THE EFFECTS OF ALUMINUM TOXICITY ON GROWTH AND THE COMPLEMENT OF POLYPEPTIDES IN THE ROOT TIPS OF WHEAT

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ABSTRACT

Aluminum toxicity is the primary limiting growth factor in acid soils. Wheat (Triticum aestivum L.) expresses differential tolerance to aluminum (Al). The wheat cultivar "Victory" is Al sensitive at 1 ug/ml Al, while the wheat cultivar "TAM W-101" is tolerant at 2 ug/ml Al.

Aniol (4) proposed that a group of proteins present only in Al tolerance plants bind with Al to render it harmless to plant cells. This hypothesis was tested by determining if induction of specific proteins was associated with 2 ug/ml Al treatment in Victory and TAM W-101 root tips. The proteins extracted from each cultivar were quantified and analyzed by SDS-PAGE. The effect of Al on the proteins of wheat root tips was not dramatic. SDS-PAGE did not reveal the presence of any new, major proteins which might be Al-binding proteins. Two-dimensional PAGE analysis of the proteins of Victory cytoplasmic and microsomal fractions was conducted. This highly sensitive protein analytic tool revealed eight major proteins in Al-treated Victory root tips that were not present in the controls, and three proteins that were present in the control and not in the Al-treated root tips.

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DETERMINATION OF AL-TOLERANCE OR INTOLERANCE IN TWO CULTIVARS OF WHEAT USING ROOT REGROWTH AS AN INDICATOR

Introduction

The Importance of Wheat

Cereals are a critical part of the human diet. In the United States cereals compose 25% of the food consumed. In Europe the influence of cereals increases to 50% of the diet, and in Asia, cereals (predominantly rice) make up 80% of the average human diet (16). Of the cereal crops, wheat is the most widely cultivated; wheat is grown in all the temperate and most of the sub-tropical countries of the world (49).

Soil Acidity

Soil acidity (water pH below 5.5) is a global problem; it is estimated that 70% of Earth's land that is potentially usable for food and biomass production is acidic. Forty percent of the arable soils of the world are acidic. Most of the acid soils are in those regions of the world where

grain is most important in the diet (the sub-tropical and tropical regions) (67).

Acid soils result from acid parent materials that are low in basic cations [calcium (Ca^{2+}), magnesium (Mg^{2+}), potassium (K^{+}), and sodium (Na^{+})]. Soils may become acidic through removal of these basic cations by leaching or crop harvesting, or the addition of acidifying agents such as nitrogenous fertilizers, or acid deposition from a polluted atmosphere (43, 68). The toxicity of acid soils is the result of a complex of factors. Soil pH, the type and amount of clay, type and amount of organic matter, levels of various salts, and plant species or genotypes must all be considered (18).

The primary growth-limiting factor in acid soils is aluminum (Al^{3+}) toxicity (30). Other growth-limiting factors identified in acid soils include H^{+} toxicity, reduced availability of Ca^{2+}, Mg^{2+}, phosphorous (P), molybdenum (Mo^{+}), and other essential elements, and manganese (Mn^{2+}) toxicity (13, 27).

Hydrogen Toxicity

Hydrogen toxicity generally occurs at pH below 4.0. Symptoms of hydrogen toxicity often resemble Al^{3+} toxicity, especially the shortening and thickening of root tips, a decrease in total root numbers, and a dull gray or brown discoloration. Hydrogen ions in toxic concentrations cause

a loss of cellular membrane integrity, increasing membrane permeability (27). Roots subjected to prolonged exposure to H^+ lose the ability to take up nutrients by passive uptake, and lose organic substances as well as K^+, Ca^{2+}, and P (17,41). Cationic transport is disrupted by H^+ competition with binding sites. These membrane effects can manifest themselves as increased requirements for Ca^{2+}, Mg^{2+}, Mn^{2+}, and copper (Cu^{2+}) (51, 54).

Manganese Toxicity

Manganese toxicity is second only to Al^{3+} toxicity in terms of limiting plant growth in acid soils (59). In most soils, Mn^{2+} reaches toxic concentrations by becoming more available in the soil solution at pH <5.5 (27).

Plant symptoms of Mn^{2+} toxicity appear in the upper parts of the plant (25). Major symptoms include marginal chlorosis and necrosis of leaves (alfalfa, lettuce), leaf puckering (snapbean, soybean, cotton), and chlorosis of young leaves (barley, lettuce, soybean) (27).

Manganese in toxic amounts appears to alter the activities of enzymes and hormones of plants (78). Some specific effects of Mn^{2+} toxicity on plants include an increase in IAA oxidase activity, an amino acid imbalance, increased potato spindle virus RNA replication, increased peroxidase and polyphenol oxidase activity, decreased activity of catalase and other enzymes, decreased ATP

contents and reduced respiration rates, and reversal of gibberellic acid inhibition on root growth (25, 30).

Aluminum Toxicity

The Earth's crust is 7.5% aluminum (Al) by weight (34, 35), making it the most abundant metal on this planet. Aluminum, a densely charged trivalent cation, is much too reactive to exist free in nature. Aluminum can exist in many different forms, though it occurs primarily as aluminosilicates.

Aluminum recently has been associated with human diseases. In 1976, Al intoxication was found to be responsible for dialysis encephalopathy, a fatal neurological syndrome (1). Aluminum has also been demonstrated to play a role in the pathogenesis of osteomalacia related to total parenteral nutrition of kidney dialysis patients, and has recently been implicated as a potential cause of Alzheimer's Disease (52).

Aluminum becomes toxic to plants when it is mobilized in the soil solution and becomes available for uptake by the plant. Aluminum mobilization and availability are dependent on the metal species present and on the distribution of the metal at the solid-liquid interface of the soil constituents (34). Foy (29) reported Al toxicity at a pH as high as 5.5 in kaolinitic soils. However, both Haug (35) and Foy (29) concur that pH 5.0 is the threshold below which Al^{3+} toxicity generally occurs.

At pH less than 5.0, clay minerals (which are aluminosilicates) become less stable. Al that is normally associated with OH^- in the alumina-octahedral clay structure moves to the exchange positions on the clay structures (29). This mobile species of Al is in the form of $Al(H_2O)_6^{3+}$ and occurs at concentrations between 1 and 30 ppm. However, Haug (34) has suggested that Al concentrations rarely exceeds 4 ppm (about 140 uM) in soil solution.

General Effects of Al on Plants

Though our understanding of the mechanisms of Al toxicity in plants has increased considerably since Hartwell and Pember (33) first identified Al as a toxic element in acid soils in 1918, the effects of Al on plants are not always easy to identify. Al toxicity symptoms often resemble P deficiency symptoms, that is, overall stunting of the plant, small, dark green leaves, late maturity, purple stems, leaves and leaf veins, and chlorosis and necrosis of leaf tips. In other plants Al^{3+} toxicity resembles Ca^{2+} deficiency (curled leaves, collapse of the growing point or petioles).

Al injury to plant roots is more uniform from species to species. Al-injured roots tend to be stubby and brittle, with many small, swollen lateral roots with little or no fine branching. The root systems of younger plants are more susceptible to injury than are those of older plants (30).

In general, Al toxicity in plant roots results in a rapid inhibition of root growth (16).

Al Tolerance in Plants

Plants exhibit a wide range of tolerances to Al. Among the cereals, and even within a single cereal species, the genetic variability relating to Al^{3+} tolerance is very high (26). It is therefore necessary to determine types and levels of response that indicate plant tolerance verses intolerance to Al.

The common indicators used to determine Al tolerance have been wheat top and root dry weights (14, 28, 31), root development (45), and wheat root growth (2, 3, 4, 65). Thomsett and Thurman (80) have suggested that root growth is a less accurate indicator of plant Al tolerance because genetic variability within and among cultivars will mask a toxic effect. The ability of the wheat root to reinitiate growth after a pulse exposure to Al is attractive as an indicator of Al tolerance because this plant growth response is quantal (reacts non-reversibly at some threshold level to a stimulus), whereas indicators such as root dry weights, development and growth are non-quantal (2, 4, 24, 61).

The objective of this present work was to measure root growth after pulse-exposure to Al in order to identify an Al-tolerant and Al-intolerant cultivar of wheat for use in investigating the mechanisms of Al toxicity. A system for

growing, treating, and extracting large numbers of plants was developed, since future experimentation into the effects of Al on wheat root protiens would require the generation of a large amount of wheat root tissue. Two winter wheat varieties, "Victory" and "TAM W-101," were tested at two concentrations of Al (40 and 80 uM). The Al concentrations tested are consistent with the levels of Al one would find in nature, (35).

Materials and Methods

Seed Germination

Approximately 500 seeds of wheat cultivars Victory and TAM W-101 were placed, 50 each, in 10 cm petri dishes on top of five to seven sheets of Whatman #4 filter paper, with one sheet of Whatman #10 filter paper on top. The filter paper was saturated with distilled water. The Whatman #4 paper retained the moisture necessary for root growth. The Whatman #10 filter paper provided a matrix for the roots to grow on, without allowing the roots to adhere to, or grow in to the paper (which would render the roots more difficult to extract.) Ten sets of germination plates were made for each cultivar. The seeds were placed in a growth chamber and moistened twice daily for 72 hours. The growth chamber was set at 25°C , 16-hour days, and 20°C, eight hour nights. The primary roots were about 1.5 cm long at the end of 72 hours.

Seedling Growth

The wheat seedlings were transferred to a hydroponic solution after the germination period was complete. Ten to twelve seedlings were measured (primary root tip to the seed, to the nearest 0.1 millimeter) then placed on a nylon net pinned to a seven to eight centimeter polystyrofoam ring (see Figure 1). The mesh on the net was large enough to allow unimpeded root growth, but just smaller than the wheat seeds. The primary root and seminal roots were placed through the net from the top facing down. Ten labelled rings of each cultivar (20 rings total) were then randomly placed in two ten-liter capacity plastic treatment containers, resulting in 50 to 60 total seedlings per treatment per cultivar. The treatment containers were filled with 6.3 l of the nutrient media of Aniol (4), with modification (see Table 1), and adjusted to pH 4.0$\pm$ 0.1. The hydroponic solutions were continuously aerated during the experiment.

Seedling Exposure to Al

Ninety hours after germination (18 hours after transfer to hydroponic solution) two more treatment containers with the appropriate concentration of Al were made up. Five rings were randomly selected from each control container, and placed in the new treatment containers; they were then labeled: "Victory Treatment" and "TAM W-101 Treatment."

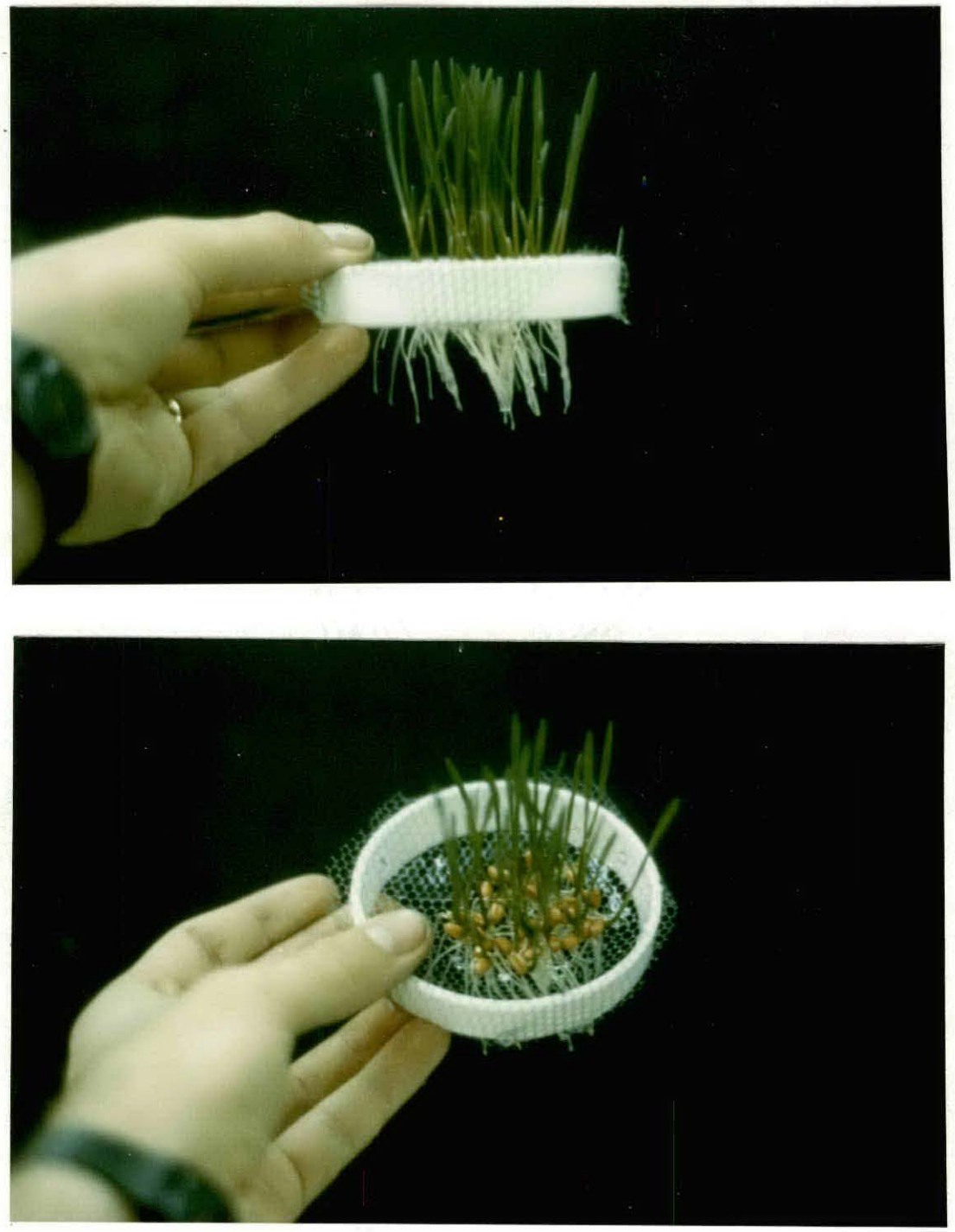

Figure 1. Net ring for hydroponic growth of wheat seedlings. The poly-styrofoam ring was cut from the top of disposable coffee cups; the nylon net was purchased at a local fabric store, as were the stainless steel pins used for holding the net in place (non-stainles steel pins rusted in the acidic environment).

TABLE 1

NUTRIENT MEDIA FOR AL^{3+} TOXICITY EVALUATION

Nutrient	Concentration
$CaCl_2$	0.4 mM
KNO_3	0.65mM
$MgCl_2$ $6H_2O$	0.25mM
$(NH_4)_2SO_4$	0.01mM
NH_4NO_3	0.04mM
pH	4.0+0.1 s.u.

The highly soluble Al salt, $AlCl_3 \cdot 6H_2O$, was utilized as the Al^{3+} source. The pH was monitored daily and maintained below 4.5 to keep the Al in solution. Treatment concentrations of 1.0 ug/ml (40 uM) Al and 2.0 ug/ml (80 uM) Al were used. At 140 hours post-germination (50 hours post-treatment) the nutrient mediums in all four treatment containers were replaced with fresh, untreated solution at pH 4.5. The plants were then grown for an additional 60-70 hours.

Root Length Measurement

The primary root length of each seedling was measured from the root tip to the coleorhiza to the nearest 0.1 mm by gently removing the seedling from the net, placing it on a moistened graduated surface for measuring, then gently replacing the seedling in the net.

The roots from all four treatment containers were measured at approximately 72, 95, 120, 140, and 212 hours post-germination. The mean root lengths at time of measurement were tabulated.

Results

The wheat cultivars "Victory" and "TAM W-101" showed differential tolerance to Al at 1 ug/ml and 2 ug/ml concentrations. The results of the root regrowth measurements made on the two cultivars at 1 ug/ml are

presented in Figure 2. The growth of primary and seminal roots of cv Victory ceased immediately after exposure to 1 ug/ml Al, and did not resume after transfer to Al-free solution. The roots of TAM W-101 exhibited a significant decrease in the rate of growth after exposure to 1 ug/ml Al, but 100% of the roots began growing again when the Al was removed.

When exposed to 2 ug/ml Al (see Figure 3), Victory roots reacted in essentially the same manner as when exposed to 1 ug/ml, while only about 44% of TAM W-101 roots exhibited regrowth (data not shown). This limited tolerance indicates that 2 ug/ml Al is near the threshold tolerance limits for cv. TAM W-101 to Al. The TAM W-101 2 ug/ml Al-treated roots that regrew did so at roughly half the rate as the 1 ug/ml-treated roots.

The rate of elongation for TAM W-101 control roots was slightly less than that of the Victory control roots after 72 hours, though both cultivars exhibited the same growth variability among the roots.

Discussion

The system developed for this experiment required measuring the complete root length of the plant roots before, during, and after a pulse of Al. The plants were grown hydroponically to allow tight control of the growth medium parameters (nutrient concentration, pH, aeration, etc.), immediate adjustment of the treatment parameters (Al

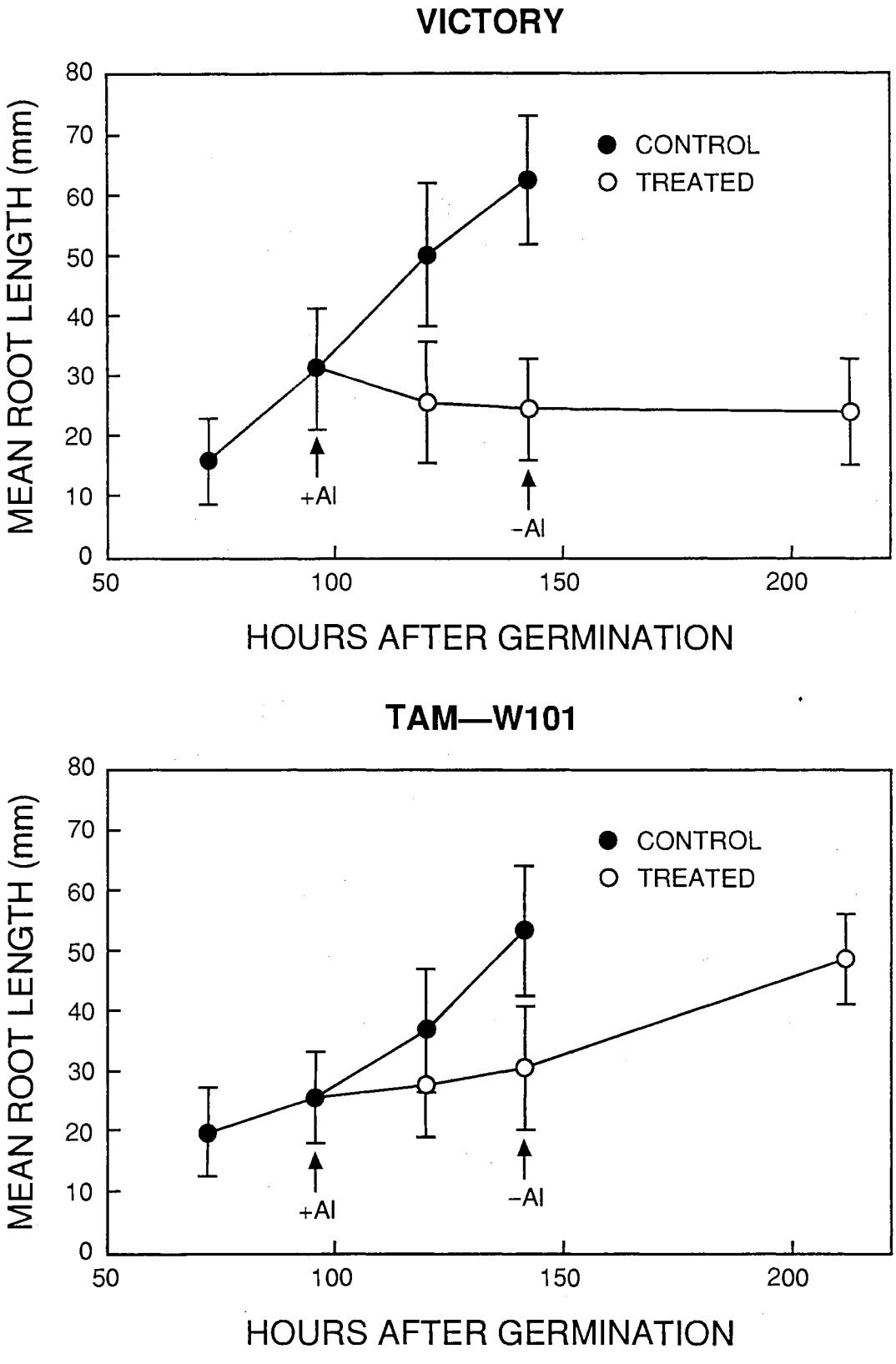

Figure 2. Root Length of Wheat Cultivars "VICTORY" and "TAM–W101" Over Time When Exposed to 1 μg/ml Al^{3+}

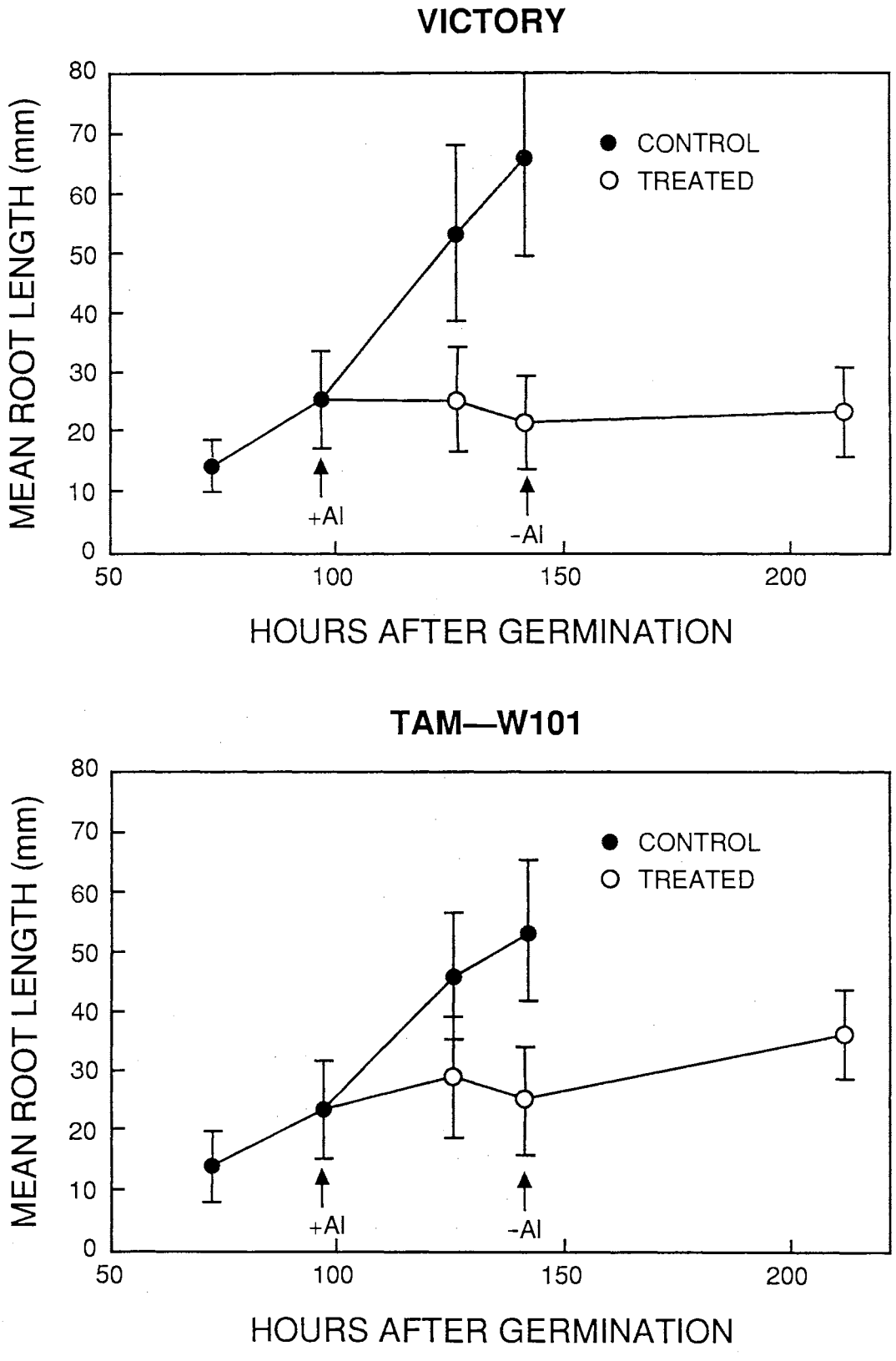

Figure 3. Root Length of Wheat Cultivars "VICTORY" and "TAM–W101" Over Time When Exposed to 2 μg/ml Al^{3+}

concentration and exposure), and easy, non-disruptive access to the plant roots for making measurements.

Careful consideration must be given to all growing conditions when seeking quantifiable results. The pH of the hydroponic solution must be maintained at 4.0 to obtain repeatable responses to Al. At this pH, the predominant form of Al is hydroxy-Al polymer ions with a valence of 3 (82). The temperature of the nutrient solution will affect the rate of Al uptake, and therefore must be constant from experiment to experiment (15). Attention must be given to the oxygen levels in the hydroponic solution; wheat grown in anoxic environments have elevated susceptabilities to Al toxicity (82). This is probably a result of the deterioration of the functioning of the Casparian strip, resulting in increased passive flow of Al to interior cells. Finally, the source of Al in the solution is a critical variable. Al sulfate, Al citrate and Al-EDTA, when added to a hydroponic solution, are not absorbed as readily as Al ions in a trivalent state (37). Al chloride was used as the source of Al for this toxicity investigation.

Wheat root growth has been accepted as an indicator of Al-tolerance since 1968 (24). However, root growth in itself is determined by many genes that are not related to Al tolerance. Thomsett and Thurman (80) suggests that this polygenic characteristic of root elongation interferes with the segregation of metal tolerant versus intolerant plants in a root growth assay.

When Victory wheat roots were exposed to toxic concentrations of Al in solution, they became thick, stunted, and brittle, which is consistent with effects reported by other investigators (29, 33, 35, 45). Cell division was inhibited (19), and undifferentiated tumorous tissue developed near the root tip (8, 19, 72) very soon after exposure to Al.

These gross morphologic effects suggest that the mechanism of Al-toxicity has a profound effect on cellular genetic activity. Metal tolerance is almost ubiquitously genetically determined (6, 45), but no convincing evidence has been presented to demonstrate whether the tolerance is simply inherited or polygenic (53).

Thomsett and Thurman (80) defined metal-sensitive (or intolerant) plants as those that die or have reduced growth rate in a particular metal concentration. Metal-tolerant plants would show little or no change in growth rate at that metal concentration. Thomsett and Thurman's (80) definition of metal tolerance is too rigorous for investigation of Al tolerance of many species of plants for the very reasons mentioned. The genetic variability within plants could mask a toxic response.

As demonstrated in Figures 2 and 3, the ability of the root to regrow after pulse exposure to Al is a more sensitive criterion for determining Al-tolerance than is the differential rate of elongation in Al solutions. The rate of elongation in the control groups of Victory and TAM W-101

was clearly different (0.68 - 0.78 and 0.48 - 0.59 mm/hr respectively) yet the response to Al-treatment was dramatic enough for the tolerant cultivar to be evident. This is consistent with observations by Moore, et al (61).

The roots of wheat cultivar Victory were irreversibly damaged by pulse exposure to as low as 1 ug/ml Al, while the roots of TAM W-101 exhibited 44% regrowth after pulse exposure to 2 ug/ml Al. Victory is thus a very Al-sensitive cultivar of wheat, while TAM W-101 exhibits Al tolerance in concentrations up to 2 ug/ml.

CHAPTER II

EXTRACTION, FRACTIONATION, QUANTIFICATION AND ONE-DIMENSIONAL ELECTROPHORESIS OF WHEAT ROOT TIP PROTEINS FROM THE TOLERANT CULTIVAR TAM W-101 AND THE INTOLERANT CULTIVAR VICTORY

Introduction

The range of tolerances to Al between cultivars in wheat (as demonstrated in Chapter 1) points to an essential Al tolerance mechanism that is differentially expressed. It is likely that this mechanism has an impact on the expression of proteins in the affected cells. In addition to the potential interaction with proteins, Al may affect other sites in the cell. The mechanism(s) responsible for Al tolerance can be illuminated by first understanding the mechanisms of Al toxicity. The present understanding of the mechanisms of Al uptake, toxicity, and tolerance are presented in the discussion below.

Al Uptake Mechanisms

The cells of the peripheral root cap are the first to take up Al (7). The primary site of Al uptake within the

peripheral root cap is the area of secretion of the root mucilage (7). The root meristematic tissue is especially sensitive to Al, and is the site of the first readily observable affects of Al toxicity (20).

The mechanisms of Al uptake have been investigated at length. Clarkson and Sanderson (20) demonstrated in 1969 that polyvalent cations such as Al are taken up by the root at the peripheral zone corresponding with the mucigel layer of the root cap. Aluminum, when present as a trivalent cation, competes with Cu, Ca, and other densely charged cations for exchange sites on the root surface (32, 42). Wagatsuma (81) discovered that Ca-desorption occurred when plant roots were exposed to ionic Al, but not Al + EDTA. Clarkson (19) proposed that Al interacts with P in the Donnan free space during uptake, but suggested that precipitation of P by Al was insignificant in P uptake.

Low temperatures do not affect Al uptake at lower concentrations. Wagatsuma (81) reported that Al is taken up at very high rates (2,000 ug Al/g dry wt. in 5 minutes) in cucumber roots at temperatures ranging from 2°C-30°C. He observed a dramatic increase of Al in plant roots immediately after initiation of treatment, suggesting that most Al is in the Donnan free space of the outer cells of the root.

The rate of cellular metabolism when suppressed by N_2 gas did not affect the rate of Al uptake in the plant roots Wagatsuma (81) investigated. However, in some plants,

anoxic conditions result in the breakdown of the non-vacuolar meristemic tissue water barrier in the root, resulting in an increase in the rate of passive uptake (81).

Mechanisms of Al Toxicity

Aluminum Interactions with DNA. Most investigators agree that the toxicity of Al is associated with the content of Al within the cell more than total Al content of the root (8, 24, 36, 37, 38, 81, 82). Clarkson and Sanderson (20) reported that the first readily observable effect of Al in roots is the inhibition of cell division in the apical meristem. Wallace and Anderson (84) discovered that Al has two affects on wheat roots: rapid inhibition of root elongation followed by inhibition of DNA sythesis.

Sampson, et al (74) hypothesized that Al has a direct role in blocking the mitotic cycle during interphase. Clarkson and Sanderson (20) proposed in 1969 that Al was binding to the DNA, introducing rigidity into the double helix, and thus interfering with replication.

Al binds with DNA in vitro (22, 44). Using pea roots, Matsumoto and Morimura (57) discovered the ratio of histone or non-histone proteins to DNA in chromatin was slightly increased by Al-treatment. Template activity of treated versus non-treated pea roots was reduced by half (62), while little, if any, binding of Al to DNA occured rapidly (57). Matsumoto found that chromatin in Al-treated roots condensed and/or aggregated (55). Matsumoto et al (56) suggested that

the primary binding site of Al in pea root cells is the phosphorous group in DNA. Matsumoto (55) concluded that Al-toxicity results from the disturbance of nuclear activity by Al in the cell.

Aluminum Interactions with the Cell Wall. The inhibition of root elongation by treatment with Al is a well-established phenomenon (24). Foy and co-workers (26, 27, 30, 31) have suggested that Al binds competitively with Ca to the non-esterified pectin carboxyl groups on the cell walls. Al associated with pectic carboxyl groups would cause strong adhesion between cell walls, restricting root elongation.

Aluminum has been localized in the cell walls of yeast by X-ray microanalysis (46). Matsumoto et al (56) have detected Al in the cell wall of pea roots. Waisel et al (83), however, used X-ray microanalysis to demonstrate that Al in bean and barley root cells was localized in the lumen but not in cell walls.

Matsumoto, et al (58), have determined that Al did not associate with pectin or uronic acids in pea roots. Therefore, the inhibition of root elongation by Al in pea roots was not related to a direct effect of Al on the pectin cross-linking. However, Matsumoto's results may have been compromised by high pH of their aqueous extract (20), resulting in the precipitation of the treatment Al as gibbsite [$Al(OH)_3$].

Effect of Al on Cell Structures. The first effect observed by Bennett et al (8) of Al on corn root cell organelles was the inhibition of the production of secretory vesicles of the Golgi apparatus, which was indicative of interference by Al in membrane transport mechanisms. The outer membranes of many vesicles disintegrated. The cisternae were distorted (curled), and overall numbers of cisternae per dictyosome were reduced. Secretory vesicles accumulated around the Golgi in the Al-treated roots, but not in the controls. The content of the vesicles were altered from a dense, granular material to a diffuse, fibrillar material.

The Golgi apparatus performs many functions, including synthesis of polysaccharides, membrane transformation and export of cellular products such as mucilage (60). The changing of cisternae number per dictyosome resulting from Al-treatment strongly suggests an alteration of the secretory activities of the Golgi apparatus (8). The disappearance of the mucilage layer observed in wheat root tips in response to Al supports this suggestion.

The activities of the Golgi apparatus include the proliferation of new membranous elements for the plasmalemma (76). Thus, the disruption of the Golgi apparutus would be expected to precede plasmlemma damage (7).

Golgi bodies are also responsible for the development of the cell plate in anaphase and telephase of mitosis (76). The disruption of the Golgi apparatus could also be

responsible for the immediate inhibition of cell division in roots exposed to Al.

It is interesting to note that Camillo Golgi first discovered dictyosomes by employing stains that contained silver, osmium, and other heavy metals (76). In essence, Golgi discovered that distyosomes bind with metals in the cell in 1906.

Al Interactions with Proteins. Aniol (4) has hypothesized that Al binds with proteins in the roots of both Al-sensitive and Al-tolerant cultivars of wheat. He monitored protein and DNA synthesis by measuring incorporation of [^{14}C] valine and [^{3}H] thymidine, respectively, and discovered that the rates of synthesis of DNA and protein actually increased in the Al-treated sensitive and tolerant cultivars. The effects of Al-toxicity were intensified when the roots were grown in solution with cycloheximide, an inhibitor of protein synthesis. From this experiment, he suggests that Al might induce proteins which bind and/or sequester Al in the cell, which could ameliorate the toxicity of Al.

The differential toxicity of Al among wheat cultivars reinforces the hypothesis that Al tolerance is a polygenic characteristic. The logical extension of this hypothesis is that tolerant cultivars would express new or different proteins not present in sensitive cultivars. These

cultivar-specific proteins would play a critical role in cellular Al-tolerance mechanisms.

Siegal and Haug (77) proposed that competitive Al binding with the Ca-binding protein,calmodulin, represents a primary toxic mechanism of Al to plants. The disruption of cellular processes such as vesiculation, cell division, root elongation, they propose, are results of the disruption of calmodulin's role in cellular metabolism (77).

Mechanisms of Al Tolerance

The mechanisms of Al-tolerance in plants are still not well understood, though much research has been devoted to identifying these mechanisms. For a plant to be Al-tolerant its root meristematic cells must express Al tolerance. Potential mechanisms of cellular tolerance to Al include Al binding to the cell wall, exclusion of Al from the cytoplasm, compartmentalization of Al within the cell, and chelation of Al to neutralize the trivalent charge. The differential expression of Al tolerance within cultivars probably results from differential actuation of various combinations of these tolerance mechanisms. These potential mechanisms are illustrated in Figure 4.

Genetic Control. The mechanisms for Al-tolerance are genetically controlled and inherited. Aniol (3) has reported that Al tolerance in corn is controlled by a single locus with multiple alleles, and in barley by a single

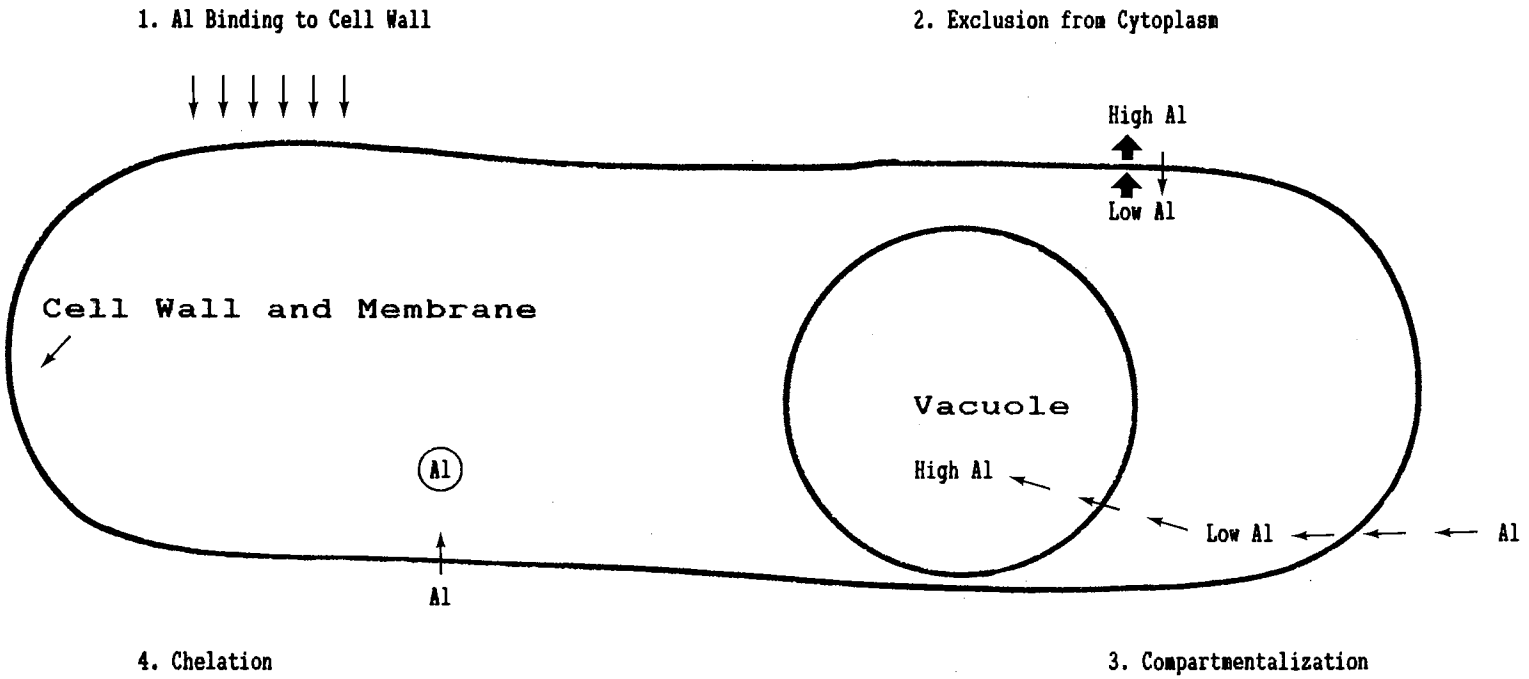

Figure 4. Potential Mechanisms of Al Tolerance in Plants
[From Thomsett and Thurman (80) with Modification]

dominant gene located on chromosome 4. Aniol and Gustafson (5) used traditional wheat breeding techniques to identify the genes responsible for Al-tolerance. They discovered that the genes for Al-tolerance in the medium-tolerant wheat variety "Chinese spring" were localized in chromosome arms 6AL, 7AS, 2DL, 3DL, 4DL, and 4BL, as well as on chromosome 7D. The actual genes responsible for confirming Al-tolerance have not been identified, nor are the mechanisms responsible for Al-tolerance in a plant well understood.

Al-Binding to Cell Wall. As previously discussed, Al has been demonstrated to bind with the root cell walls of yeast (46) and pea (56) but not with bean or barley root cell walls (83). There is no evidence that tolerant cultivars bind Al to cell walls better than sensitive cultivars. Mugwira and Elgawhary (64) reported that Al-sensitive cultivars of wheat and triticale have a higher cation exchange capacity, and generally exhibit more rapid Al uptake, than Al-tolerant cultivars.

Exclusion and/or Extrusion from Cytoplasm. Wagatsuma (81) postulated that Al-tolerance could result from plasmalemma resistance to Al transport. He observed that when metabolic inhibitors injured cellular membrane systems, Al transport across the membranes increased. Ownby et al (69) have reported the presence of Al in the cytoplasm and nucleus of Al-sensitive wheat cultivar Victory, but not in Al-tolerant wheat cultivar TAM W-101. An active Al-

extrusion system in the plasmalemma would explain this phenomenon, but no such mechanism has been identified.

Bennet et al (8) have suggested the mucilage extruded from the peripheral cap cells of grass roots is important in the expression of Al-tolerance. This polysaccharide or polysaccharide-protein complexed mucilage is responsible for binding almost half of the total Al in pea root tips (37). The mucilage has been observed to disappear from Al-sensitive cultivars of wheat, but not Al-tolerant cultivars, during Al treatment.

Compartmentalization. The cellular sequestering of Al across the tonoplast into the vacuole is a very plausible mechanism for Al-tolerance. Indeed, the progressive vacuolation of the cells of the root cap is the most readily identifiable consequence of Al toxicity (8). However, no real evidence has been presented that either localizes Al in the vacuole of Al-tolerant cells, or supports an Al transport mechanism from the extracellular environment, across the plasmalemma into the cytoplasm, through the tonoplast into the vacuole.

Chelation. There are any number of molecules that will chelate Al, including EDTA, citrate and other organic complexes and inorganic complexes such as sulfides and phosphates (80). Protein complexes such as metallothioneins and phytochelatins do not appear to bind Al, though they may play a role in heavy metal tolerance (75).

Aniol (3) has proposed that a protein or group of proteins are present in Al tolerant plants that bind with Al in such a way as to render it harmless to the cell. Furthermore, he suggests that synthesis of these Al-binding proteins was responsible for the Al tolerance induced by exposure of the plant to less-than-lethal doses of Al. When the plants were grown in the presence of cycloheximide, a protein synthesis inhibitor, induction of Al tolerance was completely inhibited. Incorporation of labeled valine and thymidine into wheat protein and DNA increased in Al-treated roots. According to Aniol's hypothesis, such Al-binding proteins could conduct Al harmlessly through the cytoplasm, to be sequestered in the vacuole. However, the evidence supporting Aniol's hypothesis is largely circumstantial, as no Al-binding protein has been identified.

The objective of this investigation was to test Aniol's hypothesis by determining if induction of specific proteins was associated with Al treatment in a cultivar (Victory) where growth was irreversibly inhibited, and a cultivar (TAM W-101) where growth was not totally inhibited. The presence of specific proteins associated with Al treatment in TAM W-101 but not Victory could represent "Al-tolerance proteins."

Methods and Materials

Growth of Wheat Seedlings

Approximately 1,000 seeds of each wheat cultivar, TAM W-101 and Victory, were germinated as described in Chapter I. At 72 hours post-germination the seedlings were transferred to growth rings as previously described. Six rings of approximately 50 seedlings were prepared for four properly labelled treatment containers resulting in a total of 24 rings. The four parameters were "Victory Control", "Victory Treated", "TAM W-101 Control" and "TAM W-101 Treated." The seedlings were then cultured and exposed to 2 ug/ml Al^{3+} as described in Chapter I Methods and Materials. At 140 hours post-germination, the seedlings were removed from the treatment containers, rinsed gently with water, and placed in fresh Al-free growth media for one hour to remove Al from the surface of the roots.

Protein Extraction

The terminal 3 mm of the primary and two seminal roots were excised from the seedlings for each of the four treatments with a chilled (4°C) razor blade and placed in chilled Al-free growth media. The root tips for each treatment were gently removed from the chilled rinse and weighed to the nearest 0.1 mg.

The root tips were then placed in a chilled morter and pestle with 2 ml of chilled extraction media (see Appendix

A, Table 1) and homogenized for four minutes. Microscopic investigation of the homogenate revealed no unbroken cells, though many intact nuclei were noted. The morter and pestle were rinsed with 2 ml of the extraction media and the homogenates were placed in four 30-ml Corex glass tubes. The homogenates were centrifuged at 300 g at 4°C for 10 minutes to remove cellular debris (cell wall material, etc.) and nuclei. The supernatant was collected for each parameter and the pellet discarded. Microscopic examination of the supernatant revealed no nuclei or cellular debris. A 60 ul aliquot was taken from each treatment for protein quantification (See Figure 5).

Protein Fractionation

The homogenate supernatant of each of the four samples were transferred to a 4.5 ml ultracentrifuge tubes (Beckman SW-61, clear), brought to 4.3 ml with extraction media, then centrifuged at 100,000 g for 30 minutes at 4°C.

The supernatant fraction resulting from the centrifugation is made up of proteins that are soluble in water, or have densities that are relatively close to water. Ribosomal proteins, for example, are often associated with membranes that are not particularly water-soluble, yet stay in the supernatant during centrifugation. This fraction represents cytoplasmic proteins.

The pellet fraction is composed of membraneous organelles such as endoplasmic reticulum, dictyosomes, mictochondria,

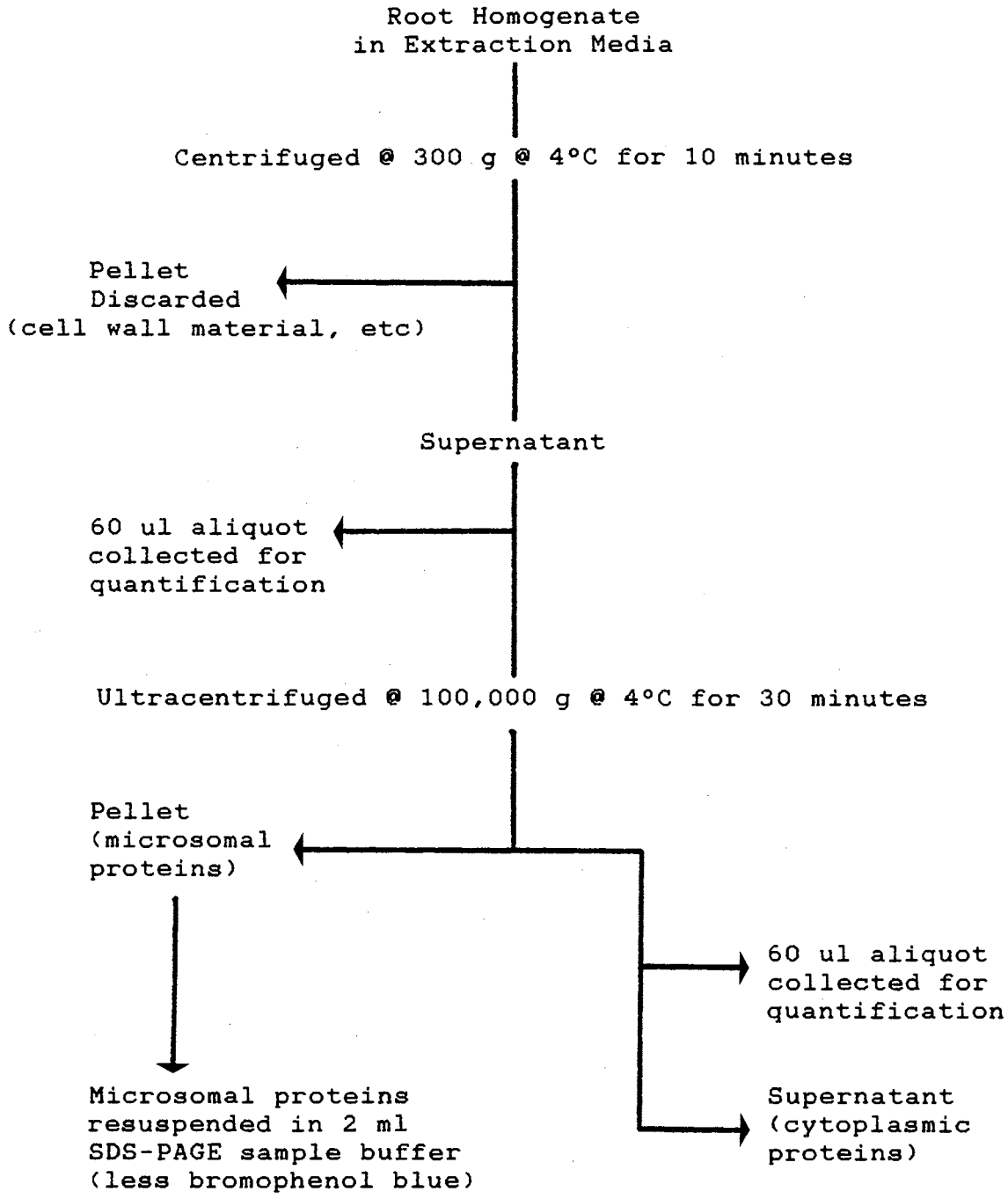

Figure 5. Protein Extraction and Fractionation Flow Chart

proplastids, and vacuoles. The proteins in this fraction are more difficult to recover; they are insoluble in water and relatively tightly bound to membranes.

The cytoplasmic protein fractions were transferred to 30-ml Corex glass tubes. The microsomal fractions were resuspended in 2 ml of SDS-PAGE sample reducing buffer, less the bromophenol blue (see Appendix A, Table 2).

Phenol Extraction

The proteins were phenol-extracted as described by Hurkman and Tanaka (39) with modification (See Figure 6). Equal volumes of phenol saturated with 50 mM Tris buffer (pH 7.5) were added to the cytoplasmic and microsomal fractions. The samples were mixed at room temperature for five minutes. The aqueous and phenol phases were then separated by centrifugation. The aqueous phases were discarded. The phenol phases were transferred to 30-ml Corex glass tubes. Proteins were precipitated from the recovered phenol phases by addition of 5 volumes of 0.1 M ammonium acetate in methanol. The samples were then incubated at -20°C overnight, then centrifuged at 9,000 g for ten minutes at 4°C to pellet the precipitates. The pellets were gently rinsed three times with the methanol/ammonium acetate solution and once with acetone, then dried with filtered air or N_2. The pelleted proteins were then solubilized by the SDS-PAGE sample reducing buffer (see Appendix A, Table 2) in

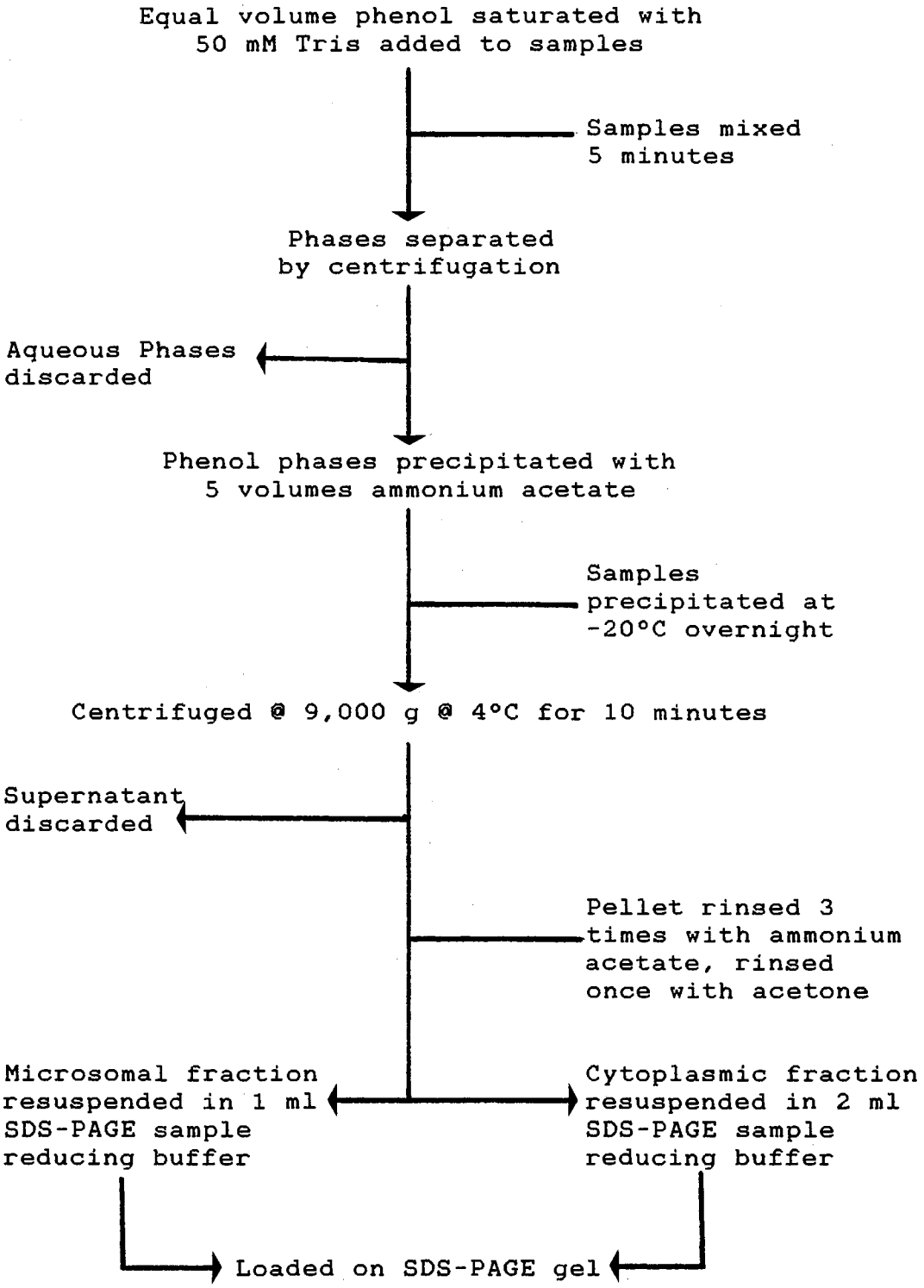

Figure 6. Protein Phenol Extraction Flow Chart

the following amounts: 2 ml for Victory Control, Victory Treated, TAM W-101 Control and TAM W-101 Treated cytoplasmic fractions and 1 ml for Victory Control, Victory Treated, TAM W-101 Control and TAM W-101 Treated microsomal fractions. The proteins were placed in eppendorf tubes and frozen at -70°C for SDS-PAGE analysis.

Protein Quantification

The protein aliquots retrieved from the whole-cell extract and the soluble fractions were quantified using the Bradford method (11). A standard curve was constructed with at least four known concentrations of bovine serum albumin (BSA). The assays were rejected if the correlation coefficients (r) of the standard curves were less than 0.990.

Aliquots of the total cell extract and cytoplasmic extract were collected from three consecutive protein fractionations (on June 4, June 10, and June 16, 1987). The aliquots were divided into 20 and 40 ug/l replicates for quantification. Microsomal protein quantities were determined by calculating the difference in total and soluble cell protein extracts, as the solubilization detergents interfered with the quantification technique. The dry weights of the source root tips were calculated from the fresh weights by multiplying with an experimentally determined conversion factor of 0.057 for control roots, and

0.048 for treated roots. The protein concentrations were converted to milligrams protein per gram dry weight of root tissue.

One-Dimensional Polyacrylamide Gel Electrophoresis

The soluble and insoluble protein fractions of TAM W-101 and Victory control and treated plant roots were analyzed by SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis) as described by Laemmli, et al (47, 86). The Protean II Slab Cell (BioRad Corporation) was utilized to cast and run the gels. All activities were carried out with electrophoresis-grade water (conductivity ~ 18 umhos).

Gel Preparation. The acrylamide matrix for differentiating the protein fractions consisted of a 12.0% acrylamide separating gel 1.5 mm thick by 20 cm long by 16 cm wide. The separating gels were allowed to polymerize for 12 hours prior to addition of the stacking gel.

A 1.5 cm 4.0% acrylamide separating gel with a 10-well comb was layered over the separating gels. The reagents and stock solutions, as well as the gel formulas, are presented in Appendix B: Reagents, Stock Solutions, and Gel Preparation for SDS-PAGE Slab Gels.

Two gels were cast simultaneously. The Protean II radiator was connected to running chilled water, assembled

to the gels, and the reservoirs were filled with the electrode running buffer described in Appendix B.

Loading the Gel. Eight protein samples were retrieved from the freezer, thawed to room temperature, heated at 95°C for four minutes, then cooled to room temperature. Each of the eight samples were gently loaded into a well, or lane, of the polymerized stacking gel with a 250 ul Hamilton syringe connected to a 25-guage needle. Molecular weight standards (Bio-Rad Low Molecular Weight Standards 10-100 kilodaltons) were loaded in the remaining two lanes.

Gel Running Conditions. The power supply, Bio-Rad Model 500/200, was adjusted to a constant 25 mA through the stacking gel, then adjusted to a constant 35 mA for the separating gel. The voltage typically started at about 90, increasing to about 280 volts at the completion of the run. The gels were run until the dye fronts were about 1 cm from the end of the gel (approximately six hours).

At completion of the run the gels were removed from the glass plates by gently twisting one of the 1.5 mm teflon spacers, lifting the separated plate from the gel, then immersing the remaining plate and gel in a water rinse. One corner of a gel was nicked for tracking through rinsing and staining activities. The plate with the gel was gently rocked to allow water between the gel and the glass plate. Care was taken to prevent tearing the gel. The gels were "floated" into labelled clean glass pans.

Gel Staining. The gels were soaked in a fresh staining solution of 40% methanol, 10% acetic acid, and 0.1% Coomassie Blue R-250 overnight. The gels were then rinsed with water and destained with 40% methanol and 10% acetic acid until the background was relatively clean, which usually required one to three hours.

The gels were photographed with a NikkorMat camera, yellow filter, and Tech-Pan black and white film, ASA 100, on a fluorescent light table.

Results

The proteins of fractions from Al-sensitive wheat cultivar Victory and Al-tolerant wheat cultivar TAM W-101 treatment and controls were quantified over a series of three extractions to determine what effect Al treatment had on protein synthesis and/or distribution in the cell.

The proteins extracted as cytoplasmic and microsomal fractions are presented in Table 2. The cytoplasmic fraction of all four treatments yielded much more protein than the microsomal fraction.

Although Al caused a 20-30% decline in cytoplasmic proteins of both cultivars, these differences were not significant due to the high variability of quantity of protein recovered. Al thus does not appear to cause a large, consistant decrease in protein accumulation over the 24 hour test period.

TABLE 2

QUANTIFICATION OF PROTEINS FROM THREE EXTRACTIONS OF THE CYTOPLASMIC AND MICROSOMAL FRACTIONS OF CONTROL AND 2 ug/ml Al-TREATED WHEAT CULTIVARS TAM W-101 AND VICTORY

	Cytoplasmic Proteins [ug/g dry weight (% control)]				
Sample	6/4	6/10	6/16	$\bar{x}$	s.d.
TAM W-101 Control	165.7 (100)	130.3 (100)	139.2 (100)	145.1 (100)	18.4 (0)
TAM W-101 Treated	131.9 (80)	79.8 (61)	133.8 (96)	115.2 (79)	30.6 (27)
Victory Control	181.8 (100)	123.0 (100)	143.7 (100)	149.5 (100)	29.8 (0)
Victory Treated	157.5 (87)	78.3 (64)	79.1 (55)	105.0 (69)	45.5 (43)
	Microsomal Proteins [ug/g dry weight (% control)]				
	6/4	6/10	6/16	$\bar{x}$	s.d.
TAM W-101 Control	6.9 (100)	8.1 (100)	18.3 (100)	11.1 (100)	6.3 (0)
TAM W-101 Treated	12.4 (180)	6.3 (78)	9.9 (54)	9.5 (86)	3.1 (33)
Victory Control	15.2 (100)	25.7 (100)	6.6 (100)	15.8 (100)	9.6 (0)
Victory Treated	15.0 (99)	20.8 (81)	1.7 (26)	12.5 (79)	9.8 (78)

SDS-PAGE of the protein fractions of TAM W-101 and Victory reveals several features of interest. The gel, shown in Figure 7, verifies that the fractionation procedure developed was successful; the microsomal proteins were different from the cytoplasmic proteins for both cultivars. The microsomal fractions of both Victory and TAM W-101 did not, in general, show the appearance of new major proteins or the disappearance of pre-existing proteins. This would suggest that Al does not cause any major shifts in the accumulation of proteins in the microsomal fraction, at least those detectable in 1-D gels.

Several series of protein bands appeared in both the cytoplasmic and microsomal fractions of the control and treated Victory and TAM W-101 extracts. The most noticable of these proteins were the three bands starting at about 50 kd.

The changes in protein bands that were most obvious were:

- A cytoplasmic protein band at about 88 kd increased in intensity in response to Al treatment in both Victory and TAM W-101 (indicated by the ✱).
- A cytoplasmic protein band present in TAM W-101 control but not in Victory control was present in TAM W-101 and Victory Al-treated roots. This protein band was about 50 kd in size (indicated by the ➧).
- A cytoplasmic band at 27 kd present in TAM W-101 control, Victory control, and TAM W-101 Al-treated was not

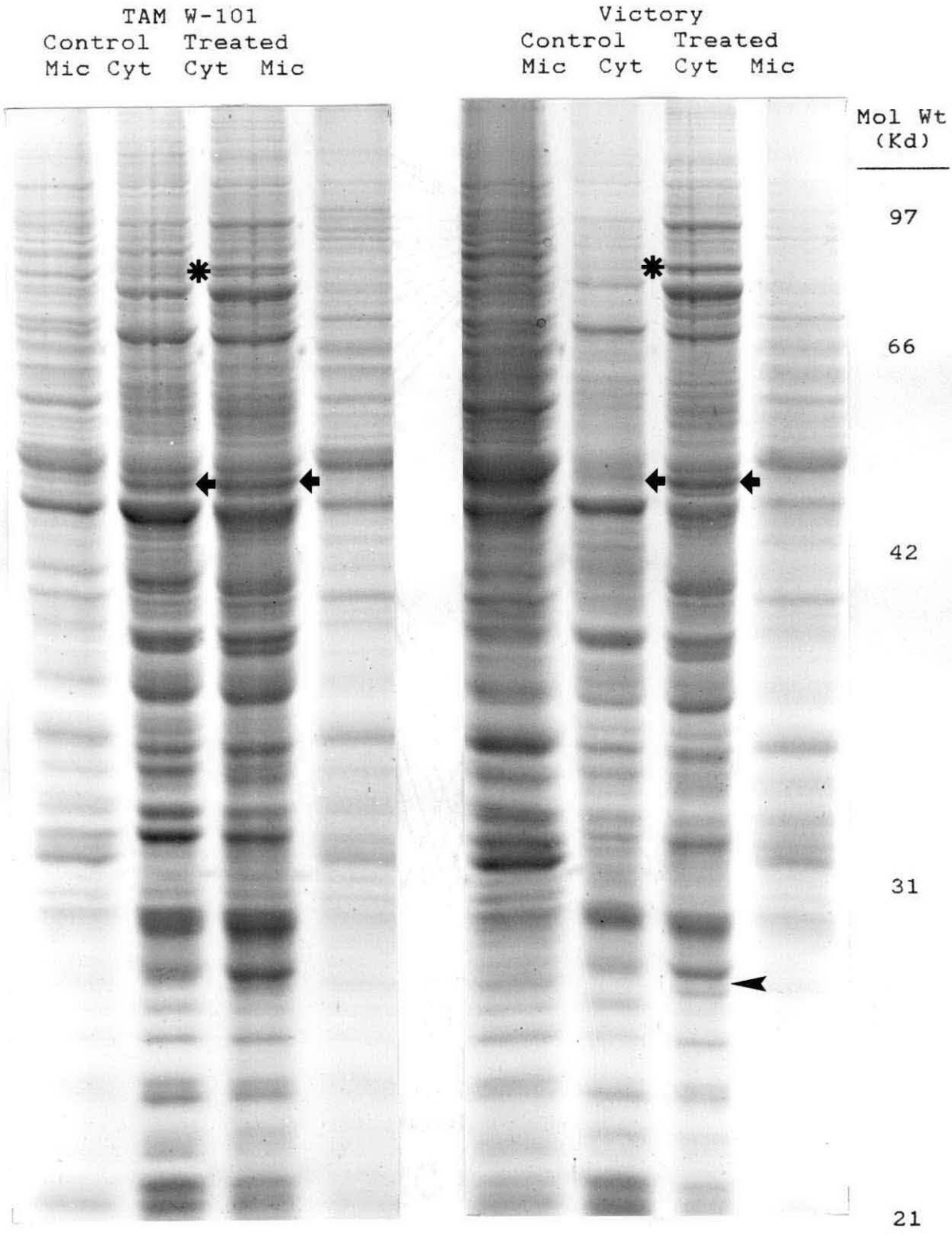

Figure 7. SDS-PAGE of Proteins from the Cytoplasmic and Microsomal Fractions of Control and 2 ug/ml Al-Treated Wheat Cultivars TAM W-101 and Victory.

present in Victory Al-treated protein fractions, but a protein unique to the Victory Al-treated fraction was present at about 28 kd (indicated by the ➤).

Discussion

The high variability of the quantity of protein recovery per gram dry weight in the three extractions presented in Table 2 are the product of several factors. A major factor was the potential interference in the protein assay by SDS which was present in the buffer used to solubilize the microsomal fractions. The Bradford method was reportedly compatible with SDS to 0.1% (9), but approximately 0.15% SDS was required to re-solubilize the microsomal pellets. Several other quantification techniques were attempted (70, 71) with unsatisfactory results. Protein concentrations in the microsomal fraction were calculated by quantifying proteins in the total cell extract and subtracting the values obtained in the cytoplasmic fraction.

The data in Table 2 did indicate, however, that no major loss of proteins occured in either the Al-sensitive or Al-tolerant cultivars in response to 2 ug/ml Al. Proteins in plants have a relatively slow turnover rate; a major change in protein synthesis might not be detected by measuring total protein, even after 24 hours. A more sensitive protein quantification method might detect a

general decrease in protein concentration in cytoplasmic proteins of Al-treated roots.

Analysis of the protein fractions by SDS-PAGE confirms that the proteins in the cytoplasmic fraction are different from the proteins in the microsomal fraction. The protein bands that did appear to occur in both the cytoplasmic fractions and microsomal fractions could be proteins which are only loosely associated with cell organelles, or proteins which occur in both cytoplasm and organelles. Wallsgrove, et al (85) found glutamine synthetase in the chloroplasts as well as the cytoplasm of pea leaves; undoubtably there are many proteins in a cell that may occur in both cytoplasm and organelles.

Based on the inforamation obtained from Figure 7, Al had a much greater impact on the cytoplasmic proteins of both the tolerant and intolerant cultivars than on the membrane-bound proteins in the microsomal fraction. One-dimensional PAGE did not reveal the presence of any new, major proteins which might potentially be Al-binding or Al-sequestering proteins (4). Likewise, no proteins were observed that parallel the heat shock response (50) (i.e. small number of low molecular weight proteins synthesized in response to temperatures above 40° - 45° C).

The effect of Al on the proteins of wheat root meristem cells is not dramatic, in contrast with the effect on root meristem growth. SDS-PAGE, capable of resolving up to 60 protein bands, however, may not have the resolution

necessary for identifying Al-induced proteins. Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE), with a resolution of up to 500 proteins, would be appropriate to incorporate into this investigation. Dunbar (21) points out that just one band on a 1-D SDS-PAGE may, in fact, be resolved as over 100 proteins by 2-D PAGE.

CHAPTER III

TWO-DIMENSIONAL GEL ELECTROPHORESIS OF CYTOPLASMIC AND MICROSOMAL WHEAT ROOT PROTEINS

Introduction

Two-dimensional polyacrylimide gel electrophoresis (2-D PAGE) provides the most detailed resolution of complex mixtures of proteins presently available. The technique, developed by O'Farrell in 1975 (66), separates proteins by two physical characteristics: isoelectric point and mass. Proteins are isoelectric-focused in a 4% acrylamide tube gel, containing ampholytes, which are complex mixtures of polyamino, polycarboxylic organic acids. In an electric field, these ampholytes migrate to their isoelectric point (pI), and, in doing so, create a pH gradient across the gel. Proteins in the sample subsequently move to their pI, and thus become resolved according to whether they are acidic or basic. The net charges (or pI) of proteins are a result of the sum of positive and negative charges on all amino acids. Protein complexes with moieties such as sulfate, carbohydrate, or which are phosphorylated, will have additional charges generated by those groups (63). After equilibration in an SDS solution, the proteins are then

separated according to molecular weight by electrophoresis in a 12% SDS-PAGE slab gel. Using this system, it is theoretically possible to resolve more than a thousand proteins from a plant cellular extraction.

Two-dimensional PAGE has proven to be a very effective tool for purification of proteins from complex cell fractions (21, 48) as well as comparison of gene expression in different organisms (12, 23, 73). 2-D PAGE is increasingly being utilized as a tool in plant genetics (79). Hurkman and Tanaka (40) employed 2-D PAGE to identify salt-stress proteins in barley roots.

The relatively few changes in wheat root proteins seen in 1-D PAGE in Chapter 2 suggested that 2-D PAGE would be needed to resolve individual proteins which might be altered by Al stress. At the time, no other investigators at Oklahoma State University were using 2-D PAGE routinely or successfully. The major limitation to the application of 2-D PAGE is the configuration of experimental parameters that must be optimized for each specific investigation. These parameters include:

- Protein concentration
- Ampholyte ratios and brands
- Gel polymerization
- Solubilization buffers
- Staining methods

This phase of the investigation was designed to test various parameters affecting the resolution of 2-D gels and

to optimize conditions so that future experiments using this technique could be carried out routinely. This experiment was conducted to determine the effect of Al treatment on the cytoplasmic and microsomal proteins of Victory root meristems by 2-D PAGE.

Materials and Methods

Protein Preparation

The proteins were extracted into phenol and precipitated as described in Chapter 2, but the pellet was resuspended in 1 ml Eppendorf tubes in IEF Solution Buffer (see Table 1 of Appendix C). The microsomal fractions were resuspended in 100 ul, and the cytoplasmic fractions were resuspended in 500 ul. To aid in resolubilization, the pellets were broken up by means of a glass rod beveled on the end to match the sample tube. The samples were allowed to sit at room temperature for one hour to facilitate solubilization. Prior to loading on the IEF gel, undissolved particulate matter was removed by centrifuging the samples at 3,000 g for three minutes.

Iso-Electric Focusing

The IEF tube gels were cast in 3 mm I.D. x 13 cm glass tubes, sealed at one end with parafilm, and held upright by a Hoefer Tube Gel Rack. The IEF acryamide solutions were made as described in Table 2 and Table 3 of Appendix C.

Immediately after adding the activators (TEMED and APS) to the acrylamide the solution was poured into a 25 ml disposable syringe with an 18 cm, 26 guage, beveled needle attached. The acrylamide solution was injected into each of 18 tubes, taking care to insure no air bubbles were trapped in the tube. The tubes were filled to 3 cm from the top. The acrylamide was distributed rapidly, as the solution polymerized within three minutes of activation. The gels were then overlaid with about 250 ul of overlay buffer (see Table 2.B in Appendix C), and allowed to polymerize for 12 hours.

After the tube gels were polymerized, the parafilm caps were removed from the bottom of the tubes. Care was taken when removing the parafilm to prevent introducing air bubbles into the gel, as the acrylamide would adhere lightly to the parafilm. The tubes were rinsed with anolyte (Table 2.D, Appendix C) and attached to the upper buffer chamber of a Hoefer Scientific GT Tube Gel Electrophoresis Unit with a cooling core. The lower buffer chamber was filled with 1.5 liters anolyte, and the upper buffer chamber was filled with 350 ml catholyte. Protein samples were loaded on the appropriate tube gel by gentle layering with a 250 ul Hamilton syringe fitted with a 4 cm 25-guage needle. For the cytoplasmic fraction, 50 ul of protein sample, corresponding to about 80 ug of protein, was loaded; for the microsomal fraction this was increased to 75 ul (80 ug of

protein). Several tubes in each run were left as blanks for pH analysis.

The GT cooling core was attached to circulating chilled water. Iso-electric focusing was conducted at 400 volts for 15 hours, and 800 volts for one hour. At completion of the 16 hours, the gels were removed from the apparatus and extruded from the glass tubes. Gel extrusion was very difficult; the primary reason for using 3 mm diameter gels was the difficulty in extruding smaller diameter gels without breakage. Gels could be consistently extruded by gently injecting fresh 10% glycerol between the gel and tube wall with a syringe and 4 cm 25-guage needle and rimming the top and bottom 4 cm of the gel.

The gels that were not run on the 2nd-dimension immediately were rapidly placed in labeled glass vials, capped, and stored at -20°C. To measure th pH gradient in the IEF gels, a blank gel was rinsed and chopped into 5 mm sections. Each section was placed into vials of 2 ml degassed ultrapure H_2O and shaken for 10 minutes. The pH of each section was determined by placing a pH probe directly into the vial.

Optimizing IEF pH Ranges

The pH range of IEF gels depends upon the ampholytes in the acrylamide solution and the ampholytes in the IEF solution buffer. In order to obtain optimum resolution of proteins, the range of the IEF pH continuum had to be

optimized. The movement and position of proteins in the IEF gel could be drastically altered by changing the ampholyte concentrations. In addition, the acrylamide polymerization reaction is very sensitive to solution pH. When the ampholyte concentration was too acidic or basic, the gels did not polymerize. The following ampholyte combinations were tested:

A. 80% pH range 5-7, 20% pH range 3-10 Biolyte brand

B. 90% pH range 5-7, 10% pH range 3-10 Biolyte brand

C. 80% pH range 5-8, 20% pH range 3-10 Servalyte brand

D. 20% pH range 5-8, 80% pH range 3-10 Servalyte brand

Second Dimension

The second dimension 12% continuous SDS-PAGE slab gels were cast as described in Chapter 2, without a stacking gel. After the gels had polymerized for 4-6 hours, they were attached to the central cooling core and placed in the lower buffer chamber, as described previously. Prior to addition of the upper running buffer, the IEF gels were loaded onto the slab gels.

To load the IEF gels, they were thawed at room temperature (if necessary) then centered on the top space between the two glass plates of the slab gel. The tube gels were not incubated in SDS prior to placement on the 2nd-dimension. The gels and glass plates were lubicated with upper running buffer, then gently pushed down between the plates to the gel surface. Care was taken not to distort,

stress, or tear the IEF gel. All air bubbles were removed from between the two gel surfaces. The IEF gel was overlaid with approximately 200 ul SDS Reducing Buffer (see Appendix B). A 4-5 mm segment of agarose-imbedded Pierce Gelcode molecular weight markers was applied directly to one side of the slab gel. The upper buffer was gently poured into the buffer chamber and the power was adjusted to 30 mA per gel. The slab gels were run, removed, and rinsed as described in Chapter 2. The gels were stained with the silver stain system described by Morrissey (63).

The gels were photographed with a Nikon FA camera and Kodak TMX 5052 film. After the gels were photographed, they were placed in labelled ziplock storage bags for storage.

Results

IEF Optimization

The pH ranges of the various ampholyte combinations tested in IEF gels are presented in Figure 8. Solution A was utilized for focusing the wheat root extracts because it provided the pH spectrum that resulted in the highest resolution of the proteins samples. The more broad pH gradient resulted in compression of the proteins in the first dimension, and therefore loss of resolution in the second dimension. Previous investigation with more narrow pH gradients resulted in the appearence of proteins as a broad smear on the cathodic end of the tube gel. Proteins

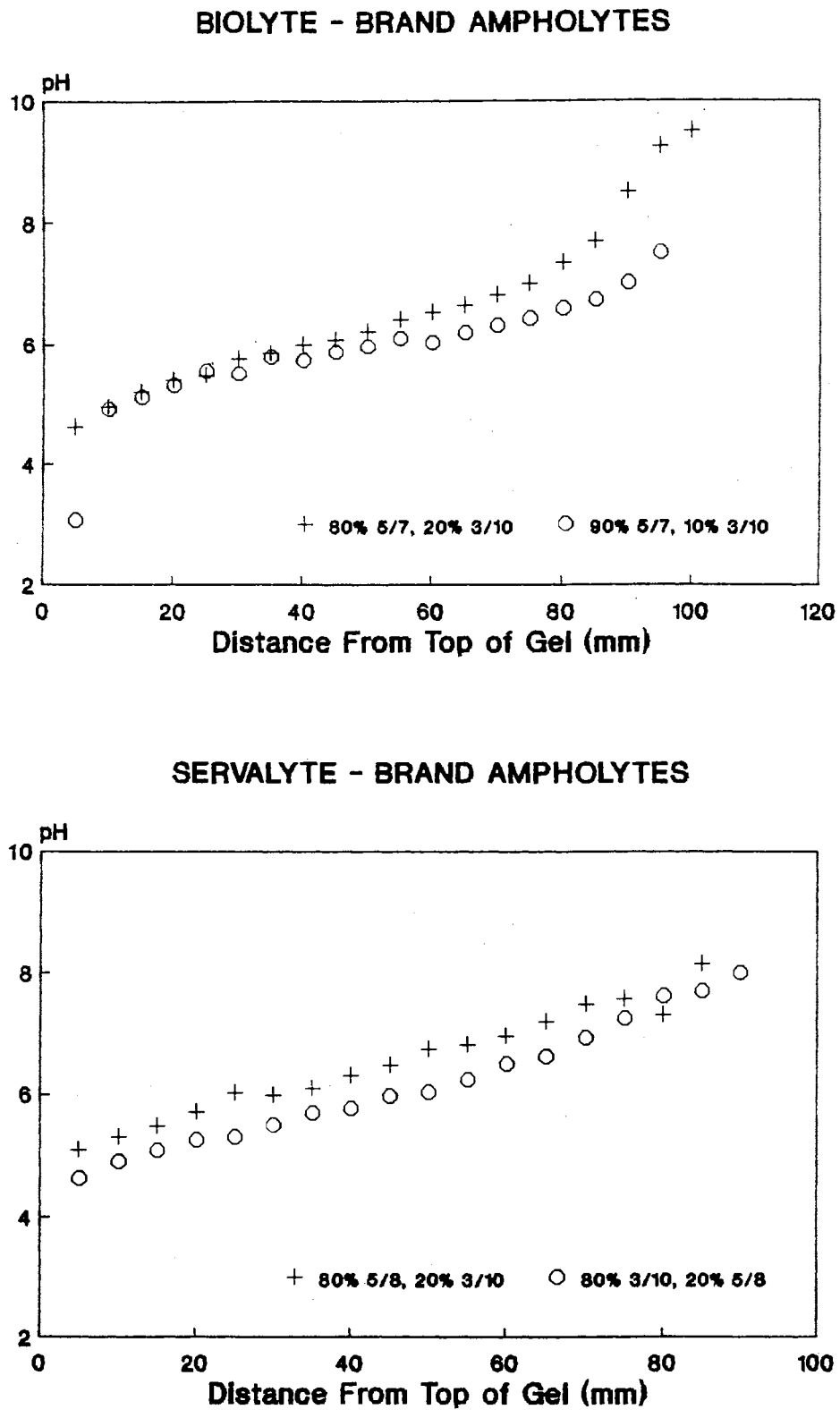

Figure 8. The Effect of Different Ratios and Brands of Ampholytes on the pH Range of IEF Gels.

which are more basic than the pH range (such as histones) do not even move into the IEF gel.

2-D-PAGE of Cytoplasmic and Microsomal Proteins

Electrophoretograms of the cytoplasmic and microsomal proteins of control and Al-treated Victory are presented in Figures 9 and 10. There were at least two major cytoplasmic proteins that appeared in control but not treated roots (indicated in Figure 9 as circles). There were multiple cytoplasmic proteins that were apparently increased or decreased in response to Al, but no quantifiable comparison were available. There were at least five major proteins that appeared in the cytoplasm of treated Victory root meristem cells that were not in the controls (indicated in Figure 10 as arrows).

The microsomal proteins of Victory did not appear to be as altered by Al treatment as were the cytoplasmic proteins. The control microsomal fraction appeared to have lost only one major protein when exposed to Al (indicated in Figure 11 as a circle). Four proteins appeared in the Al-treated fraction that were not in the control fraction (indicated in Figure 12 as an arrow).

Discussion

The discovery of eight major proteins in Al-treated Victory wheat meristem cells that are not in the controls is significant. These proteins could represent general shock

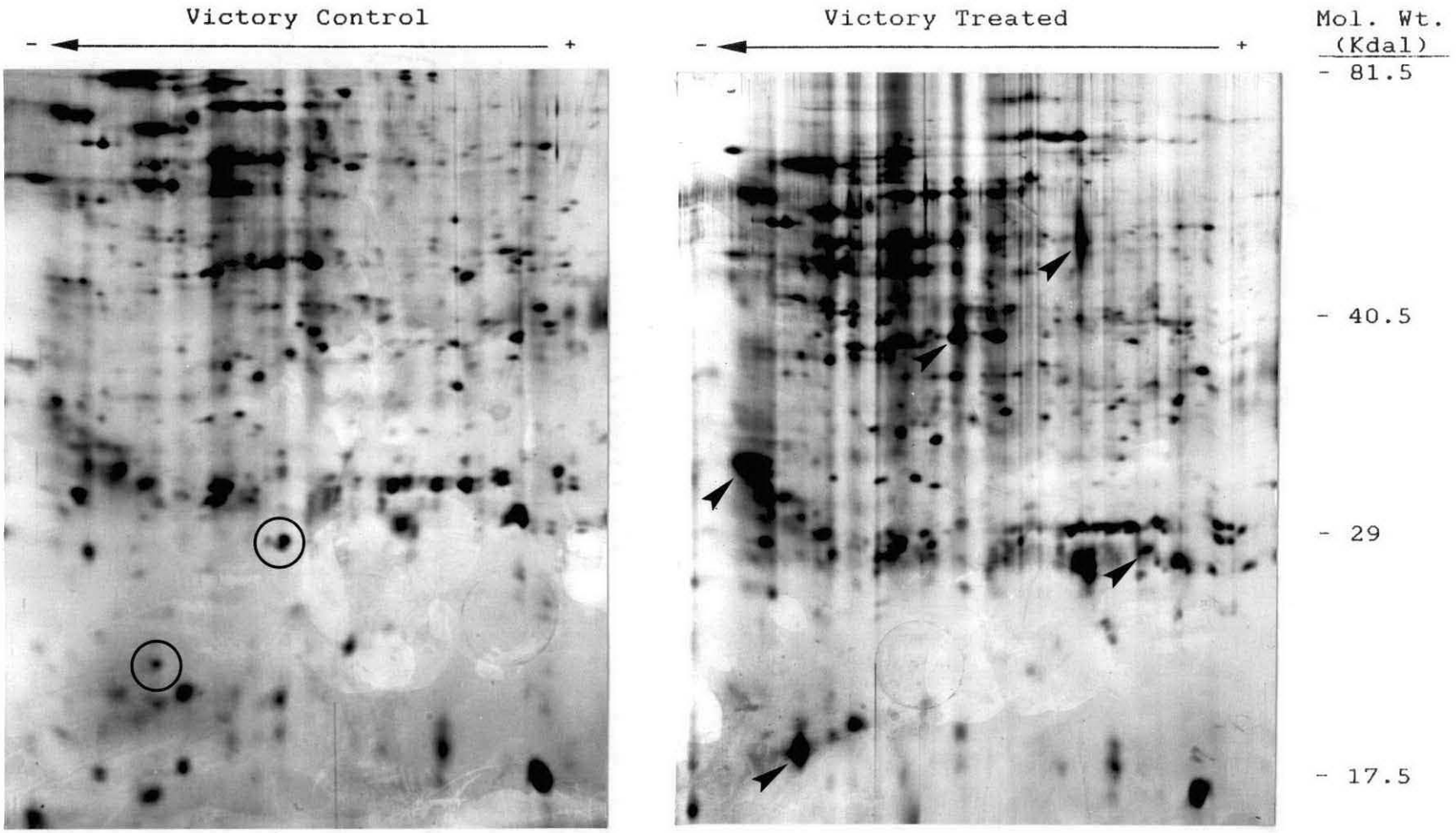

Figure 9. Two-Dimensional Gel Electrophoresis of Wheat cv Victory Cytoplasmic Proteins in Control and 2 ug/ml Al-Treated Root Tips

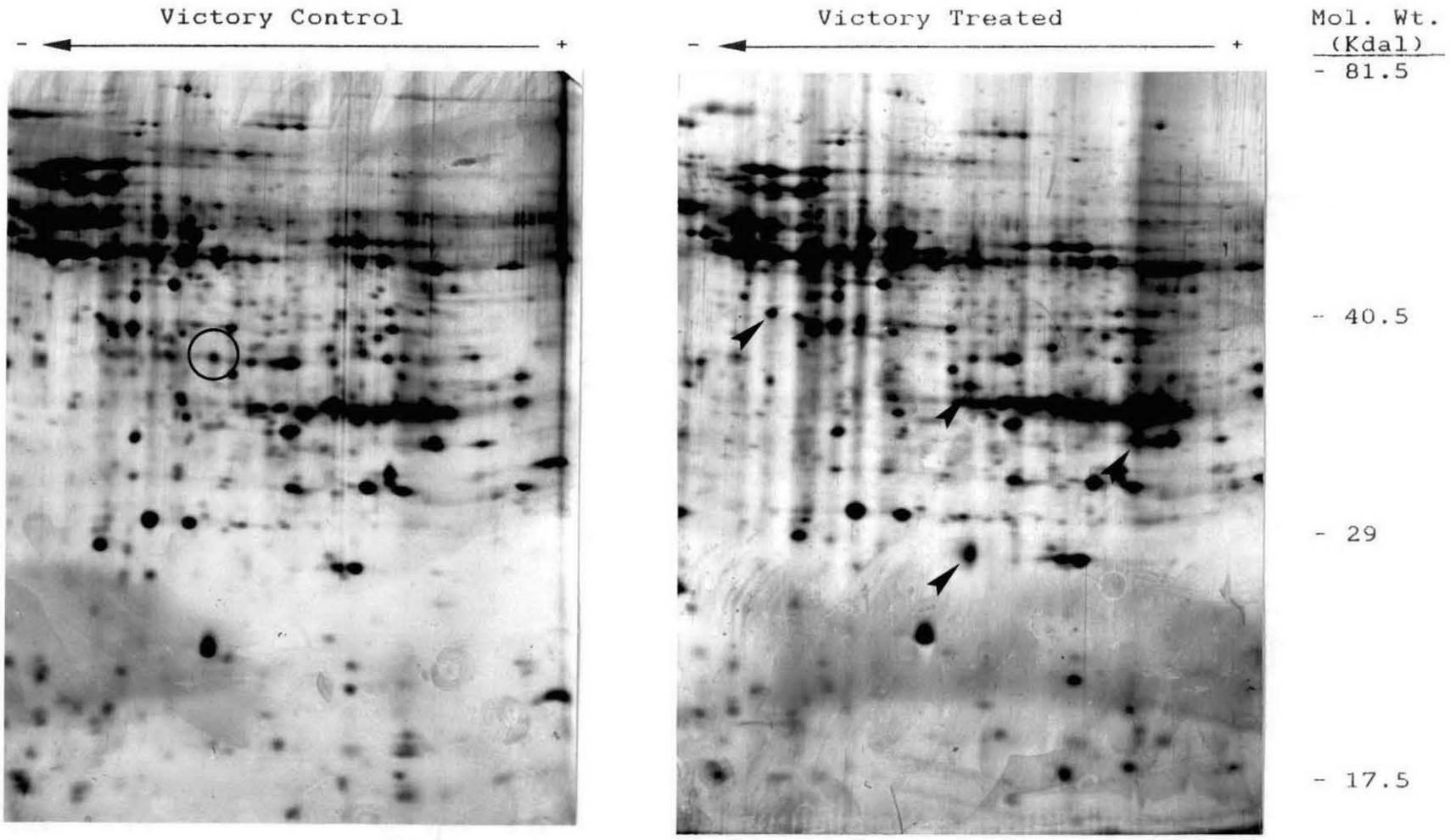

Figure 10. Two-Dimensional Gel Electrophoresis of Wheat cv Victory Microsomal Proteins in Control and 2 ug/ml Al-Treated Root Tips

response mechanisms or Al-specific response mechanisms. Victory is an Al-sensitive plant; growth of the cells in which these proteins appear in is irreversibly inhibited. If these same proteins appear in greater abundance in TAM W-101 or other Al-tolerant cultivars, they could represent proteins involved in an Al-tolerance mechanism. If they do not appear in the Al-tolerant cultivars, they might be part of the Al-toxicity mechanism.

Proteins that are present in the control and not in the treated fractions are also significant. For an Al-sensitive cultivar, these proteins represent a significant reduction or depletion of a cellular mechanism in only 24 hours. These proteins could play a critical role in the Al-toxicity mechanism.

This phase of this investigation has established the necessary conditions for conducting 2-D PAGE of wheat root meristem proteins. This procedure had not been previously utilized successfully at Oklahoma State University. The conditions that were optimized included the protein concentrations necessary for consistant resolution, the ratios and brands of ampholytes for IEF, the solubilization buffer required to keep the proteins in solution, and the staining methods that provided the highest resolution of proteins.

Two-dimensional PAGE had been demonstrated to be an effective tool in identifying changes in the genetic expression of proteins in a variety of organisms and

conditions (12, 21, 23, 39, 40, 48, 73,). This investigation has been the first step towards characterizing the effects of Al on proteins in wheat root meristems. A better understanding of the role these proteins play in Al-toxicity mechanisms can be achieved by sequencing the proteins of interest, isolating the genes which code for them, and locating these genes on the DNA.

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APPENDIXES

APPENDIX A

SOLUTIONS USED IN PROTEIN EXTRACTION

TABLE 1: EXTRACTION MEDIA FOR PROTEINS FROM ROOT MERISTEM TISSUE

Chemical	Concentration (mM)
Sucrose	250
Tris Buffer (pH 8.0)	25
EDTA	4
PMSF	2
DTT	2

TABLE 2: SDS-PAGE SAMPLE REDUCING BUFFER

Chemical	Volume (ml)
0.5M Tris-HCl (pH 6.8)	1.0
10% SDS	1.6
Glycerol	0.8
2B Mercaptoethanol	0.4
0.05% Bromophenol Blue	2.0
Distilled H_2O	4.0

APPENDIX B

STOCK SOLUTIONS FOR SDS-PAGE

SLAB GELS (LAEMMLI BUFFER SYSTEM)
[From Bio-Rad (10) with Modification]

A. Acrylamide/Bis (30% T, 2.67% C)
146 g acrylamide (29.2 g/100 ml)
4 g N'N'-Bis-methylene-acrylamide (0.8 g/100 ml)

Make to 500 ml with distilled water. Filter and store at 4°C in the dark (30 days maximum).

B. 1.5M Tris-HCl, pH 8.8
54.45 g Tris base (18.15 g/100 ml)
~ 150 ml distilled water

Adjust to pH 8.8 with 1N HCl. Make to 300 ml with distilled water and store at 4°C.

C. 0.5M Tris-HCl, pH 6.8
6 g Tris Base
~ 60 ml distilled water

Adjust to pH 6.8 with 1N HCl. Make to 100 ml with distilled water and store at 4°C.

D. 10% SDS

Dissolve 10 g SDS in water with gentle stirring and bring to 100 ml with dH_2O.

E. 5X Electrode (Running) Buffer, pH 8.3 (enough for 10 runs)

Tris base	45g	(15 g/l)
Glycine	216g	(72 g/l)
SDS	15 g	(5 g/l)

to 3 liters with dH_2O

Store at 4°C. Warm to 37°C before use if precipitation occurs. Dilute 300 ml 5X stock with 1200 ml dH_2O for one electrophoresis run.

APPENDIX C

TABLE 1: IEF SOLUTIONS BUFFER

Urea	-	9 Molar
NP-40	-	4 ml
DTT	-	0.01 ml
5/7 Biolyte	-	1.6 ml
3/10 Biolyte	-	0.4 ml

TABLE 2: 2-D STOCK SOLUTIONS FIRST DIMENSION IEF TUBE GELS

A. Triton X-100, 10 ml
H_2O, 90 ml

Stir in a beaker with 5 g AG 501-X8 mixed bed ion exchange resin. Store in brown glass bottle at room temperature.

B. Overlay Buffer

10% CHAPs X-100, 2.0 ml
Bio-Lyte 5/7, 0.45 ml
Bio-Lyte 3/10, 0.05 ml
ddH_2O, up to 10.0 ml

Store at 4°C in capped tube.

C. IEF Sample Concentrate

10% SDS, 0.1 ml (for certain applications, SDS may be omitted)
Bio-Lyte 3/10, 0.02 ml
Bio-Lyte 5/7, 0.18 ml
2-Mercaptoethanol, 0.1 ml
Triton X-100 (undiluted), 0.2 ml

Mix well and store at 4°C in capped tube. Be sure to vortex well before each use.

D. Electrolytes

Catholyte - NaOH, 4.2 g
H_2O to 1050 ml (0.1N NaOH)

Anolyte - 85% phosphoric acid, 3.06 ml
H_2O to 4.5 liters (0.06%)

The catholyte solution should be freshly degassed before use.

TABLE 3: PREPARATION OF FIRST DIMENSION IEF GELS

IEF Gel Solution

Urea, 8.1 g
ddH_2O, 4.8 ml
Acrylamide/Bis acrylamide (30% stock) 1.97 ml
10% Triton X-100, 3.38 ml
Bio-Lyte 5/7, 0.66 ml
Bio-Lyte 3/10, 0.17 ml

Warning:

Always wear gloves when performing this procedure to prevent exposure to acrylamide.

Degas for 15 minutes under vacuum. To the IEF gel solution add:

TEMED, 16 ul

Fresh 10% Ammonium Persulfate, 20 ul

TABLE 4: SEPARATING GEL PREPARATION - 0.375M TRIS, pH 8.8

	12% Acrylamide
Distilled water	33.5 ml
1.5M Tris-HCl, pH 8.8	25.0 ml
10% (w/v) SDS stock (store at room temperature)	1.0 ml
Acrylamide/Bis (30% stock) (Degas for > 15 minutes at room temperature)	40.0 ml
10% ammonium persulfate (fresh daily)	500 ul
TEMED	50 ul
TOTAL MONOMER+	100 ml

VITA

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