Characterizations of Proteins from Virulent and A-Virulent Isolates of Rhizoctonia solani

Douglas Amoo Achempong

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CHARACTERIZATION OF PROTEINS FROM VIRULENT AND A-VIRULENT ISOLATES OF RHIZOCTONIA SOLANI

A Thesis
Submitted to the School of Graduate Studies and Research
in Partial Fulfillment of the
Requirements for the Degree
Master of Science

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August 2016
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Rhizoctonia solani anastomosis group 3 (AG-3) are pathogenic to plants in the family Solanaceae. The fungus has a destructive life lifestyle therefore poses as a serious threat to the potato industry. The identification of its proteins and associated function will provide insight in devising an effective control strategy thereby ensuring food security. The purpose of this study is to identify proteins signature from virulent and a-virulent strains of Rhizoctonia solani. In this study, two isolates of Rhizoctonia solani was used. Each isolate was inoculated in soil and potato seeds were grown in them. The two isolates were also subjected to a tissue culture experiment with potato seeds. Total proteins was extracted after each treatment and their concentrations were determined. Protein extracts were also differentiated by size using protein chip analysis. Three protein extracts were selected and subjected to 2 Dimensional gel Analysis and Mass spectrophotometry for protein identification.
ACKNOWLEDGEMENTS

I am grateful to the Almighty God for his protection, unconditional love and grace upon my life. Thank you Lord for seeing me through this Journey.

I would like to thank my advisor Dr. Narayanaswamy Bharathan to whom I am greatly indebted, for all his cooperation, noble hearted help, encouragement and invaluable wealth of knowledge. Thank you for granting me the access to work in your laboratory.

To my committee members, Dr. Seema Bharathan and Dr. Daniel Widzowski, it was an honor to work with you. Thank you for your dedication, critique and invaluable suggestions to the success of this research work.

I appreciate the encouragement and support from my laboratory colleagues. You are good team players.
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<tr>
<td>2D gel</td>
<td>Two Dimensional gel</td>
<td></td>
</tr>
<tr>
<td>ABA</td>
<td>Abscisic Acid</td>
<td></td>
</tr>
<tr>
<td>AFLP</td>
<td>Amplified Fragment Length Polymorphism</td>
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<td>AG’s</td>
<td>Anastomosis Groups</td>
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<td>AG-BI</td>
<td>Anastomosis Group-Bridging Isolate</td>
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<tr>
<td>ASR</td>
<td>Abscisic Acid Stress Ripening</td>
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<tr>
<td>DC</td>
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<tr>
<td>DNA</td>
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<tr>
<td>DsRNA</td>
<td>Double-Stranded Ribonucleic Acid</td>
<td></td>
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<td>DTT</td>
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<tr>
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<td>IEF</td>
<td>Isoelectric Focusing</td>
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<td>ITRAQ</td>
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</tr>
<tr>
<td>ITS</td>
<td>Internal Transcribed Spacer Regions</td>
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</tr>
<tr>
<td>KDa</td>
<td>kilo Dalton</td>
<td></td>
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<tr>
<td>LAH</td>
<td>Acyl-Hydrolyzing Activity</td>
<td></td>
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<tr>
<td>LC-MS</td>
<td>Liquid Chromatography–Mass Spectrometry</td>
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<tr>
<td>LIT</td>
<td>Linear Ion Trap</td>
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<td>M/Z</td>
<td>Mass-To-Charge ratios</td>
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</table>
MALDI Matrix Assisted Laser Desorption/Ionization
MeO Methoxy
MS Mass Spectrometry
NCBI National Center for Biotechnology Information
OH- Hydroxyl
PAA Phenylacetic Acid
PCR Polymerase Chain Reaction
PDA Potato Dextrose Agar
pH Potential Of Hydrogen
PR-proteins Pathogenesis-Related Proteins
Q Quadrupole
QIT Quadrupole Ion Trap
RAPD-PCR Amplified Polymorphic DNA-Polymerase Chain Reaction
rDNA Ribosomal Deoxyribonucleic Acid
REP-PCR Repetitive Extragenic Palindromic Polymerase Chain Reaction
RF Radio Frequency
RFLP Restriction Fragment Length Polymorphism
S.D Standard Deviation
SDS-PAGE Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TOF Time-Of-Flight
UP-PCR Universally Primed Polymerase Chain Reaction
UV Ultra Violet
CHAPTER ONE
INTRODUCTION

*Rhizoctonia solani*, a plant pathogenic fungus has a wide range of host species (mostly agricultural and ornamental crops). These include wheat, pine, potato, tomato, grasses, tree seedlings, rice, cotton, wheat, peanuts, lettuce and strawberry (Agrois, 2005). This pathogen primarily infects roots and stems. *R. solani* contains a large diversity of extra genomic double-stranded RNA (dsRNA), which makes it a good model organism in the study of soil-born plant pathogenic fungi (Ceresini et al., 2002, Bharathan et al., 2005). It is a multinucleated filamentous fungus, with the ability to germinate in the soil either from mycelia or sclerotia (Garcia et al., 2006). *R. solani* is a facultative saprophyte that is recognized by anastomosis groups (AG’s). In each AG there are diverse group of *R. solani* isolates with a wide-variety of crops that they could infect. These isolates are classified into 14 anastomosis groups (AG’s) as, evidenced by an increased genetic variability and DNA sequence homology. Subgroups within AG’s can be isolated and differentiated by colonial morphology, virulence, genetic sequences, biochemical characteristics and host range (Bharathan et al., 2005). All the AG’s are composed of isolates whose mycelia have the capacity to interact with one another. However, in some isolates - cell death occurs at the point of contact among isolates of the same anastomosis group’s (Seema et al., 2014). *R. solani* are attracted to host plants through chemical exudates such as amino acids, sugars, organic acids and phenols from plants (Keijer, 1996). It is proposed that fungal penetration is initiated by hydrostatic pressure, although degrading enzymes such as cutinases (Bateman and Basham, 1976), pectinases (Truter and Wehner, 2004) and xylanases (Agrois, 2005), play essential roles in infection and penetration. There is evidence of homology between the dsRNA of some strains; nonetheless, its incidence is too low to provide a definite distinctive origin of the dsRNA
among majority of the strains (Bharathan et al., 2005). Currently, works are still underway to understand *R. solani* and other fungi in an attempt to develop an effective biological base control method.

**Purpose and Significance**

*R. solani* is soil borne fungal pathogen with a highly destructive lifestyle as a non-obligate parasite. It exists as a vegetative hyphae and capable of germinating from either present mycelia or sclerotia in the soil (Ceresini et al., 2002). The sclerotia are encapsulated hyphal clump that protects and preserves the fungus during adverse conditions. These fungi are genetically differentiated through a hyphal reaction known as anastomosis, or the joining of these hyphal structures, must occur (Bharathan et al., 2005; Carling, 1996). It is believed that culture filtrate proteins have predicted functions relating to modification of the plant cell wall, a major activity necessary for pathogenesis on the plant host. However, the soluble membrane-bound and culture filtrate proteins produced by *R. solani* isolates under potato infection and vegetative growth conditions have not been investigated.

In this study, various isolates (phenotypically or genetically different) of the fungi will undergo proteomic processes such as protein purification, concentration analysis, protein-on-a-chip, and 2D-gel and mass spectrophotometry. The information gathered from this investigation will help reduce the knowledge gap which surrounds how *R. solani* uses its proteins to cause infection and as part of a long term goal of devising a more effective control strategy of this plant pathogen in agriculture and ornamental plants.
Objectives

The primary objective is to

i) Isolate specific protein(s) signatures from virulent and a-virulent strains of *R. solani* following infecting the potato tubers.

ii) Characterize these proteins by protein-on-a-chip, 2-D gel analysis followed mass-spectrophotometry.
CHAPTER TWO
REVIEW OF LITERATURE

_Rhizoctonia solani_

The Genus _Rhizoctonia_ is a heterogeneous group of filamentous fungal taxa that share similarities in their anamorphic sterile state. _Rhizoctonia_ do not produce asexual spores and their teleomorphic sexual state rarely occurs (Garcia et al., 2006). _Rhizoctonia_ was defined as a Genus by De Candolle in 1815. He characterized the genus based on its production of sclerotia of uniform texture and its mycelial relationship with roots of plants (Garcia et al., 2006). Most species in the genus _Rhizoctonia_ are known pathogenic fungi affecting agricultural and ornamental plants.

_Rhizoctonia solani_ is currently the most studied species of the genus _Rhizoctonia_ due to its ability to infect a wide range of agricultural and ornamental crops such as potato, peanuts, tomato, grasses, cotton, wheat, tree seedlings, rice, pine, lettuce and strawberry (Adams, 1988; Sneh et al., 1996; Garcia et al., 2006). Julius Kühn was the first to isolate and characterize _R. solani_ (teleomorph = _Thanatephorus cucumeris_ Frank (Donk)) when working on infected potatoes in 1858 (Kühn, 1858). _R. solani_ is soil borne fungal pathogen with a highly destructive lifestyle as a non-obligate parasite. It exists as a vegetative hyphae and capable of germinating from either present mycelia or sclerotia in the soil (Ceresini et al., 2002). The sclerotia are encapsulated hyphal clump that protects and preserves the fungus during adverse conditions. The fungus is known to disperse through sclerotia, contaminated plant material or soil either by wind, water or during on farm operations such as tillage, ploughing and seed transportation. The fungi thrives on nutrients leaking from actively growing plant cells or decaying plant materials and survives as a saprophyte for long periods in the soil (Doornik, 1980).
R. solani are attracted to host plants through chemical exudates such as amino acids, sugars, organic acids and phenols from plants (Keijer, 1996). The fungus is known to enter its host plant by finding weak spot(s) on the periphery of the plant where it can break down their protective layer (Weinhold and Sinclair 1996). It is proposed that fungal penetration is initiated by hydrostatic pressure, although degrading enzymes such as cutinases (Baker and Bateman 1978), pectinases (Bertagnolli et al., 1996; Jayasinghe et al., 2004) and xylanases (Peltonen 1995), play essential roles in infection and penetration. Once inside the host, fungus grow inter- and intracellularly degrading host tissues. These are generally observed as necrotic lesions on shoots, roots and stolons (Demirci and Döken, 1998).

Classification of Rhizoctonia solani

Rhizoctonia solani have a large species diversity that comprises of many genetically and physiological diverse species (Cubeta and Vilgalys, 1997; Carling et al., 2002). Thanatephorus cucumeris is the teleomorphic, sexual state of R. solani (Banville et al., 1996). As a result of the rare nature of the teleomorphic state, characteristics of the vegetative hyphae are used in identification of the fungus. The vegetative hyphae is 7 µm in diameter and its mycelia color varies from near white to dark brown or almost black, irregularly shaped undifferentiated, light brown to dark brown sclerotia (Parmeter and Whitney 1970; Ogoshi 1987). R. solani classification is based on arranging the different fungal isolates into anastomosis groups (AGs) through hyphal anastomosis reactions. Anastomosis reactions mostly results in death of anastomosing and neighboring hyphal cells. Notwithstanding the fact that, dead hyphal cells are observed at areas where anastomosis reaction occur and easily detected under microscopic fields and considered a tool in recognizing prospective sites of anastomosis. Cell death cannot be considered as the single most important criterion used to define anastomosis points (Carling et al., 1988; MacNish et al.,
Kendrick defined anastomosis as the association between branches of the same or different hyphae (2001). Also, Anderson defined anastomosis in *R. solani* as “a manifestation of somatic or vegetative, incompatibility between hyphae of different but closely related strains (1982). Hyphal fusion has been proposed to be a reliable technique for isolating *R. solani* strains into anastomosis groups (Ogoshi, 1987). In 1994, Cubeta and Vilgalys stated that “since its inception, the anastomosis group concept has represented the single most important advance toward understanding genetic diversity in Rhizoctonia” (1997). Rhizoctonia species are difficulty to comprehend because of the absence of well define morphological features and the scarcity of knowledge about mating and mating relationships. Currently, there are 14 different anastomosis groups of isolates of *R. solani* based on hyphal anastomosis reactions (Cubeta and Vilgalys, 1997; Carling et al., 1999, 2002). Genetically, the AGs are clearly isolated, although the groups are impossible to separate by their sexual stages. This is largely due to the inability of most isolates to fruit in culture. As a result, *R. solani* is regarded as a single species divided into AGs and AG subgroups (Ogoshi, 1987).

Hyphal anastomosis reactions are not the only method employed in the classification of isolates of *R. solani*. A number of molecular techniques has been adopted in the characterization and grouping of *R. solani* isolates. These techniques used over the years has exhibited large genetic variation and low sequence homology among the Anastomosis groups. Some of the methods used are DNA/DNA hybridization, analysis of ribosomal DNA by restriction fragment length polymorphism (RFLP) or through sequencing (Carling and Kuninaga 1990; Liu and Sinclair 1993; Kuninaga 1996; Lubeck and Poulsen 2001; Gonzalez et al., 2001; Carling et al., 2002). Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR), PCR of enterobacterial repetitive intergenic consensus sequences (ERIC-PCR), and repetitive extragenic palindromic
quences (REP-PCR), universally primed PCR (UP-PCR) including amplified fragment length polymorphism (AFLP) (Duncan et al., 1993; Toda et al., 1999; Julian et al., 1999; Lubeck and Poulsen 2001; Fenille et al., 2002) has also been used.

Characterization of *R. solani* and *Rhizoctonia*-like fungi by hyphal anastomosis reactions can be time-consuming and ambiguous as a result of the presence of bridging isolates and isolates that lack the capability to anastomose (Hyakumachi and Ui 1987; Sharon et al., 2006). Nutrient availability are known to influence anastomosis reactions (Yokoyama and Ogoshi, 1988). Currently, advances in molecular techniques have enabled a more precise characterization and classification of these fungi (Sharon et al., 2006). DNA sequence analysis of the ribosomal RNA genes, specifically the internal transcribed spacer regions (ITS1 and ITS2) of the ribosomal DNA (rDNA) are regarded as the most appropriate to identify *R. solani* and *Rhizoctonia*-like fungi to AGs (Gonzalez et al., 2001; Sharon et al., 2006).

**Anastomosis Groups**

Presently, 14 AGs have been reported, namely; AG-1 through AG-13 and AG-BI (Carling et al., 2002b; Truter and Wehner, 2004; Sharon et al., 2008). The AG group (AG1 – AG13) members are known to be only capable of fusing hyphae among themselves. AG-BI (bridging isolate) also constitutes members capable of fusing hyphae among themselves and also with members of other AG. Some of the AGs (AG1, AG2, AG3, AG4, AG6, AG8 and AG9) have been further divided based on criteria different to anastomosis pairing, including pathogenicity, colony morphology, DNA complementarity, culture appearance, thiamine requirement, host range, hyphal fusion frequency, pectic zymograms, etc. Molecular studies have also revealed further division in some of the AG groups (Ogoshi 1987; Kuninaga et al., 1997; Gonzalez et al., 2001; Carling et al., 2002a, b). AG1 subdivisions (AG 1-IA to AG 1-ID) are generally based on morphology and
pathogenicity while that of AG2 subdivision (AG 2-1 to AG 2-4) is also based on hyphal fusion frequency (Ogoshi, 1987). AG2 subgroups can be further divided into cultural types (AG 2-2IIIB, AG 2-2IV, AG 2-2 LP) based on pathogenicity and morphology (Schneider et al., 1997; Hyakumachi et al., 1998; Carling et al., 2002b). AG 2-2IIIB, AG2-2IV and AG2-2LP was identified on mat rush (*Lomandra longifolia*), sugar beet and warm season turf grasses respectively. AG2-2IIIB is referred to as the rush type, AG 2-2IV as the root rot type, while that of AG 2-2LP is known as large patch type (Ogoshi 1987; Hyakumachi et al., 1998). DNA/DNA hybridization data and fatty acid analysis are generally used for the subdivisions of AG4, AG6 and AG9 (Kuninaga and Yokosawa 1994; Kuninaga 1996). Subdivisions of AG8 are based on pectic zymograms (MacNish et al., 1993). Presently, AG-BI are proposed to be a member of AG 2, which could suggest a paraphyletic origin for this AG. Carling et al. asserted that AG-BI may not be the only «bridging isolate» group due to the fact that new isolates has been identified with the same behavior in some of the classical AGs (i.e. AG 3, AG6, etc.) (2002b).

The AGs (AG1, AG2, AG3 and AG4) were the first to be described and associated with the most pathogenic forms of *Rhizoctonia* diseases across the world. The subsequent AGs were defined as less destructive pathogens and also have restricted geographical distribution (Carling et al., 2002a). *R. solani* isolates which poses major disease threat to potato (*Solanum tuberosum*) belong to AG-3 subgroup PT (AG-3PT), although AG-1, AG-2-1, AG-2-2, AG-4, AG-5 AG-7 and AG-9 have been implicated with disease symptoms on potato stems, stolons, roots and tubers around the world (Campion et al., 2003; Truter and Wehner, 2004; Rosa et al., 2005; Woodhall et al., 2007; Tsror, 2010). AG-3PT causes black scurf on tubers and stem canker disease of potatoes (Banville et al., 1996; Jeger et al., 1996; Banville and Carling, 2001).
Table 1. Anastomosis Group (AG), Subgroup, and Cultural Type of *Rhizoctonia solani* and Their Common Hosts

<table>
<thead>
<tr>
<th>AG/Subgroup</th>
<th>Hosts</th>
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<tr>
<td>AG 1 IA</td>
<td>rice, corn sorghum, bean, soybean, turf grass, camphor seedlings, crimson clover</td>
</tr>
<tr>
<td>AG 1 IB</td>
<td>bean, rice, soybean, leguminous woody plants, lettuce, Hortensia, cabbage, figs</td>
</tr>
<tr>
<td>AG 1 IC</td>
<td>buckwheat, carrot, soybean, flax, pine, lettuce</td>
</tr>
<tr>
<td>AG 2-1</td>
<td>Crucifers, strawberry, tulip, Japanese radish, subterranean clover</td>
</tr>
<tr>
<td>AG 2-2IIIB</td>
<td>rice, mat rush, ginger, turf grass, corn, sugar beet, Chrysanthemum, Gladiolus, edible burdock, tree seedlings, soybean</td>
</tr>
<tr>
<td>AG 2-2IV</td>
<td>sugar beet, turf grass</td>
</tr>
<tr>
<td>AG 2-3</td>
<td>Soybean</td>
</tr>
<tr>
<td>AG 3 (PT, TB)</td>
<td>potato, tobacco, tomato, eggplant, pepper</td>
</tr>
<tr>
<td>AG 4 (HGI, HGII, HGIII)</td>
<td>tomato, pea, potato, soybean, onion, cotton, snap bean, Loblolly pine seedlings, peanut, slash pine, cucumber, corn</td>
</tr>
<tr>
<td>AG 5</td>
<td>potato, turf grass, bean, soybeans</td>
</tr>
<tr>
<td>AG 6 (HG-I, GV)</td>
<td>non pathogenic</td>
</tr>
<tr>
<td>AG 7</td>
<td>Soybeans</td>
</tr>
<tr>
<td>AG 8 (ZG 1-1, ZG 1-2, ZG 1-3, ZG 1-4, ZG 1-5)</td>
<td>Poaceae, cereals</td>
</tr>
<tr>
<td>AG 9 (TP, TX)</td>
<td>crucifers, potato</td>
</tr>
<tr>
<td>AG 10</td>
<td><em>H. vulgarae</em></td>
</tr>
<tr>
<td>AG 11</td>
<td>Wheat</td>
</tr>
<tr>
<td>AG 12</td>
<td>cauliflower, radish</td>
</tr>
<tr>
<td>AG 13</td>
<td><em>G. hirsutum</em></td>
</tr>
<tr>
<td>AG BI</td>
<td>non pathogenic</td>
</tr>
</tbody>
</table>

Source: (Sneh et al., 1991; Tu et al., 1996; Mazzola et al., 1996; Garcia et al., 2006)
Hyphal Anastomosis Reaction

The concept of hyphal anastomosis to characterize and classify *Rhizoctonia* was reintroduced in 1969 by Parmeter and his colleagues at the University of California in Berkeley. The concept shows whether isolates of *Rhizoctonia* are genetically related or unrelated based on their ability to recognize and fuse with each other (Ceresini et al., 1999). The formation of anastomosis reaction results in four hyphal interaction phenotypes (C0, C1, C2, and C3).

C0 reaction occurs when isolates of different AGs are paired. In the C0 reaction, the hyphae of the isolates grow over and under each other. However they do not make contact or interact with each other. The “C1 reaction”, commonly known as the bridging reactions contains the bridging isolates. Bridging isolates are capable of producing hyphal anastomosis reaction with isolates from more than one AG. The C1 is similar to C2 reaction, however no membrane fusion occur in a C1 reaction. The pairing of non-identical isolates within the same AG is “C2 reaction” (Killing reaction). The C2 reaction portrays a somatic incompatibility or imperfect fusion response between genetically different individuals. (Carling 1996; Ceresini et al., 1999; McCabe et al., 1999). The wall fusion is prominent, membrane fusion is likely and anastomosing cells frequently dies in a C2 reaction. When identical isolates of the same AG are paired, the hyphal fusion is said to be “complete” and cytoplasmic mixing occurs. This reaction is known as “C3” (compatible or perfect fusion). In the C3, the wall and membrane of anastomosing cells fuse and remain alive (Worrall 1997; Johannesson and Stenlid, 2004).

Most isolates form a tuft formation when they meet with other isolates. Tufts are areas of differentiation in the confrontation zone between isolates, which is taken up by a band of hyphae elevated beyond the general level of mycelium. There are distinct difference in tuft formation
between isolates with C0 and C1 reaction. Isolates with C3 reaction have no tuft formation while isolates showing C2 reaction are known to participate in tuft formation (MacNish et al., 1997).

*Rhizoctonia solani* Infection Process

*Rhizoctonia* infection process involves cell-adhesion, penetration and colonization and host reaction. *R. solani* are attracted to host plants through chemical exudates such as amino acids, sugars, organic acids and phenols from plants (Keijer, 1996). *R. solani* infection initiates when the hypha comes into contact with a potential host. The hypha adhere and begins to grow causing hyphal branching and the formation of infection structures. The infection structures facilitates the breaking of host defense mechanisms, allowing the fungus to penetrate the host (plant tissue). Complex infection structures could be seen as a large dome-shaped infection cushions. Once the fungus penetrates the host, short swollen hyphae, apresoria or repetitive T-shaped hyphal branches are formed (Garcia et al., 2006). The swollen tips then form infection pegs, which penetrate the cuticle and epidermal cell wall of the host (Keijer, 1996). Subsequently, there is exchange of chemical substances (fungal enzymes and host exudates) between the pathogen and the host. *R. solani* AG4 are known to produce pectinolytic and cellulolytic enzymes at the early stages of infection. Endopectinylase are also produced by AG4 during late infection (Marcus et al., 1986). Isolate belonging to several anastomosis groups of *R. solani* are known to secrete enzymes such as pectinase, pectin lyase, cellulose, phosphatase or pectin methlesterase during infection (reviewed in Garcia et al., 2006).

The penetration process are accomplished through the cuticle and epidermal cell wall or across the stomata by penetration pegs (Weinhold and Sinclair, 1996). Cutinase enzymes are produced at the point of hyphal entry during early stages of infection. The fungi then colonize the host following the production of hydrolytic enzymes which degrades host cell walls beyond the
advancing hyphae. There is evidence of degradation of cell wall prior to penetration in *R. solani* AG4 (Weinhold and Motta, 1973). In addition to the cell wall degradations, there are cytological changes such as formation of reaction zones, plasmolysis and collapsing of the cytoplasm of the host tissue (Garcia et al., 2006).

*R. Solani* as a Potato Pathogen and Control

*R. solani* causes stem canker and black scurf disease in the potato industry (Wilson et al., 2008b). They are observed as necrotic lesions on the underground parts of plants and sclerotia covering progeny tubers (Carling and Leiner, 1986). *R. solani* can spread to other growing areas of the field on sclerotia-covered seed tubers (Tsror and Peretz-Alon, 2005). The fungus is known to cause necrosis in emerging sprouts, kills sprout tips and causes stem canker disease symptoms during the early growing season. The fungus attacks developing stolons and inhibits them from growing to their full potential in the late season (Banville 1989). Hide and Horrocks observed late emergence of sprouts, less stem numbers and shortening length of stolons in stem canker infection (1994). Potato tubers are mostly green due to the inability of tubers to form deep in the soil (Anderson, 1982). Tuber-borne inoculum is the primary cause of stem canker infection. Soil-borne infection is secondary because it emerges later in the season and also the fungus requires time to grow close to its potato host (Carling and Leiner, 1986).

Protection of potato from *R. solani* infection could be achieved by employing good agricultural practices in addition to chemical and/or biological plant protection techniques. Some researchers have advised potato growers to stop using sclerotia-covered seed tubers (Banville 1978; Frank and Leach, 1980). It is also proposed that planting be done in relatively dry and warm soil to facilitate accelerated emergence (Carling and Leiner, 1990). The use of crop rotation method is known to reduce inoculum levels in the soil. Cereals can be a good rotation crops in potato
productions because their AGs differ from potato (Anderson, 1982). Sprouting plants can be protected from seed-borne infection by dressing seed tubers with fungitoxic chemicals. *R. solani* has also been controlled using biological agents. Trichoderma spp. has been reported to protect plants from *R. solani* infection by production of antibiotics, antifungal chemicals and hydrolytic enzymes. *Trichoderma spp.* such as *Trichoderma harzianum* along with *Gliocladium virens* are known antagonists of the pathogen and capable of preventing or lowering the incidence of infection (Wilson et al., 2008).

**Principle, Instrumentation and Significance of Mass Spec Instrumentation**

Since the inception of proteomics, the aim is the complete characterization of all proteins. Proteomics is the study of proteins in living system, which includes the co- and post-translationally modified proteins and alternatively spliced variants. This may also include their covalent and non-covalent relationships, spatial and temporal spread within cells and the effect of modifications in extracellular and intracellular conditions. Among the techniques used to investigate proteins on a large scale, Mass spectrometry (MS) is at the center of practically all proteomics experiments (Guerrera and Kleiner, 2005). Basic application of MS are cataloging protein expression, defining protein interactions, and identifying sites of protein modification (Han et al., 2008). Mass spectrometers measure the mass-to-ratio of analytes. This includes intact proteins and protein complexes (Sobott, 2002), fragment ions produced by gas-phase stimulation of peptide ions (Bottom-up sequencing) and protein ions (top-down sequencing) (Yates, 1998; Kelleher, 2004), and peptides produced by enzymatic or chemical digestion of proteins (mass mapping) (Pan et al., 2003; Kleno et al., 2004). MS requires an interaction between mass spectrometry instrumentation (that is how analytes are ionized, activated and detected) and the gas-phase peptide chemistry
(what bonds are broken and at what rate; and how bond separation depends on peptide/protein charge state, size and composition including the sequence) (Wysocki et al., 2005).

Instrumentation

Mass spectrometers have three basic components: an ion source, a mass analyzer, and an ion detector.

Ionization

Matrix assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) are the two most commonly used techniques capable of ionizing peptides or protein (Fenn et al., 1989; Karas and Hillenkamp, 1988). In both MALDI and ESI, peptide or protein ions are created with low internal energy, which subjects them to little fragmentation. In MALDI, an organic matrix, which usually has a conjugated aromatic ring structure is used to co-crystallize samples on a metal target. The matrix is excited by a pulse laser causing the rapid thermal heating of the molecules and the disruption of ions into the gas phase. In the end, packets of ions are produced rather than a continuous beam. The MALDI technique can tolerate a high level of impurities in a sample compared to ESI technique.

In ESI, charged droplets are produced by spraying an electrically generated mist of ions through the inlet of a mass spectrometer at atmospheric pressure (Yates, 2004). This technique is generally combine with a chromatographic system (reverse phase chromatography or capillary electrophoresis). This enables the analysis of very complex samples (Takats et al., 2004).

Mass Analysis

After ionization, the samples are sent to the mass analyzer. The mass analyzer is prime to MS technology. Here, ions are separated by their mass-to-charge (m/z) ratios. The movement of ions are influenced by electric or magnetic fields to direct ions onto a detector. The number of ions
are recorded by each individual m/z value. For proteomics research, four kinds of mass analyzers are commonly used; quadrupole (Q), ion trap (quadrupole ion trap, QIT; Linear ion trap, LIT or LTQ), time-of-flight (TOF) mass analyzer and Fourier-transform ion cyclotron resonance (FTICR) mass analyzer (Domon and Aebersold, 2004; Han et al., 2008). The four mass analyzers differ sensitivity, resolution, mass accuracy and the possibility to break peptide ions (Yates, 2004). The Quadrupole, categorize mass-to-charge (m/z) by using radio frequency (RF) and DC voltages, which allows a restricted mass/charge range to reach the detector. In the case of Quadrupole ion traps, focused ions are trapped into a small volume and then scanned from the trap into the detector. This type of mass analyzer, rely primarily on RF fields. Ions with specific m/z ratios are selected in the trap for fragmentation (March, 2000). QIT is capable of determining the mass and sequence of a given peptide. The two-dimensional ion trap was recently developed with increased sensitivity, resolution and mass accuracy. However the three-dimensional ion trap is relatively cheap, robust, and sensitive but has a low mass accuracy (Hager, 2002; Schwartz et al., 2002). Time-of-flight (TOF) analyzer causes the fragmentation of ions in relation to their flight time down a field free flight tube. The time-of-flight of ions is directly related to their m/z values. The only problem to TOF, is its inability to perform true MS/MS (Medzihradszky et al., 2000; Bienvenut et al., 2002). However, TOF/TOF instrument has been developed recently to overcome such a problem. TOF/TOF instrument selects ions of one m/z ratio in the first TOF section, then fragments it in collision cell, which in turn are separated in the second TOF section (Pan et al., 2003).

Fourier transform ion cyclotron resonance (FTICR) operates by trapping ions in a high static magnetic field and cyclotron resonance to detect and excite the ions (Hopfgartner et al., 2004). The mass-to-charge is determined by measuring the frequency of motion of the ions. FTICR
is regarded as the most powerful among the four analyzers in terms of mass resolution and mass measurement accuracy (Bogdanov and Smith, 2004).

Defense Mechanism of Potato (*Solanum tuberosum* l.)

Potato is a starchy tuberous crop, which is an essential food crop consumed worldwide. Therefore the management of the tubers is of high economic importance in ensuring food security. The ability of its tubers to survive in the presence of infectious microorganisms (fungus/bacteria/virus etc.) and harsh environmental conditions is dependent on the epidermis/periderm and other functional processes that are directly or indirectly related to this structure. Its innate periderm protects the tubers from parasitic organism, pathogen and pest attack (Kolattukudy, 1980; Chaves et al., 2009). The periderm also protect the tubers from dehydration and bruising during harvest including handling and stress increase during storage (Lulai, 2007). The maintenance of tuber quality is essential for its survival when it has the capacity to quickly produce wound-periderm upon damage or bruising (Bowles, 1990). The wound-healing process of tubers incorporates dedifferentiation process, activation of metabolism and formation of mechanical barriers (Suberin) and defensive mechanisms (formation of PR-like proteins) (Lulai, 2007; Chaves et al., 2009).

Plant tissues response to wounds have been studied extensively for many years (Richards, 1896; Bowles, 1990; Lee et al., 2006). Previous studies have demonstrated that several genes are wound-inducible in potato tubers (Zhou et al., 1999; Delaplace et al., 2009) have demonstrated that several genes are wound-inducible in potato tubers. Potato slices has been a good model to study suberin formation and helped in broader study of wound induced metabolism, including healing and expression of genes upon excision which leads to suberization (Rees and Beevers, 1960; Laties, 1962; Stark et al., 1994; Chaves et al., 2009).
The production of antimicrobial compounds (PR and PR-like proteins) are essential during plant infection process (Bowles, 1998; van Loon et al., 2006; Chaves et al., 2009). Several proteins, mostly in the PR families (beta – 1, 3-glucanase (PR-2); chitinases (PR-3); osmotins (PR-5); protease inhibitors (PR-6); plant peroxidases (PR-9); PR-10 proteins) have been implicated and detected during infection processes (van Loon et al., 1999). PR proteins are activated by wounds or infection and play a critical role in plant physiological and developmental processes, including embryogenesis and abscission (Lee et al., 2006; Chaves et al., 2009; Delaplace et al., 2009).

Proteins Implicated in *R. solani* Infection Process

*Rhizoctonia solani* is composed of genetically and ecologically diverse soil-associated fungi, which functions as saprobes, mycorrhizal symbionts and pathogens of plants (Ogoshi, 1987; Cubeta and Vilgalys, 2000). Pathogenic fungi in *R. solani* species complex has been reported to infect more than 500 plant genera, however, their host range is limited within specific AG (Farr et al., 1989; Bartz et al., 2012). For example, *R. solani* AG-3 are pathogenic to Solanaceae family (eggplant, pepper, potato, tobacco and tomato) (Johnk et al., 1993; Bartz et al., 2010). Isolates in a specific AG, vary in the type of symptoms caused. Symptoms of *R. solani* reported on tomato during infection include reduced seedling emergence from soil, post-emergence damping off, root necrosis and stem cankers (McCarter, 1991). Hyphal colonization by *R. solani* on host leads to the development of symptoms and the production of phytotoxic substances (Bartz et al., 2012).

Reports have shown that *R. solani* produces phenylacetic acid (PAA) during infection. Three hydroxyl (OH-) derivatives of PAA and 3-methoxy (3-MeO-) derivatives of PAA compounds are associated in the parasitism and infection process of plants (Mandava et al., 1980; Iacobellis and Devay et al., 1987). PAA, OH-PAA and MeO-PAA derivatives are capable of damaging tomato seedling by causing both root and shoot necrosis (Bartz et al., 2012). Effector
proteins have been implicated in the pathogenicity of plant disease. Rioux et al. reported Avr-pita effector in *R. solani* AG1-IA (2011). This protein (Avr-pita effector) is similar to the fungal metalloproteases family of the deuterolysin family (Khang et al., 2008). Avr-pita gene triggers Pi–ta gene mediated resistance in host plant (Jia et al., 2000).

**Proteomics of Filamentous Fungi**

Research related to filamentous fungi has currently progress due to the availability of multiple fungal genome sequences, the arrival of next-generation nucleic acid sequencing and the possibility of powerful proteomics technologies (Tandem LC-MS) (Martin et al., 2008; Braaksma et al., 2010; Costa et al., 2010). The closing of the knowledge gap on the pathogenicity of opportunistic fungal infection (especially in immunocompromised patients), plant pathogenic nature of fungi and the biotechnological ability of fungal enzymes for biofuel production, have driven intense studies into the proteomics of filamentous fungi (Taylor et al., 2008; Dagenais & Keller, 2009; Schuster & Schmoll, 2010). A lot of studies have focused on cataloguing mycelial, organelle and secreted proteins (secretome) across a wide range of fungal species (Bouws et al., 2008; Kim et al., 2008). Most of these studies adopted technologies such as SDS-PAGE or 2D-PAGE fractionation or Shotgun proteomics. The dynamism of fungal proteomes (effects of carbon sources, antifungal drugs and gene deletion) has been explored at the proteomic level (Fernandez-Acero et al., 2010; Cagas et al., 2011). Currently, proteomics of Ascomycete have been extensively study than any other fungi due to its ability to cause animal disease and regarded as cell factories for protein secretion [e.g. *Aspergillus niger* (Aday et al., 2010)] (Doyle, 2011).

**Proteomics Studies of Pathogenic Filamentous Fungi**

*Fusarium graminearum* is a filamentous fungi pathogenic to wheat, maize and grains worldwide (Kikot et al., 2009). Zhou et al. begun studies in *F. graminearum*, which focused on
plant protein response to fungal exposure (2006). Currently there is availability of *Fusarium sp.* genome data and improvements in protein extraction techniques (Paper et al., 2007; Taylor et al., 2008). There is an online video tutorial on the complexity of protein extraction from Fusarium strains (Pasquali et al., 2010). Paper et al. identified proteins secreted by *F. graminearum* after growth on culture media and in planta during infection of wheat heads (2007). Enzymes, such as enolase, triose phosphate isomerase, phosphoglucomutase, calmodulin, aconitase and malate dehydrogenase were detected in planta. These enzymes were fungal housekeeping enzymes proposed to indicate occurrence of fungal lysis during pathogenesis. Taylor et al. adopted a Quantitative protein mass spectrometry using isobaric Tags to study *F. graminearum* protein expression in response to in vitro stimulation of biosynthesis of the mycotoxin, trichothecene (2008). The authors shown that 130 of 435 proteins detected exhibited significant expression changes with many of the proteins identified found to involve in fungal virulence. Northern analysis and reverse transcriptase-PCR was also used by Taylor et al. to confirm changes in selected protein expression following ITRAQ and 2D-PAGE analysis (2008). This highlights the relevance of fungal proteomics for identifying functions of individual proteins.

*Metarhizium spp* is entomopathogenic fungi that have potential to serve as a biological control for agricultural pest. The genome and EST sequence of *Metarhizium spp* have been reported (Wang et al., 2009; Gao et al., 2011). Current studies has explored the proteome of this fungus. Barros et al. used 2D-PAGE to detect 1130±102 and 1200±97 protein spots for *Metarhizium acridum* conidia and mycelia, respectively (2010). 94 proteins were identified by MALDI-ToF/ToF MS. Heat shock proteins and an allergen were exclusively found in conidia, while metabolic proteins (e.g. transaldolase, protein disulphide isomerase and phosphoglycerate
kinase) were detected in mycelia. The authors also observed differences in the expression of identical proteins and isoform occurrence between conidia and mycelia.

Murad et al. also used enzymatic and proteomic approach to identify the *Metarhizium anisopliae* response to the chitin-containing exoskeleton of the cowpea weevil plant pathogen (*Callosobruchus maculatus*) (2006). There was a five-fold increase in protein secretion from *M. anisopliae* with elevated chitinolytic and proteolytic exoskeleton. 2D-PAGE analysis showed seven additional proteins been expressed during exposure in this study. The authors detected N-acetyl-D-glucosamine kinase and D-glucosamine N-acetyltransferase in the *M. anisopliae* secretome, following exoskeleton co-incubation, by 2D-PAGE and MALDI-TOF/TOF MS. They proposed that chitosan adsorption by *M. anisopliae* was facilitated in by these enzymes. It is believe that chitosan is soluble and easily absorbed as nutrient by *M. anisopliae* than chitin.
CHAPTER THREE
MATERIALS AND METHODS

Rhizoctonia-solani Cultures

*R. isolates* 1AP and 123E were obtained from the laboratory of Dr. N. Bharathan in the Department of Biology, Indiana university of Pennsylvania. *R. solani* isolates 1AP and 123E was used in this experiment. *R. solani* isolates 1AP and 123E belongs to the anastomosis group 3 (AG3). AG3 is the major cause of Rhizoctonia disease of potato (*Solanum tuberosum*) (Bandy et al., 1988). 1AP is a wild type and a highly pathogenic isolates of *R. solani*. 123E has a reduced genomic complement and was derived from a heterokaryotic field isolate Rhs-1AP. *R. solani* isolate 1AP is pathogenic while *R. solani* isolate 123E is non-pathogenic.

Culture Transfers and Maintenance

Cultures were transferred and maintained using PDA plates and broth culture

Plate Transfer (PDA plates)

Cultures were grown on potato dextrose agar (BD-Difco manufacturing). Basically, it is the transfer of isolates from starter cultures to new plates to verify that pure cultures were obtained for this experiment. The cultures were transferred every 2-3 weeks to ensure proper maintenance and viability. All glassware and tools used were autoclaved before the transfers were done. For 10 agar plates, 7.8 g of Potato Dextrose Agar (PDA) was weighed and dissolved in 200 ml of distilled water (20 ml per plate) in a 1 liter Erlenmeyer flask, the resulting mixture was then autoclaved for 30 mins at 121 degrees Celsius. Approximately, 20 ml of the Potato Dextrose Agar (PDA) were poured into each plates with the lids half off in the fume-hood under the UV light and allowed to cool until no condensation was present.
The flask was then allowed to cool under the biological safety hood with the UV light on, approximately 20 ml of the PDA was poured into each of the 10 plates, and the plates were allowed to cool until no condensation was present. With the help of a sterile knife, a small sections of each of the 2 starter cultures were transferred to 5 plates each. The plates were wrapped in Para film and stored upside down in a clean dry place. Each plate were labeled with the isolate’s name, the date the starter was made, the date the new plate was formed and the initials of the experimenter. Unless otherwise mentioned all transfers of cultures to the PDA plates and Malt-extract broth (see below) was done under a-septic conditions.

**Broth Culture (liquid)**

15g of malt extract broth was dissolved in a flask containing 1L sterilized water and mixed thoroughly. 100ml of the resulting mixture were poured into 500 ml flask, sealed with a cotton swab and covered with aluminum foil. The flasks were autoclaved at 121 degrees Celsius for 30 minutes and then placed in the fume-hood under the UV light to allow it to cool. Pour into flask, place a cotton swab with aluminum foil on top and Autoclave for 30 minutes. Isolates were cut from petri plates and dropped into the flask (3 pieces per flask). The flasks were kept at room temperature and allowed to grow for at least three weeks. The flask transfer of isolates enabled the purification of fungal proteins.

**Soil Inoculation and Potato Growing**

Sterilized soils were inoculated with a plate each of 1AP and 123E isolates (120g soil/plate of isolate) in pots. Potato tubers were disinfected with Clorox by surface treatment.

**Soil-Treatments:** There were three treatments and three seeds per pot

1. Potato seeds grown in sterilized soil
2. Potato seeds grown in soil inoculated with 123E
3. Potato seeds grown in soil inoculated with 1AP

The inoculum and soil were hand mixed and planting of potato seeds were done at the same time. Five pots of soil were used for each treatment and grown under appropriate conditions necessary to enhance growth. The seedlings were watered twice daily. The seedlings were harvested two weeks after germination and the stem regions close to the roots of the plants were harvested and used for protein extraction.

![Side-by-Side image of all three treatments](image)

**Figure 1. Side-by-Side image of all three treatments (Healthy, 1AP inoculation, 123E inoculation).**
Tissue Culture

Potato seeds were placed in a petri dish with a sterilized paper towel at its base. The paper towel were watered with sterile water before the seeds were placed on it. With the help of a knife, fungal tissue from 1AP and 123E isolates were cut and place close to the “Eye” (part of the seed were germination has started). Three or four fungal tissue were placed beside each eye. As seen below in figure 3. The entire set up was then placed in a spherical glass pot, with a watered sterilized
paper towel at its base. The spherical glass pot was covered with a plastic wrap to conserve moisture. The potato seeds were left in the hood for 12 days. Watering of paper towels in the set up was done every-two days. Samples were taken at the point of contact of the fungal tissue and the “Eye”. The tissue culture process was done under a hood to avoid contamination.

Figure 3. Image of tissue culture set up (1AP fungal tissue)

![Image of tissue culture set up (1AP fungal tissue)](image)

Figure 4. 1AP and 123E in the complete tissue culture.

![1AP TISSUE CULTURE](image) ![123E TISSUE CULTURE](image)

Total Protein Isolation from *R. solani* isolates and potato

Total Proteins was isolated from the fungus, potato (healthy no treatment) and potato (inoculates both in soil and tissue culture) using Bio RAD assay.
Table 2. Buffers Required for Extraction

<table>
<thead>
<tr>
<th>Item</th>
<th>Size</th>
<th>Catalog #</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer 1</td>
<td>20mL</td>
<td>K-0025-20-1</td>
<td>-20°C</td>
</tr>
<tr>
<td>Buffer 2</td>
<td>1.0mL</td>
<td>K-0025-20-2</td>
<td>-20°C</td>
</tr>
<tr>
<td>Buffer 3</td>
<td>20mL</td>
<td>K-0025-20-3</td>
<td>-20°C</td>
</tr>
<tr>
<td>Protease Inhibitor</td>
<td>1.0mL</td>
<td>K-0025-20-4</td>
<td>-20°C</td>
</tr>
</tbody>
</table>

For 10mL of working lysis buffer; 2.0mL of buffer 1, 0.1mL buffer 2 and 0.1mL of protease inhibitor and 0.5mL of buffer 3 were thoroughly mixed. Diethyl pyrocarbonante grade water was added to the resultant mix until it reached a volume of 10mL. The resultant buffer was kept on ice. Prior to beginning extraction procedure, 1.5mg of Dithiothreitol (stored at -20°C) was added to every 10mL of working lysis buffer. The Mortar and pestle was cleaned with ethanol and allowed to air dry for 15-20 minutes then rinsed with autoclaved water and wiped with Kim wipes (after autoclaving before use).

Frozen plant tissue was placed on ice and 2.0mL of lysis buffer per gram of the tissue was added. The tissue was homogenized using mortar and pestle until no chunks were visible (care was taken to keep the sample on ice at all times). The homogenate was transferred to microfuge tubes kept on ice. The homogenate were incubated for 30 minutes on ice (vortex at least 3 times during the incubation period). After incubation, the samples were centrifuge at 6000rpm for 10 minutes at 4°C and the supernatant was transferred from the tubes into new microfuge tubes kept on ice. The centrifugation and transfer of supernatant was repeated when some pellet material was accidentally transferred into the new microfuge tube. The supernatant was stored at -80°C if not analyzed immediately.

Determination of Protein Concentration

The concentration of all protein extract were determined on a Spectrophotometer using a Bradford assay. The Bradford assay was used according to the manufacturer’s protocol. Briefly,
20ul of each of the following protein concentrations from the Bradford assay, 2mg/ml, 1.50mg/ml, 1.0 mg/ml, 0.75 mg/ml, 0.50 mg/ml, 0.25 mg/ml, and 0.125 mg/ml, was added to 1000ul of the Bradford buffer in a 2ml tube and vortex. The tubes were run on spectrophotometer to develop a standard curve with a correlation coefficient of 0.9973. 20ul of protein extracts were mixed with 1000ul of Bradford buffer to determine their concentration on a spectrophotometer using the standard curve.

The proteins from the wild type and reduced genomic compliment, viral infected and non-infected strains of *R. solani*, will be purified and quantified utilizing various protein purification processes such as protein on a chip, protein concentration analysis, 2D gel and mass spectrophotometry.

**Protein-on-Chip**

**Preparation of Sample and Ladder**

4 µl protein sample was added to 2 µl denaturing solution in a 0.5 ml vial. The sample vial and a vial containing a 6 µl aliquot of Protein 80 Ladder was heated at 95 °C for 5 min. The tubes were cooled down afterwards and spanned for 15 s. 84 µl deionized water was added to samples and ladder and vortexed.

**Loading the Gel-Dye Mix, Destaining solution, the Samples and Ladder**

The base-plate of the chip priming station was adjusted to position “A” and a syringe clipped to its middle position. A new protein chip was taken out of the sealed bag and placed on the chip priming station. 12 µl of gel-dye mix was pipetted in the well-marked “G”. The plunger was placed at 1 ml position and the chip priming station closed. The plunger was held by clip for 60 s, and then released. After 5 s, plunger was slowly pull back to 1ml position. 12 µl of gel-dye-mix was pipetted in all wells labeled with “G” and 12 µl of destaining solution in well “DS”. 6 µl
of each sample was loaded in different wells (Note: all 10 sample wells must be filled samples). Lastly, 6 µl of the prepared ladder was also loaded in the well-marked “Ladder”. The chip was then placed in the Agilent 2100 Bioanalyzer and the assay was started immediately.

Fungal Protein Processing

Sample Preparation

Precipitate 400 uL of lysate using ToPrep Kit (ITSI-Biosciences, Johnstown, PA) per manufacturer’s protocols. Re-suspend pellet in 2D gel electrophoresis compatible lysis buffer pH 8.5 (30mM Tris-HCl, 2M Thio urea, 7 M Ureal, 4% (w/v) CHAPS and 1% NP40). Sample concentrations are determined utilizing the ITSIPREP ToPA Kit (Bradford Assay, ITSI-Biosciences, and Johnstown, PA)

Protein Labeling

Label proteins (50ug) with CyDyes DIGE Fluors (GE Healthcare, Bucks, UK) according to manufacturer’s protocol (Cy3 or Cy5 fluorophores for samples and Cy2 was used as the internal standard). Internal standard (pooled sample of equal amount of all samples in experiment) is labelled with Cy2 and brought to a volume of 450 uL with 1X Rehydration Buffer (4% (w/v) CHAPS, 8 M Urea, 1% (v/v) BioLyte Buffer (BioRad, CA, USA) and 13 mM DTT).

2D Gel Electrophoresis

First dimension analysis will be performed using isoelectric focusing (IEF) in a 24-cm Immobiline Dry Strip (BioRad, CA, USA) with a linear pH 3-10 gradient using Ettan IPGphor II (GE Healthcare) at 20 degrees Celsius. Rehydrate the strips for 12 hours at 30 volts at a final volume of 450uL containing Rehydration Buffer and labelled samples. Five Steps to focusing on proteins on the rehydrated IEF strips:

1. 200 V for 1 hr
2. 500 V for 1 hr
3. 1000 V for 1 hr
4. 3 hr gradient from 1000V to 8000V
5. 8000 V until 65000 total volt hours

Equilbrate and reduce strips for 10 minutes in an Equilibration Buffer (50 mM TrisCl, pH 8.8, 6 M Urea, 30% (v/v) Glycerol, 2% (w/v) SDS containing 0.5% (w/v) DTT). After equilibration and alkylation at room temperature on a shaker for 15 minutes in the equilibration buffer containing 4.5% (w/v) Iodo acetamide, load strips onto a 24 cm x 20 cm, 12.5 SDS-PAGE gel and run for 4 hours at 16 Watts per gel.

Gel Image Analysis

Utilizing Typhoon Digital Imager, detect the Cy3 and Cy5 labelled proteins and Cy2 standard using 3 different wavelengths. DeCyder analysis software (GE Healthcare) will be used to analyze images after detection. Candidate protein spots with a 2-fold or greater threshold are then marked.

Protein Identification

Stain gels with Sypro Ruby (Invitrogen, USA) and protein spots are to be processed utilizing an Ettan Spot Handling Workstation (GE Healthcare). Destain gel plugs with 50% Methanol/20 mM Ammonium Bicarbonate. Reduce destained gel plugs in 10 mM DTT/ 20mM Ammonium bicarbonate for 15 minutes at 55 degrees Celsius then alkylate them in 20 mM IAA/20mM ammonium bicarbonate for 30 minutes at room temperature. Rehydrate gel plugs in 20 mM ammonium bicarbonate containing 1 ng/uL Trypsin Gold, Mass Spectrometry Grade (Promega, USA) and incubate overnight at 37 degree Celsius. Add 50% Acetonitrile/0.1% Trifluoroacetic acid to the plugs and incubate for 10 minutes at room temperature. Transfer the extraction solution
to a clean tube and incubate the same mixture again for an additional ten minute. Combine the two extractions and dry in the tube at 45°C for 2hrs analysis utilizing LC/MS/MS and database inquiries.

Mass Spectrophotometry

Utilize a Thermo Finnigan LCQ Deca XP Plus in positive ion mode with a spray voltage set at 1.8 kV. Utilize a Thermo Finnigan Surveyor MS Pump to deliver a linear acetonitrile gradient from 2 to 30% B over 30 minutes. Utilize the “Top Three” method to scan for the three most abundant ions to generate an experimental MS/MS spectrum. Using the following parameters, perform a NCBI-non-redundant database search to identify proteins: precursor mass tolerance of 1.4 Da and fragment ion tolerance of 1.0 Da.
CHAPTER 4

RESULTS

The potato plants were harvested two weeks after germination and total protein was successfully extracted. Total protein was also extracted from tissue culture samples. Protein concentration of all protein extract were determined using Spectrophotometer. All protein concentration were normalized to 1mg/ml before using it for protein chip analysis. The concentration of protein extract from the 1 plate/100g soil inoculation, no treatment and fungal tissue are shown in the table below.

**Table 3. Protein Concentration of Samples in Figure 1**

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy Eye (HE)</td>
<td>1.6156</td>
</tr>
<tr>
<td>Healthy Collar (HC)</td>
<td>1.6280</td>
</tr>
<tr>
<td>1AP Fungi (1AP F)</td>
<td>1.6937</td>
</tr>
<tr>
<td>123E Fungi (123E F)</td>
<td>1.9649</td>
</tr>
<tr>
<td>1AP Infection Eye (1AP E)</td>
<td>1.2423</td>
</tr>
<tr>
<td>1AP Infection Collar (1AP C)</td>
<td>1.5297</td>
</tr>
<tr>
<td>123E Infection Eye (123E E)</td>
<td>1.1689</td>
</tr>
<tr>
<td>123E Infection Collar (123E C)</td>
<td>1.3493</td>
</tr>
</tbody>
</table>

Chip analysis of Protein

The proteins extracted from plant tissues of Healthy plant, 100g Soil/ per plate isolate of 1AP and 123E, 1AP and 123E Fungi were detected on a protein chip 80 series (Figure 5). The gel shows the protein band size of each sample associated with an isolate. The sizing of proteins is a quantitative means of identification and characterization of proteins.
Figure 5. Gel image of protein extracts from Healthy plant, 100g Soil/plate of 1AP and 123 plants and protein extracts from fungal tissue. The C is “collar”, the base which a dark-green portion of the stem area closer to the surface of the soil. The E is “eye”, root which refers to the buried portion of the plant in close contact with the seeds. 1AP F and 123E F represents protein extracts directly from the fungal tissue (1AP and 123E isolates).

Figure 5, shows the difference in each sample by size in kDa. Protein size of the 1AP infection at the base of the plant (1AP C) was 14.7kDa whiles that of root of the plant (1AP E) was 13.3kDa. In the 123E infection, the protein size recorded at the base of the plant (123E C) was 9.7kDa and that of the root (123E E) was 12.0kDa. For the no treatment or healthy plant, the protein size at the base of the plant (Healthy C) was 11.1kDa, whiles that of the root (Healthy E) was 9.8kDa. The size of the protein extract directly from 1AP (1AP F) and 123E (123E F) fungus was 12.0kDa and 11.6kDa respectively (figure 5).

Three samples (Healthy C, 1AP C and 123E C) were selected for Two Dimensional gel and Mass spec analysis. Below is an electropherogram of the selected samples and their relative band size and concentration detected by the protein chip analysis Figure 6 (Healthy C), 7 (1AP C) and 8 (123C).
Figure 6. Healthy C Electropherogram

<table>
<thead>
<tr>
<th>Size [kDa]</th>
<th>Rel. Conc</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.6</td>
<td>0</td>
</tr>
<tr>
<td>3.1</td>
<td>0</td>
</tr>
<tr>
<td>4.6</td>
<td>0</td>
</tr>
<tr>
<td>7.9</td>
<td>903.2</td>
</tr>
<tr>
<td>11.1</td>
<td>3,703.00</td>
</tr>
<tr>
<td>34.9</td>
<td>19.1</td>
</tr>
<tr>
<td>39</td>
<td>3</td>
</tr>
<tr>
<td>51.9</td>
<td>3.3</td>
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<tr>
<td>84.5</td>
<td>4.2</td>
</tr>
<tr>
<td>95</td>
<td>60</td>
</tr>
</tbody>
</table>

Figure 7. 1AP C Electropherogram

<table>
<thead>
<tr>
<th>Size [kDa]</th>
<th>Rel. Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>1.6</td>
<td>0</td>
</tr>
<tr>
<td>3.3</td>
<td>0</td>
</tr>
<tr>
<td>5.7</td>
<td>62,223.80</td>
</tr>
<tr>
<td>6.3</td>
<td>9,511.20</td>
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<td>7.2</td>
<td>18,716.20</td>
</tr>
<tr>
<td>9</td>
<td>25,420.30</td>
</tr>
<tr>
<td>13</td>
<td>43,905.80</td>
</tr>
<tr>
<td>14.7</td>
<td>36,572.10</td>
</tr>
<tr>
<td>35.2</td>
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<tr>
<td>52.6</td>
<td>114.8</td>
</tr>
<tr>
<td>54</td>
<td>361.7</td>
</tr>
<tr>
<td>95</td>
<td>60</td>
</tr>
</tbody>
</table>
Protein extracts from different inoculum level of 1AP (5g, 100g, 15g and 20g per 100g Soil) were run on protein chip and each treatment differentiated by size in kDa (Figure 9). For the 5g 1AP / 100g Soil, protein size at the base (1AP 5C) and root (1AP 5E) was 12.8kDa and 13.5kDa respectively. In the 15g 1AP / 100g Soil, protein size at the base (1AP 15C) was 14.3kDa and that of the root was 15.3kDa. Protein size for 20g 1AP / 100g Soil inoculation at the base of the plant (1AP 20C) was 13.0kDa, whiles that of the root of the plant (1AP 20E) was 17.0kDa (figure 9).
Protein extracts from a 12 days tissue culture samples from each isolates were also protein chipped to determine their size on the gel. Lane 1AP 1, 1AP 2, and 1AP 3 are 1AP tissue culture protein sample while Lane 123E 1, 123E 2 and 123E 3 are 123E tissue culture protein sample (figure 10). The size of the 1AP infection protein in the tissue culture, that is 1AP 1, 1AP 2, 1AP 3 were 16.9kDa, 22.4 kDa and 15.2kDa respectively. In the 123E tissue culture experiment, the size of protein, 123E 1, 123E 2, 123E 3 was 22.6 kDa, 22.7 kDa and 23.7 kDa respectively (figure 10) after 12 days.

![Figure 10](image)

**Figure 10.** Tissue culture infection of 1AP and 123E for 12 days.

**Two Dimensional Gel (2D gel) Electrophoresis and Mass Spec Analysis**

Three samples (Healthy C, 1AP C and 123E C) were selected for 2D gel electrophoresis and Mass spec Analysis. The samples were sent to Applied Biomics, 23785 Cabot Blvd, Suite 311-313 Hayward, CA 94545, USA. The project contact at Applied Biomics was John Liao, PhD and data was reported under the Project name: ACDO160426.
Two Dimensional Gel Electrophoresis Image of Samples.

2D gel electrophoresis technique enables the separation of proteins in 2 dimensions. The first dimension (horizontal dimension) is where isoelectric focusing (IEF) is used to separate proteins based on their charge (pl). The second dimension (vertical dimension), is where SDS-PAGE is used to separate proteins based on their size (molecular weight, MW).

Figure 11. Two Dimensional Gel Electrophoresis Image with size
Figure 12. Side-by-side black/white image of each sample

Figure 13. Side-by-side black/white image of Healthy plant, 123E Fungi and a color image of their paired sample
Figure 14. Side-by-side black/white image of Healthy plant, 1AP Fungi and a color image of their pair.

Figure 15. Side-by-side black/white image of 1AP Fungi, 123E and color image of their pair.
Figure 16. Side-by-side overlay color images of each paired samples
Enlarged overlay image of each pair

**Green:** Healthy

**Red:** 123E

Figure 17. Overlay image of Healthy (No treatment) and 123E isolates with well-defined spots
The enlarged overlay has well selected-resolved protein spots. These spots were circled and numbered (1 – 40) in the overlay gel image.

**Green:** Healthy  
**Red:** 1AP

---

**Figure 18.** Overlay image of Healthy (No treatment, green spots) and 1AP (red spots) isolates with well-defined spots
Green: 1AP  
Red: 123E

Figure 19. Overlay image of 1AP (green spots) and 123E (red spots) isolates with well-defined spots
Figure 20. Shows unique protein spots identified in each sample
Decyder analysis of protein samples

In the Decyder analysis, the upper left shows the gel spot view of the samples. The yellow spots are shown in the lower panels. The upper right is the spot distribution panel. This indicates threshold level, 2 S.D, the number of spots within the threshold, the number of spots that are increased or decreased. The height of a spot indicates the volume of the spot and the curves indicates the number of spots.

Figure 21. Image of 123E / HEALTHY treatment decyder analysis

The lower left is the 3D view, which shows the characteristics of the spot of interest. Lastly the lower right gives the table view. Table view indicates all spots of interest. The spots that shows at the 3D view is highlighted. The fold change (increase or decrease) of each spot is indicated.
Figure 22. Image of decyder analysis of 1AP/HEALTHY treatment
Figure 23. 123E / 1AP image decyder analysis with 3D view

Protein Expression Ratio

Table 4. The Ratio of protein expression in each pair samples

<table>
<thead>
<tr>
<th>Assigned ID</th>
<th>Original No.</th>
<th>123E / Healthy</th>
<th>1AP / Healthy</th>
<th>123E / 1AP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>139</td>
<td>3.4</td>
<td>-1.43</td>
<td>4.91</td>
</tr>
<tr>
<td>2</td>
<td>328</td>
<td>-4.98</td>
<td>1.32</td>
<td>-6.54</td>
</tr>
<tr>
<td>3</td>
<td>640</td>
<td>2.87</td>
<td>1.56</td>
<td>1.85</td>
</tr>
<tr>
<td>4</td>
<td>700</td>
<td>-4.4</td>
<td>1.24</td>
<td>-5.43</td>
</tr>
<tr>
<td>5</td>
<td>558</td>
<td>-4.12</td>
<td>-2.64</td>
<td>-1.55</td>
</tr>
<tr>
<td>6</td>
<td>749</td>
<td>-2.72</td>
<td>2.13</td>
<td>-5.75</td>
</tr>
<tr>
<td>7</td>
<td>893</td>
<td>2.85</td>
<td>1.38</td>
<td>2.08</td>
</tr>
<tr>
<td>8</td>
<td>982</td>
<td>5.3</td>
<td>1.17</td>
<td>4.59</td>
</tr>
<tr>
<td>9</td>
<td>983</td>
<td>7.44</td>
<td>1.13</td>
<td>6.61</td>
</tr>
<tr>
<td>10</td>
<td>1047</td>
<td>9.67</td>
<td>-1.04</td>
<td>10.17</td>
</tr>
<tr>
<td>11</td>
<td>1025</td>
<td>6.97</td>
<td>-1.04</td>
<td>7.34</td>
</tr>
<tr>
<td>12</td>
<td>1014</td>
<td>8.13</td>
<td>-1.06</td>
<td>8.68</td>
</tr>
</tbody>
</table>
The ratio of protein expression are listed in the table above, the positive and negative numbers indicate up and down-regulated proteins, respectively.

**Mass Spec Analysis**

Five spots (2, 10, 14, 20, and 34) were selected for protein identification. These spots were randomly selected base on their size, pH and protein expression ratio in each paired sample. The protein identification was based on peptide fingerprint mass mapping (using MS data) and peptide fragmentation mapping (using MS/MS data). The MASCOT search engine was used to identify proteins from primary sequence databases. Using the five spots 2, 10, 14, 20 and 34; 5-
methyltetrahydropteroyltriglutamate–homocysteine methyltransferase 1 OS=Arabidopsis thaliana GN, VP3 [Epizootic hemorrhagic disease virus], Patatin-1-Kuras 2 OS = *Solanum tuberosum* GN=pat1-k2 PE=1 SV=1, Genome polyprotein (Fragment) OS=Potato virus Y (strain Chinese) PE=3 SV=1 and Abscisic stress-ripening protein 1 OS = *Solanum lycopersicum* GN=ASR1 PE=2 SV=1 respectively.

### Table 5. Size and pH of Protein Spots selected for Protein Identification

<table>
<thead>
<tr>
<th>Spot Number</th>
<th>Size (kDa)</th>
<th>PH</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>75</td>
<td>6.8</td>
</tr>
<tr>
<td>10</td>
<td>38</td>
<td>5.2</td>
</tr>
<tr>
<td>14</td>
<td>37</td>
<td>5.5</td>
</tr>
<tr>
<td>20</td>
<td>32</td>
<td>7.2</td>
</tr>
<tr>
<td>34</td>
<td>12</td>
<td>7.4</td>
</tr>
</tbody>
</table>
CHAPTER FIVE
DISCUSSION

Total protein were successfully isolated from Fungal tissues (1AP and 123E isolate, Healthy (No treatment), Section of plants growing in 1AP and 123E infected soil.

Purity and Protein Concentrations

The protein concentration of the Healthy (non-inoculated) were generally higher than the protein concentrations from other treatment (1AP infection and 123E infection) (Table 3). In addition, protein concentration from the “eye” (roots) of the potato were lower than that of the protein concentrations of the “collar” (base of the plant) across all three treatments. However, protein concentrations from the 1AP (virulent strain) infection were higher than protein concentrations from 123E infection. Protein concentration from the fungal tissue were higher than all other three treatment.

Chip Analysis of Proteins

The protein chip technique was very sensitive and allowed to detect small concentrations of the protein in a plant-microbe interaction treatment. From the three treatments, proteins from the 1AP infection had higher size (14.7 kDa) than other treatments (figure 5). The large size of the 1AP infection protein might have a role in virulence or pathogenicity. The protein size at the base of the plant from the 1AP infection was higher (14.7 kDa) than the size at the root of the plant (13.3 kDa) (figure 5). The same (Healthy C = 11.1 kDa, Healthy E = 9.8 kDa) was observed in the healthy (no treatment or inoculation). However, the size of the protein at the base (123E C = 9.7) of the plant in the 123E infection was lower that the size at the root (123E E = 12.0kDa) of the plant. The size of the protein extracts from the base of the plant were generally higher and were as result selected for 2D gel and Mass spec analysis Figure 6, 7 and 8.
The inoculum density greatly influenced the level of expression of the 15kDa protein. Analysis of the protein samples from all such treatments of 1AP (5g, 10g, 15g and 20g per 100g soil) showed that protein sizes were generally higher at the root “eye” (1AP 5E = 14.7kDa, 1AP 15E = 15.2kDa and 1AP 20E = 15.4kDa) figure 9. Generally greater expression of these protein with higher inoculum level were observed. Similar results have been reported in other infections like verticillium wilt of artichoke caused by *Verticillium dahlieextracts* (Berbegal et al., 2007) or spore density as a factor to determine infection strategy by plant pathogenic fungus *Plectosphaerella cucumerina* (http://www.plantphysiol.org/ June 13, 2016).

In the tissue culture experiment, Analysis of protein samples showed that the proteins sizes were generally higher (above 15kDa) after 12 days. This might be due to the fact that the fungus was in direct contact to the potato seeds germinating. The protein size’s from the tissue culture protein samples (figure 10) were generally higher than the size of the protein extract from the fungal tissue and the three treatments in this study (Figure 5). The protein size of the 123E tissue culture sample (123E 1, 123E 2, 123E 3) were higher than the 1AP tissue culture sample (1AP 1, 1AP 2, 1AP 3) after 12 days of fungal contact.

2-Dimensional Gel Analysis and Mass Spectrophotometry

Analysis of 2D Gel

2D gel analysis was done on three protein samples (Healthy C, 123E C and 1AP C). The protein samples were separated based on size and charge on the proteins (Figure 11, 12, 13, 14, and 15). The 1AP infection proteins and Healthy (No treatment) proteins have a lot of common spots, and not much very significant changed spots. However, 123E infection protein have some very significantly changed spots compared with other treatments or 2 samples. There were 40 well selected-resolved protein spots (Figure 16, 17, 18). Out of the 40, there were 4 unique healthy
spots, 10 unique 1AP infection spots and 26 123E infection spots (Figure 19). All spots had different size (kDa) and pH (Figure 20). The changed protein spots in the 123E infection protein could be either the new proteins from fungi, or from the plant or proteins with the expression changed due to the infection. The protein expression ratio in the 1AP/Healthy were generally lower compared to the ratio of the protein expression in 123E/Healthy and 123E/1AP. The positive and negative values indicate up and down-regulated proteins (Table 4).

Decyder analysis software were also run along the 2D gel electrophoresis analysis. This analysis is key in (2D-DIGE) analysis as it provides the option of referencing spot to an internal standard of the protein. This effectively eliminates gel-gel variation and delivers reproducible and dependable quantitation of spots on a gel (Figure 21, 22, 23).

Mass Spectrophotometry Analysis

Five spots were identified by Mass Spec analysis. The five spots 2, 10, 14, 20 and 34 were 5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase 1 OS=Arabidopsis thaliana GN, VP3 [Epizootic hemorrhagic disease virus], Patatin-1-Kuras 2 OS=Solanum tuberosum GN=pat1-k2 PE=1 SV=1, Genome polyprotein (Fragment) OS=Potato virus Y (strain Chinese) PE=3 SV=1 and Abscisic stress-ripening protein 1 OS=Solanum lycopersicum GN=ASR1 PE=2 SV=1 respectively. Almost all the protein spots identified played major role in potato infection process and disease establishment except for VP3 (Epizootic hemorrhagic disease virus) which is found to be associated with animal and human viral proteins. The Genome polyprotein (Fragment) OS=Potato virus Y (strain Chinese) PE=3 SV=1 and VP3 (Epizootic hemorrhagic disease virus) were not discussed in this project due to its non-correlation in the present study, however future works will investigate why the two proteins were detected in the present study.
Abscisic stress-ripening protein 1 OS=Solanum lycopersicum GN=ASR1 PE=2 SV=1

ABSCISTIC ACID STRESS RIPENING (ASR) gene family, ASR1, was first was isolated by screening a tomato fruit cDNA library with cDNA from stressed leaves (Lusem et al., 1993). ASR gene families are mostly found in genomes of both monocots and dicots (Carrari et al., 2004). ASR genes are known to be induced by abscisic acid (ABA) and abiotic stress (Liu et al., 2010; Phillipe et al., 2010; Joo et al., 2013). These genes have also been identified in ripening fruits and during potato tuber development (Frankel et al., 2007; Chen et al., 2011, Luo et al., 2014). ASR proteins belongs to the hydrophilic group of proteins (Battaglia et al., 2008). Tomato ASR gene family comprises of five genes (AR1 – AR5) that are localized on chromosome 4. AR1, AR2, AR3 and AR5 encodes low molecular weight proteins, although ASR4 encodes a polypeptide that is double the size of the other proteins (Frankel et al., 2006; Fischer et al., 2013). ASR1 possess Zn^{2+} dependent DNA-binding activity (Kalifa et al., 2004; Rom et al., 2006). ASR1 becomes structured, dimerizes and translocated to the nucleus once bounded to ZN^{2+} (Goldgur et al., 2007). Nuclear ASR proteins are known to modulate gene expression and it genes plays a critical role in drought and salinity stress. The overexpression of ASR genes increases the tolerance of transgenic plants to water, salinity and cold stresses (Jeannau et al., 2002; Yang et al., 2005; Saumonneau et al., 2008). ASR1 are mostly expressed in stems, roots and reproductive organs (Golan et al., 2014). The protein expression ratio of Abscisic stress-ripening protein 1 was upregulated in 123E infection/ Healthy no treatment (2.5) and 1AP infection/Healthy no treatment (3.58). However, ASR1 was down regulated in 123E infection/1AP infection (-1.42).
Patatin-1-Kuras 2 OS=Solanum tuberosum GN=pat1-k2 PE=1 SV=1

Patatin is a glycoprotein with different isoforms, found in all Solanum species (Pots et al., 1999). Patatins are known potato tuber proteins with acyl-hydrolyzing activity (LAH). Presently, patatin-related enzymes has been found to involved in different cellular functions; these includes plant responses to auxin, elicitors or pathogens, lipid mobilization during seed germination and abiotic stresses (Scherer et al., 2010). The patatin catalytic domain are generally detected in bacterial, yeast, plant and animal enzymes. High values of specific LAH activity were found in patatin samples throughout most cultivars. Currently, individual patatin forms are proposed not to have similar physiological roles. These supports the assertion that patatin does not only have storage protein capacity but also have a part of potato defense mechanism (Barta et al., 2012). Kunitz-like protease inhibitors has been associated with patatin glycoprotein in mature potato tubers (Bauw et al., 2006; Lehesranta et al., 2006). In potatoes, the patatin multigene family normally contains 10 – 18 members per haploid genome (Twell and Ooms, 1988). Patatin plays a central role in plant signaling using its LAH activity, which is related to the Phospholipase A₂ (Rydel et al., 2003). Phospholipase A₂ (PLA₂) catalyzes the hydrolysis of glycerophospholipids at the sn-2 position to liberate arachidonic acid and lysophospholipid that perform essential roles in plants and animals (Murakami and Kudo, 2004; Ryu, 2004). PLA₂ has been detected in cellular plant processes such as growth, development, stress responses and defense signaling (Wang, 2001).
5-methyltetrahydropteroylglutamate--homocysteine methyltransferase 1 OS=Arabidopsis thaliana GN

5-methyltetrahydropteroylglutamate--homocysteine methyltransferase 1 enzyme catalyzes the transfer of methyl group from 5-methyltetrahydrofolate to homocysteine resulting in methionine formation. Methionine are synthesized de novo by plants and most microorganisms (Thomas and Surdin-Kerjan, 1997). It plays a central role in cellular metabolism. Methionine belongs to the aspartate family of amino acids that constitutes lysine, threonine and isoleucine. The biochemical and molecular control study of methionine provides insight in the homeostatic regulation of amino acids in plants. S-methylmethionine (SMM) a derivative of Methionine serves a transport molecule for reduced Sulphur in some plant, connecting sink and source organs (Bourgis et al., 1999).
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