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Optimizing Growth in Low Ionic Strength Solutions and the Ameliorative Effects of Increased Ionic Strength on Copper Toxicity in *Triticum aestivum* (wheat).

by

Laura May Blair

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

Department of Biological Sciences

Edmonton, Alberta
Fall, 1997

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ABSTRACT

To obtain growth responses in the laboratory which are comparable to growth responses in soil, nutrient concentrations in solution culture experiments should closely resemble soil solution concentrations. Exponential growth of *Triticum aestivum* was optimized in low ionic strength solutions with nutrient concentrations that closely resemble soil solution concentrations using the relative addition rate (RAR) technique. This system was then used to determine if cation amelioration of copper (Cu) toxicity is a general effect of increased ionic strength or the result of specific cations. Increasing ionic strength by increasing background concentrations of plant nutrients significantly reduced Cu toxicity. In contrast, when ionic strength was increased by the addition of either Na, K, Ca or Mg, only Ca and Mg ameliorated Cu toxicity. This result suggests specific cations ameliorate Cu toxicity to a greater extent than can be explained by their effect on Cu^{2+} activity.

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LIST OF ABBREVIATIONS AND SYMBOLS

Graphite furnace atomic absorption spectrophotometry ..... GFAAS

Micro Siemen..... μS

Relative addition rate ..... RAR

Relative growth rate..... RGR

Sum of ..... Σ

1 GENERAL INTRODUCTION

It is well known that plant species differ in their tolerance to metals and the varying effects of metals on plant growth have been well documented. Despite the large body of literature documenting these differences, the physiological mechanisms of metal tolerance and sensitivity are still in question. Many studies have indicated that some species are resistant to levels of metals that would be toxic to other species (Zhu and Alva, 1993; Ouzounidou, 1995, Chiu *et al.*, 1995). Similar differences in metal tolerance within species have also demonstrated (Jan and Pettersson, 1993, Briggs *et al.*, 1989, Pettersson and Strid, 1989; Poschenrieder *et al.*, 1995). Differences in metal tolerance between or within species indicate adaptive responses to metals in the environment (Meharg, 1994; Taylor, 1988), or in the case of essential nutrients, may indicate an increased requirement for that metal (Welch, 1995). In either case, if the concentration and availability of metals in soils is increased, growth reductions can occur as a result of metal toxicity.

Although the phytotoxic impact of naturally occurring or anthropogenic inputs of trace metals depends on many factors, increases in surface soil concentrations and increased solubility of metals in acid soils are two of the major contributors to reduced plant growth in many parts of the world (Foy, 1984; Falkengren-Grerup, 1989; Ross, 1994a & b). Elevated concentrations of trace metals in the soil will not result in phytotoxicity unless metals are in the soil solution in a chemical form that plants can accumulate. Insoluble hydroxides, carbonates and organic complexes cannot normally be assimilated (Smith, 1994), while free ionic forms (eg. Cu^{2+}, Zn^{2+}, Mn^{2+}) are generally

available for absorption by plant roots (Welch, 1995). Assays of soil solutions have shown that free ionic forms of Cu^{2+}, Pb^{2+} and Mn^{2+} increases as the pH of the solution decreases (Reddy *et al.*, 1995; Temminghoff *et al.*, 1994). Increased availability of these metals at acidic pH can result in a reduction in plant growth in acid soils. For example, Smith (1994) demonstrated that decreased soil pH resulted in increased uptake of Ni, Cu and Zn and reduced growth of *Lolium perenne* (ryegrass). These results were attributed to increased metal solubility at lower pH. Similarly, Wilkinson and Duncan (1994) found that growth of *Sorghum bicolor* was significantly reduced in a Mn toxic soil as pH was reduced. The increased availability of these metal ions under acidic conditions has led many authors to suggest that acid-induced metal toxicity is a major growth limiting factor in acid soils in many parts of the world (Foy, 1984; Falkengren-Grerup, 1989).

1.1 THE STUDY OF METAL TOXICITY IN SOLUTION CULTURE

To gain a better understanding of the potential effects of metals on plant growth, an extensive amount of research has been conducted using solution culture techniques. Unfortunately, studies have used nutrient and metal concentrations many fold higher than levels found in fertile soils. For example, concentrations as high as 1.5 mM Mg^{2+} (Wang *et al.*, 1994) and 6.5 mM K^+ (Hino, 1995) have been used, even though typical soil Mg^{2+} concentrations range from 14 to 297 μM (Falkengren-Grerup, 1994) while soil K^+ ranges from 26 nM to 770 μM (Lindsay, 1979). Similarly, concentrations up to 10 μM Cd (Arduini *et al.*, 1994) and 160 μM Cu (Ouzounidou, 1994) have been used in metal

toxicity studies, although soil solution Cd is typically below 0.01 μM and soil solution Cu ranges between 0.47 and 4.7 μM (Bohn *et al.*, 1985). It is important to simulate natural soil solution concentrations in growth experiments, since use of high concentrations of nutrients or metals creates an artificial rooting environment which can potentially alter nutrient acquisition and uptake patterns (Ingestad and Lund, 1979). If experimental conditions mimic natural environments, plant responses should more closely resemble those which would be observed under natural conditions. Experimental results may then be extrapolated to natural ecosystems with more confidence (Brunet, 1994).

In addition to the concerns cited above, use of experimental solutions with high concentrations of plant nutrients (hence, high ionic strength) also results in reduced activity of metal ions in solution which decreases metal toxicity (Bard, 1966). Pavan and Bingham (1982) exposed *Coffea arabica* to five levels of nutrients at a single concentration of Al. Growth reductions were more severe at low nutrient levels, an effect which was attributed to increased Al^{3+} activity resulting from decreased ionic strength of growth solutions. In a similar study by Blamey *et al.* (1983), the toxic effect of 50 μM Al on root growth of *Glycine max* became more severe when the ionic strength of growth solutions was reduced. Similarly, Riedell and Schmid (1986) found that Mn uptake and toxicity in *Hordeum vulgare* increased when the ionic strength of growth solutions decreased. These results emphasize the importance of conducting metal toxicity studies using nutrient concentrations which resemble concentrations found in natural soil solutions.

To study plant response to metals under conditions which simulate soil solutions, plants must be grown in solutions with low nutrient concentrations (low ionic strength). However, several problems must be overcome if healthy growth of plants is to be maintained in low ionic strength solutions. These include meeting the nutrient demands of exponentially growing plants and adequate replenishment of nutrients removed by plant uptake. Although soil solutions are at low ionic strength, the exponential expansion of the root system explores an exponentially increasing soil volume, thereby maintaining nutrient supply. The increasing nutrient demand is met by the increasing root exploration of soil by the growing root system (Ingestad and Lund, 1979).

Several nutrient supply techniques, such as flowing solution culture, a non-recirculating flow-through hydroponic system and the relative addition rate (RAR) technique have been developed in an attempt to overcome the difficulties associated with the use of low ionic strength growth solutions (Asher *et al.*, 1965; Gutschick and Kay, 1991; Ingestad, 1972). Although a flowing solution culture system can provide plants with nutrient concentrations that resemble soil solution concentrations and facilitate maintenance of a constant test ion concentration (with frequent analysis and additions; Asher *et al.*, 1965), a number of problems may be encountered using this technique. When trying to impose a deficient treatment of any given nutrient, solution flow rates may have to be increased to a level that is not realistically achievable in order to prevent depletion of the test nutrient (Asher *et al.*, 1965; Asher, 1981). Similarly, high flow rates are required to meet the demands of exponential growth (Ingestad, 1982). Other

disadvantages of this technique are that specialized equipment is needed, high volumes of solutions are used and the technique is labour intensive due to the frequent solution changes which are required to prevent the depletion of basal nutrients (Asher *et al.*, 1965; Asher, 1981). The non-recirculating hydroponic system is another method developed for supplying nutrients at low levels (Gutschick and Kay, 1991). In this method, nutrient solutions are pumped through growth containers only once which limits pH shifts, microbial activity, and imbalances of nutrients. Unfortunately, non-recirculating systems are expensive to operate due to the high volumes of distilled water and nutrients which are required (Gutschick and Kay, 1991). In addition, it may be impractical to use radioactive tracers in this type of system.

1.2 THE RELATIVE ADDITION RATE (RAR) TECHNIQUE

The RAR technique is another method which has been developed to study the growth of plants in low ionic strength solutions The RAR technique can overcome problems associated with low ionic strength nutrient solutions without some of the problems associated with other methods. Adequate nutrient replenishment is achieved by frequent (daily or more often) nutrient additions to experimental containers. As solutions are not changed during the experimental period, this technique requires less labour, lower volumes of distilled water, and fewer nutrients. Nutrient additions can be exponential during the exponential phase of growth, then changed to a constant rate when plants pass out of exponential growth. In either case, the rate of nutrient additions can be tailored to

meet plant requirements (Ingestad, 1982).

In addition to overcoming some of the difficulties inherent in growing plants at low ionic strength, the RAR technique provides a means of growing plants with constant internal nutrient status (Ingestad and Lund, 1979). Maintenance of steady state nutrient status is important to ensure that the observed experimental results are due to the imposed experimental condition and not due to fluctuating internal nutrient status (Ingestad and Lund, 1986). Maintaining steady state nutrient status requires that nutrients be supplied in proportions that are found in plants at maximum growth (Ingestad and Lund, 1979; 1986). Correct proportions must be provided to prevent unseen or unwanted accumulation or depletion of any given nutrient (Ingestad and Lund, 1986). For example, Ingestad (1981) found that potassium (K^+) accumulated in solutions supporting growth of grey alder when K^+ additions were greater than plant requirements, while phosphorous (P) concentrations declined when P supply was insufficient to meet nutrient demands. When determining optimal nutrient proportions, one must recognize that some plants may accumulate high internal concentrations of nutrients even though they are not required for growth. For example, Ingestad (1972) found that Ca accumulated in old cucumber leaves resulting in reduced growth rates and deficiency symptoms in young shoots due to depletion of Ca from nutrient solutions.

In addition to correct nutrient proportions, nutrient additions must be sufficient to meet the demands of exponential growth if a constant relative growth rate (RGR) is to be

maintained. Exponentially increasing amounts of nutrients must be added to meet the requirements of exponentially growing plants (Ingestad and Lund, 1986). If plants have passed the exponential phase of growth, or if they do not show an exponential phase of growth, nutrient additions must be adjusted to a rate which meets nutrient uptake, maintains steady state growth and prevents accumulation or depletion of nutrients in growth solutions (Ingestad and Agren, 1988).

Another benefit of the RAR technique is that pH fluctuations in growth solutions can be reduced by balancing cation and anion uptake. Cation and anion uptake are normally dominated by the need to acquire nitrogen (N). Cation uptake (primarily ammonium, NH_4^+) releases H^+, decreasing solution pH. Anion uptake (primarily nitrate, NO_3^-) releases HCO_3^-, increasing solution pH (Loneragan, 1979). By varying the NH_4^+/NO_3^- ratio of growth solutions, one can balance cation and anion consumption without changing overall nutrient proportions (Ingestad and Lund, 1988; Stadt *et al.*, 1992). However, increasing the proportion of NH_4^+ can result in NH_4^+ toxicity (Ericsson, 1981; Ingestad and Stoy, 1982; Stadt *et al.*, 1992), so care must be taken to keep the NH_4^+ proportion at a non-toxic level.

1.2.1 Theory for Exponential Growth

Exponentially growing plants increase in weight according to the following formula:

1) $$W_t = W_0 \cdot e^{RGR(t-t_0)}$$

where W_t is plant weight at time t and W_0 is the initial plant weight at time = 0 (t_0), the start of the measurement period and RGR is the relative growth rate (Stadt *et al.*, 1992). The relative growth rate (RGR) is determined by:

2) $$RGR = dW/(dt \cdot W)$$

where t is time and W is plant weight (Stadt *et al.*, 1992). A nutrient that remains at a stable concentration in exponentially growing plants will increase as:

3) $$Nutr_t = C \cdot W_0 \cdot e^{RGR(t-t_0)}$$

where $Nutr_t$ is the amount of nutrient (in grams) present in the plant at time t and C is the plant nutrient content (g nutrient g plant^{-1}) (Stadt *et al.*, 1992).

The increasing nutrient demand (equation 3) of exponentially growing plants (equation 1) can be met by a relative addition rate (RAR, g nutrient g plant nutrient^{-1} unit time^{-1}; Ingestad and Lund, 1986). The relative addition rate and relative growth rate are analogous and must be equal in order to maintain steady state internal nutrient status (Ingestad and Lund, 1986). The RAR can be adjusted to support a RGR less than or equal to the maximum potential RGR (R_{max}). When RAR < R_{max}, the plant RGR declines

to match the RAR. Thus, it is possible to control the growth rate at a constant level of nutrient stress or at maximum growth while maintaining a steady nutritional state (Ingestad and Lund, 1986; Stadt *et al.*, 1992). In either case, the amount of nutrient addition required to support growth for a given time interval (A_t) can be determined by:

4) $$A_t(g) = C \cdot W_0 \cdot e^{RAR(t-t_0)}[e^{RAR}-1]$$ or

5) $$A_t(mol) = [C \cdot W_0/M]e^{RAR(t-t_0)}[e^{RAR}-1]$$

where M is the molecular weight of the nutrient (Stadt *et al.*, 1992). Nutrient additions can be calculated for nitrogen (N) alone and the remainder of the nutrients supplied in proportion to N (Ingestad, 1981; Stadt *et al.*, 1992).

The RAR technique provides a means of studying the growth of plants under low ionic strength conditions while maintaining steady-state internal nutrient status. With this technique, we can examine the response of plants to metals under conditions of nutrient supply which more closely resemble natural soil solutions.

1.3 CATION AMELIORATION OF METAL TOXICITY

High ionic strength growth solutions have been shown to reduce metal toxicity (Pavan and Bingham, 1982; Riedell and Schmid, 1986; Blamey *et al.*, 1983); however,

several studies have suggested that alleviation of metal toxicity by increased concentrations of specific cations cannot be attributed solely to reduced ion activity which accompanies increased ionic strength. For example, Alva *et al.* (1986a,b) observed an ameliorative effect of increased Ca on growth of *Glycine max*, *Helianthus annuus*, *Medicago sativa* and *Trifolium subterraneum* when grown at a constant Al^{3+} activity, which in effect removes ionic strength effects. In a similar study, Horst (1987) concluded that the ameliorative effect of increased Ca supply on Al toxicity could only be partially explained by decreasing Al^{3+} activities in growth solutions. Improved growth of two strains of *Chlamydomonas reinhardtii* was observed when H^+ concentrations were increased and ionic strength effects were removed by expressing metal availability (Cd, Co, Cu or Ni) as the concentration of the divalent ion in solution (Macfie *et al.*, 1994). Zhu and Alva (1993) also observed improved growth of *Citrus paradisi* x *Poincirus trifoliata* when the H^+ activity was increased and Cu^{2+} activity was kept constant. These studies suggest that reduced ion activities which accompany increases in ionic strength can only in part explain cation amelioration of cation toxicity.

While numerous studies have demonstrated cation amelioration of cation toxicity, the mechanism by which this amelioration occurs is still in question. Borst-Pauwels and Severens (1984) hypothesized that shifts in patterns of cation uptake could occur when inhibitory cations compete with substrate cations for carrier sites and negative binding sites on cell membranes. They proposed that the magnitude of this competition is related to the concentration of the inhibitory and substrate cations, the ionic strength of the

solution and/or the charge density of the cell membrane. They also suggested that cell membrane surface potentials in *Saccharomyces cerevisiae* were decreased due to screening of negative groups and specific binding of divalent cations, which could in turn reduce cation uptake (Borst-Pauwels and Severens, 1984).

Kinraide and Parker's (1987) external-binding-site hypothesis, which attempts to explain cation amelioration of cation toxicity, is similar to the hypothesis of Borst-Pauwels and Severens (1984). They suggested that cation amelioration reflects competition for cell-surface binding sites, with binding specificity determined by the charge of the cation. Competition for binding sites would reduce binding by the toxic cation, which would result in reduced toxicity. In a study of cation amelioration of Al toxicity, Kinraide and Parker (1987) concluded that the ameliorative effect of specific cations could be attributed to their affinity for cell-surface binding sites. Cations of higher valency appeared to have a greater affinity for binding sites and a greater ameliorative effectiveness ($Ca^{2+} > Mg^{2+} = Sr^{2+} > K^{+} = Na^{+}$; Kinraide and Parker, 1987).

Refinements to the external-binding-site hypothesis were made when new data indicated that reduced negativity of the cell-surface electrical potential contributed to cation amelioration. Negative charges located on carboxylate groups of cell wall pectins and plasma membranes, and on acidic amino acid residues and phosphate groups of phospholipids, create a cell surface electrical potential which affects ion distribution at or near the cell wall and plasma membrane (Kinraide *et al.*, 1992). The electrical potential

gradient (negative surface potential) created by cell surface charge can be reduced by i) divalent or polyvalent cation binding to the negative sites or, ii) by charge screening which occurs because coulombic attractions concentrate cations around cell-surface negative charges (Kinraide *et al.*, 1992). A reduction in the negative surface potential could result in reduced affinity of cell surfaces for toxic cations, which could in turn result in reduced toxicity.

A number of studies have suggested that interactions of cations with cell surfaces may play a role in the amelioration of toxic cations. In *Chlorella pyrenoidosa*, Parent *et al.* (1996) observed that a reduction in Al^{3+} toxicity was not proportional to reduced Al^{3+} activity in the presence of increasing fulvic acid concentrations. Reduced Al^{3+} toxicity was attributed to the binding or interaction of fulvic acid with the membrane surface which prevented binding of Al^{3+}. In an attempt to determine the mechanism of reciprocal alleviation of toxic cations (Al^{3+} and H^{+}), Kinraide (1993) measured changes in transmembrane electrical potential in *Triticum aestivum* in response to changes in pH and Al concentration. The presence of Al^{3+} helped to maintain the transmembrane electrical potential difference at low pH, which suggested that cell-surface negativity played a role in reciprocal alleviation of cation toxicity. Kinraide concluded that when two toxic cations were present, one cation reduced the cell-surface activity of the second cation, thus reducing its toxicity. The toxicity of the first cation is also reduced when the second cation simultaneously reduces the cell-surface activity of the first cation.

Additional experiments by Kinraide *et al.* (1992) and Kinraide (1994) testing the ameliorative effectiveness of cations on Al^{3+} toxicity indicated that rhizotoxicity was ameliorated by cations in the order $H^+ = C^{3+} > C^{2+} > C^+$. When membrane surface activities and activities of toxic cations in external growth solutions were calculated, membrane surface activity correlated well with growth inhibition, while no correlation was found with cation activity in the external medium (Kinraide *et al.*, 1992; Kinraide, 1994). These results suggested that accumulation of cations on negative cell surfaces ameliorates Al^{3+} toxicity by reducing the negativity of the cell-surface electrical potential by either cation binding or charge screening (Kinraide *et al.*, 1992; Kinraide, 1994).

While increased ionic strength of growth solutions can alleviate metal toxicity, amelioration can not be fully explained by decreased ion activity of the bulk solution which accompanies increased ionic strength. Ameliorating cations may reduce the negativity of the cell-surface electrical potential, which in turn reduces the deleterious effect of toxic cations. Unfortunately, direct experimental evidence to this hypothesis is still lacking. The overall objective of this study was to determine if cation amelioration is a general effect of increased ionic strength or the result of the actions of specific cations. Specific objectives were to; i) optimize growth of *Triticum aestivum* in low ionic strength growth solutions, utilizing the relative addition rate (RAR) technique, ii) utilize the optimized RAR system to determine if increasing ionic strength affects the growth response of *Triticum aestivum* to increased Cu^{2+} supply and iii) determine if any reductions in Cu^{2+} toxicity could be attributed to specific cations.

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2 OPTIMIZATION OF GROWTH OF *TRITICUM AESTIVUM* IN LOW IONIC STRENGTH NUTRIENT SOLUTIONS UTILIZING THE RELATIVE ADDITION RATE TECHNIQUE AND A COMPUTER-CONTROLLED NUTRIENT DELIVERY SYSTEM

2.1 INTRODUCTION

The majority of solution culture experiments to date have been conducted with nutrient concentrations many fold higher than levels found in most fertile soils. In order to produce growth responses in the laboratory that are similar to growth responses found in soil, it has been argued that nutrient concentrations in solution culture experiments should approximate those found in soil solutions. Unfortunately, the use of low nutrient concentrations in solution culture introduces technical difficulties associated with meeting the nutrient demands of exponential growth. This has necessitated the development of new methods such as flowing solution culture, programmed nutrient addition, and the relative addition rate (RAR) technique. Each of these methods attempts to meet the nutrient demands of exponentially growing plants, while providing nutrients in a manner which simulates the nutrient availability and nutrient acquisition that occurs in soil solutions.

To grow healthy plants in nutrient solutions with nutrient concentrations similar to soil solutions (low ionic strength) certain issues must be addressed. One must determine the relative proportion of nutrients that is required to provide optimal growth throughout

the experimental period. Since rapid nutrient depletion is a problem in low ionic strength solutions, nutrients must also be replenished at a rate that meets plant requirements. In addition, background nutrients must be provided at a concentration which allows a rate of uptake that supports healthy growth. Finally, pH fluctuations should be minimized to limit any detrimental effects of pH on growth.

Depending on the experimental species, it may be possible to determine the appropriate nutrient proportions from previously published research, with slight modifications to suit particular experimental conditions. If relevant information on a given species is not available, essential nutrients and optimal concentrations can be determined by referring to data for similar species, then testing the suitability of these conditions for the new species by performing growth studies. While most plant species require the same essential nutrients, some species may require additional elements that are detrimental to healthy growth of others. For example, halophytes require salt (NaCl) concentrations (Yeo, 1983) that are toxic to species such as citrus and snapbean (Storey, 1995; Awada *et al.*, 1995). In addition to providing essential nutrients, the concentrations and relative abundance of nutrients supplied are also important for sustaining healthy growth throughout the experimental period. For example, Ingestad (1981) showed that alder requires lower potassium and higher phosphorous concentrations than birch in order to maintain healthy growth in low conductivity nutrient solutions. Nutrient concentrations can be optimized for a particular species, but it is necessary to ensure they are appropriate for any given experimental conditions.

Low ionic strength growth solutions do not provide a reserve of nutrients that are available in traditional solution culture or in soil solutions. Therefore, nutrients must be replenished regularly to compensate for their loss as a result of plant uptake. The relative addition rate technique, which is used in this study, is a method by which replenishment is accomplished by daily (or more frequent) nutrient additions to growth solutions (Ingestad, 1982). Nutrients can be added at either an exponential rate during the period of exponential growth, or a constant addition rate where no exponential growth phase exists, or where a constant growth phase has started.

While daily additions furnish the nutrients required for growth, it is necessary to ensure the total ion concentration of the solution is sufficient to maintain healthy growth. Epstein (1976) showed that the rate of ion absorption from solution is related to the concentration of ions in the solution. When the rate of absorption of an ion is examined as a function of the external concentration of the ion, the rate of absorption of the ion increases as the external concentration increases. In low ionic strength solutions, healthy growth can only be achieved when a minimum concentration of nutrients has been provided. This required concentration would depend not only on the experimental species, but on physical factors such as the extent of stirring of the solution and the resistance of the boundary layer to nutrient diffusion (Stadt *et al.*, 1992) Ultimately, the initial solution nutrient concentration (background concentration) must be high enough to ensure the rate of ion absorption is adequate for healthy growth under the specific experimental conditions.

Once nutrient proportions and background concentrations have been determined for the experimental species, relative growth rate (RGR) can be controlled by the RAR of nutrients throughout the experimental period. If the relative growth rate and relative nutrient addition rate are equal and the uptake of cations and anions is balanced, there should be no accumulation of nutrients in the growth solution and solution pH should remain stable. To ensure that nutrient build up is not occurring and pH fluctuations are kept to a minimum, growth solutions must be monitored regularly. Measurement of pH is easily accomplished using a pH meter. Although nutrient build up could be monitored by measuring individual ion concentrations, this is labour intensive and requires a variety of analytical tools. A simple and effective alternative is to monitor electrical conductivity (EC), which is linearly related to the cation concentration of the solution (Yasuda, 1996). Increases in EC readings indicate an accumulation of nutrients.

In my study, a computer-controlled nutrient delivery system and the RAR technique were utilized to grow plants in a solution culture system that closely resembled soil solution concentrations. The overall objective of this portion of my research was to optimize the RAR technique for growth of *Triticum aestivum* in low ionic strength nutrient solutions. For the purposes of this study, optimal conditions were defined as those where the relative growth rate of plants was controlled by the relative addition rate of nutrients, with no accumulation of nutrients in the solution (stable EC), and minimal pH fluctuations. This overall objective was achieved by; i) adjusting nutrient proportions until the addition of nutrients controlled growth at a rate equal to the nutrient addition rate

(hence maintaining EC), ii) determining the background nutrient concentration which will support a rate of ion absorption which will maintain healthy growth, and iii) reducing pH fluctuations by optimization of the ammonium/nitrate (NH_4^+/NO_3^-) ratio in growth solutions.

2.2 MATERIALS AND METHODS

2.2.1 Computer-Controlled Nutrient Delivery System

A computer-controlled nutrient delivery system was utilized to provide an accurate, daily supply of nutrients to experimental containers. Two Watson Marlow multi-channel peristaltic pumps (model 202S) deliver nutrient solutions from three nutrient reservoirs to three sets of 60 valves. One set of 60 valves supplies one nutrient solution to each of 60 growth containers. When open, each valve controls the flow of one solution to one growth container. When the valve is closed the nutrient solution circulates back to the nutrient reservoir. The system allows independent delivery of three separate solutions to each growth container. Nutrient delivery (opening and closing of valves) is computer-controlled by a software program which calculates nutrient additions (A_t) with the following formula:

$$A_t(\text{mol}) = [C \cdot W_0/M]\, e^{RAR(t-t_0)}\, [e^{RAR} - 1]$$

where C is plant nutrient content (g nutrient g plant^{-1}), W_0 is initial plant weight at t_0, the start of the addition period, M is the molecular weight of the nutrient and t is the day of nutrient addition. The information required for this calculation is supplied by the operator in a computer log file specific for each experiment. Nutrients can be delivered at a constant or relative addition rate.

2.2.2 Experimental Design

In this series of experiments, two experimental designs were used; i) a time course and ii) a factorial treatment. For all experiments, nutrients were added at a 20% relative addition rate (0.2 g nutrient g plant^{-1} day^{-1}). Preliminary experiments achieved growth rates slightly higher than 20%; thus a 20% RAR was selected to ensure growth could be controlled by the rate of nutrient additions. All experiments utilized a randomized block design with three or four statistically independent replicates, for a total of 60 containers. Each experiment was performed at least twice.

Time course experiments were conducted for 21 to 30 days. To accurately determine the relative growth rate, plants were harvested every second day during the experimental period and dried to a constant weight. Dry weights (g pot^{-1}), were converted to natural logs, graphed as weight vs time including a best fit regression line. The slope of the regression line represents the average RGR for the plotted points. Alternatively, the RGR could be calculated using the following formula:

$$W_t = W_0 \cdot e^{RGR(t-t_0)}$$

Where W_t is plant weight at time t, W_0 is initial weight at t_0, the start of the measurement period. Experiments with factorial treatments consisted of combinations of NH_4^+ treatments x cultivars x replicates which are indicated in the description of each experiment. At the end of the experimental period (9 to 21 days), plants were harvested and dried to a constant weight.

The proportions of nutrients in initial growth solutions (Table 2.1) were designed based on proportions used by Ingestad and Stoy (1982), Pettersson and Strid (1989), and Stadt *et al.* (1992). Nutrient proportions are expressed as percent by weight of nitrogen (N = 100). Background nutrient levels are expressed as a concentration of N (μmol N L^{-1}). Background solutions contain that concentration of N plus the remainder of the nutrients in the weight proportions specified in Table 2.1.

2.2.3 General Growth Techniques

2.2.3.1 Nutrient Solutions

Four nutrient *stock solutions* were made up in distilled deionized water (< 18 m ohm) and stored in acid washed bottles. Three nutrient *delivery solutions* (as delivered by the computer-controlled system) were prepared by diluting four stock solutions ten fold

with deionized water. Stock solutions 1 and 4 were diluted separately to prevent precipitation of the delivery solutions while stock solutions 2 and 3 were combined to prepare one delivery solution (delivery solution 2). Nutrient delivery solutions varied with experimental design (Tables 2.2 to 2.5) and were used to make up all pre-treatment growth solutions, experimental solutions, and for daily nutrient additions.

2.2.3.2 Preparation of Plant Material (The PreTreatment Period)

Seeds of *Triticum aestivum* cv. Katepwa were surface sterilized in a 1.1% solution of sodium hypochlorite (v/v) for 20 minutes and germinated overnight in an aerated solution containing 0.005 g L^{-1} Vitavax (Uniroyal Chemical Ltd., Calgary, AB, Canada) to limit fungal growth. Seeds were then grown in aquaria (300 seeds each) on nylon mesh suspended over an aerated solution containing background nutrients (50, 200 or 500 µmol N L^{-1}), adjusted to pH 4.3 with 1.0 or 0.1 M HCl. The seedlings were thinned after 3 days, to 150 plants per aquaria. At this time, 12 seedlings were dried for two hours (to constant weight) at 60°C to determine initial dry weight (W_0) for calculation of nutrient additions for the remainder of the pre-treatment period. Nutrient additions for each day (A_t) were calculated using the formula outlined in section 2.2.1. Calculated nutrient additions were reduced by half after experiment 5, as seed reserves were providing seedlings with sufficient nutrients which accelerated the relative growth rate beyond desired levels.

As soon as plants were large enough to separate from the seed (about day 3 or 4) samples (n=8) were collected, dried, and weighed to determine RGR during the pre-treatment period. The dry weight of plants for the final day of the pre-treatment period was used to calculate W_0 for calculation of nutrient additions for the experimental period.

2.2.3.3 Experimental Period

After nine days, spent seeds were removed from plants and 8 uniform seedlings were transferred to each of 60 polyethylene containers (experimental solutions) filled with 10 L aerated growth solution. The growth solutions were prepared by computer delivery of an appropriate volume of the nutrient delivery solutions to 10 L of distilled water to achieve a background concentration of 50, 200 or 500 μmol N L^{-1}; thereafter, nutrients were supplied in exponentially increasing quantities 1-4 times per day. Seedlings were suspended on opaque Plexiglas covers, which were placed over the containers to inhibit algal growth. Distilled water was added periodically to the nutrient solutions to maintain a volume of 10 L (to compensate for water loss by evaporation and transpiration). Containers were suspended in a common water bath to limit temperature fluctuations and maintain a constant temperature across all experimental containers.

Optimization of growth conditions required that pH and electrical conductivity (EC) remained stable during the experimental period. Solution pH and EC were monitored periodically with a Radiometer pHM80 portable pH meter and a Radiometer

CDM80 portable electrical conductivity meter with a CDC104 probe. Meters were calibrated prior to every use to ensure consistent readings. Measurements were taken prior to planting, three times per week (just before nutrient additions), and immediately after harvest.

Experiments were conducted in two controlled-environment growth chambers, with 16 hr light and 8 hr darkness. Temperatures for the light period ranged from 20 to 24°C and from 16.7 to 19.5°C during darkness. Relative humidity was between 50 to 84% for the light period and 75 to 100% for the dark period. Solution temperatures varied between 19 and 23°C for the light period and 18 to 21°C during darkness. The growth chamber was illuminated by 103 cool white fluorescent lamps (25W), and 16 incandescent lamps (150W), located 1.3 m above plant bases. Photosynthetic photon flux (PPF) averaged between 332 and 471 μmol m^{-2} sec^{-1} for this series of experiments. Plants were harvested the end of the experimental period, rinsed in distilled water, separated into roots and shoots, and dried to a constant weight at 60°C.

2.2.4 Growth Techniques by Experiment

2.2.4.1 Experiment 1 (Nutrient Proportions)

This 30 day, time course experiment utilized a 200 μmol N L^{-1} background containing (μmol L^{-1}) 200 N, 5.72 P, 59.6 K, 14.28 Ca, 11.44 Mg, 2.84 S, 3.6 x 10^{-1} Fe,

2.08×10^{-1} Mn, 5.16×10^{-1} B, 5.57×10^{-2} Zn, 1.152×10^{-2} Cu, and 2.0×10^{-3} Mo. Nutrient proportions by weight were (N = 100), P = 6, K = 84, Ca = 20, Mg = 10, S = 0.033, Fe = 0.7, Mn = 0.4, B = 0.2, Zn = 0.06, Cu = 0.03, and Mo = 0.07. With the exception of phosphorous (P), these nutrient proportions are similar to those used by Ingestad and Stoy (1982), Pettersson and Strid (1989), and Stadt *et al.* (1992). The P level was reduced as phosphate has been shown to have an effect on metal toxicity. For example, it has been found to reduce Al toxicity, presumably by polymerization or precipitation of Al in studies with soybean (Blamey *et al.*, 1983). In another study Greipsson (1992), found that increased P concentrations increased toxicity of copper in rice. As this system was being optimized for the study of metal toxicity, P was kept at a lower proportion (P=6) than the proportions utilized by Ingestad and Stoy (P=13) (1982) and Pettersson and Strid (P=25.5) (1989).

In this experiment, plant RGR fell below the RAR of plant nutrients, thus nutrient solutions taken from containers on day 16 and day 22 were analyzed to determine if the N/P ratio had changed from the ratio of delivery solutions. Analyses of total P, NH_4^+/N, and NO_3^-/N were performed by the University of Alberta Limnology Laboratory using the methods described by Menzel and Corwin (1965), Stainton *et al.* (1977), and Bierhirizen and Prepas (1985), respectively. The N/P ratio was more than two-fold higher in the growth solutions than in delivery solutions (data not shown), indicating that P was being depleted at a greater rate than N.

2.2.4.2 Experiment 2 (Fine Tuning Nutrient Proportions)

In an attempt to overcome a possible P deficiency (suggested by the results of the first experiment) the P concentration was increased in the 200 µmol N L^{-1} background solution and delivery solutions for this 30 day time course experiment. This was achieved by increasing KH_2PO_4 as indicated in Table 2.3. Nutrients were changed to: (µM) 13.44 P, 73.2 K, and 5.72 S (weight proportions P = 15, K = 102.5, S = 0.065), with all other nutrients supplied at the same concentrations as in experiment 1.

2.2.4.3 Experiment 3 (Background Nutrient Concentrations)

This experiment was designed to determine if the overall growth rate would be affected by the initial background concentration of nutrients. The experiment utilized a 200 µmol N L^{-1} background for the pre-treatment period and 15 background concentrations (50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900 and 1,000 µmol N L^{-1}) for the 21 day experimental period. All nutrients were supplied in the same weight proportions as in experiment 2.

2.2.4.4 Experiment 4 (Time Course - 500 µmol L^{-1} Background)

This experiment was used to determine if a 20% relative growth rate could be maintained with a 500 µmol N L^{-1} background and a 20% RAR over a 22 day

experimental period. No changes were made to the nutrient proportions.

2.2.4.5 Experiment 5 (Calcium Background)

This experiment was the first of two separate experiments designed to determine if increasing the background calcium concentration could reduce the need for high background levels of other nutrients. In this experiment, the effect of background Ca on growth was examined. Calcium was added from a 1.0 M $CaCl_2$ stock to a 200 µmol N L^{-1} background to bring the final Ca concentration to 0.095, 0.195, 0.4, 1.0 and 2.0 mM. Plants were grown for nine days.

2.2.4.6 Experiment 6 (Background Nutrients plus 0.4 mmol L^{-1} total Calcium)

This 21 day experiment was designed to determine if a total concentration of 0.4 mM Ca in the pre-treatment (200 µmol N L^{-1}) and experimental (50 to 1,000 µmol N L^{-1}) background solutions could reduce the need for other background nutrients. In comparison to experiment 3, no other changes were made to the nutrient proportions supplied.

2.2.4.7 Experiment 7 (Nitrogen Source)

This experiment was designed to determine what NH_4^+/NO_3^- ratio would produce

the smallest pH change (plant-induced) without causing a reduction in growth. A 50 μmol N L^{-1} background with a total concentration of 0.4 mM Ca was utilized for the pre-treatment and experimental periods. Ten NH_4^+/NO_3^- treatments (5, 10, 15, 20, 25, 30, 35, 40, 45, and 50% NH_4^+ as a per cent of total nitrogen) plus five sodium chloride (NaCl) treatments (delivery solution concentrations of 0.7, 1.4, 2.1, 2.8, and 3.5 mol L^{-1} superimposed over a 25% NH_4^+/N treatment) were utilized for this 21 day experiment (Appendix 5.1). The sodium chloride treatments were included to control for expected changes in the Na and Cl counter ions and to confirm that any observed growth reductions could not be attributed to increased concentrations of Na or Cl in the growth solution. This was necessary as ammonium chloride (NH_4Cl), sodium nitrate ($NaNO_3$) and magnesium chloride ($MgCl_2$) were used in the treatment solutions to achieve the appropriate NH_4^+/NO_3^- ratios. Other stock solutions were not altered.

For all nitrogen source (NH_4^+/NO_3^- ratio) experiments, delivery solution 1 was replaced with separate nutrient solutions for each N or sodium chloride treatment. Daily additions of the N and NaCl solutions were fed by hand as the nutrient delivery system is limited to delivery of three solutions.

2.2.4.8 Experiment 8 (Cultivar Screening)

Control of solution pH by increasing NH_4^+/NO_3^- ratios could not be achieved without leading to NH_4^+ toxicity in cv. Katepwa. Thus, this experiment tested eight wheat

cultivars (Cutler, PT741, Roblin, Katepwa, Oslo, Atlas 66, Maringa and Park) to determine if one of these cultivars might be less sensitive to NH_4^+ than Katepwa (which had been utilized for all previous experiments). Cultivars were grown with a 50 µmol N L^{-1} background, a total of 0.4 mM Ca and three NH_4^+/NO_3^- ratios (20, 35, & 45% NH_4^+ as percent of total N) for 21 days. Nutrient delivery solution 2 was altered to reduce the K weight per cent from 102.5 to 82 (Table 2.4) to achieve a K concentration closer to the initial nutrient solution design. The remainder of the nutrient proportions were unchanged.

2.2.4.9 Experiment 9 (Atlas 66 - Cutler Screening)

Experiment 8 demonstrated that cultivars Atlas 66 and Cutler were most resistant to NH_4^+ toxicity. This experiment was designed to determine if the growth response of Atlas 66 and Cutler varied over a range of NH_4^+/NO_3^- ratios, including 20, 22, 24, 26, 28, 30, 32, 34, and 36% NH_4^+. The background for both the pre-treatment and experimental period was 50 µmol N L^{-1} with a total Ca of 0.4 mM.

2.2.4.10 Experiments 10, 11 and 12 (Optimization of Background Nutrients, Nitrogen Source, and Time Course of Growth for Atlas 66)

These experiments utilized Atlas 66 and were repeats of experiments previously performed with Katepwa. These experiments were repeated to ensure the growth

response of Atlas 66 was optimal under the conditions optimized for Katepwa. The experimental designs of experiments 10 and 11 were replicates of experiments 6 and 7, respectively. A 50 μmol N L^{-1} background plus Ca to total 0.4 mM was utilized for the pre-treatment period for all three experiments and for the experimental period for experiments 11 and 12. Experiment 12 was a 21 day time course experiment to determine the RGR. This final experiment utilized 36% NH_4^+/NO_3^- with no other changes to the nutrient proportions.

2.2.5 Data Analysis

Means and standard errors were calculated for each set of replicates for plant weight, pH, and EC, then graphed. All graphs were plotted using Jandel Scientific Sigmaplot 3.02. For time course experiments, dry weight means (g pot^{-1}) were log transformed, and plotted with a first order regression line along with the expected growth (RGR = RAR) regression line.

2.3 RESULTS AND DISCUSSION

2.3.1 Experiment 1 (Nutrient Proportions)

During the pre-treatment period, the 36.8% RGR (average) exceeded the 20% RAR (Fig. 2.1A). This higher rate of growth was probably due to mobilization of reserves

from seeds which provided the plants with additional nutrients above those supplied by nutrient additions. The RGR was log linear during the experimental period, but the rate (13.4%) was lower than the RAR (20%). This is illustrated by the greater slope of the expected growth line (based on a 20% RGR) compared to the slope of the observed growth (Fig. 2.1A). This inhibition of growth was accompanied by visible signs of stress. During the first week of growth, roots appeared brown and brittle with new growth being white and appearing healthy for the remainder of the experimental period. These visible signs of stress and the fact the RGR was less than the RAR, suggested that there was a deficiency of one or more nutrients in the growth solution.

With the RAR technique, reduced growth results in nutrient additions exceeding nutrient uptake. This leads to nutrient accumulation and hence an increased availability of NH_4^+ in growth solutions. As NH_4^+ is preferentially assimilated by plants and assimilation is accompanied by a release of H^+ (Loneragan, 1979), increased uptake results in a decrease in pH. Thus, reduced growth could indirectly account for the reduction in pH observed in this experiment (Fig. 2.2A) as well as the accumulation of nutrients in growth solutions (as indicated increases in EC which were observed over the experimental period; Fig. 2.2B).

Since I made use of low P levels in this experiment (to minimize potential precipitation problems in future metal toxicity experiments), it was possible the reduced growth and signs of stress were a result of insufficient P. In support of this idea, analysis

of the nutrient solutions taken on day 16 and day 22 indicated the N/P ratio of growth solutions was more than two-fold higher than that of the delivery solutions (data not shown). This indicated that P was being depleted at a greater rate than N.

2.3.2 Experiment 2 (Fine Tuning Nutrient Proportions)

Once again during the pre-treatment period the RGR of 30.5% exceeded the 20% RAR (Fig. 2.1B). When the P proportion was increased from 6 to 15, log linear growth was achieved during the first two weeks of the experimental period but the 20% RAR again exceeded the RGR of 8.4%. Subsequently the RGR accelerated to 17.1% for the remainder of the experimental period (Fig. 2.1B). Signs of root stress were once again observed during the first two weeks of the experiment (little lateral growth and brown coloration), but growth of lateral roots resumed after day 15 and new growth appeared white and healthy. The reduction of the visible root stress coincided with the increase in the RGR (Fig. 2.1B) which is shown by the increased slope of the regression line from 0.084 to 0.171. The pattern of the pH response and EC readings (data not shown) resembled that shown for experiment 1 (Figs. 2.2A & B), an observation which is consistent with a RGR < RAR.

The increase in the RGR to 17.1% suggested that when daily nutrient additions increased, thus increasing the total concentration of nutrients in solution, P was not limiting. In addition, as the level of phosphate used in this experiment was similar to the

level used in an experiment by Ingestad and Stoy (1982) where healthy growth was observed in wheat, the stress and reduced growth observed in the experiment was not likely attributable to P deficiency. Another possibility was that root stress may have been caused by an inadequate initial background concentration of nutrients. Epstein (1976) showed that ion uptake increases as the concentration of the ion increases; thus it is possible that a given background ion concentration in solution may be required to support an uptake rate which will maintain healthy growth. If the background ion concentration (200 μmol N L^{-1}) used in these experiments was insufficient, reduced uptake may have resulted in nutrient stress and reduced growth. Inadequate background concentrations in the pre-treatment period would not necessarily reduce growth as mobilization of seed reserves could provide the nutrients required for healthy growth. This was supported by the 30.5% growth rate observed in the pre-treatment.

2.3.3 Experiment 3 (Background Nutrient Concentrations)

When background concentrations were increased from 50 to 500 μmol N L^{-1}, biomass accumulation increased and the overall growth rate increased from 8% to 16.5% with little additional growth observed at concentrations greater than 500 μmol N L^{-1} (Fig. 2.3). Ingestad (1972) found growth of cucumber was reduced when nutrient concentrations were below 200 mg N L^{-1} (approx 14.2 mM). In another study, Ingestad and Lund (1979) concluded that optimum growth of birch required total N concentration in solution of at least 1.8 mmol L^{-1} but could be as high as 19.3 mmol L^{-1}. These observed

optimal background concentrations cover a wide range and are higher than the optimal background concentrations observed in the present experiment. These differences may have resulted from differences in experimental species (wheat vs. cucumber or birch) or techniques (solution culture vs. nutrient mist culture). Previous research has shown that plant genotypes differ in their ability to take up and utilize nutrients (Clark, 1983). In contrast to these studies, Stadt *et al.* (1992) reported a linear growth response of wheat with background concentrations from 0 to 360 μmol N L^{-1} with a RAR of 15% day^{-1}. This linear response in comparison to the response observed in the above experiment could have resulted from differences in nutrient proportions in nutrient solutions.

When the background was below 300 μmol N L^{-1}, pH fluctuations were minimal (data not shown). Since growth was reduced, ion uptake may have been insufficient to affect solution pH. Alternatively the cation/anion uptake may have been balanced resulting in a constant pH with time. For treatments above 300 μmol N L^{-1}, pH increased from 4.3 to over 6.3 (data not shown). Increased pH may have reflected increased nitrate uptake, as there would be a greater reserve of nitrate in high background concentrations. The EC readings for treatments above 300 μmol N L^{-1} (data not shown) remained relatively stable indicating nutrient additions and uptake were balanced. Reduced growth below 300 μmol N L^{-1} resulted in increased EC readings over the experimental period (data not shown).

2.3.4 Experiment 4 (Time Course - 500 μmol L^{-1} Background)

When the background nutrient concentration was increased to 500 μmol N L^{-1}, the RGR remained close to the 20% RAR throughout the 22 day experimental period (Fig. 2.1C). Although there were some visible signs of root stress (reduced lateral growth, brown colour) during the first week of growth, these signs disappeared after day 7 as lateral roots resumed growth and new growth was white and healthy looking. As in previous experiments, the pH of the growth solution increased to over pH 6.5 after two weeks (Fig. 2.4A), which suggested that anion uptake exceeded cation uptake. Electrical conductivity readings remained relatively constant (40-90 μS cm^{-1}, Fig. 2.4B), indicating that the rate of nutrient uptake was in balance with the nutrient supply and nutrients did not build up in the growth solution.

Even though log linear growth was achieved using a 500 μmol N L^{-1} background (Fig. 2.1C), root stress observed during the initial 7 days of the experiment suggest there was a deficiency of some essential nutrient. Symptoms, including development of brown coloration and lack of growth, resembled those associated with calcium deficiency (Loneragan *et al.*, 1969).

2.3.5 Experiment 5 (Calcium Background)

To determine if the signs of stress observed in the previous experiment could be

alleviated by increasing the Ca concentration, plants were grown at Ca concentrations ranging from 0.098 to 2.0 mmol L^{-1}. Growth increased from 0.036 to 0.073 g pot^{-1} as Ca was increased to 0.4 mmol L^{-1}. Above this concentration, little additional growth was observed (Fig. 2.5). This result suggests a minimum concentration of 0.4 mM Ca is required for healthy root growth under these experimental conditions. Pettersson (1989, 1995) also found that an initial Ca concentration of 0.5 mM was required in experiments with wheat and barley to provide adequate Ca for the entire experimental period (9 and 6 days respectively)

2.3.6 Experiment 6 (Background Nutrients plus 0.4 mmol L^{-1} total Calcium)

This experiment was designed to determine if the higher background Ca level (0.4 mmol L^{-1}) could reduce the need for higher background concentrations of other nutrients. When other background nutrients were varied over the range between 50 to 1,000 μmol N L^{-1}, growth increased linearly (Fig. 2.6A), and no visible signs of root stress were observed in any treatments. Stadt *et al.* (1992) also observed a linear response of growth when wheat was supplied with varying background nutrient concentrations.

In all treatments, pH increased from 4.3 to over 7.0 (Fig. 2.6B), once again suggesting that anion uptake exceeded cation uptake. The EC changed little during the experimental period for all background treatments, nonetheless, EC values in the lower background treatments (50 to 200 μM N), showed less variation with time than values in

the higher background treatments. For the higher background treatments (700 to 1,000 μM N), there was a greater tendency for EC readings to decline between day 9 and 19 (Fig. 2.6C), suggesting the RGR of these high background treatments was greater than the low background N treatments and greater than the RAR. This was confirmed when the overall growth rates were compared. The growth rate of the high background treatments was 0.5 to 1.5% greater than the 50 to 200 μM N treatments.

This experiment confirmed that healthy growth could be achieved with a 50 μmol N L^{-1} background with a total concentration of 0.4 mM Ca. Under these conditions nutrient consumption was in balance with nutrient supply and EC values remained within a narrow range. The remainder of the experiments in this study were conducted using this background combination.

2.3.7 Experiment 7 (Nitrogen Source)

Nutrient uptake can result in plant-induced pH fluctuations as cation uptake results in the release of H^+ ions and a decrease in solution pH, whereas anion uptake results in release of HCO_3^- which results in pH increases (Loneragan, 1979). Cation-anion balance is normally dominated by acquisition of the most abundantly required nutrient N, which can be acquired as both a cation (NH_4^+) and an anion (NO_3^-). If cation-anion uptake is balanced, there should be little change in pH of growth solutions. One of the simplest methods of changing the proportion of cations to anions in the nutrient solution,

without altering total nutrient proportions, is to change the relative supply of NH_4^+ and NO_3^-. While this approach can be used to reduce fluctuations in solution pH, NH_4^+ toxicity can result in growth reductions at high NH_4^+/NO_3^- (Taylor, 1988). Previous research has shown that increasing the NH_4^+ fraction of the nitrogen supply could reduce the observed pH increases without detrimental effects to growth (Stadt *et al.*, 1992; Ericsson, 1981).

The objective of this experiment was to gain control of plant-induced change in pH by finding an NH_4^+/NO_3^- ratio which minimizes observed pH changes without reducing growth. When NH_4^+ was increased from 5 to 50% of the total N supply, the highest levels of growth were attained when NH_4^+ was supplied between 5 and 20% of total N. Growth declined as the NH_4^+ levels were increased above 25% (Fig. 2.7A). Growth reductions were similar to reductions observed by Ingestad (1972) in cucumber when the NH_4^+/NO_3^- ratio exceeded 60/40. In a similar study with wheat, Stadt *et al.* (1992) found that growth was not affected by NH_4^+/N as high as 40%. The decline in growth observed in this experiment was accompanied by visible signs of stress, including reduced growth of lateral roots, yellowing of roots, interveinal chlorosis and tip necrosis of shoots. These visible toxicity symptoms increased as the NH_4^+ ratio increased. Similar NH_4^+ toxicity symptoms have been observed in previous studies with bean, cucumber and pea (Maynard and Barker., 1969), barley, maize and oats (Findenegg, 1987).

Plant-induced pH fluctuations decreased with increasing NH_4^+/NO_3^- ratio, with

little change in pH being obtained at NH_4^+ levels of 50% (Fig. 2.7B). Unfortunately, severe growth reductions were observed at these NH_4^+ levels, suggesting that pH control could not be achieved with this cultivar by increasing NH_4^+ levels. In experiments with a different wheat cultivar (Neepawa) Stadt *et al.* (1992) did not observe growth reductions until NH_4^+ exceeded 40% of total nitrogen. In contrast, Ingestad and Stoy (1982) found that the growth of wheat, barley, and oats was inhibited when NH_4^+ was as low as 10% of the nitrogen supply, suggesting that NH_4^+ sensitivity varies between and within species. These studies suggest that pH control may be achieved with a wheat cultivar less sensitive to NH_4^+.

Electrical conductivity was stable for all treatments below 25% NH_4^+ where growth rates were not reduced by NH_4^+ toxicity. In contrast, in treatments where growth was reduced ($NH_4^+ > 25\%$), EC increased, presumably as a result of reduced plant uptake (Fig. 2.7C).

2.3.8 Experiment 8 (Cultivar Screening)

In experiment 7, growth reductions in cv. Katepwa were observed when the NH_4^+ level exceeded 25%. In an attempt to find a more NH_4^+ resistant cultivar, I screened 8 different cultivars of wheat at three levels of NH_4^+. All 8 cultivars appeared healthy at 20% NH_4^+/N, but when NH_4^+ was increased to 35% of N, the best growth (with fewest visible signs of stress) was achieved with Atlas 66 and Cutler, 80.5 and 70.2% of control

growth at 20% NH_4^+/N, respectively (Table 2.6). These results show that Atlas 66 and Cutler had the highest NH_4^+ resistance of all cultivars tested. For these two cultivars, plant-induced pH remained below 4.3 (data not shown). Electrical conductivity in the 35% NH_4^+ treatments was relatively stable for both cultivars (data not shown).

2.3.9 Experiment 9 (Atlas 66 - Cutler Screening)

This experiment was designed to investigate the nature of growth differences between Atlas 66 and Cutler over a wider range of NH_4^+/NO_3^- than used in experiment 8. Little difference in growth was observed between the two cultivars as NH_4^+ levels increased (Fig. 2.8). Although Cutler had a greater final plant mass (attributable to higher initial weight), the RGR of Atlas 66 exceeded the RGR of Cutler by 0.3 to 0.5% in most treatments. Cutler showed some signs of stress at NH_4^+/N ratios of 28% or above, including increased branching of lateral roots and laterals closer to root tips while Atlas 66 exhibited no visible signs of root stress. This suggested a greater NH_4^+ sensitivity of Cutler than Atlas 66. The pH for both cultivars remained below 5.0 (data not shown) when the NH_4^+ ratio was 36%. These results show that pH fluctuations could be controlled in an NH_4^+ resistant cultivar by increasing the proportion of NH_4^+ to NO_3^-. Electrical conductivity remained relatively stable for all treatments (data not shown), indicating no accumulation of nutrients in growth solutions.

Atlas 66 was chosen as the experimental cultivar for the remainder of the

experiments as it appeared to have a greater tolerance to NH_4^+ than Cutler. The remainder of the experiments in this series repeated previous experiments to ensure that optimal conditions for growth of Atlas 66 would be comparable to those for cv. Katepwa.

2.3.10 Experiment 10 (Optimization of Background Nutrients for Atlas 66)

When Atlas 66 was grown over a 21 day experimental period at 15 different background concentrations (50 to 1,000 µmol N L^{-1}), growth increased linearly as background concentration increased (Fig. 2.9A), and no visible signs of stress were observed. Increased pH was observed in all treatments, but remained below 5.75 in the 50 µmol N L^{-1} background (Fig. 2.9B). Relatively stable EC was achieved at the lowest background concentrations (Fig. 2.9C), confirming that a balance between nutrient uptake and nutrient supply prevented nutrient accumulation. As in the previous background experiment (Experiment 6), a reduction in EC was observed between day 9 to 16 (RGR > RAR) in the highest backgrounds. This was attributed to the high growth rate of plants in these treatments which was higher than that observed in the lowest background treatments.

2.3.11 Experiment 11 (Optimization of Nitrogen Source for Atlas 66)

In this repeat of experiment 7, growth of Atlas 66 appeared healthy and attained the highest levels between 20 and 35% NH_4^+/N (Fig. 2.10A). Sensitivity to high NO_3^- (5

to 15% NH_4^+) and high NH_4^+ (over 40% NH_4^+) was suggested as visible signs of stress including shoot chlorosis and brown root colour were observed in these treatments and growth was reduced relative to the 20 to 35% NH_4^+ treatments (Fig. 2.10A). Reduced pH fluctuations and relatively stable EC were achieved at 35% NH_4^+/N (Fig. 2.10B & C), which suggested that a balance between cation and anion uptake, and a balance between nutrient uptake and supply had been achieved under these conditions.

2.3.12 Experiment 12 (The Time Course of Growth for Atlas 66)

A 20% RAR under the optimal conditions described above produced close control of RGR. Growth was log linear with time throughout the experimental period (Fig. 2.11A), although there may have been a slight reduction in growth rate at the end of the experimental period. The overall growth rate for the plants of the harvests of day 20 and day 22 was calculated to be 17 and 17.3% respectively. This reduction in the growth rate was accompanied by an increase in the EC and a decline in pH (Fig. 2.11B & C), but these changes occurred over a relatively narrow range in comparison with previous experiments. The increased EC at the end of the experimental period was likely a result of a greater rate of nutrient additions than nutrient uptake. Ingestad (1972) observed similar increases in electrical conductivity in cucumber and suggested the increases may also be caused by dissociated root exudates. Decreasing pH could result from nutrient accumulation (caused by reduced growth) which would result in a larger concentration of NH_4^+ available for uptake (resulting in reduced pH).

2.4 SUMMARY

The objective of this series of experiments was to optimize the RAR technique for growth of *Triticum aestivum* in low ionic strength growth solutions. With the use of the computer-controlled nutrient delivery system and the RAR technique, plants can be grown in low ionic strength growth solutions with the RGR controlled by the RAR of nutrients with little or no build-up of nutrients in growth solutions and minimal pH fluctuations. This system can now be utilized to study metal toxicity (and possible cation amelioration) in low ionic strength growth solutions.

Table 2.1. Nutrient proportions by molar concentration and by weight in a 200 μmol N L^{-1} background solution.

Element	Concentration (μM)	Weight Proportion
Nitrogen	200	100
Phosphorous	5.72	6
Potassium	59.6	84
Calcium	14.28	20
Magnesium	11.44	10
Sulphur	2.84	0.033
Iron	$3.6\ 10^{-1}$	0.7
Manganese	2.08×10^{-1}	0.4
Boron	5.16×10^{-1}	0.2
Zinc	5.57×10^{-2}	0.06
Copper	1.152×10^{-2}	0.03
Molybdenum	2.0×10^{-3}	0.07

Table 2.2. Nutrient composition of relative addition rate delivery solutions and nutrient proportions by weight used for Experiment 1.

CHEMICAL	moles L^{-1}	g L^{-1}
Delivery Solution 1		
NH_4NO_3	1.75×10^{-1}	14.007
$Mg(NO_3)_2.6H_20$	4.0×10^{-2}	10.2564
$Ca(NO_3)_2 \cdot 4H_20$	5.0×10^{-2}	11.8075
Delivery Solution 2		
KH_2PO_4	2.0×10^{-2}	2.7218
KNO_3	1.7×10^{-1}	17.1887
K_2SO_4	1.0×10^{-2}	1.7427
$MnSO_4 \cdot H_20$	7.3×10^{-4}	0.1234
H_3BO_3	1.8×10^{-3}	0.1113
$ZnSO_4 \cdot 7H_20$	9.0×10^{-5}	0.0259
$CuSO_4 \cdot 5H_20$	4.0×10^{-5}	0.010
$Na_2MoO_4 \cdot 2H_20$	7.0×10^{-6}	0.0017
Delivery Solution 3		
$FeCl_3 \cdot 6H_20$	1.25×10^{-3}	0.3379

Nutrient proportions by weight (N = 100), P = 6, K = 84, Ca = 20, Mg = 10, S = 0.033, Fe = 0.7, Mn = 0.4, B = 0.2, Zn = 0.06, Cu = 0.03, Mo = 0.07.
NH_4^+ as percent of total N - 25%

Table 2.3. Nutrient composition of relative addition rate delivery solutions and nutrient proportions by weight used for Experiments 2 to 7. Composition of delivery solution 1 was amended for the nitrogen source experiment (experiment 7) as described in Materials and Methods.

CHEMICAL	moles L^{-1}	g L^{-1}
Delivery Solution 1		
NH_4NO_3	1.75×10^{-1}	14.007
$Mg(NO_3)_2.6H_20$	4.0×10^{-2}	10.2564
$Ca(NO_3)_2 \cdot 4H_20$	5.0×10^{-2}	11.8075
Delivery Solution 2		
KH_2PO_4	4.7×10^{-2}	6.3962
KNO_3	1.7×10^{-1}	17.1887
K_2SO_4	2.0×10^{-2}	3.4854
$MnSO_4 \cdot H_20$	7.3×10^{-4}	0.1234
H_3BO_3	1.8×10^{-3}	0.1113
$ZnSO_4 \cdot 7H_20$	9.0×10^{-5}	0.0259
$CuSO_4 \cdot 5H_20$	4.0×10^{-5}	0.010
$Na_2MoO_4 \cdot 2H_20$	7.0×10^{-6}	0.0017
Delivery Solution 3		
$FeCl_3 \cdot 6H_20$	1.25×10^{-3}	0.3379

Nutrient proportions by weight (N = 100), P = 15, K = 102.5, Ca = 20, Mg = 10, S = 0.065, Fe = 0.7, Mn = 0.4, B = 0.2, Zn = 0.06, Cu = 0.03, Mo = 0.07.
NH_4^+ as percent of total N - 25%

Table 2.4. Nutrient composition of relative addition rate delivery solutions and nutrient proportions by weight used for Experiments 8 to 11. Composition of delivery solution 1 was amended for nitrogen source experiments (experiments 8, 9 and 11) as described in Materials and Methods.

CHEMICAL	moles L^{-1}	g L^{-1}
Delivery Solution 1		
NH_4NO_3	1.75×10^{-1}	14.007
$Mg(NO_3)_2.6H_20$	4.0×10^{-2}	10.2564
$Ca(NO_3)_2 \cdot 4H_20$	5.0×10^{-2}	11.8075
Delivery Solution 2		
KH_2PO_4	4.7×10^{-2}	6.3962
KNO_3	1.28×10^{-1}	12.942
K_2SO_4	2.0×10^{-2}	3.4854
HNO_3	4.2×10^{-2}	2.66 ml
$MnSO_4 \cdot H_20$	7.3×10^{-4}	0.1234
H_3BO_3	1.8×10^{-3}	0.1113
$ZnSO_4 \cdot 7H_20$	9.0×10^{-5}	0.0259
$CuSO_4 \cdot 5H_20$	4.0×10^{-5}	0.010
$Na_2MoO_4 \cdot 2H_20$	7.0×10^{-6}	0.0017
Delivery Solution 3		
$FeCl_3 \cdot 6H_20$	1.25×10^{-3}	0.3379

Nutrient proportions by weight (N = 100), P = 15, K = 82, Ca = 20, Mg = 10, S = 0.065, Fe = 0.7, Mn = 0.4, B = 0.2, Zn = 0.06, Cu = 0.03, Mo = 0.07.
NH_4^+ as percent of total N - 25%

Table 2.5. Nutrient composition of relative addition rate delivery solutions and nutrient proportions by weight used for Experiment 12.

CHEMICAL	moles L^{-1}	g L^{-1}
Delivery Solution 1		
NH_4NO_3	1.75×10^{-1}	14.007
NH_4Cl	7.7×10^{-2}	4.119
$Mg(NO_3)_2.6H_20$	4.0×10^{-2}	10.2564
$MgCl_2$	3.85×10^{-2}	7.827
$Ca(NO_3)_2 \cdot 4H_20$	5.0×10^{-2}	11.8075
Delivery Solution 2		
KH_2PO_4	4.7×10^{-2}	6.3962
KNO_3	1.28×10^{-1}	12.942
K_2SO_4	2.0×10^{-2}	3.4854
HNO_3	4.2×10^{-2}	2.66 ml
$MnSO_4 \cdot H_20$	7.3×10^{-4}	0.1234
H_3BO_3	1.8×10^{-3}	0.1113
$ZnSO_4 \cdot 7H_20$	9.0×10^{-5}	0.0259
$CuSO_4 \cdot 5H_20$	4.0×10^{-5}	0.010
$Na_2MoO_4 \cdot 2H_20$	7.0×10^{-6}	0.0017
Delivery Solution 3		
$FeCl_3 \cdot 6H_20$	1.25×10^{-3}	0.3379

Nutrient proportions by weight (N = 100), P = 15, K = 82, Ca = 20, Mg = 10, S = 0.065, Fe = 0.7, Mn = 0.4, B = 0.2, Zn = 0.06, Cu = 0.03, Mo = 0.07.
NH_4^+ as percent of total N - 36%

Table 2.6. Root growth response of 8 cultivars of *Triticum aestivum L.* (as per cent of 20% NH_4^+ treatment) with varying ratios of NH_4^+/N.

Cultivar	Root Weight (% of Control)	
	35% NH_4	45% NH_4
Cutler	70.2	19.79
PT741	46.48	14.55
Roblin	16.19	14.58
Katepwa	50.48	20.13
Oslo	18.17	15.32
Atlas 66	80.47	49.86
Maringa	68.31	27.27
Park	55.56	26.92

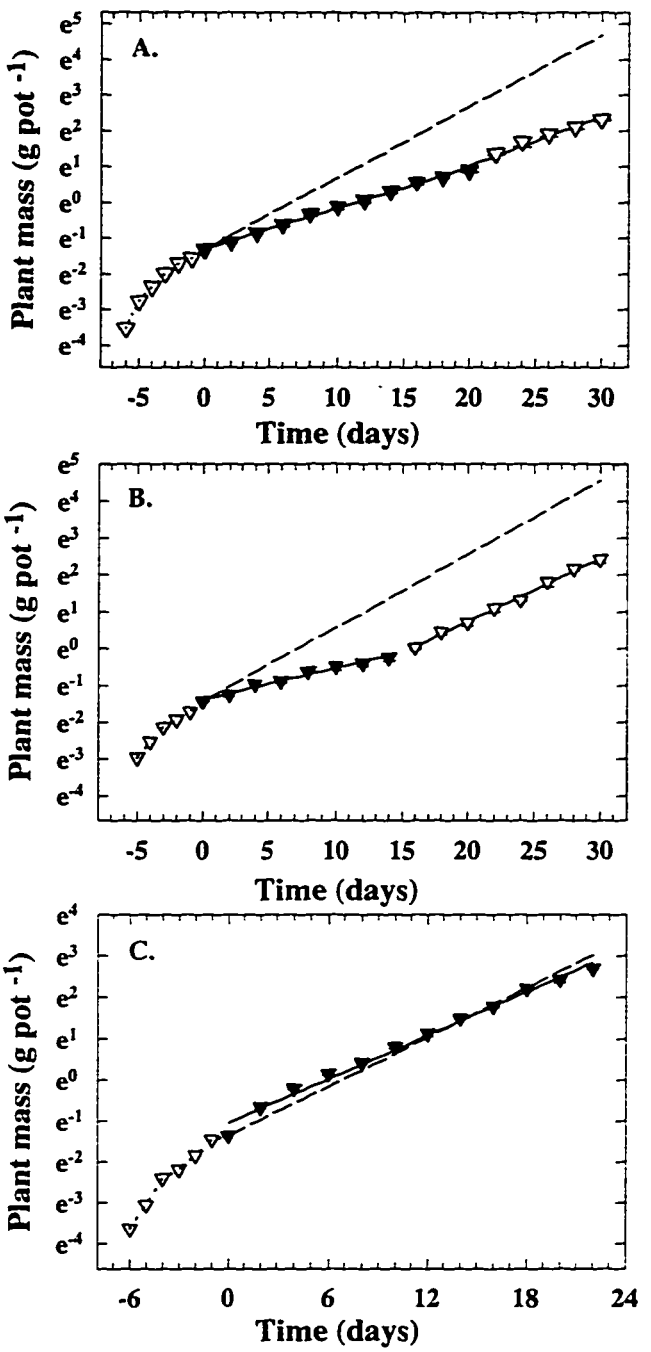

Figure 2.1. Growth of cv.Katepwa in experiments 1 (A), 2 (B) and 4 (C) with a 200 μM N (A and B) or a 500 μM N (C) background at a 20% relative addition rate. Values are means ± standard errors (n=4). Open symbols, pre-treatment period, closed symbols, experimental period, change in colour of closed symbols represents a change in growth rate. Dotted line (····), best fit regression for pre-treatment period. Solid line (—), best fit regression line for growth during the experimental period. Dashed line (- -), represents regression line for a relative growth rate of 20%.

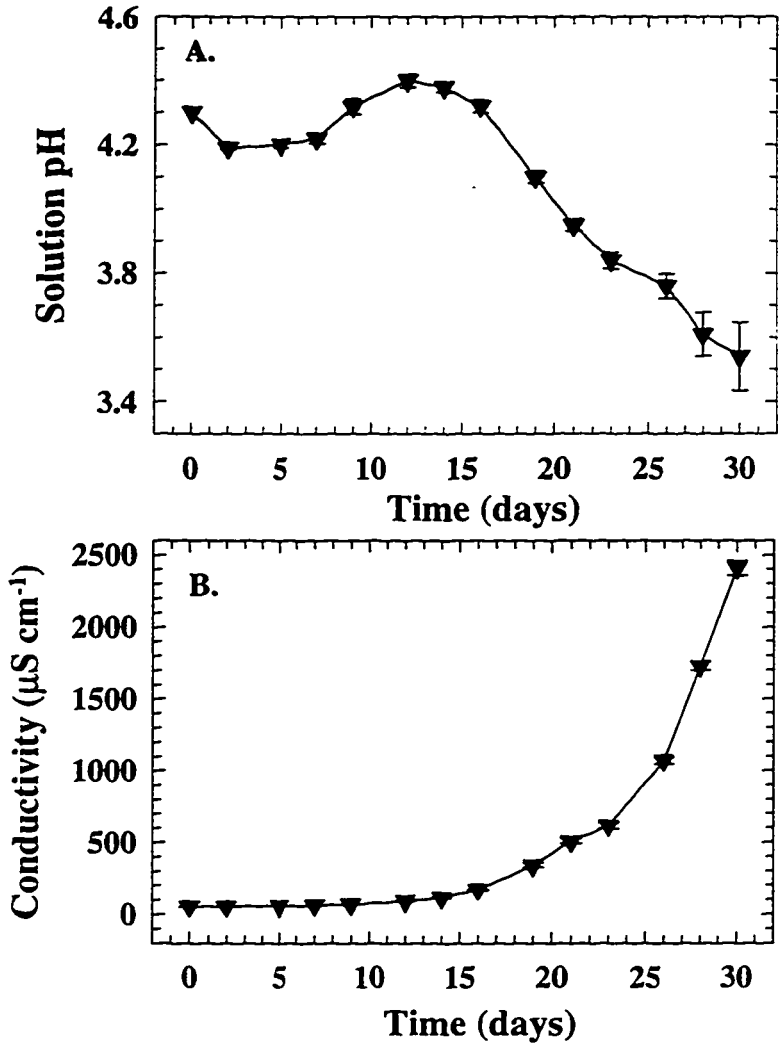

Figure 2.2. Change in solution pH (A) and in solution electrical conductivity (B)of cv Katepwa over the 30 day experimental period in experiment 1. Values are means ± SE (n=4).

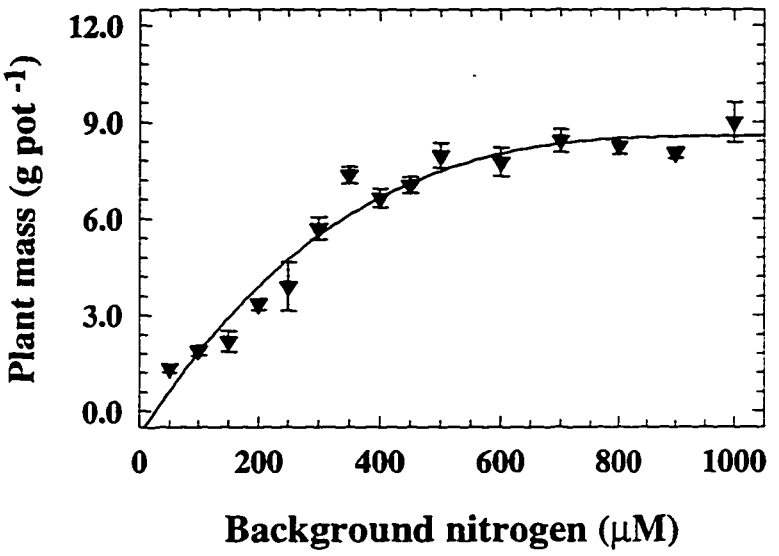

Figure 2.3. Growth of cv. Katepwa under a 20% relative addition rate and background concentrations of 50 to 1,000 μM nitrogen for the 21 day experimental period (Experiment 3). Values are means ± SE (n=4).

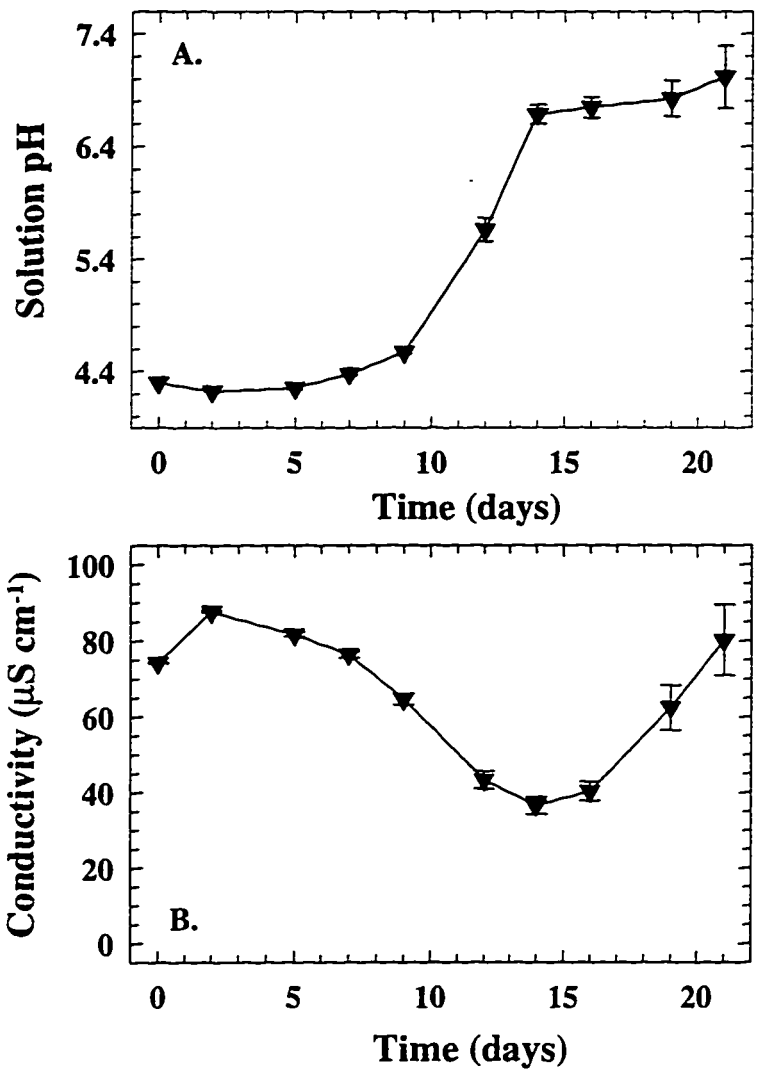

Figure 2.4. Change in solution pH (A) and in solution electrical conductivity (B) in a 500 μM N background, over the 22 day experimental period of experiment 4. Values are means ± SE (n=4).

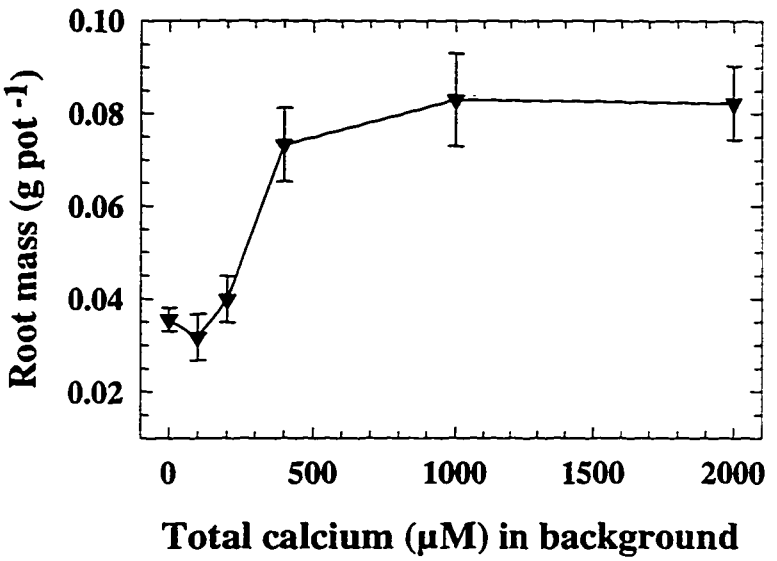

Figure 2.5. Root growth of cv. Katepwa under a 20% relative addition rate, 200 µmol N L^{-1} background nutrients and a total calcium of 0.095 to 2.0 mM for the 9 day experimental period (Experiment 5). Values are means ± SE (n=3).

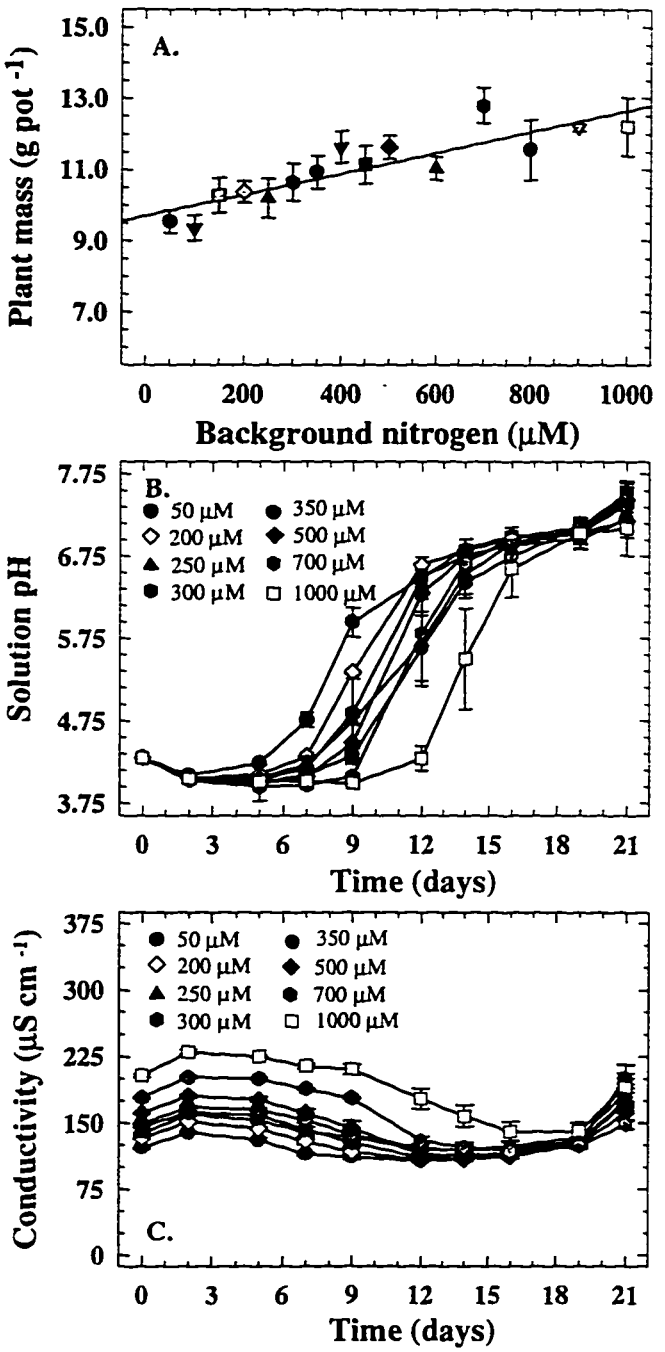

Figure 2.6. Growth of cv. Katepwa under a 20% relative addition rate, background concentrations of 50 to 1,000 µM nitrogen and 0.4 mM Ca total (A). Change in solution pH (B) and in solution electrical conductivity (C) over the 21 day experimental period (Experiment 6). Values are means ± SE (n=4).

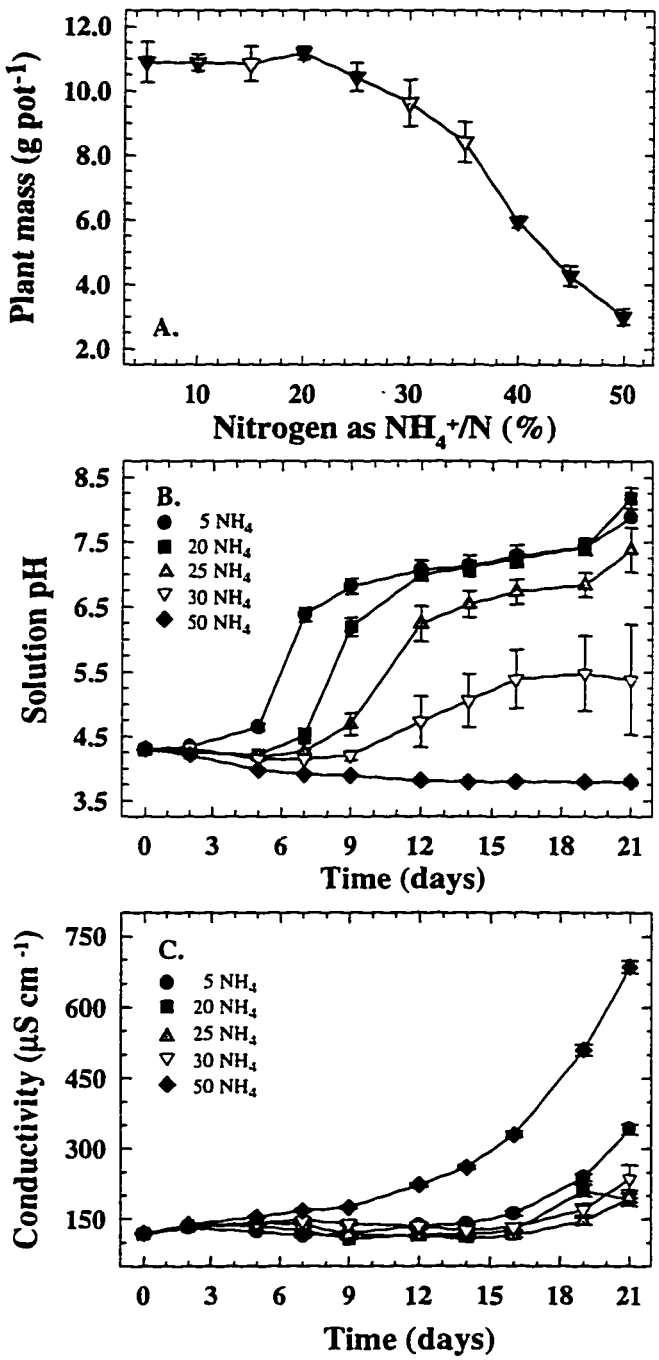

Figure 2.7. Growth of cv. Katepwa under a 20% relative addition rate with NH_4^+ as 5 to 50 % of total N (A). Change in solution pH (B) and in solution electrical conductivity (C) over the 21 day experimental period (Experiment 7). Values are means ± SE (n=4).

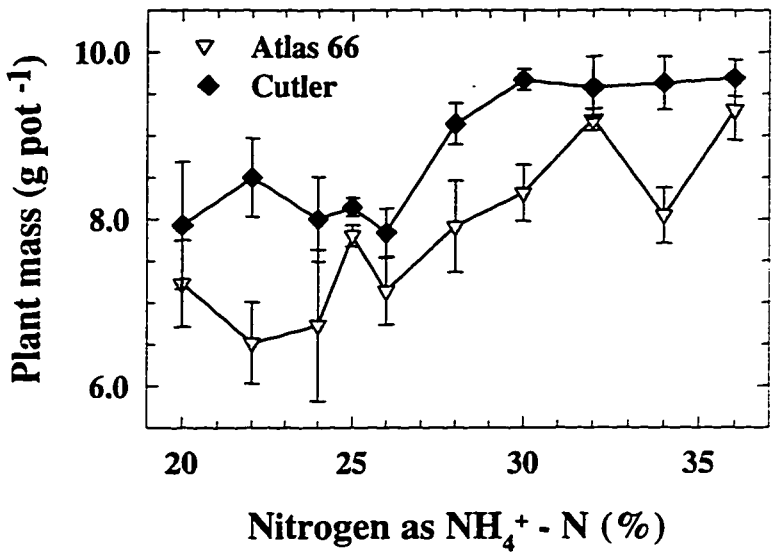

Figure 2.8. Growth of cv. Atlas 66 and Cutler under a 20% relative addition rate, with NH_4^+ as 20 to 36% of total N for the 21 day experimental period (Experiment 9). Values are means ± SE (n=3).

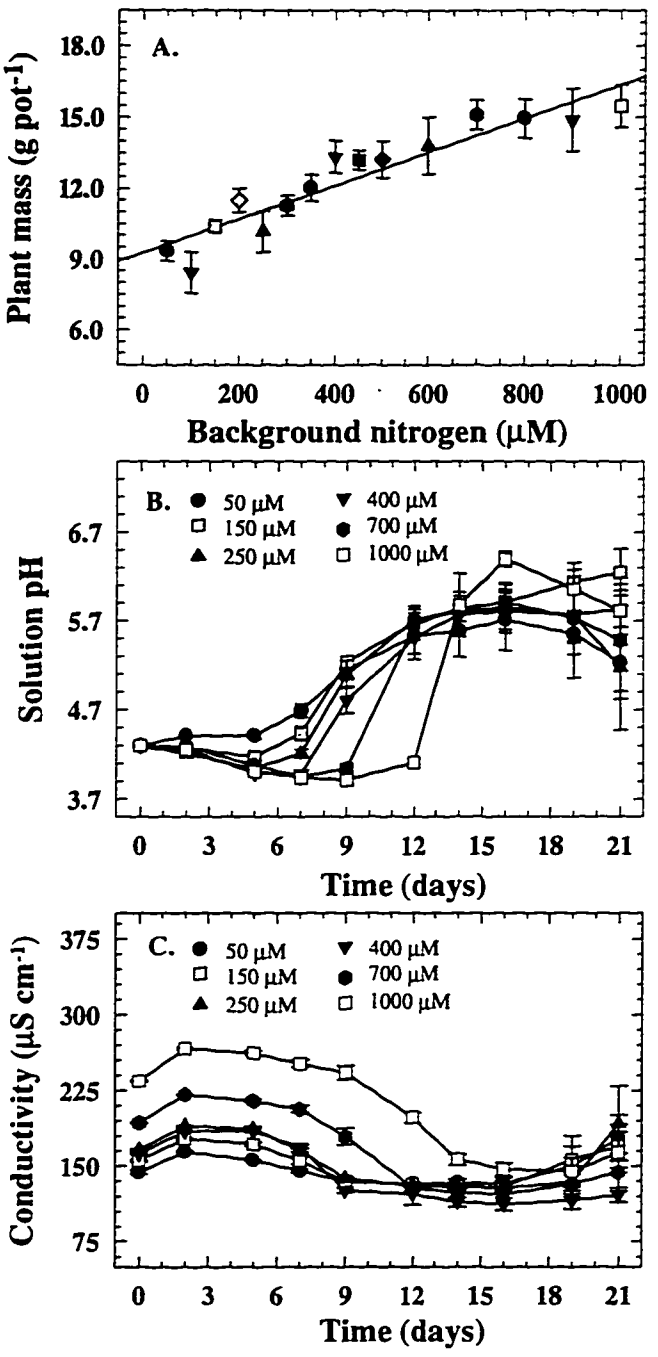

Figure 2.9. Growth of cv. Atlas 66 under a 20% relative addition rate, background concentrations of 50 to 1,000 µM nitrogen and 0.4 mM Ca total (A). Change in solution pH (B) and in solution electrical conductivity (C) over the 21 day experimental period (Experiment 10). Values are means ± SE (n=4).

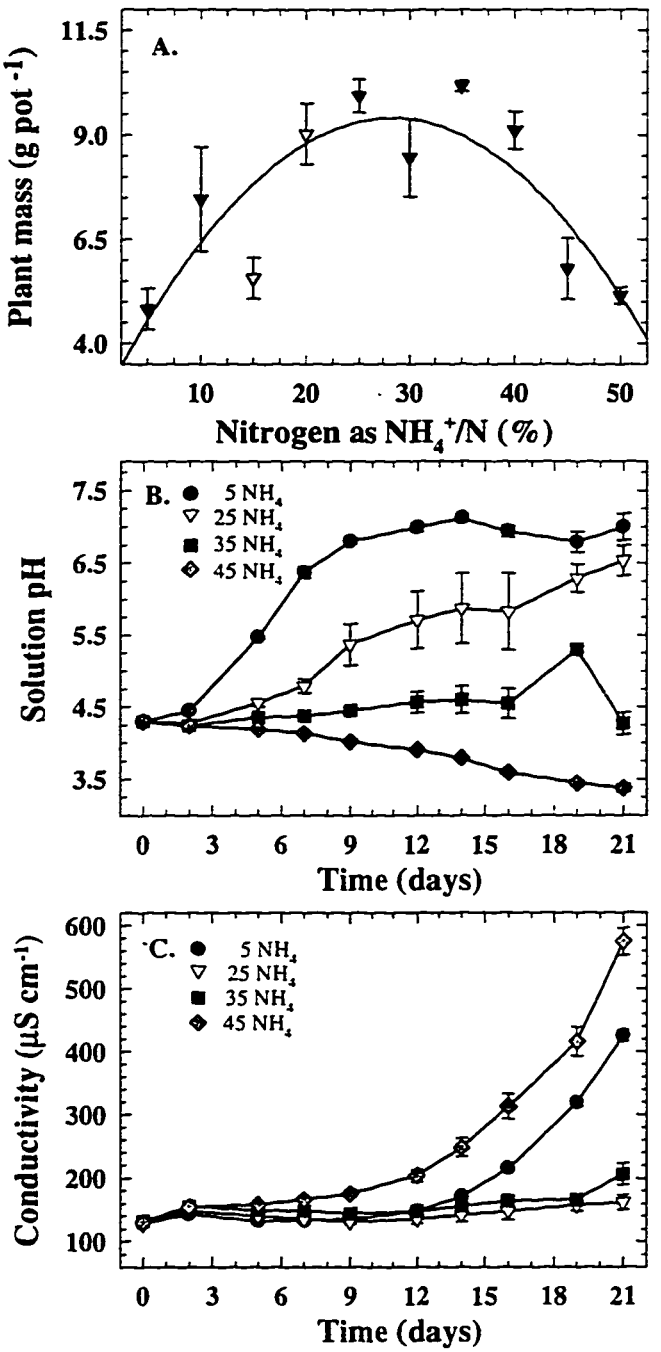

Figure 2.10. Growth of cv. Atlas 66 under a 20% relative addition rate with NH_4^+ as 5 to 50% of total N (A). Change in solution pH (B) and in solution electrical conductivity (C) over the 21 day experimental period (Experiment 11). Values are means ± SE (n=4).

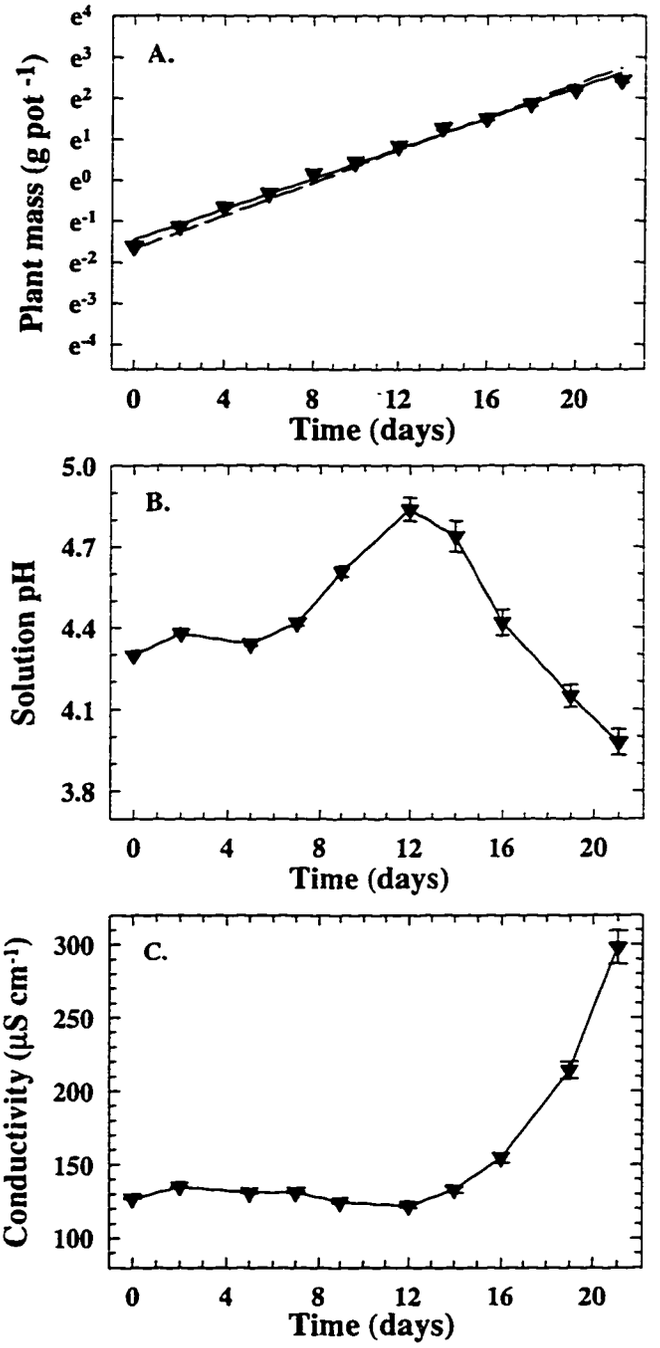

Figure 2.11. Growth of cv. Atlas 66 at a 20% relative addition rate (A). Solid line (—), best fit regression line for growth during the experimental period. Dashed line (--), represents regression line for a relative growth rate of 20%. Change in solution pH (B) and in solution electrical conductivity (C) over the 21 day experimental period (Experiment 12). Values are means ± SE (n=4).

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3 THE AMELIORATIVE EFFECTS OF INCREASED IONIC STRENGTH OR INCREASED CATION CONCENTRATIONS ON COPPER TOXICITY IN *TRITICUM AESTIVUM*

3.1 INTRODUCTION

Research on the effects of metals on plant growth has traditionally been conducted in solution culture with nutrient concentrations many fold higher than would be observed in natural ecosystems. These high nutrient concentrations could result in reduced activity of cations (Bard, 1966), which could in turn reduce cation toxicity. A number of studies have shown that increased ionic strength of growth solutions results in reduced metal toxicity (Pavan and Bingham, 1982; Riedell and Schmid, 1986; Blamey *et al.*, 1983), but this amelioration cannot always be solely attributed to reduced ion activity (Alva *et al.*, 1986a,b; Horst, 1987; Macfie *et al.*, 1994). It has been suggested that amelioration observed beyond that which can be explained by ionic strength effects is due to the reduced negativity of the cell surface electrical potential (Kinraide and Parker, 1987; Kinraide *et al.*, 1992). Negativity of cell surfaces can be decreased by either i) divalent or polyvalent cation binding to negatively charged sites or ii) by charge screening which is caused by the concentration of cations around cell surface negative charges due to coulombic attractions (Kinraide *et al.*, 1992).

Experiments investigating the ability of cations to reduce the rhizotoxicity of Al^{3+} indicated that the ameliorative effectiveness of cations was in the order of $H^+ = C^{3+} > C^{2+}$

> C^+ (Kinraide *et al.*, 1992). Experiments were conducted in which activities were calculated for cations at membrane surfaces and in the external medium (Kinraide *et al.*, 1992: Kinraide, 1994). A correlation was found between growth inhibition and predicted activities at membrane surfaces, while no correlation was found with activities in the external medium. Kinraide (1994) concluded that cation activities at membrane surfaces are a better indicator of cation toxicity than activities in growth solutions.

The first objective of this portion of my research was to determine if increased ionic strength ameliorated copper (Cu) toxicity in *Triticum aestivum* cv. Atlas 66. The second objective was to determine if amelioration could be fully explained by the reduction of Cu^{2+} activity which accompanies increased ionic strength, or if the amelioration was the result of the actions of specific cations. The objectives were achieved by performing two sets of experiments. In the first set of experiments, the ionic strength of growth solutions was increased by increasing the concentration of all ions in the nutrient solution to determine if increased ionic strength itself would reduce Cu toxicity. In the next set of experiments, ionic strength was increased by increasing the concentration of single cations (Na^+, K^+, Ca^{2+}, or Mg^{2+}) to determine if the actions of single cations altered the growth response to Cu.

3.2 MATERIALS AND METHODS

3.2.1 Preparation of Plant Material (pre-treatment period)

Seeds of *Triticum aestivum* cv. Atlas 66 were surface sterilized in a 1.1% solution of sodium hypochlorite (v/v) for 20 minutes and germinated overnight in an aerated solution containing 0.005 g L^{-1} Vitavax (Uniroyal Chemical Ltd., Calgary, AB, Canada) to limit fungal growth. Seeds were transferred to aquaria (300 seeds per aquaria), on nylon mesh suspended over a 50 μM nitrogen (N) background solution containing (μM); 50 N, 3.34 P, 15.3 K, 3.57 Ca, 2.86 Mg, 7 x 10^{-1} S, 8.93 x 10^{-2} Fe, 5.21 x 10^{-2} Mn, 1.29 x 10^{-1} B, 6.43 x 10^{-3} Zn, 2.88 x 10^{-3} Cu and 5 x 10^{-4} Mo. Additional $CaCl_2$ was added so that the background concentration was 0.4 mM Ca. Preliminary experiments suggested that this level of Ca was needed to achieve healthy root growth during the first few days of growth (see Chapter 2, section 2.3.5). Solutions were adjusted to pH 4.3 with 1.0 or 0.1 M HCl. After 3 days, seedlings were thinned to 150 per aquaria. At this time 12 seedlings were dried at 60°C for two hours (to a constant weight) to determine initial dry weight (W_0). This dry weight was used to calculate a 20% daily relative addition rate (RAR) of nutrients for the remainder of the pre-treatment period using the formula:

$$A_t(\text{mol}) = [C \cdot W_0/M]e^{RAR(t-t_0)}(e^{RAR}-1)$$

Where (A_t) is the nutrient amount to be added, C is the plant nutrient content (g nutrient g

plant^{-1}), W_0 is plant weight at time t_0 and M is the molecular weight of the nutrient (Stadt *et al.*, 1992). For this series of experiments, nutrient additions were calculated for N alone with the remainder of the nutrients supplied in a fixed relationship (weight proportion) to N (Ingestad, 1981; Stadt *et al.*, 1992). Nutrient proportions by weight when N = 100 were P = 15, K = 84, Ca = 20, Mg = 10, S = 0.065, Fe = 0.7, Mn = 0.4, B = 0.2, Zn = 0.06, Cu = 0.03 and Mo = 0.07. The nutrient additions for the remainder of the pre-treatment period were reduced to one half of calculated values as seed reserves were also supplying nutrients.

3.2.2 Experimental Period

After the nine day pre-treatment period, spent seeds were removed and eight uniform seedlings were transferred to each of 60-10 L polyethylene containers. Each container was filled with an aerated growth solution prepared by computer delivery of nutrient solutions to 10 L of distilled water to achieve the background nutrient concentration required for each experiment. Nutrient proportions in background solutions for all experiments were as described for the pre-treatment period, unless specified otherwise in the description of individual experiments. Copper treatments (described in each experiment) were superimposed over the background nutrients. Seedlings were suspended over containers mounted with foam in Plexiglas covers (which inhibited algal growth). Containers were suspended in a common water bath to maintain a constant temperature across all containers. Periodic additions of distilled water to experimental

solutions was required to compensate for water losses by evaporation and transpiration. All experiments were 18 days in duration.

Electrical conductivity and pH of experimental solutions were measured prior to planting, three times per week during the experiment and after harvest using a Radiometer CDM80 portable electrical conductivity meter and a Radiometer pHM80 portable pH meter. Meters were calibrated prior to every use to ensure consistent readings.

Experiments were conducted in a controlled-environment chamber with 16 hr light and 8 hr darkness. Temperatures ranged from 21 to 24°C for the light period and from 16 to 18.5°C during the dark period. Relative humidity was between 62 and 84% for the light period and 94 to 100% during darkness. Solution temperatures varied between 21.8 and 22.4°C during the light period and 21 to 21.8°C for the dark period. Illumination was provided by 103 cool white fluorescent lamps (25W), and 16 incandescent lamps (150W), located 1.3 m above plant bases. The photosynthetic photon flux averaged between 420 and 448 μmol m^{-2} sec^{-1} for this series of experiments. Plants were harvested at the end of the experimental period, rinsed in distilled water, separated into roots and shoots and dried to a constant weight at 60°C.

3.2.3 Growth Techniques by Experiment

3.2.3.1 Experiment 1 (Copper Add Back)

A preliminary experiment was conducted to determine if maintenance of initial copper concentrations would alter the growth response. Treatments consisted of 10 Cu concentrations (μM; 0.05, 0.5, 1, 1.5, 2, 3, 6, 9, 12 and 15), 3 replicates and plus or minus Cu add back (AB, add back; NAB, non add back). For this experiment, all nutrients were added at the beginning of the experimental period. To determine the amount of nutrients to be added, a 20% RAR was calculated for daily additions for the experimental period using the formula described for the pre-treatment period. When totalled, the daily additions equalled 2.94 mM N with all nutrients except Cu supplied in the proportions described for the pre-treatment period. Concentrations of Cu in nutrient solutions were monitored, by graphite furnace AAS (in AB treatments only) and initial concentrations of Cu were maintained using 1.0 or 10 mM $CuSO_4$. As Cu is an essential nutrient, the reduced growth observed in the NAB treatments at the lowest Cu concentrations suggested Cu was being depleted from solution to unacceptably low levels. This was supported when statistical analysis indicated significant differences in growth between AB and NAB treatments. To prevent depletion of Cu, in all subsequent experiments initial Cu concentrations were maintained.

3.2.3.2 Experiments 2 and 3 (Low versus Intermediate Ionic Strength)

These experiments were designed to determine if an increase in background nutrients from 50 to 1,000 μM N and the subsequent increase in ionic strength would reduce the toxic effect of increasing Cu concentrations. Two initial background nutrient levels were used for these experiments; low ionic strength (LIS; 50 μM N) and intermediate ionic strength (IIS; 1,000 μM N), with 10 Cu concentrations and 3 replicates. Total Ca in both treatments was 0.4 mM. In the first experiment, Cu concentrations were (μM) 0.05, 0.5, 1, 1.5, 2, 3, 6, 9, 12 and 15. In the second experiment, a narrower range of Cu concentrations was used consisting of 0.005, 0.01, 0.05, 0.1, 0.4, 0.8, 1.2, 1.6, 2 and 3 μM. Nutrients were added at a 20% RAR for all treatments. Nutrient delivery solutions did not contain Cu and initial Cu concentrations were maintained throughout.

3.2.3.3 Experiments 4 and 5 (Low versus High Ionic Strength)

These experiments were designed to determine if an additional increase in ionic strength would produce a further decrease in Cu toxicity. For both experiments, low ionic strength treatments consisted of a 50 μM N background (with a 20% daily RAR) and high ionic strength treatments (HIS) consisted of a 2.94 mM N background. Additional Ca was added so that the total concentration in each background was 0.4 mM. In HIS treatments, all nutrients were added at the beginning of the experiment as described for the preliminary experiment. This ensured that total nutrients added to experimental containers

over the course of the experiment for LIS and HIS treatments were equivalent. Only the timing of nutrient delivery (and hence ionic strength) varied. Copper concentrations used in these experiments were the same concentrations used in the two previous experiments described above. Once again, initial Cu concentrations were maintained throughout the experiment.

3.2.3.4 Experiment 6 (Amelioration Experiment)

This experiment was designed to determine if the amelioration of Cu toxicity observed in experiment 4 resulted from increased concentrations of individual cations or reduced activity of Cu caused by the increase in ionic strength. Experimental treatments were superimposed over a 50 μM N background with a total concentration of 0.4 mM Ca. This experiment was factorial in design, with 4 Cu concentrations (RAR, 0.75, 1.5 and 2.25 μM), 5 cation treatments (no added cation (control), or 3.7 mM Na, K, Ca or Mg) and 3 replicates for a total of 60 containers. The RAR Cu treatment consisted of the Cu contained in a 50 μM N background plus Cu contained in daily nutrient additions. A 20% RAR was used for all treatments and initial Cu concentrations were maintained in the 0.75, 1.5 and 2.25 μM treatments. Copper was not omitted from the delivery solutions as Cu analyses in experiments 1 to 5 indicated that Cu depletion was greater than daily Cu additions at the Cu concentrations used in this experiment. In addition, daily Cu additions would help reduce fluctuations in Cu concentrations between graphite furnace analyses.

3.2.4 Copper Analyses

In order to maintain initial concentrations of Cu in solution, samples of each experimental solution were analyzed for total Cu concentrations using a Perkin Elmer 3030 atomic absorption spectrophotometer equipped with a HGA-500 graphite furnace attachment. Analyses were done prior to planting, and on day 4, 8, 11, 14, and 16 of the experimental period. Twenty µl of nutrient solution was mixed with 20 µl 1,000 µM N nutrient solution (minus Cu) as a matrix modifier, dried at 140°C for 60 s, pre-treated at 1350°C for 50 s and atomized at 2400°C for 6 s on a L'vov platform in a pyrolytically coated graphite tube. Concentrations were calculated by integration of peak area and expressed as µmol Cu L^{-1}. If Cu concentrations were below the detection limit of the graphite furnace (approx. 0.1 µM), solutions were evaporated to dryness, re-dissolved in 2 ml deionized water and analyzed. Copper concentrations of nutrient solutions were returned to initial values by addition of 1.0 or 10 mM $CuSO_4 \cdot 5H_2O$.

When the ionic strength or composition of nutrient solutions are changed, the activity of ions in solution is affected. To predict free activity and per cent free Cu^{2+} of each of the nutrient solutions, speciation analyses of each nutrient solution were performed using the computer program GEOCHEM. Comparison of these analyses provided information about how the ionic strength of the nutrient solutions may have altered the activity of Cu^{2+}.

3.2.5 Statistical Analysis

Although roots and shoots were collected, only root biomass data were analyzed as roots are a more sensitive indicator of metal toxicity than shoots (Taylor, 1988). The response of root biomass to increased Cu^{2+} concentrations in the presence or absence of a second treatment (increased ionic strength or Cu AB vs NAB) was modeled using a modified Weibull function described by Taylor *et al.* (1992). The Weibull function is a continuous, non-linear, mathematical model underlain by a recognized statistical distribution, the Weibull distribution (Moore and Joliffe, 1987). The modified Weibull function is described by the following equation:

$$y = F(x,a,b,c,d) = a + b\, e^{-(x/c)^d}$$

In this equation, y = yield (g pot^{-1}) and x represents the metal concentration in the growth solution (μM). Parameter *a* is the absolute minimum growth, *b* is the maximum growth response above the absolute minimum (maximum growth - minimum growth) and *c* and *d* are shape parameters. If $c > 0$ and $d > 0$, the function is defined and decreases monotonically from $(a+b)$ to a. Parameter *c* is the metal concentration at which yield is reduced to $a + 0.37b$ (Taylor and Stadt, 1990; Taylor *et al.*, 1992). When comparing two functions, a significant change in only parameter *b* indicates the combined effects of the treatments altered total biomass accumulation but not the shape of the dose response curve. Whereas, changes in parameters *c* and/or *d* indicate an interaction between the

treatments affecting plant growth (Taylor *et al.*, 1992).

A non-linear model fitting procedure PROC NLIN in SAS release 6.06 was used for statistical analysis of root biomass data (Taylor and Stadt, 1990; Taylor *et al.*, 1992). R^2 was calculated as:

$$R^2 = \frac{\text{ratio of corrected sum of squares due to regression}}{\text{total corrected sum of squares}}$$

A parametric t-test (Zar, 1984), was used to test for differences in the parameters within an experiment. The resulting t values were compared to the t distribution using $\sum^{k}(n_i-m)$ degrees of freedom, where n is the number of observations for each regression, k is the number of regressions, and m is the number of parameters in each regression (Taylor *et al.*, 1992). For all experiments, $n_i = 30$, $k = 2$, and $m = 4$; therefore, df = 52 (Taylor *et al.*, 1992).

3.3 RESULTS

3.3.1 Experiment 1 (Copper Add Back)

When plants were exposed to Cu ranging from 0.5 to 15 μM in AB treatments, root growth showed a sigmoidal response, decreasing as Cu concentrations increased (Fig. 3.1A). In the NAB treatments, growth increased up to 1.0 μM Cu then decreased as Cu

concentrations increased (Fig. 3.1A). These results suggested that when initial Cu concentrations were not maintained, Cu was depleted from low Cu (0.05 to 0.5 μM) treatments inducing a deficiency response. The modified Weibull function modeled the toxic range of both curves with R^2 values ≥ 0.94 (Table 3.1). Significant differences were observed for both shape parameters, *c* and *d* (Table 3.1), indicating that the growth response to increasing Cu concentrations was altered when initial Cu concentrations were maintained compared to when initial concentrations were not maintained.

At the lowest Cu concentrations (≤ 2 μM), pH of solutions increased throughout the experimental period suggesting that anion uptake was greater than cation uptake (Fig. 3.1B and C). A stable pH was observed at the highest Cu concentrations (≥ 3 μM), indicating either a balanced cation-anion uptake or that reduced growth resulted in an overall ion uptake which was insufficient to alter the pH of growth solutions.

For the lowest Cu concentrations (≤ 2 μM) EC values decreased throughout the experimental period as nutrients were depleted from solution. At high Cu concentrations (≥ 3 μM), little change from initial EC values was observed (Fig. 3.1D and E). These results were consistent with the greater biomass accumulations and higher plant relative growth rates (RGR) observed in the lower Cu concentrations (Fig. 3.1A).

3.3.2 Experiment 2 (Low versus Intermediate Ionic Strength)

Roots showed a sigmoidal growth response to increasing levels of Cu at both ionic strength levels (50 and 1,000 μM N), with growth curves converging at concentrations of Cu greater than 2 μM (Fig. 3.2A). In both the ionic strength treatments, the growth of plants was accurately modeled by the Weibull function with R^2 values > 0.93 (Table 3.1). The greater root growth observed in the IIS treatment, compared to the LIS treatment, at concentrations of Cu 2 μM or lower, resulted in a significant increase in Weibull parameter *b* from 1.61 to 3.37 (Table 3.1). There were no significant changes in any of the other Weibull parameters (Table 3.1), suggesting that the shape of the dose response was not affected by changes in ionic strength. A RGR of 21.6% was observed in IIS treatments which exceeded the 17.9% RGR observed in LIS treatments, probably resulting from the greater availability of nutrients in the IIS background solutions.

In the LIS treatments, pH at the lowest Cu concentrations (< 1 μM) remained stable throughout the experimental period (Fig. 3.3A), suggesting that cation-anion uptake was balanced. In contrast, pH increased throughout the experimental period in the IIS treatments with 0.05 μM Cu, suggesting that plants were utilizing the NO_3^- which was present at higher levels in the 1,000 μM background (Fig. 3.3B). In this treatment, RGR > RAR so plant growth would have to be supported by nutrients present in the background. At concentrations of Cu ≥ 2 μM, pH decreased for both background levels, suggesting that cation uptake was greater than anion uptake.

Electrical conductivity in the LIS treatments remained stable until the last three days of the experimental period, when Cu concentrations were below 1 μM, and then increased. This suggested that nutrients were accumulating in the growth solutions (Fig. 3.3C). In the IIS treatments, the lowest Cu concentrations (< 1 μM) resulted in reduced EC (Fig. 3.3D). The EC's in both LIS and HIS treatments increased when Cu concentrations were greater than 1 μM, presumably as a result of a build up of nutrients in the growth solutions resulting from reduced growth imposed by Cu toxicity (Fig. 3.3C and D).

3.3.3 Experiment 3 (Low versus Intermediate Ionic Strength)

When plants were exposed to a narrower range of Cu concentrations (0.005 to 3 μM), growth decreased at both ionic strength levels as Cu levels increased (Fig. 3.2B). Once again, a greater RGR was observed at the lower Cu concentrations in the IIS treatments (20.1 to 21.4%) than in LIS treatments (17.2 to 17.6%). Although R^2 was $\geq$ 0.91 for both curves, the Weibull function was unable to model the curve for the intermediate ionic strength treatment (Table 3.1). As a result, statistical analyses could not be performed for this experiment. Electrical conductivity and pH in this experiment followed the same pattern that was observed in experiment 2 (data not shown).

3.3.4 Experiment 4 (Low versus High Ionic Strength)

A sigmoidal response was once again observed for both the LIS and HIS treatments, and the curves converged at concentrations of Cu > 2 μM (Fig. 3.4A). Growth was accurately modeled by the modified Weibull function with the R^2 values being > 0.97 (Table 3.1). The HIS treatment resulted in greater growth (RGR 18.2 to 22.9%) compared to the LIS treatment (RGR 13.2 to 19.4%) at levels of Cu below 3 μM. A significant increase was observed in parameter *b* from 2.87 to 3.88 (Table 3.1). Significant differences were also observed in Weibull parameters *c* and *d*, indicating that the HIS treatment affected the shape of the dose response curve. As little growth reduction was observed in the HIS treatments compared to the LIS treatments (at Cu concentrations < 2 μM), the significant differences in the shape parameters suggested that HIS reduced Cu toxicity.

For both treatments, pH readings exhibited a similar pattern to the ones observed in experiments 2 and 3 (Fig. 3.5A and B). For the LIS treatments (Cu < 1 μM) EC readings remained stable throughout the experimental period. Electrical conductivity readings increased at concentrations of Cu ≥ 2 μM (Fig. 3.5C). A reduction in EC was observed for the HIS treatments at concentrations of Cu < 1 μM (Fig. 3.5D), suggesting plants were depleting nutrients from solution. Where growth was inhibited (concentrations of Cu ≥ 2 μM), little reduction in EC was observed (Fig. 3.5D).

3.3.5 Experiment 5 (Low versus High Ionic Strength)

When a narrower range of Cu (0.005 to 3 μM) was used, a decrease in growth was observed in both LIS and HIS treatments as Cu levels increased (Fig. 3.4B). While the modified Weibull function modeled both curves at $R^2 > 0.94$, there were high standard errors (> 35%) observed for all of the parameters (Table 3.1); therefore, I felt statistical analysis of the results could not be relied upon for accurate interpretation of these experimental results. The pH and EC readings for LIS and HIS treatments followed a similar pattern to that observed in experiment 4 (data not shown).

3.3.6 Experiment 6 (Amelioration Experiment)

Growth varied depending on the individual ion (no ion, Na, K, Ca, Mg) present. The greatest growth was observed in the control treatment (RAR Cu with no added ion: Figs. 3.6 and 3.7). Growth decreased in the presence of Na and K as Cu levels increased, and was almost completely inhibited at the highest Cu levels (2.25 μM; Figs. 3.6 and 3.7). Growth remained relatively constant in the presence of additional Ca or Mg as levels of Cu increased (Figs. 3.6 and 3.7).

For all treatments, pH remained fairly stable throughout the experimental period (data not shown) as observed for LIS treatments in experiment 2. Electrical conductivity also remained constant until the final three days of the experimental period and then it

increased (data not shown), presumably due to an accumulation of nutrients in growth solutions. This pattern of response was similar to that observed in experiment 2.

3.4 DISCUSSION

Results from the current study supported the idea that increased ionic strength can significantly decrease Cu toxicity. Several other studies have observed similar decreases in cation toxicity as ionic strength of growth solutions is increased (Pavan and Bingham, 1982; Riedell and Schmid, 1986; Blamey *et al.*, 1983). Speciation analyses for the three ionic strength treatments used in this study indicate that Cu^{2+} activities decreased as ionic strength increased (Table 3.2). Percent free Cu^{2+} decreased as ionic strength increased, but there was no change in percent free Cu^{2+} as the Cu concentration increased within an ionic strength treatment (Table 3.2). The values predicted for percent free Cu^{2+} indicate that the majority of Cu in solution is available for plant uptake at all three ionic strengths. As the greatest change between ionic strength treatments is reduced activity, this would suggest that the reduction in activity is more likely to be responsible for reduced Cu toxicity than reduced availability of Cu^{2+} for uptake through losses by precipitation or complexation. While it is known that Cu activity will change during the course of the experiment due to uptake of nutrients by plants, the speciation analyses give an indication of the magnitude of decreases in activity that might accompany increases or decreases in ionic strength. Despite probable reductions in Cu activity resulting from plant uptake during the experimental period, I believe that frequent adjustment of Cu levels back to

initial concentrations and use of the RAR technique minimized these changes.

Use of the RAR technique should help minimize changes in speciation in growth solutions. Nutrients are added frequently in proportions which should promote healthy growth and, in my experiments, at a rate which should control the growth rate. As a result, nutrient addition and nutrient uptake should be balanced, resulting in minimal changes in the ionic composition and ionic strength of growth solutions. This should minimize changes in speciation. Monitoring electrical conductivity (EC) is a method by which nutrient accumulation in growth solutions can be detected. The stable EC observed in my experiments suggests that plant uptake and nutrient additions were balanced which should limit changes in Cu speciation.

In addition to the reduction of Cu toxicity, observed as a change in the shape of the dose response curve in HIS treatments in experiment 4, both IIS and HIS treatments with less than 2 μM Cu resulted in increased growth in comparison to LIS treatments. Increased growth was probably due to the greater availability of nutrients in the IIS and HIS background solutions. With the RAR technique, plant growth can be controlled by the relative rate of nutrient addition, provided background nutrients are low. When the concentration of background nutrients exceeds a required minimum concentration (which may vary with species), nutrient uptake increases resulting in a RGR that exceed the RAR (Ingestad, 1982). The reduction in EC values observed in both the IIS and HIS treatments supports the idea that plants were using nutrients present in the background solution. This

idea is further supported by the observation that RGR's (> 21.6%) were greater than the 20% RAR. Stadt *et al.* (1992) also observed increased RGR's as background nutrient concentrations increased. Similarly, Letchamo *et al.* (1993) observed increased biomass accumulation when nutrient levels (EC) were increased. However, increasing EC values observed in LIS treatments in the final days of an experiment (Fig. 3.3C and 3.5C) could indicate plants were coming out of exponential growth, which results in a RGR < RAR and the subsequent accumulation of nutrients. Root exudates could also contribute to the increased EC readings (Ingestad, 1972).

While results from my experiments support the idea that increased ionic strength can reduce metal toxicity and affect the shape of dose response curves, the results of my cation amelioration experiments indicate that the action of single cations can play a role in amelioration of Cu toxicity beyond that which can be explained by reduced activity alone. Similar conclusions have been drawn in studies by Horst (1987), Kinraide *et al.* (1993) and Kinraide (1994). Single cations may be effective in ameliorating the toxicity of other cations beyond that which can be explained by ionic strength effects by reducing the negativity of the cell surface potential (by binding to cell surfaces) or by charge screening (Kinraide *et al.*, 1992). Alternatively, the unique properties of the individual ameliorating cations may contribute to their ameliorative effectiveness. For example, Ouzounidou (1994) concluded that Cu toxicity in *Alyssum montanum* L. reduced photosynthesis when Cu^{2+} replaced Mg^{2+} in the chlorophyll molecule, which in turn resulted in growth reduction. In this case, Mg^{2+} may be a more effective ameliorant of Cu toxicity than other

divalent cations due to interactions with chlorophyll.

When the ionic strength of growth solutions was increased by the addition of Na, K, Ca or Mg, the calculated ionic strength and measured EC of these solutions was greater than the ionic strength of the HIS treatment in which amelioration was observed (Tables 3.2 and 3.3). If ionic strength was the only factor affecting the degree of Cu toxicity observed, amelioration should have been observed in each of these single ion treatments. Although amelioration was observed in the Ca^{2+} and Mg^{2+} treatments, K^{+} and Na^{+} were not effective (Figs. 3.6). These results are similar to those of Kinraide and Parker (1987, 1992) who found that divalent cations were more effective than monovalent cations in ameliorating Al^{3+} toxicity. They suggested that amelioration was a result of a reduction in cell-surface electrical potential caused by polyvalent or divalent cations binding to negative sites, or by charge screening which would result in reduced affinity of cell surfaces for toxic cations (Kinraide *et al.*, 1992). The effect of Ca^{2+} and Mg^{2+} in ameliorating Cu toxicity in my experiment may be the result of a reduction in cell-surface electrical potential due to the charge on these cations; however, further study is required to confirm this idea.

One result which was not anticipated was the reduction in growth (compared to the control) that was observed in the cation treatments (Na, K, Ca, Mg) when Cu concentrations were below 0.75 μM (Fig. 3.6). Reduced growth was most apparent in the K treatment and this was repeatable in three separate experiments. These results were

observed despite results from a nine day preliminary experiment which indicated that none of these cations reduced growth at concentrations up to 3.7 mM in combination with a 50 μM N background (data not shown). In addition, a previous study with *Triticum aestivum* (Kinraide and Parker, 1987) used higher concentrations than those used in this study (40 mM Na and K, 4 mM Ca and 3.6 mM Mg) without observing any detrimental effects on growth. The longer time of the amelioration experiment (18 days) may have played a role in the reduced growth in this experiment (in comparison to my shorter 9 day preliminary experiment or Kinraide and Parker's (1987) 2 day experiment). It is also possible the increased concentration of the ameliorating cations may have inhibited uptake of other nutrients which resulted in reduced growth. However, further study is required to draw a more definite conclusion.

While the results of my experiments support the idea that some mechanism in addition to ionic strength is playing a role in ameliorating Cu toxicity, I cannot determine the nature of this mechanism from my experiments. Future studies may involve looking at the effect of increasing concentrations of cations on Cu toxicity. For example, is some minimum concentration of the ameliorating cation required for amelioration and further increases in the concentration would not result in increased amelioration (no further improvement in growth). Alternatively, is the degree of amelioration dose dependent. In this case, the magnitude of amelioration (improvement in growth) would increase as the concentration of the ameliorating ion increased. Future research could also involve direct measurement of Cu activity at cell surfaces and in bulk solution. Copper activities in

nutrient solutions and at cell surfaces are presently predicted by mathematical models which rely on estimates of some of the parameter values. Therefore, the accuracy of these models is dependent on accuracy of the estimates. Development of methods which are capable of directly measuring the low levels of Cu^{2+} activities present in nutrient solutions and at cell surfaces would provide a more accurate means of determining the effects of ionic strength and the influence of cell surface charges on Cu^{2+} activity. These methods could help clarify the nature of the mechanism which plays a role in amelioration of Cu toxicity in the presence of increased concentrations of specific cations beyond the amelioration which can be attributed to ionic strength effects. Understanding this mechanism could lead to methods of improving plant growth in environments where Cu concentrations are elevated.

Table 3.1. Analyzing the effect of ionic strength on root biomass accumulation of *Triticum aestivum* L. cv Atlas 66 using the modified Weibull frequency distribution to model the response to Cu when initial Cu concentrations are or are not maintained (experiment 1), or in the presence of low, intermediate or high ionic strength growth solutions (experiments 2 to 5). Values are the four Weibull parameters ± SE and R^2 values.

	Weibull Parameters				
	a	*b*	*c*	*d*	R^2
Experiment 1					
No Cu Add Back	0.26 ± 0.09	3.37 ± 0.22	2.47 ± 0.11***	4.79 ± 1.00**	0.98
Cu Add Back	0.24 ± 0.17	3.92 ± 0.36	1.86 ± 0.20***	1.77 ± 0.49**	0.94
Experiment 2					
Low Ionic Strength	0.14 ± 0.07	1.61 ± 0.16*	1.34 ± 0.17	1.55 ± 0.43	0.93
Intermediate Ionic Strength	0.19 ± 0.08	3.37 ± 0.21*	1.20 ± 0.09	1.71 ± 0.31	0.97
Experiment 3					
Low Ionic Strength	0.45 ± 0.18	1.26 ± 0.21	1.48 ± 0.21	2.99 ± 1.39	0.95
Intermediate Ionic Strength	would not model				0.91
Experiment 4					
Low Ionic Strength	0.25 ± 0.07	2.87 ± 0.16*	1.38 ± 0.08*	2.52 ± 0.47*	0.97
High Ionic Strength	0.39 ± 0.08	3.88 ± 0.14*	1.83 ± 0.04*	9.09 ± 1.53*	0.99
Experiment 5					
Low Ionic Strength	0.001 ± 0.93	2.02 ± 0.97	1.85 ± 1.10	1.53 ± 0.86	0.94
High Ionic Strength	0.029 ± 1.94	4.18 ± 2.01	2.43 ± 0.86	2.99 ± 1.62	0.98

Significant difference between parameters within an experiment.

*** $P \leq 0.05$, $t(0.05) = 2.007$ ** $P \leq 0.01$, $t(0.01) = 2.674$\ * $P \leq 0.001$, $t(0.01) = 3.488$

Table 3.2. Geochem analysis of free activity of Cu^{2+}, per cent free activity of Cu^{2+} and calculated ionic strength in nutrient solutions with low (50 μM N), intermediate (1,000 μM N) or high (2.94 mM N) background nutrient concentrations.

	Free Activity (M)		
Cu Concentration (M)	Low	Intermediate	High
5.0×10^{-9}	4.25×10^{-9}	4.03×10^{-9}	3.78×10^{-9}
5.0×10^{-7}	4.25×10^{-8}	4.03×10^{-8}	3.78×10^{-8}
3.0×10^{-6}	2.59×10^{-6}	2.42×10^{-6}	2.26×10^{-6}
15.0×10^{-6}	1.27×10^{-5}	1.21×10^{-5}	1.13×10^{-5}
% Free Metal **	99.8	99.0	97.9
Calculated Ionic Strength	$1.27\text{-}1.30 \times 10^{-3}$	$2.12\text{-}2.15 \times 10^{-3}$	$3.48\text{-}3.51 \times 10^{-3}$

** % Free metal did not vary over the range of copper concentrations within a treatment.

Table 3.3. Geochem analysis of Cu^{2+} free activity, per cent free Cu^{2+} and calculated ionic strength in nutrient solutions containing 50 μM N background solutions with no additional cation (control), or 3.7 mM additional of one of Na, K, Ca or Mg.

	Free Activity (M)				
Cu Concentration (M)	Control	Na	K	Ca	Mg
2.88×10^{-9}	2.45×10^{-9}	2.11×10^{-9}	2.11×10^{-9}	1.80×10^{-9}	1.81×10^{-9}
7.5×10^{-7}	6.37×10^{-7}	5.49×10^{-7}	5.49×10^{-7}	4.70×10^{-7}	4.701×10^{-7}
1.5×10^{-6}	1.27×10^{-6}	1.10×10^{-6}	1.10×10^{-6}	9.40×10^{-7}	9.402×10^{-7}
2.25×10^{-6}	1.91×10^{-6}	1.65×10^{-6}	1.65×10^{-6}	1.41×10^{-6}	1.410×10^{-6}
% Free Metal **	99.8	99.1	99.1	98.7	98.7
Calculated Ionic Strength	$1.27\text{-}1.28 \times 10^{-3}$	$4.96\text{-}4.97 \times 10^{-3}$	$4.97\text{-}4.98 \times 10^{-3}$	1.24×10^{-2}	1.23×10^{-2}

** % Free metal did not vary over the range of copper concentrations within a treatment.

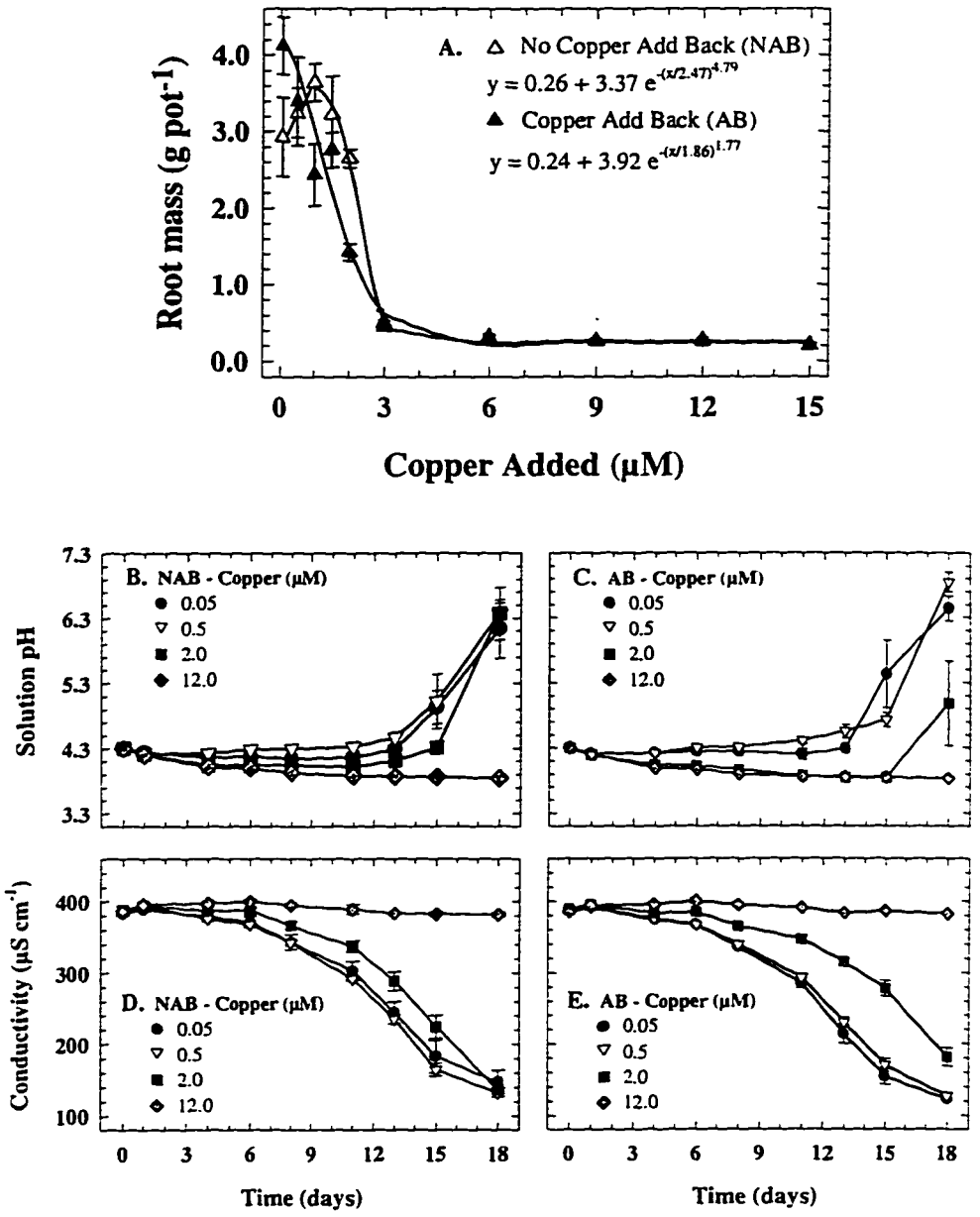

Figure 3.1. Root growth of plants exposed to 0.05 to 15 µM Cu (A) with maintenance of initial copper concentrations (add back (AB) treatments; filled symbols), or without copper add back (NAB; open symbols). Change in solution pH, NAB (B) and AB (C). Change in solution electrical conductivity, NAB (D) and AB (E). Values are means ± SE (n=3).

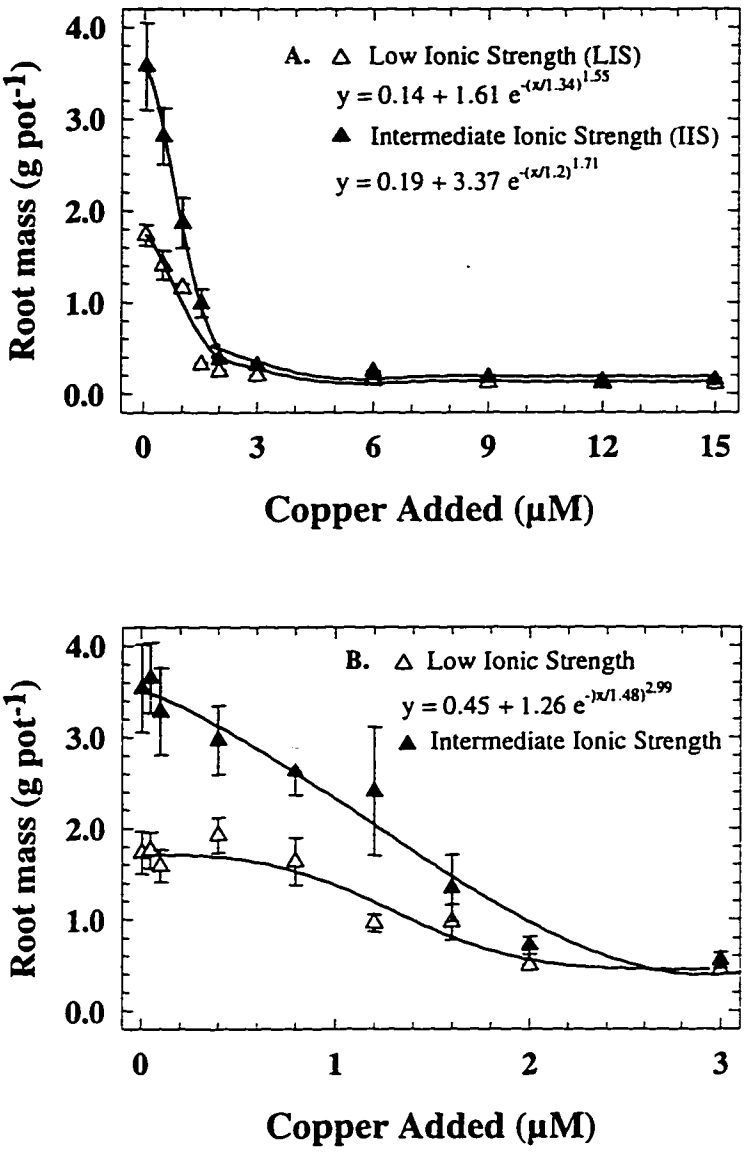

Figure 3.2. Root growth of plants exposed to 0.05 to 15 µM Cu (A) or 0.005 to 3.0 µM Cu (B), in low ionic strength (open symbols) or intermediate ionic strength (closed symbols) growth solutions. Values are means ± SE (n=3).

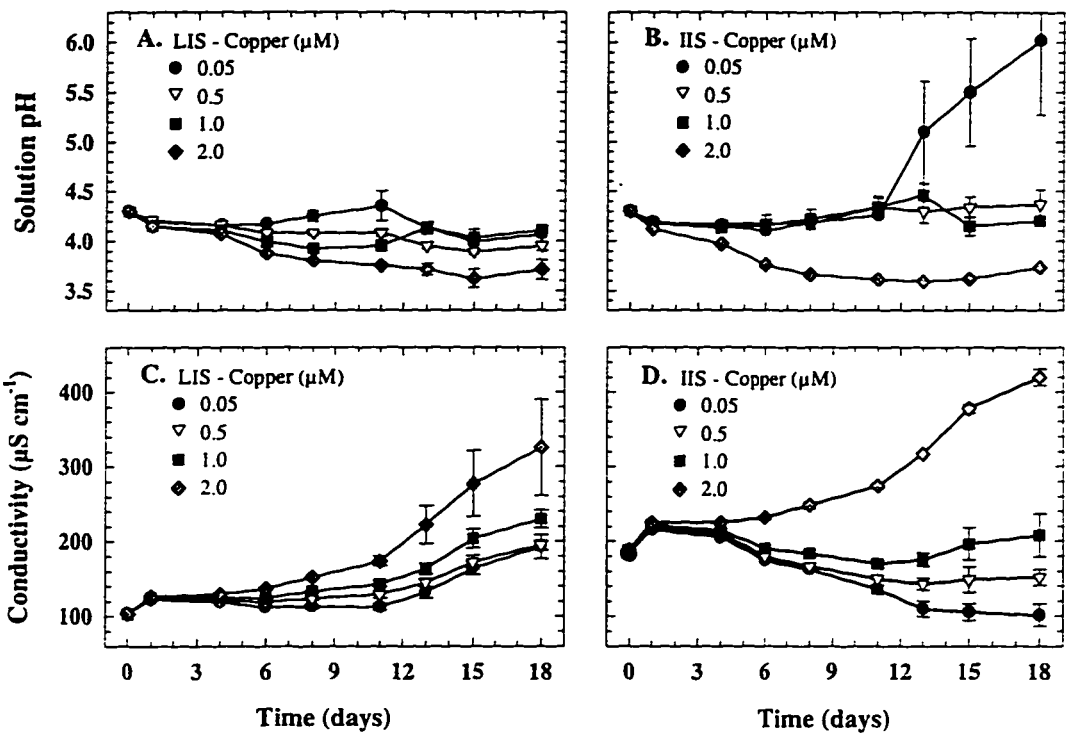

Figure 3.3. Solution pH in low ionic strength (A) and intermediate ionic strength (B) and solution electrical conductivity in low ionic strength (C) and intermediate ionic strength (D) growth solutions when exposed to varying Cu concentrations. Values are means ± SE (n=3).

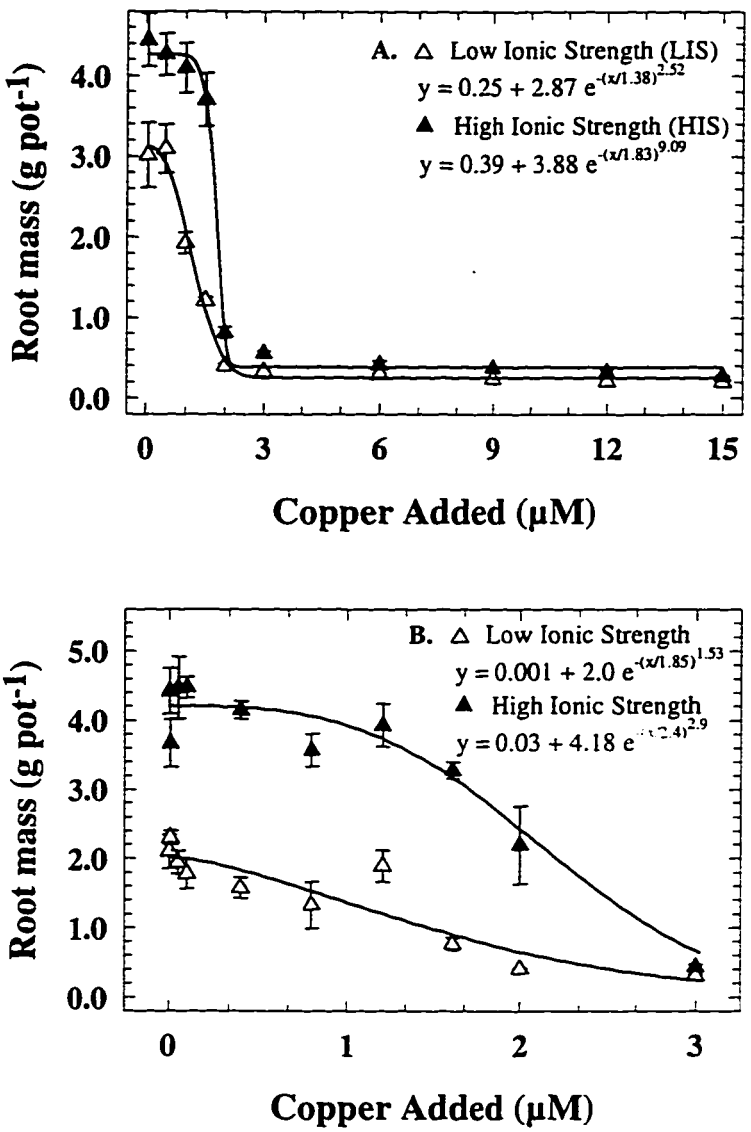

Figure 3.4. Root growth of plants exposed to 0.05 to 15 µM Cu (A) or 0.005 to 3.0 µM Cu (B), in low ionic strength (open symbols) or high ionic strength (closed symbols) growth solutions. Values are means ± SE (n=3).

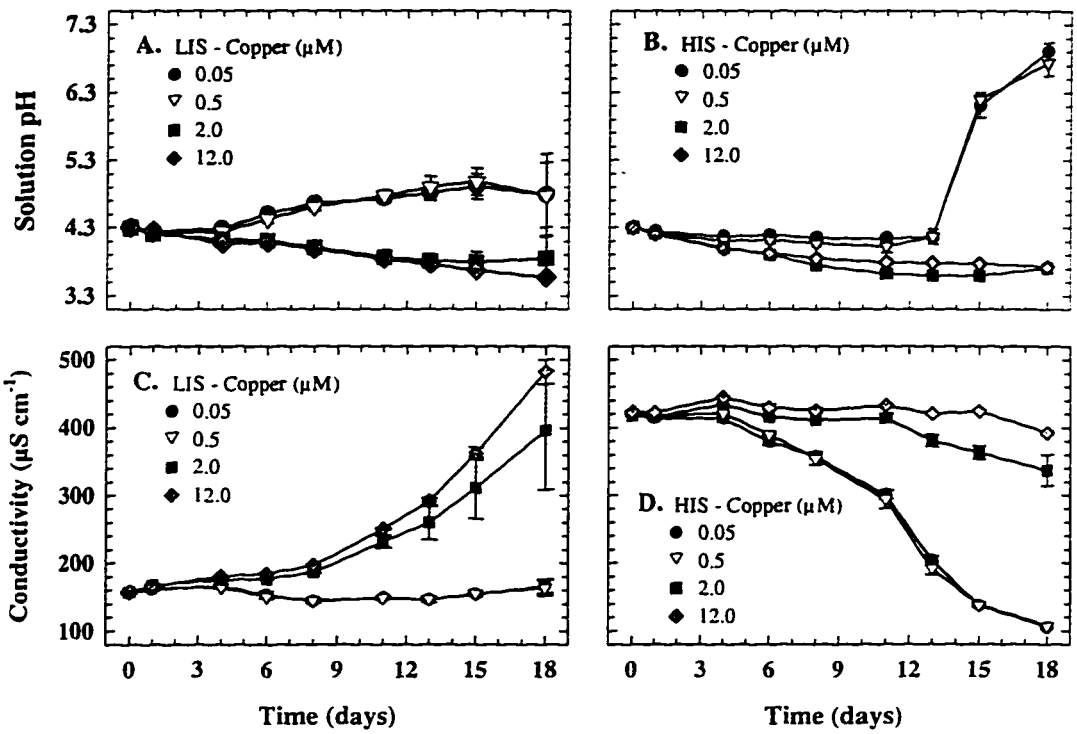

Figure 3.5. Solution pH in low ionic strength (A) and high ionic strength (B) and solution electrical conductivity in low ionic strength (C) and high ionic strength (D) growth solutions when exposed to varying Cu concentrations. Values are means $\pm$ SE (n=3).

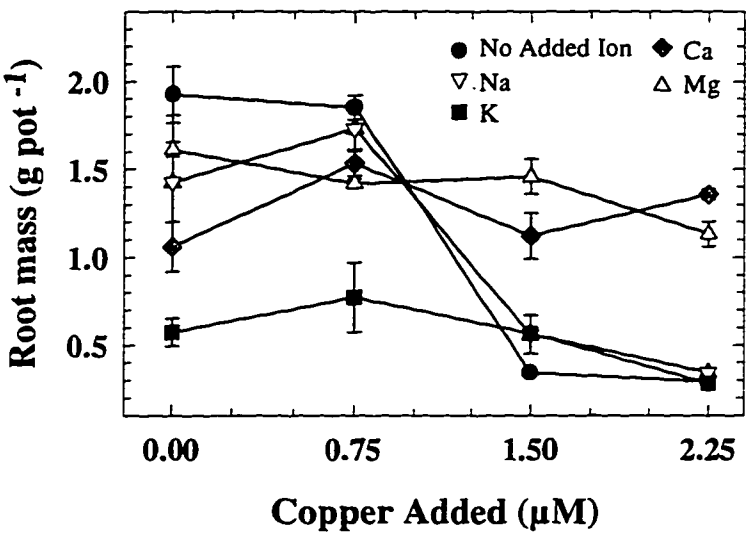

Figure 3.6. Root growth of plants exposed to 0.00288 to 2.25 µM Cu with a 50 µM N background and no added ion (control) or 3.7 mM additional Na, K, Ca or Mg.

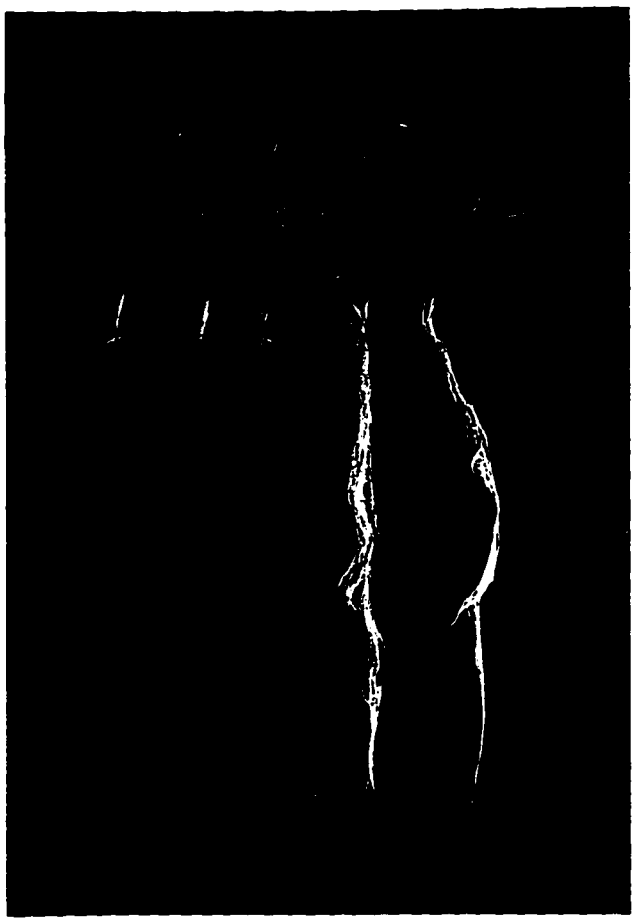

Figure 3.7. Growth of plants in experiment 6 exposed to 2.25 μM Cu with a 50 μM N background and from left to right; no added ion, Na, K, Ca or Mg.

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4 CONCLUDING DISCUSSION

The objective of my research was to determine if cation amelioration of cation toxicity is a general effect of increased ionic strength or if the actions of specific cations are responsible for amelioration. This question is of concern since our understanding of the effects of metal toxicity on plant growth is based on an extensive body of research in which the majority of experiments have used nutrient concentrations many fold higher than that which would be available in most fertile soils. High ionic strength of growth mediums have been shown to reduce the activity of ions which in turn results in reduced metal toxicity (Pavan and Bingham, 1982; Riedell and Schmid, 1986; Blamey *et al.*, 1983). However, the amelioration observed in a number of experiments can not be fully explained by decreased ion activity which accompanies increased ionic strength (Alva *et al.*, 1986a, b; Horst, 1987; Kinraide and Parker, 1987; Macfie *et al.*, 1994).

My experiments confirmed that specific cations do ameliorate Cu toxicity to a greater extent than can be explained by their effect in reducing the activity of Cu^{2+}. Before I was able to perform the amelioration experiments I had to overcome the problems of growing plants in low ionic strength solutions. This was accomplished by using a technique developed by Ingestad (1982), the relative addition rate (RAR) technique. When I started my research, few experiments had been conducted with *Triticum aestivum* using the RAR technique. Therefore, the first part of my research was the optimization of growth of *Triticum aestivum* in low ionic strength growth solutions. For these

experiments, a computer-controlled nutrient delivery system was utilized to supply nutrients to plants in daily exponentially increasing amounts. While a computer-controlled nutrient delivery system is convenient and accurate, it is not necessary for effective use of the RAR technique. Stadt *et al.* (1992) showed that the RAR technique can be effectively used with a traditional solution culture system by manual addition of nutrients.

While I was able to achieve optimal growth by adjusting relative nutrient proportions, background nutrient concentrations and total background Ca concentration with no accumulation of nutrients in growth solutions, I had difficulty reducing plant-induced pH fluctuations. Initial experiments were performed using cv. Katepwa, but growth rates were reduced at NH_4^+/NO_3^- ratios that were required to eliminate pH fluctuations of growth solutions. As prior studies had shown that changes in pH of growth solutions can vary with the cultivar being grown (Foy, 1965) and pH fluctuations can be reduced by increasing the NH_4^+ proportion of the nitrogen supply without reducing plant growth (Stadt *et al.*, 1992; Ericsson, 1981), I decided to test a number of cultivars for a greater NH_4^+ tolerance. Results of the cultivar screening indicated that Atlas 66 had the greatest resistance to increased NH_4^+ with reduced pH fluctuations. While changing cultivars enabled me to minimize pH fluctuations of growth solutions, adjustments of other nutritional requirements may have been required. Fortunately, Atlas 66 grew optimally under the conditions previously optimized for Katepwa.

The optimization portion of my research showed that it is possible to achieve healthy growth in which the RGR is controlled by the RAR of nutrients in solution culture with concentrations that closely resemble soil solution concentrations. Future nutritional and metal toxicity studies should make use of more realistic concentrations in solution culture experiments, which should provide physiological responses more closely resembling those of plants grown in soil.

Once optimization of growth had been achieved, I could proceed with the amelioration experiments. The first step was testing the effects of increased ionic strength on Cu^{2+} toxicity. In order to achieve a significant reduction in Cu toxicity, the concentration of background nutrients had to be increased from 50 μM to 2.94 mM N. While I expected there to be a reduction in Cu toxicity when the background concentration was increased to 2.94 mM N, what was most surprising was the small (less than 12%) reduction in free activity of Cu^{2+} that was predicted to occur with a greater than 55 fold increase in ionic strength (see Table 3.2). If the predicted changes in activity resulted in reduced toxicity in this study, it is probable that many metal toxicity studies using solutions with concentrations as high or higher than concentrations used in this study may also have resulted in reduced toxicity of the test ion. If studies conducted using high ionic strength have been used to set toxicity thresholds or tolerance levels for field grown plants, the tolerance of the test species may have been overestimated

While speciation analysis predicts ion activity, direct measurement would be a more effective tool in comparing differences in activities between solutions. I had planned to conduct experiments in which Cu activity was kept constant while ionic strength was increased. However, when I attempted to use a Cu-selective electrode to measure Cu^{2+} activities Cu treatments less than 3 μM were below the detection limit of the electrode or there was interference by other ions in solution. Before direct measurement is feasible, a more sensitive of directly measuring Cu^{2+} activity must be developed.

Once I had confirmed that a general increase in ionic strength would ameliorate Cu toxicity, I tested individual cations for their ameliorative effectiveness. Results of this experiment confirmed that the divalent cations Ca^{2+} and Mg^{2+} were more effective in ameliorating Cu toxicity than the monovalent cations Na^{+} and K^{+}. This result and the fact that the ionic strength of each of the cation treatments was greater that the ionic strength of the HIS treatment suggested that the amelioration observed was a result of a mechanism other than increased ionic strength. Amelioration was observed in the presence of Ca^{2+} and Mg^{2+} treatments and not Na^{+} or K^{+}, which would suggest that amelioration is associated with some property of the divalent cations. It is possible that amelioration resulted from Ca^{2+} or Mg^{2+} binding to negative sites on cell-surfaces, reducing the cell-surface potential, by charge screening or by a combination of the two (Borst-Pauwels and Severns, 1984, Kinraide *et al.*, 1992). In either case, uptake of and/or toxicity of Cu would be reduced. Unfortunately, this study was unable to clarify the associated mechanism. Presently, mathematical models and estimates of many of the

parameters are the method of determining cation activities at cell surfaces. However, these models are only as accurate as the estimates of the parameters used in the calculations. A method of directly measuring or determing the activity of cations at cell-surfaces in comparison to the activity in bulk nutrient solutions will indicate the magnitude of the influence of cell-surface charges on cation activities.

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Appendix 5.1 Composition of NH_4^+/NO_3^- and NaCl treatments used in nitrogen source experiment (experiment 7) based on a 7.0 M N *stock solution*. Values are shown as moles L^{-1}. These *stock solutions* were diluted 10 times to prepare nutrient *delivery solutions*.

	Total (M)		Chemical Composition of Stock Solutions (M)								
% NH_4^+ /N	NH_4^+	NO_3^-	NH_4NO_3	NH_4Cl	$Mg(NO_3)_2$	$Ca(NO_3)_2$	$K(NO_3)$	$NaNO_3$	$MgCl_2$	NaCl	$CaCl_2$
Nitrogen Treatments											
5	0.35	6.65	0.35	-	0.40	0.50	1.7	2.80	-	-	-
10	0.70	6.3	0.70	-	0.40	0.50	1.7	2.10	-	-	-
15	1.05	5.95	1.05	-	0.40	0.50	1.7	1.40	-	-	-
20	1.40	5.60	1.40	-	0.40	0.50	1.7	0.70	-	-	-
25	1.75	5.25	1.75	-	0.40	0.50	1.7	-	-	-	-
30	2.10	4.90	1.75	0.35	0.225	0.50	1.7	-	-	-	-
35	2.45	4.55	1.75	0.70	0.05	0.50	1.7	-	0.175	-	-
40	2.80	4.20	1.75	1.05	-	0.375	1.7	-	0.35	-	0.125
45	3.15	3.85	1.75	1.40	-	0.20	1.7	-	0.4	-	0.30
50	3.50	3.50	1.75	1.75	-	0.05	1.7	-	0.40	-	0.45
NaCl Controls											
25	1.75	5.25	1.75	-	0.40	0.50	1.7	-	0.4	0.70	-
25	1.75	5.25	1.75	-	0.40	0.50	1.7	-	-	1.40	-
25	1.75	5.25	1.75	-	0.40	0.50	1.7	-	-	2.10	-
25	1.75	5.25	1.75	-	0.40	0.50	1.7	-	-	2.80	-
25	1.75	5.25	1.75	-	0.40	0.50	1.7	-	-	3.50	-