PROTEOMIC ANALYSIS

OF APHID-WHEAT

INTERACTIONS

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INTRODUCTION

Wheat, *Triticum aestivum* L., is an extremely important agronomic crop worldwide, grown on one-seventh of the earth's arable cropland. In the U.S. it is grown in 42 states, and is Oklahoma's number one cash crop with a gross value of $326 million in 2000 (Oklahoma Dept. of Agriculture). Wheat is the most widely grown and consumed grain in the world, and contributes 10-20% of the daily caloric intake for people from over 60 countries. Over the last 20 years, wheat yields have increased by approximately half a bushel per acre per year, but there is concern that even this will not be enough to keep up with the incessantly increasing demand. Wheat is the only grain with a high enough gluten content to make leavened (raised) bread so the demand is extremely high. Based on today's yields, a single acre of Kansas wheat produces enough flour to supply an American family with bread for ten years. The problem is that the International Food Policy Research Institute projects that developing countries will need to double the amount of wheat they import by the year 2020 or find an alternative food source. The International Maize and Wheat Improvement Center (CIMMYT) has stated that while worldwide demand will increase by over 40% by 2020, the land and resources available will decrease significantly if current trends prevail (Rosegrant et al. 1997). The progress made during the first green revolution of the 60s and 70s is not going to be enough; we need to find a way to produce more grain on less land by determining the limiting factors on production. Plant pathologists are making great strides, evaluating the pathogens that limit wheat production and studying plant-pathogen interactions. Entomologists have

fallen dangerously behind, not even understanding the basic mechanisms of feeding of the most detrimental wheat pests.

One such aphid pest is the greenbug, *Schizaphis graminum* (Rondani) (Homoptera: Aphididae). In the plains states, the greenbug is a major pest of wheat, barley, oats, rye, and sorghum, with barley being its preferred host and the grain it is most successful on (Starks & Burton 1977). In Oklahoma, greenbugs are a severe pest on wheat causing 35-60% reductions in yield (Kieckhefer & Kantack 1988) and economic losses up to $135 million annually during outbreak years (Webster 1995). Greenbugs attack the winter wheat grown here throughout the fall and spring (Burton et al. 1985) and though they are typically present and damaging every year, widespread outbreaks only occur every 5 to 10 years (Hatchett et al. 1987; Porter et al. 1991).

Another aphid pest is the bird cherry-oat aphid, *Rhopalosiphum padi* (L.) (Homoptera: Aphididae). This aphid is a severe pest of cereals, particularly on winter wheat in the northern plains states (Dean 1973; Yount 1985; Kieckhefer & Kantack 1988). The bird cherry-oat aphid (BCO) attacks all small grains, but is most damaging to fall-planted wheat prior to the winter dormancy period, causing yield losses ranging from 20% in low to moderate infestation years and up to 75% during heavy infestations (Pike & Schaffner 1985; Kieckhefer & Kantack 1988; Summers et al. 2002). The BCO aphid damages plants directly as a result of feeding, but is more problematic as a vector of barley yellow dwarf virus (BYDV) (Bruehl 1961). Comparatively, BYDV infection of fall-planted wheat has been shown to cause much more severe reductions in yield than direct BCO aphid feeding damage, 46% to 91% in low to moderate BCO aphid infestation years (Palmer and Sill 1966; Fitzgerald & Stoner 1967; Carrigan et al. 1981).

The aphid and virus can induce further grain yield losses by reducing crop tolerance to environmental stresses as well (Riedell et al. 1999).

Both species, like all aphids, have piercing sucking mouthparts, which they use to penetrate plant tissue during feeding. However, the two species induce very different symptoms in plants as a result of their feeding. Greenbug feeding causes chlorotic lesions at the sight of feeding, and they are said to induce a senescence-like state in plants (Starks & Burton 1977a; Dorschner et al. 1987; Sandstrom et al. 2000). BCO aphids do not induce this senescence-like state nor do they cause any foliar symptoms, unless populations get extremely high which they rarely do under field conditions. Little is known about the biochemical processes involved in aphid feeding and even less is known about changes in protein expression as a result of aphid feeding. Only through better understanding of plant-pest interactions will we be able to combat pests effectively and meet the world's increasing demand for more wheat. The overall goal of the research conducted for this dissertation is to gain a better understanding of aphid-plant interactions by comparing two species of aphids that produce different plant symptoms.

OBJECTIVES

Objective 1. Develop two-dimensional SDS-PAGE system for mapping and evaluating the wheat proteome. The goal of this objective was to adequately separate and map proteins on two-dimensional gels, and then harvest, process and analyze those proteins using mass spectrometry to obtain identity of some of the abundant proteins in wheat extracts.

Objective 2. Evaluate changes in susceptible wheat protein profiles in response to aphid feeding. Protein expression was evaluated, following 24h or 6d of greenbug or bird-cherry oat (BCO) aphid feeding, using the two-dimensional gel system developed in objective one. The two aphids induce different symptomology, the greenbug causes visual foliar and root symptomology and the BCO aphid causes only visual root symptomology. The goal of this objective was to determine if any differences in protein expression could be observed and identified, in an attempt to elucidate potential aphid-induced plant defense-response pathways.

Objective 3. Evaluate changes in resistant wheat protein profiles in response to aphid feeding. Protein expression was evaluated, following 24h or 6d of greenbug or BCO aphid feeding, using the two dimensional gel system developed in objective one. The goal being to evaluate differential protein expression induced by the aphids in resistant wheat, and to compare those differences to those observed in the susceptible wheat protein profiles from objective two to gain better understanding of aphid-wheat interactions.

Objective 4. Evaluate different methods for removing Rubisco from wheat extracts, using preparative isoelectric focusing, immunoaffinity chromatography, and HPLC gel filtration. The goal of this objective was to remove Rubisco in an attempt to increase loading capacity of proteins of lower abundance onto two-dimensional SDS gels.

LITERATURE REVIEW

Wheat is currently grown on approximately 58.8 million acres of U.S. farmland, but it is not actually a native grass species (www.nass.usda.gov/census/census97/vol1/us-51/us1figs.pdf). Wheat was originally a wild grass, first described as an edible grain approximately 10,000 years ago in Mesopotamia and regions around the Tigris and Euphrates River Valleys. The Egyptians were the first to realize its full potential, using it to make yeast-leavened breads as early as 2000 BC. Wheat was not grown to any large extent in the US until the plains states were settled, and the first major farming of wheat was not witnessed until the early 1800s in Kansas. Russian Mennonites are credited with introducing Turkey Red Winter Wheat to Kansas between 1874 and 1884. The United States Department of Agriculture in the early 1900s made an expedition to Europe to identify other potentially suitable wheat varieties to be imported for use here. Turkey Red Winter Wheat and the varieties imported by the USDA make up the genetic stock that nearly all hard red winter wheat grown in the plains states are derived from. The varieties of wheat grown in the U.S. are grouped into six major classes: soft red winter wheat, grown in the Eastern United States; soft white wheat, grown in the Pacific Northwest; hard red spring wheat and durum wheat, grown in the Northern Plains States; and hard red winter and white wheat, grown in the Central and Southern Plains States. Hard red winter wheat is the most widely grown class in the U.S.

Wheat is not only grown for flour; in the Southern Plains of the U.S., it is also produced as an alternative forage crop for cattle. Winter wheat grown in this region is typically planted in September, and grows vegetatively until the late fall/early winter when it enters dormancy. Growth continues in the early spring, with cattle grazing on the

vegetative growth until the crop reaches first hollow-stem growth stage, usually in March. In Oklahoma, during an average year, three acres of winter wheat produces enough forage to feed a single steer throughout the winter, and approximately 50 to 55% of the planted wheat is grazed (Thompson 1990; Carver et al. 1991). The quality of winter wheat forage is highly nutritious, being almost identical to alfalfa in crude protein content and digestibility (Krenzer 1999). However, wheat is primarily used as a food crop worldwide.

Greenbug History:

The greenbug was first described in Italy in 1847 by Rondani (Burton et al. 1985), and was not observed in North America until 1882 when it was found in Virginia (Hunter 1909). Within 25 years, it spread throughout most of the United States, central Canada and northern Mexico (Webster & Phillips 1912; Wadley 1931; Leonard 1968; Porter et al. 1997). Wood (1961) was first to introduce the term biotype, which he used to describe greenbug populations that differed in their ability to damage plants. Wood (1961) determined that the formerly resistant variety of wheat, Dickinson 28-A, was no longer resistant to all greenbugs. The greenbug population that overcame the resistant Dickinson 28-A wheat was called biotype B, and the greenbug population that Dickinson 28-A was still resistant to was called biotype A (Wood 1961; Starks & Burton 1977). We now recognize biotypes A through K, although biotype A is thought to be extinct (Porter et al. 1997). Biotype E (Porter et al. 1982) was, as of a 1987 survey, the most prevalent biotype in the Southern Plains States (Kerns et al. 1987). The biotypes are virtually impossible to distinguish morphologically and have to be identified according to the

physiological characteristics such as fecundity and survival rates on various host plant varieties (Starks & Burton 1977).

Greenbug Biology:

Greenbugs are small, pale green aphids with a dark green dorsal line, black eyes and cornicles, and black tipped antennae. Greenbugs develop through four nymphal stages, not obtaining their dorsal line until they reach adulthood (Wadley 1931). The greenbug life cycle is holocyclic varying between monoecious holocycly in cooler climates and anholocycly in warmer climates (Porter et al. 1997). Greenbugs are tolerant to a wide range of temperatures, and can reproduce and develop from 40°F to 100°F but they are most fecund between 70° to 75°F (Starks & Burton 1977). Their annual life cycle typically proceeds from overwintering fertilized eggs, which all hatch into wingless fundatrix females. These females in turn give live birth to parthenogenically reproduced females called viviparae or virginoparae. The virginoparae are either alate (winged) or apterous (wingless) depending on environmental conditions, including temperature and photoperiod (Hardie 1990). If conditions become too harsh, a final sexual generation will be produced by the virginoparae giving rise to males and oviparae females which would complete the cycle by fertilizing and laying eggs that can overwinter (Hales et al. 1996). In the Southern Plains States greenbugs do not overwinter as eggs but continue parthenogenic reproduction year round (Webster & Phillips 1918; Wadley 1931; Starks & Burton 1977). All parthenogenic females can give birth to alate or apterous adults. Winged females are capable of parthenogenic reproduction within 24 to 48h while wingless females may begin reproduction almost immediately (Wadley 1931). Females may continue to reproduce for up to 20 to 30d, and can produce 50 to 100 progeny each

(Starks & Burton 1977). Each parthenogenic female contains a predetermined number of ovaries and while she is giving birth to one generation of offspring, her next generation is already in its final embryonic state. This "telescoping" of generations allows greenbugs to increase their numbers quickly (Hales et al., 1996). The number of parthenogenic generations per year depends on temperature and other factors such as drought and overall host-plant vigor (Starks & Burton 1977). Aphid parthenogenic reproduction is apomictic and thus does not involve meiotic division and recombination. The offspring produced by a single fundatrix are genetically identical and are "clones" (Hales et al. 1996). The genetic variation found in aphids comes from mutations, most often in the form of chromosomal rearrangements (Asher, 1970).

Greenbug Feeding Information:

Greenbugs, like other Homopterans, have piercing-sucking mouthparts, which they insert into plant tissue to feed. Mouthparts modified for piercing-sucking form a long stylet made up of the elongated maxillae surrounded and protected by the tougher elongated mandibles. As the aphid penetrates leaf tissue to feed, it injects saliva into the plant forming a hard protein sheath to protect and provide rigidity to the flexible stylet. The aphid ultimately penetrates into target cells, feeding on plant fluids which it sucks up its stylet. The damage inflicted by their feeding includes necrotic lesions at the sight of penetration, surrounded by chlorotic halos (Ryan et al. 1987a) (for more details see Insect-Plant Interaction section). Extensive feeding causes reduced shoot and root biomass leading to a reduction in yield (Burton 1986).

Chatters and Schlehuber (1951) conducted the first in-depth study on greenbug feeding, and concluded that the greenbug penetrates through the plant's epidermal cells

with its stylet, ultimately feeding on phloem sap found in the vascular bundle cells. They were also the first to suggest that the causal agent of tissue damage is the injection of saliva and not the mere removal of fluid or uptake of food. Saxena and Chada (1971a) later found that greenbugs do not always feed on phloem sap; they determined that biotype B actually feeds in the mesophyll parenchyma when feeding on barley. The method of stylet penetration also differs from aphid to aphid and plant to plant, and may be influenced by pH, carbohydrate concentration, osmotic pressure, and physical features of the plant (Chatters & Schlehuber 1951; Pollard 1973). Sometimes greenbugs penetrate tissue intercellularly, and other times they pierce directly through cells intracellularly (McAllen & Adams 1961; Saxena & Chada 1971a; Wood 1971; Pollard 1973; Campbell et al. 1982; Al-Mousawi et al. 1983). Intercellular penetration seems to be preferred by many aphids since intracellular penetration induces plant wound responses which leads to the release of phenolic compounds that can be toxic to aphids (Miles 1990). McAllen and Adams (1961) concluded that stylet penetration not only varies due to the plant's physiological conditions, but also differs according to the enzyme concentration in the greenbug's saliva. They concluded that aphids with high pectinase levels in their saliva have the ability to pierce through the tissue intercellularly or intracellularly, while those with low pectinase levels only penetrate intercellularly.

Tissue damage resulting from greenbug feeding can be extensive. Saxena and Chada (1971a) described two types of cellular damage, vascular bundle damage which causes the phloem parenchyma cells to look hollowed out and collapsed after being fed upon, and damage to the mesophyll cells immediately surrounding the stylet sheath, which the greenbug creates during penetration. When the greenbug's saliva comes in contact with a

cell's contents, plasmolysis occurs. The cells organelles begin to swell and rupture, the nucleus becomes a homogeneous mass with no defined shape or structure, and eventually the cell wall ruptures. The rupturing of several cells creates vacuolar spaces filled with plasmolyzed cellular fluid which the aphids then suck up through the food canal of their stylet and consume (Saxena & Chada 1971a).

The components of greenbug saliva have been studied extensively by numerous groups (McAllen & Adams 1961; Saxena & Chada 1971b; Miles 1972; Pollard 1973; Miles 1987; Campbell & Dryer 1990; Ma et al. 1990; Miles 1990; Miles & Harrewijn 1991; Baumann & Baumann 1995). Miles (1972 and 1990) determined that there are two types of aphid saliva, watery and gelatinous. The gelatinous saliva contains the proteins that form the tough salivary sheath which protects the stylet during penetration. The proteins found in the watery saliva are divided into two categories; those that help penetrate the plant tissue and those that help detoxify plant defensive compounds (Miles 1990). The pectinases and cellulases are thought to aid in the penetration of leaf tissue (Campbell & Dryer 1990; Ma et al. 1990; Miles 1990). The polyphenol oxidases and peroxidases found in aphid saliva are thought to help the aphids detoxify the plant's defensive compounds (Miles 1990; Peng & Miles 1991). The exact function of the other enzymes found in aphid saliva such as carbohydrases, esterases, lipases, and proteinases (McAllen & Adams 1961; Pollard 1973) is not known.

The true mystery of greenbug feeding is their proposed ability to alter amino acid composition of their host plants. Phloem sap itself does not provide a high enough concentration of the essential amino acids to nutritionally sustain aphids, approximately 20% of the wheat phloem sap is essential amino acids, and aphids require a diet

containing approximately 50% (Slansky & Scriber 1985; Sandstrom & Pettersson 1994; Sandstrom & Moran 1999). However, aphids are able to survive on phloem sap, and this discrepancy has lead many authors to conclude that aphids must be altering the amino acid concentration of the phloem sap in some way (Way & Banks 1967; Way & Cammell 1970; Dorschner et al. 1987; Riedell 1989). Aphids are known to compensate for the poor nutritional quality of phloem sap with the help of bacterial symbionts, *Buchnera*, that live in their gut. The *Buchnera* provide at least some of their essential amino acid requirement but not all (Douglas 1990; Sasaki & Ishikawa 1990; Douglas & Prosser 1992; Lai et al. 1994; Bracho et al. 1995; Febvay et al. 1995; Liadouze et al. 1995; Baumann et al. 1997; Douglas 1998). Researchers have shown that the induction of chlorotic lesions on leaf tissue caused by aphid feeding is followed by increases in amino acid composition (Dorschner et al. 1987; Riedell, 1989). Aphid feeding consistently induces an increase in the proportion of the essential amino acids, but whether this is part of natural resource reallocation or translocation, or whether the aphids are forcibly inducing this alteration to benefit themselves is not known at this time (Sandstrom et al. 2000). However, Sandstrom and colleagues (2000) found that the amount of change in amino acid composition differs when plants are fed on by different aphid species. Greenbugs are able to induce a much higher essential amino acid composition than the BCO aphid. Consequently, the BCO aphid does not induce the typical chlorotic halo when feeding on wheat. This would appear to strengthen the argument that greenbugs are triggering a senescence-like response in the plant, which in turn triggers the mobilization of amino acids that benefits the aphids (Starks & Burton 1977; Dorschner et al. 1987; Sandstrom et al. 2000). Perhaps the BCO aphid does not trigger the senescence-

like response since it cannot induce chlorosis, and therefore there are a lower proportion of essential amino acids available for it.

Greenbug biotypes and other aphid species that are unable to induce the chlorotic or senescence-like response are typically called avirulent. These avirulent aphids are apparently unable to modify the host plant's metabolism and as a result have lower fecundity (Ryan et al. 1987a). A greenbug biotype or other aphid species that is capable of inducing the senescence-like response and of high reproduction levels on a particular plant is considered virulent (Dorschner et al. 1987). This categorization is not static however; Dorschner and colleagues (1987) found that biotypes that were considered avirulent on a particular variety of wheat were later able to grow and reproduce successfully on that same variety if a virulent biotype fed on it first and induced the senescence-like state for them. This process is called conditioning. Conditioning can even allow aphids to develop and reproduce successfully on a plant that was formerly resistant to them (Dorschner et al. 1987).

Morgham and colleagues (1994) suggested that the definitive resistant plant may be one that the greenbug is unable to modify metabolically and therefore is not as successful on. The earliest proposed mechanisms of resistance were morphological in nature; Chatters and Schlehuber (1951) thought resistance had to do with elevated amounts of schlerenchyma in the plant leaves interfering with penetration of aphid stylets, Gibson (1971) proposed that hairy leaves were the cause, and Parry (1971) thought resistance was due to thickened plant cell walls. Al-Mousawi and colleagues (1983) compared resistant and susceptible wheat ultrastructurally and determined that there is no difference in tissue type or organization between the two. Other studies proposed that resistance

was due to the presence or absence of a particular chemical compound. Juneja and colleagues (1972) reported that resistant plants contain benzyl alcohol while susceptible plants do not, and that exogenous benzyl alcohol lowered greenbug fecundity when they were feeding on susceptible barley. These results lead them to conclude that benzyl alcohol could be responsible for greenbug resistance in barley and sorghum (Juneja et al. 1975). Argandona and colleagues (1981) reported that phenolic compounds played a role in resistance, while other studies purported that hydroxamic acid was responsible in some way for plant resistance (Todd et al. 1971; Woodhead & Cooper-Driver 1979). To add confusion to the situation, Dorschner and colleagues (1987) then stated that contrary to several reports, Todd et al. (1971), Juneja et al. (1972, 1975), Dreyer & Jones (1981), Dreyer et al. (1981), Campbell et al. (1982), Argandona et al. (1983), and Montllor et al. (1983), there is "little evidence" to support the presence of a specific feeding deterrent or other substance in resistant plants that confers resistance to greenbug feeding. Al-Mousawi and colleagues (1983) proposed that cell walls in resistant wheat may contain some structural component that greenbugs are unable to alter or affect. A similar theory was proposed by Chatters and Schlehuber (1951), who found that phloem cell walls stain differentially after greenbug feeding indicating differences in chemical composition. This theory was later explored by Ryan and colleagues (1987b), who found that resistant plants have higher levels of extractable pectins than their susceptible isogenic counterparts. More recently, various compounds like methyl salicylic acid and certain lipids (C_6 volatiles) have been found to deter aphid settling (Hardie et al. 1994) and reduce fecundity respectively (Hildebrand et al. 1993; Shulaev et al. 1997), and these compounds are known to be upregulated more quickly in resistant plants (van der

Westhuizen et al. 1998a,b; Walling 2000). These are some of the other compounds that have recently been reported to play some, as yet undetermined, role in aphid resistance: acyl sugars, glucosinolates and hydroxamic acid (Blauth et al. 1998; Giamoustaris & Mithen 1995; Gianoli & Niemeyer 1998). One thing remains clear, we still have no clear understanding of what makes a resistant plant able to prevent, tolerate or reduce greenbug feeding.

Greenbug Control:

Greenbug control is a serious concern in Oklahoma and in the rest of the Southern Plains States since this aphid causes such severe economic losses. The greenbug does have many natural enemies: lady bird beetles, damsel bugs, lacewings, syrphid flies, numerous parasitic wasps, and spiders (Royer et al 1998a) but at natural endemic levels they do little to prevent the cyclic greenbug outbreaks. There are recommended release rates for certain predators and parasites for use in Oklahoma (Royer et al. 1998b) but classic biological control has not been widely embraced. Insecticides are still the most commonly used method of greenbug control, and there are several approved for use against greenbugs; malathion, parathion, methyl parathion, imidacloprid, chlorpyrifos, dimethoate and disulfoton (Royer et al. 1998b). However, the misuse of these pesticides has lead to insecticide-resistance in greenbugs (Shotkoski et al. 1990; Wratten et al. 1990; Sloderbeck et al. 1991; Sloderbeck 1992; Peckman & Wilde 1993). For example, there has been noted greenbug resistance to organophosphate insecticides in Oklahoma as far back as 1975 (Teetes et al. 1975; Peters 1975). Because of the inherent problems associated with pesticide use, such as insect-resistance, public health concerns, and potential insecticide deregulation due to the Food Quality Protection Act, farmers and

plant breeders are looking for alternatives to protect crops from greenbugs. One of the most obvious alternatives is the use of greenbug resistant varieties of crop plants.

Painter (1951) described three types of natural or intrinsic host plant resistance, which is now commonly referred to as Painter's Resistance Triangle. The first corner of the triangle is antibiosis type resistance in which the host plant utilizes toxins or other compounds to inflict deleterious or antibiotic effects on herbivores. The second corner of the triangle is tolerance type resistance where the plant is able to withstand herbivore feeding without losing economic value. The final corner of the triangle is antixenosis type resistance in which traditional herbivore pests find a particular resistant variety unsuitable, this type of resistance is usually described as non-preference (Painter 1951). These forms of greenbug resistance occur naturally in several small grains such as barley (Jackson et al. 1964), triticale (Wood et al. 1974), and rye (Arriaga & Ree 1963). Some varieties of wheat have natural resistance as well, but these varieties impart only low levels of resistance to greenbugs (Starks & Merkle 1977).

The first truly greenbug-resistant wheat, Dickinson 28-A (DS 28A), was developed by Dahms and colleagues in 1955. This resistance was later attributed to tolerance mechanisms (Curtis et al. 1960; Painter & Peters 1956). Since that first line, which Wood had already found greenbugs virulent to by 1961, several others have been developed. Sebesta and Wood (1977) developed the wheat germplasm line Amigo, Joppa and colleagues (1980) the Largo resistant line, Martin et al. (1982) CI 17959, Tyler and colleagues (1987) CI 17882, and Porter et al. identified the GRS 1201 resistant line (Porter et al. 1991). The genes conferring resistance in the above lines are designated as g*b1*, *Gb2*, *Gb3*, *Gb4*, *Gb5* and *Gb6* for DS 28A, Amigo, Largo, CI 17959, CI 17882, and

GRS 1201 respectively (Tyler et al. 1987; Porter et al. 1994; Porter et al. 1997). These genes are all dominant except *gb1*, which is recessive (Gardenhire 1980), most confer antibiosis type resistance, and all are considered to be single genes (Porter et al. 1997).

There has been much speculation about the development of new biotypes being driven by selection pressures placed on the insects by resistant plant cultivars. Smith (1989) concluded from population simulation models (Kennedy et al. 1987; Gould et al. 1990) that the widespread use of insect-resistant plants with single genes conferring antibiosis resistance, puts too much selection pressure on greenbug populations leading to the evolution of new virulent biotypes (Porter et al. 1997). Smith (1989) further concluded that it is better to use multiple genes each with minor effects that confer antixenosis or tolerance type resistance since they impose less pressure on insect populations. However, Porter and colleagues (1997) found that the emergence of new greenbug biotypes is not correlated to the release of resistant wheat cultivars. They also reviewed data (Starks & Schuster 1976) on resistant sorghum releases and the timing of greenbug biotype emergence and again could find no direct relationship between release and new biotype emergence (Porter et al., 1997). There appears to be no consensus about what causes the occurrence of new biotypes. This inconsistency seems to indicate there is no one mechanism driving the phenomenon, and that a complex web of ecological relationships and interactions leads to the evolution of biotypes and perhaps resistant insects in general. One fact is certain however, without better understanding of how greenbugs interact with their host plants and more specifically how tolerant plants are able to withstand aphid feeding, there is little hope of ever attaining stable greenbug resistance.

Greenbug – Plant Interactions:

Characteristic macroscopic greenbug damage is observed as 1mm diameter necrotic feeding lesions surrounded by 2-3 mm diameter chlorotic halos (Wittenbach 1979). The first biochemical and microscopic symptomology of greenbug feeding is the degradation of ribulose bisphosphate carboxylase oxygenase (Rubisco) and the break down of the chloroplasts (Ryan et al. 1987b). Rubisco is involved in two competing reactions, photosynthetic CO_2 assimilation and photorespiratory carbon oxidation (Ishida et al. 1997); therefore, Rubisco concentration is correlated to CO_2 assimilation and stomatal conductance (Makino et al. 1985). Ryan and colleagues (1987b) noted that greenbug feeding triggered the inhibition of CO_2 assimilation, lowered stomatal conductance, and lowered chlorophyll concentrations. Gerloff and Ortman (1971) also observed significant reductions in chlorophyll as a result of greenbug feeding. Natural senescence also begins with the breakdown of Rubisco and the chloroplasts leading researchers to the conclusion that greenbugs induce a senescent-like state as a result of their feeding. Another consequence of natural senescence is the translocation of vast quantities of amino acids from mesophyll tissue into the phloem (Thimann 1980; Thomas & Stoddart 1980). Since Rubisco makes up greater than 50% of wheat's total protein content (Wittenbach 1979), Thomas and Stoddart (1980) concluded that a significant proportion of those amino acids come from the degradation of Rubisco. Sandstrom and colleagues (2000) found that aphid feeding (*Schizaphis graminum*, *Rhopalosiphum padi* and *Diuraphis noxia*) induces increased amino acid levels in grass species. Their group also found that aphid feeding resulted in an increase in glutamine as well. Nitrogen is translocated during natural senescence from the leaves to other sink organs in the form of glutamine (Kamachi et al.

1991; Watanabe et al. 1997). All of these facts taken together seem to indicate that greenbugs and other aphids cause plants to senesce. The common theory embraced by aphid biologists is that aphids induce this state systemically in order to nutritionally benefit from the increased amino acid levels which result from the breakdown of Rubisco and other cellular components during senescence (Starks & Burton 1977; Dorschner et al. 1987; Sandstrom et al. 2000).

Time course ultrastructural studies have also been conducted to determine what effects greenbug feeding has on wheat at the cellular level (Al-Mousawi et al. 1983; Morgham et al. 1994). The initial effect of feeding is organelle degeneration in the phloem parenchyma cells, which occurs within the first hour of feeding. The cells are dead, devoid of intact organelles, by 24h post-infestation. The mesophyll cells adjacent to the greenbug salivary sheath are visibly altered after 2d of feeding, with the chloroplasts being the first affected. They swell within their outer membrane, taking on a rounded shape, and within 3-4d their lamellae separate and granules begin to appear (Al-Mousawi et al. 1983). The granules are plastoglobuli which are thought to be caused by the condensation of degradation products from the thylakoid membranes (Jutte & Durbin 1979; Robb et al. 1977; Steinkamp et al. 1979; Morgham et al. 1994). The mitochondria are the next organelles affected, disintegrating within 4d from the onset of greenbug feeding. Within the first 4d, the number of rough endoplasmic reticulum increased significantly as well (Morgham et al. 1994). Ten days after initiation of greenbug feeding, the nucleus finally disintegrates leaving the mesophyll cells within the chlorotic halo devoid of intact organelles with the only contents being vesicles and clear vacuoles (Al-Mousawi et al. 1983). Plants resistant to greenbug feeding incur no visible damage

in their vascular tissue when fed upon, other than cell lysis where stylets punctured cells causing them to rupture. However, some symptoms can be observed in the mesophyll tissue of resistant plants found along salivary sheaths. They are often found to have granular cytoplasm, membrane damage and vesicle formation. In addition, mesophyll chloroplasts of resistant plants tend to accumulate starch when fed upon (Morgham et al. 1994). This phenomenon is not a unique response to aphid feeding, virulent bacteria and viruses are known to cause similar accumulations (Lallyett 1977; Appiano et al. 1977).

BCO Aphid Biology:

The BCO aphid is dark-green in its immature stages and ranges from olive to almost black as an adult. BCO aphids are a holocyclic/anholocyclic species that alternates between the bird-cherry tree, *Prunus padus* (Rosaceae), its winter host, and a wide range of grasses (Gramineae) in the summer in temperate regions (Wiktelius 1984). Eggs hatch in the spring on *P. padus* and the wingless aphids remain there feeding for 2-3 generations before the parthenogenic females give rise to a winged migratory generation. The migrants fly to the summer grass hosts in late spring-early summer and continue parthenogenic reproduction (Dixon 1971; Wiktelius 1984; Wiktelius et al. 1990). In the early fall, with the shortening photoperiod and dropping temperature, the parthenogenic females are induced to give birth to the males (Dixon & Dewar 1974). The males and gynoparae move back to bird-cherry tree and give birth to the oviparae, which mate and lay the overwintering eggs (Dixon 1971). In the Southern Plains States, the anholocyclic BCO aphids primarily remain on grasses year round reproducing parthenogenically. BCO aphid populations peak when grasses are in their stem elongation phase and then

decline during head emergence (Wiktelius et al. 1990). As the different grass species mature, the BCO aphids move to new, less mature, hosts (Gianoli 2000).

BCO Aphid General Information:

Unlike greenbugs, BCO aphids are not discriminatory feeders. The BCO aphids will feed on phloem cells and non-phloem cells like the mesophyll parenchyma (Zuniga et al. 1988), but does prefer to feed on the stems and basal leaves of its hosts (Leather & Dixon 1981). BCO aphid feeding does not cause any visual foliar symptomology in wheat unless infestations are extremely heavy, in which case golden yellow streaking may occur (Summers et al. 2002). BCO aphids also cause significant yield losses, up to a 45% loss of plant height (Riedell et al. 1999), and up to a 40% reduction in root biomass (Dunn et al. personal communication). The aphid does cause this direct feeding damage, but is most detrimental to wheat when acting as a vector of barley yellow dwarf virus (BYDV) (Riedell et al. 1999). This Luteovirus is found only in the sieve elements, companion cells and parenchyma of phloem cells and is ingested by the aphid during feeding (Rochow & Duffus 1981). Once ingested, the virus moves circulatively, passing through the aphid's digestive tract, crossing the hindgut epithelium, entering the hemolymph. BYDV is then translocated through the hemolymph and enters the accessory salivary glands where it is incorporated into the saliva for later injection into a new plant during aphid feeding (Sylvester 1980, Gildow 1990). The BCO aphid needs to feed for an extended period of time (greater than 4h) for virus transmission to occur, non-preferred and resistant plants are less likely to become infected with BYDV (Gibson & Plumb 1977; Scheller & Shukle 1986).

Bird-cherry oat aphid saliva is fairly similar to greenbug saliva in that it contains both watery and gelatinous components, and BCO aphids also lay down a protective sheath of protein as they penetrate plant tissues during feeding (Miles 1990). Unlike greenbugs, BCO aphids do not significantly alter the amino acid composition of plant phloem sap as a result of their feeding. Sandstrom and Moran (2001) determined that BCO aphids rely much more heavily on their symbiotic *Buchnera* to provide them with the essential amino acids they require for growth and development. Greenbugs also have *Buchnera*, but the amino acid concentration they ingest from their enriched phloem sap is sufficient for growth (Sanstrom & Moran 2001). Since BCO aphids do not alter the host amino acid concentration, they are forced to ingest much larger volumes of phloem sap to receive the required proportion of amino acids (Sanstrom & Moran 2001). The missing salivary components that prevent them from altering host phloem are unknown at this time however.

BCO Aphid Control:

Biological control of BCO aphids is very effective and has been utilized extensively in some countries (Herera & Quiroz 1988; Givovich & Niemeyer 1991). The most voracious predator of the bird-cherry oat aphid is ladybird beetle adults and larvae, but there are other natural enemies that work to various degrees, syrphid fly larvae, aphid lions, and several species of parasitic wasps (McBride & Glogoza 1993). In the U.S. we have been slow to embrace biological control, and pesticides remain our primary means of control for the BCO aphid. There are several insecticides that can be used to control BCO aphids: methyl parathion, ethyl parathion, malathion, methomyl, chlorpyrifos, di-syston, disulfoton, dimethoate, and lorsban (McBride & Glogoza 1993; Summers et al.

2002). One of the most difficult problems associated with control of BCO aphids is that even at extremely low populations, rates of BYDV transmission can still be high resulting in 40-60% yield reductions. Because of this, in years with high incidence of BYDV, the economic threshold for BCO aphids is basically zero (Zuniga 1985; McBride & Glogoza 1993; Summers et al. 2002).

BCO Aphid – Plant Interactions:

Host plant resistance to BCO aphids has been researched extensively, and numerous theories have been proposed. The plant volatile methyl salicylate (MeS) is thought to play some role in BCO aphid resistance or at least plant attractancy/repellency (Pettersson et al. 1994; Glinwood & Pettersson 2000). Shulaev and colleagues (1997) examined MeS to determine if the aphid used the volatile compound as an attractant or repellent during migration, and concluded that the chemical reduced levels of plant colonization. Unfortunately, MeS is not present in volatiles emitted by grass species that have been tested (Glinwood & Pettersson 2000). Several other chemicals that are present in cereals have been examined, hydroxamic acids (Hx's) (Argandona et al. 1983; Niemeyer 1988; Givovich & Niemeyer 1991), phenols (Leszczynski et al. 1989), gramine (Zuniga et al. 1988; Kanehisa et al. 1990; Casaretto & Corcuera 1998), and aconitic acid (Rustamani et al. 1992). The most prevalent Hx in wheat, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), was studied extensively and reported to play a role in plant resistance (Niemeyer 1988). The effects of Hx in cereals on aphids have been found to be both antibiotic (Thackray et al. 1990; Morse et al. 1991) and antixenotic (Nicol et al. 1992; Givovich & Niemeyer 1991). DIMBOA is known to inactivate acetylcholinesterase, a key enzyme in insect nerve impulse transmission and the target

enzyme of organophosphate and carbamate pesticides (O'Brien 1978; Massoulie & Bon 1982; Taylor 1991; Cuevas & Niemeyer 1993). DIMBOA's effect on greenbugs have not been examined and could be less since it was not found in phloem sap or was present at extremely low levels (Niemeyer et al. 1989). Gramine is another compound that is known to play a role in BCO aphid resistance but it is ineffective against greenbugs, most likely because it is not found in the phloem tissue where the greenbug feeds (Zuniga et al. 1988; Casaretto & Corcuera 1998).

Plant Molecular Responses to Pathogen Attack:

Plants may respond to pathogen and insect invasion in a variety of ways. They may utilize constitutive defense mechanisms, such as thickened cell walls, suberin, callose, and stored allelochemicals with antixenotic or antibiotic effects, or induce defense mechanisms via regulated gene expression or the induction of specific compounds. Little is known about how plants interact with aphids and piercing-sucking insects in general. While feeding, aphid mouthparts remain inserted in host plant tissue for extended periods of time, causing little mechanical tissue damage as they feed. In light of this, the damage they cause is distinctly different from chewing insects, it is more subtle. Walling (2000) described the aphid-plant interaction as more biochemical in nature and therefore more like a plant-pathogen interaction. In light of that, a review of plant-pathogen interactions has been added for comparing and contrasting to what is currently known about piercing-sucking insect-plant interactions.

All plant defense responses begin with pathogen/insect perception, or signal perception. In plant-pathogen interactions, signal perception is believed to be the work of resistance genes that encode for receptor proteins which detect pathogen-generated

stimuli or elicitors (Gabriel & Rolfe 1990; Keen 1992). An incompatible plant-pathogen interaction will occur when a plant perceives a pathogen's avirulence (*Avr*) genes (potential pathogen stimuli), and triggers its own disease resistance (*R*) genes (receptor proteins) (Nam 1997; Dangl et al. 2000). If the plant does not carry the proper *R* allele, fails to recognize the pathogen's *Avr* genes, or if the pathogen does not carry the *Avr* gene, the plant will not perceive or recognize the pathogen, and disease will ensue. This alternate plant-pathogen interaction is referred to as a compatible interaction (Smart 1994; Dangl et al. 2000). Part of the incompatible plant-pathogen interaction is the induction of active defense responses. There are three tiers of induced response during incompatible interactions. The primary response is localized to cells that come in direct contact with the pathogen, and typically ends in programmed cell death (PCD) or the hypersensitive response (HR) and an oxidative burst (Hutcheson 1998). The secondary defense response is induced when signal molecules, elicited from the cells undergoing the primary response, are perceived and local gene activation occurs in the vicinity of the infection site. Gene activation results in the production of secondary products like phytoalexins and in the production of compounds that strengthen cells walls (Boller 1995; Hahn 1996; Ebel & Scheel 1997; Kombrink & Schmelzer 2001). Tertiary defense responses involve the upregulation of pathogenesis-related (PR) proteins, and the hormonally induced systemic acquired resistance (SAR) response which induces resistance to the perceived pathogen throughout the entire plant (Ryals et al. 1996; Durner et al. 1997; Sticher et al. 1997).

Once a pathogen has been perceived, a number of events occur almost immediately within the plant such as ion fluxes across the plasma membrane (Nurnberger et al. 1994),

cascades of phosphorylations and dephosphorylations (Dietrich et al. 1990), and the production of reactive oxygen species (ROS) (Apostol et al. 1989; Jabs et al. 1997). Within the first hour of contact with a pathogen, plants will undergo an oxidative burst irrespective of the type of interaction that will occur. Incompatible interactions however characteristically undergo a second oxidative burst much stronger and longer lasting than the first. The second burst is thought to be responsible for the localized cell death that occurs during the HR of incompatible interactions. The first ROS produced during the oxidative burst is superoxide ($O_2^{\bullet -}$) (Doke 1983, Doke & Ohashi 1988). Molecular oxygen is converted to $O_2^{\bullet -}$ outside the cell when in the presence of NADPH. Superoxide is not very stable, nor can it cross the plasma membrane, and is therefore converted to hydrogen peroxide (H_2O_2) either nonenzymatically or via superoxide dismutase before entering plant cells (Sutherland 1991; Levine et al. 1994; Mehdy 1994; Nurnberger et al. 1994). Both $O_2^{\bullet -}$ and H_2O_2 are only moderately reactive and toxic, the damage caused by ROS is typically due to their conversion into more reactive species. One such ROS is hydroperoxyl radical ($HO_2\bullet$) which is less polar than the unprotonated $O_2^{\bullet -}$, and therefore able to cross biological membranes like H_2O_2. $HO_2\bullet$ is known to damage membranes, converting fatty acids like linolenic, linoleic, and arachidonic acids to lipid peroxides, which can be utilized as signal molecules (Halliwell & Gutteridge 1990). Hydrogen peroxide can kill pathogens directly at elevated levels, but in the presence of iron it can be converted to the extremely toxic and reactive hydroxyl radical ($OH^{\bullet}$) (Hammond-Kosack & Jones 2000). Plants use enzymes such as superoxide dismutases, catalases and peroxidases to catalyze the scavenging of ROS and maintain oxidative balance within cells (Zhang & Kirkham 1994). The success of these enzymes

depends on the availability of reduced ascorbate and glutathione which are maintained by glutathione reductase, dehydroascorbate reductase and monodehydro-ascorbate reductase using NAD(P)H as an electron donor (Roxas et al. 2000). Some of the ROS and their regulatory and scavenging enzymes have beneficial functions and roles in plant defense mechanisms. Peroxidase is thought to play a role in cell wall lignification and cross-linking of hydroxyproline- and proline-rich glycoproteins to the polysaccharide cell wall matrix, both potentially strengthening cell walls against pathogen penetration and enzymatic degradation (Hammond-Kosack & Jones 2000; Tuzun 2001). Hydrogen peroxide acts as a signal transduction molecule inducing several important enzymes like benzoic acid 2-hydroxylase which is needed for salicylic acid biosynthesis. H_2O_2 signaling is also involved in defense gene regulation such as glutathione S-transferase upregulation (Hammond-Kosack & Jones 2000).

Signal transduction involved in plant defense responses to pathogens has been well studied, but is far from completely understood. After pathogen signal perception, ROS species are produced, some of which are involved in signal transduction, then ion fluxes across the plasma membrane will occur and cascades of phosphorylations and dephosphorylations will begin (Nurnberger et al. 1994; Dietrich et al. 1990; Apostol et al. 1989; Jabs et al. 1997). An excellent example of the complexity of the signal transduction pathways involved in plant-pathogen interactions was described by Baker and colleagues (1997). They examined the effects the fungal pathogen *Phytophthora sojae* on parsley by tracing the effects of one of its elicitor molecules, Pep-13. The elicitor was recognized by a 91kDa receptor on the plasma membrane, and once bound it stimulated extensive influxes of H^+ and Ca^{2+} and effluxes of K^+ and Cl^-. The ion fluxes

in turn stimulated the activation of a mitogen-activated protein kinase (MAPK). The MAPK has two roles, it moves into the nucleus where it can stimulate a gene transcription factor or act as a transcription factor itself, or the MAPK can remain in the cytosol rapidly phosphorylating or dephosphorylating proteins. The ion fluxes also occurred in conjunction with the stimulation of membrane-bound NADPH oxidases which convert molecular oxygen to various ROS. The ROS can either cross the plasma membrane and cytosol entering the nucleus to activate gene transcription, or the ROS will remain outside the plasma membrane where it can interact with peroxidases to help cross-link cell wall polymers strengthening the walls to prevent further pathogen penetration. The binding of Pep-13 to the receptor also stimulated phospholipase action and production of jasmonic acid, which in turn moves into the nucleus to stimulate gene transcription (Baker et al. 1997). Signal transduction has been studied in numerous other organisms, and the signaling patterns described above seem to be typical in plant-pathogen interactions, at least in part. An extremely common feature of plant defense signaling is the use of salicylic acid (SA), jasmonic acid (JA) and ethylene. These three molecules are typically said to be involved in two pathways, a SA-dependent pathway and a SA-independent pathway that utilizes both JA and ethylene. The two pathways cross-talk extensively and do not function in a mutually exclusive fashion (Kunkel & Brooks 2002). SA has been shown to inhibit or at least down-regulate JA and ethylene and the gene expression they stimulate (Doares et al. 1995; Doherty et al. 1988; Pena-Cortes et al. 1993; Peirterse & van Loon 1999). However, recent studies have found that in some plant-pathogen interactions, SA and the JA/ethylene pathways may also work synergistically (Xu et al. 1994; Hutcheson 1998; Kunkel & Brooks 2002).

Salicylic acid is a phenolic signaling molecule that accumulates in plant tissue following pathogen signal perception. SA is involved in the HR and is required for induction of systemic acquired resistance (SAR) (Yalpani et al. 1991; Shirasu et al 1997; Jorda & Vera 2000). The biosynthetic pathway of SA is not yet fully elucidated. The current theory is that SA is synthesized via the phenylpropanoid pathway by way of trans-cinnamic acid β-oxidation to benzoic acid which is converted to SA in the presence of benzoic acid-2-hydroxylase (Crozier et al. 2000). Elevation in SA levels during pathogen invasion leads to the transcription of numerous defense-related genes (Ward et al. 1991; Lawton et al. 1993). The majority of these genes code for defense proteins referred to as pathogenesis-related (PR) proteins, which are further categorized into several families of proteins (Cutt & Klessig 1992; Van Loon & Van Strein 1999). The PR proteins are initially expressed in the dying tissue that is in direct contact with the pathogen where the hypersensitive response is occurring. As SA levels continue to rise, PR gene expression is induced in distal tissues, inducing SAR (Sticher et al. 1997; Jorda & Vera 2000; Metraux 2001).

Jasmonic acid (JA) is an oxylipin-like hormone, structurally similar to mammalian prostaglandins, and like the mammalian hormone, it is derived from fatty acids (Hammond-Kosack & Jones 2000). Jasmonic acid and methyl jasmonate are considered senescence-promoting compounds known to promote expression of the antifungal proteins osmotin and thionin, and the phytoalexin-related enzymes chalcone synthase, phenylalanine ammonia lyase, and hydroxymethylglutaryl-CoA reductase. Jasmonic acid is synthesized via the octadecanoid pathway from α-linolenic acid (18:3) found in membranes (Crozier et al. 2000). JA, working in conjunction with ethylene, has recently

been implicated in SAR. This SA-independent signaling pathway is induced by the plant growth-promoting rhizobacteria *Pseudomonas fluorescence*, and works independent of PR proteins in inducing SAR (Pieterse et al. 1996; Van Loon et al. 1998; Kombrink & Schmelzer 2001; Pieterse et al. 2001).

Ethylene is an endogenous plant hormone involved in many aspects of plant growth and development, such as germination, senescence, epinasty, abscission, and fruit ripening, but it is also involved in a variety of biotic and abiotic stress responses (Abeles et al. 1992). Control of these processes by ethylene involves regulation of its biosynthesis via the Methionine/Yang cycle. The Methionine cycle is primarily responsible for the conservation of endogenous sulfur; ethylene is produced as an offshoot of it. Ethylene is synthesized from 1-aminocyclopropane-1-carboxylic acid (ACC) in the presence of ACC oxidase, which in turn is synthesized from *S*-adenosyl-L-methionine (SAM) in the Methionine cycle via the action of ACC synthase. The rate of ethylene biosynthesis is limited by the concentration of ethylene and other plant hormones such as auxin which promotes ethylene synthesis, and SA which inhibits it by blocking the conversion of ACC to ethylene (Crozier et al. 2000). Ethylene is often found to work in conjunction with JA, both being required for the induction of protease inhibitors, pathogenesis-related proteins, chitinase gene transcription, and other ethylene-responsive genes (O'Donnell et al. 1996; Choa et al. 1999; Penninckx et al. 1998; van Wees et al. 1999; Hammond-Kosack & Jones 2000). Distinction between ethylene-coordinated defense responses and ethylene control over normal growth and development is believed to be under the control of different cis-elements. Defense genes regulated by ethylene have a conserved sequence referred to as a GCC box in their promoter regions

(Ohme-Takagi & Shinshi 1990; Eyal et al. 1993; Hart et al. 1993). The GCC box, required for defense gene transcription, is not found in the promoter regions of fruit ripening genes or senescence genes (Ohme-Takagi & Shinshi 1995; Shinshi et al. 1995).

A fairly recent addition to the list of plant-defense signaling molecules are the phospholipids. They have long been suspected to be involved in stress signaling, but proof of the activation of the phospholipase C pathway was only recently obtained Munnik et al. 1998; Laxalt & Munnik 2002). Phospholipids are structural components of membranes, and may also be co-factors for membrane enzymes, signal precursors or signaling molecules themselves (Laxalt & Munnik 2002). Phospholipase C (PLC) hydrolyzes phosphatidylinositol 4,5-bisphosphate into two second messengers: inositol 1,4,5-triphosphate (IP3), which diffuses into the cytosol, and diacylglycerol (DAG), which remains in the membrane. IP3 releases Ca^{2+} from intracellular stores for further signaling, and DAG is converted to phosphatidic acid (PA) via DAG kinase action (Munnik 2001). The exact role of PA in plants is unclear at this point but is believed to activate the NADPH oxidase complex as it does in other systems (McPhail et al. 1999). This theory is consistent with findings that show PA to trigger the oxidative burst response in Arabidopsis and tobacco (Munnik 2001; Sang et al. 2001). PA has also been shown to activate MAPK cascades which are known to induce further signal transduction mechanisms (Lee et al. 2001). PLC is not the only phospholipase involved in signal transduction, phospholipases D and A have also been implicated (Laxalt & Munnik 2002). PLD hydrolyzes structural phospholipids such as phosphatidyl-choline into choline and the signaling molecule PA (Munnik & Musgrave 2001). Phospholipase A2 hydrolyzes fatty acid bonds of phospholipids producing free fatty acids which can act as

signaling molecules themselves or they could be used in the octadecanoid pathway to generate JA (Six & Dennis 2000).

Whether SA, JA/ethylene, or phospholipid signal transduction pathways are utilized, the most common end result is the induction of defense genes, namely pathogenesis-related (PR) genes and proteins. These proteins may also be regulated by other signal molecules such as H_2O_2. Pathogenesis-related proteins were first described as extracellular proteins that accumulated in response to tobacco mosaic virus in susceptible tobacco. They were later found to be differentially expressed during incompatible interactions, and are known to be intra- and extracellularly localized after pathogen attack or elicitor treatment (Bowles 1990). PR proteins are grouped in a couple of ways; first they are divided into either acidic or basic subgroups. Basic PR proteins are targeted to the vacuole and tend to be more antimicrobial in nature than the acidic proteins which are secreted from the plant cells. The definitive cataloging came about in 1994 to unify the nomenclature for PRs (van Loon et al. 1994). PR proteins are now grouped into families (PR 1-14) based on shared amino acid sequences, serological relationship, and/or enzymatic or biological activity. The most well known or well characterized are families 2 and 3, the β-1,3-glucanases and chitinases respectively. The other families include the thaumatin-like proteins, proteinase-inhibitors, endoproteinases, peroxidases, ribonuclease-like proteins, defensins, thionins, lipid-transfer proteins, other chitinases (those not included in family 3), and the PR-1 proteins who's function is unknown at this time (van Loon & van Strien 1999). These proteins are thought to work together synergistically in a coordinated defense response to confer resistance to a particular pathogen (Zhu et al. 1994).

To summarize the major points of the plant-pathogen interaction, the plant may undergo either a compatible (susceptible) response, or an incompatible (resistant) response to the pathogen. The incompatible interaction begins with pathogen signal perception which triggers the hypersensitive response or walling off of the area of infection or inoculation. Concomitant with this, the plant cells surrounding the infected area will exhibit increased production of ROS species, ion fluxes across the plasma membrane, and cascades of phosphorylations and dephosphorylations. This series of events will lead to the induction of the SA-dependent, SA-independent or phospholipid-mediated signal transduction pathways, which in turn leads to the transcription of defense genes that code for pathogenesis-related proteins. The expression of pathogenesis-related proteins leads to systemic acquired resistance to the pathogen involved in the interaction.

Plant Molecular Responses to Insects:

Plants may respond to insects in a variety of ways also. Plants and insects have coexisted and coevolved for at least 100 million years, and through this time plants have developed a myriad of defense strategies to protect themselves from insect attack (Stotz et al. 1999). Their defense mechanisms may be constitutively expressed or induced upon attack. Constitutively expressed defense responses typically include compounds that either inhibit insect access to preferred tissues such as suberin, callose, and other compounds that thicken cell walls and cuticles, or compounds that function as allelochemicals (Paiva 2000). Allelochemicals are categorized as either antixenotic, which deter herbivore colonization of the plant, or antibiotic, which deter herbivore growth, reproduction, development or survival (Conn 1981; Hedin 1983; Walling 2000). Plant defensive strategies are further classified by their mode of action either direct or

indirect. Direct defenses include compounds that are antifeedants (antixenotics) or anti-nutrients (antibiotics), which work directly on the insect. Indirect defenses work in a "top-down" fashion via compounds that entice predators, pathogens, and parasites of the herbivore to the plant (Turlings et al. 1995; Takabayashi and Dicke 1996; Karban & Baldwin 1997; Kahl et al. 2000; Shen et al. 2000). These indirect defense mechanisms are often mediated through the release of volatile organic compounds (VOCs) (Baldwin et al. 2001). However, VOCs may have various roles, functioning as a direct defense mechanism when repelling oviposition of herbivores (DeMoraes et al. 2001; Kessler & Baldwin 2001), or they may be beneficial to the herbivores when used to distinguish between host and nonhost plants or to assess the density of herbivores already feeding on a particular plant (Bolter et al. 1997, Quiroz et al. 1997).

Some of the most common VOCs utilized are the family of C_6 volatiles from the lipoxygenase and hydroperoxide lyase-dependent pathways, indole and methyl-SA from the shikimic acid and tryptophan pathway, cyclic and acyclic terpenoids from the isoprenoid pathway, and oximes and nitriles which are derived from amino acids (Dicke 1999, Dicke et al. 1999, Pare & Tumlinson 1999). Other more specialized VOCs may also be utilized like the partially volatile glucosinolates which are emitted by the Brassicacae (Halkier & Du 1997; Walling 2000). These VOCs may stimulate the expression of wound and defense response genes, or may act directly on the insects by reducing fecundity as with aphids and spider mites or they may act as feeding deterrents as witnessed with some caterpillars (Avdiushko et al. 1997, Hildebrand et al. 1993, Kasu et al. 1995). Unfortunately, these same VOCs can also work as attractants to specialized

herbivores such as the Colorado potato beetle and specialist aphids (Bolter et al. 1997, Visser et al. 1996).

Most of the VOC research done to date was conducted on chewing insects, like caterpillars and spider mites. In comparison, relatively little is known about the volatile blends induced by aphid feeding. Certain aphids stimulate the release of volatiles that attract parasitic wasps (Du et al. 1998), while other VOCs are utilized to discern host plants and aphid density (Bernasconi et al. 1998, Quiroz et al. 1997). The corn leaf aphid, *Rhopalosiphum maidis*, is known to stimulate the release of the following VOCs when feeding on maize: β-ocimene, linalool, 4,8-dimethyl-1,3,7-nonatriene, α-farnesene, β-farnesene, 4,8,12-trimethyl-1,3,7,11-tridecatetraene, and acetylated C_6 volatiles (Bernasconi et al. 1998, Pare & Tumlinson 1999, Quiroz et al. 1997). These same VOCs are commonly released in response to caterpillar and spider mite feeding, but it is not known at this time whether other aphids induce the same VOC response (Walling 2000).

All defense mechanisms, whether constitutive or induced, are costly (Baldwin & Preston 1999) (Walling 2000). Defense responses drain energy from developmental processes such as vegetative and reproductive growth (Conn 1981, Hedin 1983, Walling 2000). Thus, the plant must be certain that this resource reallocation is warranted and efficacious. Plant responses to pathogens, insects and wounding may utilize many of the same pathways and defense mechanisms; however the temporal and spatial pattern of expression in relation to the onset of induction may differ widely depending on the elicitor of the response (Agrawal 2000; Hatcher 1995; Karban & Baldwin 1997; Bostock et al. 2001). The elicitors of the defense response are not believed to be the actual organisms causing the damage per se, but are biochemical agents involved in their

interaction with the plant, and may be either of herbivore or plant origin. Many of the elicitors characterized thus far are oligosaccharides such as β-1,3-1,6 glucans, xyloglucans, oligogalacturonides, and chitin-derived oligomers (Côté & Hahn 1994; Klarzynski et al. 2000). Some specific insect elicitors have been determined; volicitin (*N*-17-hydroxylinolenyl-L-glutamine) which was identified in regurgitant of the beet armyworm (Turlings et al. 1993; Pare et al. 1998), β-glucosidase from Pieris caterpillars (Mattiacci et al. 1995), and bruchins (long chain diols) from weevils (Doss et al. 2000). Determination of the elicitors involved in insect-plant interactions is crucial if we are to gain further insight into these complex relationships, however at this time little is known about general insect elicitors and even less is known about the elicitors involved in aphid-plant interactions.

Once a plant perceives pathogen attack (i.e. it recognizes the elicitor), it will often undergo HR (the hypersensitive response) as part of its resistant defense response. This mode of defense has also been observed in insect-plant interactions, but to a lesser extent. Brassica spp. have been shown to undergo HR in response to oviposition of Pieris butterflies (Shapiro & Devay 1987), wheat initiates HR to cordon off Hessian fly larvae (Dweikat et al. 1997; Schulte et al. 1999), and HR is known to be initiated in response to galling insects on a legume species (Fernandes 1998; Stotz et al. 1999). The HR is extremely effective against pathogens, but it is costly and it is imperative that the elicitor recognition be accurate or the response will be deployed inappropriately. This may be why we see HR employed less frequently in insect-plant interactions. The HR may work effectively against a more stationary feeder like aphids, but with active feeders like Lepidopteran larvae that can merely move to an alternate leaf if they sense induced

toxins, the HR may be a waste of resources. If the two types of feeders utilize the same elicitors, plants may have already realized the futility of HR against insects, or perhaps we see it used less frequently merely because we have not studied aphid-plant interactions thoroughly yet.

We may be uncertain about the use of HR in insect-plant interactions, but plant responses involving toxins have been fairly well characterized. A plant compound is considered a toxin if it has any negative effect on the growth, development or survival of the organism feeding on it (Wittstock & Gershenzon 2002). Some of the well characterized toxins include: saponins which disrupt cellular membranes (Osbourn 1996), hydrogen cyanide which inhibits cellular respiration (Jones et al. 2000), and cardenolides which are specific inhibitors of the Na+/K+-ATPase (Schatzmann 1959; Repke & Portius 1963 both reviewed in Wittstock & Gershenzon 2002). Some compounds that act as toxins in defense against insects are actually part of the plant's normal metabolism. Phytic acid is one such compound; known for its action as a cation chelator, it is the primary mechanism for phosphorus storage in seeds and fruits of many plants. However, when ingested by some Lepidopteran larvae, phytic acid will bind to essential nutrients blocking their digestion (Green et al. 2001). The use of its own metabolites as defensive compounds is ideal for plants. There is no additional energy cost and the compounds are obviously not harmful to the plant so they do not require sequestration. That is not the case with all toxins, some have deleterious effects on the plant as well as the insect. These injurious compounds must be synthesized and stored in a manner that won't poison the plant as well. Plants deal with this in a variety of ways; some toxins are synthesized as precursors that are only toxic when exposed to their activating enzymes as is the case

with glycosides (Jones & Vogt 2001). Other compounds are stored in specialized structures such as resin ducts, laticifers or glandular trichomes and are only released when these structures are ruptured during herbivore feeding (Dussourd & Hoyle 2000; Duke et al. 2000; Hallahan 2000). Because of the innate toxicity of many of these compounds and their location, they may work well for chewing herbivores, but they will be relatively ineffective against the phloem feeding aphids.

The plants utilize different defensive strategies for insects from the different feeding guilds (i.e. chewing insects versus piercing-sucking insects). The mechanical damage caused during chewing insect feeding is obviously much more severe, and because of this, plant responses to the more traditional herbivore (chewing insects) are often compared to plant responses to wounding. However, this is not entirely accurate either. When herbivores feed, their mastication not only causes mechanical wounding, but also introduces saliva to the wound. As previously mentioned, elicitors have been found in insect saliva, the two best characterized are volicitin and β-glucosidase (Baldwin et al. 2002). Volicitin is a fatty-acid-amino-acid conjugate (FAC) whose conjugation appears to be mediated microbially (Spiteller et al. 2000), and is thought to function as a surfactant aiding in digestion in the insect (Baldwin et al. 2001). How this elicitor triggers defense genes and produces volatile terpenoids is unknown. Bruchins are long chain diols that have been mono- and diesterified with 3-hydroxypropanoic acid. In peas, bruchins trigger neoplastic growth in the pods which lifts the weevil's egg out of the oviposition site and impedes larval entry (Doss et al. 2000). Even less is known about β-glucosidase; it is known to cleave terpenoids stored as β-glucosides in cabbage plants (Mattiacci et al 1995), but its role in the insect is unclear at this time (Shen et al. 2000).

All in all very little is understood about the elicitation of plant defense responses by insects, chewing or otherwise. Other elicitation theories have been proposed such as glucose oxidases increasing H_2O_2 production, which then acts as the trigger or elicitor of the defense response (Felton & Eichenseer 1999), but the number of insects carrying glucose oxidases in their saliva is unknown (Kessler & Baldwin 2002). Pathogen triggered defense responses are known to be under gene-for-gene control with *avr* proteins acting as the pathogen elicitor and plant *R* genes acting as receptors for those proteins. No such general mechanism has been found in chewing insects thus far.

Signal transduction pathways leading to the regulation of defense genes and mechanisms have been studied much more extensively than the elicitation of the responses. Signal transduction involved in wound responses has been well characterized. Mechanical wounding is known to generate electrical or hydraulic signals at the site of damage (Rhoades et al 1999). These signals then stimulate the local and systemic release of oligogalacturonide, which is liberated from pectin through the action of polygalacturonase, and the peptide systemin to further amplify the signal transduction cascade (Bergey et al. 1999). Oligogalacturonide and systemin induce increases in cytosolic calcium, membrane depolarization, K^+ and H^+ fluxes, MAP kinase activity, generation of ROS, phospholipases A_2 and D activation, as well as inactivation of H^+-ATPase (Bergey et al. 1996; Stratmann & Ryan 1997; Moyen et al. 1998; Schaller 1999; Ryan 2000; Walling 2000). The wound signal transduction cascade ends with systemin activating phospholipase A_2, which releases linolenic acid from the plasma membrane triggering the initiation of the octadecanoid pathway and hence JA synthesis (Ryan 2000). The synthesis of JA leads to the transcription of systemic wound-related proteins

or *SWRPs* (Walling 2000). Herbivores cause mechanical damage as they feed, so it is not surprising that there is an upregulation of *SWRPs* as a result of their feeding. The surprising factor was that the initiation of *SWRP* transcription was activated more quickly in response to herbivore feeding than mechanical wounding (Korth & Dixon 1997). When volatile release patterns were compared, they were found to be significantly different leading researchers to believe that different elicitors or alternate signaling cascades were being utilized to initiate transcription of *SWRPs* (Alborn et al. 1997; Paré & Tumlinson 1997; Wasternack & Parthier 1997).

Herbivore signal transduction pathways are not fully elucidated at this time. Herbivory stimulates increases in lipoxygenase (LOX), which catalyzes the oxygenation of linolenic acid. Linolenic acid can then be utilized to synthesize JA, but LOX has many roles so it is unclear whether it is directly triggering the production of JA (Creelman & Mullet 1997; Schaller 2001). JA is known to be upregulated by many herbivores: beetles, caterpillars, thrips and spider mites all show significant upregulations of JA and JA mediated responses (Ryan 2000). However, the signal transduction cascades that lead to this upregulation are unclear at this time. Herbivore salivary secretions contain chitosan and/or polygalacturonase (Bronner et al. 1989, Miles 1999). Polygalacturonase, when in the presence of cell wall pectins, triggers the production of oligogalacturonides. Oligogalacturonides and chitosan are known to trigger JA synthesis (Baydoun & Fry 1988; Walling 2000).

Ethylene has also been implicated in some herbivore-induced defense signal transduction (Kahl et al. 2000). *Manduca sexta*, a specialist on tobacco, is thought to be "recognized" by the plant (McCloud & Baldwin 1997). Once the plant recognizes the

insect, or its salivary secretions, tobacco changes its defense strategy. The plant will forego its typical direct defense mechanism, the production of nicotine, and switch to an indirect type of defense where alternate volatile emission patterns have been observed. This switch from nicotine to VOC production is triggered by an ethylene burst (Kahl et al. 2000). Ethylene and JA often work in conjunction as exhibited here, but ethylene's role in defense signaling against other herbivores is unclear at this time. Herbivores seem to primarily utilize JA-mediated signaling pathways, with little evidence that SA signaling is being utilized. This seems to be the major difference between the traditional herbivore and piercing-sucking insect defense signal transduction mechanisms.

As previously stated, there are many similarities between wound responses and herbivore defense responses. The two are not identical though; herbivores do induce wound-response genes, but they also trigger the expression of plant genes not exhibited during wound responses (Korth & Dixon 1997; Baldwin et al. 2001). Herbivores seem to alter normal plant process more than wounding. Some of the additional transcripts affected by herbivory include those involved with photosynthesis, electron transport, the cytoskeleton, carbon and nitrogen metabolism, signaling and those involved in other stress responses (Hermsmeier et al. 2001). Hermsmeier and colleagues (2001) found that genes regulating photosynthesis were strongly downregulated, while those involved with stress responses and the shifting of carbon and nitrogen to defense were strongly upregulated. The effects of these metabolic shifts on insects, if any, are not known at this time.

During wound responses proteinase inhibitors, polyphenol oxidases, and leucine aminopeptidase are upregulated. Not surprisingly, plant mastication by herbivores

induces the accumulation of these same compounds both locally and systemically as well (Green & Ryan 1982; Pautot et al. 1993; Stout et al. 1996; Karban & Baldwin 1997). These aren't the only defensive compounds triggered during herbivory however. Defensive compounds are typically categorized by their mode of action (Duffey & Stout 1996). For example, proteinase inhibitors (PIs) are considered antidigestive proteins; they work by inhibiting insect digestive enzymes (Koiwa et al. 197; Tamayo et al. 2000). While polyphenol oxidases are classified as antinutritive enzymes because of the way they cross-link proteins and/or polymerize quinones (Kessler & Baldwin 2002). Polyphenol oxidase catalyzes the oxidation of phenolic secondary metabolites converting them into reactive quinones which polymerize into a gluey substance that can trap insects if walked across or if ingested, can reduce the nutritional quality of the food by cross-linking proteins effectively making them indigestible (Constabel et al. 2000).

Protease inhibitors (PIs) target proteolytic digestive enzymes (proteinases) in larval midguts, and are capable of slowing growth and even causing death from starvation (Pechan et al. 2000). PIs act primarily against trypsin and chymotrypsin (Boisen et al. 1981; Koiwa et al. 1997), but have recently been found inhibit both elastases and chymotrypsin in the midgut of *Spodoptera littoralis* larvae feeding on corn (Tamayo et al. 2000). A correlation has been found between PI levels and resistance to grasshoppers in barley; cultivars expressing higher levels of chymotrypsin inhibitor activity were found to be more resistant (Weiel & Hapner 1976). Their effectiveness depends on the PIs' affinity and specificity for specific gut proteinases (Koiwa et al. 1997). Some herbivores are able to overcome plant PIs by increasing proteolytic activity (Girard et al. 1998), utilizing alternate proteolytic enzymes that are insensitive to the plant PIs being

expressed (Gruden et al. 1998), or by upregulating their own proteinases that degrade the plant PIs (Giri et al. 1998). Alternatively, herbivores were able to overcome a genetically modified over-expression of PI in *Brassica* simply by eating more leaf material (Winterer & Bergelson 2001). Plants may also contain other enzyme inhibitors such as the α-amylases which act on key gut digestive hydrolases (Konarev 1996; Chrispeels et al. 1998). α-amylases (α-1,4-glucan-4-glucanohydrolases) catalyze the hydrolysis of α-D-(1→4)-glucan linkages in starch, glycogen and other carbohydrates, and is necessary for carbohydrate metabolism (Franco et al. 2002). Without α-amylase, insects that live primarily on starch will starve to death.

Enzyme inhibitors are not the only resource plants have to defend themselves against herbivores. Glycoalkaloids found in potatoes are known to have inhibitory effects on some herbivores (Jadhav et al. 1981). Phenylalanine ammonia-lyase, a key enzyme in the biosynthesis of phenolics, is upregulated in response to spider mites (Arimura et al. 2001). Barley utilizes alkaloid gramine, flavonoids and phenolic compounds to defend itself against herbivory (Casaretto & Corcuera 1998). Corn upregulates indole in response to herbivory, which is then converted to the hydroxamic acid 2,4-dihyroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) (Frey et al. 1997; Sicker et al. 2000). Some plants produce β-glucosides to defend themselves, but herbivores are known to reduce their toxicity by decreasing the β-glucosidase activity in their midgut, effectively blocking their transformation into toxic hydrolyzed compounds (Baker & Woo 1992; Mainguet et al. 2000). Arabidopsis utilizes glucosinolate synthesis and breakdown to defend itself against some herbivores (Mauricio 1998). Certain plants utilize phytochemicals in response to herbivory such as alkaloids in wild parsnip (Zangerl 1990;

Zangerl & Berenbaum 1995), and furanocoumarins in swede, kale and oilseed rape (Birch et al. 1992). While tomato plants use a suite of compounds to ward off herbivory: proteinase inhibitors, polyphenol oxidase, phenylalanine ammonia lyase, lipoxygenase, peroxidase and the alkaloid tomatine are all upregulated, and two phenolics, chlorogenic acid and rutin are downregulated (Stout et al. 1994; Stout 1996). This last defense strategy, describing a plant utilizing more than one method of defense, is actually more common than defense strategies involving single compounds. Plants usually upregulate these compounds in response to a single herbivore, but in doing so they may provide systemic resistance throughout the plant to numerous pests, including pathogens (McIntyre et al. 1981; Karban et al. 1987; Benedict & Chang 1991; Kogan & Fischer 1991; Inbar et al. 1998; Inbar et al. 2001). This phenomenon is not typical though, and there are some plants where the opposite is true (Apriyanto & Potter 1990; Ajlan & Potter 1992; Thaler et al. 1999).

Insects with piercing-sucking mouthparts such as aphids cause limited tissue damage as they feed. They probe briefly until they gain access to target cells, most typically phloem cells, and then remain in this feeding position for a substantial amount of time. Because of the unobtrusive way these piercing-sucking insects (PSIs) interact with the plant, it is not surprising that plant responses are distinct from those induced by chewing insects. As previously mentioned, some have claimed that the response PSIs induce is more like that of a pathogen-insect interaction (Walling 2000). The actual response appears to be intermediate to the chewing insect-plant interaction and the pathogen-plant interaction, and there is actually great overlap between all of the plant responses. HR has long been thought to be characteristic of the plant-pathogen interaction alone, but as

discussed earlier, some chewing insects are capable of inducing HR, and Russian wheat aphids (RWA) are known to induce a response similar to HR as well (Belefant-Miller et al. 1994; van der Westhuizen et al. 1998). There are other areas that overlap; both chewing insects and PSIs are capable of inducing wound-response genes (Kessler & Baldwin 2002).

The elicitors of PSI-induced defense responses are largely a mystery. PSIs are constantly secreting salivary enzymes as they probe and feed, and most believe the elicitors are in their saliva, as is the case for chewing insects (Korth & Dixon 1997; Páre & Tumlinson 1999; Walling 2000; van de Ven et al. 2000). Some researchers feel that signals may be the result of physical damage and/or mechanical stress. During probing, PSIs may damage cells along the stylet path, releasing defensive compounds or signaling molecules, or their intercellular probing may disrupt essential cell-to-cell communication which could be perceived as stress or invasion (Walling 2000). Probing-induced elicitation of the defense response seems unlikely since many of the PSI-plant interactions are species and sometimes biotype specific, and we know that biotypes probe in the same manner (Wood 1971; Pollard 1973; Campbell et al. 1982; Al-Mousawi et al. 1983; van de Ven et al. 2000). PSI saliva is not well characterized for many species, but the aphid saliva studied contained pectinases, cellulases, amylases, proteases, lipases, alkaline and acidic phosphatases, and peroxidases, any of which could be acting as elicitors (Miles 1999). Oligogalacturonides generated via pectinase activity, and ROS from peroxidases, both found in their saliva, are known to be elicitors of wound- and/or defense-signaling pathways (Walling 2000).

Elicitation of plant defense responses to pathogens is purported to be a gene-for-gene interaction (Baron & Zambryski 1995; Hammond-Kosack & Jones 1996; Baker et al. 1997; De Wit 1997). No such interaction has been witnessed in the chewing insect-plant interaction, but PSIs are thought to be using some sort of gene-for-gene mechanism in their elicitation/recognition interaction. Aphids, which are known to feed on specialized cell types, have exhibited such a mechanism (Glazebrook 1999, van Helden et al. 1993, Roche et al. 1997; Rossi et al. 1998; Vos et al. 1998). The *Mi* gene, first discovered in tomato, was found to confer resistance/recognition to a root knot nematode (Kaloshian et al. 1995). This same gene, containing a nucleotide-binding site and leucine-rich repeat domain, was then found to confer the same resistance recognition to the potato aphid, *Macrosiphum euphorbiae* (Milligan et al. 1998; Rossi et al. 1998). Other single *R* genes in plants have been found to respond similarly to different avirulence genes or elicitors (Bisgrove et al. 1994; Grant et al. 1995), so it was not surprising to find a single gene conferring resistance to such diverse pests. Other *R*-like genes have been found exhibiting gene-for-gene resistance against PSIs: the *Nr* gene in lettuce against the aphid *Nasonovia ribisnigri* (Eenink et al. 1982a,b; van Helden et al. 1993); the *Sd1* gene in apple against the rosy leaf curling aphid, *Dysaphis devecta* (Roche et al. 1997); and several genes from wheat against the Hessian fly, *Mayetiola destructor* (Say) (Dweikat et al. 1994; Dweikat et al. 1997).

Once the PSIs attack is perceived, signal transduction cascades are induced which leads to the transcription of defense genes. There are numerous examples of PSI utilizing both SA-dependent and JA-dependent signal transduction cascades to trigger plant defense responses (Shulaev et al. 1997; Chao et al. 1999; Walling 2000; van de Ven et al.

2000; Moran & Thompson 2001). PSIs can induce the expression of PR genes, and are reportedly capable of inducing SA-mediated systemic acquired resistance (SAR) like a pathogen (Shulaev et al. 1997; Moran & Thompson 2001). As previously mentioned, PSIs induce the expression of both pathogen-responsive-SA-regulated and wound-responsive-JA-regulated genes (Walling 2000). The PSI-induced signal transduction mechanisms and defense responses seem to include many features of pathogen, chewing insect and wound responses. Silver leaf whiteflies, *Bemisia tabaci* (Gennadius), trigger the upregulation of a SLW3 gene that is not responsive to any known wound or defense signaling mechanism, suggesting that there could be alternative signal transduction pathways being stimulated by PSIs that have yet to be elucidated (van de Ven et al. 2000; Kessler & Baldwin 2002).

As with the signal transduction mechanisms, there is significant overlap in the defense proteins expressed in response to PSIs. *PR* proteins, typically associated with pathogen defense responses, are also upregulated in response to PSIs (Bronner et al. 1991; Mayer et al. 1996; Broderick et al. 1997; van der Westhuizen et al. 1998a,b; Fidantsef et al. 1999; Stout et al. 1999; Walling 2000; Kessler & Baldwin 2002). *PR* proteins are divided into families of enzymes (van Loon & van Strien 1999). Some of the PR families upregulated in response to PSI feeding include: β-glucanases (Broderick et al. 1997, Bronner et al. 1991, Mayer et al. 1996, van der Westhuizen et al. 1998a,b), chitinases (Boijsen et al. 1993; van der Westhuizen et al. 1998), protease-inhibitors (PIs) (Casaretto & Corcuera 1998), peroxidase (Stout et al. 1998, Fidantsef et al. 1999), ribonuclease-like proteins (Broderick et al. 1997, Mayer et al. 1996), and defensins (Moran & Thompson 2001).

PSI-induced β-glucosidases may have several different substrate specific defensive functions (Walling 2000). They may affect development by catabolizing glycosylated forms of phytohormones (Brzobohaty et al. 1993), and hydrolyzing cell wall polysaccharides (Wallner & Walker 1975); or they may aid in defense via the release of phenols, isoflavanoids, SA, and cyanogenic compounds from glucosylated storage products through its enzymatic action (Miller 1973; van de Ven et al. 2000). The upregulation of chitinase is inexplicable at this time since there is no clearly defined defense function for chitinases against PSI attack (van der Westhuizen et al. 1998). Chitinase is thought to disrupt midgut peritrophic membranes in chewing insects (Mayer et al. 1995), but fluid feeding insects typically lack a peritrophic membrane (Gullan & Cranston 1994). The induction of PIs in response to PSIs is not universal; they are upregulated in response to some PSIs but not others (Casaretto & Corcuera 1998; Fidantsef et al. 1999; Stout et al. 1999). Also, only chymotrypsin inhibitor activity and not trypsin inhibitor activity has been exhibited (Casaretto & Corcuera 1998). The effects of PIs on PSIs is unknown at this time, especially considering the dogma that most PSIs do not possess proteases in their midguts since they are unable to digest complex proteins (Casaretto & Corcuera 1998). Peroxidase upregulation is common during plant-pathogen interactions as well. However, as previously mentioned, its specific function here is difficult to ascertain since peroxidases are involved in so many processes: signal transduction, oxidative stress regulation, cell wall lignification, direct toxicity to foreign organisms, and indirect injury to herbivores via oxidative damage to dietary substances (Bowles 1990; Felton et al. 1994; Mehdy 1994; van der Westhuizen et al. 1998).

Other defense compounds triggered in response to PSIs include lipoxygenase (Hildebrandt et al. 1989), polyphenol oxidase (Felton et al. 1994) and stress-related monosaccharide symporter genes (Moran & Thompson 2001). Several additional compounds are reportedly involved in PSI-resistance as well: acyl-sugars (Blauth et al. 1998), glucosinolates (Giamoustaris & Mithen 1995), hydroxamic acids (Niemeyer 1988; Gianoli & Niemeyer 1998), gramine (Zuniga et al. 1988; Kanehisa et al. 1990), and aconitic acid (Rustamani et al. 1992).

It is important to understand that these aforementioned pathways, proteins and compounds regulated during plant-pathogen, -chewing insect, and -PSI interactions are not static. Not all PSIs or pathogens discussed induce all of the response mechanisms mentioned. Differential expression is exhibited within families, species and even biotypes. The responses described here are merely trends exhibited by some individuals that have been examined. What has emerged from the research conducted is that the dominant signaling pathways and cellular responses to these signals are extremely complex and diverse. Only through continued research utilizing new global analysis tools, will we gain the understanding required to develop sustainable resistance mechanisms to the plant pests described here.

Proteomics:

The trend of the last 25 years has been the evaluation of organisms at the genomic level, resulting in several plant genomes being fully or partially sequenced. Unfortunately, elucidation of an organism's genome does not tell us what the organism is expressing at the functional level. The genome may contain stretches of viable DNA, not retrotransposon or non-coding DNA, which are never expressed. Researchers have also

determined that there is a low correlation between mRNA expression levels and protein abundance (Anderson & Seilhamer 1997; Gygi et al. 1999). This phenomenon could in part be due to posttranslational modifications. Proteins may undergo hundreds of possible posttranslational modifications, such as phosphorylation, glycosylation, methylation, oxidation, carboxylation, hydroxylation, or the addition of lipids, and these modifications can differ depending on the proteins location within the organism (Gooley & Packer 1997). Because of these many possibilities, it may be difficult to tell what the functional protein will be by mere examination of its gene. The work conducted for this dissertation examines plant-insect interactions at the protein level in an attempt to better understand the biochemical nature of aphid-plant interactions.

Proteomics, as defined by Wilkins and colleagues (1995), is the systematic analysis of a protein population in a given organism, tissue, cell, or subcellular compartment. Proteomics allows researchers to examine and compare global protein expression profiles that have been manipulated in some manner (i.e. stressed vs. non-stressed tissue). Much of the proteomic research conducted has focused on bacterial and animal tissues thus far as clearly seen by the number of corresponding protein databases (reviewed in Lopez 1999). The first review of plant proteomics (Thiellement et al. 1999) extensively discussed and outlined the current literature up to that point, indicating that most research did not involve the use of mass spectrometry (MS) to obtain protein identity, but merely compared protein expression levels and patterns. Protein identification obtained up to 1999 was primarily via Edman sequencing (Thiellement et al. 1999). Proteomics projects today primarily involve protein separation via two-dimensional (2-D) sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), *in situ* enzymatic digestion and

elution of peptides from the gels, MS analysis, and protein identification by matching observed peptide masses and/or amino acid sequence tags to expected masses derived *in silica* from known protein or nucleic acid sequences in public databases (Shevchenko et al. 1996; Molloy et al. 1998; Yates 1998). There has been a wealth of plant proteomic research published recently that follows this basic plan (Kehr et al. 1999; Komatsu et al. 1999; Sherrier et al. 1999; Chang et al. 2000; Ferro et al. 2000; Natera et al. 2000; Panter et al. 2000; Rakwal & Komatsu 2000; Vener et al. 2000; Porulbeva et al. 2001; Yamaguchi & Subramanian 2000; Peltier et al. 2001; Rossignol 2001; Watson et al. 2003).

Plant proteomics is slightly behind bacterial and animal proteomics for several reasons not the least of which is difficulties associated with protein extraction and solubilization (Molloy et al. 1998; Tsugita & Kamo 1999). Comparatively, plant tissue has far lower protein concentration than bacterial and mammalian tissue, and Tsugita and Kamo (1999) recommend precipitating proteins from plants with trichloroacetic acid (TCA) in acetone to help compensate (Tsugita & Kamo 1999). Plant proteins are also notoriously difficult to solubilize. Molloy and colleagues (1998) proposed differential solubilization, sequentially solubilizing proteins from ground leaf tissue using increasingly stringent solutions. Their research first utilized a Tris-based extraction buffer containing protease inhibitors as recommended by Rabilloud (1996) to minimize proteolysis, another severe problem associated with plant proteomics. The second phase of their extraction protocol utilized the conventional two-dimensional electrophoresis (2DE) solubilization solution containing high quantities of urea and a reducing agent. The final step of their procedure brings together several recent advances in protein

solubilization, the addition of a second chaotropic reagent (thiourea), replacement of the charged reducing agent with an uncharged one (tributyl phosphine) as per Hebert et al. (1998), and the addition of a sulfobetaine surfactant (SB3-10) as per Rabilloud et al (1997). Two-dimensional electrophoresis discriminates against strongly hydrophobic proteins (van Wijk 2001) and to overcome this Seigneurin-Berny and colleagues (1999) extracted hydrophobic plant proteins with organic solvents followed by traditional 1-D SDS-PAGE and examined the non-hydrophobic proteins using a TCA precipitation followed by 2DE.

Two-dimensional gels have been used to analyze protein profiles for over 25 years, separating proteins according to their isoelectric point (pI) in the first dimension, and by their molecular weight in the second dimension (O'Farrell 1975). Two-DE has been used since the 1970s, but reproducibility and protein capacity were poor until Bjellqvist and colleagues (1982) developed immobilized pH gradient (IPG) strips for use in the first dimension. The IPG strips revolutionized isoelectric focusing by immobilizing a carrier ampholyte induced pH gradient in acrylamide polymerized to a strip of plastic. There are still some inherent problems associated with the use of 2-D gels however, not all proteins can be separated using this technique. Proteins with molecular weights greater than 100kDa can not be separated on commercially available IPG strips, and pouring the strips without mechanical quality control leads to reproducibility problems. Proteins that are hydrophobic also can not be separated on 2-D gels, which includes many important cell-surface proteins (Molloy et al. 1998). Membrane proteins present another problem. They are nearly impossible to solubilize prior to isoelectric focusing, and even if they can be solubilized, they often precipitate in the IPG strips during isoelectric focusing (Adessi et

al. 1997, Rabilloud et al. 1997). Another limitation of 2DE was demonstrated by Gygi and colleagues (2000) who analytically determined that only the most abundant proteins can be represented on 2D gels. Part of the problem stems from the availability of sensitive dyes that do not interfere with MS, and to that end many researchers have been working to find more suitable dye techniques (Steinberg et al. 2000; Sumner et al. 2002). In an attempt to analyze more than just the abundant proteins, researchers have tried to find a way to load more protein onto the gels while maintaining sufficient resolution for spot detection, removal, and MS analysis. Several researchers have proposed using gels with expanded separation ranges, such as smaller pH gradient ranges on the IPG strips (Gorg et al. 2000; Wildgruber et al. 2000); however, this approach necessitates running multiple gels, compounding the time it takes to analyze a single sample (Griffin & Aebersold 2001). Clearly there are limitations associated with the use of 2DE, and these limitations are the driving force behind recent advances in alternate protein separation techniques.

Protein separation research has been striving to end our dependence on 2DE. Many researchers have proposed the use of isoelectric focusing in solution rather than on an IPG strip to further separate and subfractionate proteins (Egen et al. 1988; Righetti et al. 1989; Hochstrasser et al. 1991). However, the most commonly used alternative to traditional 2DE is multidimensional chromatography, which is either used in place of, or prior to, 2DE (Corthals et al. 1997; Blackstock & Mann 2000). Chromatography prior to electrophoresis can pre-fractionate protein samples, enabling greater protein loading onto the gels while not decreasing resolution. Multidimensional chromatography is more commonly used in place of 2DE, and is often coupled directly to a mass spectrometer.

Techniques used recently include heparin, high-capacity cation exchange columns (Fountoulakis et al. 1997; Fountoulakis & Takacs 1998; Karlsson et al. 1999); hydrophobic interaction chromatography (HIC) on phenyl columns (Fountoulakis et al. 1999a); hydroxyapatite chromatography (Fountoulakis et al. 1999b); and ion exchange chromatography (Fountoulakis et al. 1998) (reviewed by Righetti et al. 2001). Complex mixtures of proteins can also be enzymatically digested then fractionated using high-performance liquid chromatography linked directly to a mass spectrometer (Opiteck et al. 1997; Link et al. 1999; Gygi et al. 1999; Spahr et al. 2000; Washburn & Yates 2000; Regnier et al. 2001). However, this signature peptide technology is still being developed, and requires the use of complex search algorithms to interpret the data, such as the pioneer algorithm Sequest developed by Eng and colleagues (1994) (Beavis & Fenyo 2000).

Current proteomic analysis typically ends with generation of mass spectrometry data. Mass spectrometry, originally conceptualized in the late 19th century (Thomson 1897), went through a series of advancements throughout the 1900s. However, protein and peptide mass spectrometry, often referred to as biological mass spectrometry, was not truly feasible until quite recently (Karas & Hildenkamp 1988; Tanaka et al. 1988; Fenn et al. 1989). Two types of mass spectrometry are commonly used in proteomics projects to obtain data leading to protein identification, matrix-assisted laser desorption/ionization time of flight (MADLI-TOF) and electrospray ionization (ESI) mass spectrometers. Where MS analysis used to require specialists, the two newer mass spectrometers are user friendly and cost-effective enough that they have become the mainstay of many biochemistry facilities and departments, and are commonly used by amateur mass

spectrometrists. The profusion of proteomic research recently published is in large part due to the advancement and commercial availability of these MS tools (van Wijk 2001).

The two mass spectrometers yield similar results in that they both measure the mass/charge (*m/z*) ratio of a given molecule generating a mass spectrum, but their capabilities are quite varied. MALDI can be used to obtain super-high-accuracy molecular weights for intact proteins or for individual peptide fragments generated from enzymatic digestion, the spectra from these are referred to as the molecule's mass fingerprint (Karas & Hildenkamp 1988). MALDI cannot be used successfully on complex protein mixtures, and can only be used on intact proteins with molecular weights up to approximately 100-150kDa. Electrospray ionization involves tandem mass spectrometry or MS/MS. The machine first measures the full range of masses of a molecule/s introduced and generates a spectrum similar to the MALDI mass fingerprint. Individual peptide ions are then selected, or trapped, and sent into the second mass spectrometer to undergo collision-induced dissociation (CID) which generates internal amino acid sequence information, or sequence tag, for that peptide ion (Papayannopoupos 1995; Wilm et al. 1996; Wilm & Mann 1996). The observed peptide fingerprint masses and/or amino acid sequence tags are compiled and used to search databases on the web potentially matching them to expected masses derived *in silica* from known protein or nucleic acid sequences. Data from the peptide mass fingerprint and the ESI sequence tags are usually enough to obtain protein identity (Mann et al. 1993; Henzel et al. 1993; Shevchenko et al. 1996; Roepstorff 1997; Ducret et al. 1998; Gavaert & Vandekerckhove 2000). Database searching and bioinformatics in general is the limiting step of proteomics especially when the genome of the organism in question

has not been fully sequenced. Proteomics projects generate enormous amounts of data and there are a plethora of bioinformatics tools now available for managing and analyzing this data. Researchers are now required by most of the premier journals to submit protein sequences to the international protein databases when they publish, so the protein databases are growing dramatically every month. However, the majority of the information being submitted is still bacterial and mammalian, and the plant databases are severely lacking in comparison. There are two basic types of database searches that can be conducted, a search of the protein databases where the protein amino acid sequences are converted *in silica* to theoretical peptides masses; and expressed sequence tag and genomic database searches where the nucleic acid sequences are converted to either theoretical amino acid sequence or peptide masses *in silica*. There are numerous search engines available for free on the web that allow users to interface with these databases such as http://prospector.ucsf.edu/ and www.matrix-science.com/ (for a more complete listing see Rowley et al. 2000).

METHODS & MATERIALS

Objective 1. **Develop two-dimensional SDS-PAGE system for mapping and evaluating the wheat proteome.**

Experimental Plan. The goal of this research objective was to separate and map abundant wheat leaf proteins on two-dimensional gels then analyze them using mass spectrometry to obtain putative identity. Preliminary data showed that the use of a single gel system did not provide adequate separation and resolution, so a four-gel system was developed and used in all of the following experiments. Narrow range immobilized pH gradient (IPG) strips (4-7pH or 6-11pH) were used to separate the acidic and basic proteins from each other and two different acrylamide percentages (11% or 14%) were utilized in the second dimension to effectively separate the low and high molecular weight proteins. Preliminary data showed that coomassie blue was not sensitive enough to stain wheat profiles, only detecting approximately half of the proteins. A MALDI-TOF friendly silver staining protocol was developed in order to detect proteins of low abundance while not interfering with mass spectrometry, as many silver staining methods do. Once proteins were detected, removed and digested, MALDI-TOF mass spectrometry was performed in an attempt to obtain protein identity.

Plants. All experiments were conducted using a hard red winter wheat (TXGBE307) obtained from Dr. Mark Lazar's laboratory at Texas A & M. Wheat was planted singly in 3.8cm-diameter x 20.4 cm-high Cone-tainers (Ray Leach Cone-tainer Nursery, Canby, OR) containing Scotts Terra-Lite® Redi-earth® (Marysville, OH). Cone-tainers were held in racks in water pans, and spaced every other row for a total of 48 seedlings per tray. Plants were grown in chambers at Oklahoma State University in Stillwater, OK with a

22°:18°C day:night temperature cycle and a 14:10 day:night photoperiod until they reached the 1-2 leaf stage, approximately 7-10d after planting. Wheat was harvested by cutting it at the base of leaf number one, quickly wrapping it in an aluminum foil pouch, and immediately submerging it in liquid nitrogen to stop proteolytic activity until it was placed in a -80°C freezer. Wheat samples were stored at -80°C no longer than six months.

Water. Water purity is extremely important when proteomic applications are utilized. Impurities may cause streaking of protein spots during 2D SDS-PAGE, yellowing or cloudiness during silver staining, and may impede protein identification from MALDI analysis. Water used in the following experiments was type I, 18 megahom purified with a Barnstead Nanopure Infinity water system, and then further purified via distillation.

Protein Precipitation & Solubilization. Wheat leaf tissue (5g) was ground with a ceramic mortar and pestle (Coors 2¾ in.) with liquid nitrogen. The resulting powder was suspended in chilled (-20°C) 10% trichloroacetic acid (TCA) in acetone containing 0.07% β-mercaptoethanol (ME) and 1% plant protease inhibitor cocktail (Bio-Rad P9599). Ground tissue was added to the suspension solution at a ratio of 1g/5ml. The mixture was incubated at -20°C for at least 1 h then centrifuged at low speed (16,000rpm) (Beckman J2-HS) before harvesting the pellet. The pellet was washed three times (5 ml) with chilled (-20°C) acetone containing 0.07% ME and 1% plant protease inhibitor cocktail (BioRad P9599). The pellet was centrifuged again at 16,000 rpm for 30min between each rinse, then harvested, and slowly dried under nitrogen. The protein pellet was solubilized in 8M urea, 2% Triton X-100 and 60mM dithiothreitol (DTT) (1:30 powder to solution, w/v). The mixture was incubated at 37°C for 30min, centrifuged

(45,000 rpm) (Beckman L8-M Ultracentrifuge) and the supernatant harvested. If lyophilized powder was not solubilized immediately, it was stored at -80°C for later use.

Protein Quantification. The protein quantification assay used is from Ramagli (1999) and is based on the standard Bradford protein assay. The 8M urea and 60mM DTT used in the protein solubilization solution interfere with typical Bradford-based assays. Ramagli and colleagues (1999) have overcome the interference of those substances with the addition of hydrochloric acid (HCl). All standard solutions (ovalbumin 1mg/ml) and samples were prepared by adding 10µl of standard or sample in solubilization solution to a mixture of 10µl 0.1N HCl / 80µl H_2O. Bio-Rad's Protein Assay dye (500-0006) was added to 3 volumes of water and mixed with the standards and samples (180µl dye: 20µl standard or sample) as per the Ramagli & Rodriguez (1985) protocol. Absorbance was read on a Bio-Rad Model 3550 Microplate Reader at 595nm.

2-D SDS-PAGE. 2-D electrophoresis was performed using Amersham Pharmacia's (Uppsala, Sweden) Multiphor II for the isoelectric focusing, and Bio-Rad's Protean II xi Cell, large gel format (16 cm X 20 cm) for the SDS-PAGE. All protein samples were analyzed using a four-gel system: a 4-7pH immobilized pH gradient (IPG) strip (13 cm) on an 11% SDS gel, a 6-11pH IPG strip on an 11% SDS gel, a 4-7pH IPG strip on a 14% SDS gel, and a 6-11pH IPG strip on a 14% SDS gel. Immobilized pH gradient strips (Amersham Pharmacia) were reswelled overnight with 250µl of solubilized protein sample containing 2% 4-7pH or 6-11pH carrier ampholyte (Pharmalyte, Amersham Pharmacia), which is added just before reswelling commences. Low range (4-7 pH) IPG strips were run on the Multiphor II at 300 Volts (V) for 3h then ramped up to 3500V for 18 h. Upper range (6-11 pH) IPG strips were run for 3h at 300V then ramped up to

3500V for 21h. The second dimension SDS gels were run at constant current (35mAmp per gel) until the tracking dye line was 1-2mm from the bottom, approximately 12-15h. Proteins were visualized with silver stain using a modified version of Blum et al. (1987). Gels were fixed in 50% methanol and 12% acetic acid overnight, then rinsed with 50% ethanol (two times for 20min) and water (20min) before treating for one minute with sodium thiosulfate (0.2g/L). Gels were rinsed with water then incubated in silver nitrate (2.0g/L) for 30min. Incubated gels were rinsed with water and developed in a solution of sodium carbonate (60g/L) and sodium thiosulfate (4.0mg/L). Development was stopped by washing in 5% acetic acid and gels were stored in this solution until they could be processed and the spots removed from them.

In-Gel Digestion. Proteins of interest were cut from the gels and placed into 96-well microtiter plates. In-gel digestion of those removed spots was conducted following protocols from Jensen et al. (1999), Shevchenko et al. (1996), and the Keck Biotechnology Resource Laboratory at Yale University (www.info.med.yale.edu/wmkeck). A BioMek 2000 robot was programmed to conduct the extensive pipetting involved in the in-gel digestion protocol to increase throughput and reduce human error. The first step of in-gel digestion is to remove the silver from the gel pieces using 30mM potassium ferricyanide and 100mM sodium thiosulfate then rinsed with 25mM ammonium bicarbonate in 50% acetonitrile (ACN) according to Yale's protocol. The reduction and alkylation of the cysteine disulfide bonds was performed according to Jensen et al. (1999) in 10mM DTT and 55mM iodoacetamide. The reswelling of the gel pieces and tryptic digestion of the proteins followed a slightly modified version of the Shevchenko et al. (1996) protocol where the proteins were digested overnight at 37°C in

20µl of 0.25µg/µl trypsin (Promega V5111) with no additional ammonium bicarbonate added. The supernatant was harvested the following day and the fluid further extracted from gel pieces with 0.1% trifluoroacetic acid (TFA) in 50% ACN and then with 100% ACN. All fluid was pooled with the trypsin supernatant and transferred to 600µl tubes to be slowly dried to approximately 0.5-1.0µl under nitrogen to prevent proteins from binding to the sample tubes.

Protein Identification. Protein identification was attempted using a MALDI-TOF mass spectrometer (2000 Applied Biosystem Pro-Star MALDI-TOF in the OSU Department of Biochemistry and Molecular Biology Core Facility). The digested proteins (above) were mixed with the α-cyano-4-hydroxycinnamic acid (Sigma 14,550-5) matrix required for MALDI-TOF analysis. The matrix needed to be purified via recrystallization prior to use. Recrystalization of the matrix was accomplished by first rinsing it several times with 100% ethanol, and then heating the matrix to 50˚C in 10ml of straight ethanol. The solution was transferred to a clean vile and 5ml of a 1:2 water/ethanol solution was added before incubating the mixture at 4˚C overnight. The α-cyano-4-hydroxycinnamic acid precipitated out of the solution and was harvested and dried. The matrix was then solubilized in 0.1% TFA in 50% ACN (10mg/ml), vortexed, and centrifuged (12,000 rpm) (Stratagene ProFuge™ 10K) to pellet any unsolubilized material. Digested protein samples were mixed with matrix (1:1 v/v) then spotted on a MALDI plate (0.5µl). MALDI-TOF analysis was conducted at the following settings: reflector mode, positive ion mode, acceleration voltage 20,000, grid voltage 77, guide wire voltage 0.002, delay time 300nsec, with 50 shots per acquisition. An accumulated spectrum or peptide mass fingerprint (PMF) was produced for each sample by gathering

50 shots from four separate locations on a given spot for a total of 200 shots per sample. A close external calibration was applied to all samples using bradykinin (0.106ng/μl) (Sigma B3259), adrenocorticotropic hormone (ACTH) (8.36ng/μl) (Sigma A2407), and insulin B chain (42ng/μl) (Sigma I6383). Once the accumulated spectra were gathered and calibrated, they were deisotoped and an internal calibration was applied using the autolytic peaks of trypsin. A list of the peptide masses was generated from each PMF and these peak lists were saved for database analysis.

Bioinformatics. The peak lists from the PMFs generated from MALDI analysis were used to search a local version of NCBI's wheat unigene which is updated monthly. If a suitable match was not found within the wheat unigene, the same PMF was used to search local versions of the rice, barley and corn unigenes which are also downloaded monthly. Hits obtained from the local unigene databases are scored based on several criteria. A putative identification is considered acceptable if at least four peptides from the PMF match the unigene and those peptide matches must cover at least 10% of the putative protein the unigene is coding for. Once a suitable hit is found in one of the unigene databases, the sequence of that gene is copied and submitted to NCBI for a BLAST search (blastx) at http://www. ncbi.nlm.nih.gov/blast/. The putative protein identity obtained from the BLAST search must score over e-10 to be considered acceptable. If protein identification could not be obtained from our local unigene databases, the PMF was submitted to NCBI for a protein database search using the ProFound search engine (http://129.85.19.192/ profound_bin/ WebProFound.exe) and the Mascot search engine http://www.matrixscience.com/cgi/index.pl?page=../home.html. PMF are used to search proteins in the Viridiplantae taxon only with the following search

parameters: One missed cleavage, Tryptic digestion, Iodoacetamide Complete Modification with Methionine Oxidation as a Partial Modification, and a MH^+ charge state. Search tolerance was set at 100ppm according to the specifications of our MALDI. A putative match to a protein in the database is considered successful if the PMF matches at least 4 peptides in the protein, and a significantly high score is given (Porubleva et al. 2001). In ProFound a Z Score of 1.00 or higher is acceptable, with 1.282 being in the 90% confidence interval and 3.090 being equal to 99.9%. If the Mascot search engine was employed, then scores over 63 are deemed significant according to the Mascot scoring algorithm.

Objective 2. **Evaluate changes in susceptible wheat protein profiles in response to aphid feeding.**

Experimental Plan. The goal of this research objective was to determine if the feeding of two different aphids, each inducing unique symptomology, would cause proteins to be differentially expressed in wheat leaves. Secondarily, if differences in protein expression could be visualized, how did the protein profiles change over time? The two aphids evaluated were the greenbug, which causes visual foliar and root symptomology, and the bird-cherry oat (BCO) aphid which causes only visual root symptomology. The overall goal was to compare differentially expressed proteins identified in the aphid-affected profiles to proteins found in known plant defense pathways, in an attempt to elucidate the biochemical effects and mechanisms of aphids feeding on wheat.

The four-gel system developed in objective one was used to map the wheat proteins before and after aphid feeding. The BCO aphids and greenbugs were allowed to feed for

24h and for 6d to evaluate how protein profiles change over time. The proteins that differ were removed, digested and analyzed using MALDI mass spectrometry in an attempt to ascertain protein identity.

Insects. The greenbugs used in the following experiments were biotype E greenbugs reared in continuous culture at the USDA-ARS Laboratory in Stillwater, OK. Insects were reared on potted winter malt barley (13cm diameter pots) in a chamber with a 14:10 photoperiod and 22:18°C day to night temperature. Colonies are tested at the USDA-ARS periodically to ensure they are the correct biotype. All greenbugs used were non-alate adult insects. BCO aphids were reared under the same conditions using only adult insects.

Plants. Wheat (TXGBE307) was grown in Cone-tainers under the same conditions described previously; however, once the wheat reached the 1-2 leaf stage it was infested with 10 greenbugs or 10 BCO aphids for 24h and 6d. The aphids were caged over individual seedlings in Cone-tainers with a clear plastic sheath (18cm high) that has two mesh-covered holes. Once the aphids fed for the predetermined amount of time, they were removed and the wheat was harvested and stored as described above. Control seedlings with no aphids, but still covered by the plastic sheaths, were placed every other row in the trays of Cone-tainers in order to compare differences in protein expression.

Protein Analysis & Identification. Proteins were precipitated, solubilized, run using the four-gel system and visualized as described above. Gels were compared to determine differences in protein expression between the control and experimental gels as well as differences in protein expression between the two different aphid species. Proteins that differed in presence or absence, or substantial up- or downregulation on the gels were cut

from the gels, digested and analyzed as described above in an attempt to determine protein identity.

Objective 3. **Evaluate changes in resistant wheat protein profiles in response to aphid feeding.**

Experimental Plan. The goal of this research objective was to determine if greenbug feeding causes proteins to be differentially expressed in the resistant wheat when compared to susceptible wheat (Objective 2). The overall goal of this objective was to determine how aphid-wheat interactions are expressed at the protein level in the two different varieties of wheat. The protein profiles of resistant wheat fed on by greenbugs or BCO aphids for 24h and 6d were generated using the four-gel system developed in objective one. Proteins that differed were removed from the 2-D gels, digested, and analyzed using MALDI mass spectrometry in an attempt to identify them.

Insects. The greenbugs were reared in growth chambers at Oklahoma State University in Stillwater, OK as described above. BCO aphids were reared under the same conditions using only adult BCO aphids being used for the experiments.

Plants. Resistant wheat (TXGBE273), containing the *Gb3* gene which confers resistance to biotype E greenbugs, was grown in Cone-tainers under the same conditions described above, and infested as previously described. Wheat was harvested and stored as described above.

Protein Analysis & Identification. Proteins were precipitated, solubilized, separated with the 2-D PAGE four-gel system described in Objective 1, and visualized as described above. Gels were compared to determine differences in protein expression between the control and experimental gels as well as differences in protein expression between the

two different aphid species and wheat varieties. Proteins exhibiting differential expression patterns were cut from the gels, digested and analyzed as described above in an attempt to determine protein identity.

Objective 4. **Evaluate different methods of Rubisco removal from wheat extracts.**

Experimental Plan. Rubisco is an extremely abundant protein in wheat extracts, representing up to 80% of the total protein. One of the limitations of IPG strips is that they can only hold about 1mg of protein and if 80% of that is Rubisco, there are a lot of proteins that will not be of high enough abundance to visualize. Protein profiles created on 2-D gels will never be able to visualize an organism's complete proteome. However, if we can remove a protein of exorbitant abundance, it should enhance visualization of scarce proteins. The goal of this objective was to evaluate three methods for Rubisco removal, preparative isoelectric focusing, immuno-affinity chromatography, and HPLC gel filtration in an attempt to increase visualization of proteins of lower abundance.

Preparative Isoelectric Focusing. Preparative isoelectric focusing of leaf proteins was carried out in free solution via Bio-Rad's Rotofor System (Hercules, CA) in an attempt to remove Rubisco. Lyophilized powder from the TCA/acetone precipitation was solubilized in the 8M urea solution as described above (1:30). The solubilized proteins were added (9ml) to 31ml of an 8M urea solution containing 2% BioLyte 3-10 ampholyte (Bio-Rad, Hercules, CA) and 10mM DTT for a total volume of 40 ml which was injected into the Rotofor. Rotofor fractions were collected and proteins separated on 6-18% gradient SDS polyacrylamide (7cm) gels to confirm the location of Rubisco. All gels were run at constant voltage (180V) until tracking dye was approximately 3mm from the bottom. The pH of each Rotofor fraction was determined on a Fisher AR15 pH meter.

Fractions not containing Rubisco were divided and pooled to form two new fractions according to their pH; fractions at a pH of 0-6 were pooled, and those with a pH of 6 or higher were pooled. Fractions containing Rubisco were also pooled and stored at 4°C for later use. Fractions were extremely dilute so several concentration methods were attempted prior to 2-D SDS-PAGE. The first attempt was made with CentriCon Plus 20s (Milipore) which included a buffer exchange to reduce salt levels in the concentrated samples. Attempts were next made to precipitate proteins from the urea solution using TCA in acetone (34ml of chilled TCA/acetone per ml urea solution). Samples were incubated in TCA/acetone at -20˚C overnight, centrifuged at 16K to pellet the protein. Protein was resuspended in resolubilization solution (1µg/10µl) and run on 8-16% SDS polyacrylamide gels. Concentration was then attempted using methanol (34.5ml of chilled methanol per 0.5ml urea solution) as described for the TCA/acetone precipitation. The final concentration method attempted was a double methanol precipitation. Proteins were first precipitated in methanol as previously described, resolubilized in 500µl or solubilization solution and then subjected to a second methanol precipitation (500µl sample into 34.5ml of chilled methanol). Once proteins were concentrated, they were resuspended in resolubilization solution, run on 8-16% SDS polyacrylamide gels, and visualized with coomassie blue or silver stains.

Generation of Rubisco Antibody. Extraction of wheat proteins was accomplished by grinding wheat leaf tissue (5g) in liquid nitrogen then sonicating (1min) and incubating in Tris buffer (50mM Tris-HCl pH 8.0, 7mM EDTA, 1% Plant Protease Inhibitor and 0.04% ME) for 1h. The solution was centrifuged twice at 16,000 rpm (Beckman J2-HS) for 45min at 4°C, harvesting the supernatant after each successive spin. Protein

supernatant was mixed 4:1 with sample buffer (125mM Tris-HCl, pH 6.8, 1% SDS, 1% DTT, 10% glycerol and bromophenol blue) and 50µl was added per well (approximately 280µg total protein per gel). Six 8-16% gels were run (180V) and bands from both the large and small subunits of Rubisco were removed. Six pore limiting gels were also run to obtain purified Rubisco in its non-denatured form. The pore limiting gels were loaded with the same extract (minus the 0.04% ME) and run for 24h at 125V. The running buffer for the pore limiting gels was TBE buffer (90mM Tris, 80mM boric acid and 3mM EDTA). All gels were stained with Gelcode® Blue Stain Reagent (Pierce No. 24592). Once bands were cut from the gels, the proteins were extracted by grinding them in liquid nitrogen then incubating in elution buffer (6ml of 50mM Ammonium Bicarbonate and 0.1% SDS) for 1h at 37°C. The resultant slurry was centrifuged at 7,000 rpm at 4°C for 30 min then aliquotted (1ml) and concentrated (to approximately 25µl) under vacuum centrifugation (Savant Speed-Vac, -50°C cold trap). Once concentrated to 250µl, an additional 250µl of water was added to each tube. The tubes were vortexed continuously for 2min then combined and vortexed for an additional 2min.

The mice used to generate the antibody need to be injected with 50µg of protein three times, with two additional booster injections of 50µg each for a total of 250µg per mouse (3 mice used, 750µg of Rubisco required). Quantification of Rubisco was carried out using BioRad's DC Protein Assay, a modified Bradford assay that compensates for the increased SDS levels. The assay indicated that there was not enough protein (0.72µg/µl in 500µl, 360µg total) so six additional IPG gels were run. Each IPG gel was loaded with 0.75ml of Tris extract (0.7mg/ml for total of 525µg per gel). The protein assay was run again (0.82µg/µl in 500µl, 410µg). The two concentrated Rubisco purifications were

pooled (770μg total) and sent to the Oklahoma State University Hybridoma Center for polyclonal antibody generation from mice. IgG was purified from the mouse blood serum using Protein A Affinity Chromatography on 5ml Pharmacia Biotech HiTrap Columns according to the manufacturer's instructions. Titer of the antibody was tested via immunoblotting. Wheat leaf protein extracts were run on SDS gels, blotted onto nitrocellulose overnight at 25V in 4°C using a 25mM Tris/192mM glycine transfer buffer. Western blots were attempted using a serial dilution of the Rubisco antibody ranging from 1:10 (antibody:blotto) up to 1:1000. Membranes were stained using Chemiluminescence (Pierce 34080).

HPLC Gel Filtration. Wheat leaf proteins were extracted into a non-denaturing Tris buffer (50mM Tris pH 8.0, 7mM EDTA, 1% plant protease inhibitor and 0.04% ME) as described for antibody generation. Tris extracts were then injected (200μl) onto the HPLC gel filtration column (Superdex 200, Amersham Pharmacia 17-1088-01) and run at a flow rate of 0.25ml/min. Protein peaks were detected by UV absorption at 280nm (Waters LambdaMax 481 LC Spectrophotometer), and fractions were collected every 8min.

Fractions from several runs were pooled and each fraction was concentrated in dialysis tubing (3,000 molecular weight cut-off) using Aquacide II (Calbiochem® 17851). Concentrated fractions were run on SDS gels to determine which contained the highest Rubisco concentrations.

RESULTS & DISCUSSION

Develop two-dimensional SDS-PAGE system for mapping and evaluating the wheat proteome. Two-dimensional (2D) gel electrophoresis enables researchers to analyze protein expression patterns at a global level, and has been widely used to map plant proteomes (reviewed in Thiellement et al. 1999). Several of these plant studies have been conducted on wheat: analysis of genetic variation in wheat lines (Zivy et al. 1983), a comparative study of wheat gliadins (Branlard 1983), analysis of storage proteins in wheat seeds (Anderson et al. 1985), a heat-shock protein comparison in wheat lines (Zivy et al. 1987), characterization of several varieties of durum wheat (Picard et al. 1997), allergen analysis of several wheat cultivars (Weiss et al. 1997), and a large-scale comparative analysis of the Triticeae to better understand their phylogeny (Bahrman et al. 1988). The advent of affordable and user friendly protein mass spectrometry (MS) has enabled us to take the mapping of these proteomes one step further, adding the capability of high-throughput protein identification.

2D gel systems have become much more sophisticated since their conception over 25 years ago (O'Farrell 1975; Klose 1975). IPG strips, first developed in the early 1980s (Bjellqvist et al. 1982), have now been developed for a variety of pH ranges, enabling researchers to design their 2D gel systems specifically for organism or tissue type they are working on. However, before the gel system could be designed for this project, the protein extraction/precipitation method that is most efficacious for wheat leaf proteins had to be determined. Two methods were evaluated: protein extraction into an aqueous buffer of 50mM Tris, which in theory is capable of extracting soluble proteins from the leaf tissue, and protein precipitation via TCA in acetone which is capable of precipitating

total protein from the wheat leaves. Protein quantification assays were conducted on both methods. The Tris extraction yielded a protein concentration of 0.7μg/μl, while the TCA/acetone precipitation yielded a protein concentration of 2.55μg/μl initially. In Figures 1 and 2, protein extracts were run on SDS polyacrylamide gels and visualized with coomassie blue. The gels clearly indicate that the TCA/acetone method yields not only higher protein concentration but better preservation of the proteins as exemplified by the greater extent of smearing under the 55kDa band of the Tris gel. Similar results were found by Damerval and colleagues (1986) and Tsugita and colleagues (1994), who found that precipitation of proteins from plant leaf tissue via TCA/acetone yielded the best results in terms of protein quantification and gel quality.

TCA/acetone is capable of precipitating total protein from tissue, soluble proteins as well as membrane-associated and membrane-bound proteins. However, the membrane-associated and -bound proteins are difficult to resolubilize and are often poorly represented in later SDS-PAGE analyses (Molloy et al. 1998). Several techniques were evaluated to enhance protein solubilization into a urea solution. Proteins were initially incubated for two hours at ambient temperature, vortexing every 15min. The mixture was centrifuged and the supernatant was analyzed, yielding a protein concentration of 2.55μg/μl (Figure 2). Resolubilization efficiency was then analyzed by incubating the urea/protein mixture in a warm water bath (37°C) for 1h, followed by incubation at ambient temperature for 1h, vortexing every 15min. This resolubilization method yielded a protein concentration of 3.1μg/μl (Figure 3). The final resolubilization method entailed incubation of the urea/protein solution in a 37°C water bath for 1h, vortexing every 15 min, followed by ultrasonication with a microtip at 35% (full power for the microtip) for

2min (Fisher Sonic Dismembrator Model 300), and incubation at ambient temperature for 1h. The protein concentration following this resolubilization was 3.6μg/μl (Figure 4). The resulting gels comparing resolubilization using the warm water incubation only and the ultrasonication/water bath combination (Figures 3 & 4) clearly show the enhanced protein loading capabilities with this final method. When the amount of protein loaded onto the gel was reduced, to more effectively compare the two methods (Figure 3 & 5), one can see that the number of proteins resolubilized increased as well as the protein concentration. One final attempt was made to enhance the protein profile using a second resolubilization step. The proteins were resolubilized using the bath followed by ultrasonication and centrifugation. The supernatant was removed and an additional 200μl of urea solution was added to the pellet. The mixture was incubated in the warm water bath and ultrasonicated again. Protein quantification was not performed on this second resolubilization since the results would only indicate the concentration and not population, and the goal was to get additional proteins back into solution not merely a higher concentration of the same proteins. The resulting gel of the second resolubilization alone (Figure 6) shows only a couple of additional proteins were solubilized, and they were of such low quantity that when the two extracts are added together they will most likely be too dilute to visualize. Hence, the resolubilization method utilized for the remainder of the project was the water bath followed by ultrasonication.

Preliminary SDS-PAGE was conducted utilizing wide range 3-10pH IPG strips in the first dimension and 14% SDS polyacrylamide gels in the second (Figure 7). Results indicated that this single gel format was not going to yield optimal separation of wheat

leaf proteins in several areas of the gel. If individual, non-overlapping, proteins cannot be removed from the gel, the MALDI analysis and protein database searching will be unsuccessful. The protein extracts were then run using a series of gels, four in total, to obtain greater resolution (Figure 8). Figure 8 shows a composite of the four-gel system developed to enhance separation, with the highlighted boxes indicating the areas of maximized separation for each gel. Proteins were removed for further analysis from the highlighted areas only. The four-gel system entails separating each protein extract on two 4-7pH IPG strips and two 6-11pH IPG strips. Each pH range strip is then placed on both an 11% and a 14% SDS polyacrylamide gel to separate proteins in the second dimension (Figures 9-12). All proteins of substantial intensity are assigned a number, and their experimental isoelectric points (pIs) and experimental molecular weights are cataloged (Table 1). Also included in Table 1 is a relative concentration or intensity (1-5), which was assigned to each protein based on their approximate diameter with 1 being equal to approximately 0-1mm, 2 being equal to 1-2mm etc. up to 5 which is any protein spot with a diameter over 4mm. The table also indicates whether a particular protein was identified or not, and if not was the spectra obtained good (G) (containing more than eight non-trypsin peaks) or poor (P) and hence the likely reason the protein was not identified. Initially, the proteomes were going to be mapped and analyzed for both the susceptible TXGBE307 line and the resistant TXGBE273 line, but that was not economically feasible or required for the achievement the primary objective, the evaluation of the aphid-wheat interaction. However, preliminary results indicated that there are numerous differences between the susceptible and resistant lines even though the lines are purported to be nearly isogenic, and should differ only by a single gene.

Figures 13 and 14 highlight some of the examples of proteins differentially expressed in the two lines. Proteins circled in red are those present in the resistant line only, whereas the black empty circles correspond to proteins of that pI and molecular weight that are only present in the susceptible line. The proteins identified from the resistant line are included in the identification table (Table 2).

Preliminary results indicated that silver staining the gels, as opposed to coomassie staining, enabled visualization of proteins of low abundance (gels not shown). However, some silver staining procedures are thought to interfere with MALDI analysis (Scheler et al. 1998; Gharahdaghi et al. 1999), so a MALDI-compatible silver stain method was developed to allow for the enhanced visualization silver stain offers, while not reducing the quality of the MALDI mass fingerprints significantly. Blum and colleagues (1987) developed the silver staining protocol that served as the basis for the methods developed here. Their protocol calls for the addition of formaldehyde in the fixative, the silver solution use to impregnate the gels, and the developer. Formaldehyde is believed to be the major contributing factor to the reduction of sensitivity during MALDI analysis, and was hence removed from the fixation and impregnation steps. Unfortunately it is mandatory for proper development, and could not be removed from this step. The use of methanol (MeOH) can potentially modify the proteins, which needs to be avoided for proper protein identification. The Blum protocol utilizes MeOH in the fixative, the development-stopping solution, and the storage solution. The MeOH was first removed from the storage solution, switching to 5% acetic acid, which also helped reduced some of the cloudiness associated with storage times greater than 24h. The company Protana (www.protana.com/services/protocols/) recommends an ethanol (EtOH) fixative, a 5%

acetic acid solution for stopping development, and a 1% acetic acid solution for gel storage. The EtOH fixative resulted in gels with high background noise and slightly cloudy appearance. The 5% acetic acid storage solution was continued, but Blum's MeOH/acetic acid fixative was retained. Protana also recommended impregnating the gels at 4°C, which was found to cause no difference in MALDI mass fingerprint quality or gel image quality, and was therefore discontinued. The final silver stain protocol produces better image quality, and MALDI spectra that contain a sufficient number, intensity and quality of peptide fragments for protein identification (Figures 15 & 16).

Figures 15 and 16 show the quality of spectra for protein spots of varied abundance. Figure 15 is a peptide mass fingerprint of an extremely abundant protein, Rubisco (spot #252). The intensity of the peaks (their height) as well as the number of peaks is similar to what would be expected from a protein stained with the non-interfering stain coomassie blue. Figure 16 is a peptide mass fingerprint of a nucleotide-binding site-leucine-rich repeat region (NBS-LRR) protein of extremely low abundance (spot #253). The peaks are less intense and their number is low, but this protein is so scarce it would not have been visualized if coomassie staining had been utilized. The relative abundance of protein number 253 is approximately the lower limit of detection-identification for this system of silver stained gels followed by MALDI analysis, when three spots from three different gels are combined and analyzed. Proteins of lower abundance on the wheat leaf proteome were not cataloged or analyzed, nor were proteins not reproducibly observed.

Silver stain visualization, followed by MALDI MS is purported to yield peptide mass fingerprints with reduced sensitivity and sequence coverage (Scheler et al. 1998; Gharahdaghi et al. 1999). This can be overcome, at least partially, by effective

precipitation of the silver from the gel pieces prior to MALDI analysis. There are numerous enzymatic digestion protocols available to do so, and three were hybridized and modified for use in this project. A protocol from Yale's website (www.info.med.yale.edu/wmkeck) was used to destain the gel pieces utilizing Farmer's reducing reagents, potassium ferricyanide and sodium thiosulfate (Gharahdaghi et al. 1999), to solubilize the silver which is then rinsed away. The proteins were then reduced and alkylated using Jensen and colleagues (1999) protocol. The addition of trypsin for the enzymatic digestion initially followed Shevchenko and colleagues protocol (1996), which added 0.8μg of trypsin to each protein spot (pieces approximately 3mm in diameter by 1.5mm thick or $7mm^3$). The mass fingerprints resulting from this digestion had too many autolytic trypsin peaks which squelch the intensity of the peptide fragments. Yale's protocol called for 0.1μg of trypsin per $15mm^3$ of gel which was not enough trypsin, resulting in incomplete cleavage of the proteins and hence poor identification capability. The protocol developed for this project contains 0.025μg/μl of trypsin, with 30μl added to three pooled spots ($7mm^3$ x 3 or $21mm^3$) for a final ratio of approximately 0.25μg per $7mm^3$ of gel. The resulting mass fingerprints contain autolytic trypsin peaks that are comparable in intensity to the peptide fragment peaks from the sample, and can be used to internally calibrate the spectra (peptide fragments 844.5973, 2223.3223 and 2240.3467 from Figure 16).

Proteins of sufficient abundance (those larger than spot 253, Figure 16) were removed from the gels, destained, digested, and analyzed using MALDI MS. The resulting peak mass lists generated from the peptide mass fingerprints were then submitted to NCBI's protein database using both the Mascot and ProFound search engines. For the aphid-

plant interaction objectives (2 and 3), the proteins were first submitted to a local database (http://139.78.139.117/) containing the NCBI Unigenes for wheat, barley, rice and corn, and if identity was not obtained there, then the protein databases were utilized.

The genome of wheat is not fully elucidated, which in the past meant database searching with MS data was not very effective. However, the abundance of plant EST research submitted to the databases in the last few years has enabled proteomic success to no longer be contingent upon the presence of complete genomic sequence information in the databases. NCBI has developed an algorithm to form contigs of aligned, non-redundant sequence from all the submitted ESTs and full-length mRNAs for a given plant species, generating what they call a Unigene. NCBI has completed the Unigenes for the following plant species: *Pinus taeda*, *Glycine max*, *Lycopersicon esculentum*, *Medicago truncatula*, *Solanum tuberosum*, *Vitus vinifera*, *Sorghum bicolor*, barley (*Hordeum vulgare*), corn (*Zea maize*), *Arabidopsis thaliana*, rice (*Oryza sativa*), and wheat (*Triticum aestivum*). The wheat Unigene is composed of 297,684 ESTs and 715 full-length mRNAs. I did not learn of the wheat Unigene until I was already done with the first objective of this project. However, I went back through the archived peak lists of proteins that were not identified, and was able to successfully identify 58 additional proteins bringing the identification success rate from 33% to 54%. From hence forth, the Unigene databases were searched prior to the protein databases.

In total, 404 proteins were visualized on the four gels of the susceptible wheat leaf proteome. Two-hundred and seventy-seven proteins were removed from the protein profile, and of those removed, 91 were initially identified using the protein databases and 58 using the local EST databases for a 54% identification success rate. The protein

identity, the organism the protein identity came from, the pI of the protein in the database, the molecular weight of the protein in the database, the database the identity was obtained from, the score of the identification, how many peptide fragments submitted to the database matched the protein identified (hit #), and what percentage of the protein's sequence those peptide fragments covered are listed in Table 2.

The identified proteins were grouped by their functions according to Bevan and colleagues' (1998) criteria. The functional annotation classes are as follows: metabolism, energy, cell growth and division, transcription, protein synthesis, protein destination and storage, transport, intercellular traffic, cell structure, signal transduction, disease and defense, and secondary metabolism. Figure 17 shows the functional annotation ratios of the wheat leaf proteome. Most of the proteins identified are involved in energy and metabolism as would be expected (Porubleva et al. 2001, Watson et al. 2003). The proteins annotated under energy production (23%) include the enzymes involved with glycolysis, gluconeogenesis, the pentose phosphate pathway, the TCA cycle, respiration, fermentation, electron transport and photosynthesis (Bevan et al. 1998). While the proteins grouped under metabolism include those involved with the metabolism of amino acids, nitrogen and sulfur, nucleotides, phosphate, sugars and polysaccharides, lipids, sterols and cofactors. The proteins annotated in the disease and defense category include resistance proteins, defense-regulated proteins, those involved with cell death, cell rescue, stress responses, detoxification and others (Bevan et al. 1998). The percentages of functional annotations are comparable to those published by Porubleva and colleagues (2001) on maize, and by Watson and colleagues (2003) on *Medicago truncatula* utilizing Bevan and colleagues' (1998) Arabidopsis annotations. The most significant difference

between the findings of this study and the studies on maize and *M. truncatula* was observed in the percentage of proteins whose function remains unclear. Proteins in this group matched gene sequences in the database whose functions are unknown. The database hit will come back as an unknown, hypothetical, or putative protein. In the two years between the 2001 maize study and the 2003 *M. truncatula* study, the percentage of unknown and hypothetical proteins declined from 59% in 2001 to 3% in 2003. In this project 12% of the proteins identified were unknown, hypothetical or putative proteins. Part of this increase in successful identification is due to the abundant EST data submitted to NCBI with their annotated functions. Watson and colleagues (2003) increased their identification success rate from 25% to 55% by searching the EST databases, which is comparable with the 33% to 54% increase in success rate observed with the dual protein/EST searching methods utilized for this research. The benefit of EST data is clear; in the *M. truncatula* project the researchers were able to increase their success rate substantially because they have generated a tremendous EST library as part of their program, showing the advantages of studying systems holistically from both the genome and proteome points of view.

The data presented here can also be viewed at www.ento.okstate.edu/labs/jwd (site is under construction). The four-gel system may be viewed, with the protein numbers hyperlinked to the cataloging data (pI, molecular weight and identification if obtained with all of the scoring data), as well as the peptide mass fingerprint and mass peak list generated.

Evaluate changes in susceptible and resistant wheat protein profiles in response to aphid feeding (Objectives 2 and 3). Aphids induce varied symptomology in plants, which could be caused by numerous facets of their biology, physiology and biochemistry such as: different probing techniques, inter- versus intracellularly; feeding preferences, removing fluid strictly from phloem cells or indiscriminately from phloem or mesophyll cells; where on the plant they feed, on stems, leaves or roots; the time of day they feed could induce different stress responses; preferential feeding on particular life stages; and/or their different salivary enzymes which could induce various responses. The induction of these varied responses is a complex web that will take years to decipher. Before induction can be understood, the biochemistry of the various aphid-induced plant responses must be analyzed.

The two aphids examined in this project induce different visual symptomology, but the biochemistry of the plant responses in the wheat was unknown. The greenbug induces necrotic lesions at the probing site surrounded by chlorotic halos on wheat leaves (Ryan et al. 1987a). After 24h of greenbug feeding, the necrotic lesions are not yet observable in the susceptible line, some cell damage can be seen in the form of "wetness" at the feeding sites though. By 6d the necrotic lesions and chlorotic halos are present as is a general yellowing of the leaves. The BCO aphid induces no visual symptomology on susceptible or resistant wheat leaves at 24h or after 6d of feeding. However, both aphids cause reduction in root biomass and yield reductions (Burton 1986; Dunn et al. personal communication), so they must have affected the plant biochemically. The results presented here clearly indicate that the two aphids induce different biochemical responses as well (Figures 18-81).

The four-gel system and techniques developed for mapping the wheat leaf proteome were also utilized to evaluate differential protein expression induced by greenbug and BCO aphid feeding. All gels were silver stained and evaluated, to identify proteins that differed between control and aphid fed-on tissues. The two aphids caused substantially different patterns of protein upregulation and downregulation (Figures 18-81). These gels were grouped pair-wise for comparison, with the stressed tissue on the left and the corresponding control tissue on the right. Proteins were considered differentially expressed only in terms of presence and absence, unless the up or downregulation was unequivocally obvious and not believed to be a mere staining artifact. Differentially expressed proteins are circled in the gel Figures 18 to 81, and are numbered beginning at 400 for those not found on the wheat leaf constitutive proteome with a letter following that signifies where the protein was found more highly expressed: a C indicates the protein was more abundant in the control tissue whereas an A indicates the protein was present at a higher level in the aphid fed-on tissue.

The differentially expressed proteins were cataloged by assigned protein number with their experimental pI and molecular weight, relative protein intensity or concentration was also assigned using the same criteria as described for the constitutive protein expression (1-5 based on approximate diameter) (Table 3). Table 3 also indicates whether the protein was identified or not, and whether the lack of protein identity was due in part to the lack of acceptable spectra (G = good; P = poor, based on the spectra containing 8 non-trypsin peaks or more). The differentially expressed proteins identified

are listed in Table 4 (greenbug-induced expression) and Table 5 (BCO aphid-induced expression). The proteins identified were cataloged with their putative identity, the pI of the protein matched in the database, and the molecular weight of the protein in the database. The database identity was obtained from, the score, the number of peptide fragments submitted that match the protein in the database (hit #), and the percentage of the protein sequence that the peptide fragments cover were also recorded with the database cataloging information. The figure number indicating which gel the differential expression was observed on is also listed in Tables 4 and 5. Table 6 lists all the aphid-induced differentially expressed proteins with their expression pattern. The results clearly indicate the two aphids cause very different protein expression patterns as would be expected from two insects that cause such radically different visual symptomology.

Not only did the responses differ between the two aphid species, the aphid-wheat interaction also differed between the susceptible and resistant lines. Peroxidase, chitinase and germin, all common defense proteins, were upregulated in response to greenbug feeding at 24h and 6d; however, they were upregulated in the susceptible line only. These defense proteins are typically upregulated in resistant tissue in response to stress (van der Westhuizen et al. 1998; Roxas et al. 2000; Tuzun 2001), and these results seem to indicate that the resistant wheat could potentially be utilizing an alternate defense strategy against the greenbug. The BCO aphid did not induce the upregulation of any of these defense proteins in either the susceptible or resistant lines.

Peroxidase, one of the defense proteins upregulated in response to greenbug feeding in the susceptible tissue (Table 6), may be involved in a number of biochemical reactions. Its specific function in the greenbug-wheat interaction is difficult to ascertain since

peroxidases are involved in many processes: signal transduction, oxidative stress regulation, cell wall lignification to protect against pathogen penetration and enzymatic degradation, direct toxicity to foreign organisms, and indirect injury to herbivores via oxidative damage to dietary substances (Bowles 1990; Felton et al. 1994; Mehdy 1994; van der Westhuizen et al. 1998; Hammond-Kosack & Jones 2000; Tuzun 2001). Peroxidases are one of the families of pathogen-related (PR) proteins, commonly upregulated during plant-pathogen interactions (van Loon & van Strien 1999). Peroxidase levels also increase in response to other insects, the caterpillars *Helicoverpa zea* and *Manduca sexta*, the beetle *Leptinotarsa decemlineata*, the leaf minor *Liriomyza trifolii*, the mite *Aculops lycopersici*, and the aphid *Diuraphis noxia* (Green & Ryan 1982; Stout et al. 1994; Stout et al. 1998, Fidantsef et al. 1999; Ni et al. 2001).

The chitinase family of PR proteins are also commonly upregulated in response to pathogen attack (van Loon & van Strien 1999); however, the upregulation observed in response to greenbug feeding (Table 6) is inexplicable at this time since there is no clearly defined defensive function for chitinases against piercing-sucking insects (PSIs) (van der Westhuizen et al. 1998). Chitinase is thought to disrupt midgut peritrophic membranes in chewing insects (Mayer et al. 1995), but fluid feeding insects typically lack a peritrophic membrane (Gullan & Cranston 1994). The greenbug is not the only PSI to induce chitinase upregulation however, *D. noxia* and the whitefly *Bemisia tabaci* will also induce the upregulation of chitinase, but in all examples this occurred in resistant tissue indicating that plants do not respond to all aphids with the same defense mechanisms (Bronner et al. 1991; Boijsen et al. 1993; Mayer et al. 1996; Broderick et al. 1997; van der Westhuizen et al. 1998).

The germin protein was upregulated in response to the greenbug during both the early and late susceptible responses (Table 6). Germin proteins in cereals function as oxalate oxidases, and are involved in cell wall synthesis. This protein is known to be upregulated in response to salt stress and during powdery mildew infection in wheat as well (Hurkman & Tanaka 1995; Schweizer et al. 1999). The plant may be using both peroxidase and germin to help strengthen or repair cell walls during greenbug feeding.

Other proteins involved in the susceptible greenbug-wheat interaction are bisphosphoglycerate-independent phosphoglycerate mutase, ATP synthase and peptidyl-propyl isomerase (Table 6). All of these enzymes are involved in the early susceptible response, but they are back to non-stressed (control) expression levels after 6d of greenbug feeding. The bisphosphoglycerate-independent phosphoglycerate mutase was upregulated in response to greenbug feeding at 24h. This enzyme is involved in the later portion of the glycolytic pathway and is not considered a regulatory enzyme; it is responsible for the conversion of glyceraldehyde 3-phosphate to glyceraldehyde 2-phosphate which is then converted into pyruvate. This upregulation at the early response time could potentially indicate that the plant is stimulating the glycolytic pathway in response to the increased energy demands a defense response requires. The upregulation of ATP synthase also points toward increased energy production. Increases in energy production and the reallocation of energy reserves were also observed in response to herbivory by the chewing insect *M. sexta* (Hermsmeier et al. 2001).

In contrast to the bisphosphoglycerate-independent phosphoglycerate mutase and the ATP synthase, peptidyl-prolyl isomerase is downregulated during the plant's early response to greenbug feeding in the susceptible wheat line. This enzyme functions like a

chaperone, aiding in protein folding. It accelerates the rate-limiting step in folding, the *cis-trans* isomeration of Xaa-Proline peptide bonds (Xaa = any amino acid proceeding proline) (Tan et al. 1997; Yang et al. 1997). Why the plant would want to slow the folding of some of its proteins is unclear at this time, but it suggests that perhaps the aphid is stimulating this downregulation for its own benefit.

There were two other enzymes, glucan synthase and NADP-specific isocitrate dehydrogenase, that were involved only in the susceptible wheat-greenbug interaction, but these were downregulated during both the early and late responses. Glucan synthase is a general family of proteins, this particular synthase could be cellulose synthase or callose synthase. Callose synthase is commonly upregulated during defense and wound responses (McCormack et al. 1997), while cellulose synthase is involved in cell wall synthesis (Richmond & Somerville 2000). Either function appears to contradict the results presented earlier, with cell wall synthesis (germin-induced) and defense proteins (peroxidase and chitinase) being upregulated during the susceptible wheat-greenbug interaction. This is an excellent example of the complexity of the aphid-wheat interaction, and a good indication that though we may be discovering pieces involved in the interaction we are a long way from full elucidation. NADP-specific isocitrate dehydrogenase has many possible functions; it can act as an electron donor, it may be involved in the production of reduced glutathione, or the enzyme may work to reduce thioredoxin, which in turn regulates several enzymes such as alternative oxidase. The function of these enzymes and their role in the greenbug interaction is unclear at this time, but both are enzymes that regulate functions crucial to defense and should be examined further.

Two other proteins involved in the susceptible wheat-BCO aphid interaction are glycine decarboxylase and an HR-induced protein (Table 6). The BCO aphid downregulates glycine decarboxylase in susceptible wheat during the 24h interaction. Glycine decarboxylase is a light-inducible, multi-enzyme complex involved in photorespiratory carbon cycle. The function of the carbon cycle is to salvage the carbon in glycolate-2-phosphate which is formed during the oxygenation of ribulose-1,5-bisphosphate during photosynthesis. Most of the carbon is recycled during the carbon cycle but approximately 25% is released as CO_2 in a reaction catalyzed by glycine decarboxylase (Wingler et al. 1997). This was the only photosynthetic-related enzyme suppressed during the susceptible wheat-BCO aphid interaction. Rubisco activase is downregulated in the resistant interaction with both aphids (see below), but it is unclear whether the downregulation of glycine decarboxylase is enough of an indication to say that the BCO aphid is suppressing photosynthesis in susceptible wheat. In plant-pathogen interactions, glycine decarboxylase is the target protein of victorin, a host-specific toxin produced by the fungus, *Cochliobolus victoriae* (Navarre & Wolpert 1995). Perhaps glycine decarboxylase is acting as a receptor for a BCO aphid elicitor as well and the aphid may be trying to suppress the protein so the plant is unaware of its presence. However, the upregulation of the HR-induced protein in response to BCO aphid feeding indicates that the plant is aware that it is under attack, and points toward the downregulation of glycine decarboxylase being a function of suppressed photorespiration.

The HR-induced protein is being upregulated in response to the BCO aphid in susceptible wheat during the 24h interaction (Table 6). The HR in plant-pathogen

interactions is typically induced during the early part of that interaction as well; however, the HR is commonly induced in resistant plants only. Other insects are known to induce a HR-like response, such as Pieris butterflies in Brassica spp. (Shapiro & Devay 1987), Hessian fly larvae in wheat (Dweikat et al. 1997; Schulte et al. 1999), and galling insects on legume species (Fernandes 1998; Stotz et al. 1999). In these situations the HR-like induced response is occurring in resistant wheat, but it is important to remember that both the susceptible and the resistant lines used in this research are tolerant to BCO feeding; the resistant line was developed to be resistant to biotype E greenbugs.

Some of the enzymes identified were involved only in wheat's resistant response to the aphids, such as the downregulation of rubisco activase, which stimulates photosynthesis. This downregulation of photosynthesis was only observed in resistant tissue at the early response time (24h), but it was repressed by both the greenbug and the BCO aphid. One of the first greenbug-induced biochemical changes in wheat leaf tissue is the degradation of Rubisco and the break down of the chloroplasts, resulting in the suppression of photosynthesis (Ryan et al. 1987b). Ryan and colleagues (1987b) observed this greenbug-induced shutdown of photosynthesis in a susceptible line of wheat. No such breakdown of the chloroplasts has been reported in resistant wheat however. Also, the BCO aphid, which does not induce chlorosis, was not expected to downregulate photosynthesis. Hermsmeier and colleagues (2001), however, found that genes regulating photosynthesis were also strongly downregulated in response to the herbivore *Manduca sexta,* which does not induce a chlorotic response either. The fact that this response was only observed in resistant wheat during the early response time, points toward this enzyme having a role in the resistant wheat defense response and not a

general stress-induced response or it would be downregulated in the susceptible wheat early time point as well. This downregulation of photosynthesis, or at least of Rubisco activase, did not appear to affect the concentration of Rubisco however. There was no observed decrease in Rubisco in any of the treatments, even the 6d susceptible greenbug interaction where a decrease was expected as Ryan and colleagues found (Ryan et al. 1987b). This absence of degradation should be qualified though; Ryan and colleagues evaluated Rubisco degradation using enzyme activity assays. It is possible that the Rubisco is degrading in the greenbug treatments but because of its overabundance, small shifts in its concentration are not detectable on 2D gels.

Another protein with a similar response pattern was the reversibly glycosylated polypeptide, which was downregulated in resistant plants only in response to greenbugs and BCO aphids at both 24h and 6d. Reversibly glycosylated polypeptides are proposed to act in the synthesis of hemicellulosic polysaccharides, specifically xyloglucans, and are believed to be involved in cell wall synthesis (Dhugga et al. 1997). Why suppression of cell wall synthesis occurs during the resistant defense response is unclear at this time. Logic would lead one to believe that thickened cell walls would be beneficial during a defense response, although it does not seem to help the susceptible wheat which upregulates cell wall synthesis in response to greenbugs at 24h and 6d to no avail.

Other proteins involved in the resistant wheat-aphid interaction only are a RAS-related protein, malate dehydrogenase, alternative oxidase, and a mitogen-activated protein kinase (MAPK). The RAS-related protein was downregulated in resistant wheat during the 24h greenbug interaction. RAS-related proteins are part of the family of low molecular weight G-proteins, which act as switches activating and deactivating many

signaling molecules. This particular RAS-related protein's role is unclear, but many molecules would be switched on or off during defense responses and further research will need to be conducted to determine its explicit role. This protein would be an excellent candidate for gene silencing. If the gene was turned off in susceptible wheat, would that enhance its resistance to the greenbug? Malate dehydrogenase is one of the enzymes of the TCA cycle, and is responsible for the last step of the cycle the conversion of malate into oxaloacetate. This enzyme has a peculiar expression pattern; it was downregulated during the resistant wheat-BCO aphid interaction at the early response time (24h), but was upregulated in the resistant wheat by the late response time (6d). The reason for this fluctuation of the TCA cycle during the BCO aphid interaction is unclear at this time.

The alternative oxidase is upregulated during the resistant wheat-BCO aphid interaction at 24h but not 6d. Alternate oxidase is a membrane bound protein that functions as an alternate electron acceptor during plant electron transport (animals do not have alternate oxidase). The function of the alternate oxidase is not clear; it is active in all plant tissues, but has very high activity in roots. Energy passed through the alternate oxidase instead of complex III during electron transport generates heat instead of ATP. This heat is used by some plants to thermo-regulate themselves during spring germination, however the heat may also be used to help volatilize certain compounds. Why it is upregulated in response to BCO feeding in resistant wheat is unclear at this time (Hammond-Kosack & Jones 2000). The presence of this membrane bound protein indicates that the system developed for this project does have the capability to evaluate some membrane bound proteins even though they are difficult to resolubilize and tend to run poorly on IPG strips (Molloy et al. 1998).

Mitogen-activated protein kinases (MAPKs) are signaling molecules often upregulated during plant-pathogen interactions (Hammond-Kosack & Jones 2000). The MAPK was upregulated in response to BCO aphid feeding after 6d and only in resistant wheat. The MAPK has two potential roles, it may move into the nucleus where it can stimulate a gene transcription factor or act as a transcription factor itself, or the MAP kinase can remain in the cytosol to rapidly phosphorylate proteins (Baker et al. 1997).

Some of the enzymes identified are not alternately expressed in terms of resistant and susceptible interactions but rather late and early responses. A high affinity phosphate transporter was upregulated in response to greenbug feeding in both susceptible and resistant wheat but only at the early response time (24h). High affinity phosphate transporters are membrane-associated proteins that regulate phosphate uptake (Huang et al. 2000). Aldehyde dehydrogenase was upregulated in both susceptible and resistant tissue but only during the early response (24h) to greenbug. Aldehyde dehydrogenase may have many roles including detoxification, intermediary metabolism, osmotic protection and NADPH generation (Perozich et al. 1999). Many of these functions would be logically upregulated during greenbug-induced stress responses, but its exact role is unclear at this time. A calmodulin-related protein was also upregulated in response to greenbug feeding in both susceptible and resistant wheat; however, the upregulation only occurred during the late response (6d). Calmodulin, when bound to Ca++, interacts with various target proteins. Activated calmodulin wraps around the target domain of a calmodulin-sensitive protein altering the proteins activity. A wpk4 protein kinase was downregulated during the late response of resistant wheat to greenbug feeding. These wheat kinases are known to be upregulated by light and cytokinins and downregulated by

increasing sucrose concentrations. The function of this kinase is thought to stabilize carbon assimilation rates during periods of stress (Ohba et al. 2000). The expression pattern indicates that some inhibitory agent responsible for the kinase suppression must be present by the late response time but not during the early response. Sucrose levels increase during reallocation of carbon resources, which could be causing the suppression. Whether this reallocation would be enough to trigger the kinase suppression and why it is suppressed in resistant tissue when reallocation should not be occurring is unknown.

Protein expression patterns were similar for both aphids in a few instances. One example of identical expression pattern was observed for a protein kinase (#274). The kinase was upregulated during the early response of both susceptible and resistant wheat to both aphids; however, the kinase was downregulated in the late response in resistant wheat to both aphids. Unfortunately the function of this protein is unknown, but with the unusual expression pattern, this protein is an excellent candidate for further research.

Another example of similar protein expression being induced by both aphids was observed for cyclin B which was downregulated in resistant wheat during the early response (24h) to both aphid species. Cyclin B interacts with cdc2 protein kinase to form a serine/threonine kinase holoenzyme complex also known as the maturation promoting factor. The complex accumulates rapidly during the G2 phase of the cell cycle stimulating the beginning of mitosis (Draetta et al. 1989). Cyclin B is only downregulated during the early resistant response; however, the expression of cdc2 protein kinase is downregulated in all greenbug treatments and both susceptible and resistant early responses to the BCO aphid. Even though cyclin B is only suppressed in the resistant line, mitosis will be shut down for all greenbug and all early BCO aphid

interactions because the two enzymes work as a complex and both are required to trigger mitosis. The plant may have suppressed the cell cycle due to stress and/or energy conservation, but whether this is any benefit to the insect is unclear.

Both aphids induce the downregulation of 3-dehydroquinate dehydratase, the third enzyme in the shikimate or prechorismate pathway. Greenbugs suppress the expression of this enzyme at 24h and 6d in susceptible tissue, and the BCO aphids suppress its expression at 24h in the resistant wheat. The shikimate pathway is the biosynthetic pathway for chorismate (Figure 82). Chorismate is the building block for many defensive compounds with ring structures such as the alkaloids, phytoalexins, glucosinolates, hydroxycinnamic acids, lignins, flavonoids, and isoflavonoids, as well as the ringed amino acids tryptophan, phenylalanine, and tyrosine. The suppression of this single enzyme can shut down the production of all of these compounds, which is obviously beneficial to the aphids. The role this enzyme plays in the synthesis of so many defense compounds makes it an excellent candidate for further research to try and deduce its exact role in the aphid-wheat interaction.

Some of the enzyme expression activity was aphid species-specific. Cytochrome P450 monooxygenase and the ribosomal L12 protein were upregulated in response to BCO aphid feeding in all treatments. Membrane associated cytochrome P450s have numerous roles in plant stress responses, but the exact role of this P450 is unknown. This enzyme would be an excellent candidate for further research to determine its function and precise role in the BCO aphid-wheat interaction since it was so clearly upregulated in all BCO aphid treatments but none of the greenbug treatments. The ribosomal L12 protein is part of a protein complex that forms the protein moiety of the GTPase domain in the

eukaryotic ribosome. The protein is the main component of the ribosomal stalk, which is directly involved in the interaction of elongation factors (Briones et al. 1998).

Another species-specific response occurred with the greenbug-induced suppression of starch and fructan biosynthesis via the downregulation of ADP-glucose pyrophosphorylase and sucrose:fructan fructosyltransferase respectively. ADP-glucose pyrophosphorylase was downregulated in all greenbug treatments while the sucrose:fructan fructosyltransferase was downregulated during the early response in susceptible wheat only. ADP-glucose pyrophosphorylase is the regulatory enzyme of starch biosynthesis (Figure 83). Suppression of starch biosynthesis during a stress response seems logical; the plant's resources are being utilized in defense and are not available for storage. However, Al-Mousawi and colleagues (1983) found accumulations of starch granules in resistant wheat cells following greenbug attack, indicating greenbugs are also capable of inducing the production of starch. Why a plant would store starch when those resources are needed for defense is unclear. The conflicting data could be the result of aphid biotype diversity or wheat line variation; Al-Mousawi used biotype C greenbugs and TAM W 101xAmigo wheat and this project evaluated biotype E greenbugs on TXGBE273 wheat. This varied response clearly demonstrates the complexity of aphid-wheat interactions, and the improbability that a single aphid species could ever serve as a model for all aphid-plant interactions.

The sucrose:fructan fructosyltransferase is involved in fructan biosynthesis. Most plant species accumulate starch and sucrose as temporary storage carbohydrates. Cereals however have the ability to store their carbohydrates not only as sucrose and starch, but also as fructan (Wang et al. 2000). In barley, leaves will accumulate fructan more

prevalently than starch when undergoing nitrogen or phosphorous deficiency (Wang & Tillberg 1996, 1997). The downregulation of this enzyme and ADP-glucose pyrophosphorylase is clearly advantageous to the greenbug which has limited access to complex compounds such as starch or fructan, and would benefit from the carbohydrates remaining in simple sugar form.

Evaluate different methods for removing Rubisco from wheat extracts. Rubisco catalyzes the initial steps of the photosynthetic and photorespiratory pathways (Pierce 1988). Rubisco is the most abundant protein on earth (Ellis 1979), and is reported to make up 25-60% of total leaf protein (Ku et al. 1979). Because of the protein's abundance in leaf extracts, much of the protein capacity of the IPG strips it taken up with Rubisco. This severely limits the concentration of other proteins on the 2D gels, and hinders the detection of proteins of low abundance. If Rubisco could be removed from the protein extracts, the concentration of other proteins could in theory be doubled. In an attempt to increase the concentration of proteins of lower abundance, three methods of Rubisco removal were evaluated: preparative isoelectric focusing using the Rotofor, immuno-affinity chromatography utilizing a Rubisco antibody, and HPLC gel filtration fractionation.

Rotofor preparative isoelectric focusing. The first method utilized the Rotofor, which fractionates complex protein samples in free solution using preparative isoelectric focusing. The preparative isoelectric focusing separates proteins according to their isoelectric point, which is the pH at which the protein carries no net charge. Prior to focusing in the Rotofor, the proteins were precipitated with TCA/acetone and resolubilized as previously described. The first attempt at Rotofor fractionation was

carried out with approximately 10.9mg of protein in 50ml of urea solution; 100mg lyophilized powder was resolubilized in 3ml urea solution yielding a protein concentration of 3.6mg/ml, which was added to 47ml of urea solution containing 2% carrier ampholyte. This solution was focused on a 3-10 linear pH gradient and then fractionated into 20 semi-discrete fractions of 2.5ml. Each fraction was analyzed on an 8-16% SDS gel to determine which fractions contained Rubisco (Figure 84). The small subunit of Rubisco was found in fractions 6 & 7 at pHs of 6.578 and 6.598 respectively. The large subunit of Rubisco was in fractions 8-10 at pHs of 6.624, 6.874 and 7.328. These fractions were removed and the remaining fractions were divided and pooled, with fractions at a pH of 0-6 pooled, and those with a pH of 6 or higher pooled. The two pooled groups of fractions could then be run on complementary IPG strips (4-7pH and 6-11pH). This effectively doubles the loading capacity by removing the proteins that are not within the pH range of the strip but that still must be considered in the 1.0mg loading capacity of the strip. The pooled fractions were first run on a one dimensional SDS gel to evaluate protein quality and concentration (Figure 85); the gel was oddly streaked and the protein concentration was too low to run on a 2D gel. Additional wheat leaf proteins were fractionated on the Rotofor at approximately double the concentration, 21.8mg of protein in the 50ml of solution. The fractions were again visualized on 8-16% SDS gels (Figures 86 & 87), and at this concentration there was a substantial increase in the number of proteins present within the fractions. However, when the pooled fractions were run on a 2D gel, protein concentration was insufficient to visualize. Protein concentration was tripled, approximately 43.6mg in 35ml of solution, and then separated and fractionated on the Rotofor. The fractions were visualized on 8-16% SDS gels

(Figures 88 & 89). The fractions contained a substantially higher quantity of protein but the 2D gels still only showed a few proteins.

The pooled fractions were then concentrated using CentriCon Plus 20s in an attempt to reduce the solution volume. Pooled fractions (12.5ml of 4-7pH pooled fractions and 25ml of 6-11pH pooled fractions) were first brought up to 1M sodium chloride to dissociate the ampholyte from the proteins, and then concentrated. The fractions were each concentrated down to approximately 0.5ml, brought back up to 20ml with the urea solution two times to buffer exchange the salt and ampholyte away, then concentrated to approximately 1.0ml, and run on an 8-16% SDS gel (Figure 90). The resulting gel was streaked and unusable at both low and high concentrations and in both fractions. The cause of the streaking was unclear, but was thought to be either excess salt, protein degradation, or excess ampholyte. Figure 91 shows a gel evaluating the cause of streaking. The first lane is a crude non-concentrated Rubisco fraction that contains the normal 2% carrier ampholyte. The second lane is a Rubisco fraction that has been brought up to 40% carrier ampholyte, double the expected amount of ampholyte in the concentrated fractions if the salt dissociation did not work. The third lane is a Rubisco fraction that was concentrated exactly as described above for the pooled fractions. The fourth lane is a crude non-concentrated Rubisco fraction that was brought up to 1M sodium chloride in case the salt was not buffer exchanged away in the CentriCon Plus 20s. As the results clearly indicate, the cause of the smearing is an overabundance of carrier ampholyte.

The next step was to attempt a protein precipitation of the pooled fractions to remove the ampholyte contamination since ampholytes should not precipitate. Again the Rubisco

fractions were utilized for testing. TCA/acetone was attempted first, adding 1ml of Rubisco fraction to 34ml of TCA in acetone, but nothing precipitated from the solution. MeOH precipitation was attempted next, adding 0.5ml of Rubisco fraction to 34.5ml of MeOH. The proteins precipitated this time; however, the streaking was still present. The final precipitation method tried was a double MeOH precipitation. The proteins were precipitated as described, the resulting pellet was resolubilized in 0.5ml of urea solution, and precipitated again in 35ml of MeOH. The pellet was resuspended in 250µl of urea solution and run on an 8-16% SDS gel (Figure 92). The gel was still extremely streaked and smeared.

The ampholyte problem in the Rotofor fractions was never resolved, but the crude extract was tested on 2D gels with the abundant ampholyte remaining. The pooled fractions were run on 4-7pH or 6-11pH IPG strips accordingly, and then on 14% SDS gels in the second dimension. The 7cm IPG strips used can hold up to 125µl of solution, this amount was loaded onto the strips, but the excess ampholyte caused them to spark and burn. The quantity of pooled fraction solution was dropped to 100µl and then 75µl before any of the strips would focus without burning, the 6-11pH range strips never focused even down to 25µl of solution. The 4-7pH 14% gel with 75µl of pooled fraction had insufficient protein quantity and quality when compared to a normal 4-7pH gel (Figure 93 compared to 94). The concentration of proteins did not seem greater after fractionation and concentration. The removal of the Rubisco fractions (the right side of the gel) caused the loss of too many other proteins. The ampholyte is unable to dissociate from the proteins when solubilized in urea, however when the preparative isoelectric focusing was attempted in an aqueous buffer, all proteins precipitated in the Rotofor

before focusing was complete. The removal of Rubisco does not seem possible using this technique.

Rubisco antibody. Prior to antibody production, preliminary research was conducted to determine if a Rubisco antibody would be reactive across different species. The expense of making an antibody would be far less cost prohibitive if the antibody could be used on different plant species. A small amount of antibody, which was generated to Rubisco from barley, was obtained from Terence Murphy's lab at UC Riverside. Tris-aqueous extracts of barley, wheat and the legume *Medicago truncatula* were run on 8-16% SDS gels (Figure 95), the proteins were transferred to nitrocellulose and probed with the barley Rubisco antibody (Figure 96). The results clearly show that the antibody is cross reactive. The antibody reacted more strongly with the wheat Rubisco than the *Medicago truncatula* Rubisco indicating the Rubisco from the two grass species are probably more closely related. This also indicated that this technique could be widely applicable with a single Rubisco antibody.

An attempt was made to generate a wheat Rubisco antibody at the Oklahoma State University Hybridoma Center in mice. The antibody in culture media was concentrated in dialysis tubing (3,000 molecular weight cut-off) using sucrose as recommended by the Hybridoma Center. The IgG from the antibody was then purified and concentrated further using a Protein A column. The western blots probed with the wheat Rubisco antibody did not show any reactivity to wheat, barley or *Medicago truncatula.* The serum obtained did not have a high enough antibody titer to visibly react with the plant extracts. Antibody production should be attempted again, there was not enough data to support abandoning this technique. Perhaps the next antibody could be generated in

rabbit instead of mice, since the antibody obtained from UC Riverside was generated in a rabbit and exhibited excellent titer level. The removal of Rubisco via affinity chromatography could still be successful.

HPLC gel filtration fractionation. Low pressure gel filtration chromatography is not a high-resolution technique; however, high performance liquid chromatography (HPLC) offers better resolution. The Superdex gel filtration column used in this study was first calibrated, and a molecular weight standard curve was generated (Figure 97) with standards ranging from 669kDa to 29kDa. The Tris extract of the wheat leaves was then loaded on the column and run at a flow rate of 0.25ml/min. The resulting chromatogram (Figure 98) shows peaks substantially past the bed volume of the column (V_t) which was 24ml. The collected fractions (2ml each) were analyzed on an 8-16% SDS gel (Figure 99), and showed that all of the proteins came off the column within the appropriate time according to the standard curve and the total column volume (i.e. between V_o and V_t). The problem is that Rubisco is in fractions 6-9 instead of one single fraction, indicating that Rubisco could not be successfully separated from the other wheat leaf proteins because of the insufficient resolution of gel filtration chromatography.

An interesting result of the gel filtration chromatography was the indication that there are a substantial number of peaks past fraction 9 on the chromatogram (Figure 99). Further research should be conducted to ascertain if these are peptides that did not show up on the 8-16% gel or if this is merely a chromatography artifact due to the proteins interacting with the Superdex column. If these are peptide peaks, the peptides could be collected and analyzed further to potentially identify some of the peptides in the wheat

leaves. Perhaps these peptides could then be analyzed to determine how their expression changes due to aphid feeding.

CONCLUSIONS

The initial goal of this project was to determine if proteomic techniques could be used to evaluate aphid-wheat interactions. Would differences between stressed and non-stressed tissue be visible, and if so would they be identifiable with the current state of technology and database capabilities? The results clearly indicate that proteomic approaches can be applied to this system efficaciously to identify proteins involved in aphid-wheat interactions. The practical benefits of this type of global analysis are self evident. No longer hypothesis driven, global analysis can take the guess-work out of interaction studies. A single gel can elucidate hundreds of proteins involved in an interaction, when previously scientists would have hypothesized about a particular protein's role in an interaction, and then tracked it down from there. This technology has the potential to advance interaction studies much more quickly than the old hunt-and-peck methods.

This study clearly shows the protein and genomic/EST databases are no longer an insurmountable hindrance to plant proteomic research. Proteins with unknown, hypothetical or putative annotations are still fairly common (11%), but the rate has dropped 53% since 2001 (Porubleva et al. 2001) and will continue to do so. One important fact to remember though is that the identifications obtained from the protein and EST databases are all putative assignments. Until the protein is sequenced or some other confirmation technique is used, the identifications are all putative and should be treated accordingly. One should also remember that the proteome elucidated in this research is not the complete proteome of wheat leaves, but rather the proteome as visualized by the gel system designed and is therefore limited by the techniques applied.

The problems associated with the solubilization of lipophillic proteins limits the representative number of membrane-bound proteins in the proteome. Silver staining does not visualize all proteins, and is also partially responsible for the incomplete nature of the proteome. 2D electrophoresis also restricts the scope of the proteome, with proteins of low abundance falling below the technique's limit of detection. There are alternate technologies being developed now to enhance mass spec protein analysis. In particular, the use of HPLC coupled to an electrospray mass spectrometer to separate proteins in solution and then inject them directly into the MS. The theory is that proteins of lower abundance will not be excluded from this type of analysis enabling researchers to evaluate more complete proteomes. The wheat leaf proteome elucidated here, which may be viewed at www.ento.okstate.edu/labs/jwd (site under construction), may not be complete but it can serve as a comparative template for all types of future research both in wheat and other cereal crops and is extremely beneficial.

The aphid-wheat interaction research exemplified the complexity of these interactions. The two aphids induced radically different patterns of protein expression when feeding on the same tissue. The BCO aphid-wheat interaction seemed more subtle, inducing fewer changes in protein expression, which could be expected since it also induces fewer changes in the physical appearance of the wheat leaves. The BCO aphid feeding did not induce the upregulation of any of the typical defense proteins except the HR-induced protein during the susceptible interaction, which is not where defense proteins are traditionally upregulated. The greenbugs on the other hand induce many of the traditional plant defense proteins, but again they were not expressed in the resistant tissue where they were expected to. This research has elucidated many proteins involved

in the aphid-wheat interaction, but we are still a long way from understanding the broad spectrum of what is going on in wheat biochemically in response to these insects.

There was an interesting trend that I have speculated about, but this should not be interpreted as fact without further research. The wheat's susceptible interaction with the greenbug was surprising in several aspects. The upregulation of the defense proteins was obviously not expected nor was it effective; the greenbugs were still able to kill these susceptible plants. The plant utilized precious resources on these compounds to no avail, which is surprising since plants are so well adapted to conserve their resources. So why are they doing it? Is the greenbug sophisticated enough to know that if the plant wastes it resources fighting, it will be easier to overcome? Would the greenbug be able to overcome or kill wheat if it weren't wasting its resources fighting? In other words, would wheat be tolerant of aphid feeding if it wasn't depleting its resources trying to fight? The resistant wheat is tolerant of greenbug feeding, it does not contain a feeding deterrent that discourages feeding. There was no observed upregulation of defensive compounds in the resistant line in response to aphids; in fact the interaction suppressed the only semi-defense protein found, the reversibly glycosylated polypeptide. In the susceptible interaction the wheat upregulates cell wall thickening, whereas the resistant wheat downregulates cell wall thickening. Could this be because the resistant wheat realizes the thickening is unnecessary or futile? The literature and breeders claim the TXGBE307 and TXGBE273 wheat used were nearly isogenic lines however there were so many changes in constitutive expression it didn't seem the case. Perhaps the single gene blocks the induction of the more traditional plant defense response like the one observed in the susceptible line. The BCO aphid did not induce the defense response in either line

though. Conceivably, the aphids elicit a response in the wheat which some have purposed is a gene-for-gene mechanism similar to the plant pathogen *R* gene-*avr* gene mechanism (Milligan et al. 1998; Rossi et al. 1998). Perhaps the resistance mechanism of the *Gb3* gene in the TXGBE273 line is related to this elicitor-recognition mechanism in some respect. The gene could block the elicitor-recognition mechanism effectively keeping the plant from wasting its resources defending itself; or the gene is the elicitor-recognition mechanism which then triggers the plant to not undergo the defense response. Either way, there is no clear way to tell if this speculation is on target or not from the results, further research needs to be conducted.

Other studies evaluating different pairs of susceptible and resistant wheat lines need to be conducted to see if the same trends hold true. Evaluating this set of wheat lines using different aphids would also be beneficial to the confirmation and further elucidation of the aphid-wheat interaction. The most beneficial approach may be to look at the lines of wheat used here stressing them with the different greenbug biotypes. TXBGE273 is only resistant to biotypes C, E, H, I and K; by evaluating protein expression in TXBGE273 wheat exposed to these aphids and comparing that to expression-induced by the other biotypes, we could confirm the greenbug-TXBGE273 interaction at least. The biggest gap in our understanding of aphid-wheat interactions is the lack of knowledge of elicitor-recognition mechanisms. Until we know what the aphid elicitors are, we will never fully understand aphid-wheat interactions. It is difficult to understand the biochemistry of an interaction without knowing how it began.

The data gathered here should be analyzed further as well, beginning with the proteins of unknown function. Traditional biochemical and molecular methods should be

applied to ascertain their true identity. Proteins of interest could be sequenced, and then using that information, gene silencing techniques could be applied to determine if the removal of that particular protein confers or debilitates resistance to the aphids. Other knockout techniques could also be applied to those same ends, but the knockout technology seems less refined and hence the results would be less conclusive.

There are many ways to build upon the data gathered here, this project was designed to be the initial phase of aphid-wheat interaction evaluation, but it will be years before the aphid-wheat interaction is fully elucidated.

Figure 1. Aqueous extraction of wheat leaf proteins on 8-16% SDS gel.

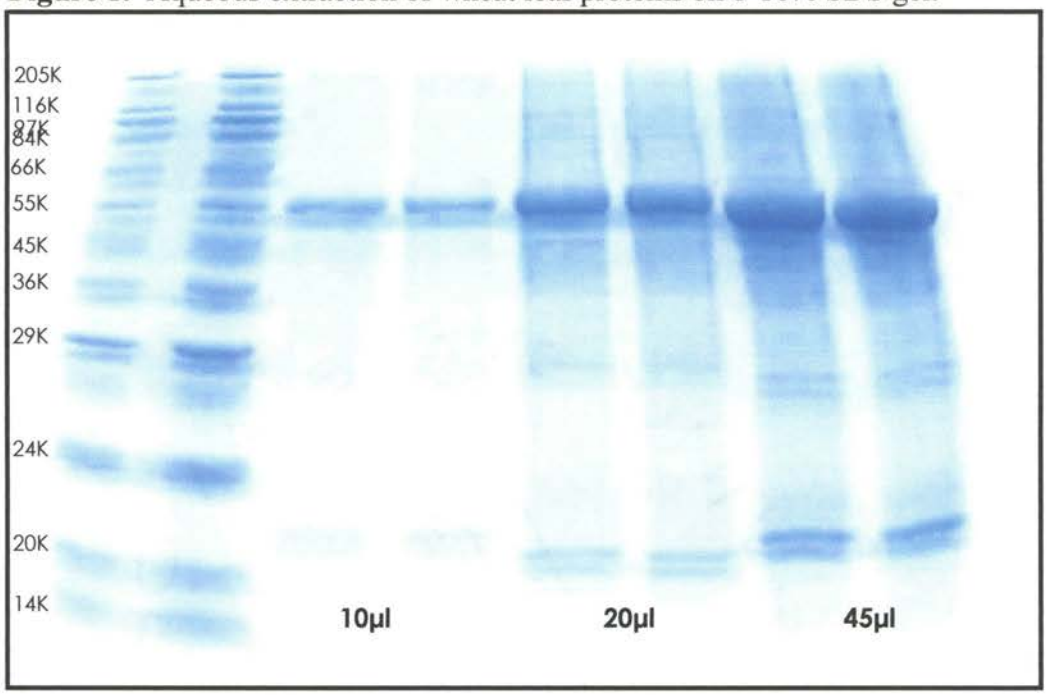


Figure 2. TCA/acetone extraction of wheat leaf proteins on 4-20% gradient SDS gel.

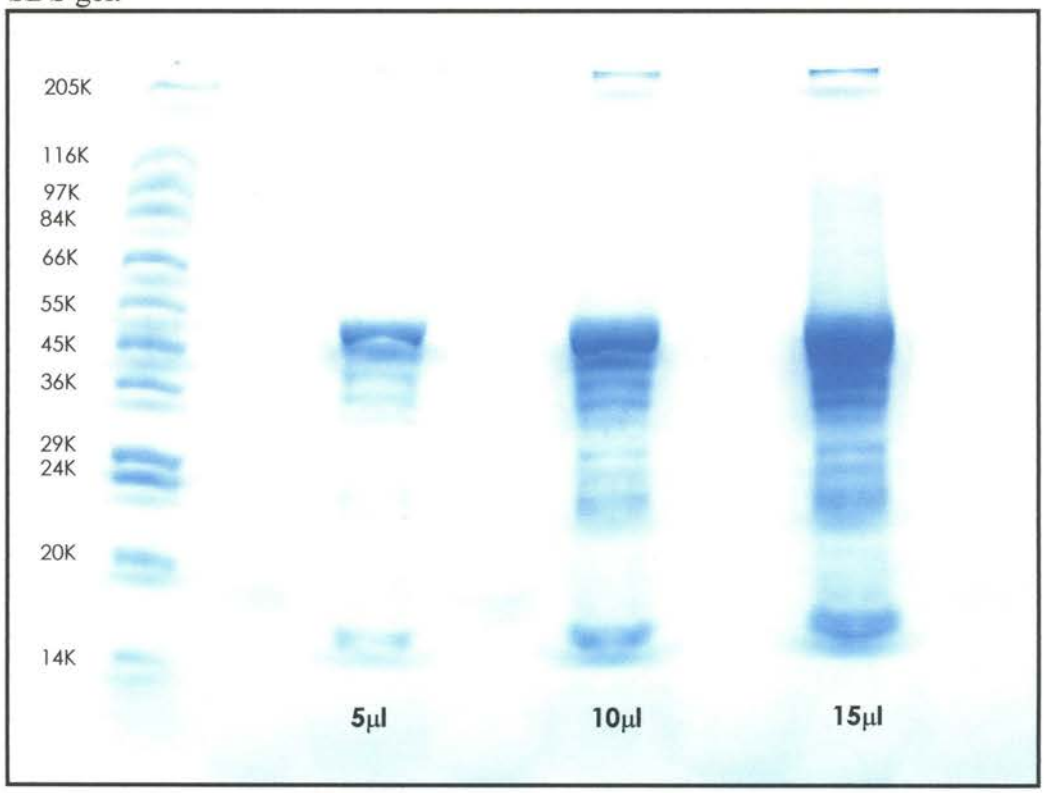


Figure 3. TCA/Acetone extraction of wheat leaves, followed by resuspension into urea buffer (1µg powdered extract /30µl resolubilization solution; loaded 125µl of solution onto an 11% gel) using only a warm water bath to aid in resolubilization.

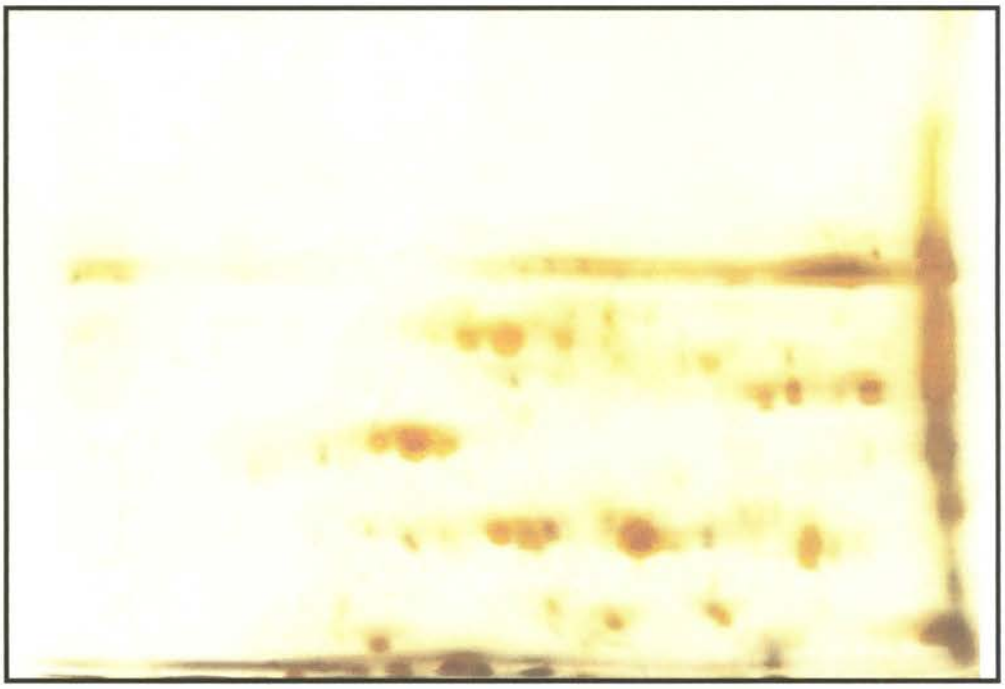


Figure 4 TCA/acetone extract, resolubilized in urea (1µg powdered extract/ 30µl resolubiliztion solution; loaded 125µl of solution onto gel) using ultrasonication to aid in protein resolubilization.

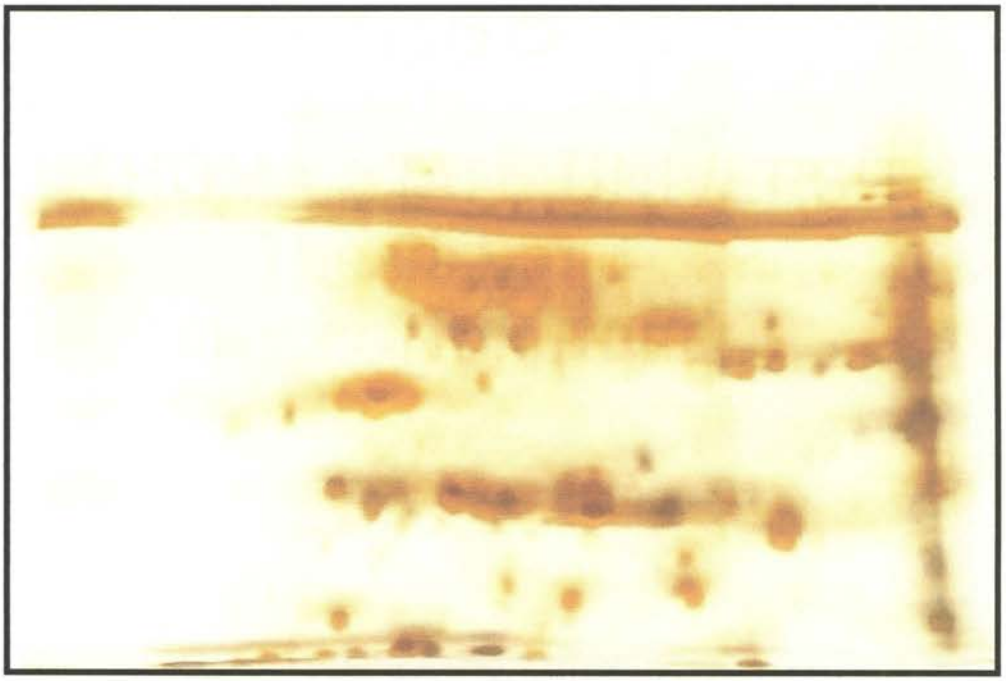


Figure 5. TCA/acetone extraction, ultrasonicating during resolubilization (1µg/30µl), loaded 90µl of solution instead of 125µl on a 4-7 IPG strip and run on an 11% SDS gel.

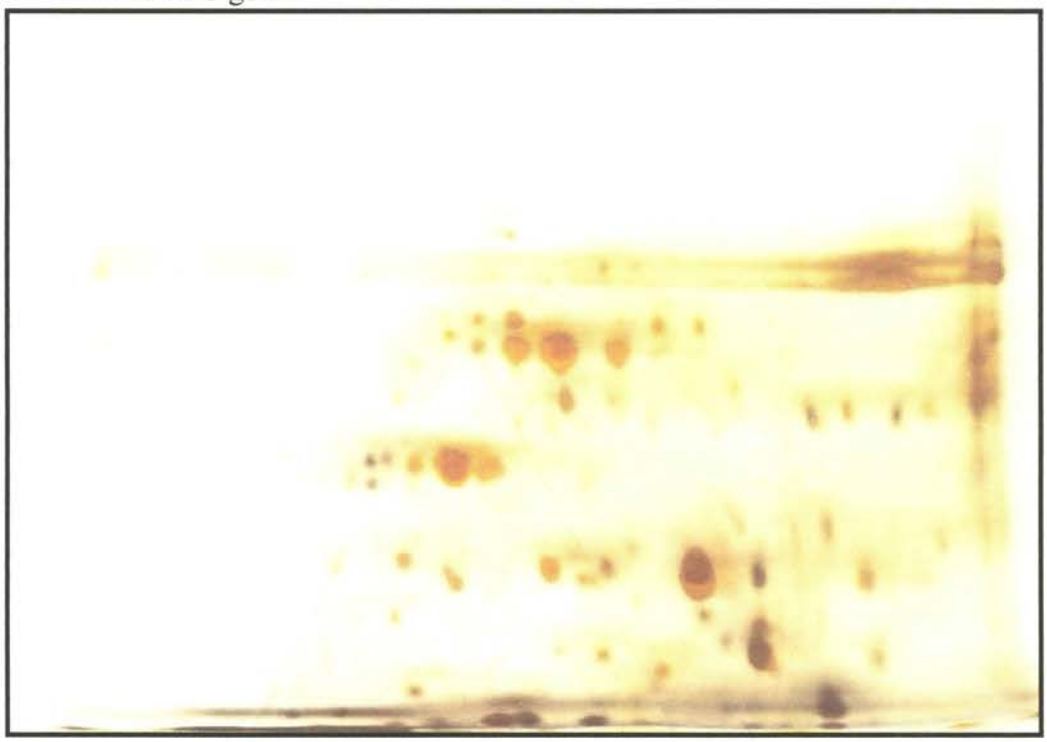


Figure 6. Second solubilization of protein pellet (not added to original extract) in urea solution to enhance resolubilization run on an 4-7 IPG strip and an 11% SDS gel.

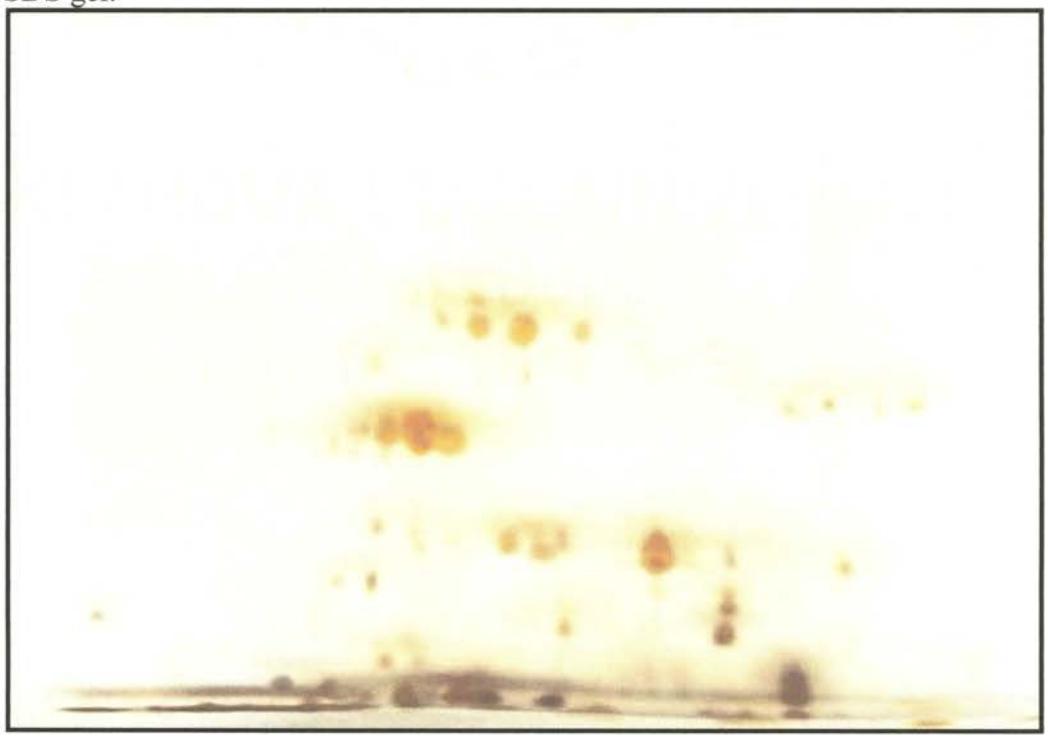


Figure 7. SDS-PAGE analysis of wheat leaf proteins utilizing a 3-10 IPG strip and a 14% SDS gel.

Figure 8. Four-Gel System; 4-7pH 11% SDS (A), 6-11pH 11% SDS (B), 4-7pH 14% SDS (C), 6-11pH 14% SDS (D).

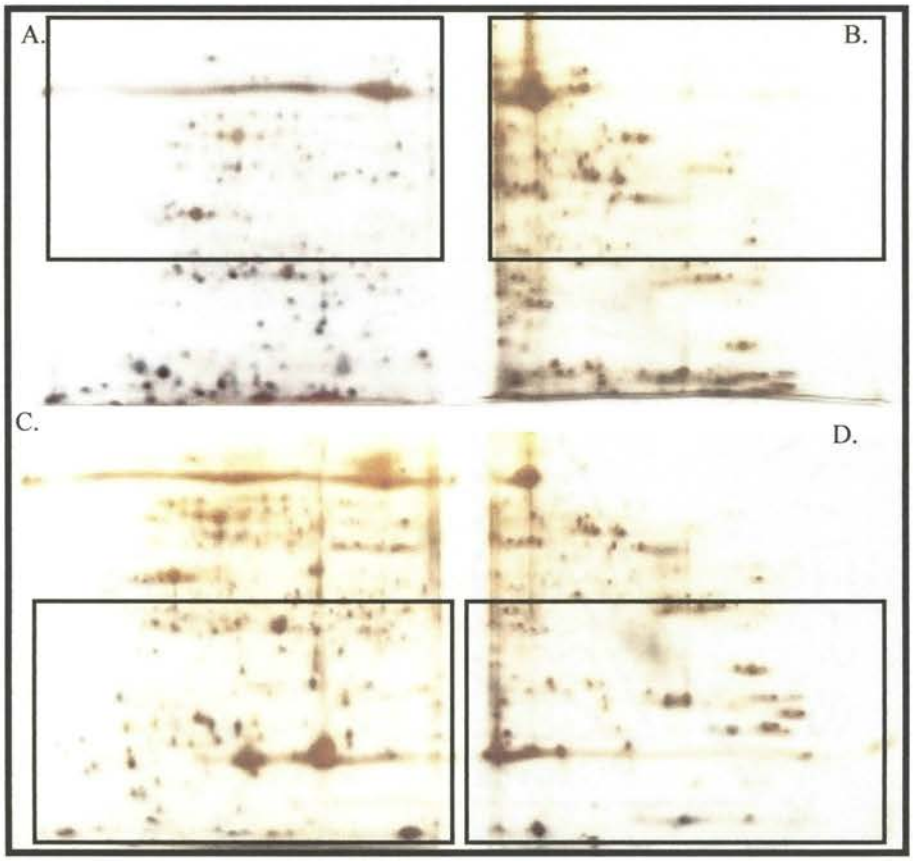


Figure 9. 2D-gel separation of a susceptible wheat leaf extract (4-7pH range, 11% SDS-PAGE).

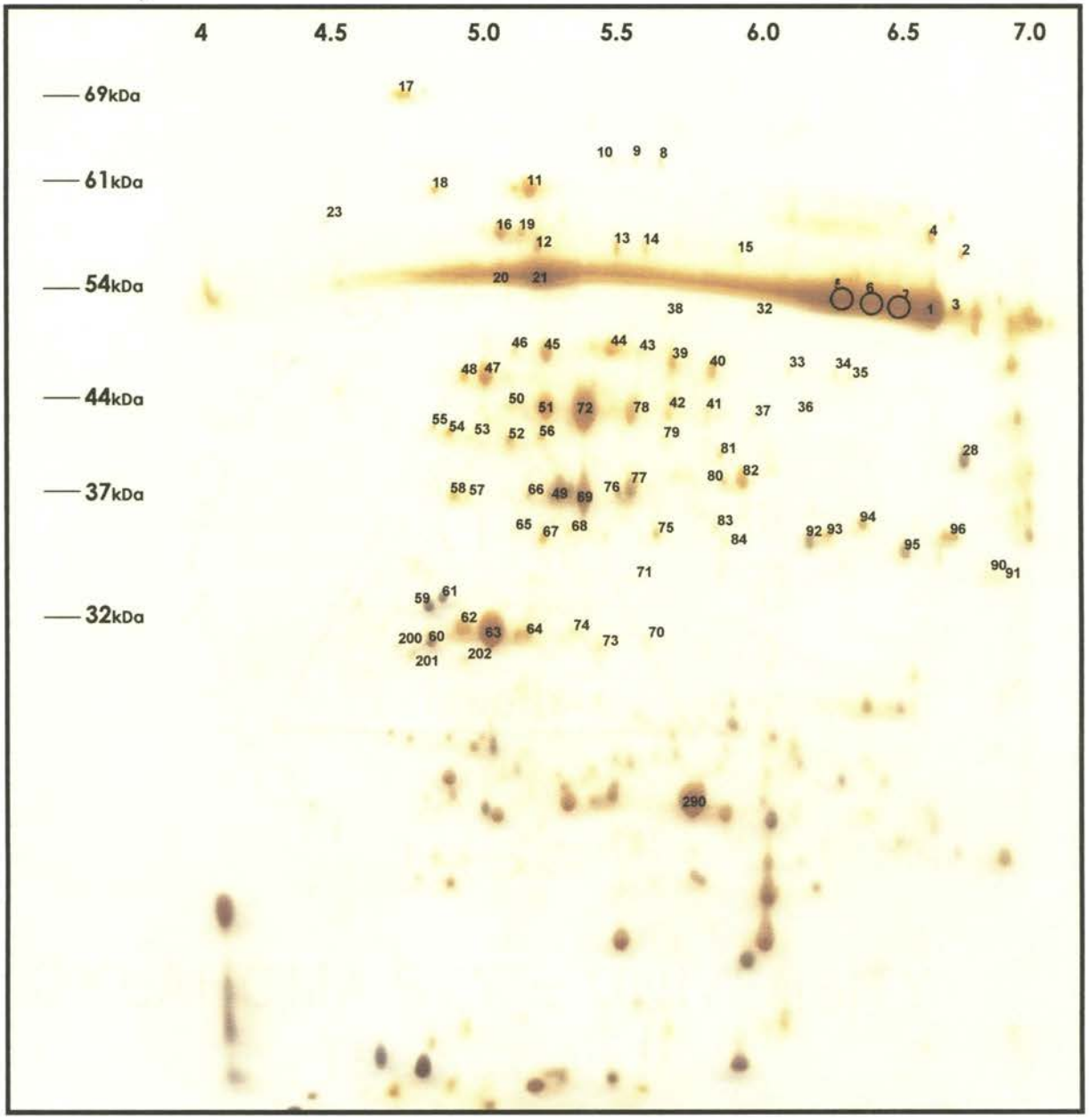


Figure 10. 2D-gel separation of a susceptible wheat leaf extract (4-7pH range, 14% SDS-PAGE).

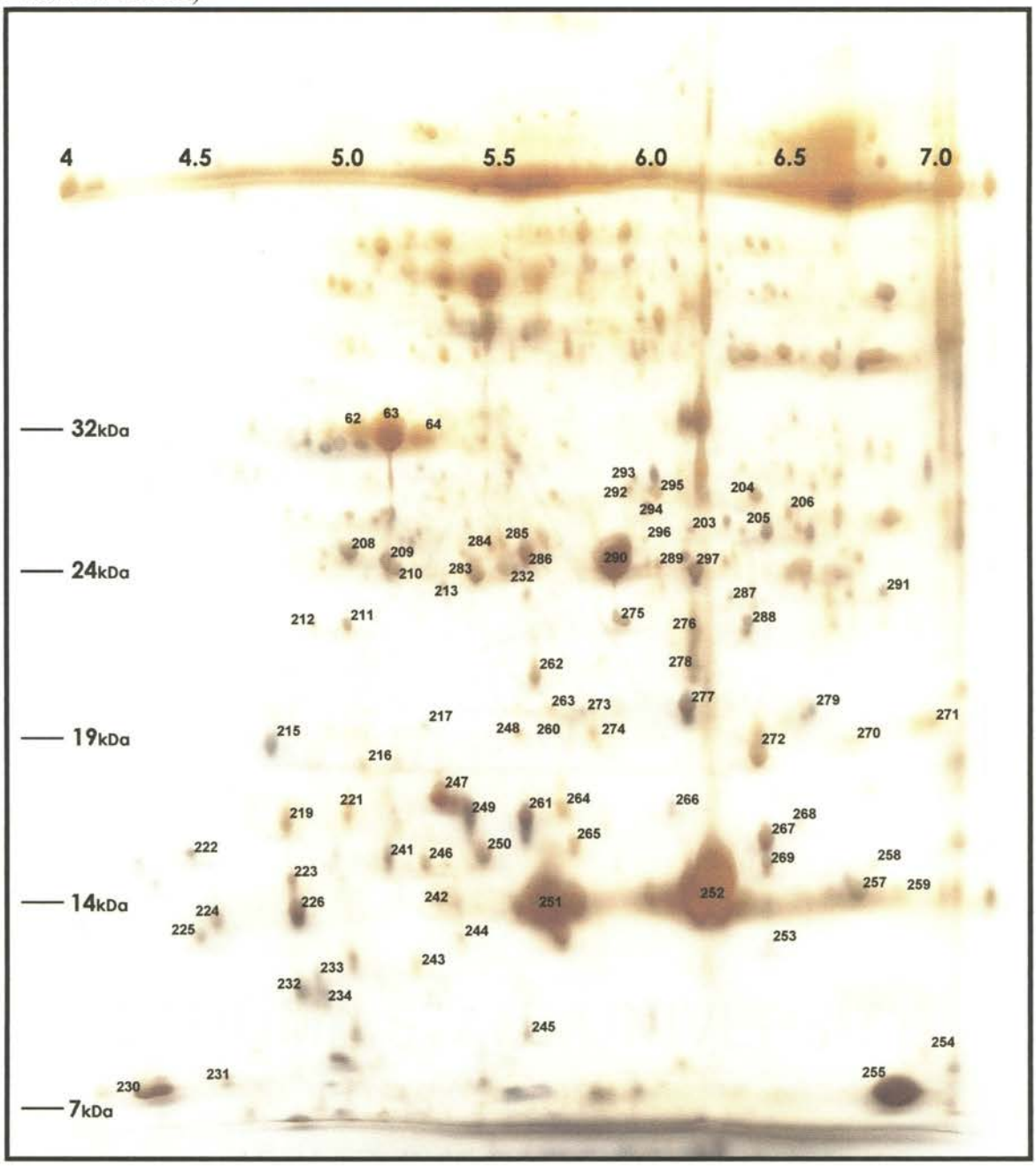


Figure 11. 2D-gel separation of a susceptible wheat leaf extract (6-11pH range, 11% SDS-PAGE).

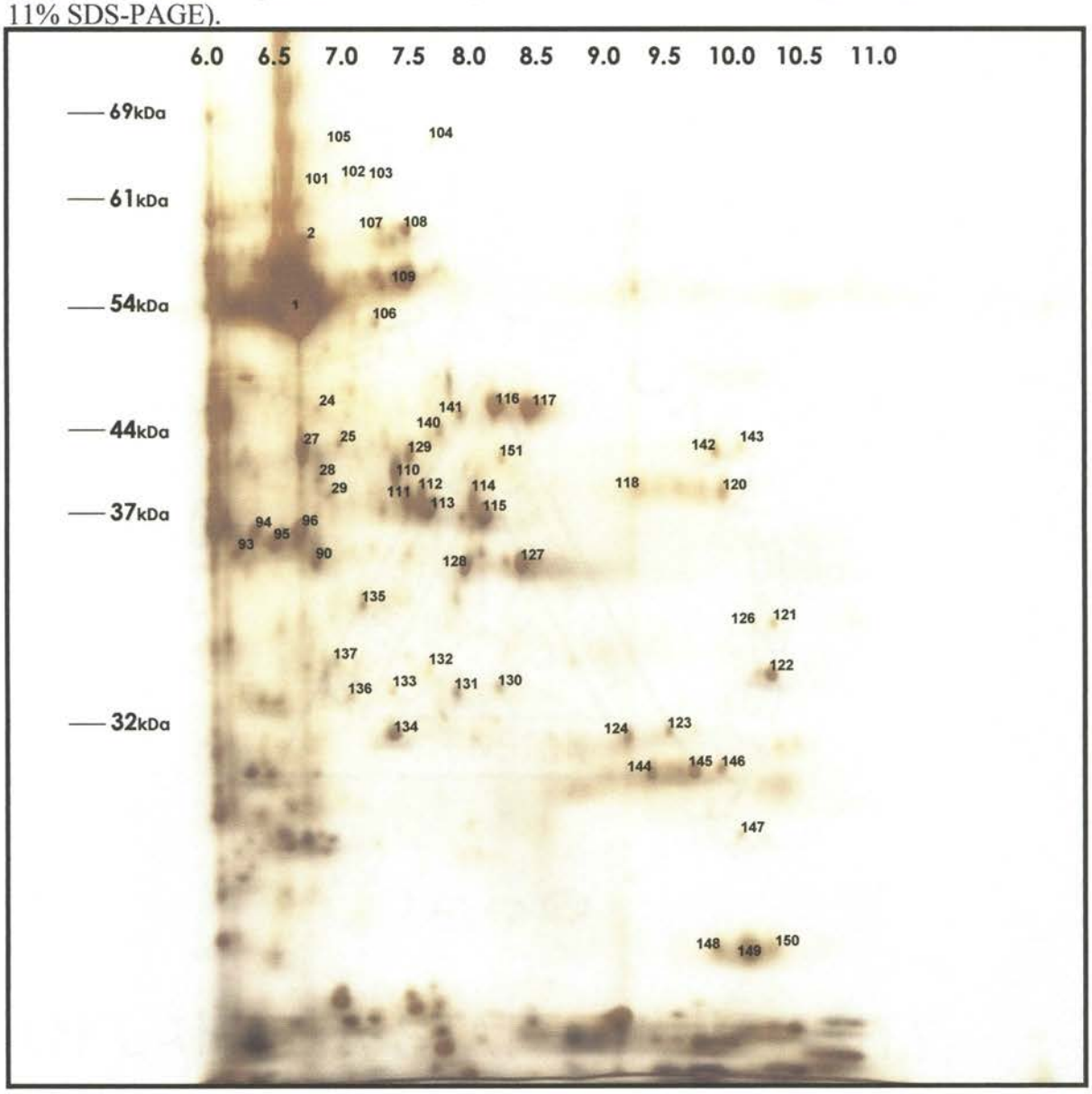


Figure 12. 2D-gel separation of a susceptible wheat leaf extract (6-11pH range, 14% SDS-PAGE).

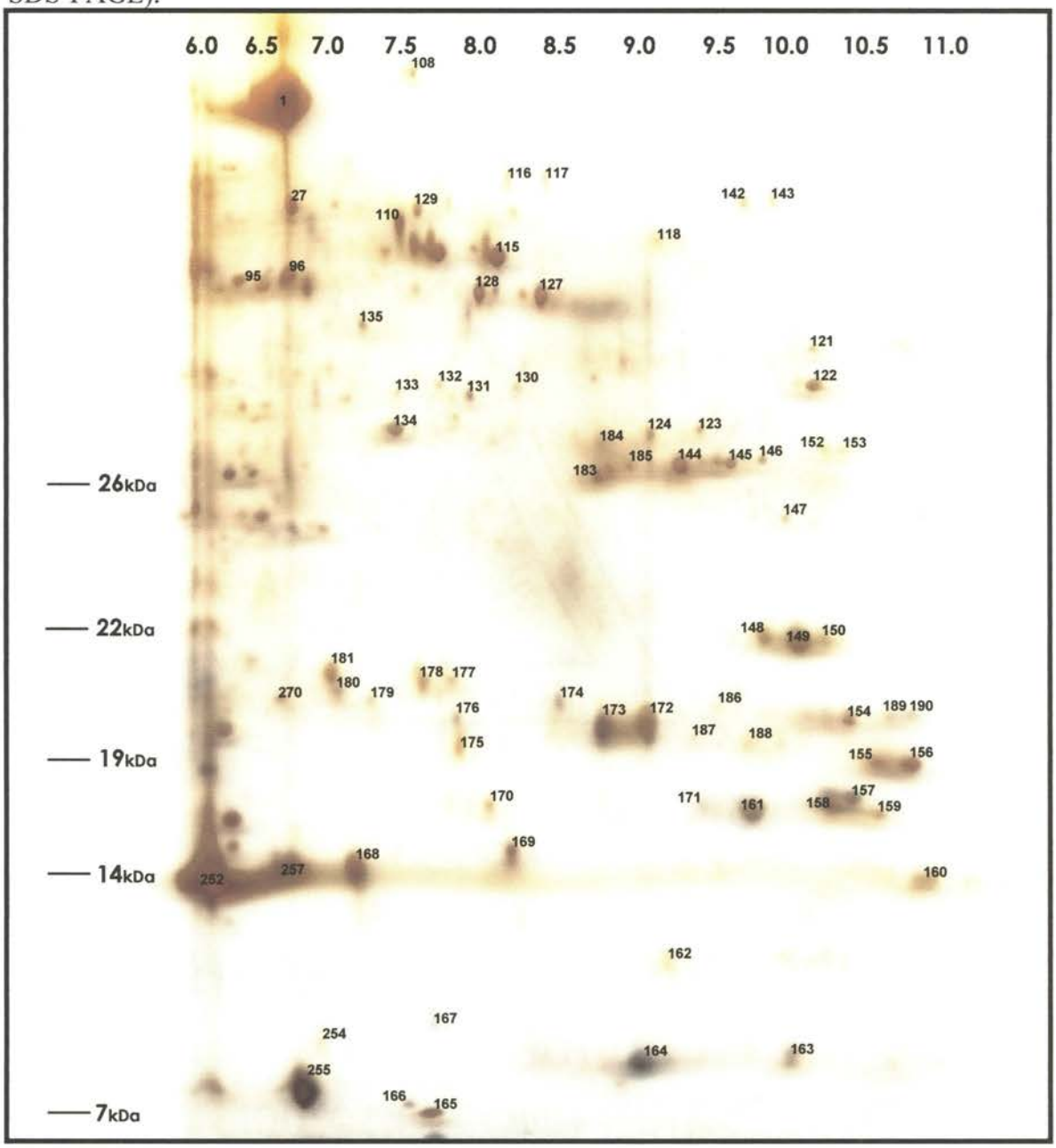


Table 1. Catalog of greenbug susceptible wheat leaf proteins at the two leaf stage with pI, molecular weight, protein intensity, identification indicator, and spectra quality. Protein numbers correspond to the spot numbers on gels shown in Fig 9 to Fig 12. Protein concentration refers to relative size and intensity of protein spot. Spectral quality refers to quality of MALDI spectra and is expressed as G = good, P = poor, and x = adequate for identification.

#	pI	Mol Wt (kDa)	Prot Conc	ID	Spec Qual	#	pI	Mol Wt (kDa)	Prot Conc	ID	Spec Qual
1	6.60	54	5	Y	x	**45**	5.25	48.5	2	N	P
2	6.70	58.5	1	Y	x	**46**	5.15	48.5	1	Y	x
3	6.70	54	2	Y	x	**47**	5.00	46	2	Y	x
4	6.60	61	1	Y	x	**48**	4.95	46	1	Y	x
5	6.30	54	5	Y	x	**49**	5.25	37.5	3	Y	x
6	6.40	54	5	Y	x	**50**	5.10	43.5	1	Y	x
7	6.50	54	5	Y	x	**51**	5.25	43.5	2	Y	x
8	5.65	65	1	Y	x	**52**	5.10	42	1	Y	x
9	5.55	65	1	Y	x	**53**	5.00	42	1	N	P
10	5.45	64.5	1	Y	x	**54**	4.90	42	1	Y	x
11	5.20	61	2	Y	x	**55**	4.80	42.5	1	N	P
12	5.20	57	1	Y	x	**56**	5.20	42	1	N	G
13	5.50	57	1	Y	x	**57**	4.95	37	1	N	G
14	5.60	57	1	Y	x	**58**	4.90	37	1	Y	x
15	5.95	57	1	Y	x	**59**	4.80	33	2	Y	x
16	5.05	58	2	Y	x	**60**	4.90	31.5	2	Y	x
17	4.70	69	2	Y	x	**61**	4.80	33.5	2	Y	x
18	4.80	61	1	Y	x	**62**	4.95	32	3	Y	x
19	5.15	58	1	Y	x	**63**	5.05	32	4	Y	x
20	5.05	54	5	Y	x	**64**	5.15	32	2	N	G
21	5.20	54	5	Y	x	**65**	5.15	36	1	N	G
23	4.45	60.5	1	Y	x	**66**	5.20	37.5	1	Y	x
24	6.75	43.5	1	N	P	**67**	5.25	35.5	1	Y	x
25	6.90	40.5	1	N	P	**68**	5.30	36	1	N	G
26	6.70	38	1	N	P	**69**	5.35	37	3	Y	x
27	6.65	40	2	N	G	**70**	5.60	32	1	N	P
28	6.70	39.5	2	Y	x	**71**	5.60	34	1	N	P
29	6.85	39	1	N	G	**72**	5.30	43.5	4	Y	x
32	6.00	53.5	1	Y	x	**73**	5.45	32	1	N	P
33	6.10	48.5	1	Y	x	**74**	5.30	32.5	1	N	G
34	6.25	48.5	1	N	G	**75**	5.60	35.5	1	Y	x
35	6.30	48.5	1	Y	x	**76**	5.50	37.5	1	N	P
36	6.10	45	1	Y	x	**77**	5.55	38	2	Y	x
37	6.00	44.5	1	Y	x	**78**	5.55	43.5	2	Y	x
38	5.65	52	1	Y	x	**79**	5.65	42	1	N	G
39	5.70	48.5	2	Y	x	**80**	5.90	38	1	Y	x
40	5.80	48.5	2	Y	x	**81**	5.90	39	1	N	P
41	5.80	46	1	Y	x	**82**	5.95	38	2	Y	x
42	5.65	46	1	Y	x	**83**	5.85	36	1	Y	x
43	5.55	49	1	Y	x	**84**	5.90	35.5	1	Y	x
44	5.50	49	2	Y	x	**90**	6.80	34	1	N	P

#	pI	Mol Wt (kDa)	Prot Conc	ID	Spec Qual	#	pI	Mol Wt (kDa)	Prot Conc	ID	Spec Qual
91	6.90	34	1	Y	x	**146**	9.75	26	1	N	G
92	6.15	36	2	Y	x	**147**	9.85	24.5	1	Y	x
93	6.20	36	1	Y	x	**148**	9.70	22	2	Y	x
94	6.35	36.5	2	Y	x	**149**	9.95	22	3	Y	x
95	6.55	35.5	2	Y	x	**150**	10.15	22	2	Y	x
96	6.70	36	2	Y	x	**151**	8.10	40	1	N	P
97	10.00	21	1	Y	x	**152**	10.15	26.5	1	N	P
98	10.20	21	1	Y	x	**153**	10.30	26.5	1	N	P
99	10.30	20.5	1	N	G	**154**	10.30	19.5	2	N	G
101	6.75	62.5	1	N	G	**155**	10.50	18	3	Y	x
102	7.00	62.5	1	Y	x	**156**	10.75	18	3	N	P
103	7.20	62	1	Y	x	**157**	10.85	16.5	2	N	G
104	7.55	66.5	1	N	P	**158**	10.75	16.5	3	N	G
105	6.85	66.5	1	N	G	**159**	10.55	16	1	N	G
106	7.20	51.5	1	N	G	**160**	10.80	14	3	Y	x
107	7.20	58.5	2	N	P	**161**	9.75	16.5	3	N	G
108	7.40	58.5	2	N	G	**162**	9.20	11	1	N	G
109	7.45	55	4	Y	x	**163**	10.00	8	2	N	P
110	7.30	39	2	N	G	**164**	9.00	8	3	Y	x
111	7.40	38	2	N	G	**165**	7.65	6.5	2	N	P
112	7.55	38.5	2	N	P	**166**	7.50	7	1	N	P
113	7.55	37	3	N	G	**167**	7.65	9	1	N	P
114	7.90	38	2	N	G	**168**	7.15	14	4	Y	x
115	8.00	36.5	3	Y	x	**169**	8.20	14.5	2	Y	x
116	8.10	44.5	3	Y	x	**170**	8.00	16.5	1	N	P
117	8.35	44.5	3	Y	x	**171**	9.40	16	1	N	P
118	9.10	38	1	N	G	**172**	9.05	19.5	4	Y	x
120	9.75	38	1	Y	x	**173**	8.75	19.5	4	N	G
121	10.10	32	1	N	P	**174**	8.45	20	1	N	G
122	10.10	30	2	Y	x	**175**	7.75	18.5	1	Y	x
123	9.30	28	1	Y	x	**176**	7.75	19.5	1	N	G
124	9.10	28	2	N	P	**177**	7.70	20.5	1	N	P
127	8.25	35	2	Y	x	**178**	7.55	20.5	2	N	G
128	7.80	35	2	N	G	**179**	7.25	20	1	N	P
129	7.50	39.5	2	Y	x	**180**	7.05	20.5	1	N	P
130	8.15	29.5	1	N	P	**181**	7.00	21	2	N	G
131	7.75	29.5	1	N	G	**182**	10.65	8	1	Y	x
132	7.60	30	1	N	P	**183**	8.80	25.5	2	N	P
133	7.30	29.5	1	N	P	**184**	8.80	26.5	2	N	P
134	7.30	28	2	N	P	**185**	8.95	26	2	N	P
135	7.15	33	1	N	P	**186**	9.50	20	1	N	P
136	7.00	29	1	N	G	**187**	9.80	19	1	N	P
137	6.85	30.5	1	N	P	**188**	9.70	19	1	N	G
138	9.80	10	1	N	G	**189**	10.65	20	1	N	P
139	9.75	24	1	Y	x	**190**	10.75	20	1	N	P
140	7.65	42.5	1	N	G	**200**	4.70	31.5	1	Y	x
141	7.75	43.5	1	N	P	**201**	4.75	31	1	Y	x
142	9.65	41	1	N	P	**202**	4.90	31	1	Y	x
143	9.90	41.5	1	N	G	**203**	6.30	26	1	Y	x
144	9.75	26	2	N	G	**204**	6.40	27	2	N	G
145	9.55	26	2	N	G	**205**	6.45	26	2	Y	x

#	pI	Mol Wt (kDa)	Prot Conc	ID	Spec Qual	#	pI	Mol Wt (kDa)	Prot Conc	ID	Spec Qual
206	6.50	26.5	2	Y	x	**256**	6.55	18	1	Y	x
208	4.95	25	2	N	G	**257**	6.75	14	5	Y	x
209	5.10	24.5	2	Y	x	**258**	6.85	15.5	1	Y	x
210	5.15	24	1	Y	x	**259**	6.90	14	1	N	G
211	4.90	22.5	1	Y	x	**260**	5.65	19.5	1	Y	x
212	4.80	22.5	1	N	G	**261**	5.60	17	2	Y	x
213	5.30	23.5	1	Y	x	**262**	5.65	21	2	N	P
214	4.70	21.5	1	Y	x	**263**	5.70	20	1	Y	x
215	4.70	19	2	Y	x	**264**	5.75	17	2	Y	x
216	5.00	18	1	Y	x	**265**	5.80	16	2	Y	x
217	5.25	19.5	1	N	G	**266**	6.15	16.6	1	N	G
218	4.90	18	2	Y	x	**267**	6.45	16	2	Y	x
219	4.75	16.5	2	N	G	**268**	6.55	16.5	1	N	P
220	4.05	17	2	Y	x	**269**	6.45	15	2	N	G
222	4.45	15.5	1	N	G	**270**	6.75	19	1	N	G
223	4.80	15	1	N	G	**271**	7.00	19.5	1	Y	x
224	4.55	13.5	2	Y	x	**273**	5.80	20	1	N	G
225	4.45	13	2	N	G	**274**	5.85	19.5	1	Y	x
226	4.80	13.5	2	Y	x	**275**	5.95	22.5	2	N	G
227	5.05	16.5	1	N	P	**276**	6.15	22	1	Y	x
228	5.20	16.5	1	Y	x	**277**	6.15	20	3	N	P
230	4.30	7.5	2	N	G	**278**	6.15	21.5	2	N	P
231	4.55	8	1	N	P	**279**	6.55	19.5	2	N	P
232	4.80	11	2	Y	x	**280**	6.95	17.5	1	Y	x
233	4.95	12	1	N	G	**282**	5.50	24	2	N	P
234	4.90	11	2	Y	x	**283**	5.40	24	2	Y	x
241	5.10	15.5	2	Y	x	**284**	5.45	25.5	1	Y	x
242	5.40	14	1	Y	x	**285**	5.50	25.5	1	Y	x
243	5.25	12	1	Y	x	**286**	5.55	25	2	N	G
244	5.35	13	1	N	G	**287**	6.30	23	1	N	P
245	5.60	9.5	1	Y	x	**288**	6.35	22.5	2	Y	x
246	5.25	15.5	2	N	G	**289**	6.05	24.5	1	Y	x
247	5.30	17.5	3	Y	x	**290**	5.90	24.5	5	N	P
248	5.55	19.5	1	Y	x	**291**	6.80	23.5	1	Y	x
249	5.40	17	2	Y	x	**292**	6.05	28.5	1	N	G
250	5.45	15.5	2	Y	x	**293**	5.95	28.5	1	N	P
251	5.70	14	5	Y	x	**294**	6.00	27	1	Y	x
252	6.20	14	5	Y	x	**295**	6.05	28	1	Y	x
253	6.40	13	1	Y	x	**296**	6.00	26	1	N	P
254	7.00	8.5	1	N	G	**297**	6.20	24	2	Y	x
255	6.85	7.5	5	Y	x						

Figure 13. 2D-gel separation of a resistant wheat leaf extract (4-7pH range, 11% SDS-PAGE).

Figure 14. 2D-gel separation of a resistant wheat leaf extract (4-7pH range, 14% SDS-PAGE).

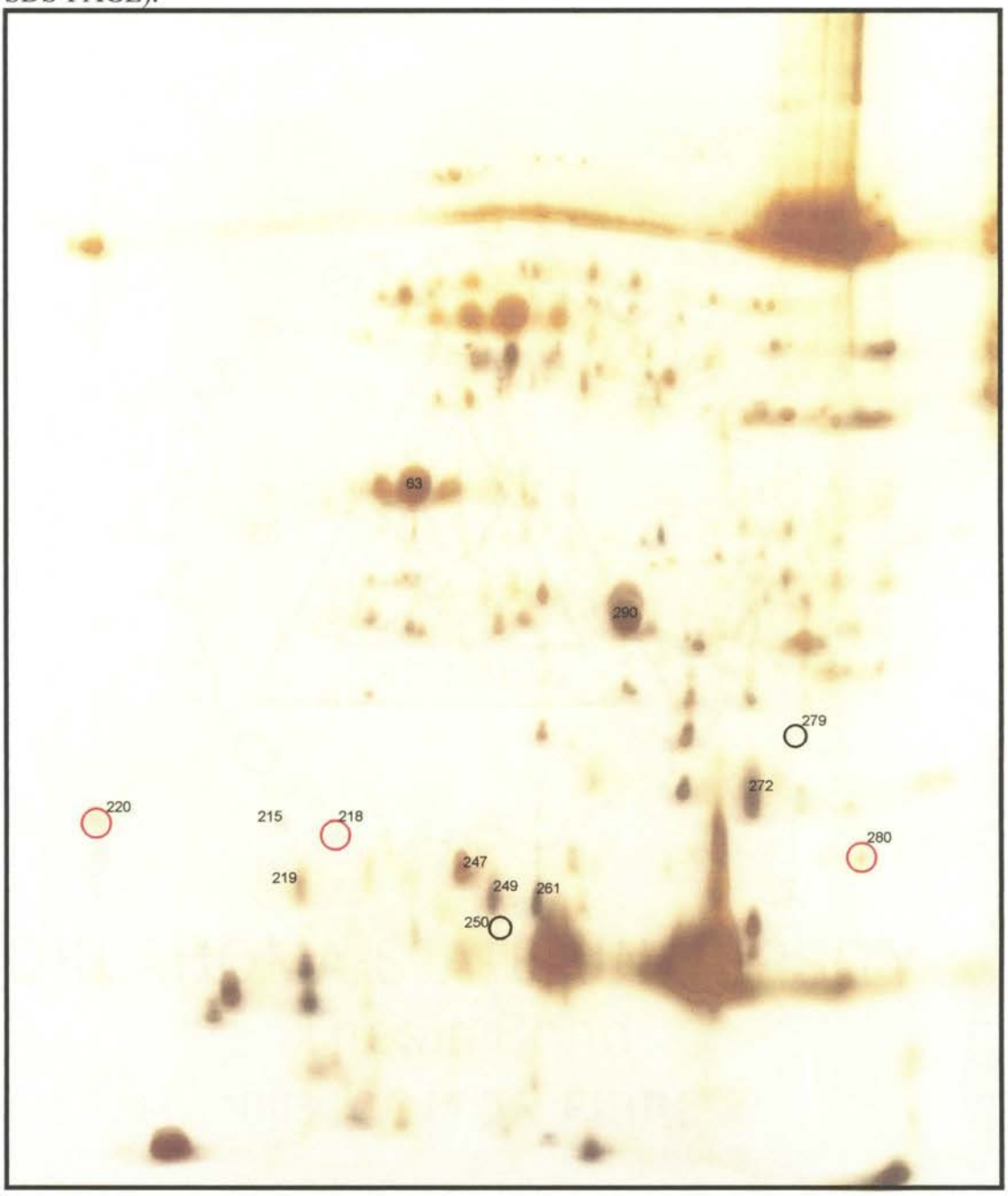


Figure 15. Peptide mass fingerprint for spot 252, a protein of higher abundance.

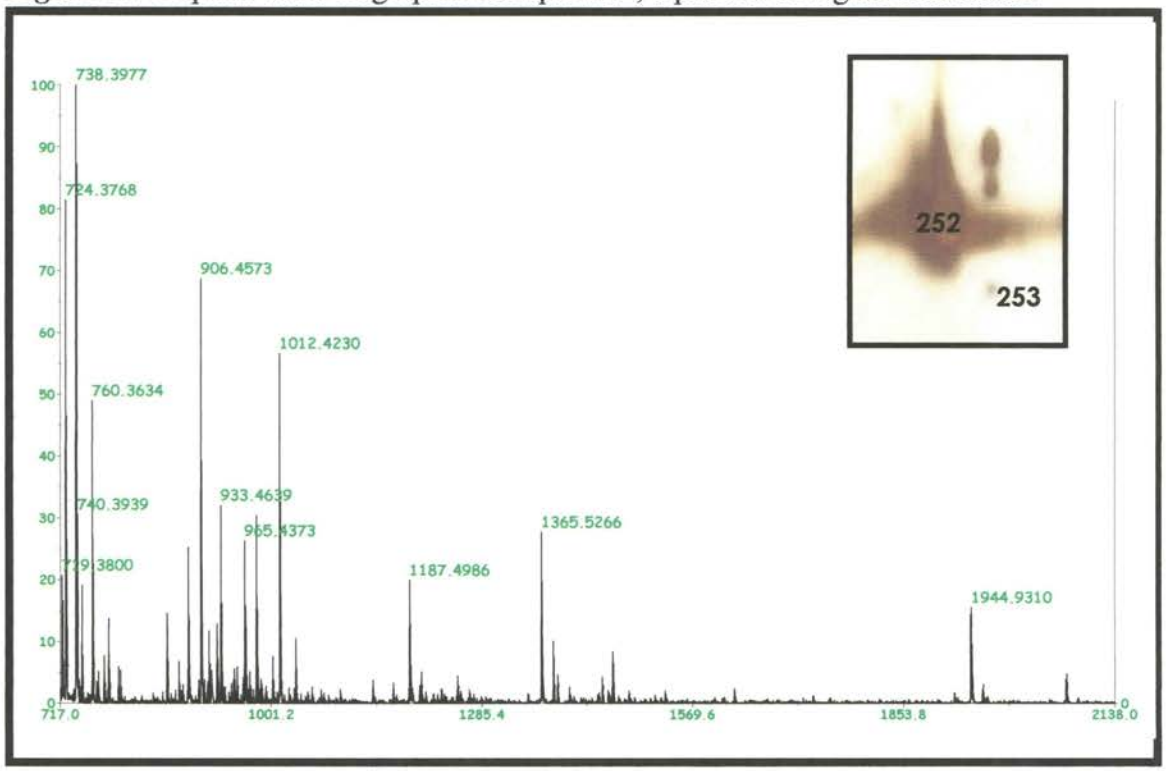


Figure 16. Peptide mass finger print for spot 253, a protein of low abundance.

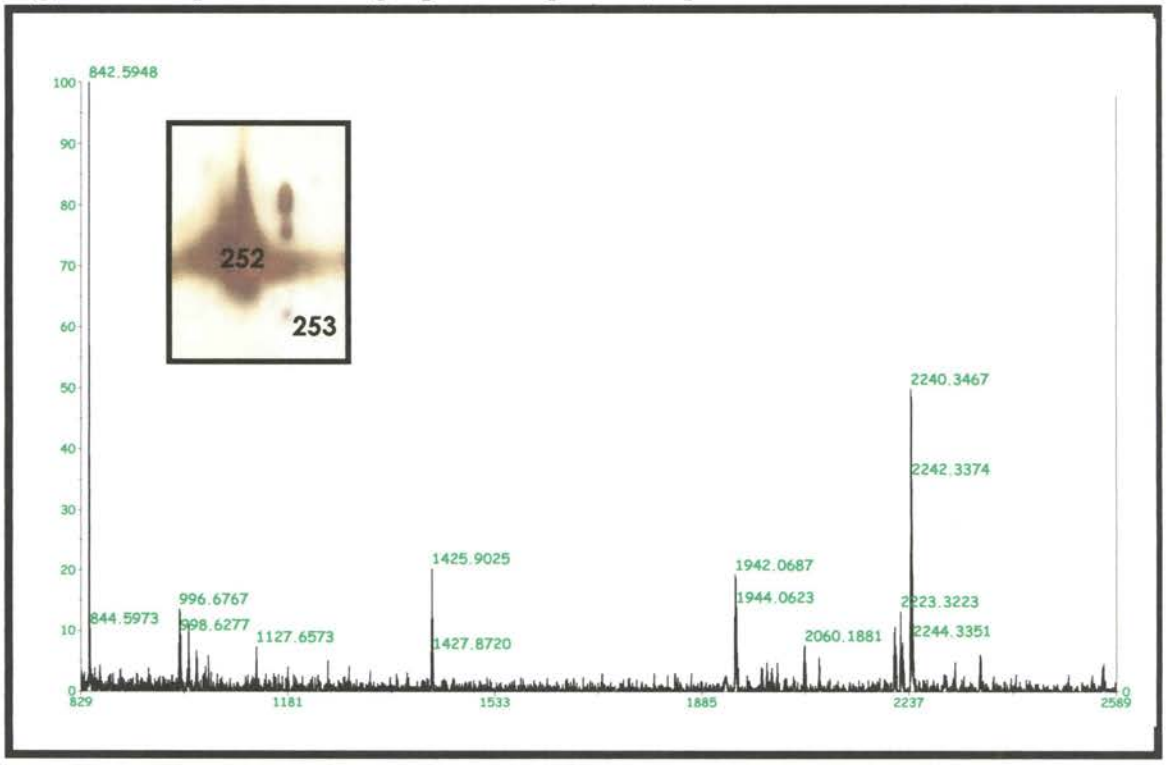


Table 2. Proteins identified in the wheat leaf proteome.

#	Theoretical Protein Name	Organism	pI	Mw (kDa)	Database	Score	Hit #	Cov
1	Rubisco Large Subunit	*Elyophorus globularis*	6.2	50140	ProFound	2.43	9/10	15
2	Isoprene Synthase	*Populus canescens*	5.3	68880	ProFound	1.17	8/44	25
3	Rubisco Large Subunit	*Tacca palmata*	6.6	50728	Mascot	65	9/16	20
4	Rubisco Large Subunit	*Coleocarya gracilis*	6.4	51555	Mascot	93	11/19	22
5	Rubisco Large Subunit	*Kabuyea hostifolia*	6.5	49870	ProFound	0.42	7/13	25
6	Rubisco Large Subunit	*Phragmites australis*	6.6	48740	ProFound	0.12	4/5	7
7	Rubisco Large Subunit	*Isolepis bicolor*	6.3	52450	ProFound	0.26	6/12	17
8	Fimbrin 1	*Arabidopsis thaliana*	6.1	67820	ProFound	1.35	9/67	22
9	Bisphosphoglyc.-Independ. Phosphoglyc. Mutase	*Triticum aestivum*	5.5	60996	EST/BLAST	e-131	8/24	27
10	Bisphosphoglyc.-Independ. Phosphoglyc. Mutase	*Triticum aestivum*	5.5	60996	EST/BLAST	e-131	9/24	31
11	Reversibly Glycosylated Polypeptide	*Triticum aestivum*	5.8	41499	EST/BLAST	0	4/31	16
12	H+-Transporting ATP Synthase Beta Chain	*Triticum aestivum*	5.6	59340	ProFound	2.32	13/46	32
13	Cytochrome P450	*Triticum aestivum*	5.5	77429	EST/BLAST	0	5/55	16
14	At1g19370/F18O14_17	*Arabidopsis thaliana*	6.2	56860	ProFound	1.10	8/55	24
15	Polyphenol Oxidase (Catechol Oxidase)	*Ipomoea batatas*	5.8	55340	ProFound	1.36	5/43	15
16	H+-Transporting ATP Synthase Beta Chain	*Triticum aestivum*	5.1	53824	Mascot	102	20/40	44
17	Proliferating-Cell Nucleolar Antigen	*Arabidopsis thaliana*	6.6	76710	Mascot	61	9/24	18
18	Putative Phosphoenolpyruvate Carboxykinase	*Oryza sativa*	6.3	71380	ProFound	0.35	7/40	18
19	ATP Synthase Beta Chain	*Aegilops columnaris*	5.2	53880	ProFound	1.82	14/34	36
20	ATP Synthase Beta Chain	*Aegilops columnaris*	5.2	53880	ProFound	1.82	14/34	36
21	ATP Synthase Beta Chain	*Triticum aestivum*	5.6	59340	ProFound	2.43	10/52	47
23	Glucosyltransferase IS5a	*Nicotiana tabacum*	5.8	54050	ProFound	2.43	8/68	22
28	LIFtsZ	*Triticum aestivum*	7.7	49167	EST/BLAST	1e-85	5/37	36
32	BCS1 Protein-Like Protein	*Arabidopsis thaliana*	6.1	55010	ProFound	1.37	5/50	16
33	D-Type Cyclin	*Zea mays*	5.5	38837	EST/BLAST	e-179	4/22	15
35	Mitogen-Activated Protein Kinase	*Triticum aestivum*	5.7	70633	EST/BLAST	0	7/79	16
36	Unknown Protein P0529E05.15	*Triticum aestivum*	6.3	70619	EST/BLAST	e-151	4/29	13
37	Transcription Factor	*Triticum aestivum*	6.9	51787	EST/BLAST	0	5/32	14
38	Unknown Protein	*Zea mays*	5.9	49273	EST/BLAST	0	4/26	14

#	Theoretical Protein Name	Organism	pI	Mw (kDa)	Database	Score	Hit #	Cov
40	S-Adenosylmethionine Synthetase 2	*Hordeum vulgare*	5.5	42842	EST/BLAST	0	6/40	26
41	DNA-Binding Protein 3	*Triticum aestivum*	6.9	34841	EST/BLAST	3 e-18	5/24	21
42	S-Ribonuclease Binding Protein SBP1	*Arabidopsis thaliana*	5.2	37530	ProFound	1.11	8/27	26
43	Unknown Protein	*Arabidopsis thaliana*	9.1	52116	Mascot	48	6/20	14
44	Eukaryotic Translation Initiation Factor 4B	*Triticum aestivum*	5.7	47572	EST/BLAST	e-178	5/32	13
46	Hypothetical Protein	*Arabidopsis thaliana*	4.4	44850	Mascot	47	8/31	28
47	G2/Mitotic-Specific Cyclin 2 (B-Like Cyclin)	*Oryza sativa*	5.7	47572	EST/BLAST	0	4/21	15
48	Protochlorophyllide Reductase (ChlN subunit)	*Mesostigma viride*	5.5	50990	ProFound	0.36	9/77	32
49	Unknown Protein	*Prunus armeniaca*	5.9	42240	ProFound	1.56	7/33	24
50	Ribulose Bisphosphate Carboxylase Activase B	*Triticum aestivum*	6.9	47815	EST/BLAST	0	5/9	13
51	Pathogen-Related Protein	*Triticum aestivum*	5.9	17174	EST/BLAST	3 e-78	5/16	22
52	Sedoheptulose-1,7-Bisphosphatase	*Triticum aestivum*	6	42560	ProFound	1.31	10/48	29
54	Protoporphyrin IX Magnesium Chelatase	*Hordeum vulgare*	4.9	36530	ProFound	1.36	9/47	35
58	Farnesyl Pyrophosphate Synthase	*Gossypium arboreum*	5.6	39980	ProFound	0.64	7/30	22
59	Maturase K	*Mirabilis jalapa*	9.9	33829	Mascot	59	6/20	20
60	ATP Synthase Beta-Subunit	*Pandorina morum*	5.5	40770	ProFound	1.34	8/26	34
61	Hypothetical Protein	*Arabidopsis thaliana*	6.1	34220	ProFound	0.26	6/35	22
62	Ras-Related Protein ARA-5	*Oryza sativa*	6.5	29440	ProFound	0.73	6/45	51
63	Putative Oxygen Evolving Protein of Photosystem II	*Oryza sativa*	6.1	35070	ProFound	1.38	6/19	23
66	Gibberellin 20-Dioxygenase	*Triticum aestivum*	6.1	40293	EST/BLAST	0	5/67	16
67	Putative Plastidic Cysteine Synthase 1	*Triticum aestivum*	6.1	43585	EST/BLAST	e-110	7/17	38
69	Caffeic Acid O-Methyltransferase	*Triticum aestivum*	5.5	38755	EST/BLAST	e-112	5/83	66
72	Rubisco Activase	*Hordeum vulgare*	5.6	47510	ProFound	1.35	4/9	12
75	MYB40 - putative transcription factor	*Arabidopsis thaliana*	5.4	30780	ProFound	0.51	5/32	28
77	ADP-Glucose Pyrophosphorylase	*Zea mays*	6.6	55560	EST/BLAST	0	6/45	12
78	Ribulose-Bisphosphate Carboxylase Activase	*Hordeum vulgare*	5.6	47510	EST/BLAST	0	6/33	16
80	26S Proteasome Regulatory Particle Triple-A ATPase	*Oryza sativa*	8.9	47223	EST/BLAST	0	7/65	23
82	Putative Protein	*Arabidopsis thaliana*	5.5	53610	ProFound	1.32	8/61	19
83	N-Acetylornithine Deacetylase-Like Protein	*Arabidopsis thaliana*	5.1	44520	ProFound	2.43	6/42	12
84	NADP-Specific Isocitrate Dehydrogenase	*Zea mays*	6.3	46043	EST/BLAST	0	7/50	18
91	ATP Synthase Beta Subunit	*Pinguicula lutea*	5.4	39920	ProFound	2.43	11/35	38
92	Ribosomal Protein L1	*Triticum aestivum*	9.3	37568	EST/BLAST	e-109	6/37	26

#	Theoretical Protein Name	Organism	pI	Mw (kDa)	Database	Score	Hit #	Cov
94	Rubisco Activase B	*Triticum aestivum*	6.9	47815	EST/BLAST	0	5/52	16
95	GTP-Binding Protein	*Triticum aestivum*	8.4	68031	EST/BLAST	e-110	5/17	14
96	Starch Branching Enzyme Isoform RBE3	*Oryza sativa*	5.7	92757	EST/BLAST	0	5/44	14
97	Glutathione S-Transferase (GST6)	*Arabidopsis thaliana*	8.5	29270	ProFound	1.47	9/69	51
98	rps4	*Voitia hyperborea*	10.1	21760	ProFound	2.43	4/36	23
102	H+ Transporting Two-Sector ATPase	*Triticum aestivum*	5.6	59249	EST/BLAST	0	11/52	25
103	High-Affinity Phosphate Transporter PT1	*Triticum aestivum*	8.8	43520	EST/BLAST	0	4/13	10
109	Rubisco Large Subunit	*Triticum aestivum*	6.2	52817	Mascot	73	11/19	18
115	3-Dehydroquinate Dehydratase	*Zea mays*	6.1	56906	EST/BLAST	e-162	4/36	19
116	Ribulose Bisphosphate Carboxylase Activase B	*Hordeum vulgare*	7.6	47228	EST/BLAST	0	4/22	12
117	Putative Glucan Synthase	*Triticum aestivum*	8.8	190781	EST/BLAST	4e-30	5/19	21
120	NADPH-Protochlorophyllide Oxidoreductase B	*Zea mays*	9.5	42148	EST/BLAST	0	7/21	33
122	Arm Repeat Containing Protein	*Triticum aestivum*	8.3	28818	EST/BLAST	9e-54	4/30	20
123	Outer Mitochondrial Membrane Protein Porin	*Triticum aestivum*	8.4	28904	EST/BLAST	e-117	6/18	37
127	Malate Dehydrogenase Glyoxysomal Precursor	*Zea mays*	8.1	37385	EST/BLAST	5e-77	4/10	27
129	Glyceraldehyde 3-Phosphate Dehydrogenase	*Hordeum vulgare*	6.7	36061	ProFound	1.14	7/16	24
139	40 S Ribosomal Protein S2	*Picea abies*	10.6	25370	ProFound	0.74	5/28	24
147	IB1C3-1 Protein	*Arabidopsis thaliana*	9.6	28280	ProFound	1.05	6/29	20
148	SERK1	*Helianthus annuus*	9.1	25750	ProFound	0.95	7/74	41
149	Mitochondrial Aldehyde Dehydrogenase	*Oryza sativa*	6.3	58903	EST/BLAST	0	5/35	14
150	Photosystem I Reaction Center Subunit II (PSI-D)	*Hordeum vulgare*	9.8	21970	ProFound	1.71	7/13	33
155	Unknown Protein AF435650 1	*Oryza sativa*	6.9	55800	EST/BLAST	0	5/35	14
160	Stripe Rust Resistance Protein Yr1C	*Triticum aestivum*	7.2	93219	EST/BLAST	0	7/46	14
164	NADPH-Cytochrome P450 Reductase	*Triticum aestivum*	5.0	72950	EST/BLAST	0	6/51	16
168	Rubisco Small Subunit	*Triticum aestivum*	8.8	19449	Mascot	101	13/38	68
169	Glutelin Precursor	*Triticum aestivum*	9.2	56309	EST/BLAST	0	4/58	20
172	Photosystem I Chain IV Precursor	*Hordeum vulgare*	9.8	15447	Mascot	65	5/12	30
175	High-Affinity Phosphate Transporter PT1	*Oryza sativa*	9.0	60087	EST/BLAST	0	6/59	18
182	40 S Ribosomal Protein S2	*Picea abies*	10.6	25370	ProFound	0.74	5/28	24
191	Alternative Oxidase	*Arabidopsis thaliana*	6.3	33130	ProFound	2.43	5/26	25
200	Eukaryotic Initiation Factor 4A	*Oryza sativa*	5.5	47065	EST/BLAST	0	5/37	19
201	Unknown Protein B1147B04.8	*Zea mays*	4.7	48017	EST/BLAST	e-117	5/54	29

#	Theoretical Protein Name	Organism	pI	Mw (kDa)	Database	Score	Hit #	Cov
202	Unknown Protein AT5g51140	*Arabidopsis thaliana*	6.5	42060	ProFound	2.43	5/24	19
203	Protein Kinase	*Arabidopsis thaliana*	9.6	52844	Mascot	55	10/36	55
205	Ascorbate Peroxidase	*Hordeum vulgare*	5.8	27530	ProFound	0.48	4/7	22
206	Alternative Oxidase	*Triticum aestivum*	8.7	36658	EST/BLAST	e-165	5/21	25
209	Putative Calcium Sensor Protein	*Oryza sativa*	5.0	31440	ProFound	0.54	8/69	26
210	NADPH-Cytochrome P450 Reductase	*Triticum aestivum*	5.0	72950	EST/BLAST	0	6/51	16
211	Cinnamyl-Alcohol Dehydrogenase ELI3-2	*Arabidopsis thaliana*	6.8	39440	ProFound	0.41	7/80	33
213	Hypothetical Protein	*Oryza sativa*	10.6	52701	Mascot	59	8/20	30
214	RAS-Related Protein RAB2BV	*Beta vulgaris*	6.4	23940	ProFound	2.43	6/56	35
215	Cytochrome P450	*Triticum aestivum*	8.4	59872	EST/BLAST	0	5/29	14
216	F-box Protein Family, AtFBX5	*Arabidopsis thaliana*	6.0	100578	Maseot	42	7/9	10
218	Hypothetical Protein	*Arabidopsis thaliana*	8.5	51777	Mascot	69	6/11	26
220	Calcineurin-Like Protein	*Oryza sativa*	4.5	19997	EST/BLAST	5e-47	4/28	33
221	Unknown Protein At3g48860.1	*Triticum aestivum*	5.8	63725	EST/BLAST	1e-33	5/26	23
224	Putative Glycine Decarboxylase Subunit	*Triticum aestivum*	5.0	21250	EST/BLAST	3e-83	5/23	46
226	RAS-Related Protein RAB7	*Glycine max*	5.5	23430	ProFound	2.43	5/55	35
228	Unknown Protein P0031D02.12	*Triticum aestivum*	5.4	19477	EST/BLAST	3e-37	4/25	39
232	Putative Phosphoenolpyruvate Carboxykinase	*Oryza sativa*	6.3	71380	ProFound	0.35	7/40	18
234	Thioredoxin	*Arabidopsis thaliana*	5.9	18770	ProFound	0.96	5/34	39
241	Polyadenylate-Binding Protein	*Mesemb. crystallinum*	4.8	19020	ProFound	1.17	5/51	49
242	RAB11G	*Lotus japonicus*	5.2	24600	ProFound	0.98	8/61	40
243	Protein Import Receptor TOM20, Mitochondrial	*Solanum tuberosum*	5.3	22799	Mascot	59	7/15	56
245	V-ATPase G-Subunit Like Protein	*Arabidopsis thaliana*	5.8	13270	ProFound	2.21	9/55	57
247	Calmodulin	*Triticum aestivum*	4.1	16087	ProFound	2.43	5/36	54
248	Pyruvate Kinase-Like Protein	*Triticum aestivum*	6.3	53442	EST/BLAST	2e-24	7/74	53
249	Origin Recognition Complex Subunit 4	*Zea mays*	6.8	48001	Mascot	45	9/19	38
250	GTP-Binding Protein RAB1	*Petunia x hybrida*	5.3	22700	ProFound	2.43	7/67	30
251	Rubisco Small Subunit	*Triticum aestivum*	5.8	13270	ProFound	2.36	6/27	44
252	Rubisco Small Subunit	*Triticum aestivum*	5.8	13270	ProFound	2.16	10/54	60
253	NBS-LRR-Like Protein	*Mentha longifolia*	5.3	20330	ProFound	1.32	5/53	35
255	Putative RING Zinc Finger Protein	*Arabidopsis thaliana*	7.0	12670	ProFound	0.60	5/42	32
256	PRLI-Interacting Factor E	*Arabidopsis thaliana*	7.2	13820	ProFound	1.22	5/63	68

#	Theoretical Protein Name	Organism	pI	Mw (kDa)	Database	Score	Hit #	Cov
257	Rubisco Small Subunit	*Secale cereale*	9.0	18475	Mascot	54	7/25	54
258	Small Heat Shock Protein	*Triticum aestivum*	6.2	23463	EST/BLAST	1e-99	5/39	15
260	Glutathione S-Transferase (GST Class-Zeta)	*Triticum aestivum*	6.1	24020	ProFound	1.10	6/29	35
263	Unknown Protein At5g1110.1	*Triticum aestivum*	9.1	23897	EST/BLAST	1e-47	11/42	38
264	Actin-Depolymerizing Factor 3	*Triticum aestivum*	5.7	15946	EST/BLAST	3e-49	6/27	40
265	Triosephosphate-Isomerase	*Hordeum vulgare*	5.4	26950	ProFound	1.12	8/42	37
267	Putative Protein	*Arabidopsis thaliana*	5.2	16270	ProFound	1.77	6/57	44
271	Ferritin 2 Precursor	*Zea mays*	5.7	27870	ProFound	1.41	5/38	26
272	MADS Box Transcription Factor AP3-2	*Asarum europaeum*	5.8	24490	ProFound	1.62	4/14	18
274	Protein Kinase-Like Protein	*Oryza sativa*	6.3	39494	EST/BLAST	9e-95	4/19	25
276	Calcium-Dependent Protein Kinase	*Oryza sativa*	7.6	57584	EST/BLAST	0	6/18	13
280	Rubredoxin Putative	*Arabidopsis thaliana*	6.3	22140	ProFound	1.19	4/28	29
283	Phosphatidylinositol Bisphosphate Phosphodiesterase	*Zea mays*	6.4	66942	EST/BLAST	0	8/43	16
284	Proteasome Subunit Alpha Type 2	*Triticum aestivum*	5.4	25844	EST/BLAST	e-128	6/34	23
285	Triosephosphate-Isomerase	*Hordeum vulgare*	5.4	26950	ProFound	1.12	8/42	37
288	Beta-Glucosidase	*Oryza sativa*	6.9	58539	EST/BLAST	0	6/74	16
289	NBS-LRR-Like Protein	*Triticum aestivum*	8.8	90702	EST/BLAST	7e-47	5/34	10
291	Superoxide Dismutase	*Triticum aestivum*	7.9	25259	Mascot	36	4/16	36
294	Putative Selenocysteine Methyltransferase	*Arabidopsis thaliana*	5.5	37999	Mascot	48	7/23	28
295	Hypothetical Protein	*Arabidopsis thaliana*	7.6	43610	Mascot	53	8/23	25
297	Dehydroascorbate Reductase	*Triticum aestivum*	5.9	23343	Mascot	86	8/19	56

Figure 17. Functional annotation of wheat leaf proteome.

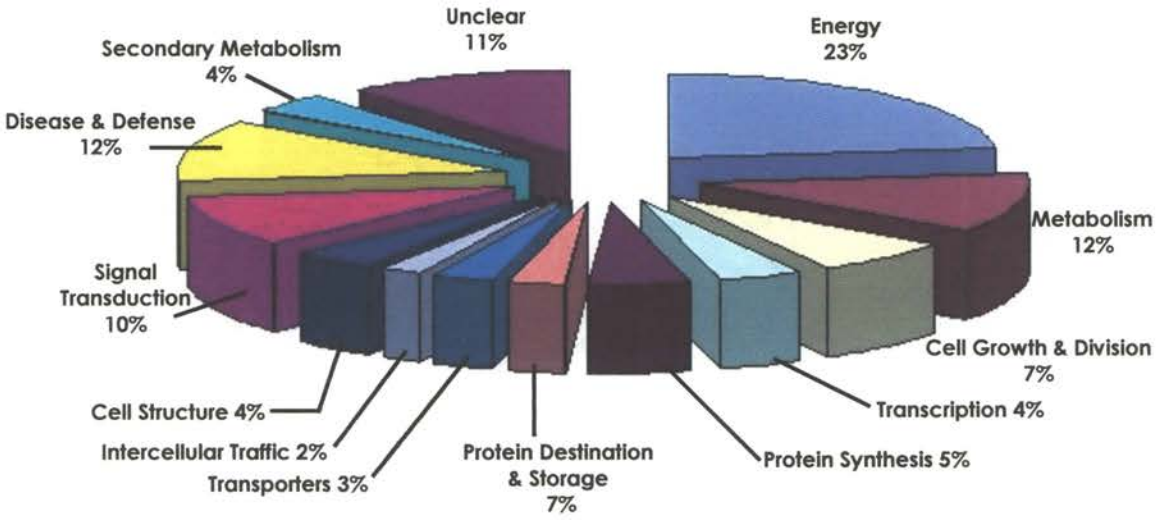


Table 3. Cataloged proteins differentially expressed due to greenbug or BCO feeding with pI, molecular weight, protein intensity, identification indicator, and spectra quality.

#	pI	Mol Wt	Prot Conc	ID	Spec Qual	#	pI	Mol Wt	Prot Conc	ID	Spec Qual
10Ag	5.45	64.5	2	Y	x	**411Ab**	6.20	40	2	N	G
11Cgb	5.20	61	2	Y	x	**500Cg**	9.60	23	3	Y	x
47Cgb	5.00	46	2	Y	x	**503Ag**	6.80	27.5	3	Y	x
49Cgb	5.25	37.5	3	Y	x	**610Ag**	4.90	32	3	N	P
50Cgb	5.10	43.5	2	Y	x	**611Cg**	5.75	25	2	N	P
77Cg	5.55	37.5	2	Y	x	**613Cg**	5.60	37.5	2	N	P
102Ag	7.00	62.5	2	Y	x	**614Cg**	5.65	37	1	N	P
115Cgb	8.00	36.5	3	Y	x	**616Cg**	5.80	37	1	N	P
116Cgb	8.10	44.5	3	Y	x	**617Cg**	5.80	36.5	1	N	G
117Cg	8.35	44.5	3	Y	x	**619Ag**	4.80	54	5	Y	X
127Cb	8.25	35	2	Y	x	**620Ag**	5.00	54	5	Y	X
128Cg	7.80	35	2	N	G	**621Ag**	5.20	54	5	Y	X
141Cg	7.75	43.5	1	N	P	**622Ag**	5.50	54	5	Y	X
149Ag	9.95	22	3	Y	x	**705Ag**	6.20	25	3	Y	X
215Ab	4.70	19	3	Y	x	**706Ag**	6.75	23	1	N	P
218Ab	4.90	18	3	Y	x	**707Cg**	6.25	27	2	N	P
222Cb	4.45	15.5	1	N	G	**708Cg**	6.25	26	2	N	P
224Cb	4.55	13.5	3	Y	x	**709Cg**	6.40	27	1	N	G
225Cb	4.45	13	2	N	G	**710Cg**	6.50	26.5	1	N	G
226Cg	4.80	13.5	2	Y	x	**801Ag**	9.55	26	1	N	P
228Ab	5.20	16.5	2	Y	x	**900Cg**	6.90	16	2	Y	X
274Cg	5.85	19.5	2	Y	x	**903Ag**	5.90	17.5	1	Y	X
400Cg	5.40	24.5	1	Y	x	**950Ag**	6.85	9	2	Y	X
408Cb	4.25	13.5	1	N	G	**975Ab**	6.25	26	2	N	P
409Ab	5.70	13.5	2	N	G	**976Ab**	6.25	25	2	N	P
410Ab	5.35	21.5	1	Y	x						

A = Upregulated in response to aphid feeding
C = Downregulated in response to aphid feeding
g = Induced by greenbug feeding
b = Induced by BCO feeding

Table 4. Proteins differentially expressed in susceptible and resistant wheat in response to 24h and 6d of greenbug feeding. Protein numbers correspond to spot numbers on gels shown in Fig 18 to Fig 81. Theoretical protein name was obtained from putative database identification of the MALDI peptide masses, organism refers to the species the putative protein was identified in. The pI and molecular weight refer to the isoelectric point and molecular weight of the protein the MALDI peptide masses matched in the database. The database refers to where the identification was obtained and the score, hit, and coverage are the scoring criteria evaluated to determine identification acceptability. The Figure numbers refer to the gels the protein differences were observed on.

#	Theoretical Protein Name	*Organism*	pI	Mw (kDa)	Database	Score	Hit #	Cov	Figure #
10	Bisphosphoglyc.-Independ. Phosphoglyc. Mutase	*Triticum aestivum*	5.5	60996	EST/BLAST	e-131	4/20	20	18
11	Reversibly Glycosylated Polypeptide	*Triticum aestivum*	5.8	41499	EST/BLAST	0	4/31	16	50, 58
47	G2/Mitotic-Specific Cyclin 2 (B-Like Cyclin)	*Oryza sativa*	5.7	47572	EST/BLAST	0	4/21	15	50
49	Protein cdc2 Kinase	*Oryza sativa*	8.9	34604	EST/BLAST	e-177	4/28	16	18, 26, 50, 58
50	Rubisco Activase B	*Triticum aestivum*	6.9	47815	EST/BLAST	0	5/13	13	50
77	ADP-Glucose Pyrophosphorylase	*Zea mays*	6.6	55560	EST/BLAST	0	6/45	12	18, 26, 50, 58
102	H+ Transporting ATP Synthase B	*Triticum aestivum*	5.6	59249	EST/BLAST	0	11/52	25	18
115	3-Dehydroquinate Dehydratase	*Zea mays*	6.1	56906	EST/BLAST	e-162	4/36	19	22, 30
117	Putative Glucan Synthase	*Triticum aestivum*	8.8	190781	EST/BLAST	4e-30	5/19	21	22, 30
149	Mitochondrial Aldehyde Dehydrogenase	*Oryza sativa*	6.3	58903	EST/BLAST	0	5/13	11	22, 54
226	RAS-Related Protein (RAB7)	*Glicine max*	5.5	23430	ProFound	2.43	5/55	35	52
400	Unknown Protein At2g25280.1	*Triticum aestivum*	6.4	32644	EST/BLAST	e-109	4/12	23	28
500	Peptidyl-Prolyl Isomerase	*Triticum aestivum*	9.4	28306	EST/BLAST	2e-74	5/12	37	24
503	Peroxidase 1 Precursor	*Hordeum vulgare*	6.1	33410	ProFound	0.15	4/14	15	18, 26
614	NADP-Specific Isocitrate Dehydrogenase	*Oryza sativa*	6.3	46043	EST/BLAST	0	6/40	18	18, 26
616	Sucrose:Fructan 6-Fructosyltransferase	*Triticum aestivum*	5.3	68463	EST/BLAST	0	4/21	26	18
619	High-Affinity Phosphate Transporter PT1	*Triticum aestivum*	8.8	43520	EST/BLAST	0	6/39	13	18, 50
620	High-Affinity Phosphate Transporter PT1	*Triticum aestivum*	8.8	43520	EST/BLAST	0	4/15	10	18, 50
621	High-Affinity Phosphate Transporter PT1	*Triticum aestivum*	8.8	43520	EST/BLAST	0	6/22	14	18, 50
622	High-Affinity Phosphate Transporter PT1	*Triticum aestivum*	8.8	43520	EST/BLAST	0	6/15	13	18, 50
705	Germin Homolog Ger3	*Triticum aestivum*	6.2	23470	ProFound	0.70	4/8	23	20, 28
900	wpk4 Protein Kinase	*Triticum aestivum*	8.8	58637	EST/BLAST	0	4/21	22	30, 64
903	Chitinase Cht2b Precursor	*Hordeum vulgare*	6.1	26900	ProFound	1.75	5/21	29	20, 28
950	Calmodulin-Related Protein	*Oryza sativa*	4.8	17546	EST/BLAST	7e-14	6/34	42	32, 64

Table 5. Proteins differentially expressed in susceptible and resistant wheat in response to 24h and 6d of BCO aphid feeding. Protein numbers correspond to spot numbers on gels shown in Fig 18 to Fig 81. Theoretical protein name was obtained from putative database identification of the MALDI peptide masses, organism refers to the species the putative protein was identified in. The pI and molecular weight refer to the isoelectric point and molecular weight of the protein the MALDI peptide masses matched in the database. The database refers to where the identification was obtained and the score, hit, and coverage are the scoring criteria evaluated to determine identification acceptability. The Figure numbers refer to the gels the protein differences were observed on

#	Theoretical Protein Name	*Organism*	pI	Mw (kDa)	Database	Score	Hit #	Cov	Figure
11	Reversibly Glycosylated Polypeptide	*Triticum aestivum*	5.8	41499	EST/BLAST	0	4/31	16	66
47	G2/Mitotic-Specific Cyclin 2 (B-Like Cyclin)	*Oryza sativa*	5.7	47572	EST/BLAST	0	4/21	15	66
49	Protein cdc2 Kinase	*Oryza sativa*	8.9	34604	EST/BLAST	e-177	4/28	16	34, 66
50	Rubisco Activase B	*Triticum aestivum*	6.9	47815	EST/BLAST	0	5/13	13	66
115	3-Dehydroquinate Dehydratase	*Zea mays*	6.1	56906	EST/BLAST	e-162	4/36	19	70
116	Rubisco Activase	*Hordeum vulgare*	7.9	47228	EST/BLAST	0	4/22	12	70
127	Malate Dehydrogenase Glyoxysomal Precursor	*Zea mays*	8.1	37385	EST/BLAST	5e-77	4/10	27	72, 80
215	Cytochrome P450 CYP86-TA	*Triticum aestivum*	8.4	59872	EST/BLAST	0	5/29	22	36, 44, 68, 76
218	Ribosomal Protein L12	*Hordeum vulgare*	5.3	18736	EST/BLAST	2e-47	6/8	35	36, 44, 68, 76
224	Puative Glycine Decarboxylase Subunit	*Triticum aestivum*	5.0	21250	EST/BLAST	3e-83	5/23	46	36
228	Unknown Protein P0031D02.12	*Triticum aestivum*	5.6	19477	EST/BLAST	3e-37	4/25	39	44, 76
274	Protein Kinase-Like Protein	*Oryza sativa*	6.3	39494	EST/BLAST	9e-95	4/19	25	36, 44, 68, 76
400	Unknown Protein At2g25280.1	*Triticum aestivum*	6.4	32644	EST/BLAST	e-109	4/12	23	36, 44
410	Mitogen-Activated Protein Kinase (FLRS)	*Triticum aestivum*	5.5	45302	EST/BLAST	0	4/14	16	76
550	Alternative Oxidase	*Triticum aestivum*	8.7	36658	EST/BLAST	e-165	7/39	29	34
551	Hypersensitive-Induced Response Protein	*Zea mays*	5.2	31367	EST/BLAST	e-156	4/28	21	66
614	NADP-Specific Isocitrate Dehydrogenase	*Oryza sativa*	6.3	46043	EST/BLAST	0	6/40	18	68

Figure 18. Susceptible wheat fed on by Greenbugs for 24h; 4-7pH 11% SDS.

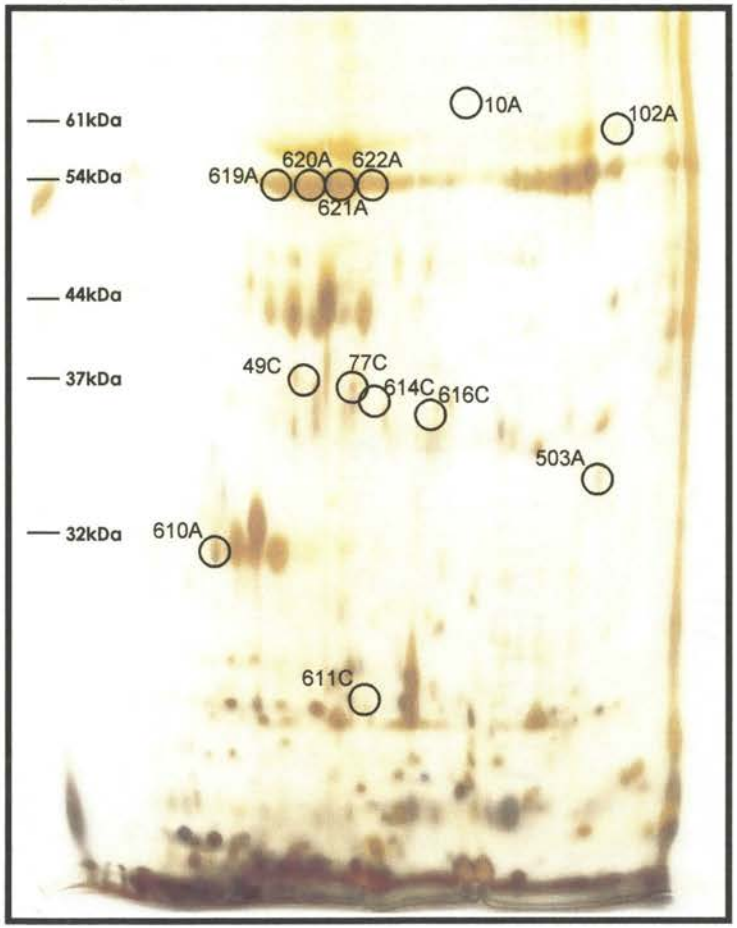


Figure 19. Control for susceptible wheat fed on by Greenbugs for 24h; 4-7pH 11% SDS.

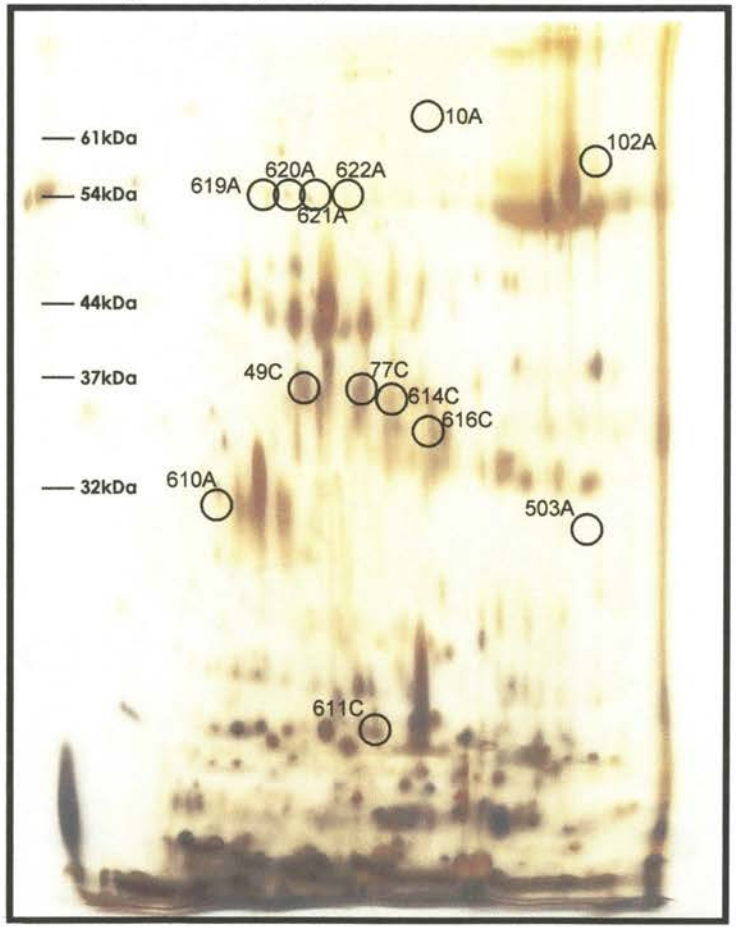


Figure 20. Susceptible wheat fed on by Greenbugs for 24h; 4-7pH 14% SDS.

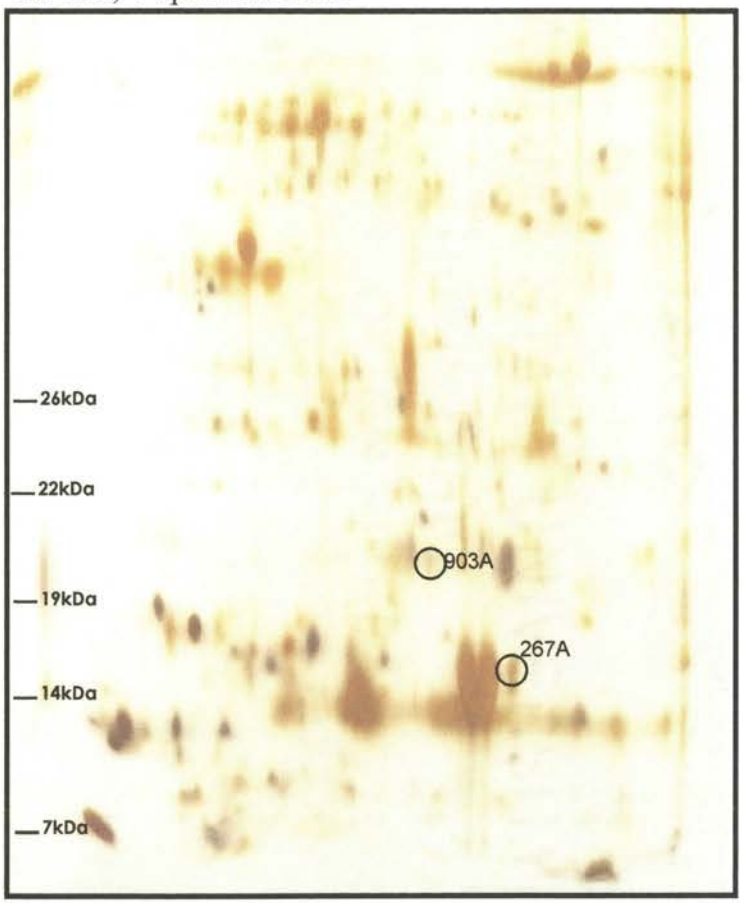


Figure 21. Control for susceptible wheat fed on by Greenbugs for 24h; 4-7pH 14% SDS.

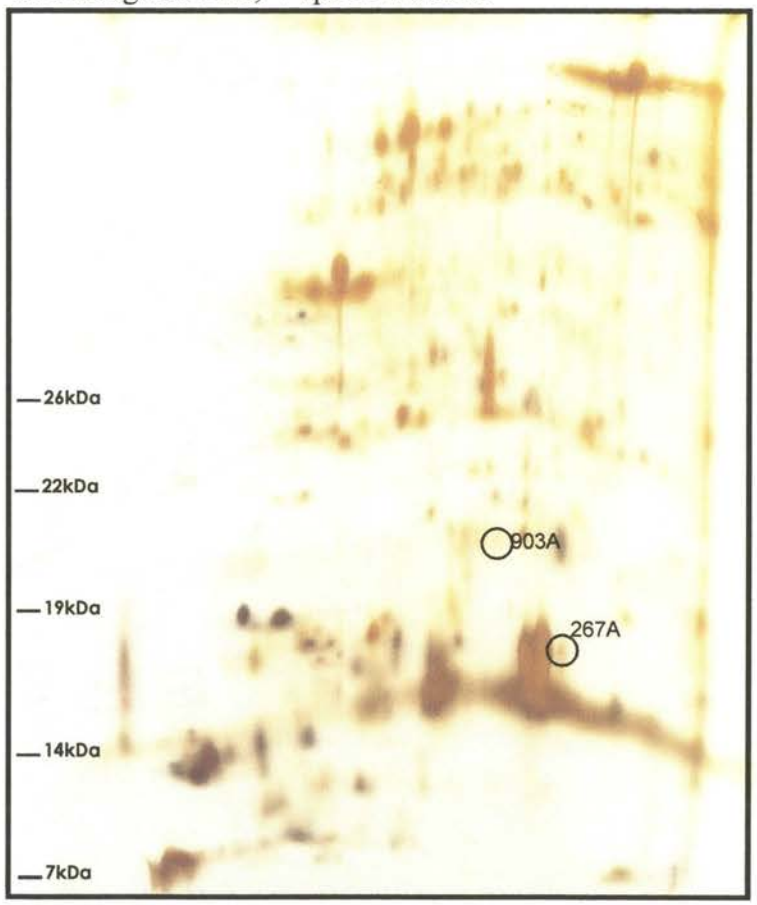


Figure 22. Susceptible wheat fed on by Greenbugs for 24h; 6-11pH 11% SDS.

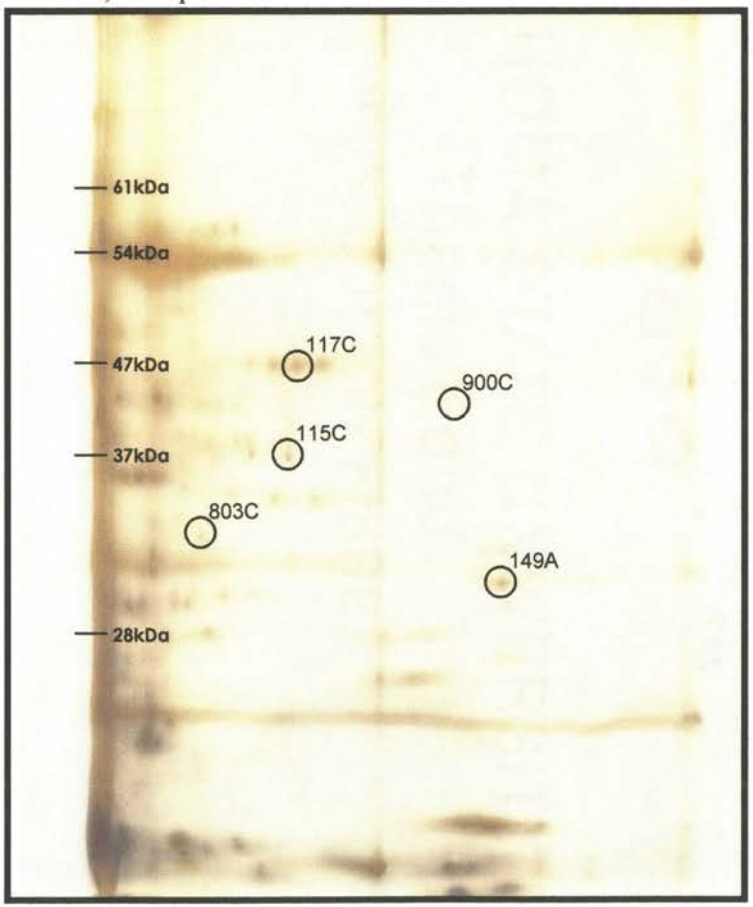


Figure 23. Control for susceptible wheat fed on by Greenbugs for 24h; 6-11pH 11% SDS.

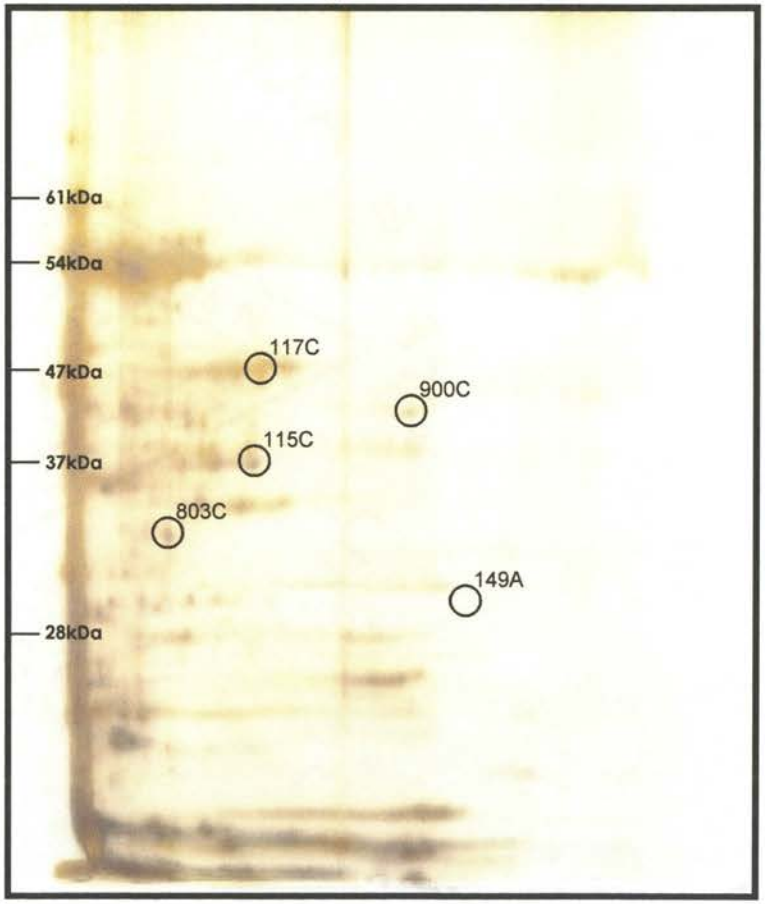


Figure 24. Susceptible wheat fed on by Greenbugs for 24h; 6-11pH 14% SDS.

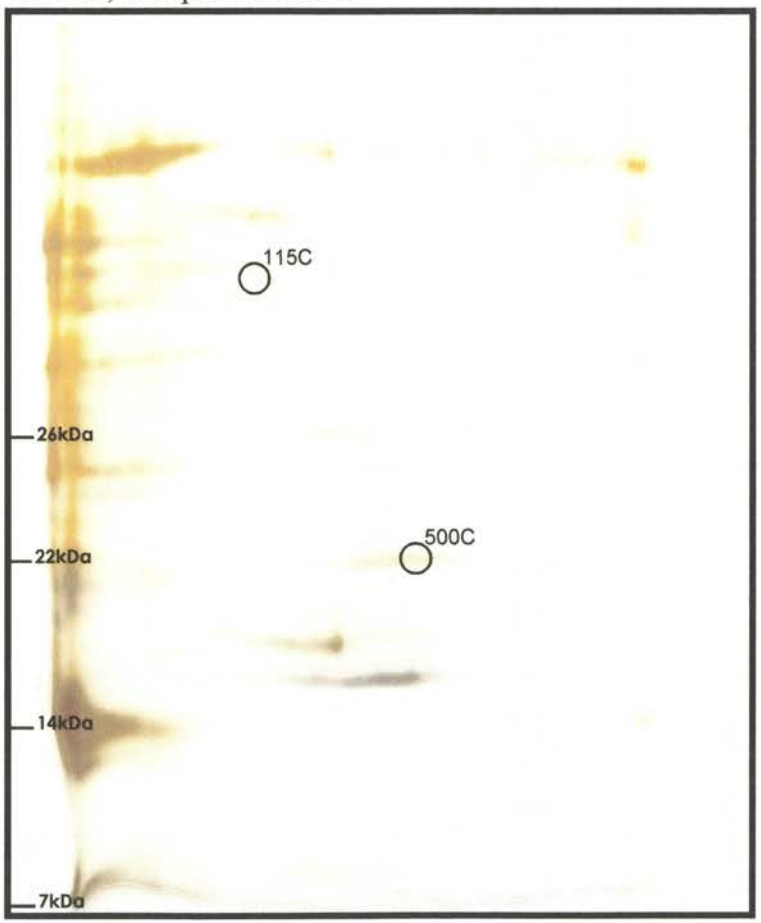


Figure 25. Control for susceptible wheat fed on by Greenbugs for 24h; 6-11pH 14% SDS.

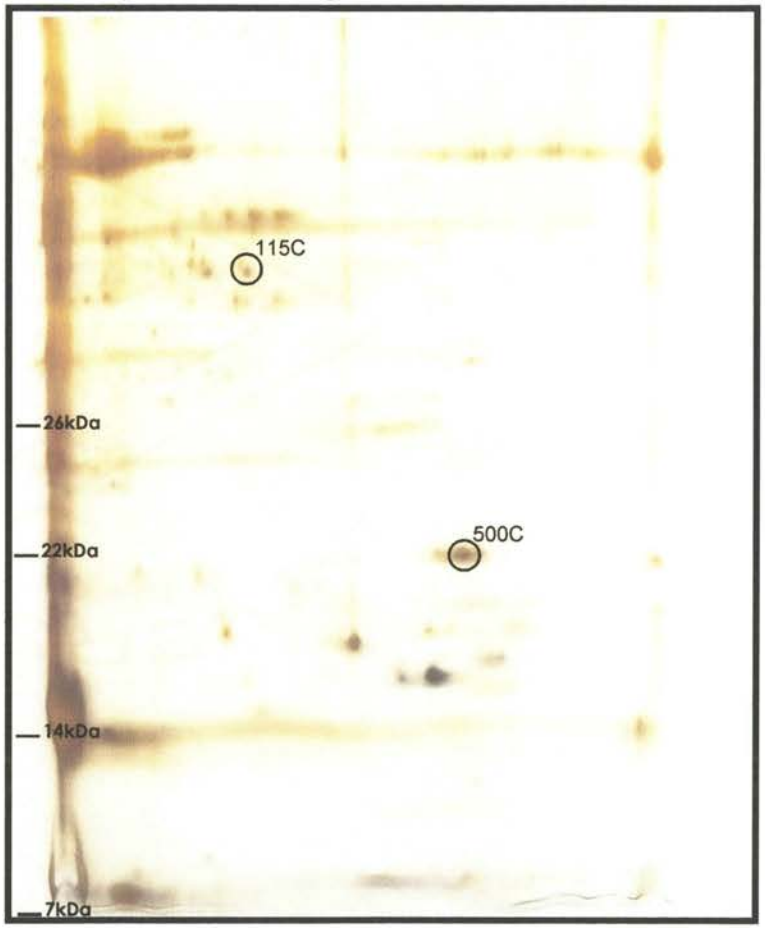


Figure 26. Susceptible wheat fed on by Greenbugs for 6d; 4-7pH 11% SDS.

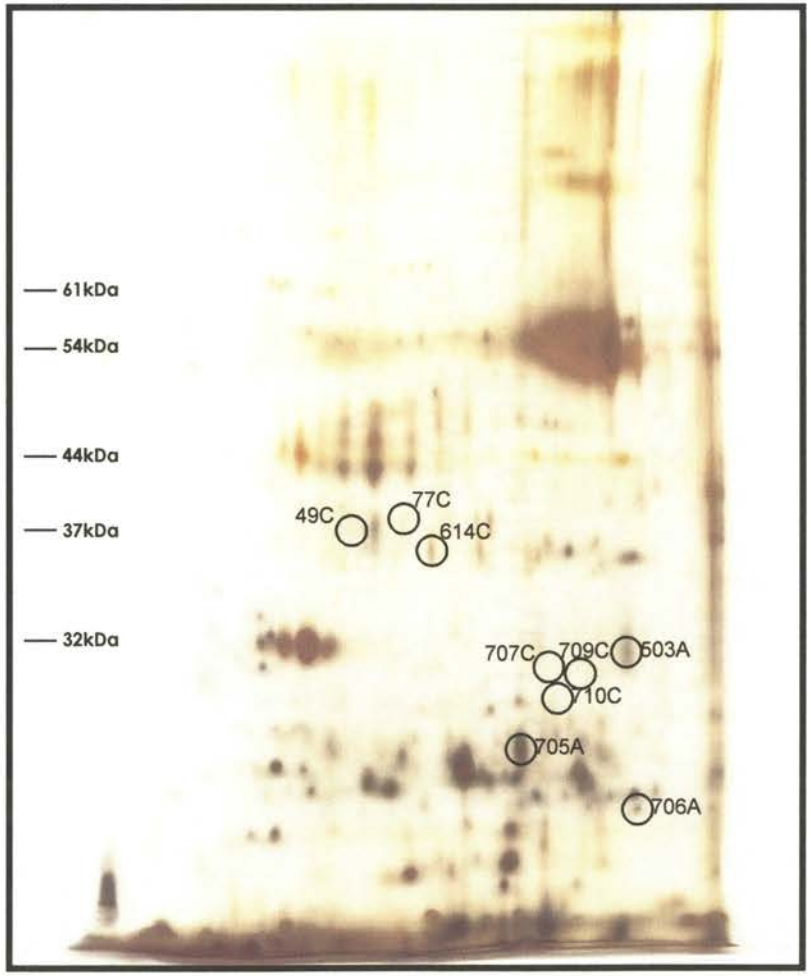


Figure 27. Control for susceptible wheat fed on by Greenbugs for 6d; 4-7pH 11% SDS.

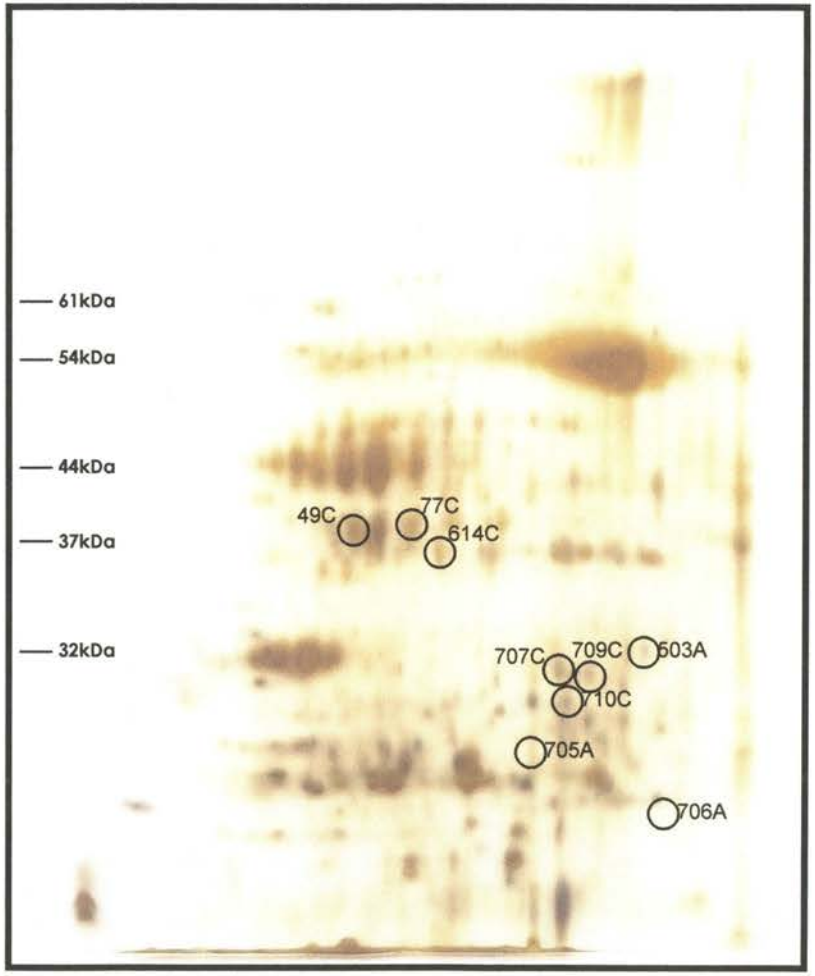


Figure 28. Susceptible wheat fed on by Greenbugs for 6d; 4-7pH 14% SDS.

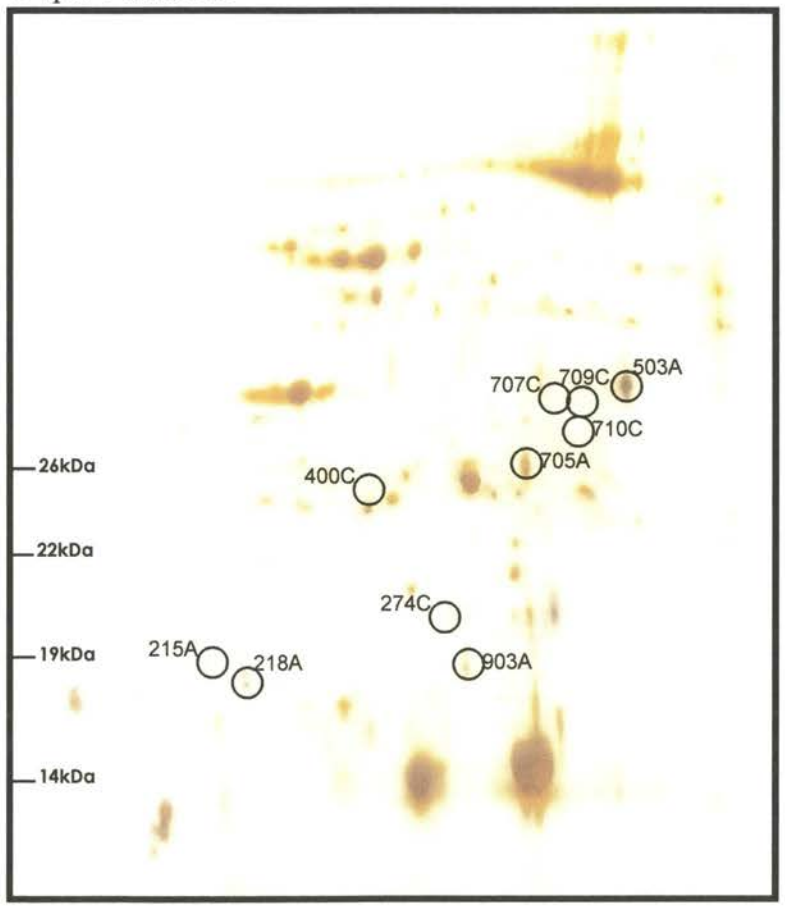


Figure 29. Control for susceptible wheat fed on by Greenbugs for 6d; 4-7pH 14% SDS.

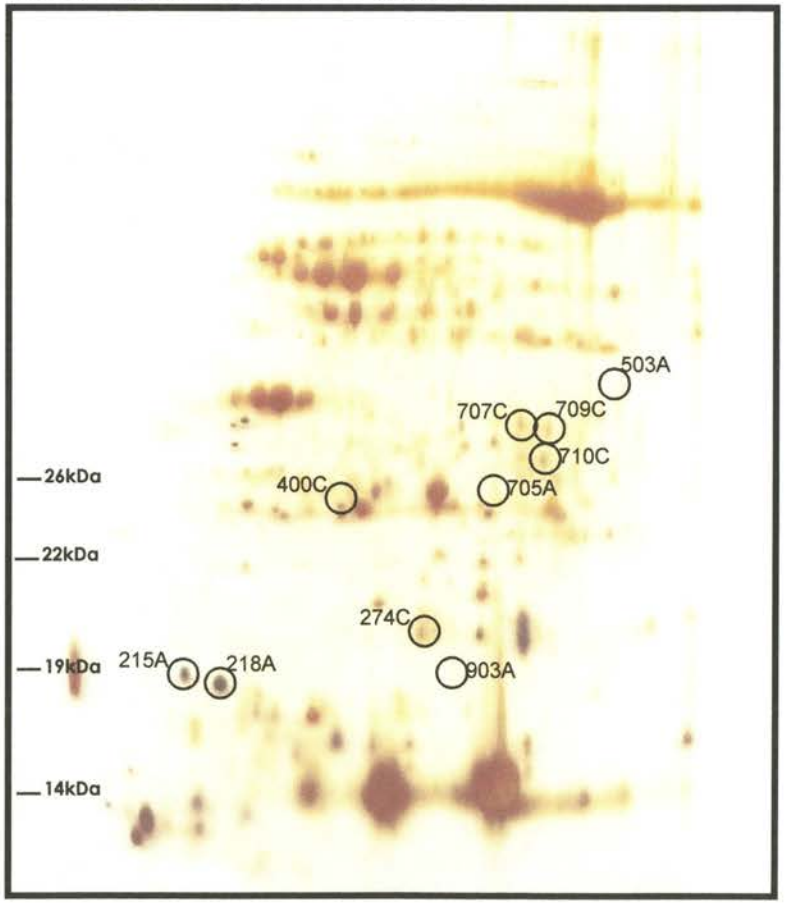


Figure 30. Susceptible wheat fed on by Greenbugs for 6d; 6-11pH 11% SDS.

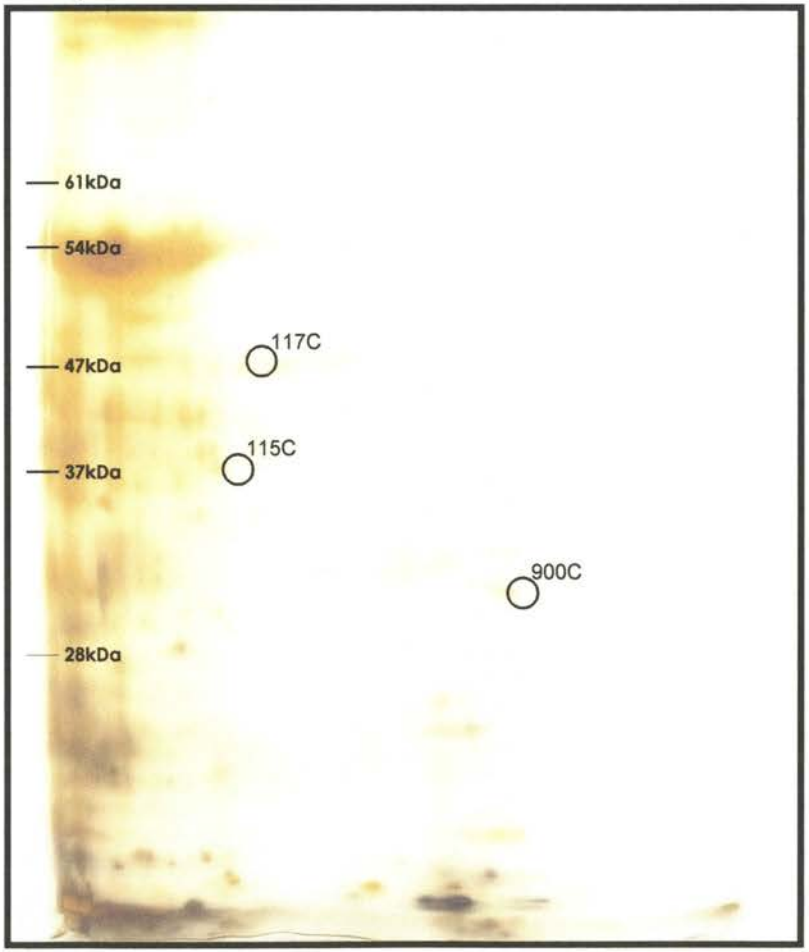


Figure 31. Control for susceptible wheat fed on by Greenbugs for 6d; 6-11pH 11% SDS.

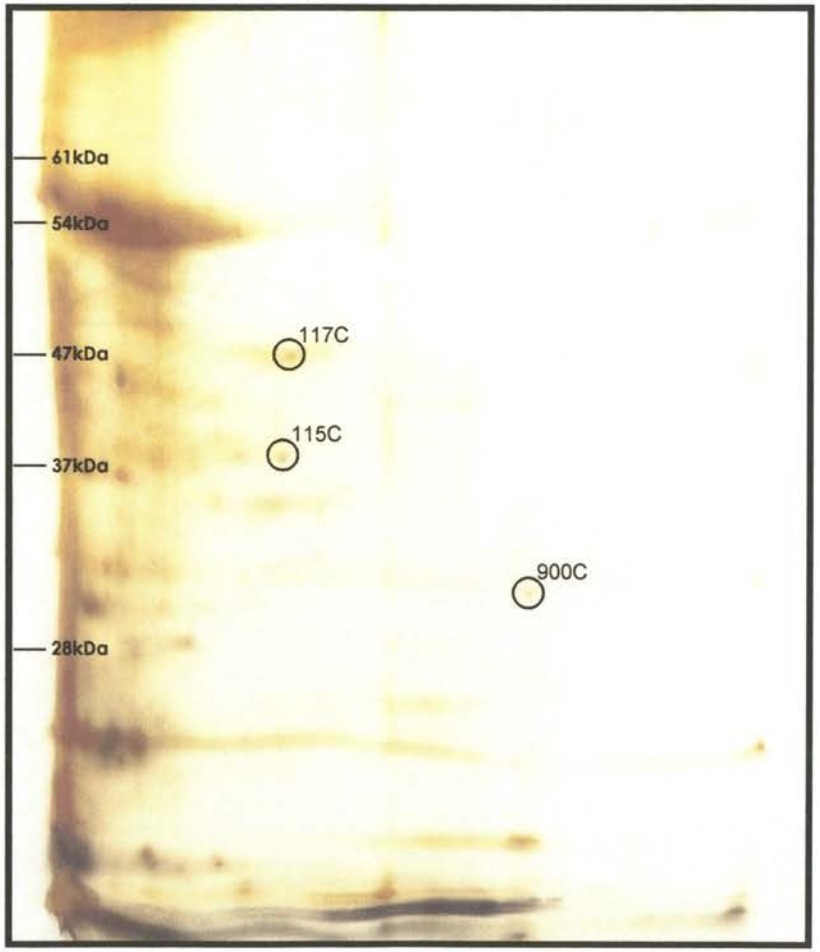


Figure 32. Susceptible wheat fed on by Greenbugs for 6d; 6-11pH 14% SDS.

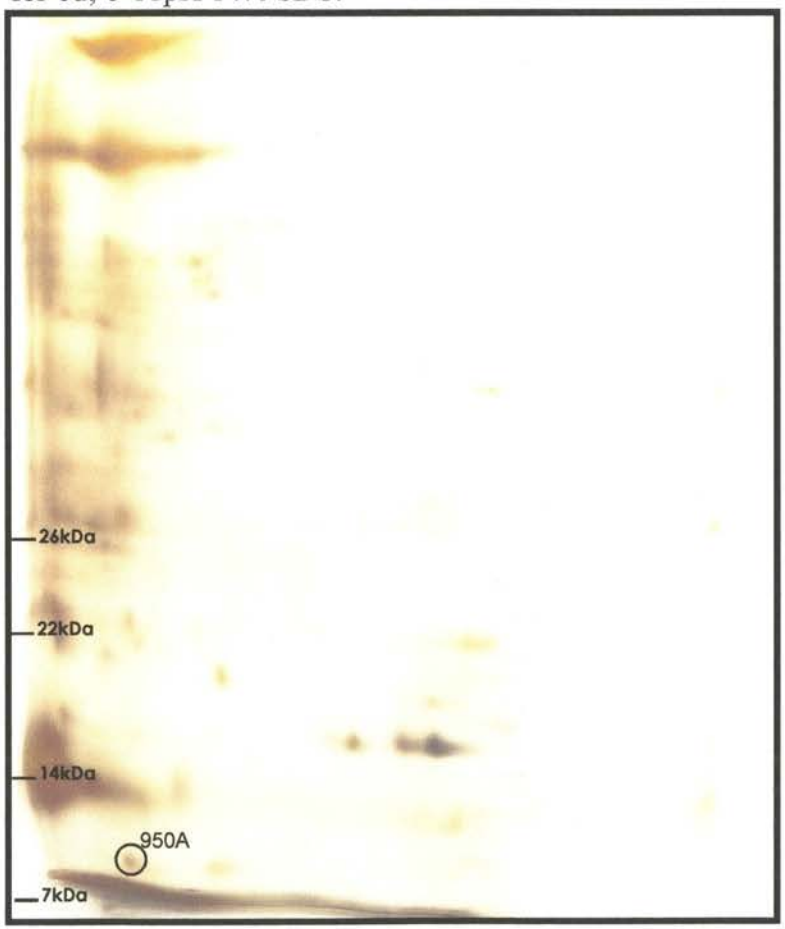


Figure 33. Control for susceptible wheat fed on by Greenbugs for 6d; 6-11pH 14% SDS.

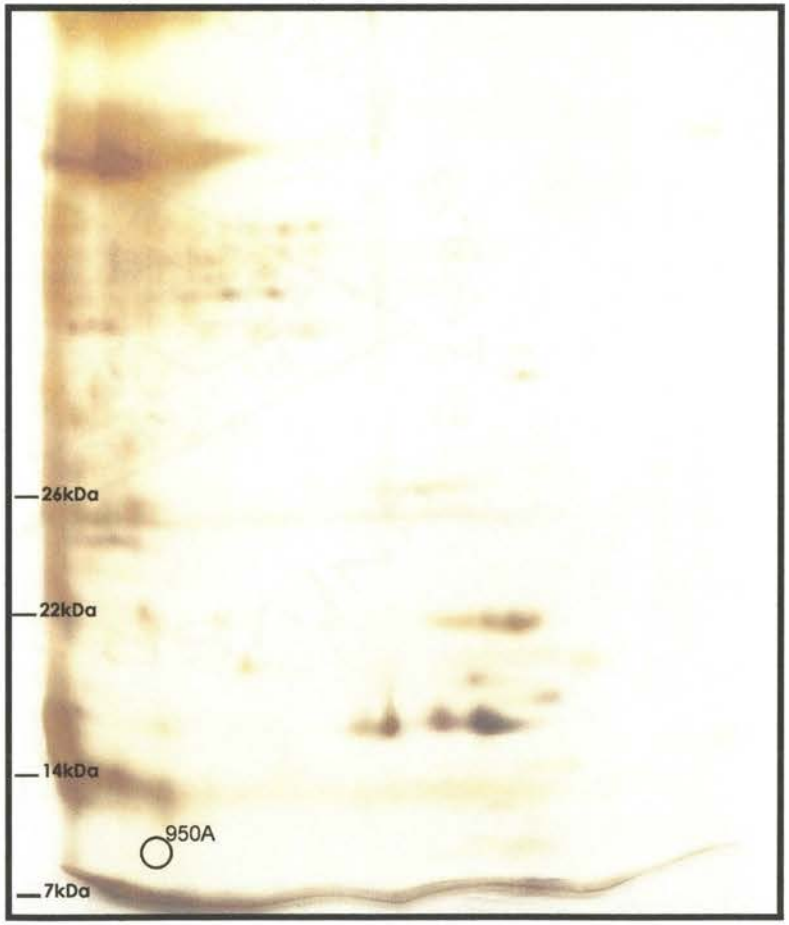


Figure 34. Susceptible wheat leaves fed on by BCOs for 24h; 4-7pH 11% SDS.

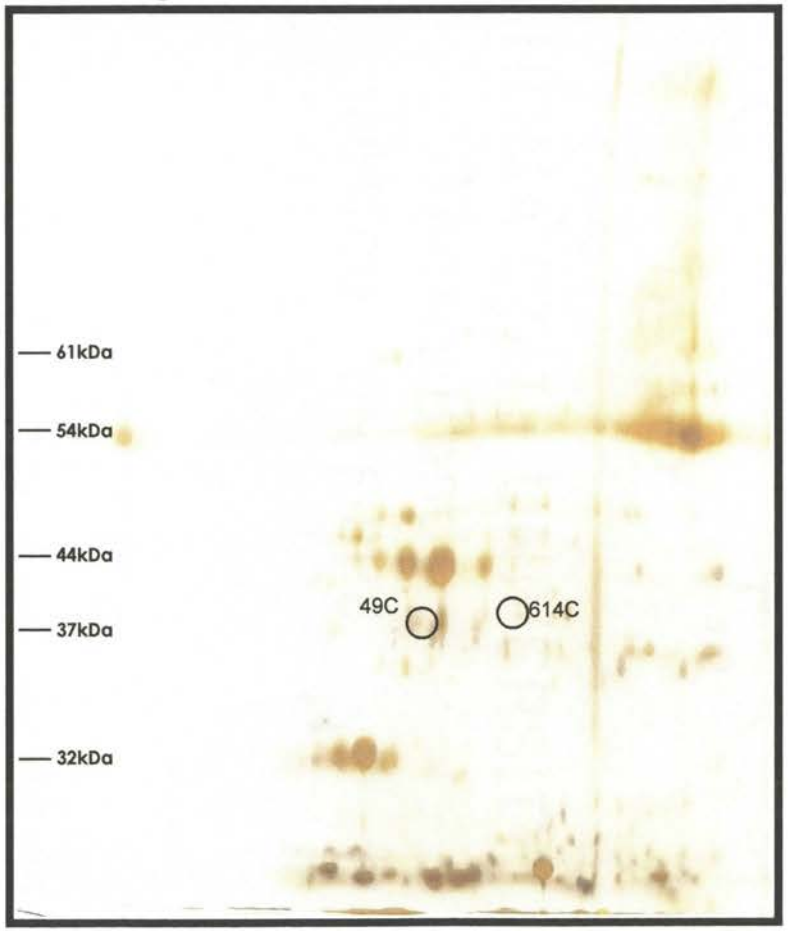


Figure 35. Control for susceptible wheat leaves fed on by BCOs for 24h; 4-7pH 11% SDS.

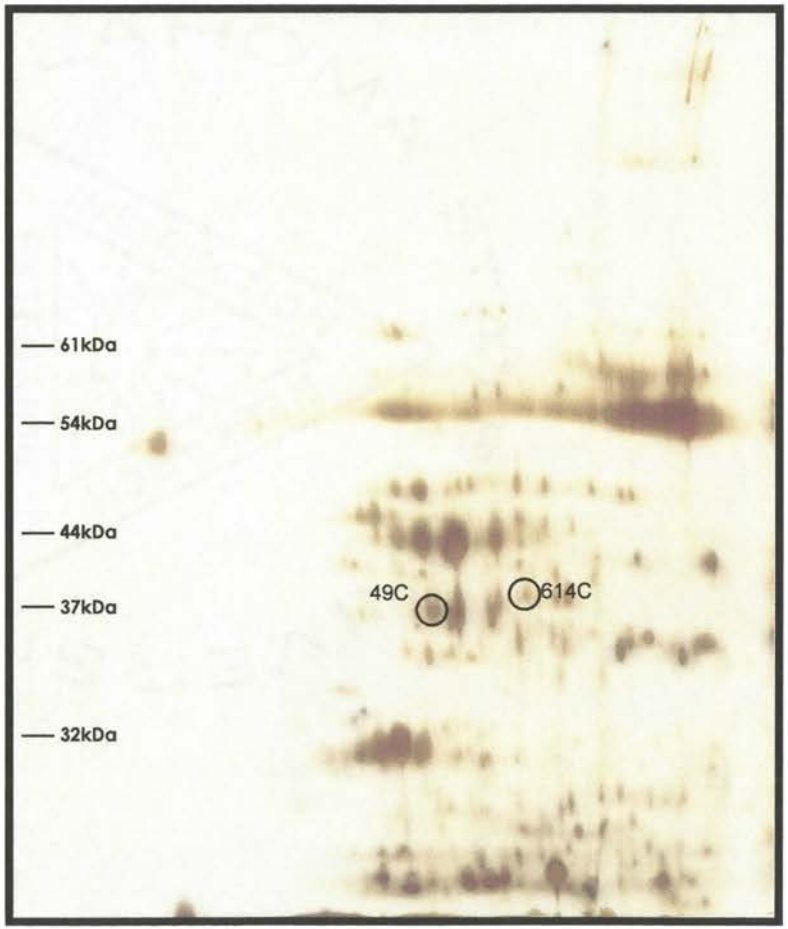


Figure 36. Susceptible wheat leaves fed on by BCOs for 24h; 4-7pH 14% SDS.

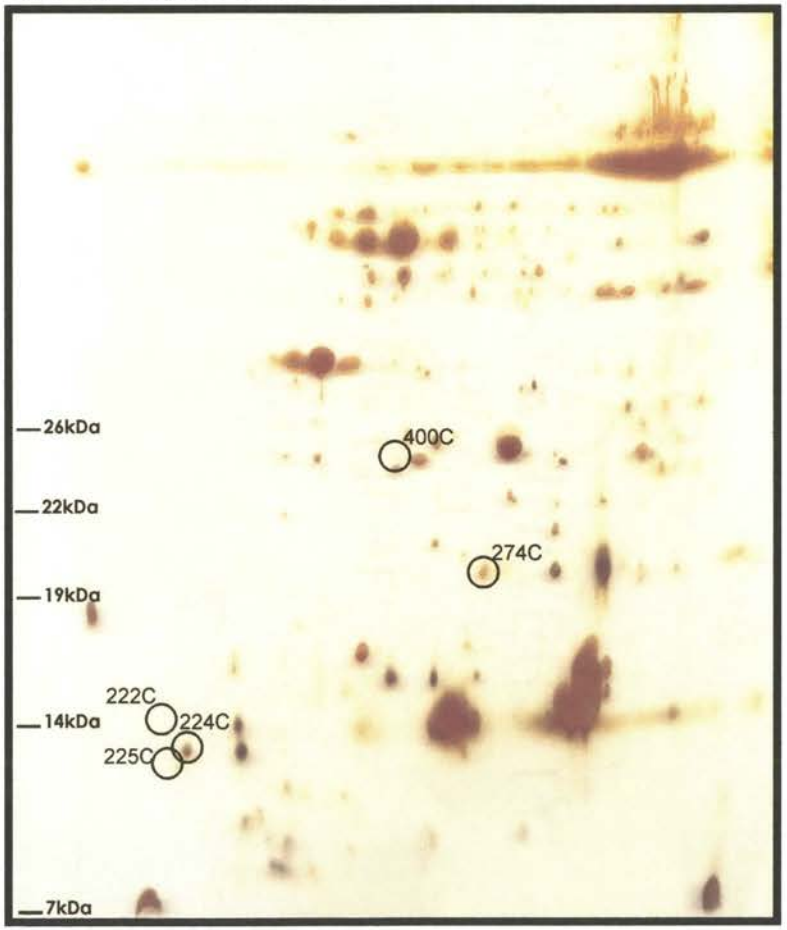


Figure 37. Control for susceptible wheat leaves fed on by BCOs for 24h; 4-7pH 14% SDS.

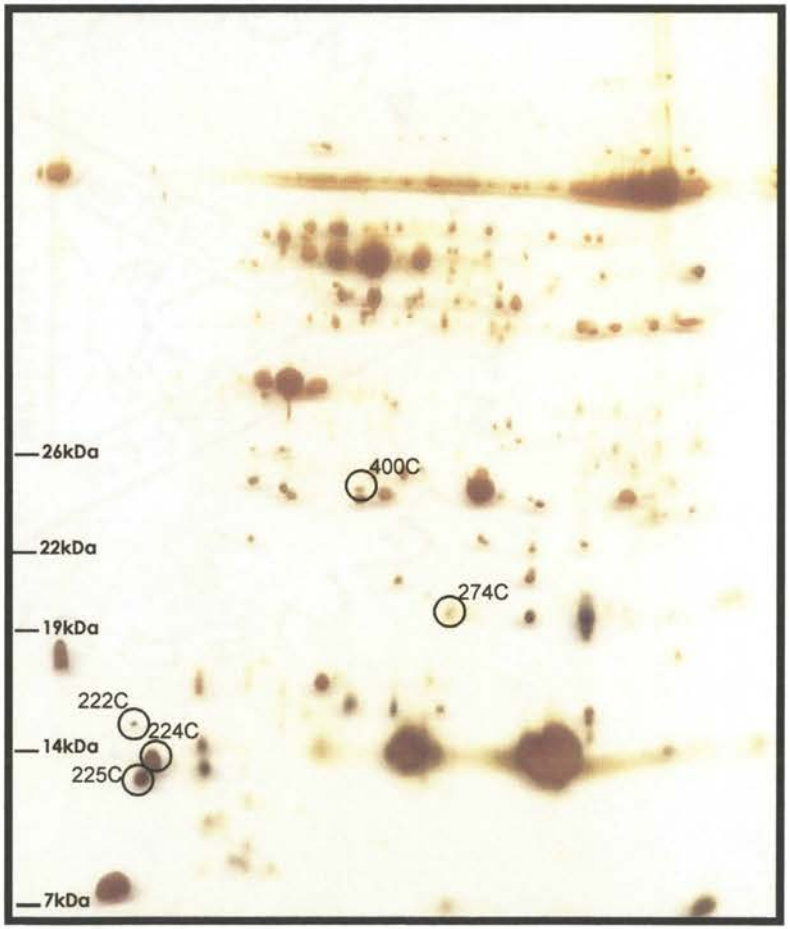


Figure 38. Susceptible wheat leaves fed on by BCOs for 24h; 6-11pH 11% SDS.

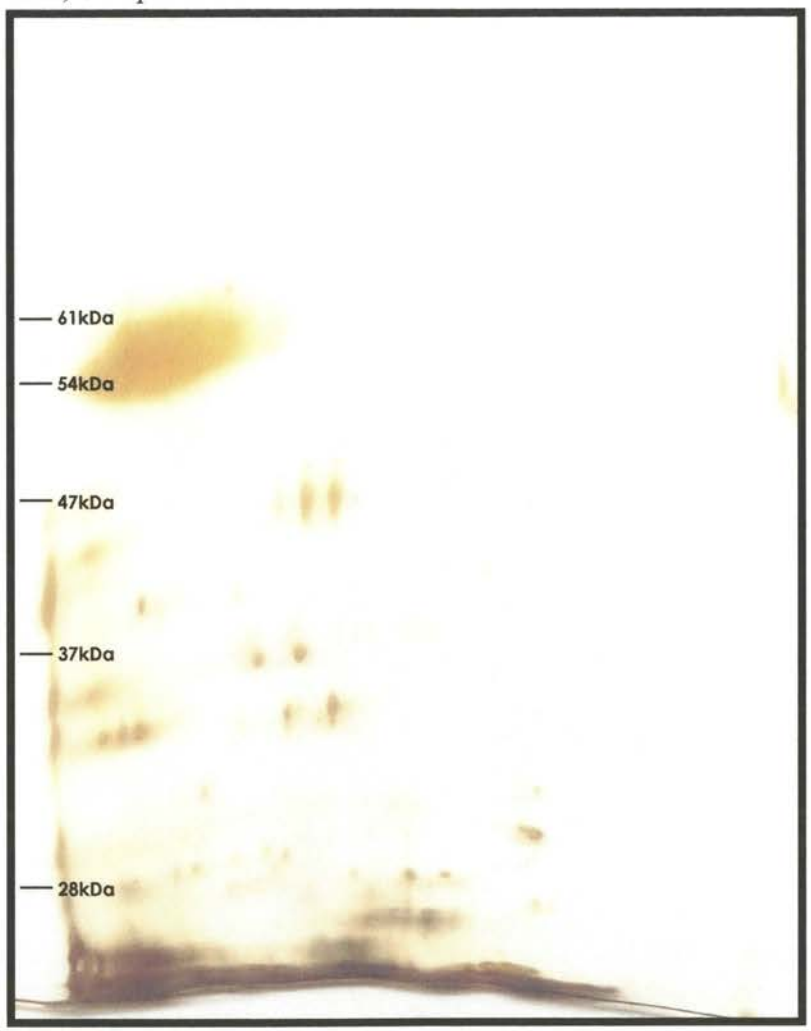


Figure 39. Control for susceptible wheat leaves fed on by BCOs for 24h; 6-11pH 11% SDS.

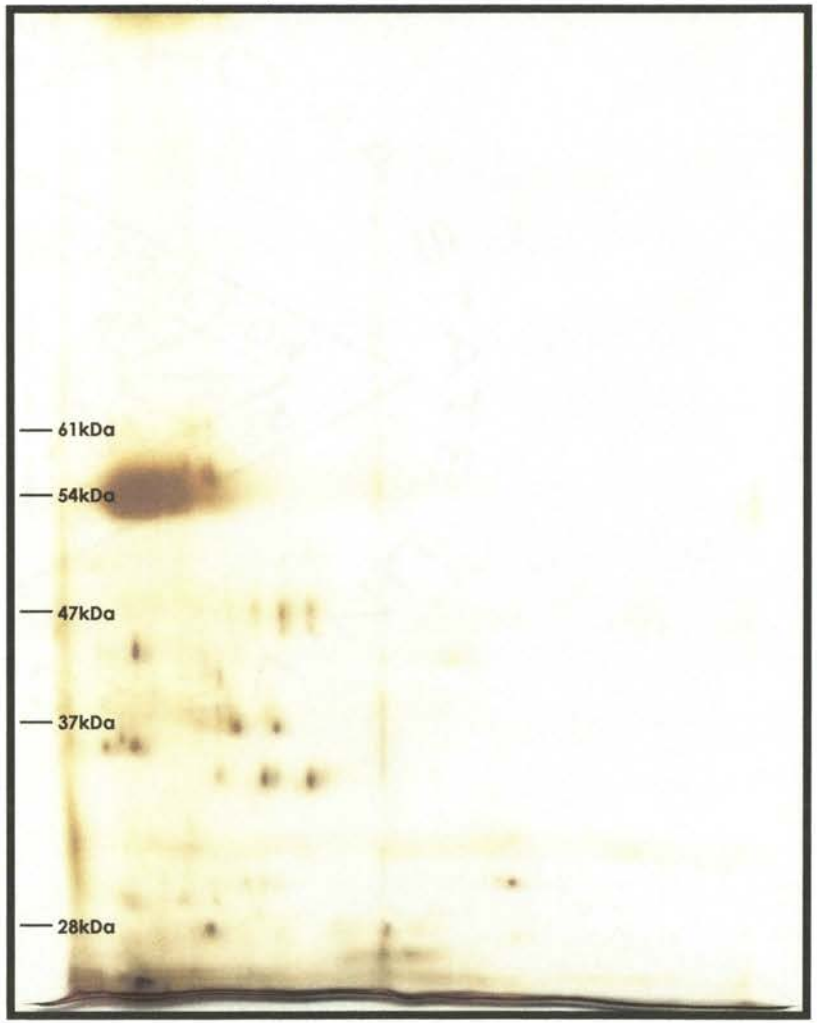


Figure 40. Susceptible wheat leaves fed on by BCOs for 24h; 6-11pH 14% SDS.

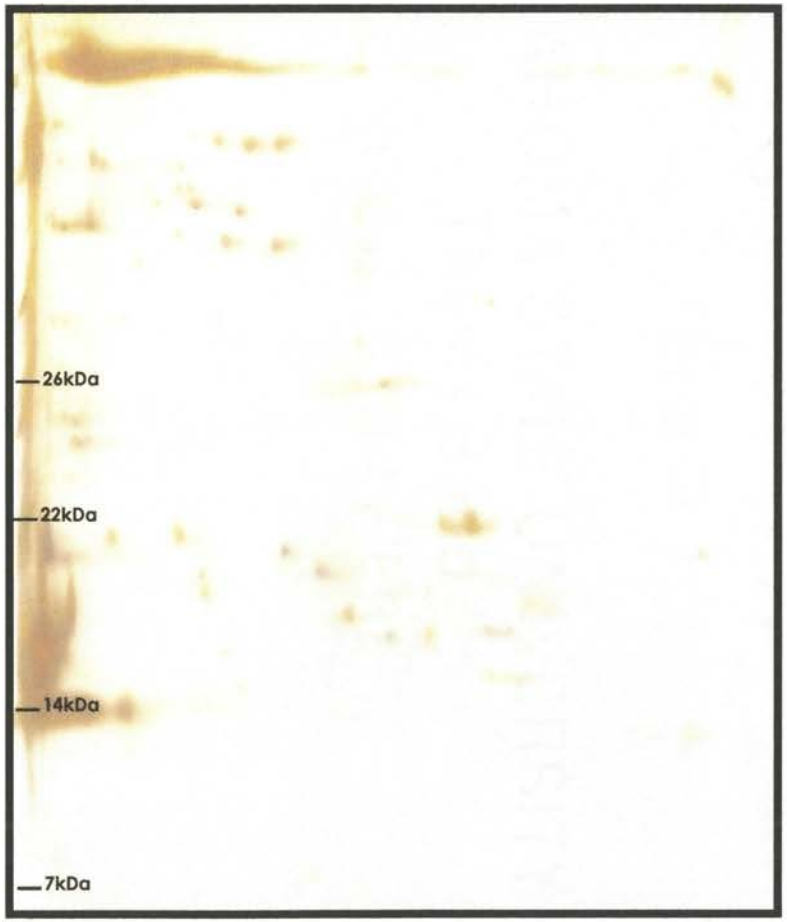


Figure 41. Control for susceptible wheat leaves fed on by BCOs for 24h; 6-11pH 14% SDS.

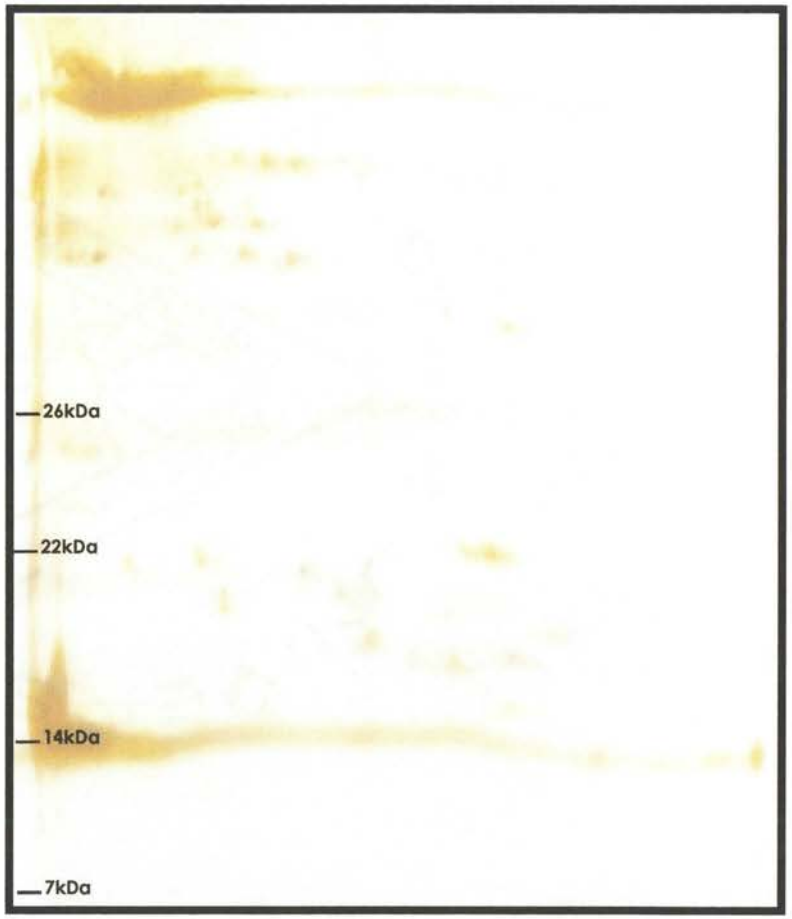


Figure 42. Susceptible wheat leaves fed on by BCOs for 6d; 4-7pH 11% SDS.

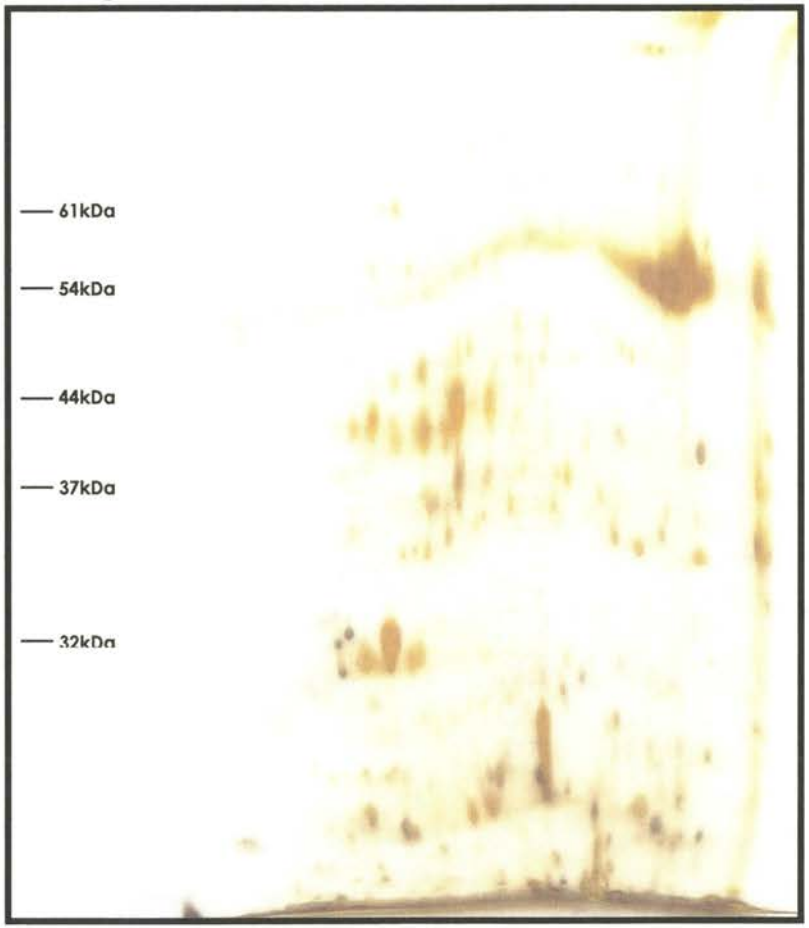


Figure 43. Control for susceptible wheat leaves fed on by BCOs for 6d; 4-7pH 11% SDS.

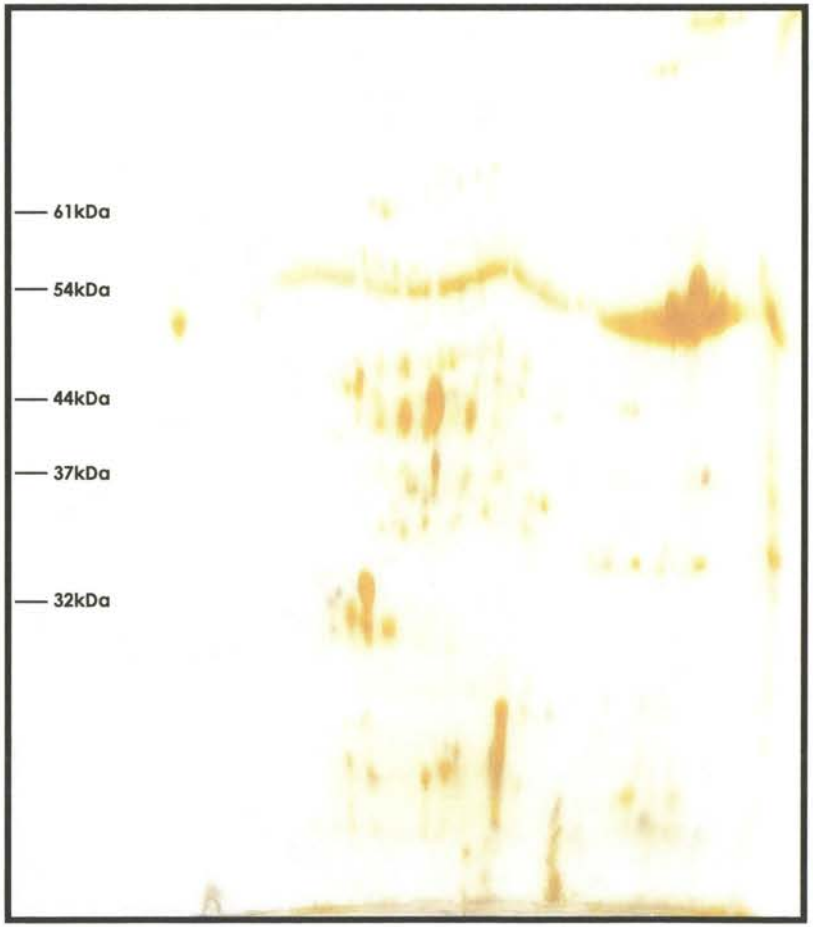


Figure 44. Susceptible wheat leaves fed on by BCOs for 6d; 4-7pH 14% SDS.

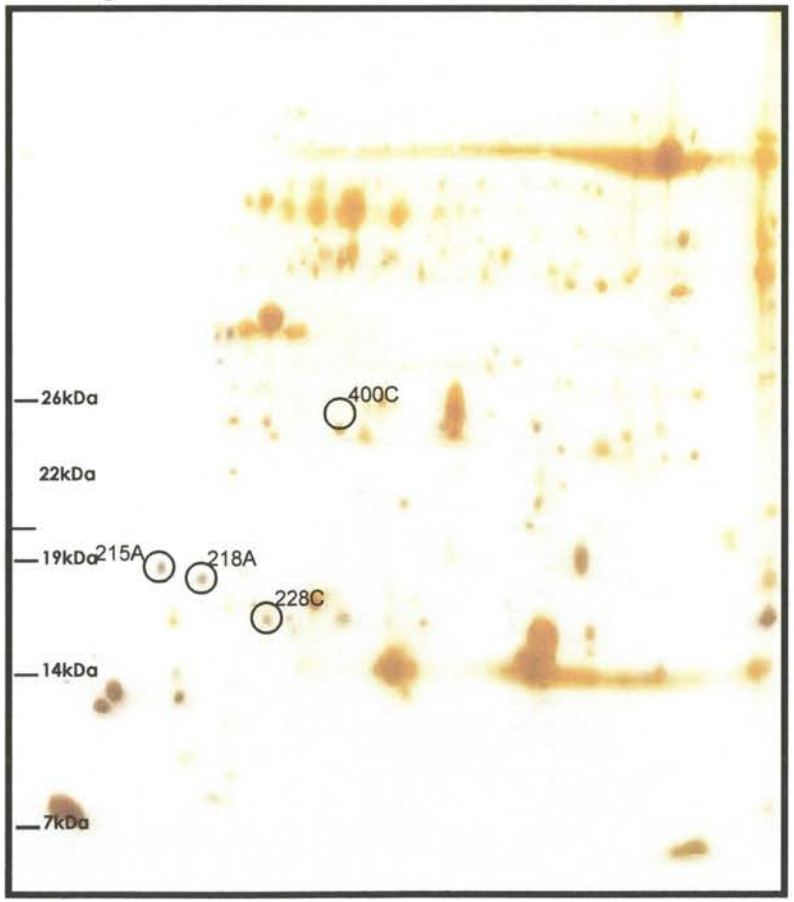


Figure 45. Control for susceptible wheat leaves fed on by BCOs for 6d; 4-7pH 14% SDS.

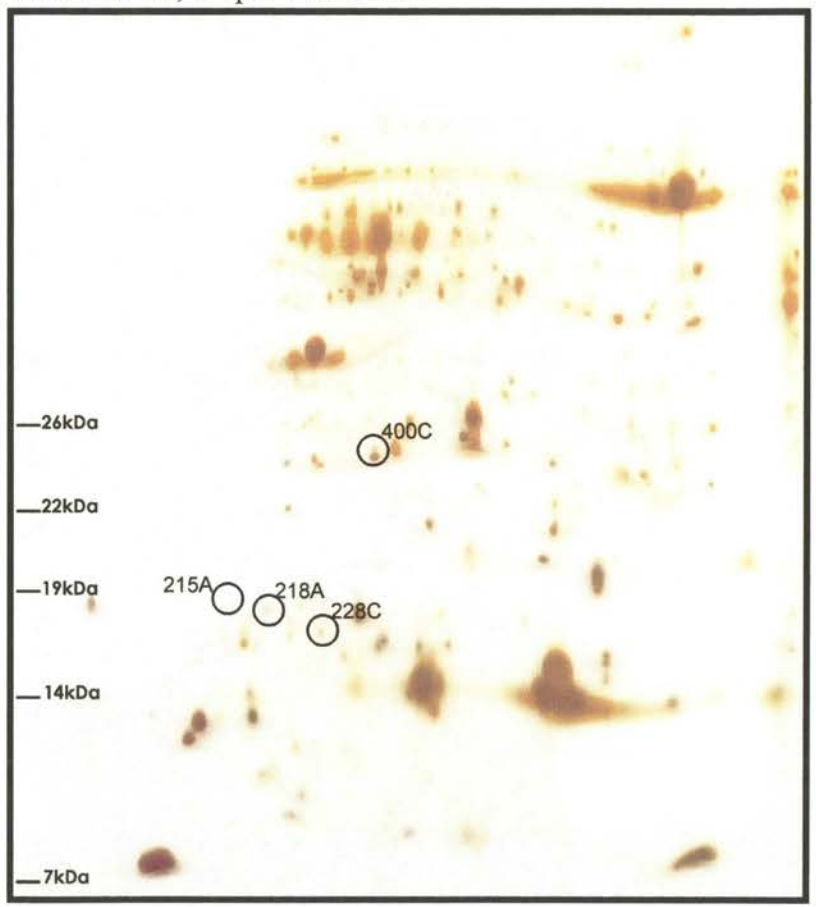


Figure 46. Susceptible wheat leaves fed on by BCOs for 6d; 6-11pH 11% SDS.

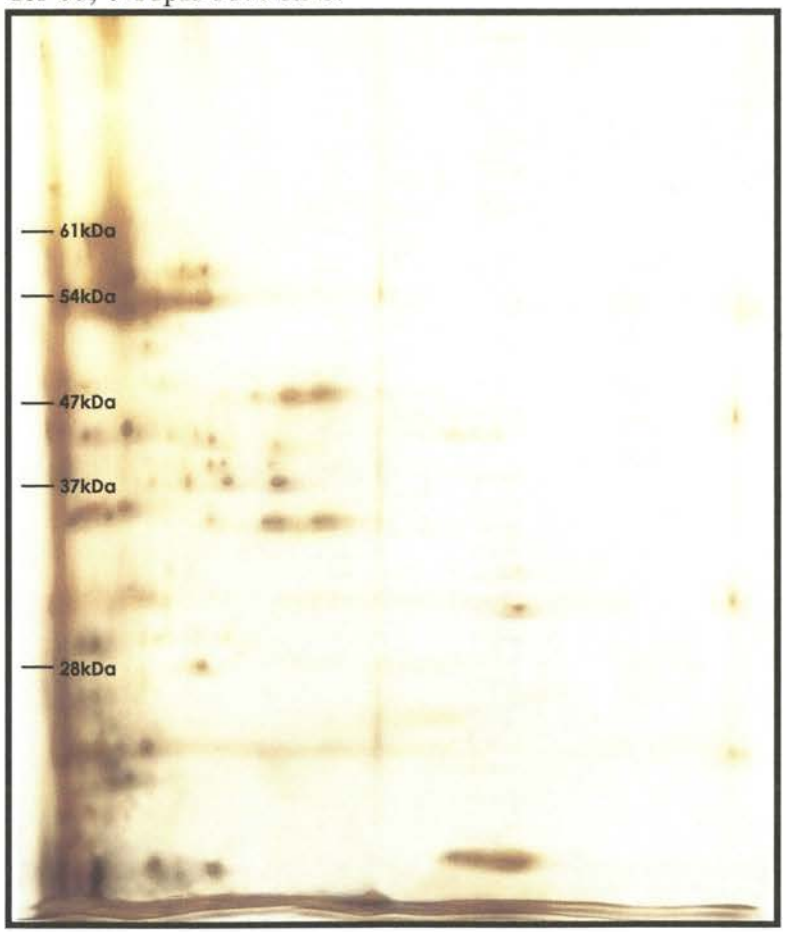


Figure 47. Control for susceptible wheat leaves fed on by BCOs for 6d; 6-11pH 11% SDS.

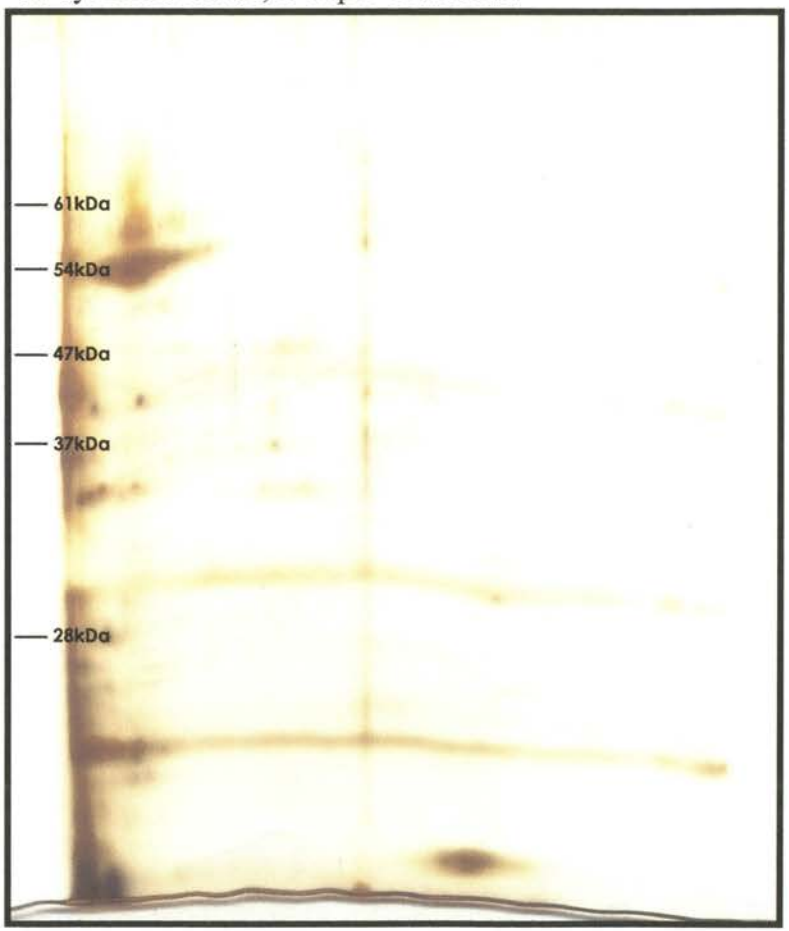


Figure 48. Susceptible wheat leaves fed on by BCOs for 6d; 6-11pH 14% SDS.

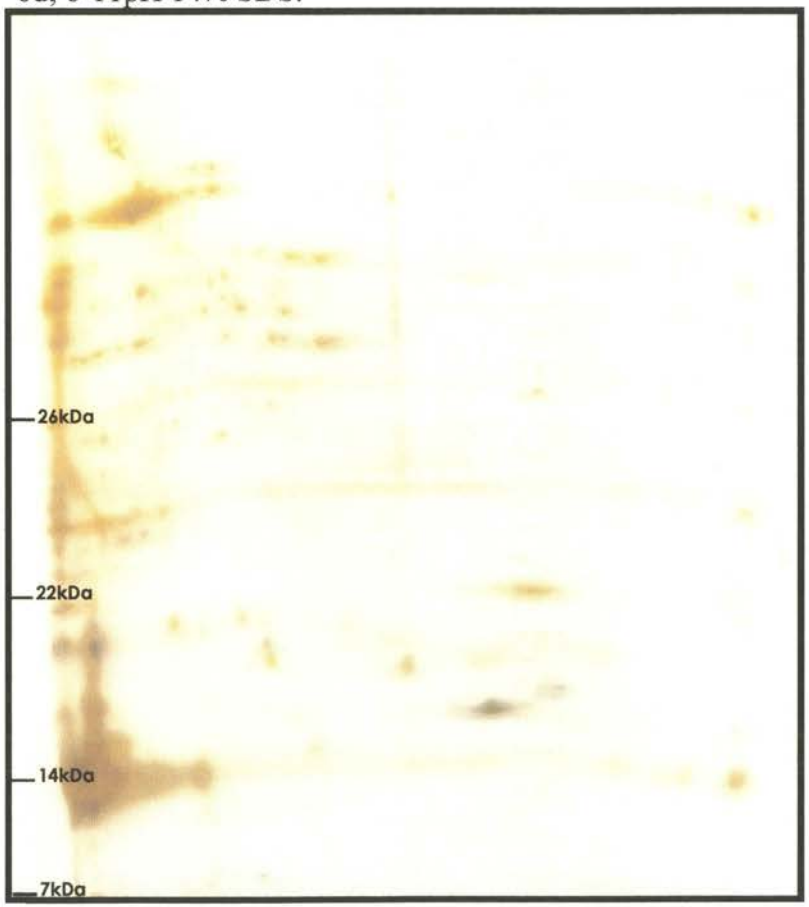


Figure 49. Control for susceptible wheat leaves fed on by BCOs for 6d; 6-11pH 14% SDS.

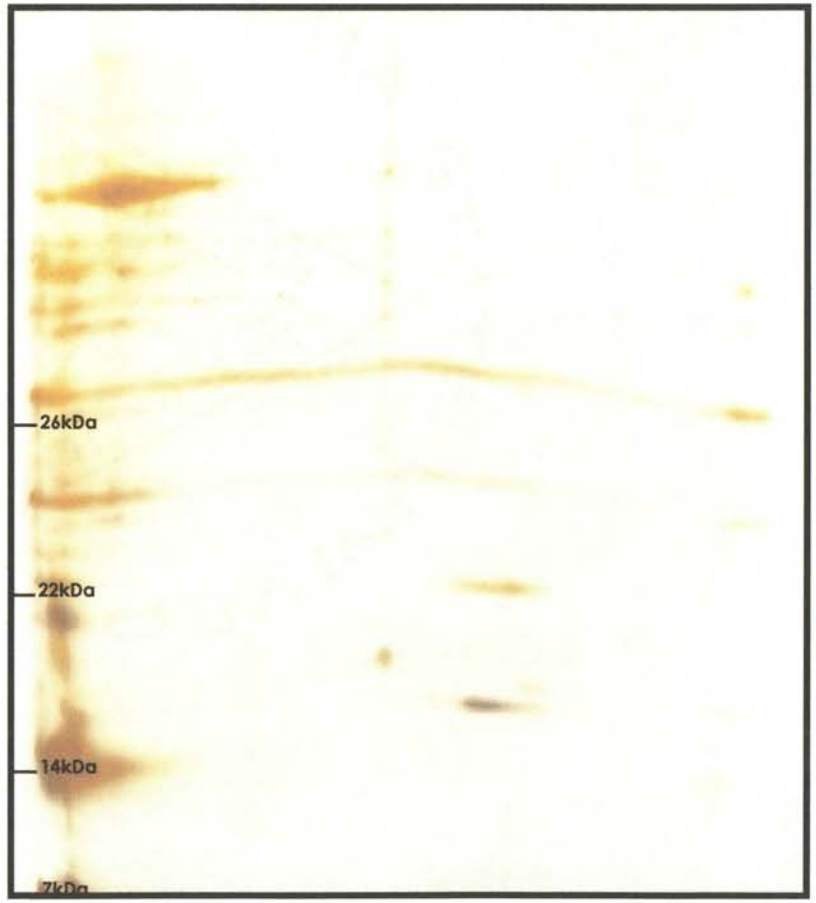


Figure 50. Resistant wheat fed on by Greenbugs for 24h; 4-7pH 11% SDS.

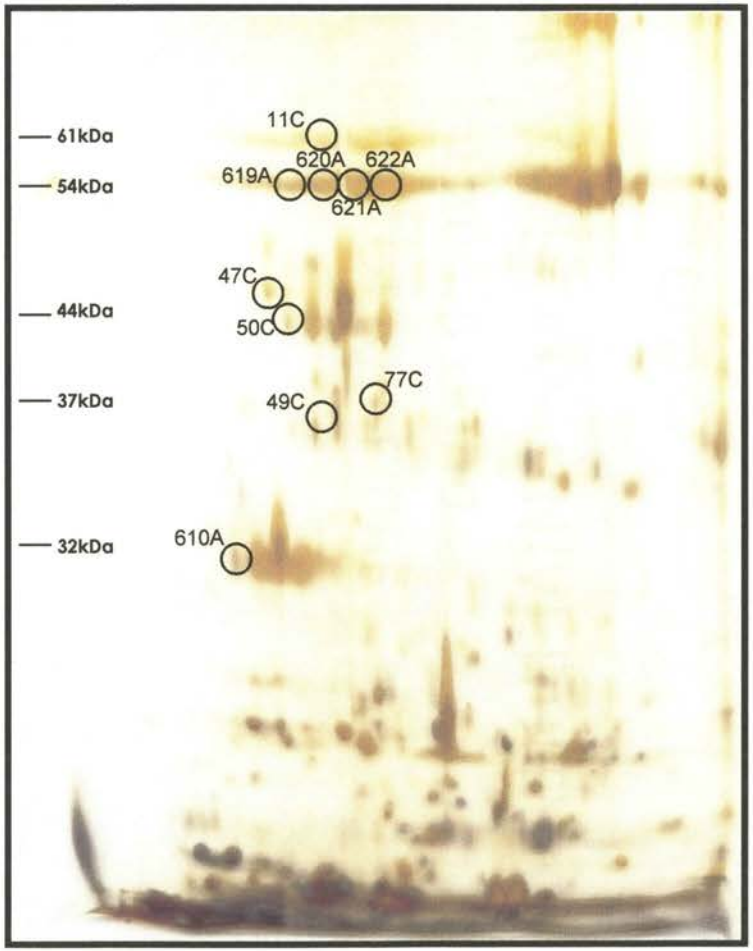


Figure 51. Control for resistant wheat fed on by Greenbugs for 24h; 4-7pH 11% SDS.

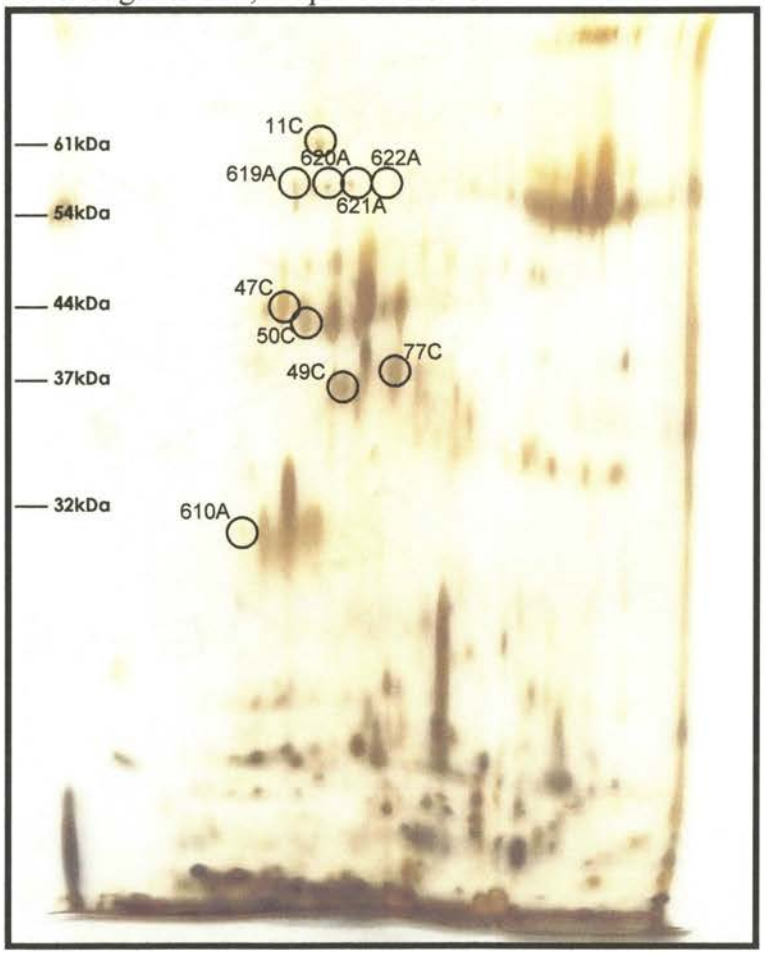


Figure 52. Resistant wheat fed on by Greenbugs for 24h; 4-7pH 14% SDS.

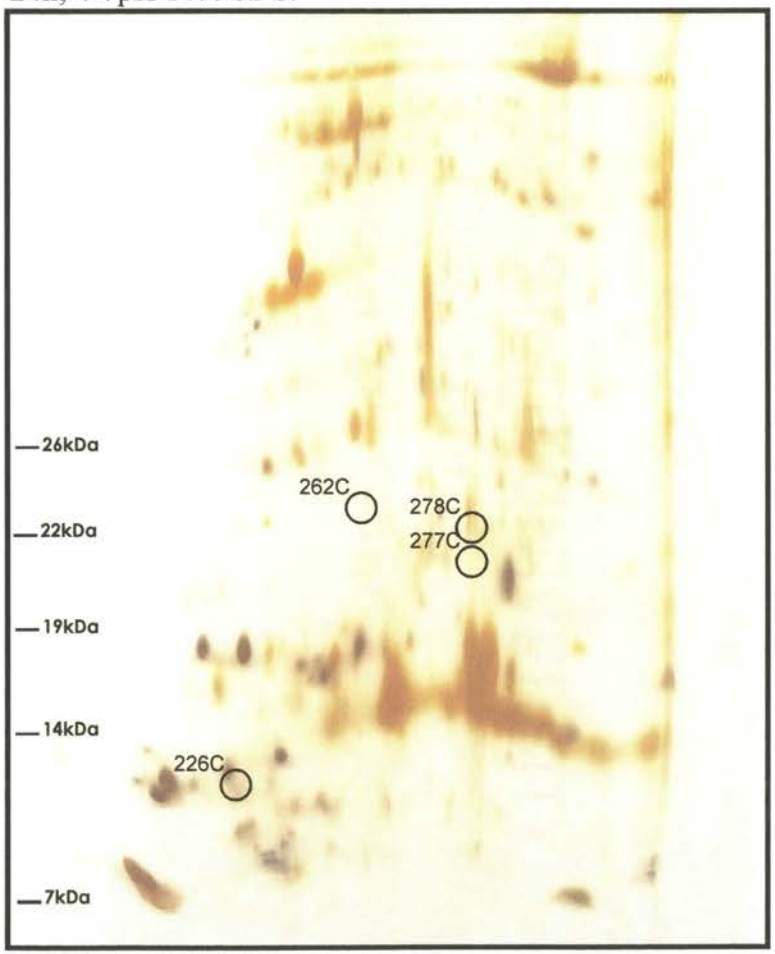


Figure 53. Control for resistant wheat fed on by Greenbugs for 24h; 4-7pH 14% SDS.

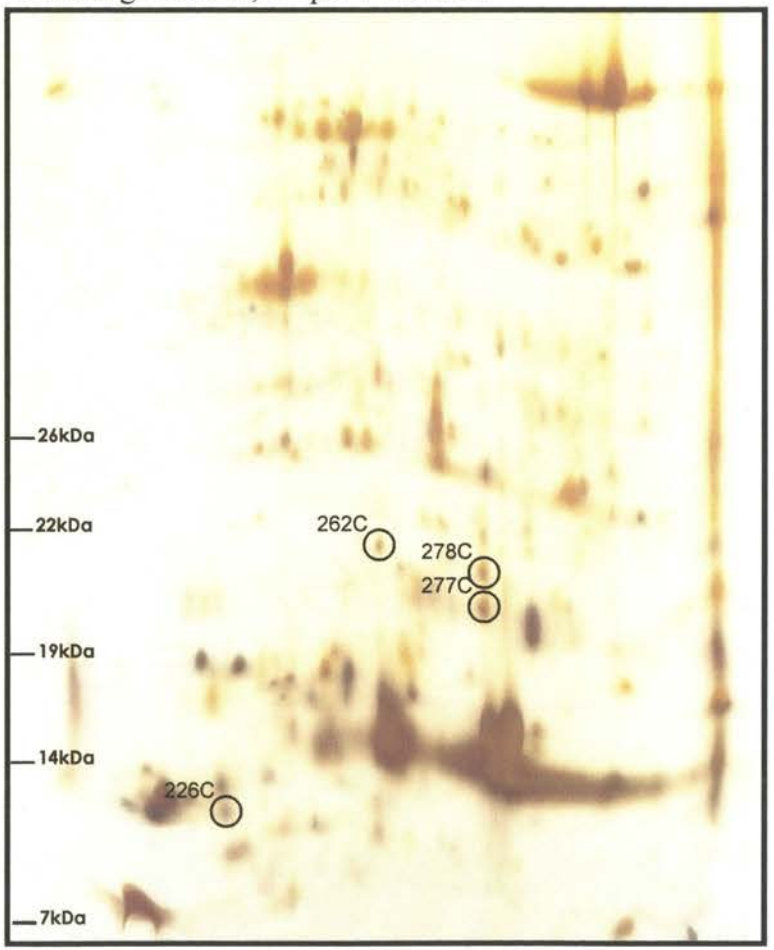


Figure 54. Resistant wheat fed on by Greenbugs for 24h; 6-11pH 11% SDS.

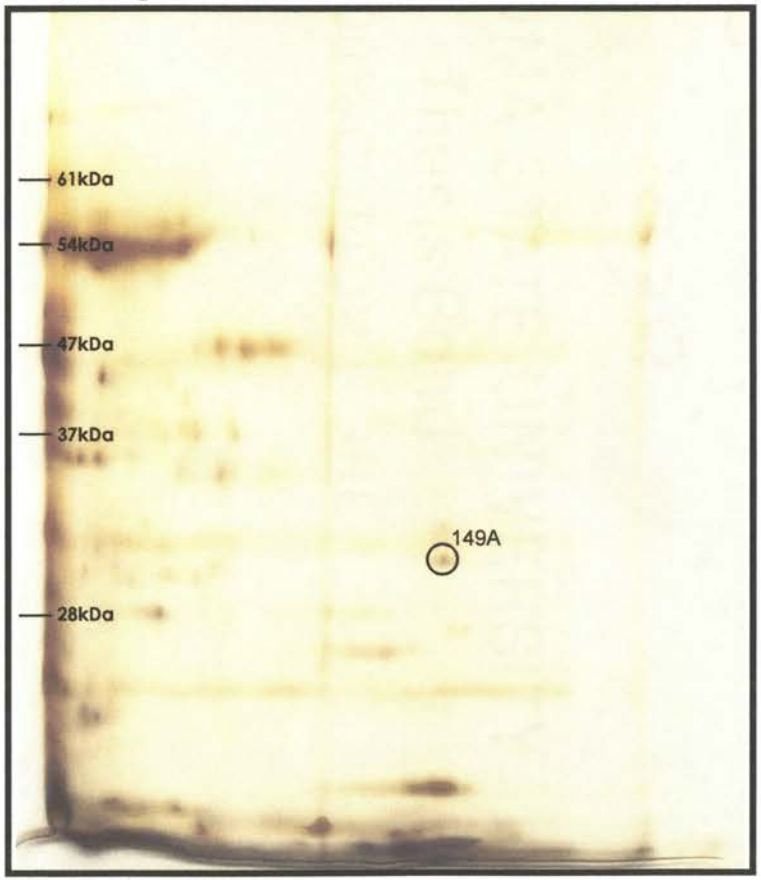


Figure 55. Control for resistant wheat fed on by Greenbugs for 24h; 6-11pH 11% SDS.

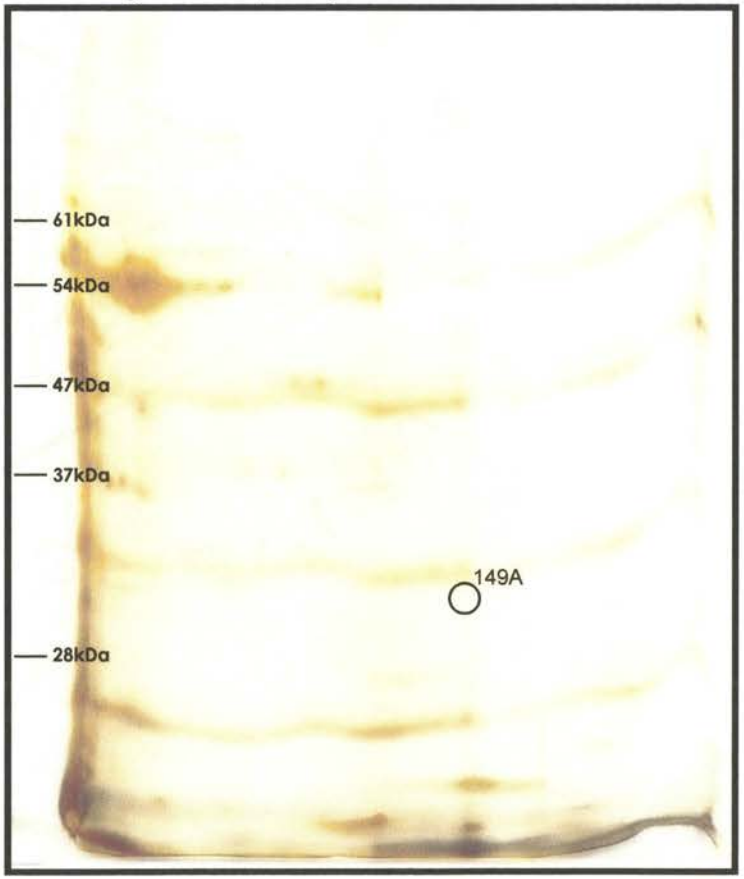


Figure 56. Resistant wheat fed on by Greenbugs for 24h; 6-11pH 14% SDS.

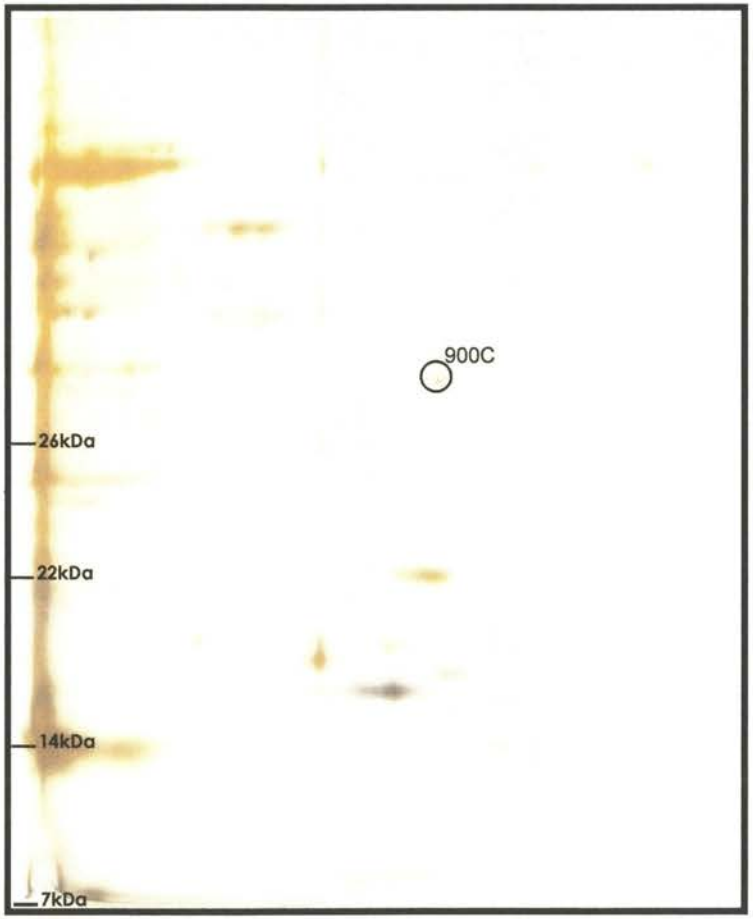


Figure 57. Control for resistant wheat fed on by Greenbugs for 24h; 6-11pH 14% SDS.

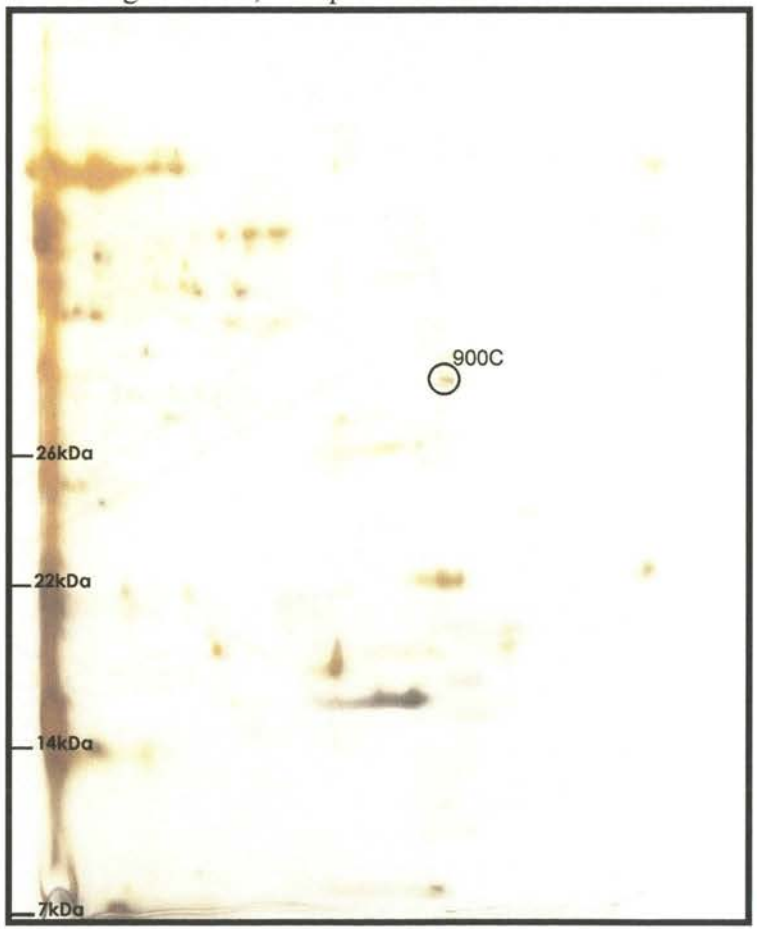


Figure 58. Resistant wheat fed on by Greenbugs for 6d; 4-7pH 11% SDS.

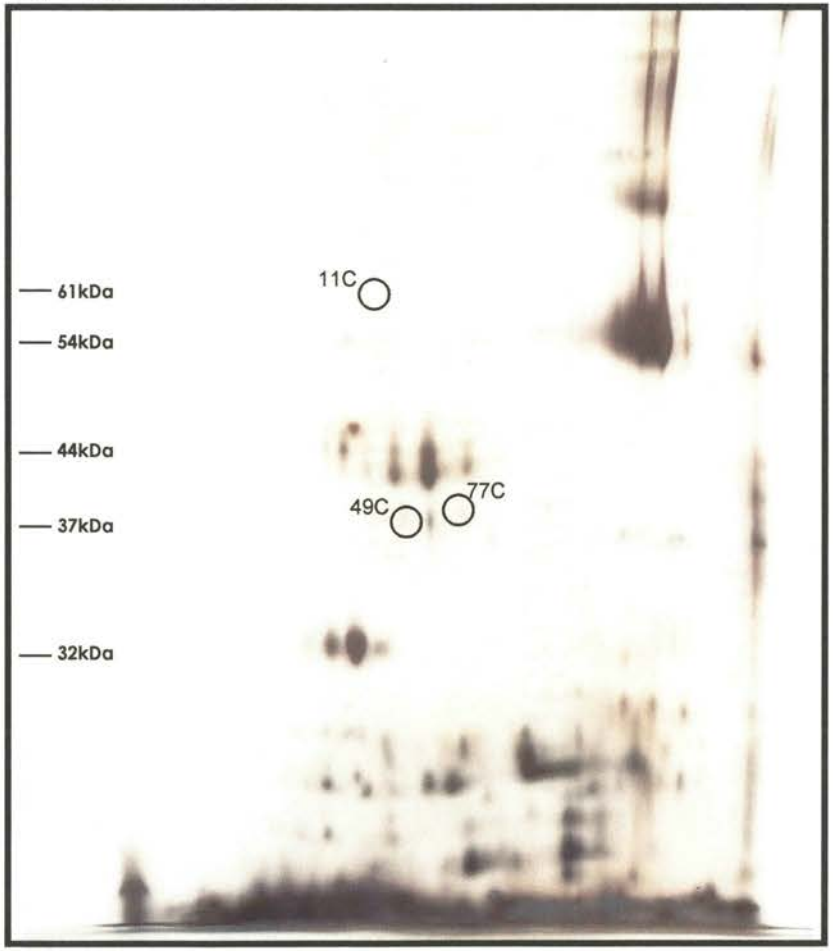


Figure 59. Control for resistant wheat fed on by Greenbugs for 6d; 4-7pH 11% SDS.

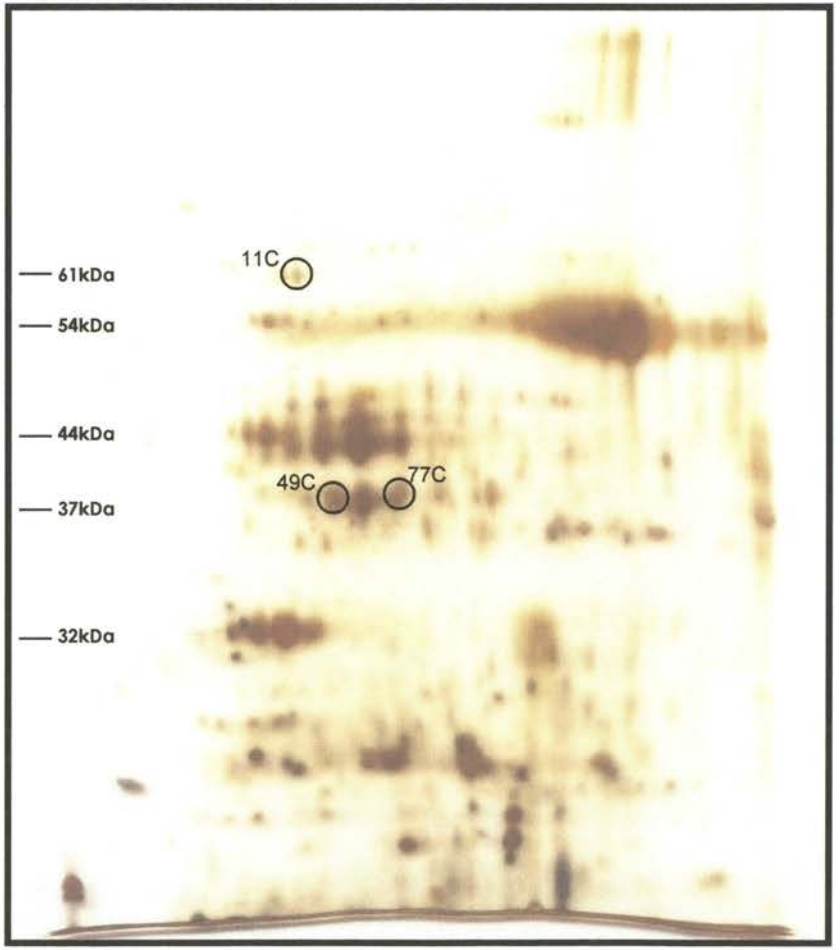


Figure 60. Resistant wheat fed on by Greenbugs for 6d; 4-7pH 14% SDS.

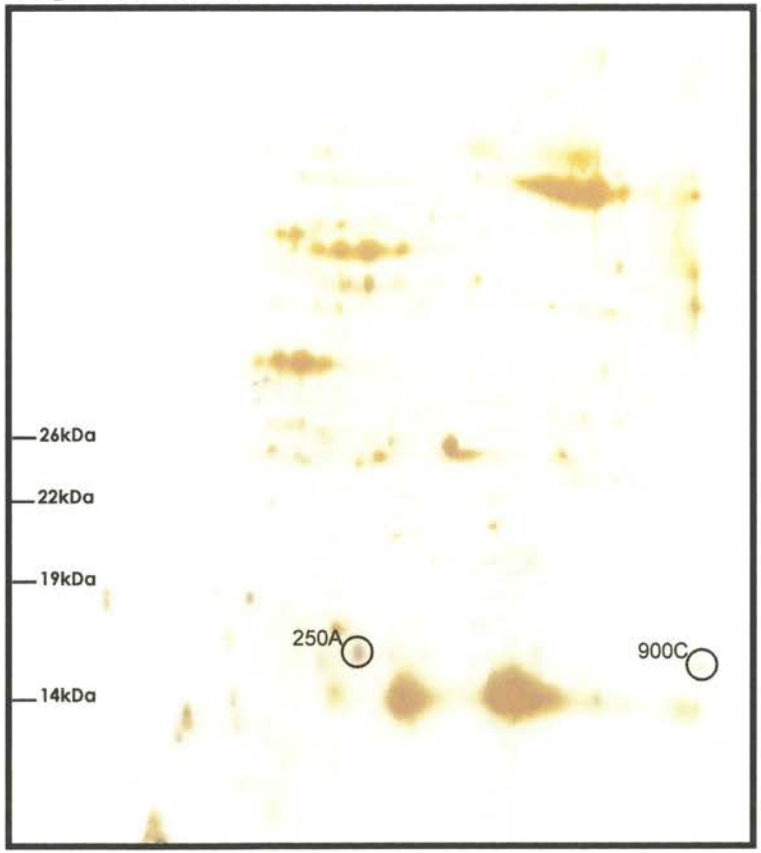


Figure 61. Control for resistant wheat fed on by Greenbugs for 6d; 4-7pH 14% SDS.

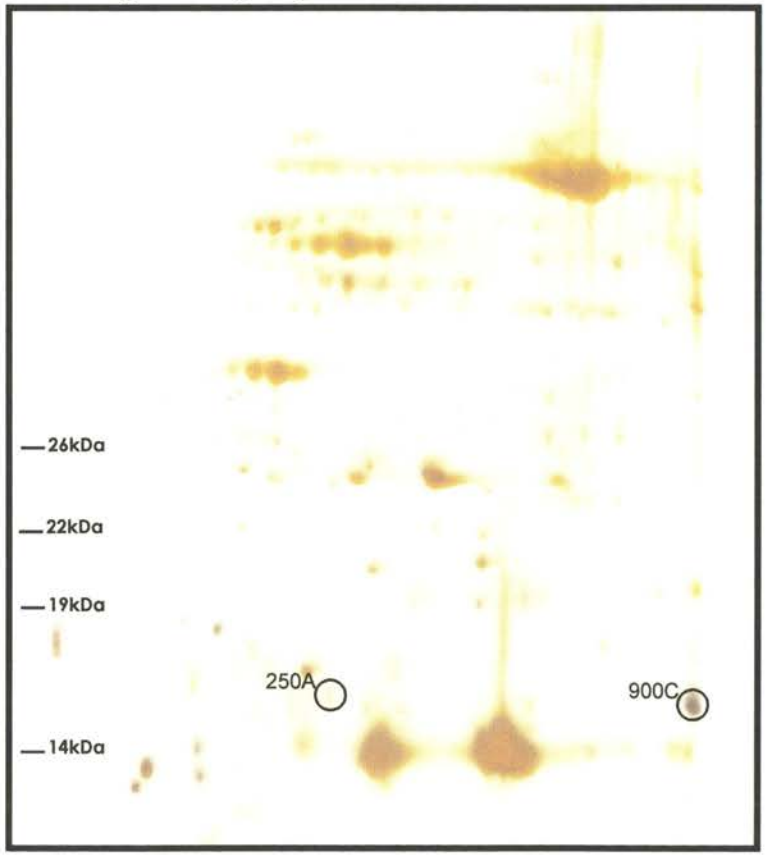


Figure 62. Resistant wheat fed on by Greenbugs for 6d; 6-11pH 11% SDS.

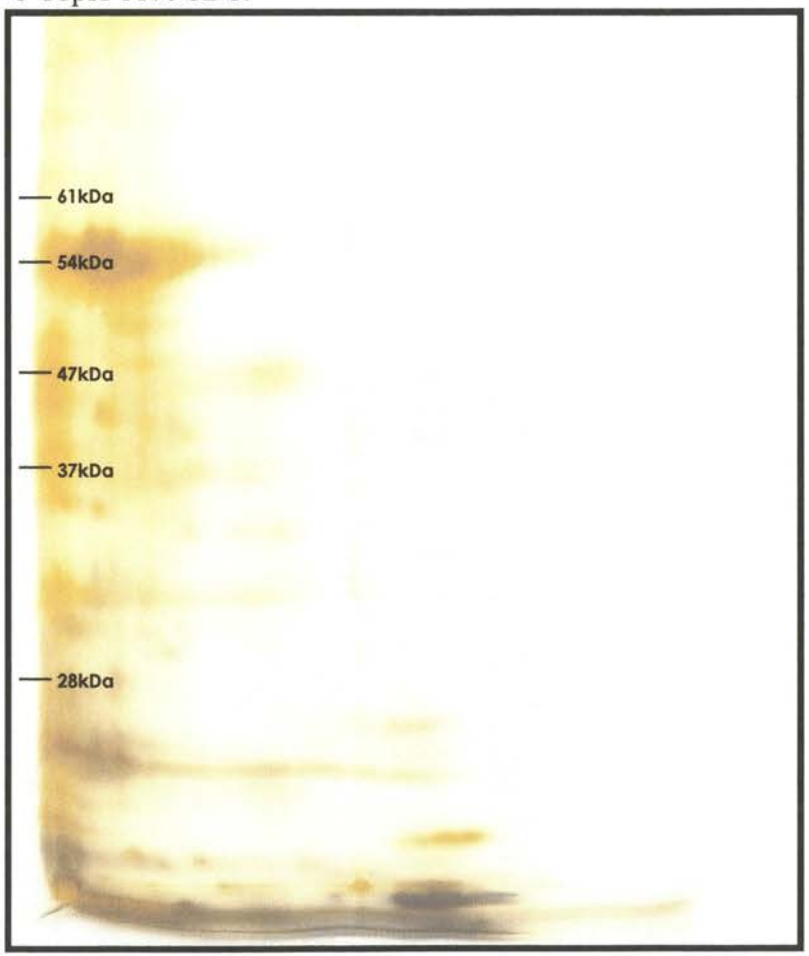


Figure 63. Control for resistant wheat fed on by Greenbugs for 6d; 6-11pH 11% SDS.

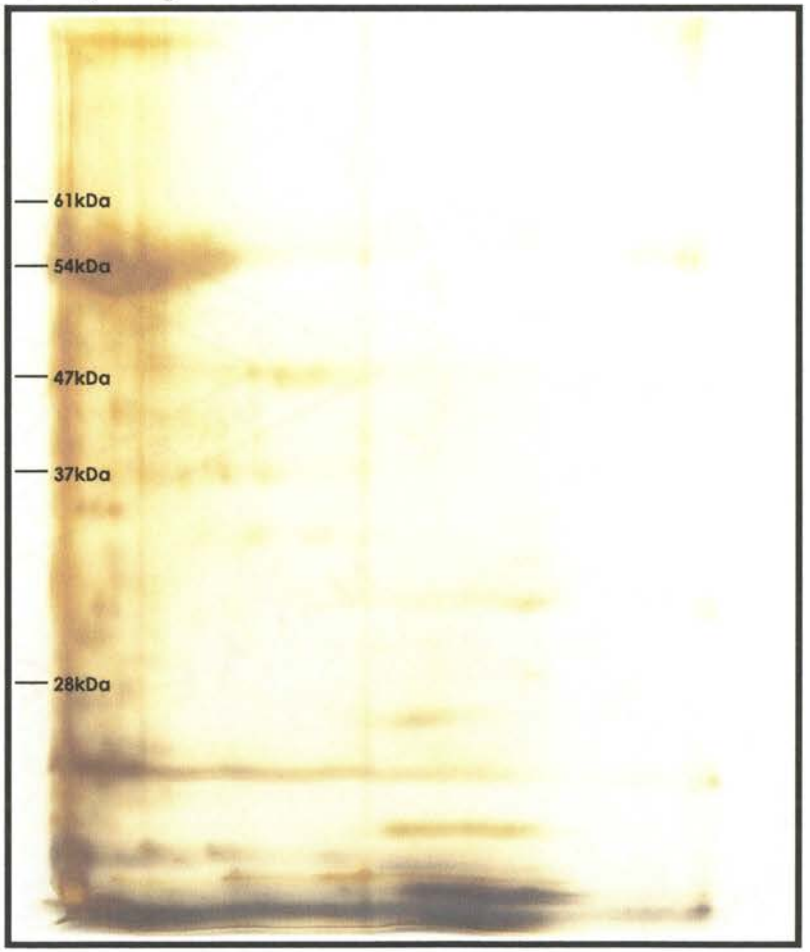


Figure 64. Resistant wheat fed on by Greenbugs for 6d; 6-11pH 14% SDS.

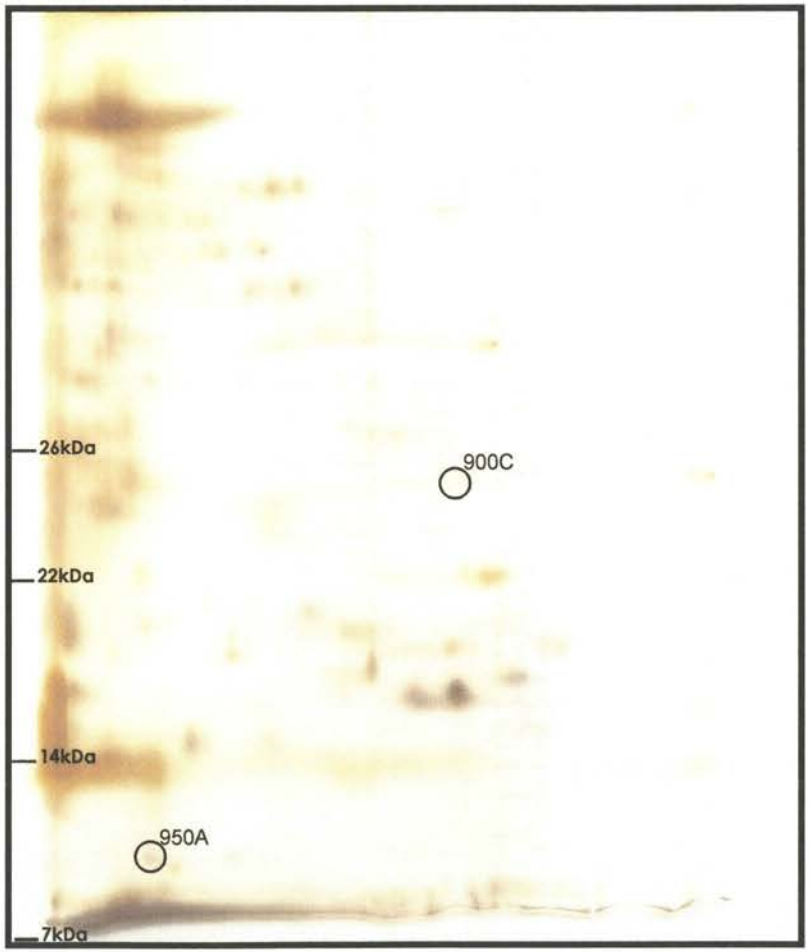


Figure 65. Control for resistant wheat fed on by Greenbugs for 6d; 6-11pH 14% SDS.

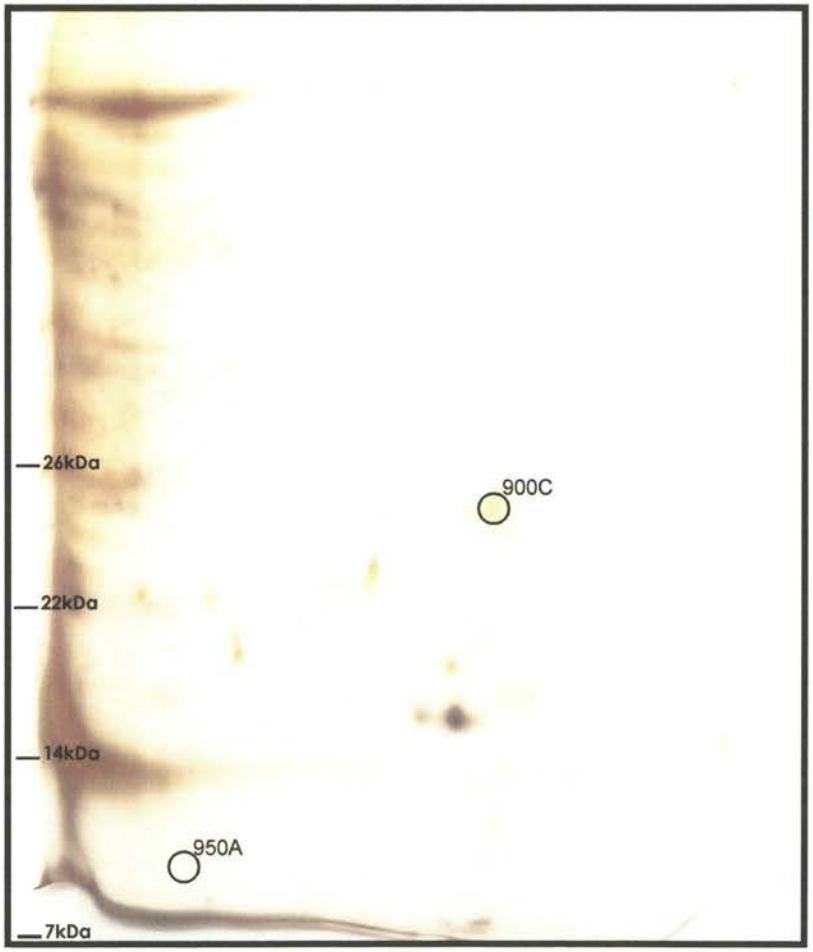


Figure 66. Resistant wheat leaves fed on by BCOs for 24h; 4-7pH 11% SDS.

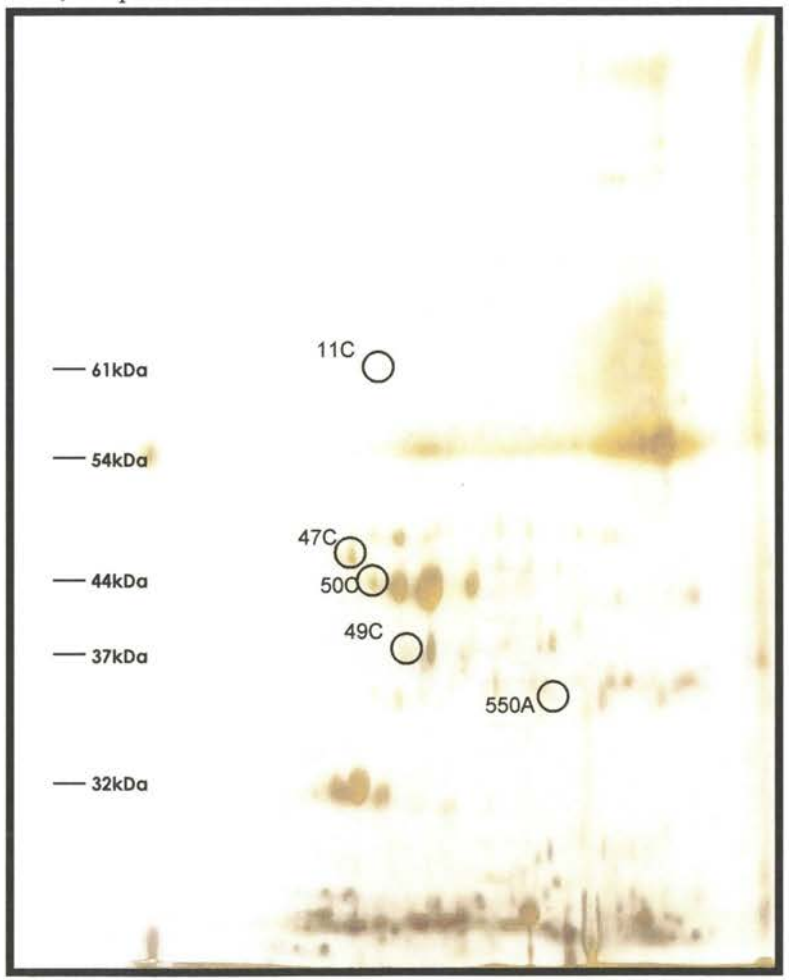


Figure 67. Control for resistant wheat leaves fed on by BCOs for 24h; 4-7pH 11% SDS.

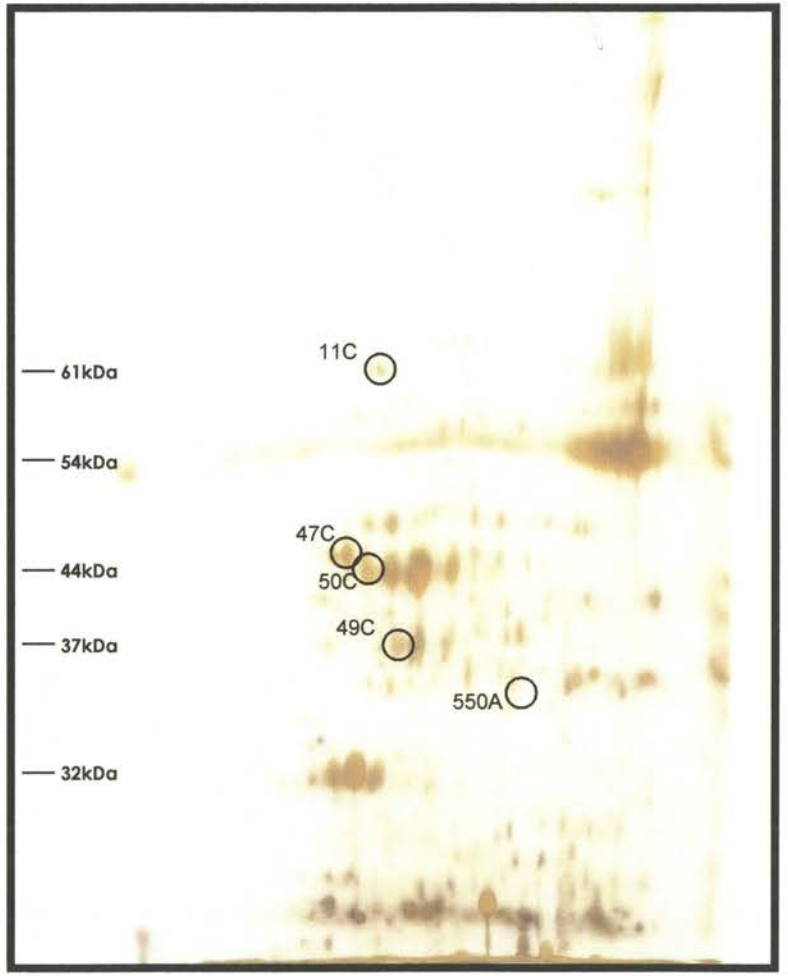


Figure 68. Resistant wheat leaves fed on by BCOs for 24h; 4-7pH 14% SDS.

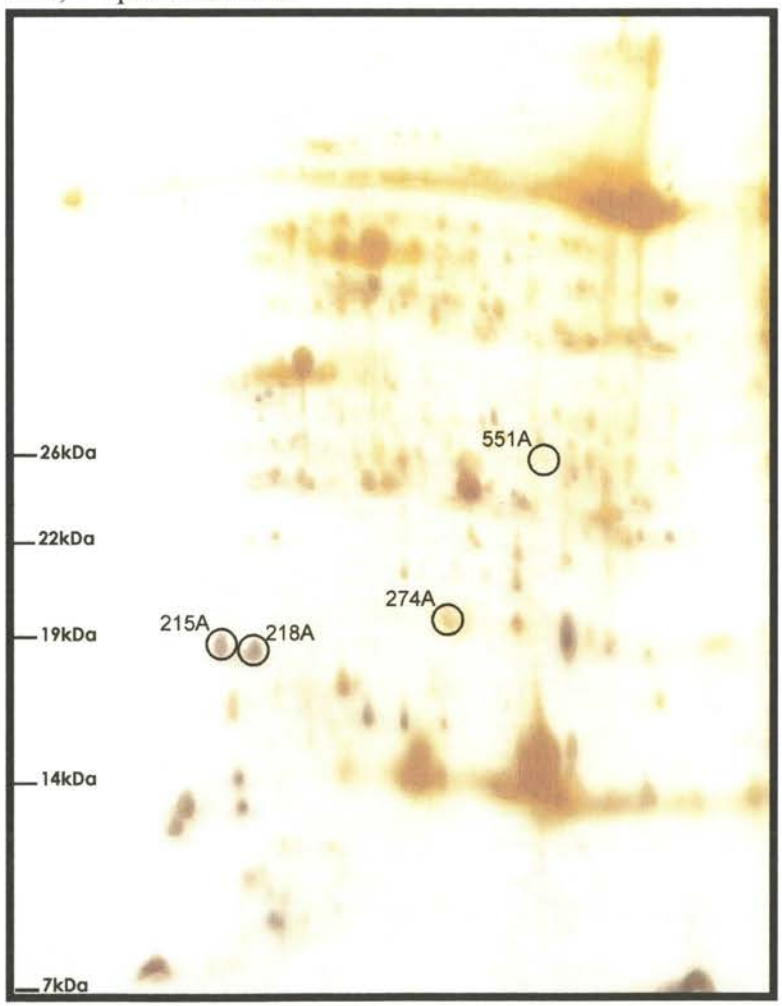


Figure 69. Control for resistant wheat leaves fed on by BCOs for 24h; 4-7pH 14% SDS.

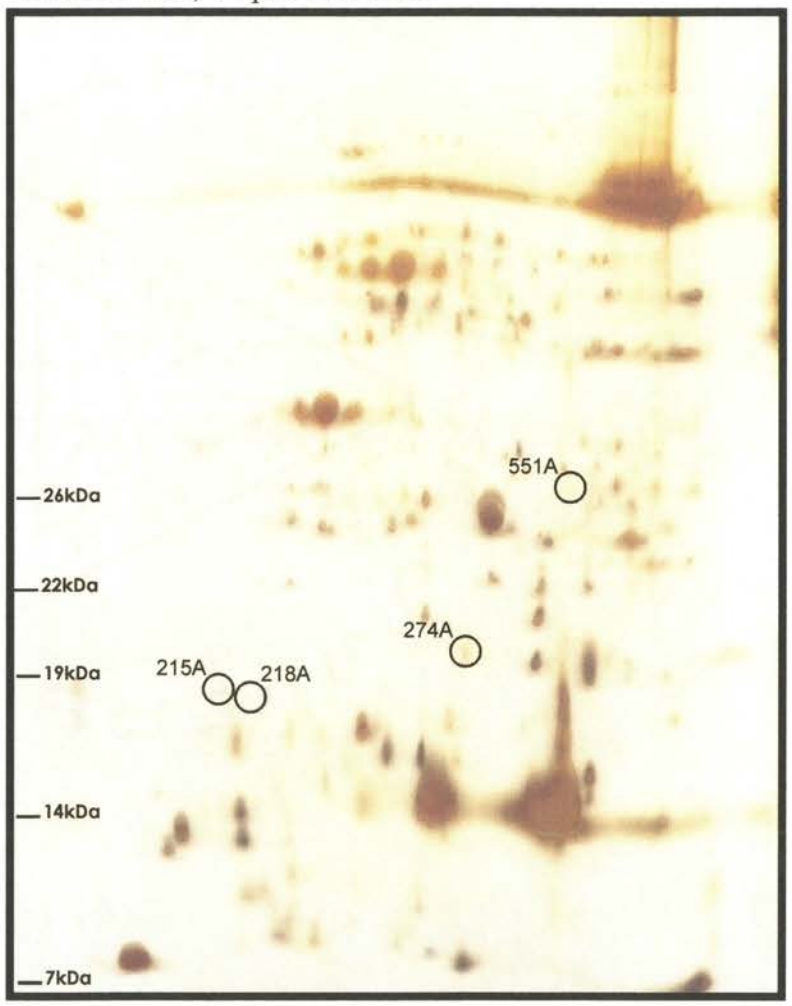


Figure 70. Resistant wheat leaves fed on by BCOs for 24h; 6-11pH 11% SDS.

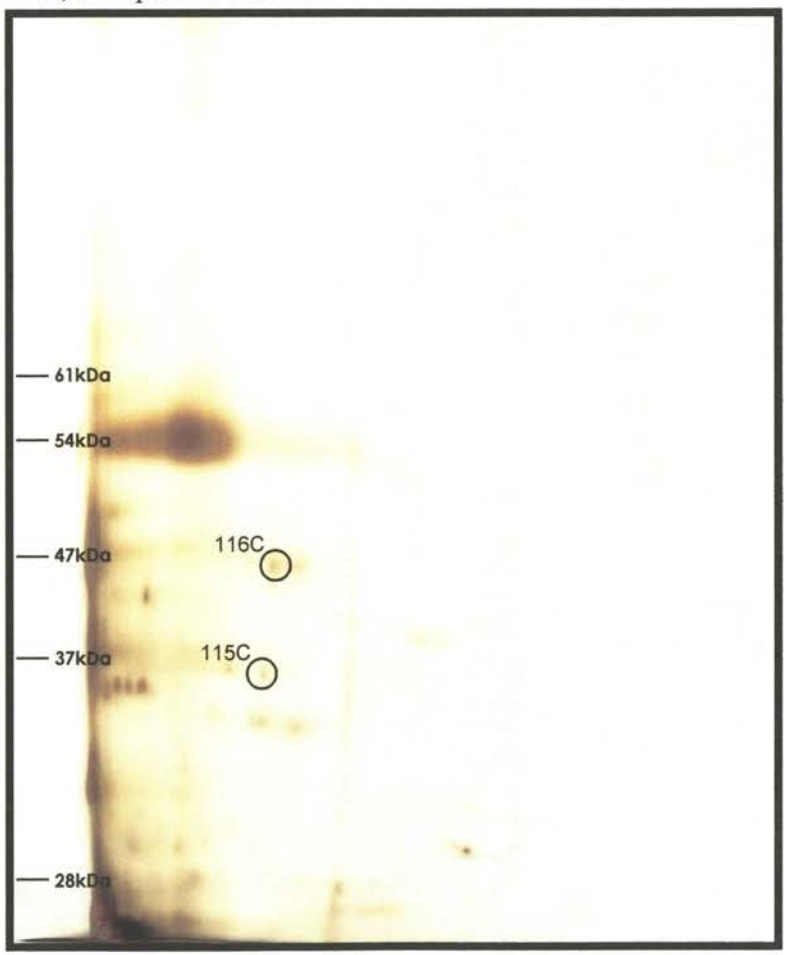


Figure 71. Control for resistant wheat leaves fed on by BCOs for 24h; 6-11pH 11% SDS.

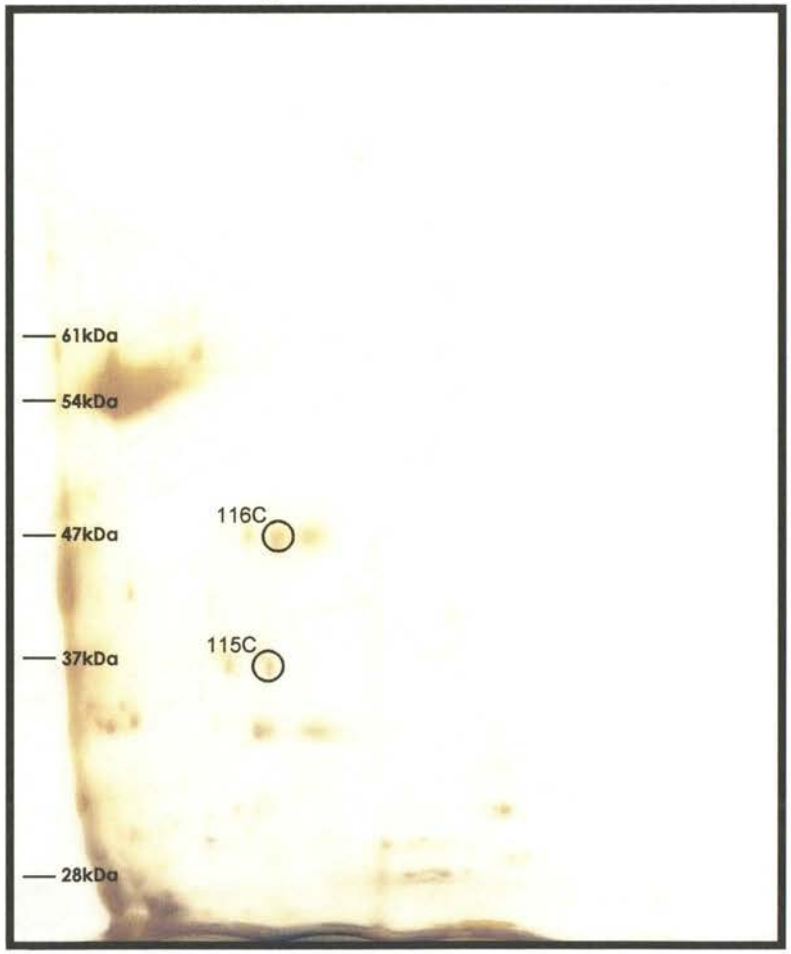


Figure 72. Resistant wheat leaves fed on by BCOs for 24h; 6-11pH 14% SDS.

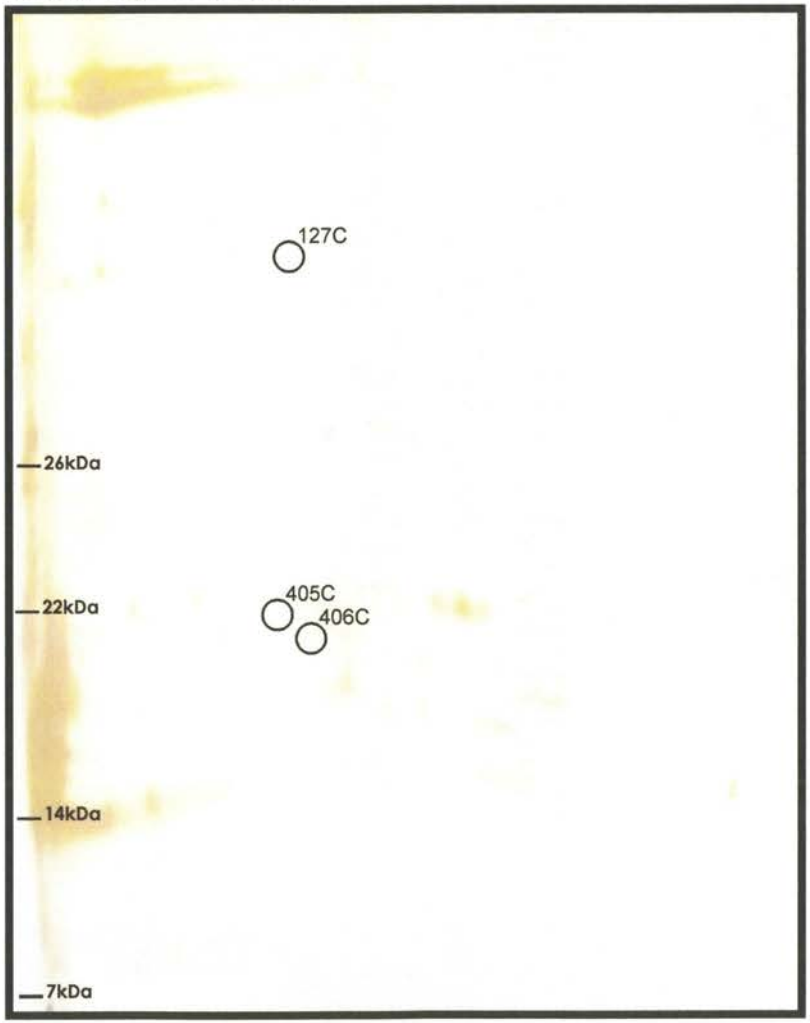


Figure 73. Control for resistant wheat leaves fed on by BCOs for 24h; 6-11pH 14% SDS.

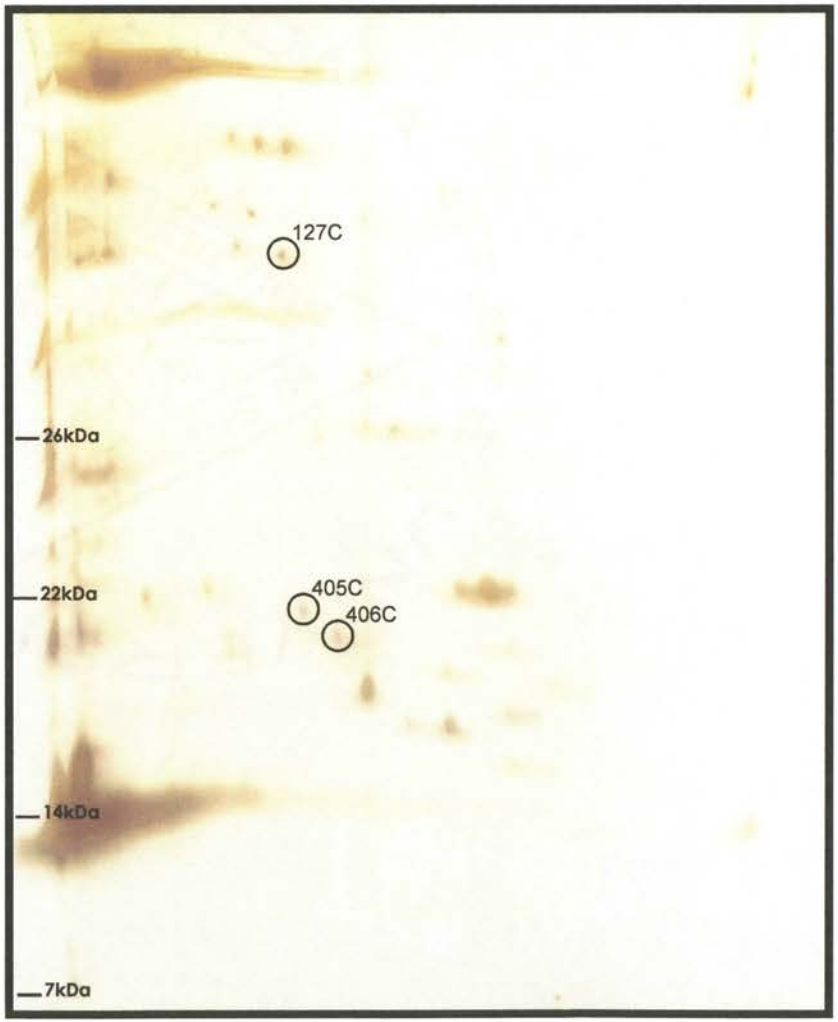


Figure 74. Resistant wheat leaves fed on by BCOs for 6d; 4-7pH 11% SDS.

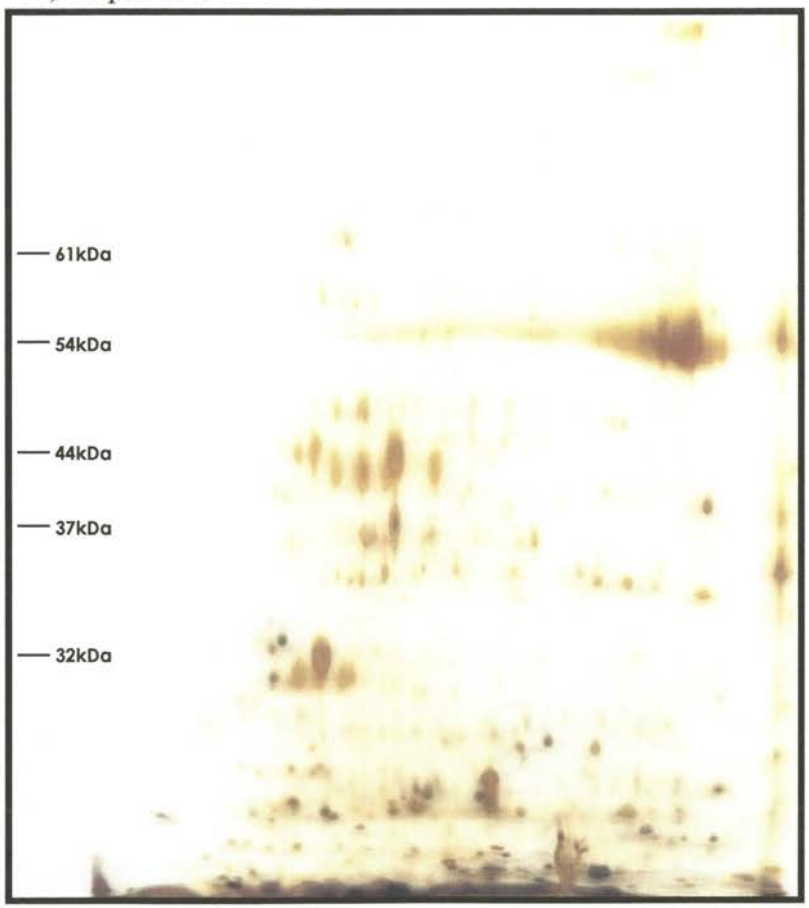


Figure 75. Control for resistant wheat leaves fed on by BCOs for 6d; 4-7pH 11% SDS.

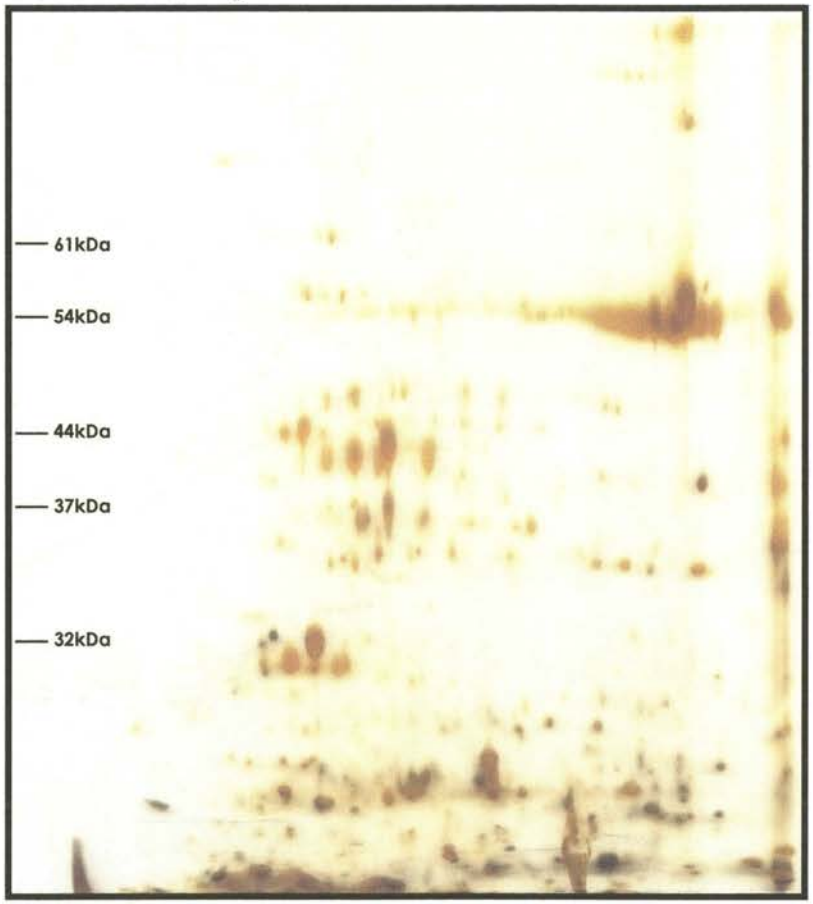


Figure 76. Resistant wheat leaves fed on by BCOs for 6d; 4-7pH 14% SDS.

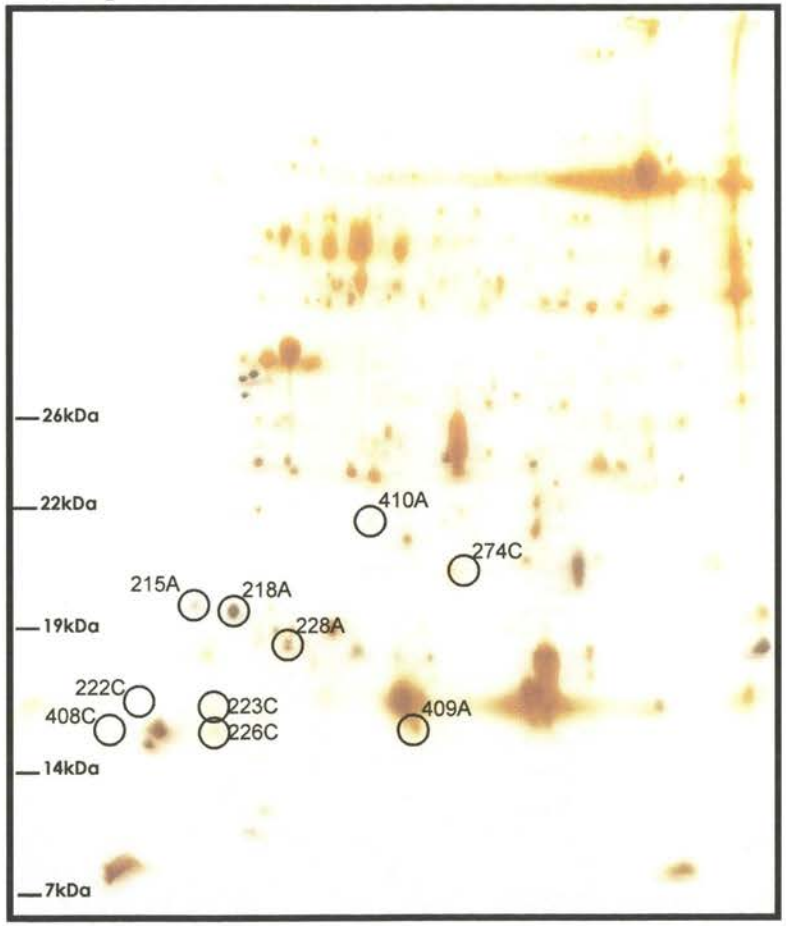


Figure 77. Control for resistant wheat leaves fed on by BCOs for 6d; 4-7pH 14% SDS.

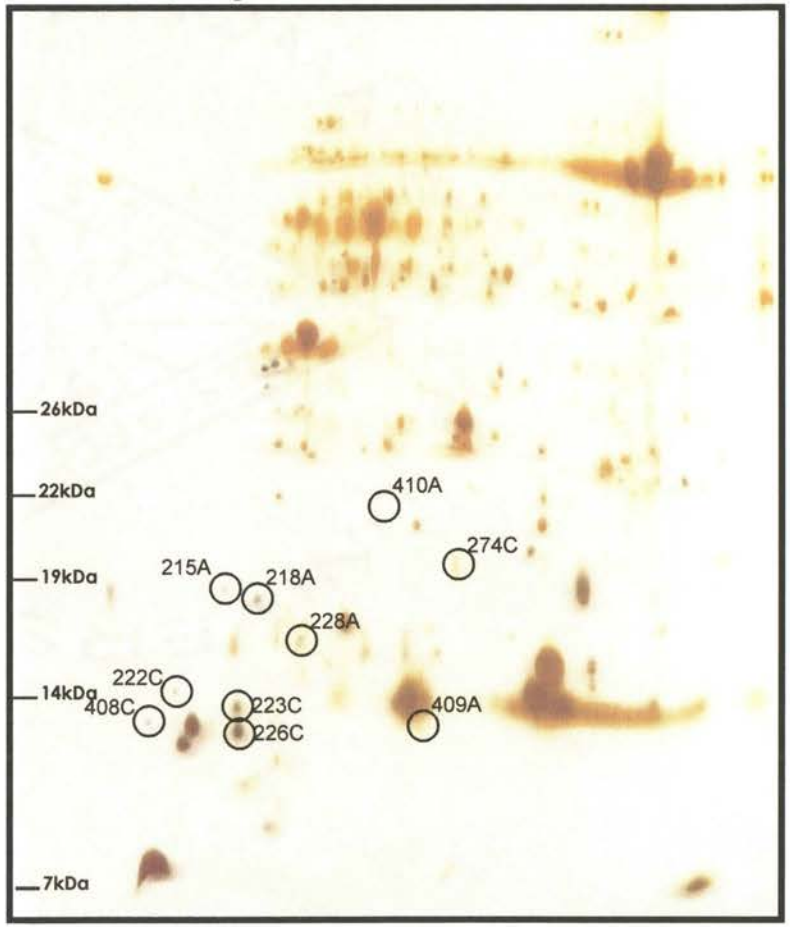


Figure 80. Resistant wheat leaves fed on by BCOs for 6d; 6-11pH 14% SDS.

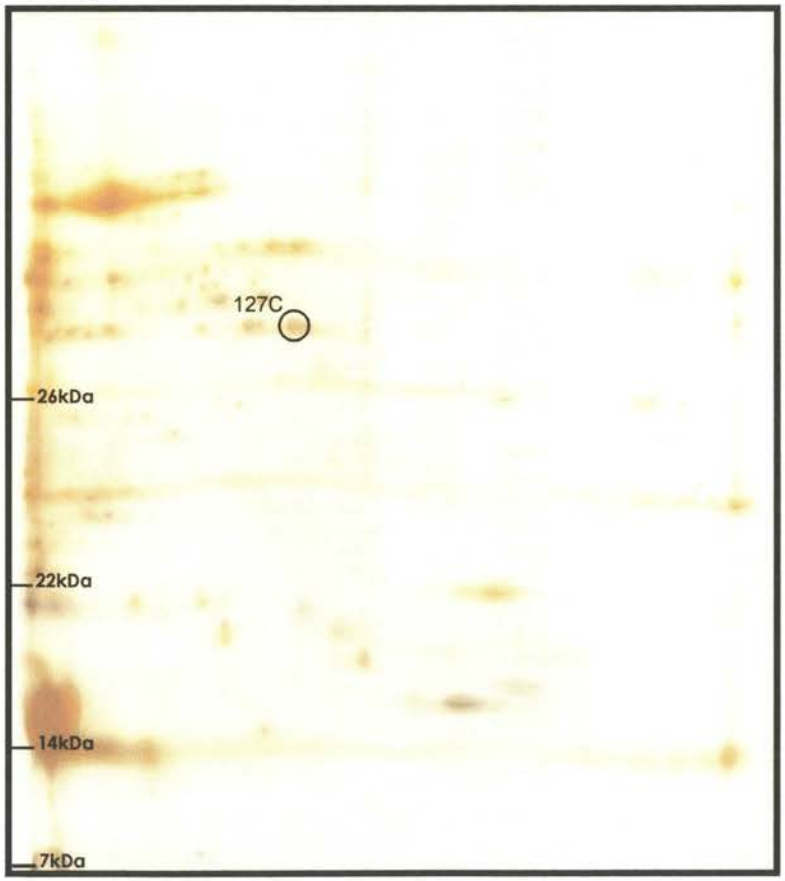


Figure 81. Control for resistant wheat leaves fed on by BCOs for 6d; 6-11pH 14% SDS.

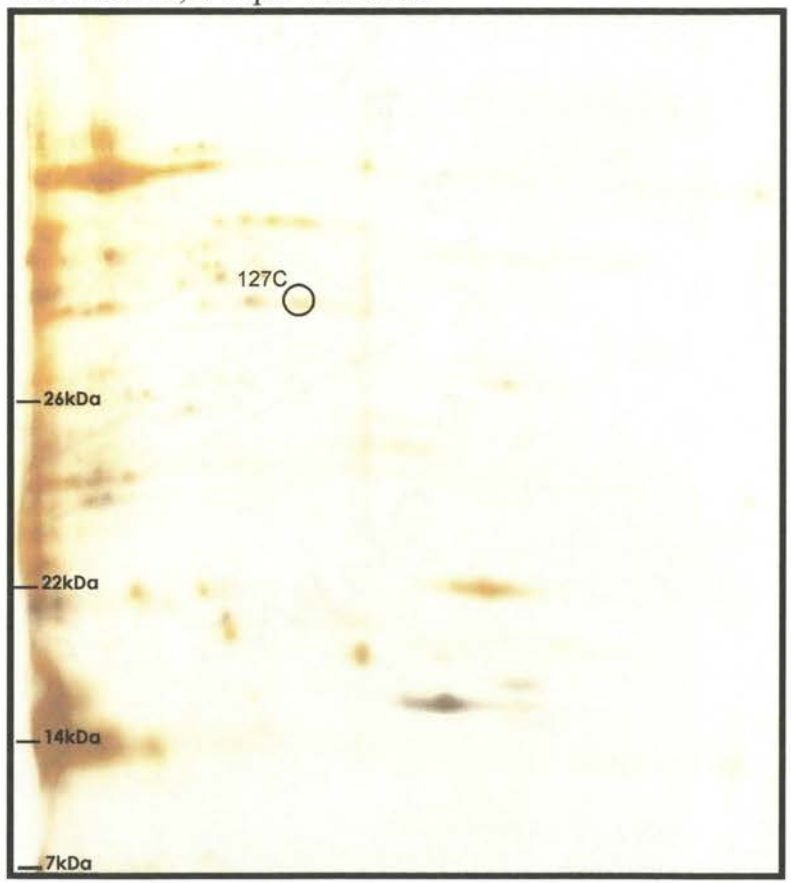


Figure 78. Resistant wheat leaves fed on by BCOs for 6d; 6-11pH 11% SDS.

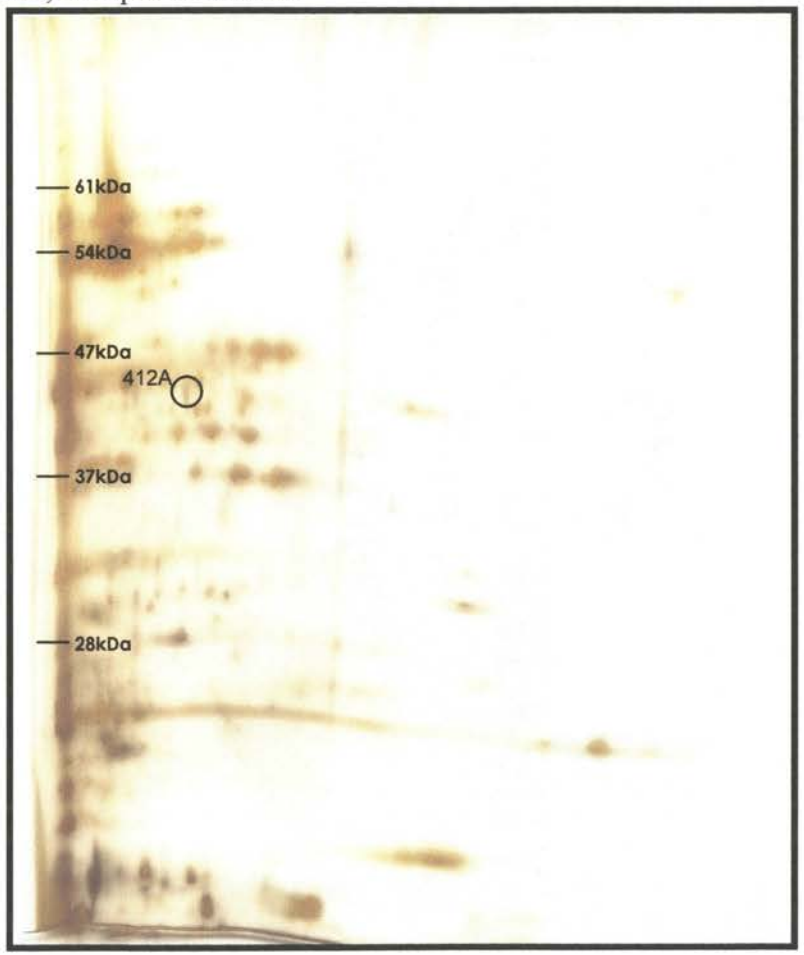


Figure 79. Control for resistant wheat leaves fed on by BCOs for 6d; 6-11pH 11% SDS.

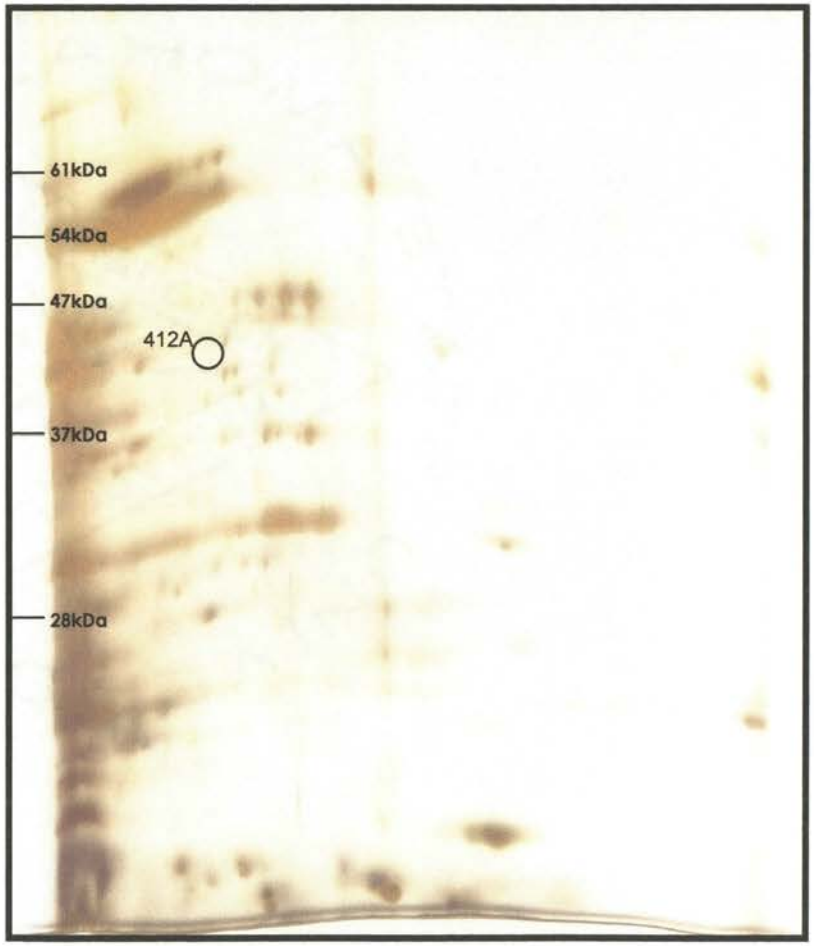


Table 6. Protein expression patterns for aphid-induced differential expression.

Protein	Greenbug-Induced Expression				BCO-Induced Expression			
	Susceptible		Resistant		Susceptible		Resistant	
	24h	6d	24h	6d	24h	6d	24h	6d
Peptidyl-Prolyl Isomerase	■	x	x	x	x	x	x	x
Reversibly Glycosyl. Polypept.	x	x	■	■	x	x	■	■
Bisphosphoglyc.-Independ. Phosphoglyc. Mutase	●	x	x	x	x	x	x	x
Peroxidase	●	●	x	x	x	x	x	x
Chitinase	●	●	x	x	x	x	x	x
Germin	●	●	x	x	x	x	x	x
Cyclin B	x	x	■	x	x	x	■	x
Protein cdc2 Kinase	■	■	■	■	■	x	■	x
Rubisco Activase B	x	x	■	x	x	x	■	x
ADP-Glucose Pyrophosphorylase	■	■	■	■	x	x	x	x
H+ Transporting ATP Synthase B	●	x	x	x	x	x	x	x
3-Dehydroquinate Dehydratase	■	■	x	x	x	x	■	x
Rubisco Activase	x	x	■	x	x	x	■	x
Glucan Synthase	■	■	x	x	x	x	x	x
RAS-Related Protein (RAB7)	x	x	■	x	x	x	x	x
NADP-Spec. Isocitrate Dehydrog.	■	■	x	x	■	x	x	x
Sucrose:Fructan Fructosyltransfer.	■	x	x	x	x	x	x	x
High-Affinity Phosph. Transporter	●	x	●	x	x	x	x	x
wpk4 Protein Kinase	x	■	x	■	x	x	x	x
Calmodulin-Related Protein	x	●	x	●	x	x	x	x
Aldehyde Dehydrogenase	●	x	●	x	x	x	x	x
Alternative Oxidase	x	x	x	x	x	x	●	x
HR-Induced Protein	x	x	x	x	●	x	x	x
Malate Dehydrogenase	x	x	x	x	x	x	■	●
Cytochrome P450 CYP86-TA	x	x	x	x	●	●	●	●
Ribosomal Protein L12	x	x	x	x	●	●	●	●
Glycine Decarboxylase	x	x	x	x	■	x	x	x
Protein Kinase (274)	●	x	●	■	●	x	●	■
MAPK (Kinase)	x	x	x	x	x	x	x	●

■ = **Downregulated in response to aphid feeding**
● = **Upregulated in response to aphid feeding**
X = **Unchanged**

Figure 82. Biosynthesis of chorismate and its byproducts via the Shikimate or prechorismate pathway.

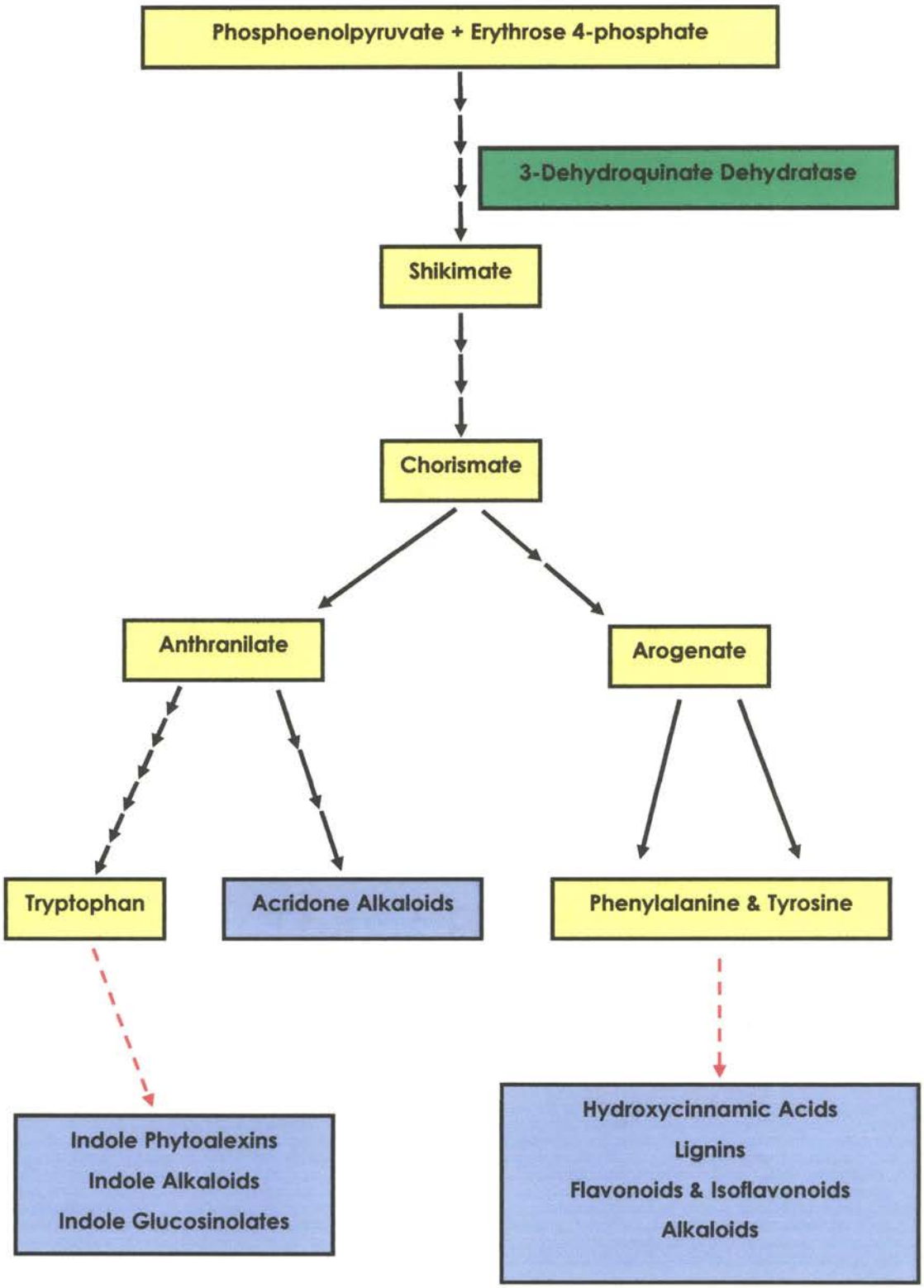


Figure 83. Starch biosynthetic pathway.

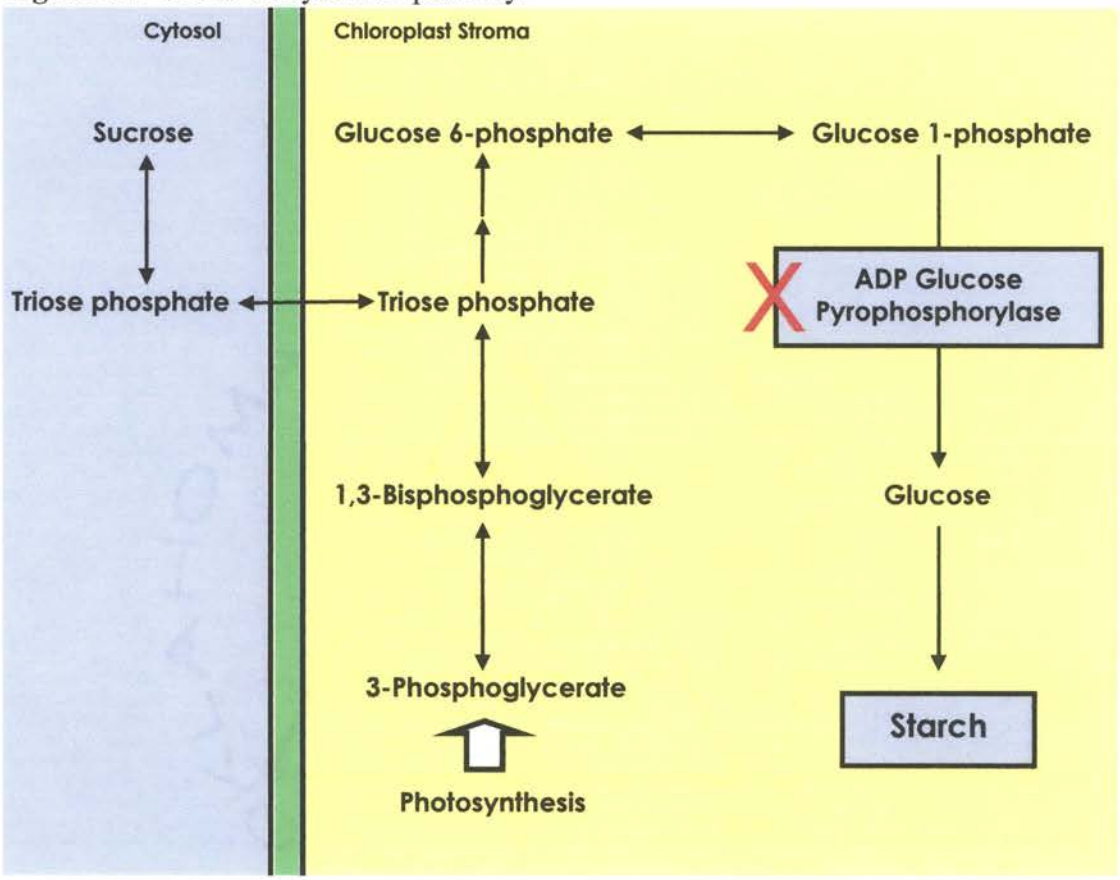


Figure 84. Rotofor fractions and pH values for fractions containing Rubisco (15µl loaded per well; 10.9mg of total protein separated into 20 fractions).

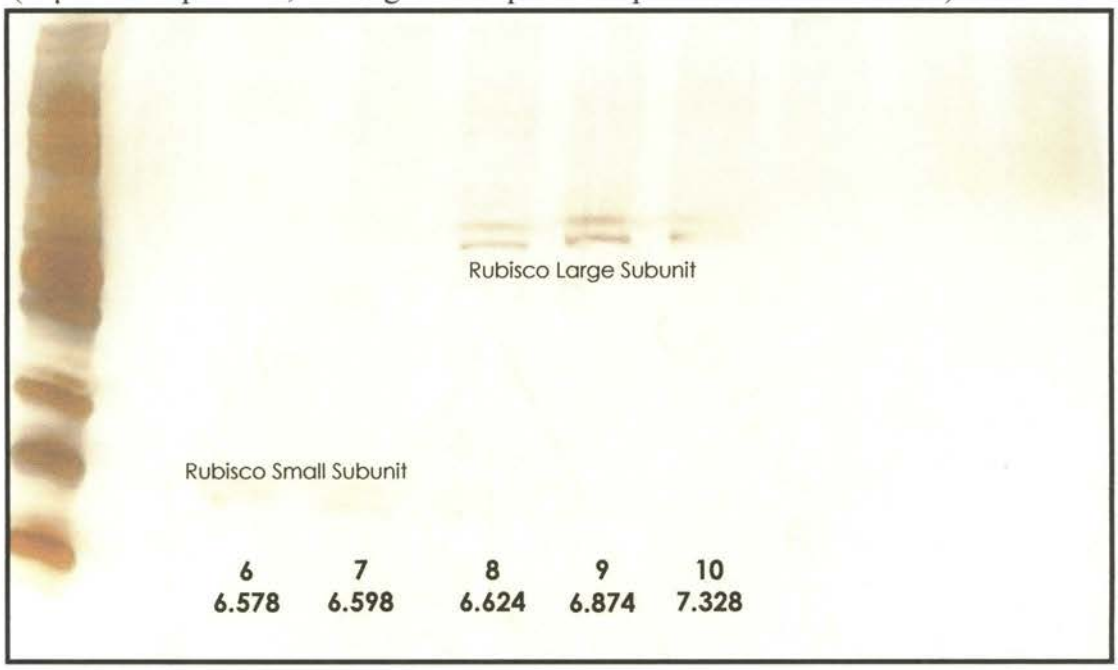


Figure 85. Rotofor fractions separated into two groups, 4-7pH range fractions and 6-11pH range fractions, 15µl of each pooled group was loaded.

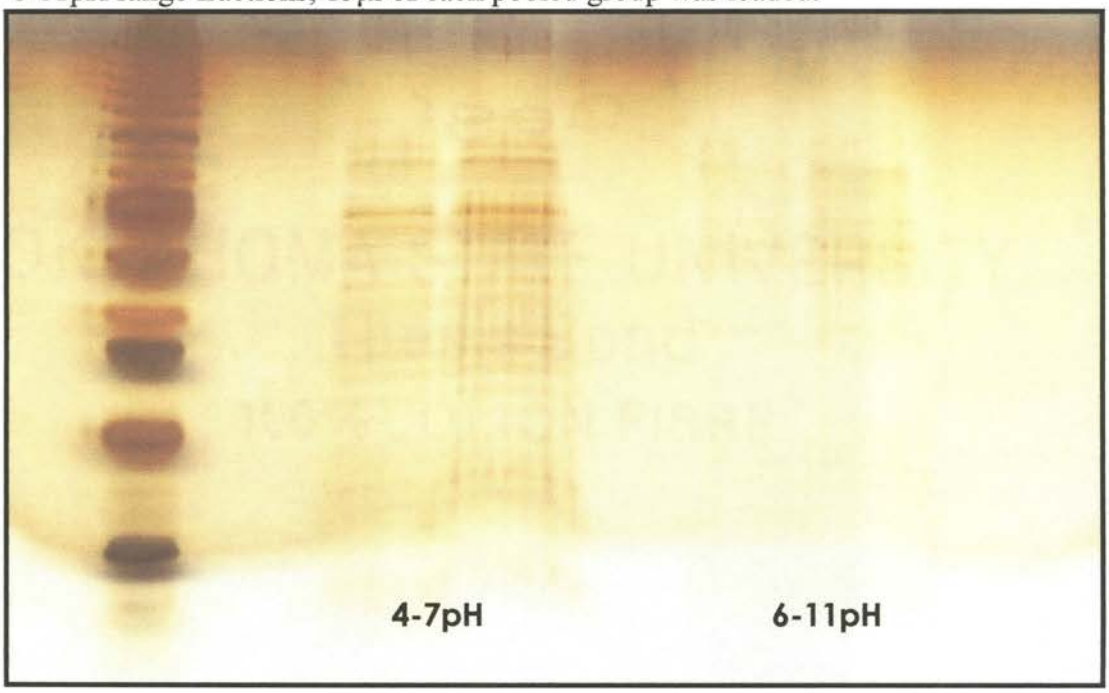


Figure 86. Rotofor fractions 4-11 on an 8-16% SDS gel with 15µl of each fraction per well (21.8mg total protein separated into 20 fractions).

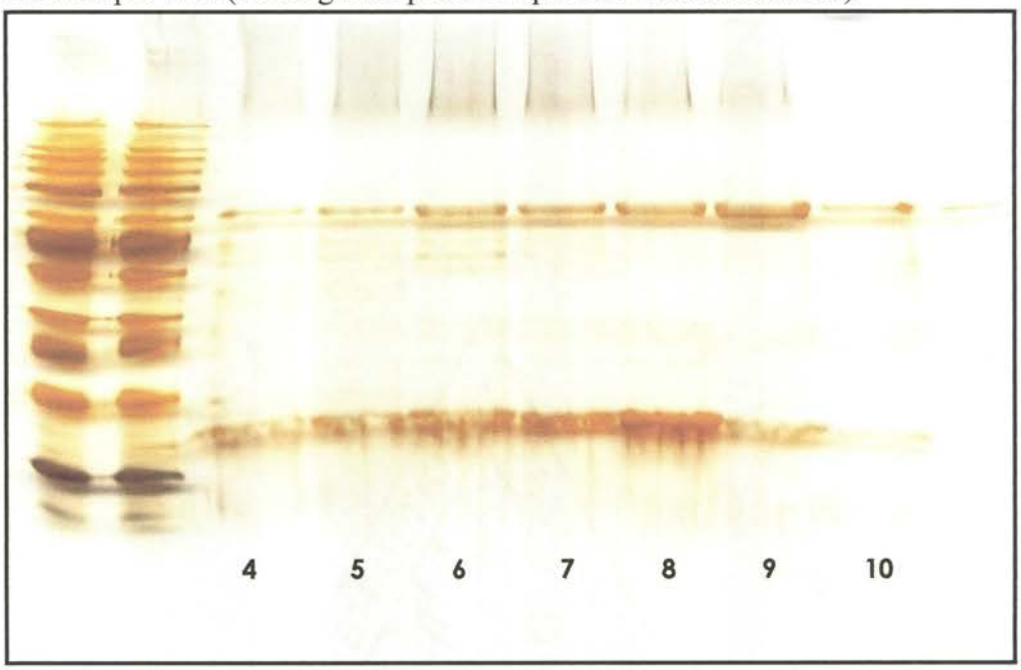


Figure 87. Rotofor fractions 11-19 on an 8-16% SDS gel with 15µl of each fraction per well (21.8mg total protein separated into 20 fractions).

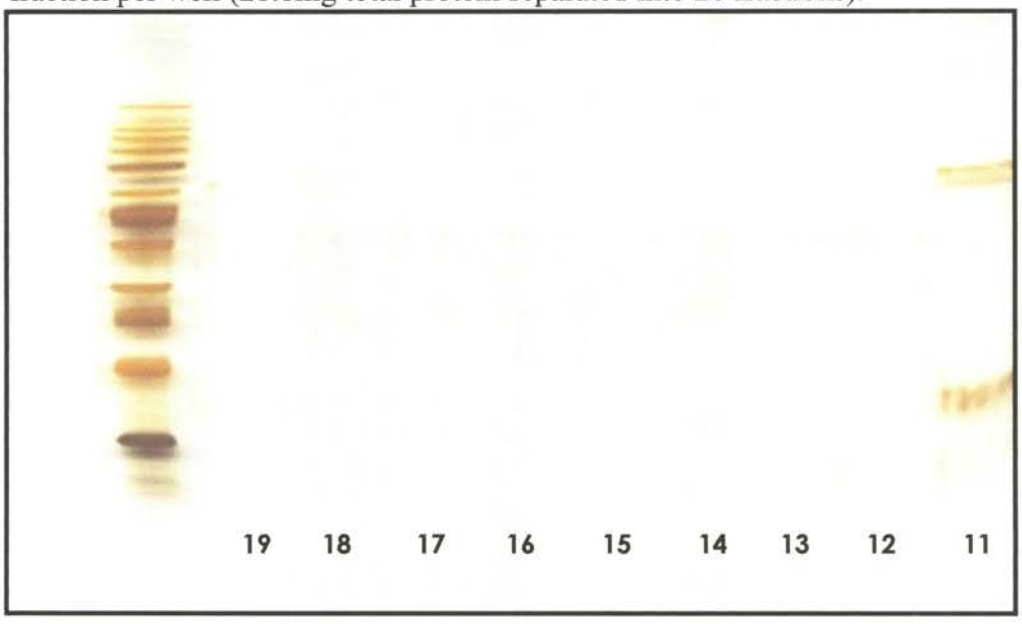


Figure 88. Rotofor fractions 5-11 on an 8-16% SDS gel with 15µl of each fraction per well (43.6mg total protein separated into 20 fractions).

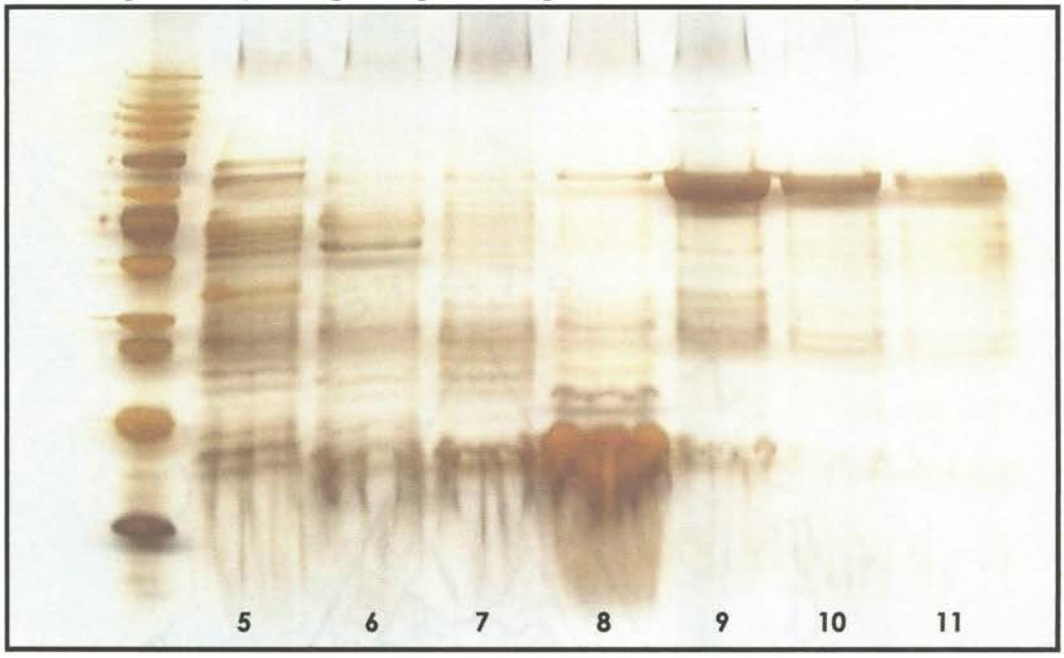


Figure 89. Rotofor fractions 12-18 on an 8-16% SDS gel with 15µl of each fraction per well (43.6mg total protein separated into 20 fractions).

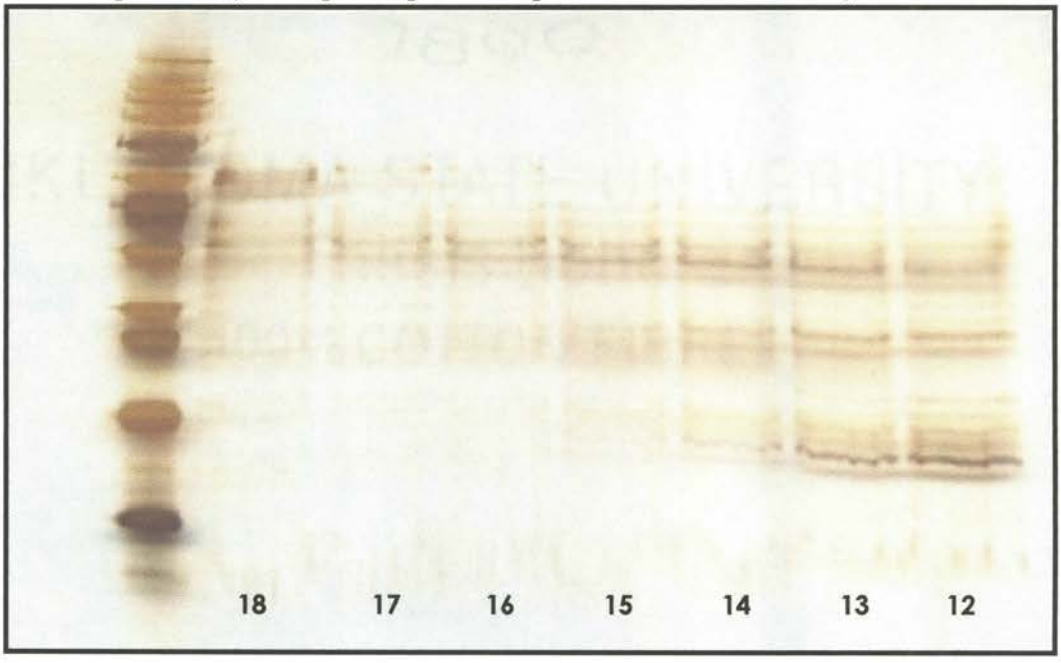


Figure 90. Rotofor fractions concentrated using CentriCon Plus 20.

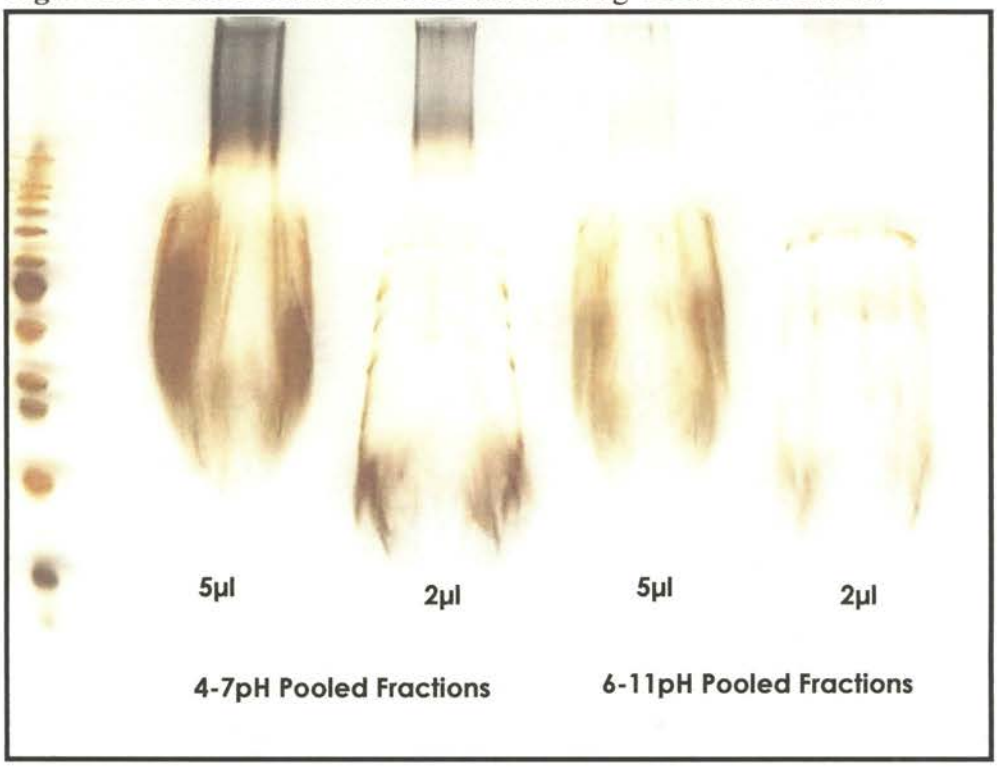


Figure 91. Gel analysis to determine cause of smearing after Rotofor fraction concentration.

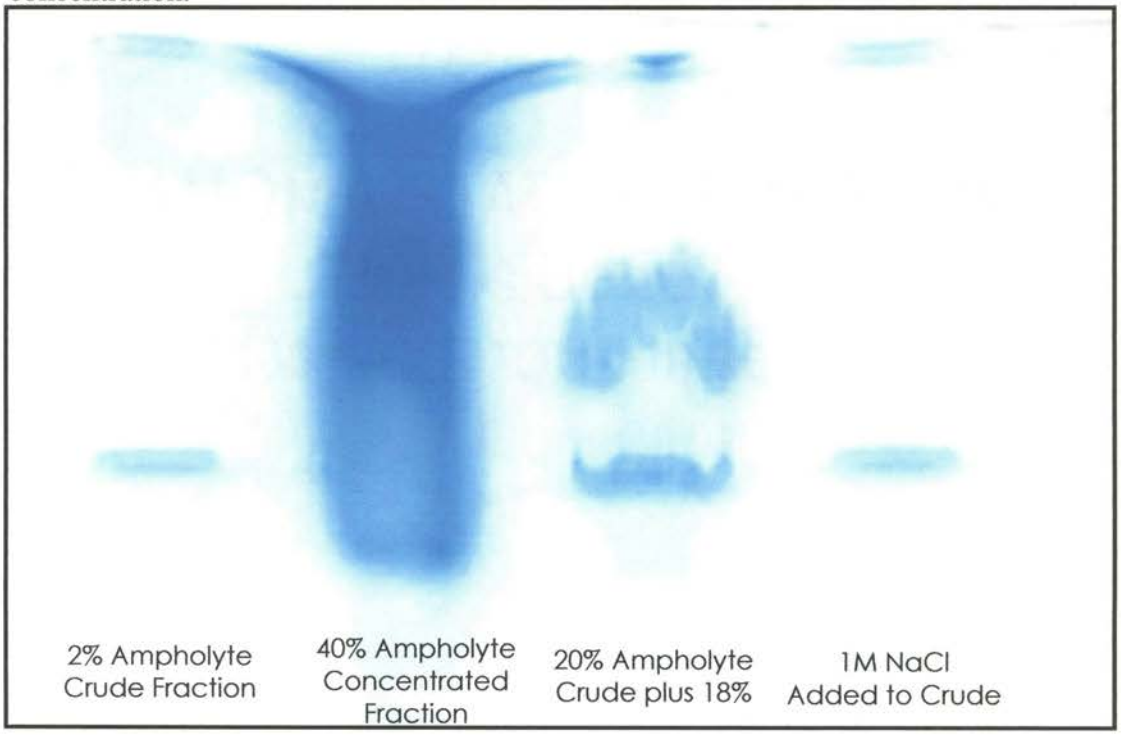


Figure 92. Methanol precipitation of pooled Rotofor fractions to remove ampholyte on an 8-16% SDS gel.

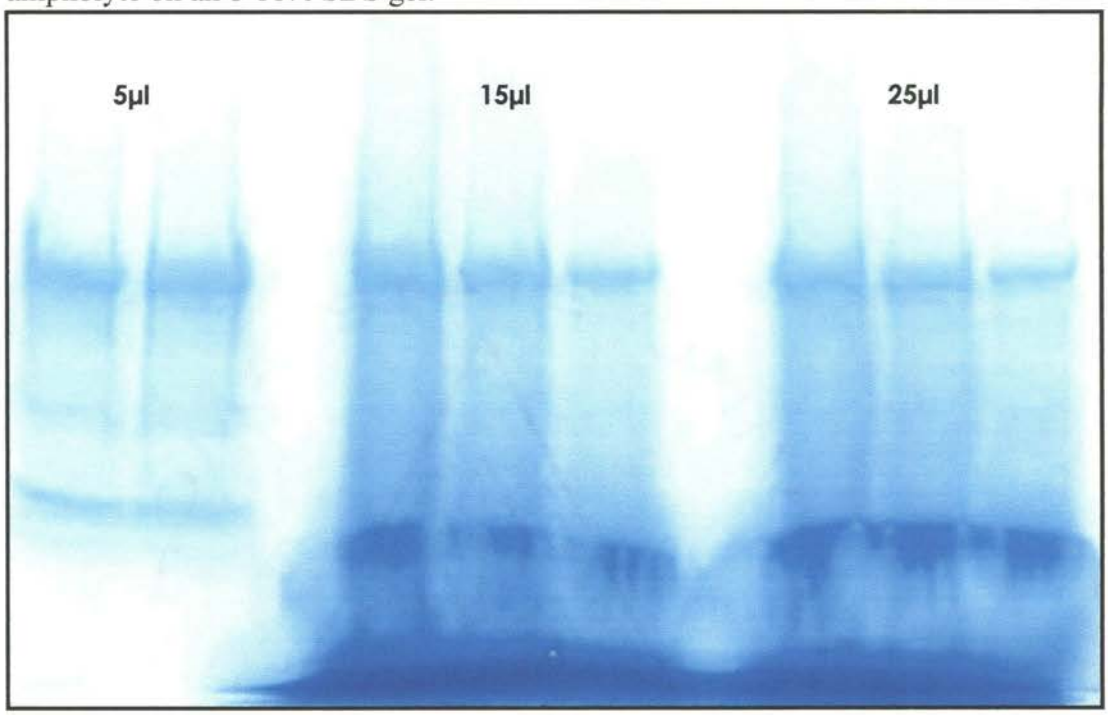


Figure 93. 2-D gel of 4-7pH range pooled Rotofor fractions; 75µl of concentrated solution loaded on IPG strip then run on an 8-16% SDS gel.

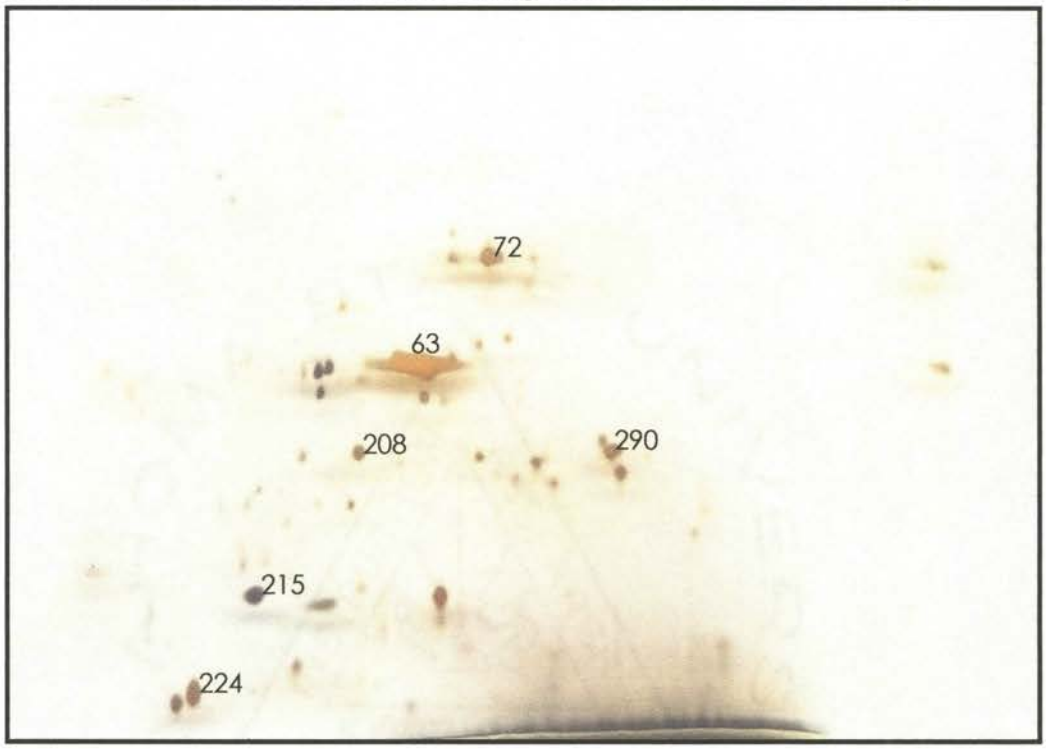


Figure 94. 2-D gel of TCA/acetone extract prior to fractionation; 125µl of concentrated solution loaded on IPG strip then run on an 11% SDS gel.

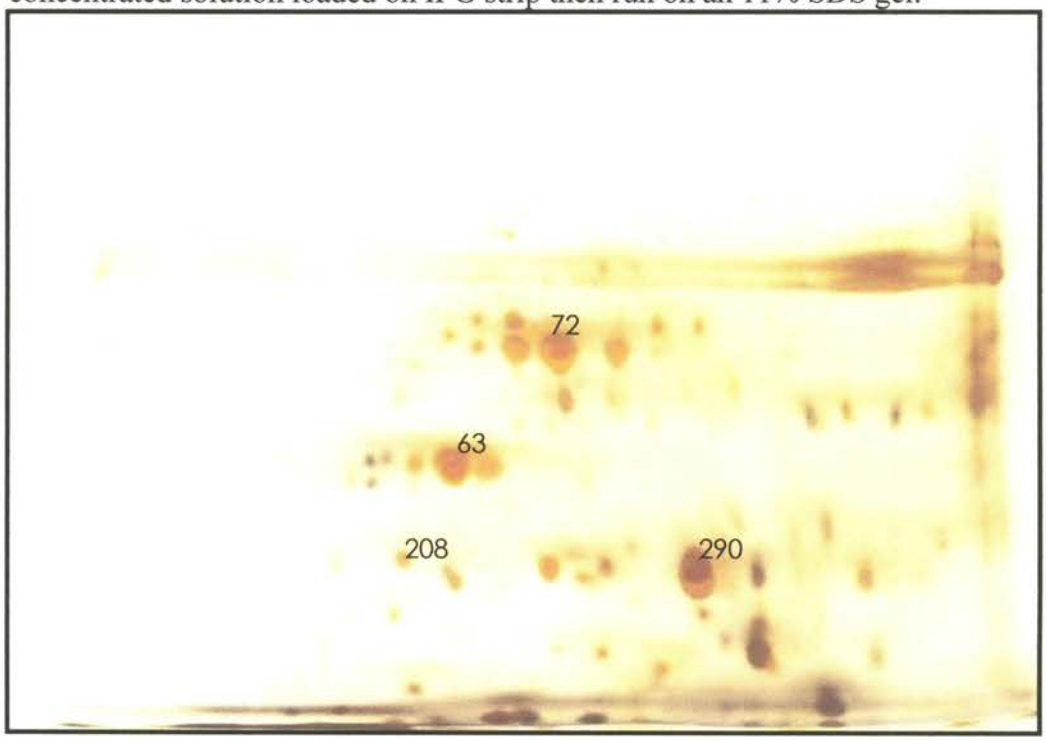


Figure 95. 8-16% SDS gel of wheat, *Medicago truncatula*, and barley leaf extracts (30µl loaded per well).

Figure 96. Western blot of wheat, *Medicago truncatula*, and barley leaf extracts (Figure 95) probed with a barley Rubisco antibody.

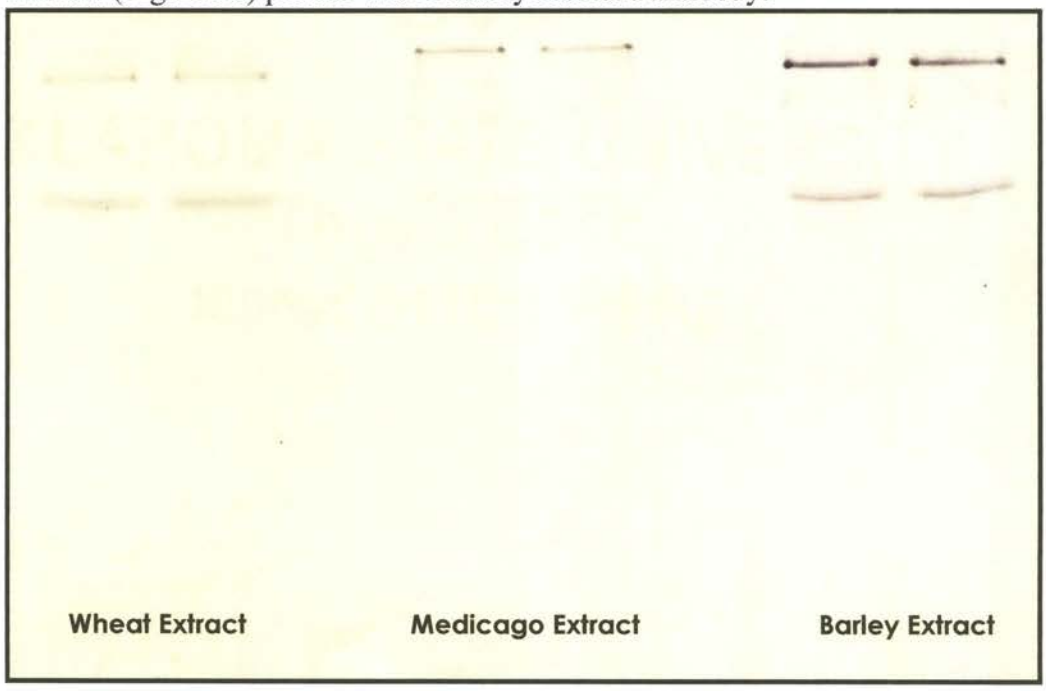


Figure 97. Standard curve for HPLC gel filtration on Superdex 200.

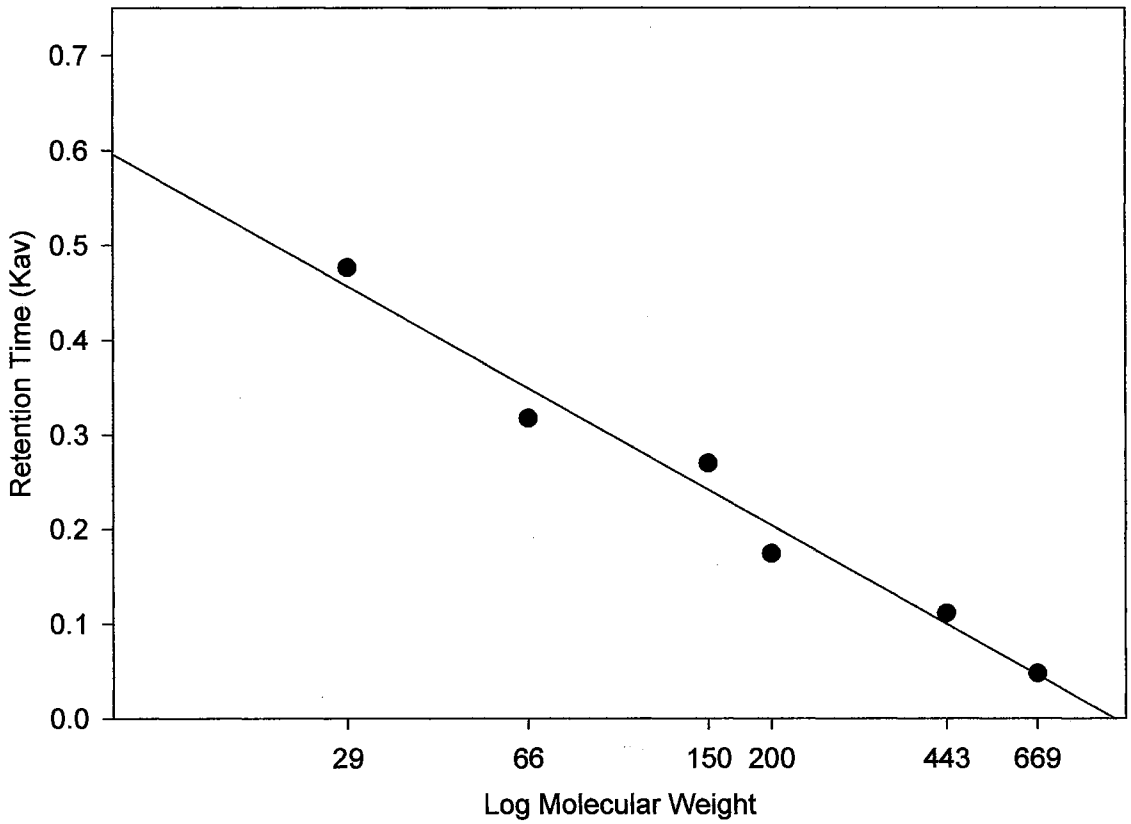


Figure 98. HPLC chromatogram of native Tris extraction of wheat leaves separated on a Superdex 200 gel filtration column at an absorbance of 280nm.

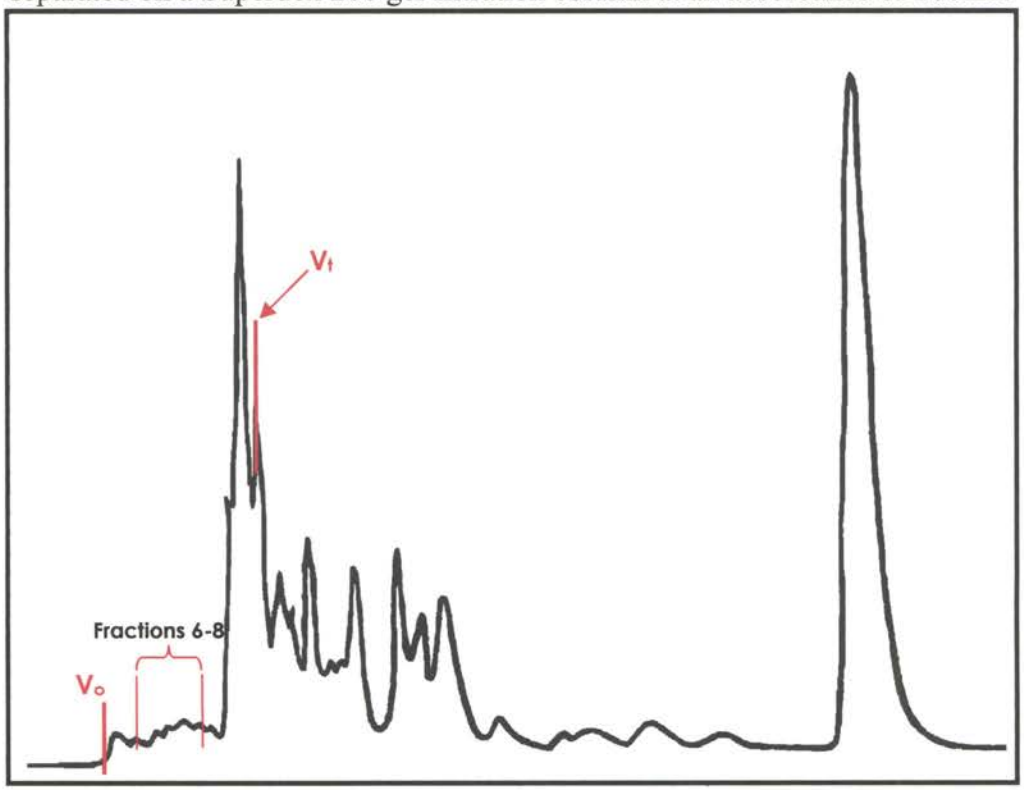
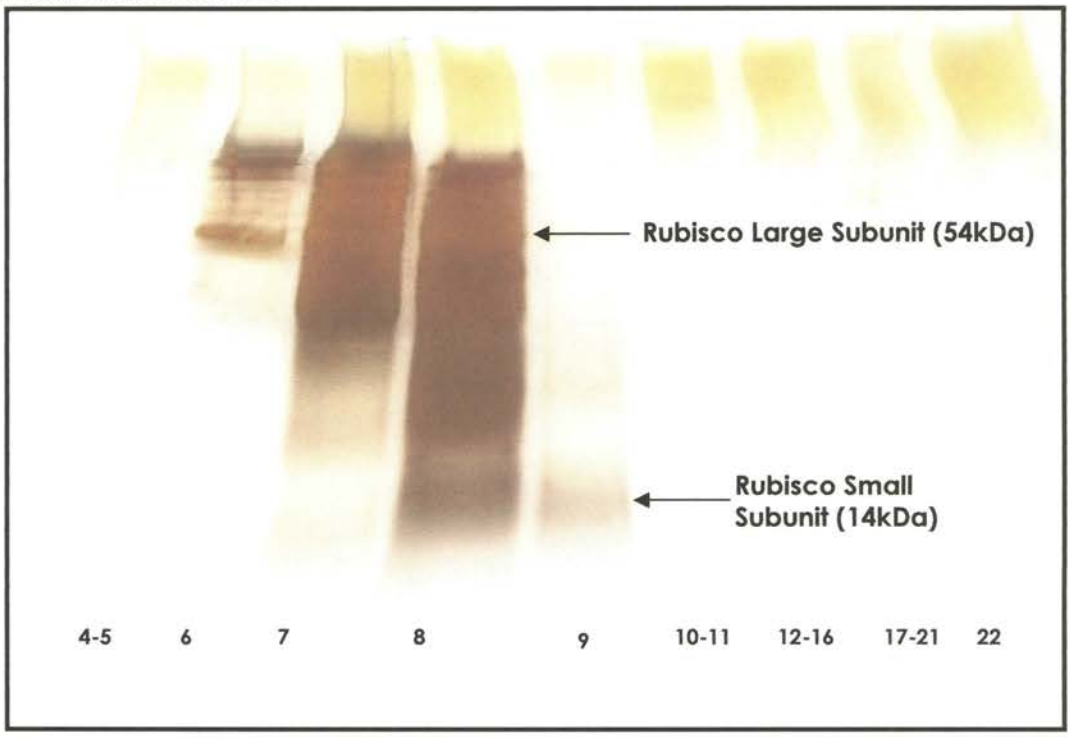


Figure 99. HPLC fractions 4-22 (8min or 2ml fractions) with Rubisco present in fractions 6-9.



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