederer, 2003; Gorg *et al*., 2004). This compound is able to denature polypeptides by disrupting the non-covalent and ionic bonds between amino acid residues (Shaw and Riederer, 2003), and in theory is supposed to denature proteases as well, even more so at such low temperatures. Protein samples before electrophoresis were resolubilised in most cases in ULB before being mixed with Laemmli buffer and loaded onto acrylamide gels. We found ULB to be a better suited resolubilising buffer even for analysis at the 1-DE level, as it was able to efficiently dissolve protein pellets and produce well resolved protein bands in comparison to resuspension in a SDS-loading buffer. Furthermore, even though our protein samples were resuspended in ULB and stored at -80^{o}C, we still cannot accurately explain the loss of protein over time at these conditions. It is apparent though that for further proteomic platform analyses on *X. viscosa* root tissues, protein extractions need to be performed just before proteomic analysis to avoid possible proteolysis during cold storage and to ensure that stable good quality protein is extracted.

3.3) OFFGEL Fractionation Analysis (maize and *X. viscosa* root tissue)

To overcome the hurdles involved with 2-DE analysis especially with regards to proteins from *X. viscosa* root tissues, we investigated a novel proteomic analysis technique developed by Agilent Technologies, called OFFGEL fractionation. This technique was able to separate proteins according to their isoelectric points (p*I*) by electrofocussing samples in a multiwell device with the advantage of recovering fractionated protein samples in a liquid form to be used for further analysis (Michel *et al.*, 2003; Horth *et al*., 2006; Lam *et al*., 2007; Ernoult *et al*., 2008). The advantages of using the OFFGEL fractionation technique over 2-DE analysis included the ability to load protein samples directly onto the IPG strips into different wells placed along the IPG strip, instead of soaking and rehydrating the strips in protein solution for 16 hours. Electrofocussing is then applied to protein samples and proteins are able to migrate from the wells in which they were loaded, to the wells where their respective p*I* points are situated. Once electrofocussing is complete, protein samples present in the various p*I*-based wells are then recovered in solution. These liquid samples are then precipitated down to pellet form using a simple, but effective cold acetone precipitation subsequent to resolubilisation in a SDS-loading buffer (namely, Laemmli buffer). The resuspended protein samples are then separated by SDS-PAGE and visualised with Coomassie brilliant blue stain. This allows proteins to be totally recovered after electrofocussing and eliminates the potential loss of protein occurring due to technical issues that are characteristic to 2-DE analysis.

We firstly wanted to test this technique on maize root protein extracted using Method C to observe the fractionated protein profile present in maize roots, and furthermore conduct optimisation procedures on these results before proceeding to *X. viscosa* root proteins. In Fig. 10, we were able to observe the fractionated protein samples from maize roots in a pH range of 5-8. Since OFFGEL fractions 1 and 2 had a low protein yield after protein quantification for both maize and *X. viscosa* root protein, we decided to combine these two fractions and apply this procedure in all of our following attempts at OFFGEL analysis.

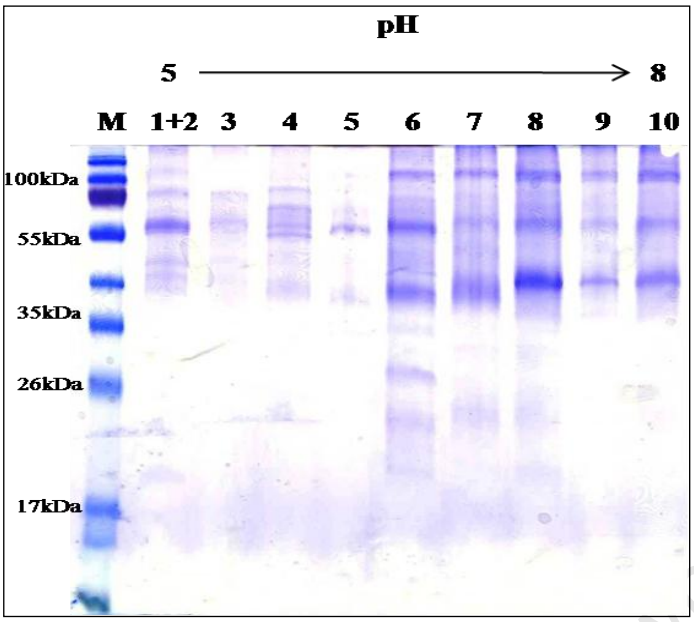

Fig. 10 SDS-PAGE profile of the OFFGEL fractionated proteins present in maize roots. Lane M: MW marker (Fermentas); MW are given in kDa. Lanes 1-10: OFFGEL fractions of maize root proteins in a pH 5-8 range. Fractionated protein samples were resuspended in Laemmli buffer, before being heated at 80^{o}C for 5 minutes and loaded at an amount of 20 µg onto 15% polyacrylamide gels. Fractionated protein bands stained with Coomassie brilliant blue (CBB) stain.

The protein bands in Fig. 10 represent the proteins present at their respective p*I*s and set molecular weights in maize root tissue. The protein profile displayed was well resolved and showed a good distribution of protein samples that has significantly improved in comparison to results obtained when using 2-DE analysis (Fig. 8A and 8B). The most predominant protein bands were seen from OFFGEL fraction 6 onwards, which corresponds to a pH of approximately 6.5. There are reproducible protein bands at 100, 55 and 43 kDa that were observed in all fractions with a more neutral pH (from pH 6.5-8 or OFFGEL fractions 6-10) indicating that these protein fractions have a neutral to basic nature. In another study recently performed by Vincent and Solomon (2011), using the OFFGEL fractionation technique, they were able to show a much broader range of fractionated proteins present in wheat leaves that had a more acidic to neutral pH, while the resolution of fractionated proteins in a slightly basic range were less enhanced. To date, they are one of the few research groups that have managed to demonstrate the OFFGEL fractionation technique in plant tissues.

Some of the optimisation procedures that allowed the production of improved OFFGEL analysis results in the current study were firstly, resolubilisation of the dry protein pellets after the extraction directly in very diluted state in OFFGEL buffer. This buffer, which is very similar to ULB, contains urea at a concentration of 6 M, 2 M thiourea and 2% (w/v) DTT, and

is able to efficiently denature and dissolve protein pellets in a short period of time. The diluted aliquots of samples allow salts to be diluted out of solution and are hence less of an interference to the electrophoretic run (Vincent and Solomon, 2011). This desalting effect could also potentially decrease the time required for electrofocussing to be complete and avoids the likelihood of proteins moving out of solution as well as the potential loss of protein solubility at their respective p*I*-points (Berkelman *et al*., 2000). Secondly, the protein pellets, after an ice cold precipitation in acetone, were usually small in size and very light in colour, indicating that the majority of interfering compounds were removed (Wang *et al.*, 2008). Thirdly, these samples were more accurately quantified using the Pierce BCA protein assay kit (Thermoscientific, USA), instead of the Bradford quantification method as the reagents used for resuspension, particularly SDS (added at a concentration of 5% (w/v)), were only tolerated by the BCA protein assay kit. By using these reagents, minute concentrations of proteins were detected especially in fractions where protein migration is low. Fourthly, the BCA-compatible resuspension solution was able to facilitate better resolubilisation of fractionated protein pellets due to the higher SDS concentration. Lastly, when it came to sample loading onto 15% polyacrylamide gels, it was found that those samples that were the highest in concentration and hence the smallest volume when being loaded, gave the best resolution and separation when SDS-PAGE was applied. Thus it was our objective to keep resuspended protein samples as concentrated as possible by resuspending protein pellets in a minimal amount of resuspension solution to ensure high-quality separation. The only disadvantage to this approach was that samples then took much longer to fully resolubilise.

In conjunction with all the positive approaches using this technique, the time required to reach focusing completion was much less in comparison to 2-DE analysis (Lam *et al*., 2007) which provided less opportunity for proteolysis to occur. To our knowledge, the only disadvantage that we encountered with OFFGELL fractionation was that it is more time consuming and laborious in comparison to 2-DE analysis, especially with regard to protein quantification. Each protein fraction along the pH gradient has to be individually aliquoted into a well-labelled microcentrifuge tube, precipitated down to pellet form, fully resolubilised and individually quantified to ensure uniformity in concentrations when conducting SDS-PAGE. To this point however, OFFGEL fractionation demonstrates excellent protein fractionation ability and good-quality resolving power, though it is necessary to optimise the fractionation procedure in order to obtain the best protein recovery and result (Lam *et al*., 2007).

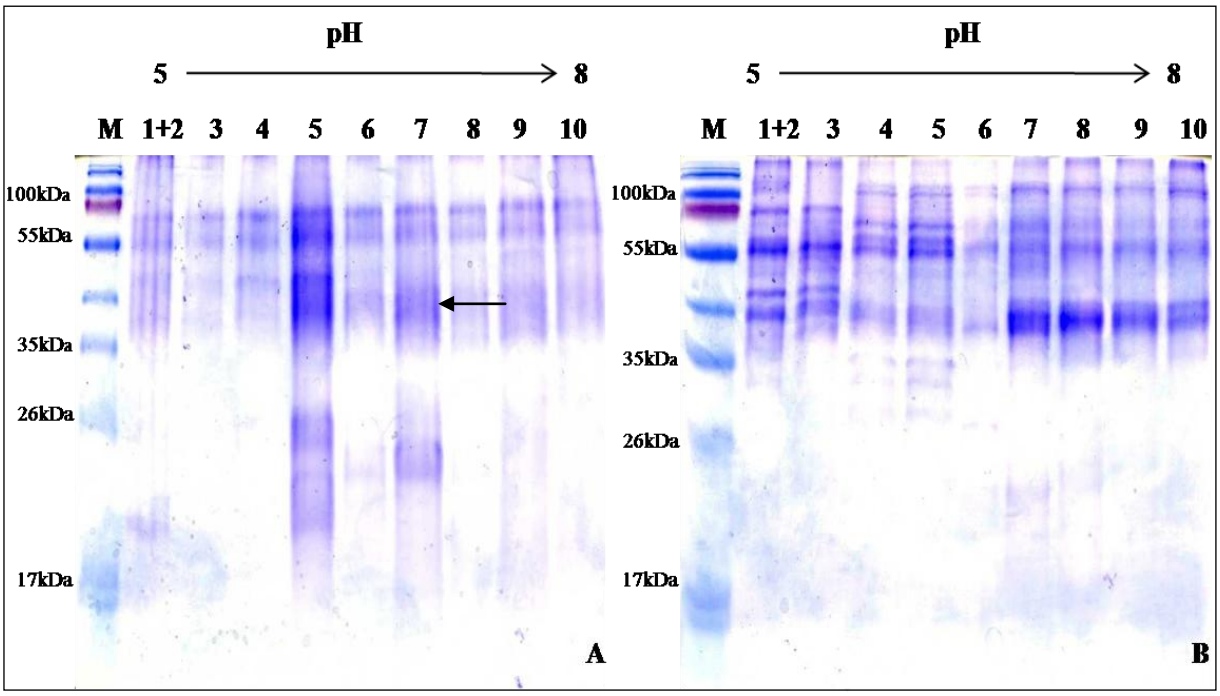

Fig. 11 SDS-PAGE profiles of the OFFGEL fractionated proteins present in *X. viscosa* roots (A) and maize roots (B). Lane M: MW marker (Fermentas); their MW are given in kDa. Lanes 1-10: OFFGEL fractions of *X. viscosa* root proteins in a pH 5-8 range (A). Lanes 1-10: OFFGEL fractions of maize root proteins in a pH 5-8 range (B). Fractionated protein samples were resuspended in Laemmli buffer, before being heated at 80^{o}C for 5 minutes and loaded at a concentration of 20 µg onto 15% polyacrylamide gels. Fractionated protein bands stained with Coomassie brilliant blue (CBB) stain.

The OFFGEL fractionated protein profiles of *X. viscosa* roots and those of the positive control, maize root fractionated proteins (Fig. 11A and 11B, respectively), shows well-resolved electrophoretic patterns and protein bands in each fraction loaded. This was a significant result, especially with regards to protein analysis from *X. viscosa* roots. Fig. 11A shows common protein bands consistently spread along OFFGEL fractions 1-10 at both 70 and 55 kDa. The most predominant protein bands were present in OFFGEL fractions 5 and 7 (Fig. 11A), especially at approximately 47 kDa (arrow). These two fractions displayed even lower molecular weight proteins at 26, 20 and 18 kDa, while all other OFFGEL fractions were able to display slight protein bands between 55 and 35 kDa. This could potentially indicate the presence of acidic and basic fractions in *X. viscosa* roots, however most protein bands centre around a neutral pH. From the results obtained, both low and high molecular weight proteins were distinguishable, this not being achieved when using 2-DE analyses.

Furthermore there was considerable enhancement of protein bands that were very faint utilizing standard 2-DE analyses as reported by Vincent and Solomon, (2011).

OFFGEL analysis on maize root proteins (Fig. 11B) showed highly-resolved and more distinct protein bands in all the fractions loaded. We were able to make accurate comparisons between this and previous data (compare Figs. 8B, 9B and 10). The slightly acidic OFFGEL fractions in a pH range of 5-6 (fraction 1-5), were more clearly distinguishable and displayed much better separation with more protein bands present than in Fig. 10. The amount, quality and protein profile of Fig. 11B coincide with what has been previously shown to be present in maize roots with regards to 2-DE analysis (Requejo and Tena, 2005, Prinsi *et al*., 2009), with some differences, as what we are observing are distinct bands at p*I* points instead of discrete spots. The fact that these protein bands (Fig. 11B) are much more enhanced and highly-resolved when OFFGEL analysis was utilized, indicates just how variable the 2-DE analysis technique can be, when taking into account all the technical hurdles associated with poor 2-DE results (Fig. 8A and 8B).

Nevertheless, the high-quality results could be due to our small, but evident optimisation procedures or the product of using OFFGEL fractionation as an excellent protein p*I*-based separation tool. From here we can clearly make decisions about how to proceed further when analysing the root proteomes of maize, and more so *X. viscosa,* without the concerns of protein degradation, sample contamination, long focusing times, insufficient resolubilisation and poor resolution. We now obtained a result that clearly depicted the p*I*- separated proteins present in the root tissues of *X. viscosa,* which could potentially be used for further proteomic analyses.

3.4) Identification of hydrated root proteins using OFFGEL fractionation analysis and LC- MS/MS

From the results displayed displayed in Fig. 11, four protein fractions were chosen for further comprehensive proteome analysis using liquid chromatography-tandem mass spectrometry (LC-MS/MS) in order to identify the proteins present in the root tissues of hydrated *X. viscosa* and maize (OFFGEL fractions 5 and 7 from *X. viscosa* (Fig. 11A) and OFFGEL fractions 1 and 7 from maize (Fig. 11 B)). These fractions (Fig. 11) were chosen on the basis of relative abundance and protein band intensity. For the purpose of this study, it was decided to concentrate on the protein identities from *X. viscosa* root proteins only and not the identities generated from maize (fractions 1 and 7) as the original aim of the current study was the identification of proteins from *X. viscosa* roots. The complete list of identified proteins for maize root fractions 1 and 7 are presented in the supplementary tables (S.1 and S.2).

All the data gathered during analysis were subjected to a homology-based search using the protein database (NCBInr). For *X. viscosa*, the protein identity threshold score was set at 84 and proteins were identified with a 95% confidence level ($P<0.05$). The lists of protein identities are shown in Table 2.1 and Table 2.2. From the proteins identified, it was found that 128 peptide matches out of 150 peptides identified were above the identity threshold with a false discovery rate of 3.1% (in fraction 5) (Table 2.1), while in fraction 7, 80 peptide matches out of 109 peptides identified were above the identity threshold with a false discovery rate of 8.8% (Table 2.2). Additionally, the identified proteins were functionally classified based on their annotated biological processes (Fig. 12A and 12B) according to the PANTHER classification system (PANTHER: a library of protein families and subfamilies indexed by function, Thomas *et al.*, 2003).

These functional categories include: carbohydrate metabolism (representing 24 and 35% of the total proteins present in OFFGEL fractions 5 and 7, respectively); energy metabolism (4 and 5%); protein metabolism (15 and 26%); antioxidant mechanisms (22 and 9%); apoptosis (5 and 9%); unknown proteins (18 and 5%); nucleasome assembly (1 and 2%); protein transport and signalling (5 and 5%) as well as stress response proteins (1 and 2%). In both fractions proteins involved in certain biological processes only, were also classified such as: nucleotide and nucleic acid metabolism (1%, OFFGEL fraction 5); metabolic process (3%, OFFGEL fraction 5); cellular protection (1%, OFFGEL fraction 5) and cell communication (2%, OFFGEL fraction 7). Some of these functional categories are further discussed.

Table 2.1 Identification of *X. viscosa* root total proteins present in OFFGEL fraction 5 by LC-MS/MS analysis

MASCOT Score	Expectation	Protein ID	Protein Name	MW (Da)	%Coverage	Biological Process
595	4.10E-53	gi|326490934	predicted protein [*Hordeum vulgare subsp. vulgare*]	48201	26.1	Nucleasome assembly
566	3.50E-50	gi|90110845	enolase; 2-phospho-D-glycerate hydro-lyase	47942	23.8	Carbohydrate metabolism
496	3.80E-43	gi|8919731	enolase [*Spinacia oleracea*]	48137	18.2	Carbohydrate metabolism
483	7.40E-42	gi|115451911	Os03g0248600 [*Oryza sativa Japonica Group*]	47942	24	Carbohydrate metabolism
481	1.00E-41	gi|115466256	Os06g0136600 [*Oryza sativa Japonica Group*]	47908	24.7	Carbohydrate metabolism
401	1.10E-33	gi|15222848	GAPC2 (GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE C2); NAD or NADH binding [*Arabidopsis thaliana*]	36890	27.5	Carbohydrate metabolism
369	1.70E-30	gi|231586	ATP synthase subunit beta, mitochondrial	60221	20.6	Energy metabolism
369	1.90E-30	gi|222632492	hypothetical protein OsJ_19476 [*Oryza sativa Japonica Group*]	55275	21	Carbohydrate metabolism
358	2.40E-29	gi|258642943	glyceraldehyde-3-phosphate dehydrogenase 2 [*Festuca arundinacea*]	36481	22	Carbohydrate metabolism
327	2.70E-26	gi|34850871	glyceraldehyde 3-phosphate dehydrogenase [*Torenia hybrida*]	21374	30.5	Carbohydrate metabolism
306	3.20E-24	gi|225457450	hypothetical protein [*Vitis vinifera*]	53854	11.9	unknown
300	1.40E-23	gi|115459078	Os04g0486600 [*Oryza sativa Japonica Group*]	36750	27.9	Carbohydrate metabolism
284	5.70E-22	gi|114411	ATP synthase subunit alpha, mitochondrial	55310	14.2	Energy metabolism-ATP synthesis
273	7.40E-21	gi|281341811	hypothetical protein PANDA_012419 [*Ailuropoda melanoleuca*]	39324	15.7	unknown
271	1.10E-20	gi|326467057	glyceraldehyde-3-phosphate dehydrogenase [*Litchi chinensis*]	33892	18.1	Carbohydrate metabolism
252	7.90E-19	gi|19423862	1-cys peroxiredoxin [*Xerophyta viscosa*]	24253	27.4	Antioxidant mechanisms
247	2.60E-18	gi|224133862	predicted protein [*Populus trichocarpa*]	36747	23.7	unknown
242	9.10E-18	gi|147801802	hypothetical protein VITISV_023718 [*Vitis vinifera*]	64895	8.5	unknown
241	1.00E-17	gi|148554276	elongation factor Tu [*Sphingomonas wittichii RW1*]	42917	17.4	Protein metabolism
238	2.20E-17	gi|20322	unnamed protein product [*Oryza sativa Indica Group*]	41926	19.6	unknown
226	3.20E-16	gi|2829755	actin-1	41819	16	Protein transport
220	1.30E-15	gi|147778328	hypothetical protein VITISV_040027 [*Vitis vinifera*]	41644	17.8	unknown

(Continued)

215	4.70E-15	gi|11181616	translational elongation factor EF-TuM [*Zea mays*]	48518	11.1	Protein metabolism
213	7.60E-15	gi|15231715	fructose-bisphosphate aldolase, putative [*Arabidopsis thaliana*]	38516	17.9	Carbohydrate metabolism
212	8.60E-15	gi|262316938	glyceraldehyde-3-phosphate dehydrogenase [*Manihot irwinii*]	13303	27.2	Carbohydrate metabolism
211	1.10E-14	gi|326806921	Class III peroxidase [*Cynara cardunculus var. scolymus*]	21432	18.5	Antioxidant mechanisms
202	8.70E-14	gi|217976769	elongation factor Tu [*Methylocella silvestris BL2*]	43107	14.4	Protein metabolism
202	9.40E-14	gi|145588228	elongation factor Tu [Polynucleobacter necessarius subsp. asymbioticus	42986	16.4	Protein metabolism
197	2.80E-13	gi|225460961	hypothetical protein [*Vitis vinifera*]	109987	5.8	unknown
195	4.30E-13	gi|1743354	aldehyde dehydrogenase (NAD+) [*Nicotiana tabacum*]	59298	9.6	Carbohydrate metabolism
194	5.10E-13	gi|224077754	predicted protein [*Populus trichocarpa*]	41226	14	unknown
186	3.20E-12	gi|1389835	peroxidase [*Linum usitatissimum*]	38171	8.7	Antioxidant mechanisms
186	3.60E-12	gi|255583617	aconitase, putative [*Ricinus communis*]	108304	7.3	Carbohydrate metabolism
184	5.00E-12	gi|77745438	unknown [*Solanum tuberosum*]	39669	20.1	unknown
182	8.70E-12	gi|285309965	aconitate hydratase 1 [*Citrus clementina*]	98424	6.9	Carbohydrate metabolism
170	1.30E-10	gi|62526573	aldo/keto reductase AKR [*Manihot esculenta*]	37684	11.9	Metabolic processes
169	1.80E-10	gi|168014669	predicted protein [*Physcomitrella patens subsp. patens*]	71027	11.6	Protein metabolism
165	4.40E-10	gi|311332744	hypothetical protein PTT_02545 [*Pyrenophora teres f. teres 0-1*]	74180	8.7	unknown
164	5.00E-10	gi|13274150	putative cytosolic CuZn-superoxide dismutase [*Populus tremula x Populus tremuloides*]	15186	16.4	Antioxidant mechanisms
164	5.70E-10	gi|2827080	malate dehydrogenase precursor [*Medicago sativa*]	35832	12.2	Carbohydrate metabolism
163	7.50E-10	gi|170180310	heat shock protein 70 [*Daphnia magna*]	70318	8.7	Stress response
153	7.30E-09	gi|110639548	elongation factor Tu [*Cytophaga hutchinsonii ATCC 33406*]	42994	10.1	Protein metabolism
150	1.30E-08	gi|33346927	ubiquitin/actin fusion protein 1 [*Bigelowiella natans*]	26376	15.9	Protein transport
149	1.70E-08	gi|171462864	translation elongation factor Tu [*Polynucleobacter necessarius subsp. necessarius STIR1*]	43088	11.6	Protein metabolism
145	4.20E-08	gi|56199601	dehydration up-regulated putative membrane pore protein [*Xerophyta humilis*]	18880	25.3	Protein transport
143	6.40E-08	gi|417745	adenosylhomocysteinase; AdoHcyase; S-adenosyl-L-homocysteine hydrolase	53402	9.1	Protein metabolism
142	8.60E-08	gi|194701874	unknown [*Zea mays*]	34969	11	unknown

(Continued)

131	0.0000011	gi|225451235	hypothetical protein isoform 2 [*Vitis vinifera*]	34348	16	unknown
128	0.0000021	gi|83701236	glyceraldehyde-3-phosphate dehydrogenase [*Taliparitiliaceum*]	14442	49.6	Carbohydrate metabolism
127	0.0000027	gi|113436	alcohol dehydrogenase	41432	11.1	Apoptosis
125	0.0000045	gi|297562685	adenosylhomocysteinase [*Nocardiopsis dassonvillei subsp. dassonvillei DSM 43111*]	52001	6.7	Protein metabolism
125	0.0000048	gi|24421235	superoxide dismutase [*Brassica juncea*]	15201	15.1	Antioxidant mechanisms
122	0.000009	gi|116778798	unknown [*Picea sitchensi*s]	38202	11.8	unknown
120	0.000014	gi|242063776	hypothetical protein SORBIDRAFT_04g001130 [*Sorghum bicolor*]	53834	11.5	Cellular protection
117	0.000028	gi|283101466	alcohol dehydrogenase 1 [*Hygroryza aristata*]	26343	16.7	Apoptosis
117	0.000028	gi|225452831	hypothetical protein [*Vitis vinifera*]	43723	9	unknown
115	0.000048	gi|167966208	type II peroxiredoxin [*Xerophyta viscosa*]	17476	32.7	Antioxidant mechanisms
111	0.0001	gi|224121066	predicted protein [*Populus trichocarpa*]	53473	6.1	Protein signalling
111	0.00011	gi|134612	superoxide dismutase [Cu-Zn] 1	15294	15.1	Antioxidant mechanisms
109	0.00016	gi|553107	triosephosphate isomerase [*Oryza sativa Japonica Grou*p]	27588	13	Carbohydrate metabolism
106	0.00038	gi|255570416	alcohol dehydrogenase, putative [*Ricinus communis*]	41297	6.6	Apoptosis
105	0.00039	gi|37811994	cytosolic triosephosphate isomerase [*Euglena gracilis*]	27779	9.4	Metabolic process-Fatty acid biosynthesis
104	0.00053	gi|37594590	catalase 2 [*Zantedeschia aethiopica*]	56946	9.1	Antioxidant mechanisms
93	0.0073	gi|1170141	putative exoglucanase type C; 1,4-beta-cellobiohydrolase; Beta-glucancellobiohydrolase	54669	6.6	Carbohydrate metabolism
90	0.014	gi|68138959	alcohol dehydrogenase [*Citrus x paradisi*]	41313	6.6	Apoptosis
89	0.018	gi|108706511	proteasome subunit alpha type 6, putative, expressed [*Oryza sativa Japonica Group*]	32244	16.1	Protein metabolism
89	0.017	gi|134300981	F0F1 ATP synthase subunit beta [Desulfotomaculum reducens MI-1]	51270	5.7	Energy metabolism-ATP hydrolysis
89	0.017	gi|5531937	acetoacetyl CoA thiolase [*Zea mays*]	22213	14	Protein metabolism
86	0.031	gi|1170506	eukaryotic initiation factor 4A-2; eIF-4A-2;ATP-dependent RNA helicase eIF4A-2	46799	6.8	Nucleotide and Nucleic acid metabolism
86	0.035	gi|1346180	glycine-rich RNA-binding protein GRP1A	16006	14.5	Protein metabolism

Table 2.2 Identification of *X. viscosa* root total proteins present in OFFGEL fraction 7 by LC-MS/MS analysis

MASCOT Score	Expectation	Protein ID	Protein Name	MW (Da)	% Coverage	Biological Process
515	4.30E-45	gi|115459078	Os04g0486600 [*Oryza sativa Japonica Group*]	36750	26.7	Carbohydrate metabolism
378	2.20E-31	gi|312192239	glyceraldehyde-3-phosphate dehydrogenase [*Ananas comosus*]	36575	32.3	Carbohydrate metabolism
343	6.80E-28	gi|238015186	unknown [*Zea mays*]	36472	25.8	unknown
326	3.70E-26	gi|307136112	glyceraldehyde-3-phosphate dehydrogenase [*Cucumis melo subsp. melo*]	36357	26.4	Carbohydrate metabolism
322	9.20E-26	gi|21388550	putative mitochondrial NAD-dependent malate dehydrogenase [*Solanum tuberosum*]	36144	15.7	Carbohydrate metabolism
313	6.70E-25	gi|258642943	glyceraldehyde-3-phosphate dehydrogenase 2 [*Festuca arundinacea*]	36481	25.8	Carbohydrate metabolism
303	6.60E-24	gi|114408	ATP synthase subunit alpha, mitochondrial	55562	16.8	Apoptosis
291	1.10E-22	gi|114411	ATP synthase subunit alpha, mitochondrial	55310	17.9	Apoptosis
263	6.80E-20	gi|192910916	catalase 2 [*Elaeis guineensis*]	56922	17.3	Antioxidant mechanisms
256	3.50E-19	gi|15231715	fructose-bisphosphate aldolase, putative [*Arabidopsis thaliana*]	38516	21.2	Carbohydrate metabolism
236	3.10E-17	gi|115679	Catalase isozyme 1	56841	11.2	Antioxidant mechanisms
225	3.90E-16	gi|74486738	translation elongation factor 1A-6 [*Gossypium hirsutum*]	49336	12.3	Protein metabolism
223	7.30E-16	gi|302920406	hypothetical protein NECHADRAFT_91822 [*Nectria haematococca mpVI 77-13-4*]	21737	24.5	Protein metabolism
221	1.00E-15	gi|168014627	predicted protein [*Physcomitrella patens subsp. patens*]	38380	11.6	Cell communication
216	3.30E-15	gi|3309243	aconitase-iron regulated protein 1 [*Citrus limon*]	98027	10.2	Carbohydrate metabolism
198	2.10E-13	gi|1351856	aconitate hydratase, cytoplasmic; aconitase; Citrate hydro-lyase	97943	8.7	Carbohydrate metabolism
196	3.50E-13	gi|326490934	predicted protein [*Hordeum vulgare subsp. vulgare*]	48201	12.5	Nucleasome assembly
194	6.00E-13	gi|162458207	enolase 1 [*Zea mays*]	48033	13.5	Carbohydrate metabolism
193	6.60E-13	gi|90110845	enolase; 2-phospho-D-glycerate hydro-lyase; 2-phosphoglycerate dehydratase; OSE1	47942	15	Carbohydrate metabolism
192	7.90E-13	gi|83283995	fructose-bisphosphate aldolase-like protein [*Solanum tuberosum*]	38594	19.8	Carbohydrate metabolism
185	4.40E-12	gi|255549601	5-methyltetrahydropteroyltriglutamate homocysteine methyltransferase, putative [*Ricinus communis*]	90131	6.4	Protein metabolism
178	2.10E-11	gi|8439545	methionine synthase [*Solanum tuberosum*]	84613	6.7	Protein metabolism

(Continued)

178	2.30E-11	gi|115466256	Os06g0136600 [*Oryza sativa Japonica Group*]	47908	15.7	Carbohydrate metabolism
171	1.10E-10	gi|148524155	elongation factor 1-alpha [*Chara australis*]	47628	10.7	Protein metabolism
170	1.50E-10	gi|154300849	conserved hypothetical protein [*Botryotinia fuckeliana B05.10*]	34340	12.7	Protein metabolism
162	7.80E-10	gi|326806921	Class III peroxidase [*Cynara cardunculus var. scolymus*]	21432	18.5	Antioxidant mechanisms
161	1.10E-09	gi|1389835	peroxidase [*Linum usitatissimum*]	38171	8.7	Antioxidant mechanisms
148	2.00E-08	gi|168017225	26S proteasome regulatory complex, ATPase RPT4 [Physcomitrella patens subsp. patens]	43975	13	Protein metabolism
139	1.80E-07	gi|8919731	enolase [*Spinacia oleracea*]	48137	7.2	Carbohydrate metabolism
136	3.60E-07	gi|293336155	hypothetical protein LOC100384174 [*Zea mays*]	26382	13.3	unknown
133	6.20E-07	gi|1100223	glyceraldehyde-3-phosphate dehydrogenase [*Pinus sylvestris*]	46111	7.4	Carbohydrate metabolism
124	0.0000055	gi|12585492	V-type proton ATPase subunit E;V-ATPase subunit E; ClVE-1; Vacuolar proton pump subunit E	26327	8.3	Energy metabolism-ATP hydrolysis
122	0.0000084	gi|1762914	alcohol dehydrogenase A [*Washingtonia robusta*]	33085	13.7	Apoptosis
120	0.000013	gi|149391359	polyubiquitin containing 7 ubiquitin monomers [*Oryza sativa Indica Group*]	22628	21.4	Protein metabolism
119	0.000017	gi|21672565	glyceraldehyde 3-phosphate dehydrogenase [*Buchnera aphidicola str. Sg (Schizaphis graminum)*]	36461	6.9	Carbohydrate metabolism
112	0.000084	gi|33346945	ubiquitin/actin fusion protein [*Gymnochlora stellata*]	49352	5.7	Protein transport
106	0.00036	gi|15219805	oxidoreductase [*Arabidopsis thaliana*]	36329	13.6	Stress response
105	0.00043	gi|269838652	alcohol dehydrogenase [*Salvia miltiorrhiza*]	40738	6.9	Apoptosis
102	0.00087	gi|541632	glutamine synthetase [*Solanum lycopersicum*]	18735	13.9	Protein metabolism
101	0.0012	gi|261746206	manganese-superoxide dismutase [*Bambusa oldhamii*]	25203	16	Antioxidant mechanisms
99	0.0016	gi|56199601	dehydration up-regulated putative membrane pore protein [*Xerophyta humilis*]	18880	14.6	Protein transport
94	0.0054	gi|7592102	elongation factor 1 alpha [*Xyleborus affinis*]	31858	9.6	Protein metabolism
93	0.0064	gi|1143394	V-type proton-ATPase [*Arabidopsis thaliana*]	26054	10	Energy metabolism-ATP hydrolysis
85	0.045	gi|1532214	cyclophilin A [*Trypanosoma brucei brucei*]	18751	8.5	Protein metabolism

(Mascot score, score obtained from the Mascot ™ search engine and is the protein score (or Mowse score) that is -10x log (*P*), where *P* is the probability that the observed match is a random event; Expectation value, the number of matches that are expected to occur by chance alone($P < 0.05$); Protein ID, accession number of the matched protein from the NCBI database; Protein name, matched protein description; MW, predicted molecular weight of the deduced amino acid sequence of the matched protein; %Coverage, percentage covered of the identified peptide sequence in the matched region. Protein identities with a % coverage < 5 % were omitted from the data. Almost all proteins identified had tentative 1 significant hit. Proteins were categorised based on their annotated biological process).

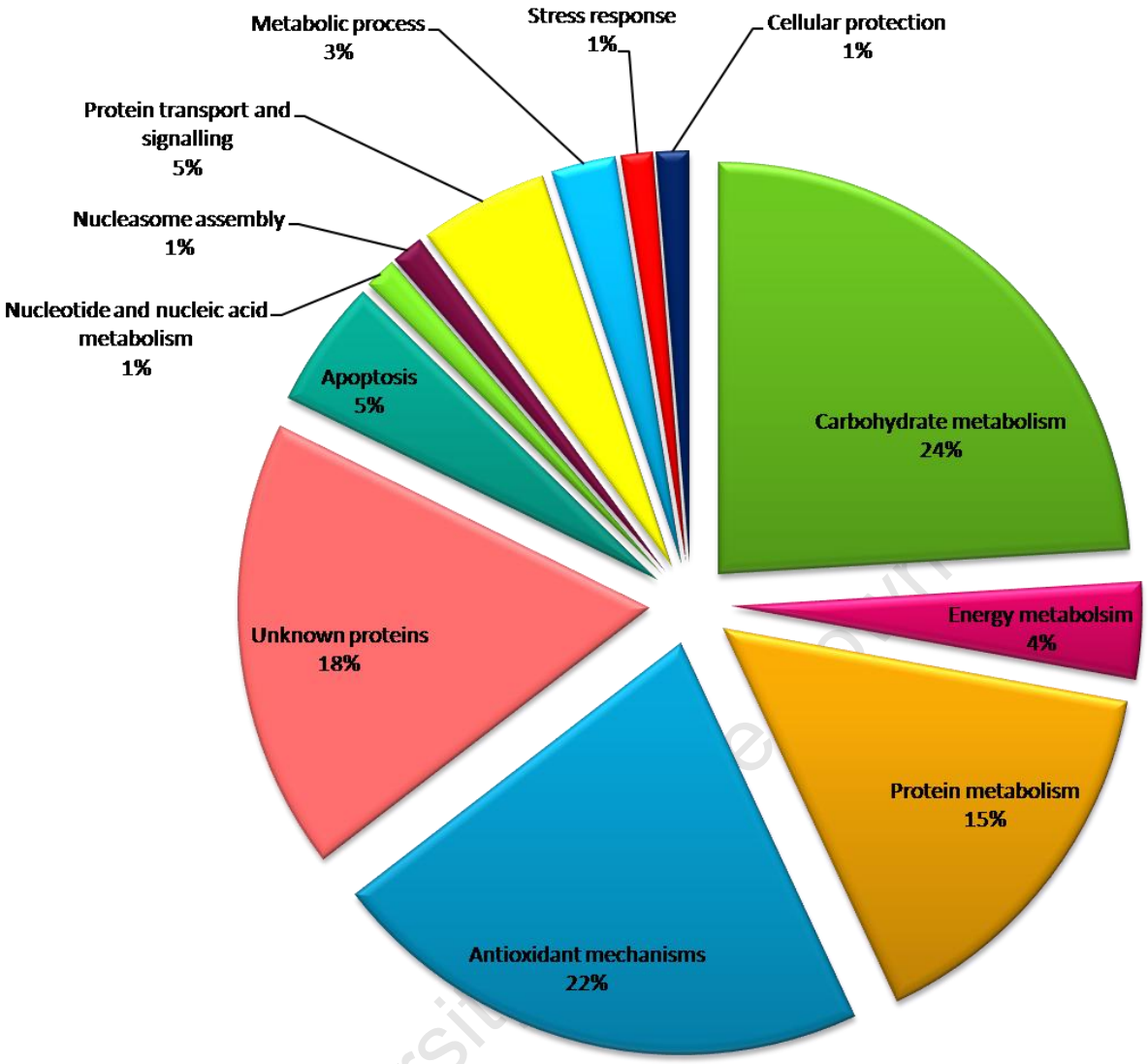

Fig. 12A Functional classification of the identified proteins present in OFFGEL-LC-MS/MS analysis of *X. viscosa* root protein in fraction 5.

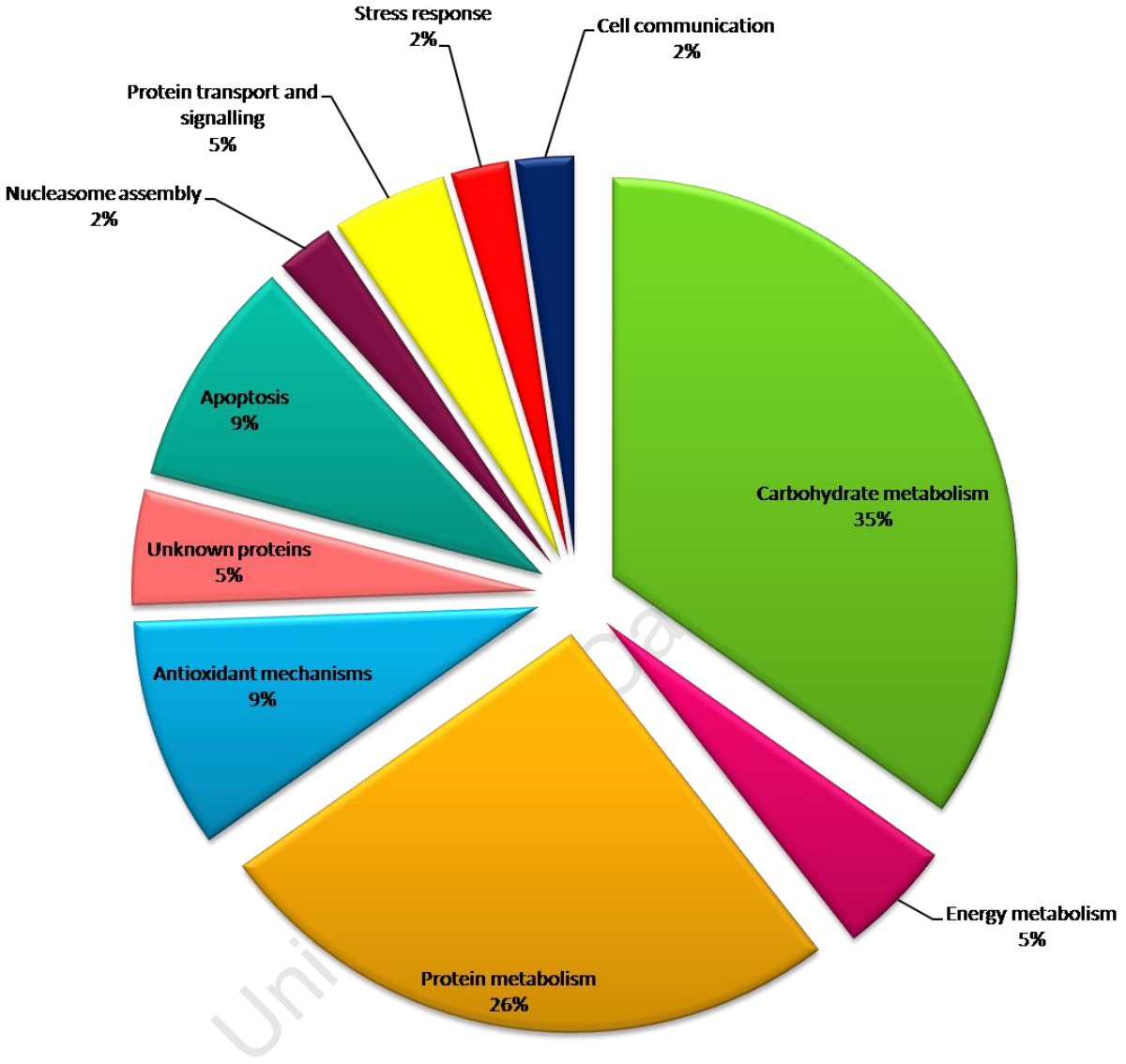

Fig. 12B Functional classification of the identified proteins present in OFFGEL-LC-MS/MS analysis of *X.viscosa* root protein in fraction 7.

3.4.1) Carbohydrate metabolism (24 and 35%)

A number of proteins or enzymes involved in carbohydrate metabolism were identified in the roots of *X.viscosa*, for both fractions 5 and 7 (Tables 2.1 and 2.2, respectively). Most of these enzymes identified are involved in the glycolytic pathway, some of which include: glyceraldehyde-3-phosphate dehydrogenase (GAPDH); fructose-bisphosphate (FBP) aldolase; and enolase. These enzymes are of key importance in glycolysis and function in the

breakdown of glucose to yield energy in the form of ATP and precursors essential for the synthesis of primary metabolites such as amino acids and fatty acids (Plaxton, 1996; Muñoz-Bertomeu *et al*., 2010). In addition to catalysing reactions in glycolysis, GAPDH has been reported to have protein kinase activity and the ability to decrease the production of reactive oxygen species (ROS) and enhance ribozyme and phosphotransferase activities (Duclos-Vallee *et al.,* 1998; Baek *et al.,* 2008; Xu and Huang, 2008). FBP aldolase has been reported to be up-regulated in maize leaves during water deficit (Riccardi *et al.,* 1998). The question, whether the same effect occurs in maize roots is still left unanswered, as little is known about the function of FBP aldolase in plant stress response (Xu and Huang, 2008). The enzyme, enolase is one of the major enzymes in glycolysis, catalysing the synthesis of phosphoenolpyruvate from 2-phosphoglycerate and has been previously reported to be responsive to various environmental stresses such as salt, drought, cold and anaerobic stresses in different plant species (Riccardi *et al.,* 1998; Lee *et al.,* 2002; Manaa *et al*., 2011).

Aconitase (aconitate hydratase) and malate dehydrogenase were identified (Tables 2.1 and 2.2) and are both involved in the citric acid cycle/gluconeogenesis, catalysing the isomerisation of citrate to isocitrate and malate to oxaloacetate respectively. Recently, aconitase was suggested to play an important role in modulating resistance to oxidative stress in *Arabidopsis* by acting as a biosensor for oxidants (Moeder *et al*., 2007; Chevalier and Rossignol, 2011). Some enzymes involved in carbohydrate metabolism were detected in fraction 5 only (Table 2.1), such as aldehyde dehydrogenase (NAD^+), triosephosphate isomerise and putative exoglucanase type C. Triosephosphate isomerase (TIM) is an essential, ubiquitous enzyme that functions not only in glycolysis but plays a role in gluconeogenesis, the pentose phosphate pathway and fatty acid biosynthesis as well (Table 2.2), while putative exoglucanase type C functions in the degradation of cellulose.

Aldehyde dehydrogenase (ALDH), which usually functions in carbohydrate metabolism in the oxidation of aldehydes to their corresponding carboxylic acids using NAD^+ or $NADP^+$ as a co-factor, is able to reduce the build up of toxic aldehydes that cause lipid peroxidation (Yoshida *et al.,* 1998). Interestingly, this enzyme has also been suggested to be induced in response to oxidative stress caused by certain abiotic stress treatments such as drought, high salinity and abscisic acid (ABA) in barley roots (Nakamura *et al*., 2001) and in resurrection plants (Kirch *et al*., 2001; Chen *et al*., 2002; Collett *et al*., 2004). This effect has also been

observed in *M. truncatula* seeds, where aldehyde dehydrogenase (amongst the free radical scavenger, 1-cys peroxiredoxin) was accumulated in response to desiccation (Leprince and Buitink, 2010; Farrant *et al*., 2011) and is believed to play an important role in attaining desiccation tolerance (Farrant *et al.*, 2011). In *Arabidopsis*, over expression of aldehyde dehydrogenase (*ALDH3*) has been suggested to improve stress tolerance by acting as an antioxidant and scavenging toxic aldehydes that cause lipid peroxidation (Sunkar *et al*., 2003).

Although these proteins function predominantly in glycolysis and subsequent energy yielding pathways, it can be theorised that they somehow offer a measure of protection in the roots of *X. viscosa* once stress occurs. It is well known that sucrose and raffinose family oligosaccharides (RFOs) accumulate in response to dehydration in desiccation tolerant tissues, where they are believed to act as water replacement molecules and/or stabilizers of the subcellular milieu in the dry state by facilitating cytosolic vitrification. Furthermore, these sugars serve as an energy source for recovery during rehydration (reviewed in Farrant 2007; Farrant *et al*., 2011). Antioxidant properties (discussed further below) are also considered vital for survival of abiotic stresses and upregulation of these, or their activities during dehydration, would undoubtedly facilitate survival of desiccation.

3.4.2) Energy metabolism (4 and 5%)

The proteins involved in energy metabolism include different forms of ATP-synthase (α, β-subunits) (Table 2.1) and V-type proton ATPase, (Table 2.2) and are predominantly related to the production and hydrolysis of ATP in the electron transport chain. F_0-F_1–ATP synthase (Table 2.1), is a large multi-subunit complex, situated within the mitochondrial membrane in the root tissues of plants, and is the site where ATP is synthesised from complex carbohydrates into energy via oxidative phosphorylation (Robison *et al.*, 2009). The root tissues of plants in most part are predominantly carbon and energy storing organs and energy is mostly required for general maintenance of the plant when not exposed to stress. Not much is known about ATP synthase and its role in stress response. However, recently in rice leaves, ATP synthase activity was reduced in response to heat stress, whereby the activity decreased by phosphorylating ATP synthases (Chen *et al.*, 2011). V-type ATPase however, has previously been shown to be highly active in plant tolerance to increased salinity concentrations (Manaa *et al*., 2008; Chen *et al*., 2011), especially in the roots and leaves of *M.*

crystallinum where the transcriptional activity of V-type ATPase was enhanced in response to salinity stress (Dietz *et al*., 2001). V-type ATPase has also been involved in relation to increased exposure to heavy metals, drought, cold, and acid stress where it has been proposed that maintenance or adjustment of the levels of this compound facilitates survival of such stresses (Dietz *et al*., 2001).

3.4.3) Protein metabolism (15 and 26%)

A number of enzymes related to protein biosynthesis and catabolism were identified in the roots of *X. viscosa*, some of which include: elongation factor Tu (EF-Tu); adenosylhomocysteinase; acetoacetyl CoA thiolase, and a glycine-rich RNA binding protein GRP1A (Table 2.1). Other enzymes involved in protein metabolism are given in Table 2.2 and these include: translation elongation factor 1A-6; glutamine synthetase; methionine synthase; 26S proteasome regulatory complex; polyubiquitin as well as cyclophilin A. Although these two protein fractions (fraction 5 and 7) only differ by a pH range of approximately 0.5, there seems to be a distinct difference in the proteins identified in each of these.

Elongation factor Tu (EF-Tu) is a protein that plays an important role in the elongation cycle of protein biosynthesis and functions by promoting the GTP-dependent binding of aminoacyl tRNA to the free A-site of a ribosome (Choi *et al*., 2000). Although chloroplasts are absent from root tissues, it is possible that the EF-Tu transcripts were either plastid, mitochondrial or cytoplasmically derived. Indeed the homologue identified in Table 2.1 was related to the mitochondrial form of Elongation Factor Tu (EF-TuM) from maize as well as the cytoplasmic elongation factor-1 (EF-1α) (Table 2.2). EF-Tus have previously been shown to have heat shock polypeptide (HSP) activity in maize leaves when exposed to heat stress (Bhadula *et al*., 2001). In this study, a chloroplast EF-Tu from thermotolerant transgenic maize was shown to increase in transcript and polypeptide levels when the plants were exposed to heat stress (Bhadula *et al*., 2001). The ability of EF-Tu to act as an HSP during stress conditions does not necessarily indicate thermotolerance in an actively metabolising root, however if heat stress was applied to the roots, it would be interesting to observe whether constitutive EF-Tu transcripts are translated in response. Since *X.viscosa* plants are situated on rocky outcrops and shallow soils (Porembski and Barthlott, 2000) where the temperatures rapidly cycle between -4^0 C and 60^0 C in a one day (Mundree *et al*., 2002; Farrant, 2007), it makes sense

that these EF-Tu-HSP transcripts are constitutive and increase to modulate root thermotolerance.

The amino acid synthesising enzymes glutamine synthetase and methionine synthase were identified (Table 2.2). The presence of these two enzymes in *X. viscosa* roots is not unusual as our protein extractions and subsequent analysis were performed on an actively metabolising root where the synthesis of polypeptides are expected, especially those critically involved in amino acid biosynthesis. Glutamine synthetase plays a central role in plant nitrogen metabolism and functions by assimilating ammonia produced from nitrogen fixation or nitrate reduction. This reaction is the only reported method whereby plant roots take up inorganic nitrogen from the surrounding environment for subsequent assimilation into an organic form before transporting it via the xylem to higher parts of the plant (Miflin and Lea, 1980; Miflin and Habash, 2002; Ishiyama *et al.,* 2004). Methonine synthase is an enzyme that catalyses the biosynthesis of the sulphur containing amino acid, methionine, which plays an important role in not only initiating mRNA translation and protein synthesis, but indirectly regulates various cellular processes in its precursor form, via *S*-adenosylmethionine synthetase (SAM) (Ravanel *et al*., 1998; Hacham *et al*., 2002). SAM acts as a major methyl-group donor in transmethylation reactions and is an intermediate in the biosynthesis of plant metabolites such as polyamines and the phytohormone, ethylene (Ravanel *et al*., 1998). In addition to this the enzyme, 5-methyltetrahydropteroyltriglumate, which participates in methionine metabolism, was also detected (Table 2.2).

A number of proteins classically involved in protein catabolism via the ubiquitin-proteasome pathway were also identified. These include the 26S proteasome regulatory complex, polyubiquitin (Table 2.2) and the proteasome subunit α-type-6 (Table 2.1). The presence of these degradational enzymes could potentially explain the lack of protein bands and spots in our 1-DE and 2-DE analysis (described above in section 3.1 and 3.2). Although this is simply a speculation, the presence of these catabolic enzymes could play a role in our inability to consistently isolate the same repertoire of proteins from *X. viscosa* roots during hydrated conditions. If the roots are constantly synthesising and degrading amino acids and hence polypeptides, this could very well be the case. Protein degradation occurs as a normal cellular activity in plant tissues which has been reported to increase in times of stress to remove

damaged proteins from the cell and maintain cellular function (Ferguson *et al*., 1990; Walford, 2008).

The cell wall protein, glycine-rich RNA binding protein (GRP1 A), was also identified (Table 2.1). Many of the cell wall proteins identified to date (extensions and proline-rich proteins), have been known to strengthen the plant cell wall, by providing elasticity required for cell growth and extension (Mousavi and Hotta, 2005). GRPs in particular are able to induce certain post-transcriptional regulation of genes in plants under various stress conditions and may act as part of the plants defence mechanism (Mousavi and Hotta, 2005). Interestingly, when tomato plant roots were exposed to salt stress, GRP 7 transcripts were up regulated with increased salinity (Manaa *et al*., 2008), indicating that while glycine rich proteins might be putatively involved with cell wall stability, they could potentially function as a defence mechanism under stress conditions. Cyclophilin A (Table 2.2), part of the cyclophilin group of proteins, has the ability to accelerate the correct folding of proteins by having peptidyl-prolyl cis-trans isomerase (rotamase) activity (Meza-Zepeda *et al.*, 1998). In addition to previously reported immunophilic properties, cyclophilin mRNA transcripts increased when exposed to low temperatures, drought, wounding and abscisic acid in *Solanum commersonii* (Meza-Zepeda *et al.*, 1998).

3.4.4) Antioxidant mechanisms (22 and 9%)

From the data gathered, a significant portion of the proteins identified are actively involved in antioxidant mechanisms and protection against reactive oxygen species (ROS). The antioxidant enzymes indentified include: 1-cys peroxiredoxin; Class III peroxidase; superoxide dismutase (SOD), Cu-Zn SOD and catalase 2 (Table 2.1) as well as catalase isozyme 1, peroxidase and Mn-SOD (Table 2.2). Antioxidant enzyme activity and accumulation as a result of stress conditions are the most widely reported free radical scavenging systems to date. Their function is to neutralise reactive species that contain lone electrons such as singlet oxygen, superoxide radical (O_2^-), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2) (Noctor and Foyer, 1998). The antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD), are mostly involved in general "housekeeping" of plant tissues (Illing *et al*., 2005), and typically function in day to day cellular homeostasis as well as in protection against a variety of abiotic and biotic stresses (Estner and Oswlad, 1994).

SOD catalyses the conversion of O_2^- to H_2O_2 and although there are only three known classes of SOD enzymes that differ by the active site metal cofactors (Fe, Mn, or Cu and Zn) (Kliebenstein *et al.,* 1998), both Cu-Zn SOD and Mn-SOD were identified in the roots of *X. viscosa.* The two SOD isozymes differ in subcellular location in plant tissues, Mn-SOD is located in the mitochondria of plant cells while Cu-Zn SOD is mostly found in the chloroplasts or starch storing organelles, plastids (Kliebenstein *et al.,* 1998). It is interesting that Cu-Zn SOD is detected in plant roots, when they are known to mostly be found in the chloroplasts of leaves as a result of photosynthetically produced ROS, however it is possible that the Cu-Zn enzymes detected could have originated from plastids present in root tissues. Catalase then further catalyses the destructive H_2O_2 radical to molecular oxygen and water (Noctor and Foyer, 1998), together these enzymes are able to function in a synchronised manner to stop free radical damage.

In addition to protection by SOD and CAT, peroxidases are an alternative mode of H_2O_2 detoxification and are commonly found throughout the cell, having a high affinity to the H_2O_2 radical (Noctor and Foyer, 1998). In plants, various protection functions for peroxidases have been reported such as toxic reductant, oxidation, the production and degradation of lignin in plant cell walls, stress response, auxin catabolism, in defence against pathogen and insect attack and against wounding (Noctor and Foyer, 1998; Hiraga *et al*., 2001; Yoshida *et al.*, 2003, Xu and Huang, 2008).

The presence of 1-cys peroxiredoxin (Table 2.1) is a significant finding. To date this antioxidant has only been reported in dry desiccation tolerant tissues of resurrection plants and orthodox seeds (Mowla *et al.*, 2002; Illing *et al.*, 2005; Farrant *et al*., 2007) and is absent in desiccation sensitive vegetative tissues. The cellular function of 1-cys peroxiredoxin is not yet fully understood, however it is believed to play a central role in maintaining orthodox seed dormancy and as a protective antioxidant in nuclei of resurrection plant vegetative tissues (Mowla *et al*., 2002). The fact that this protein was isolated from *X. viscosa* root tissues implies that its presence along with all the other antioxidant enzymes identified, is constitutive. We have previously shown the antioxidant activity and accumulation in the roots of *X. viscosa* in response to desiccation stress (Kamies *et al*., 2010), and found that although antioxidant activity decreased during initial water deficit the antioxidant potential was maintained throughout desiccation, even below 10% RWC.

3.4.5) Stress response proteins (1 and 2%)

Heat shock (Hsp 70) (Table 2.1) and oxidoreductase (Table 2.2) proteins were identified in the roots of *X. viscosa.* These proteins are known to play an important role during stress response in plants. The proteins belonging to the Hsp group are known for their involvement in binding to polypeptide chains of other proteins, initiating the correct folding of polypeptides and hence offering a measure of protection from denaturation and degradation (Craig, 1993; Frydman *et al.*, 1994; Ndimba *et al*., 2005). Hsp 70 has also been reported to be involved in the transportation of proteins across organelle membranes, suggesting that they play a role in osmotic stress tolerance by maintaining the integrity of other proteins and facilitating the intercellular transportation of vital cellular enzymes. (Ndimba *et al.*, 2005). It is also believed that Hsp 70 functions in protection against heat stress by acting as molecular chaperones when transporting proteins against organelle membranes (Vierling, 1991; Waters *et al*., 1996; Bhadula *et al.*, 2001).

In resurrection plants, it has previously been reported that small heat shock proteins might aid in vitrification of the cytoplasm, or formation of the "glassy state" which helps to stabilise the subcellular milieu during desiccation stress (Almogeura and Jordano, 1992; Vertucci and Farrant, 1995; Wehmeyer *et al*., 1996; Farrant, 2007). In the resurrection plant *C. plantagineum,* small heat shock proteins were shown to be constitutively present in the vegetative tissues during hydrated conditions but increased in response to desiccation stress and heat shock (Alamillo *et al.*, 1995; reviewed in Farrant *et al*., 2011). A similar effect was observed in the resurrection plant *X. humilis,* when water stress was applied (Walford, 2008; Farrant *et al*., 2011). We propose that this occurs in the root tissues of *X. viscosa* as well, where Hsp 70 is constitutively expressed and is upregulated in response to stress conditions, however this theory can only be confirmed once we observe the proteins upregulated in the dry state.

Oxidoreductase enzymes are found in all eukaryotic cells and have various functions, such as catalysing different thiol-disulfide exchange reactions, including oxidation, reduction and isomerisation (Onda *et al*., 2011). In addition to general catalysis reactions involved in the glycolytic pathway (Suarez-Rodriguez *et al*., 2010), oxidoreductase enzymes also display chaperone activity (Hatahet and Ruddock, 2009). In a study conducted by Walford (2008),

transcriptomic data generated from *X. humilis*, showed an increase in transcript abundance in the vegetative tissues and seeds in response to desiccation. Amongst those transcripts identified, the oxidoreductase transcript was shown to be upregulated in stressed vegetative tissues and seeds (Walford, 2008). Interestingly when microarray profiling and cluster analysis was performed it was found that of the five grouped expression profiles, two groups (each containing 82 and 48 genes, respectively) showed an increase in gene expression in desiccated leaves and seeds, and constitutive expression in roots (Walford, 2008). The author goes on to speculate that a possible constitutive protection mechanism resides in the roots of *X.humilis* that is only active in the leaf tissues during desiccation (Walford, 2008).

When further comparisons were made between the transcriptomic data generated from *X. humilis* (Walford, 2008), and proteins present in *X. viscosa* roots, it was found that 1-cys peroxiredoxin and heat shock proteins were common to both species. It could be that the transcripts common to both species in the root tissues are constitutive and during times of stress, these transcripts are then translated in response. Furthermore, analyses on root proteins in the dry state could be performed to observe and verify this effect.

3.4.6) Unknown proteins (18 and 5%)

A number of proteins with unknown biological functions were detected (Table 2.1 and Table 2.2). These proteins encompass a wide range of those sequenced. This was not unusual when working with a non-model organism such as *X. viscosa* and particularly since no molecular based root studies on resurrection plants has yet been reported. However, results from this study can be considered a starting point for further proteomic analysis in resurrection plant roots where identification and subsequent characterisation of function could result in greater understanding of their role in these plants.

3.4.7) Other metabolic processes

A number of proteins with various biological functions were identified in the roots of *X. viscosa* that are reported to function mainly in general cellular homeostasis and maintenance of the cell (Tables 2.1 and 2.2). In the category of apoptosis (5 and 9%), the predominant enzyme identified was alcohol dehydrogenase, whose reported enzymatic activity mostly lies in anaerobic metabolism in plants. However, from our data it was interestingly classified under the functional category of apoptosis, implying that this protein could potentially have a

dual function in the degradation of proteins that has not yet been classified. In the category of protein transport and signalling, the proteins actin, ubiquitin/actin protein fusion protein as well as different forms of dehydration upregulated putative membrane pore proteins, were identified. These enzymes catalyse the transport of proteins across organelle membranes and maintain cellular homeostasis of the cell. A cytosolic form of triosephosphate isomerase was also detected, the presence of which is reportedly linked to the biosynthesis of fatty acids and gluconeogenesis. Aldo/keto reductase enzyme was also identified, this enzyme generally has a widespread function in glucose metabolic processes and aldehyde catabolic processes.

4.) Conclusions and future work

In this study, aeroponically grown *X. viscosa* plant roots were subjected to various proteomic techniques. The aeroponic method of growing plants provided an opportunity to observe the proteins present in the root tissues of *X. viscosa* under hydrated conditions without the hindrances associated with soil grown plants. Three protein extraction methods were investigated to extract protein from hydrated *X.viscosa* root tissues, designated as Method A, B and C, respectively. Of the three methods attempted, it was found that only one method (Method C) was uniquely suited for total protein isolation from the root tissues. This was not unusual since it is widely known that root tissues are highly recalcitrant and generally difficult to work with, even after extensive optimisation.

When conducting optimisation procedures, maize root tissue was used as a positive control and as a testing material for all troubleshooting efforts. This allowed the establishment of a modified protocol for efficient root protein extraction of roots in the hydrated state. Once good quality protein was isolated, proteins were analysed using various protein separation techniques such as 2-DE and OFFGEL fractionation analysis. Here again, technical challenges were encountered that required considerable optimisation procedures. However using OFFGEL fractionation analysis as an alternative method of protein separation to 2 DE allowed highly resolved, good-quality protein separation to be achieved. The technique provided an opportunity to analyse the hydrated root proteome of *X. viscosa* without the concerns of protein degradation, sample contamination, long focusing times, insufficient resolubilisation and poor resolution that were characteristic to our 2-DE analysis results.

Following successful protein fractionation, two protein fractions from *X. viscosa* roots were chosen and identified using LC-MS/MS analysis. These results provided information on the proteins present within hydrated root tissues prior to desiccation stress being imposed. Many of those identified are involved in general metabolism commonly associated with an actively metabolising root under normal hydrated conditions. These included the identification of proteins involved in carbohydrate metabolism and the glycolytic pathway, energy metabolism and proteins linked to the synthesis and hydrolysis of ATP, protein metabolism and the enzymes associated with the biosynthesis and catabolism of amino acids, as well as enzymes involved in antioxidant mechanisms. A number of proteins related to stress response and other metabolic processes were also identified. Although the majority of these proteins might

function in general metabolism and its maintenance, several have been widely reported to have multiple activities when exposed to stress conditions. In these instances it is possible that such enzymes or proteins are constitutively expressed in the root tissues of *X.viscosa*, but when stress conditions such as desiccation, high salinity, cold and heat stress occur, their activity or levels might be upregulated. This should be tested in future studies (as discussed below).

Among the potentially stress related proteins identified were the *de novo* synthesised (only by resurrection plants and orthodox seeds) antioxidant 1-cys peroxiredoxin, class III peroxidases, the "housekeeping" antioxidants, superoxide dismutase and catalase as well as proteins known to function in response to stress such as Hsp 70, oxidoreductase and aldehyde dehydrogenase. A number of unknown proteins were also identified which could also potentially function as protectants during stress conditions. However, due to the limited data available on proteins isolated from root tissues and none so far on the root tissues of *X viscosa*, identification was not possible. These unidentified proteins could potentially be important to resurrection plant survival when exposed to biotic and abiotic stresses.

Initially the aim of this study was to observe the proteins present in the root tissues of *X. viscosa* under both hydrated and dehydrated conditions. However, due to time constraints and technical difficulties associated with root proteomic observation, studies in the desiccated state were not conducted. In future, proteomic studies on *X. viscosa* roots exposed to different stress conditions, particularly desiccation, should be conducted. These proteins could be extracted using Method C and subjected to a separation technique such as OFFGEL analysis and identified using mass spectrometry. Alternatively, the proteins extracted in the dry and hydrated state could be analysed by a more sophisticated tool such as isobaric tags for relative and absolute quantification (iTRAQ), where the multiplex nature of this methodology would not only identify and quantify root proteins, but allow comparison of the dehydrated root proteome to the hydrated in response to stress conditions.

This is the first report of a proteomic study on the roots of a resurrection plant. This study has revealed important and valuable information that could potentially be used for further proteomic analyses of roots in *X .viscosa* and other resurrection plants.

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1.) Supplementary Data

Table S.1) Identification of maize root total proteins present in OFFGEL fraction 1 by LC-MS/MS analysis

MASCOT Score	Expectation	Protein ID	Protein Name	MW(Da)	% Coverage
1545	0	gi|226493589	ATP synthase beta chain [*Zea mays*]	58943	74.1
1481	0	gi|162462751	ATP synthase subunit beta, mitochondrial precursor [*Zea mays*]	59067	69.6
1428	0	gi|226531470	vacuolar ATP synthase subunit B [*Zea mays]*	54055	69
1349	0	gi|162458207	enolase 1 [*Zea mays*]	48033	65.7
1188	2.30E-112	gi|212275438	hypothetical protein LOC100191846 [*Zea mays*]	52146	67.2
1165	4.50E-110	gi|4582787	adenosine kinase [*Zea mays*]	36009	72.2
1161	1.10E-109	gi|212274479	hypothetical protein LOC100191561 [*Zea mays*]	41699	62.3
1111	1.20E-104	gi|224088196	actin 3 [*Populus trichocarpa*]	41674	63.4
975	4.40E-91	gi|321437407	actin 2 [*Musa acuminata AAA Group*]	38348	64.2
959	1.80E-89	gi|226530373	actin-7 [*Zea mays*]	41607	46.3
950	1.50E-88	gi|226510248	succinyl-CoA ligase beta-chain [*Zea mays*]	45165	60.2
939	1.70E-87	gi|293333684	hypothetical protein LOC100382805 [*Zea mays*]	67389	44.2
914	5.50E-85	gi|326516786	predicted protein [*Hordeum vulgare subsp. vulgare]*	41629	48.3
854	5.40E-79	gi|115466256	Os06g0136600 [Oryza sativa Japonica Group]	47908	41.3
837	2.50E-77	gi|302799655	hypothetical protein SELMODRAFT_233752 [Selaginella moellendorffii]	41690	36.4
823	6.30E-76	gi|3746938	actin 2 [Anemia phyllitidis]	41600	39.3
793	6.30E-73	gi|302807614	hypothetical protein SELMODRAFT_269014 [Selaginella moellendorffii]	53525	36.7
785	4.60E-72	gi|226496139	hypothetical protein LOC100279351 [Zea mays]	35464	54.3
763	6.10E-70	gi|112490284	Chain A, Crystal Structure Of The Maize Glutamine Synthetase Complexed With Adp And Methionine Sulfoximine Phosphate	39198	54.5
728	2.40E-66	gi|293334811	hypothetical protein LOC100383576 [Zea mays]	49627	39.4
719	1.50E-65	gi|223947755	unknown [Zea mays]	72155	29.6
717	2.50E-65	gi|51969596	vacuolar-type H+-ATPase subunit B3 (VHA-B3) [Arabidopsis thaliana]	31589	57.4
715	4.80E-65	gi|162463755	glutamine synthetase root isozyme 3 [Zea mays]	39270	50.6

714	5.90E-65	gi|293331731	hypothetical protein LOC100381283 [Zea mays]	50084	45.2
700	1.40E-63	gi|194699842	unknown [Zea mays]	41614	33.2
677	2.70E-61	gi|195615416	pathogenesis-related protein 1 [Zea mays]	16960	86.2
677	2.80E-61	gi|226500228	succinyl-CoA ligase beta-chain [Zea mays]	45173	45.7
676	3.40E-61	gi|162461063	protein disulfide isomerase [Zea mays]	56838	45.9
663	6.50E-60	gi|242083856	hypothetical protein SORBIDRAFT_08g018750 [Sorghumbicolor]	70990	29.9
663	7.40E-60	gi|223942631	unknown [Zea mays]	37980	39.2
653	7.00E-59	gi|255684856	actin [Malus x domestica]	28035	69.8
645	3.90E-58	gi|195620516	pro-resilin precursor [Zea mays]	42657	27.2
643	7.40E-58	gi|293331695	HSP protein [Zea mays]	80312	25.5
610	1.20E-54	gi|3287956	RecName: Full=Actin-3	41582	19.7
609	1.80E-54	gi|219885633	unknown [Zea mays]	71118	33.4
608	2.30E-54	gi|56202189	putative heat shock protein 82 [Oryza sativa Japonica Group]	70659	26.2
595	4.20E-53	gi|2944389	actin 4 [Glycine max]	41399	31.4
579	1.80E-51	gi|226501216	LOC100286091 [Zea mays]	27302	58.1
572	8.30E-51	gi|311303102	heat shock protein 90 [Pennisetum glaucum]	80220	24.2
567	3.00E-50	gi|242045124	hypothetical protein SORBIDRAFT_02g028050 [Sorghum bicolor]	80199	21.2
525	4.50E-46	gi|18033230	UDP-glucosyltransferase BX9 [Zea mays]	50217	33.8
520	1.50E-45	gi|6911551	heat shock protein 70 [Cucumis sativus]	71444	24.1
494	6.10E-43	gi|242090773	hypothetical protein SORBIDRAFT_09g022580 [Sorghum bicolor]	70958	21
491	9.90E-43	gi|194705984	unknown [Zea mays]	37629	29.2
489	1.70E-42	gi|242036895	hypothetical protein SORBIDRAFT_01g046840 [Sorghum bicolor]	89890	24
488	2.30E-42	gi|255582806	heat shock protein, putative [Ricinus communis]	79904	17.9
480	1.50E-41	gi|85823009	actin [Labyrinthula sp. N8]	34488	32.5
477	2.60E-41	gi|110289141	Cell division cycle protein 48, putative, expressed [Oryza sativa Japonica Group]	89755	21.3
463	6.40E-40	gi|162464059	pyruvate dehydrogenase2 [Zea mays]	39787	38.3
445	4.10E-38	gi|223947829	unknown [Zea mays]	62253	14.9
443	6.80E-38	gi|194690236	unknown [Zea mays]	22630	34.1
430	1.40E-36	gi|162458813	pyruvate dehydrogenase E1 beta subunit isoform 3 [Zea mays]	39937	27.8

425	4.50E-36	gi|29367547	adenosine kinase-like protein [Oryza sativa Japonica Group]	40206	28.1
415	4.50E-35	gi|224031309	unknown [Zea mays]	53200	20.8
404	5.80E-34	gi|226502632	LOC100282052 [Zea mays]	23804	38.2
390	1.50E-32	gi|162461230	protein disulfide isomerase2 [Zea mays]	56648	20.5
387	2.90E-32	gi|226505300	LOC100283392 [Zea mays]	17312	69.1
374	5.00E-31	gi|226507194	ATP synthase D chain, mitochondrial [Zea mays]	19915	53.5
371	9.90E-31	gi|212274681	hypothetical protein LOC100191638 [Zea mays]	35252	32.6
370	1.30E-30	gi|194703130	unknown [Zea mays]	28366	43.1
369	1.80E-30	gi|226499304	pathogenesis-related protein 10 [Zea mays]	17016	54
346	3.20E-28	gi|28317	unnamed protein product [Homo sapiens]	59492	13.2
343	6.80E-28	gi|195628018	enoyl-[acyl-carrier-protein] reductase [NADH] [Zea mays]	39153	18.6
338	2.00E-27	gi|556560	rice homologue of Tat binding protein [Oryza sativa Japonica Group]	47767	21
335	4.70E-27	gi|162457723	luminal-binding protein 2 precursor [Zea mays]	73040	16.9
323	6.80E-26	gi|3907620	actin 1 [Penaeus monodon]	41716	17.6
323	6.90E-26	gi|194702698	unknown [Zea mays]	32401	33.6
319	1.80E-25	gi|162460800	peroxidase 42 precursor [Zea mays]	32990	29.3
311	1.20E-24	gi|18483235	methionine synthase protein [Sorghum bicolor]	83736	14.3
307	2.70E-24	gi|195650763	fructokinase-1 [Zea mays]	34582	28.5
302	9.20E-24	gi|226529884	10-deacetylbaccatin III 10-O-acetyltransferase [Zea mays]	45455	33.6
296	3.70E-23	gi|194703542	unknown [Zea mays]	28095	27.2
293	6.20E-23	gi|22748337	Putative GLN1_ORYSA GLUTAMINE SYNTHETASE ROOT ISOZYME [Oryza sativa Japonica Group]	38494	22.3
289	1.90E-22	gi|162459533	actin-depolymerizing factor 3 [Zea mays]	15890	43.9
281	1.20E-21	gi|226531007	proteasome subunit alpha type 1 [Zea mays]	29997	25.2
280	1.30E-21	gi|1170506	eukaryotic initiation factor 4A-2	46799	23.7
280	1.50E-21	gi|17017263	methionine synthase [Zea mays]	84400	9.1
279	1.60E-21	gi|219886233	unknown [Zea mays]	49928	10.1
272	7.90E-21	gi|226491116	copper chaperone [Zea mays]	8732	82.1
266	3.70E-20	gi|242039457	hypothetical protein SORBIDRAFT_01g020010 [Sorghum bicolor]	61037	12.5

253	7.10E-19	gi|226498098	hypothetical protein LOC100280280 [Zea mays]	53660	22
252	9.20E-19	gi|226499800	hypothetical protein LOC100279833 [Zea mays]	40570	23.1
251	1.20E-18	gi|195619126	triosephosphate isomerase, cytosolic [Zea mays]	26684	50.4
251	1.20E-18	gi|28863909	UTP:alpha-D-glucose-1-phosphate uridylyltransferase [Solanum tuberosum]	51903	10.5
250	1.50E-18	gi|1352830	V-type proton ATPase catalytic subunit A;	61913	13.7
246	3.20E-18	gi|148224327	hypothetical protein LOC779096 [Xenopus laevis]	41940	11.7
246	3.70E-18	gi|226494478	LOC100284287 [Zea mays]	39158	18.6
243	6.80E-18	gi|5031145	ATP synthase beta subunit [Tetracentron sinense]	53601	7.4
243	7.00E-18	gi|242077366	hypothetical protein SORBIDRAFT_06g030270 [Sorghum bicolor]	80854	6.3
243	7.50E-18	gi|7706852	ATP synthase beta subunit [Apium graveolens]	52922	9
240	1.50E-17	gi|226493400	hypothetical protein LOC100273295 [Zea mays]	20596	20.1
238	2.10E-17	gi|226506764	LOC100284080 [Zea mays]	46217	13.8
229	1.80E-16	gi|195610706	inorganic pyrophosphatase [Zea mays]	31717	22.6
224	5.40E-16	gi|226497844	shepherd-like1 [Zea mays]	83019	8.1
223	7.50E-16	gi|3255943	PP2A1 protein [Catharanthus roseus]	35990	18.5
218	2.10E-15	gi|1168582	ATP synthase subunit beta;	51753	7.7
218	2.20E-15	gi|195637964	transaminase/ transferase, transferring nitrogenous groups [Zea mays]	43503	24.4
211	1.00E-14	gi|226529563	alpha-soluble NSF attachment protein [Zea mays]	32377	13.1
203	7.10E-14	gi|212275574	hypothetical protein LOC100192106 [Zea mays]	67993	11.3
202	8.90E-14	gi|194701624	unknown [Zea mays]	19336	43.9
201	1.00E-13	gi|226493643	hypothetical protein LOC100272829 [Zea mays]	41549	17.9
201	1.10E-13	gi|224293	histone H4	11329	39.2
199	1.80E-13	gi|224865	histone H3	15155	42.2
196	3.20E-13	gi|226492619	transaldolase 2 [Zea mays]	46103	7.9
195	4.10E-13	gi|162460525	fructokinase-2 [Zea mays]	35459	16.1
193	6.20E-13	gi|115466004	Os06g0114000 [Oryza sativa Japonica Group]	64046	6.3

189	1.60E-12	gi|162463575	LOC732740 [Zea mays]	65130	12.3
189	1.70E-12	gi|195637494	ML domain protein [Zea mays]	16741	32.5
187	2.50E-12	gi|195619190	fructokinase-2 [Zea mays]	41152	23.5
185	3.90E-12	gi|194699294	unknown [Zea mays]	35356	26.7
184	5.20E-12	gi|242053347	hypothetical protein SORBIDRAFT_03g025720 [Sorghum bicolor]	127086	5
181	1.00E-11	gi|219887925	unknown [Zea mays]	54992	14.3
178	2.00E-11	gi|114800518	F0F1 ATP synthase subunit beta [Hyphomonas neptunium ATCC 15444]	50868	6.1
177	2.60E-11	gi|223946977	unknown [Zea mays]	54518	19.6
177	2.60E-11	gi|226491271	3-isopropylmalate dehydrogenase 2 [Zea mays]	43465	17.4
174	5.10E-11	gi|3334320	40S ribosomal protein SA	33885	10
173	6.60E-11	gi|195630027	ruBisCO large subunit-binding protein subunit beta [Zea mays]	64403	5.3
169	1.80E-10	gi|15233740	ATP binding / unfolded protein binding [Arabidopsis thaliana]	94146	4.9
168	2.10E-10	gi|242075420	hypothetical protein SORBIDRAFT_06g011100 [Sorghum bicolor]	55179	12
168	2.10E-10	gi|226506256	ribokinase [Zea mays]	37486	9.3
164	5.60E-10	gi|2266662	14-3-3 protein [Hordeum vulgare]	29924	21.7
161	1.10E-09	gi|46805841	putative serine/threonine protein phosphatase PP2A-3 catalytic subunit [Oryza sativa Japonica Group]	33688	15.3
157	2.40E-09	gi|1352081	Beta-glucosidase, chloroplastic	64197	10.4
154	5.10E-09	gi|226498820	heat shock 70 kDa protein 4 [Zea mays]	92276	7.7
153	6.50E-09	gi|1170141	Putative exoglucanase type C	54669	5.4
151	1.00E-08	gi|62546209	protein disulfide isomerase [Oryza sativa Japonica Group]	56820	14.5
147	3.00E-08	gi|226508278	wound/stress protein [Zea mays]	19680	32.6
147	3.00E-08	gi|3925225	6-phosphogluconate dehydrogenase isoenzyme B [Zea mays]	18932	25.8
144	5.50E-08	gi|22773257	Putative probable submergence induced, nickel-binding protein 2A [Oryza sativa Japonica Group]	29552	18.9
142	9.30E-08	gi|194697952	unknown [Zea mays]	26120	25.6
140	1.50E-07	gi|293336965	hypothetical protein LOC100383563 [Zea mays]	81632	10.4
140	1.30E-07	gi|224028729	unknown [Zea mays]	27020	30.8

140	1.50E-07	gi|226528471	hsc70-interacting protein [Zea mays]	43359	21.6
139	1.50E-07	gi|226498360	hypothetical protein LOC100272696 [Zea mays]	33651	10.4
137	2.60E-07	gi|166012624	beta-actin [Maruca vitrata]	7574	60.3
136	3.70E-07	gi|162459159	betaine aldehyde dehydrogenase [Zea mays]	54941	20
135	4.40E-07	gi|242066822	hypothetical protein SORBIDRAFT_04g035840 [Sorghum bicolor]	61722	7.6
134	6.00E-07	gi|55741057	putative alanine aminotransferase [Zea mays]	56419	8.1
131	9.90E-07	gi|226491558	hypothetical protein LOC100279996 [Zea mays]	53443	10.1
128	0.0000023	gi|212722354	hypothetical protein LOC100192890 [Zea mays]	42878	6.1
127	0.000003	gi|223975935	unknown [Zea mays]	43170	10.5
126	0.0000036	gi|226504710	LOC100281192 [Zea mays]	60529	9.9
124	0.0000051	gi|212274373	hypothetical protein LOC100191237 [Zea mays]	38258	11.8
124	0.0000049	gi|22168	adenine nucleotide translocator [Zea mays]	42242	8
124	0.0000059	gi|75994143	maize protease inhibitor [Zea mays subsp. parviglumis]	7429	75.7
123	0.0000065	gi|226498936	anamorsin homolog [Zea mays]	27470	27.4
123	0.0000068	gi|195642478	glycine-rich RNA-binding protein 2 [Zea mays]	15646	19
121	0.0000097	gi|219362573	hypothetical protein LOC100216995 [Zea mays]	36179	11.7
119	0.000018	gi|194701240	unknown [Zea mays]	29296	18
118	0.000024	gi|195613358	non-cyanogenic beta-glucosidase precursor [Zea mays]	56681	5.2
117	0.00003	gi|226530007	hypothetical protein LOC100280334 [Zea mays]	80643	4.8
116	0.000032	gi|212723798	hypothetical protein LOC100194227 [Zea mays]	25249	15.6
114	0.000056	gi|195611322	hypothetical protein [Zea mays]	51694	9
113	0.000063	gi|226507816	hypothetical protein LOC100274480 [Zea mays]	17973	16.9
112	0.000095	gi|39546245	OSJNBa0089N06.15 [Oryza sativa Japonica Group]	37433	5.6
111	0.00011	gi|194707550	unknown [Zea mays]	20376	15.2
111	0.00012	gi|294954688	Tubulin alpha chain, putative [Perkinsus marinus ATCC 50983]	50329	7.5

110	0.00015	gi|226504734	hypothetical protein LOC100272320 [Zea mays]	22856	25
109	0.00016	gi|242046452	hypothetical protein SORBIDRAFT_02g040640 [Sorghum bicolor]	72284	13.3
108	0.00022	gi|226497010	LOC100283781 [Zea mays]	38016	10.1
106	0.00037	gi|45685267	peroxidase [Zea mays]	38298	8.7
105	0.00043	gi|226529177	hypothetical protein LOC100272866 [Zea mays]	36168	13.2
104	0.0006	gi|226494616	LOC100284721 [Zea mays]	45920	15.2
103	0.00074	gi|115467850	Os06g0325500 [Oryza sativa Japonica Group]	40460	5.8
102	0.00084	gi|115449183	Os02g0793700 [Oryza sativa Japonica Group]	10819	22.5
99	0.0018	gi|225442531	PREDICTED: hypothetical protein [Vitis vinifera]	64970	6.1
97	0.0028	gi|212722336	hypothetical protein LOC100193584 [Zea mays]	23568	20.5
93	0.0071	gi|167860184	LOC100136885 [Zea mays]	20883	17
92	0.0094	gi|226506856	hypothetical protein LOC100272352 [Zea mays]	30799	22.1
92	0.0096	gi|162458198	soluble inorganic pyrophosphatase [Zea mays]	24354	15.9
91	0.012	gi|293333997	hypothetical protein LOC100382162 [*Zea mays*]	33954	6.3
90	0.015	gi|194701098	unknown [*Zea mays*]	40439	9.3
89	0.016	gi|115447465	Os02g0634500 [*Oryza sativa Japonica Group*]	31941	9.7
89	0.017	gi|226508498	hypothetical protein LOC100272880 [*Zea mays*]	38417	20.3
89	0.017	gi|194701654	unknown [*Zea mays*]	23493	13.5
88	0.022	gi|308080274	hypothetical protein LOC100502334 [*Zea mays*]	29419	22.5
87	0.025	gi|223942763	unknown [*Zea mays*]	13724	8.5
86	0.031	gi|194693054	unknown [*Zea mays*]	10120	13.5
86	0.036	gi|47497805	putative D-protein [*Oryza sativa Japonica Group*]	34175	7
85	0.046	gi|226508482	LOC100282555 [*Zea mays*]	27721	10.4
85	0.042	gi|531379	RCI1B [*Arabidopsis thaliana*]	28125	4.8
84	0.057	gi|9998903	putative membrane protein [*Zea mays*]	31340	10.9

Table S.2) Identification of maize root total proteins present in OFFGEL fraction 7 by LC-MS/MS analysis

MASCOT Score	Expectation	Protein ID	Protein Name	MW (Da)	% Coverage
1043	6.30E-98	gi|162462949	glyceraldehyde-3-phosphate dehydrogenase, cytosolic 3 [*Zea mays*]	36426	59.3
1010	1.40E-94	gi|238015186	unknown [*Zea mays*]	36472	64.7
981	1.20E-91	gi|226503399	LOC100285098 [*Zea mays*]	93862	41.8
974	6.10E-91	gi|162461501	glyceraldehyde-3-phosphate dehydrogenase, cytosolic 2 [*Zea mays*]	36519	70.3
961	1.20E-89	gi|194690156	unknown [*Zea mays*]	38566	63.7
957	2.50E-89	gi|195648024	lipoxygenase 2 [*Zea mays*]	98049	38.7
860	1.50E-79	gi|226492743	fructose-bisphosphate aldolase cytoplasmic isozyme [*Zea mays*]	38435	66.8
854	5.00E-79	gi|242054379	hypothetical protein SORBIDRAFT_03g034200 [*Sorghum bicolor*]	93830	39.4
802	9.40E-74	gi|212722794	hypothetical protein LOC100194034 [*Zea mays*]	37471	53.5
774	6.10E-71	gi|226507242	hypothetical protein LOC100274379 [*Zea mays*]	38371	64.8
695	3.90E-63	gi|195641104	peroxidase 1 precursor [*Zea mays*]	38304	42.8
675	4.80E-61	gi|212722456	hypothetical protein LOC100192461 [*Zea mays*]	51571	40.8
627	2.70E-56	gi|162460508	allene oxide synthase1 [*Zea mays*]	53029	27.6
619	1.60E-55	gi|162459661	annexin2 [*Zea mays*]	35237	48.1
602	8.50E-54	gi|212275574	hypothetical protein LOC100192106 [*Zea mays*]	67993	23.4
535	4.70E-47	gi|226502434	hypothetical protein LOC100279332 [*Zea mays*]	44006	34.7
528	2.20E-46	gi|212722150	hypothetical protein LOC100193260 [*Zea mays*]	25045	62.9
512	8.40E-45	gi|8809764	exoglucanase precursor [*Zea mays*]	66900	21.4
490	1.40E-42	gi|226510588	LOC100282843 [*Zea mays*]	25156	39.4
455	3.90E-39	gi|226490863	CBS domain protein [*Zea mays*]	22484	53.7
455	4.20E-39	gi|226500532	hypothetical protein LOC100274592 [*Zea mays*]	46194	27.4

450	1.40E-38	gi|94502565	ATPase subunit 1 [*Zea mays subsp. mays*]	55146	23
428	2.10E-36	gi|9408184	F0-F1 ATPase alpha subunit [*Sorghum bicolor*]	47612	23.8
427	2.80E-36	gi|226531053	hypothetical protein LOC100274499 [*Zea mays*]	44173	21.6
425	4.30E-36	gi|78099751	Fructose-bisphosphate aldolase cytoplasmic isozyme	38839	39.1
425	4.80E-36	gi|162460928	peroxidase 66 precursor [*Zea mays*]	33399	44.1
410	1.40E-34	gi|226496896	hypothetical protein LOC100273417 [*Zea mays*]	26613	29.1
409	1.60E-34	gi|212721760	hypothetical protein LOC100194311 [*Zea mays*]	30135	33.6
405	4.80E-34	gi|219887485	unknown [*Zea mays*]	84565	16.6
401	1.00E-33	gi|194705850	unknown [*Zea mays*]	49192	29.3
399	1.60E-33	gi|162462940	anionic peroxidase [*Zea mays*]	37751	22.2
399	1.60E-33	gi|224589757	glyceraldehyde-3-phosphate dehydrogenase [*Dimocarpus longan*]	36533	24.4
393	6.60E-33	gi|3851005	pyruvate dehydrogenase E1 alpha subunit [*Zea mays*]	42889	24.5
380	1.40E-31	gi|226502058	hypothetical protein LOC100274264 [*Zea mays*]	35264	42.9
376	3.20E-31	gi|195629804	peroxidase 12 precursor [*Zea mays*]	38044	29.1
373	7.50E-31	gi|226493663	peroxidase 39 [*Zea mays*]	35358	43.3
361	1.20E-29	gi|308081758	hypothetical protein LOC100501719 [*Zea mays*]	43415	26.4
352	9.30E-29	gi|242057247	hypothetical protein SORBIDRAFT_03g013290 [Sorghum bicolor]	52923	19.8
345	4.00E-28	gi|195619166	vacuolar ATP synthase subunit E [Zea mays]	26597	29.1
338	2.40E-27	gi|1345683	Catalase isozyme 3	56760	22
335	4.50E-27	gi|219884127	unknown [Zea mays]	52397	18.2
332	7.80E-27	gi|293332239	hypothetical protein LOC100381602 [Zea mays]	16820	63.1
329	1.80E-26	gi|162459667	annexin p33 [Zea mays]	35539	34.1
327	2.90E-26	gi|212275542	hypothetical protein LOC100192105 [Zea mays]	33661	27.8
324	5.00E-26	gi|242080811	hypothetical protein SORBIDRAFT_07g005390 [Sorghum bicolor]	107832	12.3

324	5.30E-26	gi|195613496	cytochrome P450 CYP74A19 [Zea mays]	53071	11.4
323	7.30E-26	gi|226533530	hypothetical protein LOC100272772 [Zea mays]	53732	14.2
321	1.10E-25	gi|242046968	hypothetical protein SORBIDRAFT_02g043230 [Sorghum bicolor]	37893	19.6
320	1.30E-25	gi|195605264	histone H2A [Zea mays]	16213	47.4
319	1.90E-25	gi|242044850	hypothetical protein SORBIDRAFT_02g026140 [Sorghum bicolor]	80436	20.2
318	2.20E-25	gi|194705966	unknown [Zea mays]	28639	28
317	3.10E-25	gi|162462799	lipoxygenase [Zea mays]	98285	16.2
306	3.50E-24	gi|212275951	hypothetical protein LOC100191862 [Zea mays]	39285	27.4
299	1.80E-23	gi|242059525	hypothetical protein SORBIDRAFT_03g042450 [Sorghum bicolor]	98193	12.6
297	3.00E-23	gi|194703024	unknown [Zea mays]	48970	26.2
290	1.50E-22	gi|162463728	maize 20S proteasome alpha subunit [Zea mays]	27386	28.5
285	4.40E-22	gi|145342477	predicted protein [Ostreococcus lucimarinus CCE9901]	14950	30.8
284	6.00E-22	gi|45685267	peroxidase [Zea mays]	38298	30.8
276	3.70E-21	gi|168407	alcohol dehydrogenase [Zea mays]	40857	20.8
276	3.80E-21	gi|214014552	chitinase [Zea mays subsp. parviglumis]	29144	25.5
275	4.00E-21	gi|242052311	hypothetical protein SORBIDRAFT_03g008050 [Sorghum bicolor]	41655	18.9
273	6.90E-21	gi|212723566	hypothetical protein LOC100193561 [Zea mays]	39993	23.3
273	7.20E-21	gi|194704678	unknown [Zea mays]	21546	41.9
272	9.00E-21	gi|195615008	glucose-6-phosphate 1-dehydrogenase 2 [Zea mays]	67014	19.1
269	1.70E-20	gi|223974989	unknown [Zea mays]	25957	37
268	2.00E-20	gi|156322163	hypothetical protein NEMVEDRAFT_v1g225293 [Nematostella vectensis]	20183	44.4
261	1.00E-19	gi|226499006	LOC100284590 [Zea mays]	61873	16

260	1.50E-19	gi|126066	RecName: Full=L-lactate dehydrogenase; Short=LDH	38527	23.2
258	2.40E-19	gi|194701280	unknown [Zea mays]	44840	17.6
256	3.40E-19	gi|162458207	enolase 1 [Zea mays]	48033	17.7
255	4.70E-19	gi|162464321	malate dehydrogenase, cytoplasmic [Zea mays]	35567	17.8
252	8.80E-19	gi|224293	histone H4	11329	58.8
251	1.00E-18	gi|20192	catalase [Oryza sativa Indica Group]	56601	7.9
251	1.20E-18	gi|115465579	Os05g0574400 [Oryza sativa Japonica Group]	35414	24.1
247	2.90E-18	gi|162464281	glutathione S-transferase12 [Zea mays]	25760	35.1
243	7.20E-18	gi|226493289	LOC100285468 [Zea mays]	22448	21.9
235	3.90E-17	gi|242081745	hypothetical protein SORBIDRAFT_07g023230 [Sorghum bicolor]	41840	9.7
232	8.50E-17	gi|162463282	NADPH producing dehydrogenase of the oxidative pentose phosphate pathway [Zea mays]	53022	9.1
232	9.40E-17	gi|194707788	unknown [Zea mays]	56193	9.1
230	1.30E-16	gi|226491860	succinyl-CoA ligase alpha-chain 2 [Zea mays]	34187	36.6
226	3.30E-16	gi|115435850	Os01g0267200 [Oryza sativa Japonica Group]	34317	27
226	3.60E-16	gi|68655466	putative S-adenosylhomocystein hydrolase 2 [Hordeum vulgare subsp. vulgare]	53224	13.2
225	4.60E-16	gi|226530957	LOC100283929 [Zea mays]	24106	18.6
223	6.40E-16	gi|37625525	ribosomal protein L11-like protein [Solanum palustre]	18693	36.2
221	1.10E-15	gi|195643554	germin-like protein subfamily 1 member 17 precursor [Zea mays]	24574	52.2
220	1.30E-15	gi|242083266	hypothetical protein SORBIDRAFT_08g008400 [Sorghum bicolor]	26410	22.4
220	1.50E-15	gi|148524155	elongation factor 1-alpha [Chara australis]	47628	12.5
218	2.10E-15	gi|212276302	hypothetical protein LOC100191877 [Zea mays]	45264	11.8
218	2.20E-15	gi|226509426	LOC100283595 [Zea mays]	38154	30.5
217	2.50E-15	gi|231586	ATP synthase subunit beta, mitochondrial; Flags: Precursor	60221	12.5
217	2.80E-15	gi|17017271	formate tetrahyrofolate ligase [Zea mays]	31594	39.5
216	3.60E-15	gi|226493800	hypothetical protein LOC100273341 [Zea mays]	17684	35.5

210	1.50E-14	gi|17298147	26S proteasome regulatory particle triple-A ATPase subunit4b [Oryza sativa Japonica Group]	41980	14.3
208	2.20E-14	gi|226508498	hypothetical protein LOC100272880 [Zea mays]	38417	10.3
207	2.80E-14	gi|195616434	histone H2A [Zea mays]	16434	30.2
207	2.90E-14	gi|194704098	unknown [Zea mays]	44384	17.1
207	3.00E-14	gi|162458131	LOC100037815 [Zea mays]	27211	21.7
206	3.10E-14	gi|189054178	unnamed protein product [Homo sapiens]	65980	8.5
206	3.60E-14	gi|194695892	unknown [Zea mays]	43273	18.7
204	5.10E-14	gi|229256	dehydrogenase,glyceraldehydephosphate	35694	9
203	7.20E-14	gi|226509775	LOC100282447 [Zea mays]	25770	29.6
202	9.30E-14	gi|162463403	6-phosphogluconate dehydrogenase2 [Zea mays]	52919	13.3
202	9.70E-14	gi|224117236	predicted protein [Populus trichocarpa]	98485	7.5
201	1.10E-13	gi|194703542	unknown [Zea mays]	28095	19.8
200	1.50E-13	gi|195622962	histone H2B.1 [Zea mays]	16351	22.4
195	4.10E-13	gi|226509666	hypothetical protein LOC100272870 [Zea mays]	32515	17.9
193	6.20E-13	gi|226528389	GTP-binding protein PTD004 [Zea mays]	44215	24.7
192	8.20E-13	gi|195612788	stress responsive protein [Zea mays]	22929	25.4
188	2.00E-12	gi|226491894	hypothetical protein LOC100274481 [Zea mays]	34215	21.8
188	2.30E-12	gi|20601	aspartate aminotransferase [Panicum miliaceum]	47604	15
187	2.60E-12	gi|242086519	hypothetical protein SORBIDRAFT_09g000350 [Sorghum bicolor]	26176	18.9
187	2.90E-12	gi|242048780	hypothetical protein SORBIDRAFT_02g020340 [Sorghum bicolor]	16750	34.5
186	3.60E-12	gi|136429	RecName: Full=Trypsin; Flags: Precursor	24394	16.5
185	4.50E-12	gi|115450595	Os03g0136900 [Oryza sativa Japonica Group]	106235	6.8
181	1.10E-11	gi|226491656	peptidyl-prolyl cis-trans isomerase [Zea mays]	26201	25
180	1.50E-11	gi|326514166	predicted protein [Hordeum vulgare subsp. vulgare]	35282	16.6
177	3.00E-11	gi|226528403	NADP-dependent oxidoreductase P1 [Zea mays]	38911	18.4

176	3.20E-11	gi|162458737	cysteine synthase [Zea mays]	34185	16.9
176	3.50E-11	gi|226509682	LOC100281617 [Zea mays]	61495	12.1
174	5.80E-11	gi|226492253	hypothetical protein LOC100272855 [Zea mays]	60262	8
173	7.60E-11	gi|219886541	unknown [Zea mays]	53565	10.2
171	1.00E-10	gi|226530866	UDP-sulfoquinovose synthase [Zea mays]	52477	17.4
169	1.60E-10	gi|281341811	hypothetical protein PANDA_012419 [Ailuropoda melanoleuca]	39324	13.3
169	1.90E-10	gi|212723854	hypothetical protein LOC100192949 [Zea mays]	16256	37.2
168	2.20E-10	gi|194701318	unknown [Zea mays]	16421	77.1
168	2.30E-10	gi|194703724	unknown [Zea mays]	34096	14.4
167	2.50E-10	gi|212723604	hypothetical protein LOC100193240 [Zea mays]	22183	31.2
166	3.60E-10	gi|226491782	hypothetical protein LOC100276151 [Zea mays]	12525	29.7
165	4.90E-10	gi|190895700	esterase/lipase/thioesterase [Populus tremula]	44653	21.6
164	5.20E-10	gi|12620877	lipoxygenase [Zea mays]	96416	7.4
164	5.70E-10	gi|195639070	lactoylglutathione lyase [Zea mays]	35140	15.6
159	1.80E-09	gi|195644888	ATP synthase gamma chain [Zea mays]	35791	13.6
159	1.80E-09	gi|13160653	ATP:citrate lyase [Capsicum annuum]	65692	5.1
158	2.10E-09	gi|195619638	C-1-tetrahydrofolate synthase, cytoplasmic [Zea mays]	30575	30.5
158	1.90E-09	gi|259490641	hypothetical protein LOC100304315 [Zea mays]	74267	9.2
158	2.40E-09	gi|242089565	hypothetical protein SORBIDRAFT_09g004130 [Sorghum bicolor]	45047	9.9
157	2.90E-09	gi|194699568	unknown [Zea mays]	16246	37.2
157	2.90E-09	gi|260782839	hypothetical protein BRAFLDRAFT_75126 [Branchiostoma floridae]	21834	17.2
154	6.00E-09	gi|162460904	voltage-dependent anion channel protein1a [Zea mays]	29466	15.9
151	9.80E-09	gi|226500378	LOC100284565 [Zea mays]	32070	20.1
151	1.10E-08	gi|226509797	hypothetical protein LOC100274579 [Zea mays]	42413	20.1
151	1.20E-08	gi|195622620	serine hydroxymethyltransferase [Zea mays]	56499	9.7
150	1.20E-08	gi|226491029	proliferation-associated protein 2G4 [Zea mays]	43204	11.7

148	2.40E-08	gi|162459613	voltage-dependent anion channel protein1b [Zea mays]	29630	25.7
144	5.40E-08	gi|195640660	formate dehydrogenase 1 [Zea mays]	41393	15.4
143	7.40E-08	gi|14276718	T-cytoplasm male sterility restorer factor 2 [Zea mays]	59431	8.6
141	1.10E-07	gi|33304714	ubiquitin/actin fusion protein 2 [Bigelowiella natans]	51097	5.4
140	1.50E-07	gi|242051062	hypothetical protein SORBIDRAFT_02g041030 [Sorghum bicolor]	29630	10.6
138	2.30E-07	gi|194706014	unknown [Zea mays]	40248	9.2
138	2.10E-07	gi|225437455	hypothetical protein [Vitis vinifera]	53498	5.5
138	2.40E-07	gi|226532936	jasmonate-induced protein [Zea mays]	22953	16.3
137	2.90E-07	gi|115453877	Os03g0577000 [Oryza sativa Japonica Group]	25419	22.4
136	3.30E-07	gi|226494959	hsp20/alpha crystallin family protein [Zea mays]	22602	13.5
133	7.60E-07	gi|226532468	LOC100282529 [Zea mays]	17547	14
132	8.40E-07	gi|584706	Aspartate aminotransferase, cytoplasmic	44479	10.8
129	0.0000018	gi|224034807	unknown [Zea mays]	58615	10.3
129	0.0000018	gi|226508302	LOC100283246 [Zea mays]	58407	6.6
129	0.0000019	gi|194708148	unknown [Zea mays]	11773	27.3
128	0.0000021	gi|194705804	unknown [Zea mays]	11917	28.3
127	0.0000026	gi|195654249	homoserine kinase [Zea mays]	38315	10.3
124	0.0000051	gi|195609112	universal stress protein [Zea mays]	18184	37.1
123	0.0000065	gi|307778472	elongation factor-1 alpha [Isepeolus cortesi]	27055	10
123	0.0000066	gi|226510596	hypothetical protein LOC100273659 [Zea mays]	53088	6.3
122	0.0000081	gi|194699980	unknown [Zea mays]	28850	20.2
122	0.0000091	gi|194704880	unknown [Zea mays]	22191	35.2
120	0.000013	gi|226507466	LOC100285601 [Zea mays]	19156	19.2
120	0.000013	gi|162463414	alpha-1,4-glucan-protein synthase [UDP-forming] [Zea mays]	41178	19.5
119	0.000019	gi|166872	S-adenosylmethionine synthetase [Arabidopsis thaliana]	43117	6.4
118	0.000022	gi|226509807	3-isopropylmalate dehydrogenase [Zea mays]	39883	8.5
114	0.000052	gi|2982289	60S ribosomal protein L17 [Picea mariana]	14233	27.1
114	0.000054	gi|163838732	ZCN14 protein [Zea mays]	19301	11.6

113	0.000069	gi|242075420	hypothetical protein SORBIDRAFT_06g011100 [Sorghum bicolor]	55179	5.5
112	0.000084	gi|194705168	unknown [Zea mays]	38803	11.7
111	0.0001	gi|5499732	germin-like protein 2 precursor [Oryza sativa Japonica Group]	24211	9.8
109	0.00017	gi|162460322	glucose-6-phosphate isomerase, cytosolic [Zea mays]	62198	6.9
109	0.00017	gi|212274867	hypothetical protein LOC100191764 [Zea mays]	46870	7.3
109	0.00018	gi|194701006	unknown [Zea mays]	63249	7.6
108	0.00023	gi|9372	ubiquitin [Giardia intestinalis]	8616	25
106	0.00032	gi|238014172	unknown [Zea mays]	39576	11.1
105	0.00044	gi|195628630	elongation factor 1-gamma 3 [Zea mays]	47129	11.8
105	0.00047	gi|2345154	ribsomal protein S4 [Zea mays]	30152	7.5
104	0.0005	gi|242080813	hypothetical protein SORBIDRAFT_07g005430 [Sorghum bicolor]	67293	5.3
104	0.00054	gi|212721636	hypothetical protein LOC100194089 [Zea mays]	19380	11.1
103	0.00068	gi|19880027	glyceraldehyde-3-phosphate dehydrogenase [Oryza sativa]	54172	7.5
103	0.00071	gi|226533140	hypothetical protein LOC100274292 [Zea mays]	33338	5.9
102	0.00082	gi|2492519	26S protease regulatory subunit 7	47689	12
102	0.00092	gi|195659213	pyruvate dehydrogenase E1 component alpha subunit [Zea mays]	43134	5.4
101	0.001	gi|194701748	unknown [Zea mays]	39930	9.4
100	0.0014	gi|162459030	legumin-like protein [Zea mays]	37818	13.6
99	0.0019	gi|223947861	unknown [Zea mays]	27556	9.1
97	0.0028	gi|53749383	putative ferredoxin sulfite reductase [Oryza sativa Japonica Group]	64078	5
97	0.0025	gi|226507004	LOC100282837 [Zea mays]	25550	13.3
97	0.003	gi|226509640	hypothetical protein LOC100274318 [Zea mays]	33126	8.1
96	0.0034	gi|212275800	hypothetical protein LOC100191165 [Zea mays]	31727	8.9
96	0.0032	gi|1730158	Glyceraldehyde-3-phosphate dehydrogenase	33511	12.5

96	0.0037	gi|38350579	cysteine synthase [*Nicotiana plumbaginifolia*]	34015	7.4
94	0.0049	gi|29367403	elongation factor 1 gamma-like protein [*Oryza sativa Japonica Group*]	46460	12
94	0.0049	gi|226491474	LOC100280467 [*Zea mays*]	36491	19.8
94	0.0052	gi|224030347	unknown [*Zea mays*]	36214	7.6
93	0.0067	gi|4454799	translation initiation factor 4A2 [*Zea mays*]	25013	20.9
93	0.0072	gi|115481492	Os10g0320400 [*Oryza sativa Japonica Group*]	35238	7.7
92	0.0079	gi|131772	40S ribosomal protein S14	16248	24.2
90	0.014	gi|195636088	aspartate aminotransferase [*Zea mays*]	49336	6.1
90	0.015	gi|90185060	histone H3a [*Ceratobatrachus guentheri*]	12327	39.4
89	0.016	gi|1173187	RecName: Full=40S ribosomal protein S23; AltName: Full=S12	15766	7.7
89	0.016	gi|163838700	Asr protein [*Zea mays*]	11783	20.2
89	0.017	gi|162458324	60S ribosomal protein L17 [*Zea mays*]	19494	11.1
87	0.025	gi|2267006	endosperm lumenal binding protein [*Oryza sativa*]	73495	7.8
86	0.033	gi|194697338	unknown [*Zea mays*]	26002	14.3
84	0.061	gi|2981135	family F xylanase [*Fusarium oxysporum f. sp. lycopersici*]	41192	6.2

(Mascot score, score obtained from the Mascot ™ search engine and is the protein score (or Mowse score) that is -10x log (*P*), where *P* is the probability that the observed match is a random event; Expectation value, the number of matches that are expected to occur by chance alone($P < 0.05$); Protein ID, accession number of the matched protein from the NCBI database; Protein name, matched protein description; MW, predicted molecular weight of the deduced amino acid sequence of the matched protein; %Coverage, percentage covered of the identified peptide sequence in the matched region. Protein identities with a % coverage < 5 % were omitted from the data. Almost all proteins identified had tentative 1 significant hit).