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UNIVERSITY OF ALBERTA

MANGANESE TOXICITY IN *Triticum aestivum* L. (WHEAT)

BY

SHEILA MARGARET MACFIE

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY.

IN

ECO-PHYSIOLOGY

DEPARTMENT OF BOTANY

EDMONTON, ALBERTA

FALL 1991

ISBN 0-315-69997-3

UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR: **Sheila Margaret Macfie**

TITLE OF THESIS: **Manganese toxicity in *Triticum aestivum* L. (wheat)**

DEGREE: **Doctor of Philosophy**

YEAR THIS DEGREE GRANTED: **1991**

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled MANGANESE TOXICITY IN *Triticum aestivum* L. (WHEAT) submitted by SHEILA MARGARET MACFIE in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in ECO-PHYSIOLOGY.

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ABSTRACT

Manganese (Mn) and aluminum (Al) toxicities are potentially important growth-limiting factors for crops on acid soils. Until recently, most studies related to metal toxicity on acid soils have focussed on Al. This dissertation contains important information on Mn-tolerance and toxicity in *Triticum aestivum* L. (wheat). A screen of thirty cultivars of wheat identified a broad range of tolerance of Mn (in 500 μM Mn, growth ranged from 8 to 88% of control) confirming that Mn toxicity may be as important as Al toxicity for wheat growing on acid soils. In addition, a positive correlation was found between Mn- and Al-tolerance, indicating potential for developing lines better suited to areas with acid soils. Two cultivars (Mn-tolerant 'Norquay' and Mn-sensitive 'Columbus') were chosen to investigate the physiology of differential tolerance of Mn. Precipitation of Mn occurred on the roots of the tolerant cultivar when grown in excess Mn, whereas roots of the sensitive cultivar remained white. Plant-induced changes in pH of nutrient solutions appeared to control precipitation of Mn onto root surfaces; however, this precipitation did not confer tolerance of Mn. This result, along with results of other studies, suggests that internal tolerance mechanisms are more important than external tolerance mechanisms in determining tolerance of Mn in wheat.

In wheat, Mn toxicity involves an array of physiological responses, including reduced photosynthetic rates and chlorosis. When grown in a range of concentrations of Mn (0-1000 μM), the tolerant cultivar maintained higher photosynthetic rates and concentrations of chlorophyll than the sensitive cultivar despite accumulation of greater concentrations of Mn in leaves. Examination of photosynthetic rate per unit chlorophyll indicated important differences between the cultivars. The primary toxic effect of Mn in the tolerant cultivar was a reduction in chlorophyll content which, in turn, resulted in a lowered rate of photosynthesis. In contrast, Mn exerted its toxic effect in the sensitive cultivar on photosynthesis and chlorophyll content independently. It has been suggested that tolerance of high internal concentrations of toxic metals may be achieved *via* complexation with organic acids, either in the cytosol or in the vacuole. In this study, growth in excess Mn increased concentrations of aconitate, α-ketoglutarate, and succinate in leaves of the sensitive cultivar only. However, incubation of leaf slices with [2-^{14}C] sodium acetate indicated that these organic acids were not derived from acetyl CoA feeding into the citric acid cycle. These results suggest that biosynthesis of organic acids is not a primary mechanism of Mn-tolerance in wheat.

In summary, differential tolerance of Mn has been demonstrated in wheat, and appears to be related to chlorophyll content and/or the ability to maintain photosynthesis. In addition, this study has generated some ideas for future research which should help clarify the Mn toxic response.

ACKNOWLEDGEMENTS

This work was funded by the Natural Sciences and Engineering Research Council of Canada and the University of Alberta. I would like to give special thanks to my supervisor, Dr. Gregory J. Taylor, for his time, advice and endless support. I would also like to thank the members of my supervisory committee: Dr. Mark Dale, Dr. David Gifford and Dr. Jane King for their time and helpful suggestions. Research equipment was borrowed from Dr. Edwin Cossins, Dr. David Gifford and Dr. John Hoddinott, to each I owe thanks. I am also indebted to Dr. Keith Briggs for providing wheat seeds, Dr. Edwin Cossins and Kenneth Stadt for reviewing manuscripts and Katherine Courtney, Jason Kerr and Kenneth Stadt for assistance in the laboratory.

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1. General introduction

Like many other Ph.D. candidates, I had no idea of the directions my dissertation research would take me. When I first arrived in Dr. Gregory Taylor's lab, he and his students were all working on the physiology of the aluminum (Al) stress response in wheat. I had not yet decided what my research topic would be, although I was determined to work on a stress other than Al. Not wanting to wander too far from the interests of my coworkers, I decided to investigate another metal stress, manganese (Mn) toxicity. For reasons which should become apparent as you read this dissertation, I soon discovered that I had stumbled across an intriguing topic.

1.1 Manganese toxicity: a problem of international importance

Manganese toxicity is part of the larger issue of soil acidity. Soil acidity is a major growth-limiting factor for plants in many parts of the world (Foy 1983) and can be caused by natural soil properties or by agricultural, manufacturing and waste disposal practices (Westerman 1987, Foy *et al.* 1978). Acid soil toxicity comprises a complex array of factors which may affect the growth of plants through several physiological pathways, including mineral deficiency, mineral toxicity or drought stress (Foy 1983). In particular, Al and Mn toxicities have been identified as major growth-limiting factors for crops on acid soils (Reisenauer 1988, Westerman 1987, Foy 1983). Aluminum toxicity can be severe in soils with pH $\leq$ 5.0, but may also occur at pH 5.5 in kaolinitic (Al silicate) soils (Foy *et al.* 1978). It is a particular problem in areas with strongly acidic subsoils that are difficult to lime and may be exacerbated through the use of nitrogenous fertilizers. Manganese toxicity may arise under a variety of conditions including; high total soil Mn, pH of substrate below 5.5, low soluble calcium (Ca) in relation to Mn, low soil oxygen caused by poor drainage, high temperature, soil compaction and excessive irrigation or rainfall (Ritchie 1989, Reisenauer 1988, Foy 1983). Manganese toxicity is of particular concern on acid soils since Mn in acid soil solutions is mainly in an uncomplexed form (Mn^{2+}) which can be readily taken up by plants (Ritchie 1989).

Given the worldwide distribution of acid soils, it is not surprising that Mn toxicity is an important concern for production of wheat (*Triticum aestivum* L.) on a worldwide basis. In Fig. 1.1, the distribution of acid soils is superimposed on the distribution of areas of wheat production . Large areas of Canada, the USA, eastern Europe and China have potential Mn toxicity problems for wheat. Amelioration of Mn-toxic soils in these areas is not always feasible or economical, especially in developing countries. It therefore becomes important to develop cultivars which are better suited to such growing conditions (Foy *et al.* 1988). In order to breed plants for growth on Mn-toxic soils,

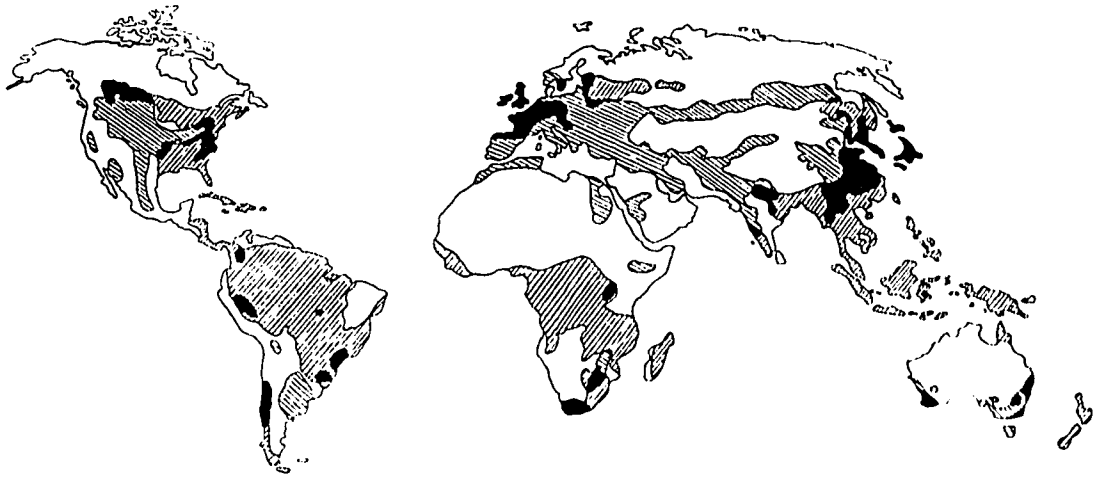

Figure 1.1 Worldwide distribution of acid soils (////) and major areas of production of wheat (\\\\). Areas of overlap are shaded black. Adapted from Van Wambeke (1976) and Briggle and Curtis (1987).

there must be appropriate genetic variation. Differential tolerance of Mn has been identified in many plant species and cultivars (Evans *et al.* 1987, Foy and Campbell 1984, Osborne *et al.* 1981, Foy *et al.* 1969). Cultivars of wheat also exhibit a range of tolerance to Mn (Neenan 1960, Foy *et al.* 1988), offering potential for growing wheat in acidic soils where Mn and Al toxicity may be growth-limiting factors. Furthermore, the genetic potential of wheat for improvement through plant breeding seems promising. Control of Mn-tolerance in many species appears to be multigenic (Heenan *et al.* 1981, Brown and Devine 1980). While tolerance of Mn in wheat is quantitatively inherited (Moroni 1991), it seems to have a relatively simple pattern of inheritance (Scott and Fisher 1989, Foy *et al.* 1988).

The factors outlined above are only part of the reason I elected to work with wheat as an experimental species. Wheat is an interesting crop in its own right. It is grown around the world in a wide range of environments; in fact, more land is devoted worldwide to the production of wheat than to any other commercial crop (Briggle and Curtis 1987). Wheat provides more nourishment for humans than any other food source (Johnson *et al.* 1978) and significant amounts are used for animal feed (Briggle and Curtis 1987). In Canada, wheat is the predominant crop grown, and 95% of all Canadian wheat is grown in the southern portions of the prairie provinces (Peterson 1965). As the global population increases, there will be a need to increase production of food crops. Greater production of wheat can be achieved in two ways, by (i) expanding the area sown and/or (ii) improving the yield per unit area sown (Briggle and Curtis 1987). The extent to which wheat can be grown on marginal lands depends on the nature of the stresses involved. Several cultivars of wheat have already been developed (through a cooperative program between Brazil and the International Maize and Wheat Improvement Center in Mexico) that yield twice as much as traditional cultivars under Al-toxic conditions (Briggle and Curtis 1987). The results of Chapter 2 (Macfie *et al.* 1989) indicate that a similar potential exists for Mn-tolerant lines.

1.2 Manganese toxicity: symptoms and toxic effects

Symptoms of Mn toxicity are most noticeable in aerial tissues and include marginal leaf chlorosis, interveinal chlorosis, crinkle leaf and necrotic spots, particularly on older leaves (Ritchie 1989, Horst 1988, Westerman 1987, Foy and Campbell 1984). Generally, damaged tissues have higher concentrations of Mn than surrounding tissues (Vlamis and Williams 1973). The first report of Mn toxicity was published in 1909 by Kelley who described toxicity for *Ananas comosus* growing on manganiferous soil in the drier regions of the Hawaiian Islands (Schlichting and Sparrow 1988). More recently, Mn-toxic soils have been noted in sections of Puerto Rico, Africa, Brazil, Australia and the USA. This list is likely incomplete, however, since little data have been published for

temperate, continental regions. In general, Mn-toxic soils are characterized by the weathering of parent material rich in bases (*eg.* basalt and limestone) in hot to warm regions, whereas the influence of ground water is also a factor in warm to cool regions (Schlichting and Sparrow 1988).

While differential tolerance of Al has been well described (see for example Taylor 1988, Foy 1983), relatively little attention has been paid to Mn-tolerance. This probably reflects the fact that, until recently, Al was considered to be of greater concern for plants growing on acid soils (Foy *et al.* 1978). Prior to this dissertation, the most recent comprehensive report of differential Mn-tolerance in wheat was published over 30 years ago (Neenan 1960) and did not include information on Canadian cultivars. To address this issue, my investigations began with a screen of 30 cultivars (Chapter 2, Macfie *et al.* 1989). While the majority of the cultivars screened were sensitive of Mn, the range of differential tolerance measured was comparable to previously documented differences in tolerance of Al (Briggs *et al.* 1989). This broad range of tolerance of Mn has been confirmed by more recent investigations at the University of Alberta (Moroni 1991). These results indicate that Mn toxicity is potentially as important as Al toxicity for plants growing on acid soils.

The physiology of Mn toxicity has received attention in the recent literature (see review by Horst 1988); however, the mechanisms of Mn phytotoxicity and differential tolerance remain unclear. The biochemistry of Mn in plants is equally ill-defined, although it is clear that Mn plays an important role in many biochemical processes, especially the water-splitting reaction in photosynthesis. The lack of understanding of the specific effects of Mn deficiency and /or toxicity results from the fact that, unlike other essential trace elements (such as Cu, Fe, Mo and Zn), Mn usually acts as a cofactor for enzyme-catalyzed reactions rather than as an integral component of enzymes (Burnell 1988). The implication of this is that Mn often replaces, or is replaced by, other divalent cations. Nieboer and Richardson (1980) classified metal ions based on their chemical properties and biological activity. According to their classification, one would expect Mn^{2+} to have phytotoxic effects similar to those of other "borderline" metals (eg. Cd^{2+}, Co^{2+}, Cr^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+}, Pb^{2+}, Sn^{2+}, Ti^{2+}, V^{2+} and Zn^{2+}). However, in some biological systems, Mn^{2+} can substitute for Ca^{2+} while in others it can substitute for Mg^{2+} (Hughes and Williams 1987), both of which were classified as "class A" metals by Nieboer and Richardson (1980). Indeed, one of the difficulties in understanding the biochemistry of Mn^{2+} is the identification and differentiation of those systems which require it and not Mg^{2+} (Hughes and Williams 1987). It is not my intention to elucidate the biochemistry of Mn toxicity; however, the results of experiments presented in this dissertation do help to identify pathways which are prime candidates for further investigation.

1.3 Manganese tolerance: physiological and biochemical aspects

The physiological bases for differential metal tolerances are also ill-defined; however, two broad categories of metal tolerance mechanisms are currently recognized, (i) exclusion mechanisms, where the toxic metal is excluded from the symplasm and (ii) internal tolerance mechanisms, where the toxic metal crosses the plasma membrane but is either detoxified or removed from sensitive metabolic sites. General examples of exclusion mechanisms include (i) exudation of chelate ligands into the rhizosphere, (ii) selective permeability of the plasma membrane, (iii) metal ion efflux, (iv) compartmentation of the metal in the cell wall, (v) plant-induced pH barrier in the rhizosphere and (vi) plant-induced redox barrier in the rhizosphere (Cumming and Taylor 1990), not all of which can explain tolerance of Mn. In soils, Mn forms chelates with citrate and oxalate at pH $\leq$ 6, with malate and malonate at pH $\leq$ 5.7 and with succinate at pH $\leq$ 5.4 (Cline *et al.* 1983). There has been no evidence, however, that exudation of organic ligands acts as a Mn-tolerance mechanism. Indeed, elevated concentrations of Mn found in tissues of Mn-tolerant plants (Foy 1984) would indicate that Mn is taken up by the plant. Selective permeability of the plasma membrane is unlikely to account for tolerance of Mn, since Mn enters the cell readily (Graham 1979) and there have been no reports on possible Mn efflux pumps in plant membranes. Similarly, there is no evidence of compartmentation of Mn in the cell wall (Memon and Yatazawa 1982). However, plant-induced oxidation of Mn^{2+} to Mn^{3+} has been well-documented for many wetland plants (Armstrong 1970). Plant-induced changes in pH of the rhizosphere can also be sufficient to render Mn insoluble (Foy *et al.* 1988). The relative effectiveness of these plant-induced changes would appear to be genotype-specific. In wheat, roots of plants grown in excess Mn turned a brown colour (Horiguchi 1987, Foy 1984), possibly reflecting precipitation of Mn on the root surface. During the dose-response experiments which I ran as part of Chapter 2 (Macfie *et al.* 1989), I noticed that the Mn-tolerant cultivar induced a rise in pH of solution by the end of the experimental period, and that the roots of these plants turned brown. Chapter 3 (Macfie and Taylor 1989) describes an experiment in which pH of solution was manipulated by altering supply of ammonium to test whether precipitation of Mn on root surfaces could account for differential tolerance of Mn. Plant-induced changes in pH of the nutrient solution were correlated with precipitation of Mn on the root surface; however, it was clear that this phenomenon did not confer tolerance of excess Mn. Thus, the majority of evidence indicates that high internal tolerance is more important than exclusion in determining Mn-tolerance of genotypes within species such as wheat (Foy *et al.* 1988, Scott and Fisher 1989).

As is the case with exclusion mechanisms, a variety of internal mechanisms might operate to detoxify or immobilize metals in the cytosol. These mechanisms include; (i) metal-binding proteins,

(ii) evolution of metal-tolerant enzymes, (iii) increases in enzyme activity, (iv) compartmentation of the metal in the vacuole (or another site removed from metabolic activity) and (v) chelation in the cytosol with organic ligands (Taylor 1991). Once again, not all of these mechanisms are applicable to tolerance of excess Mn. Memon and Yatazawa (1982) found that Mn is not complexed with proteins in the cytosol in leaves of Mn-accumulating plants, indicating the lack of specific Mn-binding proteins which confer tolerance of Mn. Information on the effects of Mn on enzyme activity is scarce. Insufficient studies have been done to conclude whether or not enzyme tolerance could explain Mn-tolerance. On the other hand, there is ample evidence that compartmentation is an important mechanism of Mn-tolerance. For example, Mn in leaves of a Mn-tolerant cultivar of *Vigna unguiculata* was uniformly distributed and in an easily extractable form, whereas in a Mn-sensitive cultivar, Mn was localized and not as easily extractable (Horst 1980). In excised *Zea mays* root tissue, Mn was irreversibly sequestered in the vacuole (Pfeffer *et al.* 1986). Also, Memon *et al.* (1980) noted that the highest concentrations of Mn occurred in the epidermis, collenchyma and bundle sheath cells in the Mn-accumulator *Acanthopanax sciadophylloides*, further indicating that Mn-tolerance likely involves some form of compartmentation.

I became interested in whether the phytotoxic effects of Mn were directly related to concentrations of Mn in the leaf, or if a tolerant cultivar could grow normally despite high internal concentrations of Mn. In Chapter 4, I explored the relationship between concentrations of Mn in leaves, photosynthetic rate and chlorophyll content (Macfie and Taylor Submitted). The results were genotype specific. Mn toxicity affected concentrations of chlorophyll and photosynthetic rate independently in a Mn-sensitive cultivar, whereas reduced photosynthetic rate in a Mn-tolerant cultivar could be accounted for simply by the toxic effect of Mn on chlorophyll content. Results from this experiment clearly demonstrated that the Mn-tolerant cultivar could maintain higher concentrations of chlorophyll and rates of photosynthesis than the Mn-sensitive cultivar in the face of higher concentrations of Mn in leaves. It is not clear from these results, however, whether differential tolerance is a result of differential compartmentation of Mn, detoxification of Mn or differential enzyme response to Mn.

Complexation with organic ligands in the cytosol is another potential mechanism for Mn-tolerance (Horst 1988). Organic ligands could chelate with Mn in the cytosol, effectively removing Mn from metabolic activity. In my final study (Chapter 5), I investigated the relationship between concentrations of organic acids, biosynthesis of organic acids and tolerance of excess Mn. The results indicated that concentrations of organic acids varied in response to excess Mn; however, after exposure to excess Mn, the concentrations of some organic acids were higher in the Mn-sensitive

cultivar than in the Mn-tolerant cultivar. Thus, the production of organic acids is not likely a primary mechanism of Mn-tolerance in wheat.

The experiments reported in this dissertation have contributed towards our understanding of the toxic effects of Mn in wheat. A broad range of Mn-tolerance was found which indicates that Mn toxicity is potentially an important growth-limiting factor for wheat in many areas of the world. In addition, I have narrowed the field by more clearly identifying potential mechanisms of Mn-tolerance. The results of my research indicate that differential tolerance of Mn is probably related to some component of photosynthesis, be it enzyme activity or detoxification of Mn within the cell. My work has also raised several important questions which remain unanswered. In the general discussion of this thesis I present directions for future research which I believe will further our knowledge of Mn toxicity and tolerance.

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2. Differential tolerance of manganese among cultivars of *Triticum aestivum*[1]

2.1 Introduction

Manganese (Mn) and aluminum (Al) toxicities are two important growth-limiting factors for plants in acid soils (Foy 1983). Toxic levels of these elements may develop if pH falls below 5.5 in the case of Mn (above 5.5 under waterlogged conditions) or 5.0 in the case of Al (Osborne *et al.* 1981). Plant species, cultivars and ecotypes respond differently to toxic levels of Mn or Al in their substrate. For example, differential tolerance of Al has been identified in over 40 plant species including wheat (*Triticum aestivum*, see review by Taylor 1988). While Mn-tolerance has received less attention, differential tolerance has been reported in *Amaranthus tricolor* (Foy and Campbell 1984), *Gossypium hirsutum* (Foy *et al.* 1969), *Medicago sativa* (Ouellette and Dessureaux 1958), *Trifolium subterranean* (Evans *et al.* 1987, Osborne *et al.* 1981) and wheat (Neenan 1960). Recent work on wheat, and information on North American cultivars of wheat are lacking.

Because Al and Mn toxicities are related to soil acidity, plants growing in acid soils might be expected to be tolerant of both Al and Mn (Foy *et al.* 1973). Tolerances of Mn and Al were related in *Medicago sativa* ecotypes selected for acid soil tolerance (Ouellette and Dessureaux 1958). However, Mn-tolerance was not related to Al-tolerance in studies with *Amaranthus tricolor* (Foy and Campbell 1984) or *Gossypium hirsutum* (Foy *et al.* 1969). Foy *et al.* (1973) found that a cultivar of wheat tolerant of Al was sensitive of Mn (and *vice versa*), yet there is a high probability of obtaining this result in an experiment restricted to two cultivars.

The objectives of this study were (i) to determine the range of Mn-tolerance in 30 cultivars of wheat, (ii) to identify and characterize Canadian standards for Mn-tolerance and Mn-sensitivity and (iii) to determine the relationship between Mn- and Al-tolerance in wheat.

[1] A version of this chapter has been published. Macfie, S.M., Taylor, G.J., Briggs, K.G. and Hoddinott, J. 1989. Differential tolerance of manganese among cultivars of *Triticum aestivum*. Can. J. Bot. 67:-1305-1308.

2.2 Methods

2.2.1 *Screening for manganese tolerance in solution culture*

Thirty cultivars of wheat were selected for experimentation. These included three winter wheat cultivars ('Atlas 66', 'Monon', 'Scout 66'); two cultivars of Brazilian origin ('Maringa', 'PF7748'); four of Kenyan origin ('K.Kongoni', 'K.Nyumba', 'K.Tembo', 'Romany'); nine registered Canadian Western Red Spring (CWRS) cultivars ('Benito', 'BW92', 'Columbus', 'Conway', 'Katepwa', 'Kenyon', 'Lancer', 'Neepawa', Park') and 12 experimental lines or cultivars with established high yield potential. The winter wheats were selected because 'Atlas 66' was known to be Mn-sensitive and Al-tolerant, 'Monon' was known to be Mn-tolerant and Al-sensitive (Foy *et al.* 1973) and 'Scout 66' was known to be Al-sensitive (Taylor and Foy 1985) and expected to be Mn-tolerant. Seventy seeds of each cultivar were surface sterilized in 1.2% sodium hypochlorite for 20 min and germinated overnight in an aerated solution of 0.005 g L^{-1} Vitavax to prevent fungal growth. Seedlings were placed on nylon mesh suspended over 10 L of nutrient solution and grown for 3 days in a solution containing (μM): Ca, 1000; Mg, 300; NO_3, 2900 and NH_4, 300; then for 5 days in a complete nutrient solution containing (μM): Ca, 1000; Mg, 300; K, 800; NO_3, 3300; NH_4, 300; PO_4, 100; SO_4, 101; Cl, 64; Na, 20; Fe, 10; B, 6; Mn, 2; Zn, 0.5; Cu, 0.15 and Mo, 0.1 (Briggs *et al.* 1989). Iron was supplied as Fe-EDTA prepared from equimolar amounts of $FeCl_3$ and Na_2EDTA. All growth solutions were acidified to pH 4.8 with HCl.

Sixteen uniform, nine-day-old seedlings of each cultivar were mounted with strips of polyurethane foam on Plexiglas frames which covered each of 60 polyethylene containers of 10 L capacity. Each frame supported eight plants in four groups of two and shielded growth solutions from light to inhibit algal growth. Plants were grown in a controlled environment room with temperature maintained between 19 and 20°C during a 16-h light period and between 15 and 16°C during darkness. Relative humidity was maintained between 50 and 60% during the light period and between 74 and 86% during darkness. Solution temperatures were maintained between 18 and 19°C by immersing all containers in a common water bath. The growth room was illuminated by 12 HID mercury halide (400W) and 4 HID high pressure sodium (400W) lamps located 1.3 m above the plant bases. The photosynthetic photon flux density (PPFD) was 325±29 μmol m^{-2} s^{-1} at plant base level.

A randomized, block factorial design with 30 cultivars, two Mn treatments and three replicates (totalling 180 containers) was used. Due to space constraints, threefold replication was achieved in time. All treatments involved the complete nutrient solution described above, except that

the concentrations of NH_4 and NO_3 were increased to 600 µM and 3200 µM respectively. The control treatment consisted of unaltered nutrient solution (as described above). For the Mn treatment, 500 µM Mn, supplied as $MnCl_2.4H_2O$, was superimposed over the basal nutrient solution. Control and Mn treatments for each cultivar within each replicate were blocked together to minimize variation. The pH of aerated nutrient solutions were adjusted initially to 4.8 with HCl or KOH and were recorded three times weekly. Nutrient solutions were adjusted periodically to 10 L with distilled water to compensate for water loss by evaporation and transpiration.

After a 14-day treatment, the plants were harvested, divided into roots and shoots, dried at 60°C, and weighed. A root weight index (RWI) and shoot weight index (SWI) were calculated by dividing the root or shoot dry weight of plants grown with an extra 500 µM Mn by the respective values for control plants. Tolerances of Mn were compared to tolerances of Al determined by Briggs *et al.* (1989). Data were analyzed using Duncan's Multiple Range Test and simple regression with significance defined at α=0.05.

2.2.2 *Standards for manganese tolerance and sensitivity.*

For the purposes of this dissertation, the terms 'Mn-tolerant' and 'Mn-sensitive' are used to describe relative growth under conditions of Mn stress, a tolerant plant will grow better than a sensitive one at any given concentration of Mn in solution. Based upon the results of the screening experiment, two Canadian cultivars were selected as standards for Mn tolerance ('Norquay') and Mn sensitivity ('Columbus'). These cultivars were grown over a range of concentrations of Mn (0, 5, 10, 25, 50, 100, 250, 500, 750, 1000 µM) superimposed on a basal nutrient solution which lacked Mn. All other techniques were as described in the screening experiment. Treatments were randomly assigned within each of three blocked replicates. After a 14-day treatment, plants were harvested, divided into roots and shoots, dried at 60°C and weighed.

The standards were further characterized in a time course study using a randomized, block factorial design with two cultivars, two treatments, eight harvest dates and three replicates. Treatments included a control (basal nutrient solution) and 500 µM Mn superimposed on the basal nutrient solution. Control and Mn treatments within each replicate were blocked together to minimize variation. Plants were harvested at 0, 2, 4, 6, 8, 10, 12 and 14 days after treatment. All other procedures were as described in the screening experiment. Data were analyzed using analysis of variance (ANOVA) with significance defined at α=0.05.

2.3 Results

The 30 cultivars of wheat differed in tolerance of Mn based upon both RWI and SWI (Table 2.1). Both root and shoot biomass were depressed in the presence of 500 µM Mn; however, symptoms of Mn toxicity were most evident on shoots. Roots appeared healthy while shoots were chlorotic and speckled with necrotic spots, especially in the more sensitive cultivars. Toxicity symptoms were similar to those described in Foy *et al.* (1973). Despite the lack of diagnostic symptoms on roots, the RWI was deemed to be a more suitable measure of tolerance since it provided a better separation of tolerant and sensitive cultivars (0.08 to 0.88) than the SWI (0.41 to 0.89). This is in agreement with previous studies which suggested that RWI was a more reliable indicator of Al-tolerance in wheat (Briggs *et al.* 1989, Zhang and Taylor 1988, Taylor and Foy 1985).

Based upon the RWI, all nine CWRS cultivars tested were sensitive of Mn ($0.08 < RWI < 0.40$). The Brazilian and Kenyan cultivars had RWI values between 0.24 and 0.58. Cultivars with high yield potential showed moderate tolerance of Mn ($0.50 < RWI < 0.70$). 'Norquay' (RWI = 0.88), a delicensed, high yield cultivar, was the only cultivar with a RWI above 0.70. Twenty nine of the cultivars tested for Mn-tolerance in this study were previously screened for Al-tolerance by Briggs *et al.* (1989) using a similar experimental design. Using results from both studies, a positive, but weak correlation between Al- and Mn-tolerance was observed ($r = 0.57$, Fig. 2.1).

The cultivars 'Norquay' and 'Columbus' were selected to serve as standards for Mn tolerance and Mn sensitivity. Root dry weight of both cultivars decreased under Mn-deficient and Mn-toxic conditions, however, Mn-sensitive 'Columbus' reached a maximum root weight at an external Mn concentration of 10 µM, whereas roots of Mn-tolerant 'Norquay' increased in weight up to 100 µM Mn (Fig. 2.2). The mean root weight for 'Norquay' did not reach 100% of maximum because of variation between replicates. In one replicate, maximum growth was achieved at an external concentration of 50 µM Mn, while in the other two replicates, maximum growth occurred at 100 µM Mn. At 500 µM Mn, root weight of 'Columbus' was 20 % that of 'Norquay'. Roots of 'Norquay' maintained significantly greater biomass than 'Columbus' up to 1000 µM Mn. Analysis of variance (ANOVA) of root weight data indicated significant main effects attributable to cultivar and concentration of Mn, as well as a significant cultivar by concentration of Mn interaction ($p < 0.001$).

Table 2.1. Differential tolerance of 30 cultivars of *Triticum aestivum* grown in solution culture as measured by root weight index (RWI) and shoot weight index (SWI). The ANOVA was performed on the arcsine square root of the raw data. Means followed by the same letter are not significantly different at the 5% level according to Duncan's Multiple Range Test.

Cultivar	RWI ± s.e.	SWI ± s.e.
Lancer	0.08 ± 0.01 a	0.40 ± 0.04 a
Columbus	0.09 ± 0.01 a	0.41 ± 0.04 ab
Neepawa	0.15 ± 0.04 ab	0.56 ± 0.11 abcd
Conway	0.16 ± 0.01 ab	0.53 ± 0.01 abcd
Katepwa	0.16 ± 0.03 ab	0.59 ± 0.10 abcde
Kenyon	0.18 ± 0.06 ab	0.60 ± 0.11 abcde
Scout 66	0.19 ± 0.07 abc	0.43 ± 0.06 ab
Marshall	0.21 ± 0.01 abc	0.47 ± 0.05 cdefg
Glenlea	0.21 ± 0.05 abc	0.64 ± 0.09 abc
Benito	0.23 ± 0.02 abc	0.69 ± 0.11 defghij
Maringa	0.24 ± 0.04 abcd	0.66 ± 0.05 cdefghi
BW92	0.24 ± 0.02 abcd	0.65 ± 0.03 cdefg
K.Nyumba	0.32 ± 0.12 bcde	0.62 ± 0.05 bcdef
Atlas 66	0.38 ± 0.02 cdef	0.70 ± 0.07 defghij
QT8132	0.39 ± 0.09 cdef	0.62 ± 0.0 8 bcdef
Park	0.39 ± 0.06 cdef	0.71 ± 0.04 defghij
Oslo	0.43 ± 0.11 defg	0.60 ± 0.08 abcde
K.Kongoni	0.46 ± 0.11 efgh	0.80 ± 0.14 ghijk
K.Tembo	0.50 ± 0.10 efgh	0.69 ± 0.05 defghi
Owens	0.52 ± 0.08 efgh	0.81 ± 0.04 fghijk
PT325	0.53 ± 0.13 efgh	0.84 ± 0.07 hijk
HY320	0.53 ± 0.14 efgh	0.71 ± 0.09 defghij
Monon	0.55 ± 0.12 fgh	0.77 ± 0.05 efghijk
Romany	0.55 ± 0.09 fgh	0.78 ± 0.08 efghijk
PT741	0.58 ± 0.13 fgh	0.85 ± 0.09 ijk
PT7748	0.58 ± 0.09 fgh	0.88 ± 0.01 jk
PT329	0.61 ± 0.19 gh	0.77 ± 0.19 efghij
PT726	0.66 ± 0.12 h	0.88 ± 0.08 jk
PT742	0.67 ± 0.13 h	0.83 ± 0.03 ghijk
Norquay	0.88 ± 0.03 i	0.89 ± 0.07 k

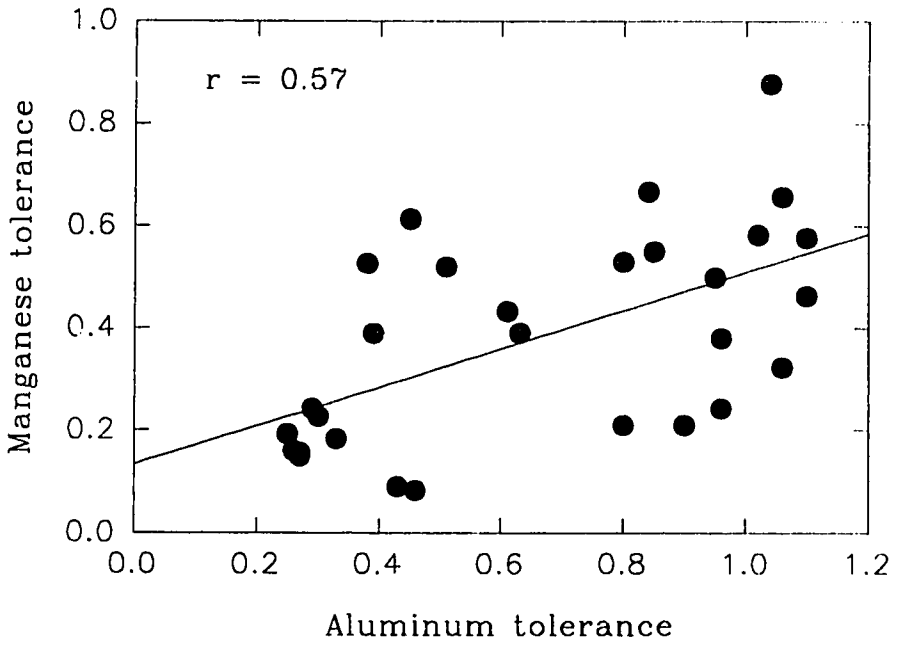

Figure 2.1. Correlation between aluminum-tolerance (Briggs *et al.* 1989) and manganese-tolerance (based on root weight index) for 29 cultivars of *Triticum aestivum* grown in solution culture.

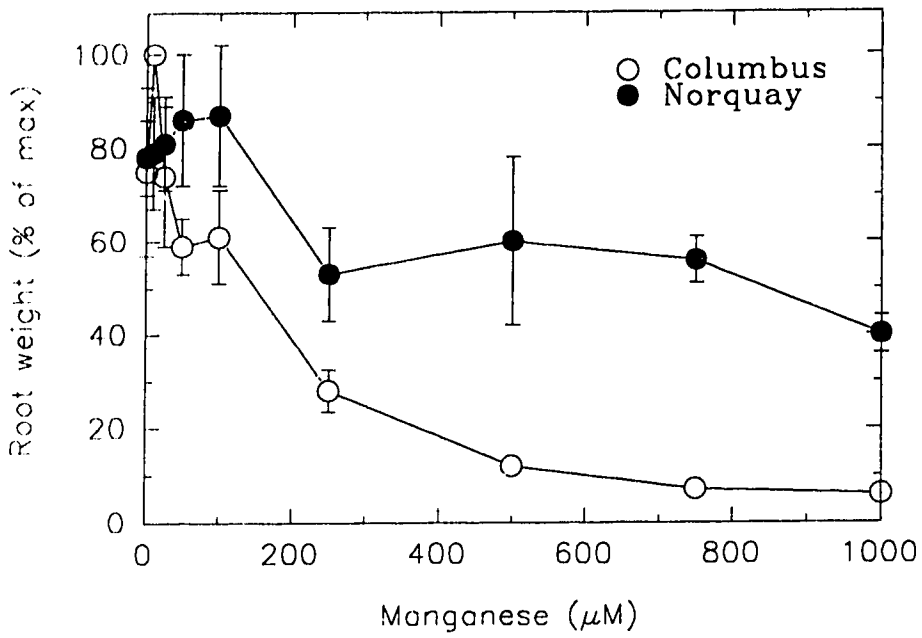

Figure 2.2. The effect of concentration of manganese (μM) on the root weight of Mn-tolerant ('Norquay') and Mn-sensitive ('Columbus') cultivars of *Triticum aestivum* grown in solution culture. Mean root dry weight is expressed as a percent of maximum weight for each cultivar.

The differences between root weights of 'Norquay' and 'Columbus' were also demonstrated in the time course study (Fig. 2.3). In the first eight days of treatment, root weights of 'Norquay' grown in 500 μM Mn did not differ from control, but declined to 50 % of control by the fourteenth day. In contrast, root weights of 'Columbus' grown in 500 μM Mn were less than control by the fourth day, and little additional growth was observed for the remainder of the experimental period. By the fourteenth day, root weight was only 10 % of control.

2.4 Discussion

Differences in tolerance of Mn among cultivars of wheat have been reported (Foy *et al.* 1973, Neenan 1960); however, these studies utilized a narrow range of germplasm, and differences between cultivars were small. For example, Neenan (1960) reported variation in the relative root growth of four cultivars from Ireland. Root growth in soils containing 137 μg g^{-1} Mn ranged from 20 to 37% of control. Foy *et al.* (1973) reported differences between two cultivars from the USA. Root growth in solutions containing 75 mg L^{-1} Mn were 33 and 41% of control. The data reported here are the first to document large variation in cultivar tolerance of Mn among a wide range of germplasm developed in four countries. The range of tolerance of Mn (RWI = 0.08 to 0.88) was similar to the range measured for Al-tolerance (RWI = 0.25 to 1.10; Briggs *et al.* 1989).

The results of the screening test were interesting because the RWI values were related to cultivar origin. Cultivars of Brazilian and Kenyan origin were only moderately tolerant or sensitive of Mn despite the predominance of acid soils in these regions. Canadian Western Red Spring (CWRS) cultivars were uniformly sensitive of Mn, whereas cultivars with documented high yield potential were more tolerant.

These results paralleled findings of Briggs *et al.* (1989) with respect to Al-tolerance. The CWRS cultivars were derived from repeated backcrossing with a narrow germplasm base lacking Al (and likely Mn) tolerance, whereas the high yield cultivars came from a broader base of germplasm grown under generally high fertility field conditions (Briggs *et al.* 1989). Briggs *et al.* (1989) speculated that the relationship between Al-tolerance and high yield potential may be related to nitrogen (N) use efficiency, although the hypothesis has yet to be tested. The same situation may hold true for Mn; however, Mn-tolerance has often been associated with tolerance of waterlogged soils (see Foy 1983). Perhaps Mn-tolerance is also related to root oxidizing potential (Taylor 1987).

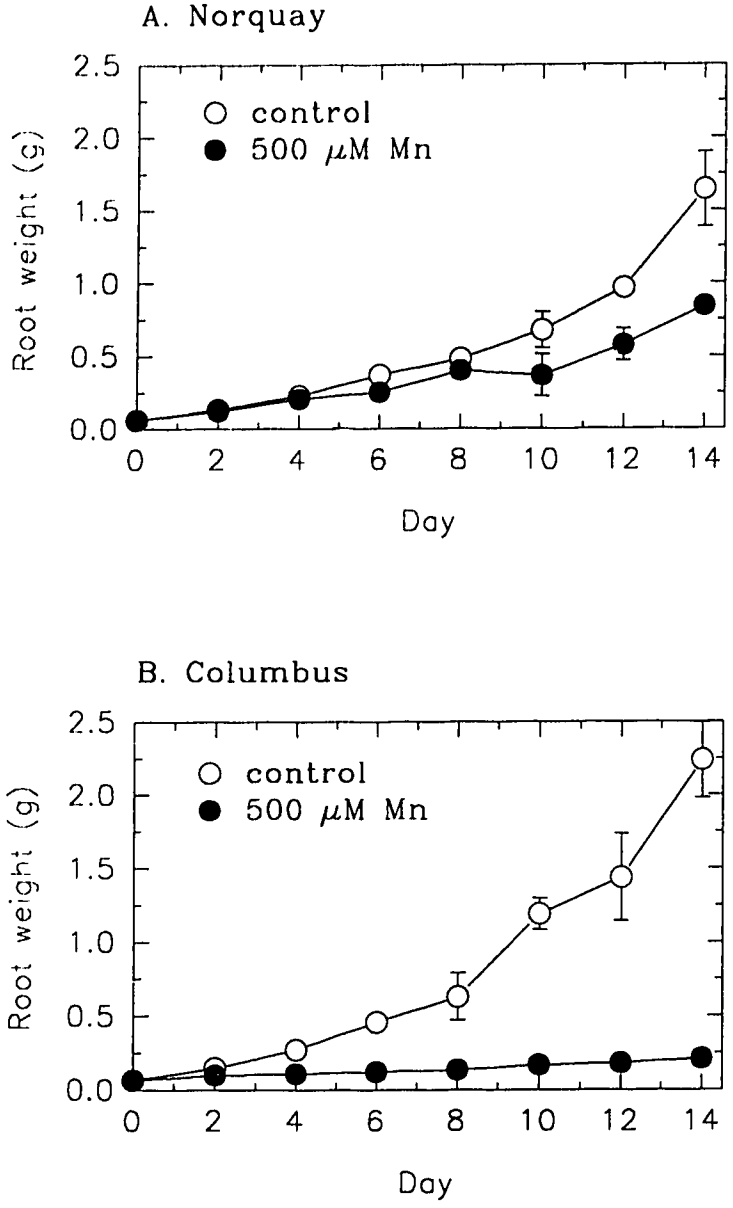

Figure 2.3. The effects of time and concentration of manganese on root dry weight ± s.e. of (A) Mn-tolerant 'Norquay' and (B) Mn-sensitive 'Columbus' cultivars of *Triticum aestivum* grown in solution culture.

The selection of cultivars which had been previously screened for Al-tolerance (Briggs *et al.* 1989) permitted investigation of the relationship between Mn- and Al -tolerance in wheat. Many of the Mn-tolerant cultivars identified in this study were identified as Al-tolerant by Briggs *et al.* (1989). Likewise, many of the Mn-sensitive cultivars were identified as Al-sensitive. In light of the similarity between the results of this study and Briggs *et al.* (1989), the positive correlation between Al- and Mn- tolerance reported here (Fig. 2.1) was not surprising. Nonetheless, this finding contradicts a previous report in which Al-tolerance was associated with Mn-sensitivity and Al-sensitivity was associated with Mn-tolerance (Foy *et al.* 1973). Although the results of Foy *et al.* (1973) for two cultivars were confirmed, their findings cannot be extrapolated to wheat in general; tolerance of Al and Mn were not opposite in the 30 cultivars studied here. While tolerances of Al and Mn were correlated, the relationship was weak ($r = 0.57$). The weakness of this correlation may be explained by variation in soil conditions in which the cultivars were developed, or by different mechanisms of metal tolerance under different genetic control(s).

The two cultivars characterized in this study would appear to be suitable standards for Mn-tolerance and sensitivity. Differences in tolerance were observed over a broad range of concentrations, similar to those previously reported for differential Al-tolerance (Briggs *et al.* 1989, Zhang and Taylor 1988). The Mn-tolerant cultivar, 'Norquay', exhibited maximum root growth at a concentration of Mn which was toxic to the Mn-sensitive cultivar, 'Columbus'. This suggests that Mn-tolerance in 'Norquay', and perhaps other Mn-tolerant cultivars, may be associated with an increased requirement for Mn. Previous studies have reported an increased need for metals in metal-tolerant ecotypes of many species (Shaw 1987, Foy *et al.* 1973, Antonovics *et al.* 1971). The wide range and co-occurrence of Al and Mn tolerance in certain cultivars suggests that the development of wheat adapted to Al- and Mn-toxic soils is feasible using conventional breeding techniques.

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3. The effects of pH and ammonium on the distribution of manganese in *Triticum aestivum* grown in solution culture[2]

3.1 Introduction

Many symptoms of manganese (Mn) toxicity have been described for aerial plant parts including; (i) interveinal chlorosis resembling iron (Fe) deficiency (Foy 1984, Foy and Campbell 1984, Kohno *et al.* 1984, Kohno and Foy 1983b, White 1970), (ii) brown spots consisting of precipitates of Mn oxides (Arnold and Binns 1987, Wissemeier and Horst 1987, Foy and Campbell 1984, Sirkar and Amin 1974, Kang and Fox 1980, Mengel and Kirkby 1980, Vlamis and Williams 1973, White 1970), (iii) crinkle leaf (Sirkar and Amin 1974, Foy 1984, Foy and Campbell 1984, Kiesling *et al.* 1984), (iv) stunting and stiffness of leaves (Kiesling *et al.* 1984, Ohki 1984), and (v) necrotic leaf tips (Kiesling *et al.* 1984, Ohki 1984). Despite the prevalence of studies using solution cultures, most reports of Mn toxicity symptoms have focused upon aerial parts, with little attention paid to roots. Several studies have reported that severe Mn toxicity causes plant roots to turn brown, usually after the tops have been injured (Foy 1984). Aoba *et al.* (1977) reported that roots of *Citrus reticulata* injured by excess Mn were stained dark brown. Roots of *Hordeum vulgare*, *Lycopersicon esculentum*, *Medicago sativa*, *Oryza sativa* and *Triticum aestivum* (wheat) also turned a brown colour when grown in culture solutions with high concentrations of Mn, whereas roots of *Cucurbita moschata* did not (Horiguchi 1987). These reports have implied that browning of roots is a symptom of Mn toxicity, however, the authors did no more than describe root colour. There has not been discussion of cause or effect of root discolouration.

Brown colouration, identified as oxidized Mn, accounted for over half of total Mn in roots of *Hordeum vulgare* and wheat (Horiguchi 1987). There has been discussion of precipitated metal oxides conferring metal tolerance in some species, including *Aster tripolium* (Otte *et al.* 1987), *Typha domingensis* (Hocking 1981), *Typha latifolia*, and *Oryza sativa* (Crowder *et al.* 1987, Marschner *et al.* 1987), but such a mechanism cannot explain tolerance in other species. While oxidation of metals on root surfaces may affect distribution of metals between tops and roots (Horiguchi 1987), tolerance of Mn in some species and cultivars is observed despite high tissue concentrations of Mn (Evans *et al.* 1987, Miller 1987, Kohno *et al.* 1984, Foy *et al.* 1973). To date, there have been no published studies

[2] A version of this chapter has been published. Macfie, S.M. and Taylor, G.J. 1989. The effects of pH and ammonium on the distribution of manganese in *Triticum aestivum* grown in solution culture. Can. J. Bot. 67:3394-3400.

relating the effect of Mn precipitates on plant roots to tolerance of Mn.

In a preliminary study with wheat, a precipitate of Mn was observed on roots of a Mn-tolerant cultivar grown with excess Mn, after pH of solution rose above 5.0 (approx. 10 days in culture). The plant-induced rise in pH coincided with depletion of NH_4^+, followed by an increase in the uptake of NO_3^- (Taylor 1988b, Foy 1984). In contrast, a Mn-sensitive cultivar did not increase pH of solution above 5.0, and its roots remained white. From these results one might suggest that 'brown roots' are not a symptom of toxicity. They may, in fact, be an indicator of tolerance. The objectives of this study were: (i) to determine the effect of pH and ammonium supply on the formation of a precipitate of Mn on roots of wheat, (ii) to determine the effect of Mn precipitation on the distribution of Mn between roots and leaves and (iii) to determine the relationship between the presence of a Mn precipitate and relative tolerance of Mn.

3.2 Methods

3.2.1 *Effect of ammonium supply*

A Mn-tolerant cultivar ('Norquay') and a Mn-sensitive cultivar ('Columbus') of wheat were selected for experimentation. Seven hundred seeds of each cultivar were surface sterilized in 1.2% sodium hypochlorite for 20 min and germinated overnight immersed in an aerated solution of 0.005 g L^{-1} Vitavax to prevent fungal growth. Seedlings were grown for 3 days in a solution containing (μM): Ca, 1000; Mg, 300; NO_3, 2900 and NH_4 300; then for 5 days in a complete nutrient solution containing (μM): Ca, 1000; Mg, 300; K, 800; NO_3,3300; NH_4,300; PO_4,100; SO_4,101; Cl, 34; Na, 20; Fe, 10; B, 6; Mn, 2; Zn, 0.5; Cu, 0.15 and Mo, 0.1. Iron was supplied as Fe-EDTA prepared from equimolar amounts of $FeCl_3$ and Na_2EDTA. All growth solutions were acidified to pH 4.8 with HCl.

Nine-day-old seedlings of each cultivar were mounted on Plexiglas frames which covered polyethylene containers of 10 L capacity. Each frame supported 12 plants in four groups of three, and shielded growth solutions from light to inhibit algal growth. Plants were grown in a controlled environment room with temperature maintained between 22 and 23°C during a 16-h light period and between 16 and 18°C during darkness. Relative humidity was maintained between 70 and 76% during the light period and between 86 and 90% during darkness. Solution temperatures were maintained between 18 and 19°C by immersing all containers in a common water bath. The growth room was illuminated by 12 high-intensity discharge (HID) mercury halide (400W) and four HID

high pressure sodium (400W) lamps located 1.3 m above the plant bases. The photosynthetic photon flux density (PPFD) was 334 $\pm$ 27 μmol m^{-2} s^{-1} at plant base level.

A randomized block factorial design with two cultivars, three concentrations of Mn, three treatments affecting solution pH, and three replicates (totalling 54 containers) was used. All containers were filled with the complete nutrient solution described above. The pH of aerated nutrient solutions were adjusted initially to 4.8 with HCl or KOH and were recorded three times weekly. Nutrient solutions were adjusted periodically to 10 L with distilled water to compensate for water loss by evaporation and transpiration. Three concentrations of Mn were superimposed over the basal nutrient solution (0, 200, and 500 μM Mn, supplied as $MnCl_2.4H_2O$). The three treatments affecting pH included; (i) an unaltered solution with no addition of extra NH_4NO_3 or adjustment of pH (ii) addition of a further 0.3 mM NH_4NO_3 and a reduction in the number of plants to eight per container, and (iii) control of pH at 4.8 by twice daily addition of either HCl or KOH . Treatments (ii) and (iii) were designed to prevent precipitation of Mn on root surfaces by keeping pH below 5.5.

After a 14-day treatment, plants were harvested, rinsed for approximately 4 min in distilled water, desorbed for 30 min in 1 mM $CaSO_4$, rinsed in distilled water for another 4 min, divided into roots and leaves, dried at 60°C, and weighed (Zhang and Taylor 1988). Plant material was prepared for Mn analysis by ashing approximately 0.5 g (dry weight) at 500°C for two days. The ash was dissolved in 0.4 ml concentrated reagent-grade HNO_3 and 0.4 ml 51% H_2O_2, and brought to 20 ml with deionized H_2O (after Taylor and Foy 1985a). Concentrations of Mn were determined by atomic absorption spectrophotometry (AAS) using Fisher Scientific atomic absorption standard Mn reference solution. All concentrations of Mn measured exceeded the detection limit of 5 μg g^{-1} by at least a factor of 20. Data were analysed using analysis of variance (ANOVA) and Scheffe's test with significance defined at α=0.05.

3.2.2 *Dose response*

To test the effect of precipitation of Mn on roots on the distribution of Mn in the plant, dose response experiments were conducted. 'Norquay' and 'Columbus' were grown over a range of Mn concentrations (0, 5, 10, 25, 50, 100, 250, 500, 750, and 1000 μM) superimposed upon a basal nutrient solution which lacked Mn. In one experiment, the basal nutrient solution described previously (which allowed a Mn precipitate to form on the root surface) was used. In a second experiment, the concentration of NH_4^+ was increased to 0.6 mM to prevent precipitation of Mn. For each dose response experiment, a randomized block factorial design with two cultivars, 10

concentrations of Mn, and three replicates was used. Eight plants were grown in each container, all other techniques were as described for the control plants in the ammonium supply experiment. Data were analysed using ANOVA and simple linear regression with significance defined at α=0.05.

3.3 Results

3.3.1 *Effect of ammonium supply*

When grown with 0.3 mM NH_4^+, the two cultivars studied had different patterns of pH change over the experimental period (Fig. 3.1). For all three concentrations of Mn added, the Mn-tolerant cultivar ('Norquay') reduced solution pH from 4.8 to 4.0 by days 7 to 9, after which pH rose rapidly to 6.5 (Fig. 3.1A). The initial decline in pH reflects uptake of NH_4^+ by the plant; depletion of NH_4^+ and a subsequent increase in the uptake of NO_3^- coincides with the point of inflection for the rapid rise in pH (Taylor and Foy 1985c). A similar pattern was observed for the Mn-sensitive cultivar ('Columbus') grown in control solution; however, pH of solutions with excess Mn declined steadily throughout the experimental period (Fig. 3.1B). Roots of 'Norquay' grown in excess Mn turned brown by day 12 at which time the pH was approximately 5.5. Roots of 'Columbus' from all concentrations of Mn remained white and the only visual symptom of Mn toxicity was a reduction in biomass. The observed rise in pH was prohibited both by doubling of NH_4^+ to 0.6 mM (treatment 2) and by maintenance of pH at 4.8 (treatment 3; data not shown).

An ANOVA for concentration of Mn in roots indicated significant main effects due to concentration of Mn in solution, cultivar, and pH treatment. Interaction effects were not significant. An ANOVA for roots of 'Norquay' alone, indicated significant main effects of concentration of Mn in solution and pH treatment. In contrast, an ANOVA for 'Columbus' indicated a significant main effect of concentration of Mn in solution only. In both cultivars, concentrations of Mn associated with roots increased with concentration of Mn in solution, regardless of amount of ammonium added to solution (Fig. 3.2). Roots of 'Norquay' grown in excess Mn and 0.3 mM NH_4^+ contained higher concentrations of Mn, reflecting the precipitation of Mn on root surfaces (Fig. 3.2A). No precipitate formed on roots of 'Columbus', accounting for the lack of a significant pH treatment effect. For each concentration of Mn in solution, roots of 'Norquay' contained higher concentrations of Mn than did roots of 'Columbus'. An ANOVA for concentration of Mn in leaves indicated significant main effects due to concentration of Mn in solution and cultivar, but not for pH treatment. Interaction effects were not significant; however, the interaction between cultivar and concentration of Mn was nearly significant (p = 0.056). Concentrations of Mn associated with leaf tissues increased with

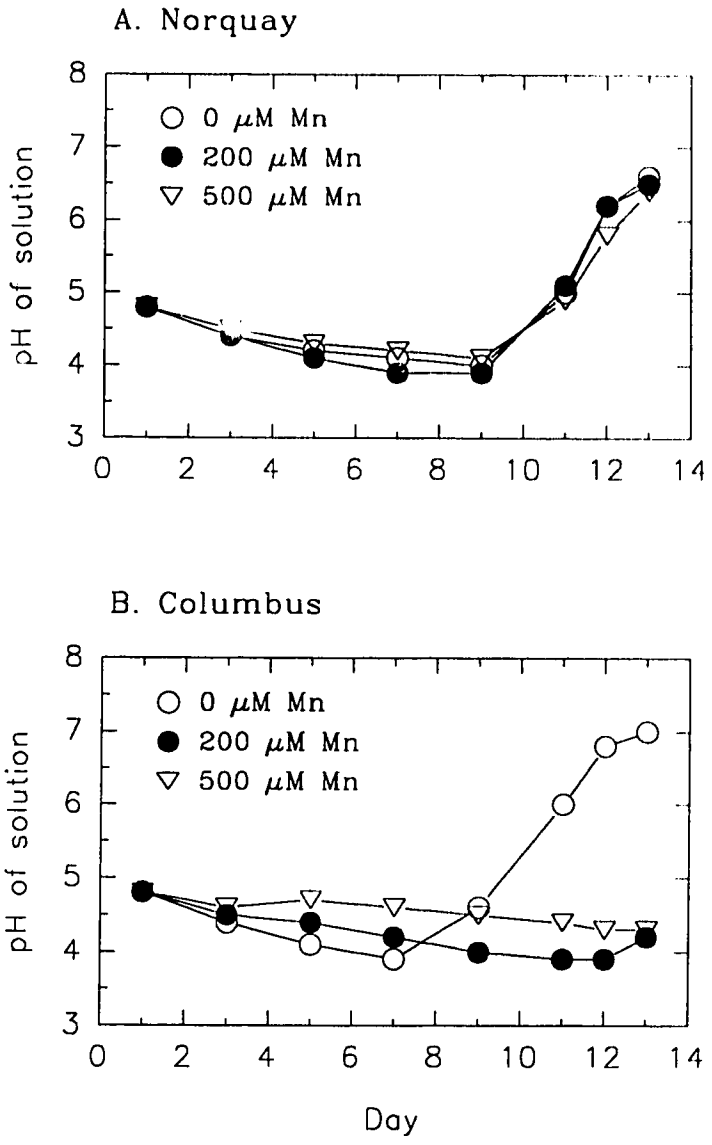

Figure 3.1. Pattern of plant-induced changes in pH of solution for (A) Mn-tolerant 'Norquay' and (B) Mn-sensitive 'Columbus'. Plants were grown in solutions containing 0.3 mM NH_4^+ to which 0, 200, or 500 μM Mn were added.

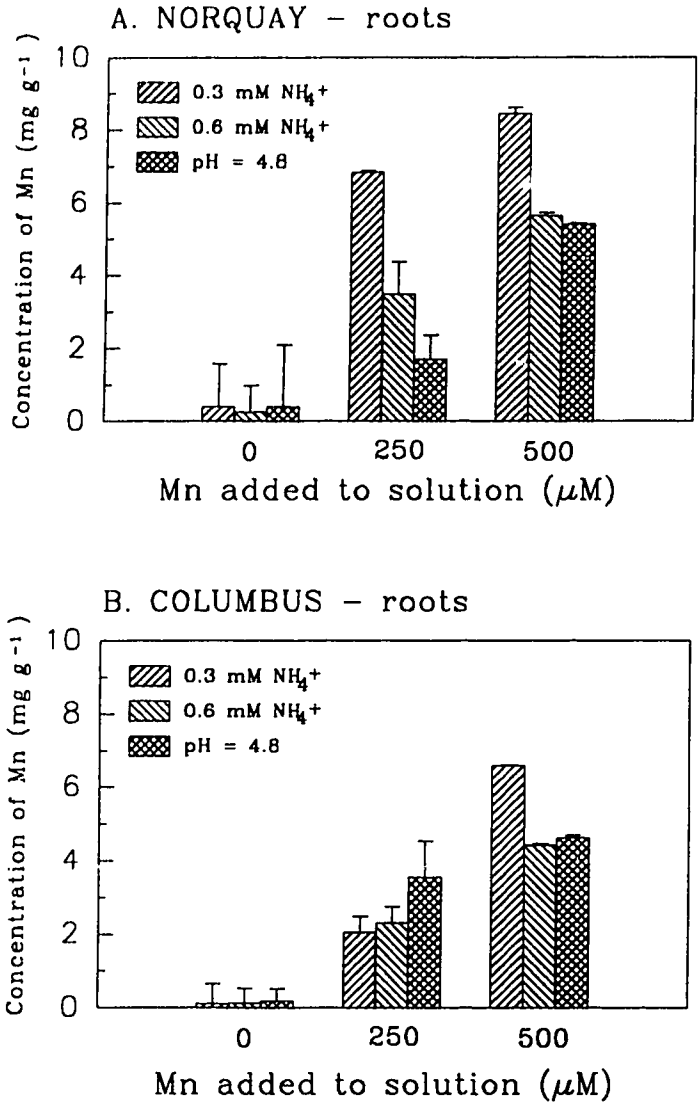

Figure 3.2. Mean concentrations of Mn ± s.e. in roots of (A) Mn-tolerant 'Norquay' and (B) Mn-sensitive 'Columbus'. Plants were grown in solutions with three concentrations of Mn and three treatments to control pH change.

concentrations of Mn in solution for both cultivars (Fig. 3.3). 'Norquay' contained greater concentrations of Mn than did 'Columbus'.

Analyses of variance for root and leaf dry weights indicated significant main effects due to cultivar, concentration of Mn in solution, and pH treatment, as well as an interaction between cultivar and concentration of Mn. Root and leaf dry weights of 'Norquay' declined with concentration of Mn in solution, but to a lesser extent than those of 'Columbus' (Table 3.1). The effect of pH treatment may reflect reduced biomass of plants grown in 0.6 mM NH_4^+ as a result of mild NH_4^+ toxicity. The significant interaction term was due to greater biomass of 'Columbus' at low concentrations of Mn and maintenance of greater biomass of 'Norquay' at high concentrations of Mn.

3.3.2 *Dose Response*

With 0.3 mM NH_4^+ in solution, roots of 'Norquay' accumulated more Mn than roots of 'Columbus' over a range of concentrations of Mn (50-1000 µM) in solution (Fig. 3.4A). Concentrations of Mn in leaves showed little difference between cultivars (Fig. 3.4B). Increasing NH_4^+ to 0.6 mM NH_4^+ in solution prevented precipitation of Mn on roots, hence less Mn was associated with root tissues, and more was found in leaves (Fig. 3.5). Nonetheless, with 0.6 mM NH_4^+ in solution, Mn-tolerant 'Norquay' had higher tissue concentrations of Mn than Mn-sensitive 'Columbus' when concentrations of Mn in solution were greater than 100 µM.

Root dry weight decreased with increased concentrations of Mn in roots for both cultivars ('Norquay' r = -0.59, 'Columbus' r = -0.85; Fig. 3.6A). Similarly, leaf dry weight decreased with concentrations of Mn in leaves ('Norquay' r = -0.75, 'Columbus' r = -0.89; Fig. 3.6B). Above 2.0 mg/g Mn in roots and leaves, 'Norquay' plants had greater dry weights than 'Columbus' plants. Roots and leaves of 'Columbus' with more than 3.0 mg/g Mn had the same biomass at the end of the experiment as they did at time of planting whereas 'Norquay' continued to grow despite higher concentrations of Mn in its tissues (up to 12.0 mg/g in roots, 7.3 mg/g in leaves).

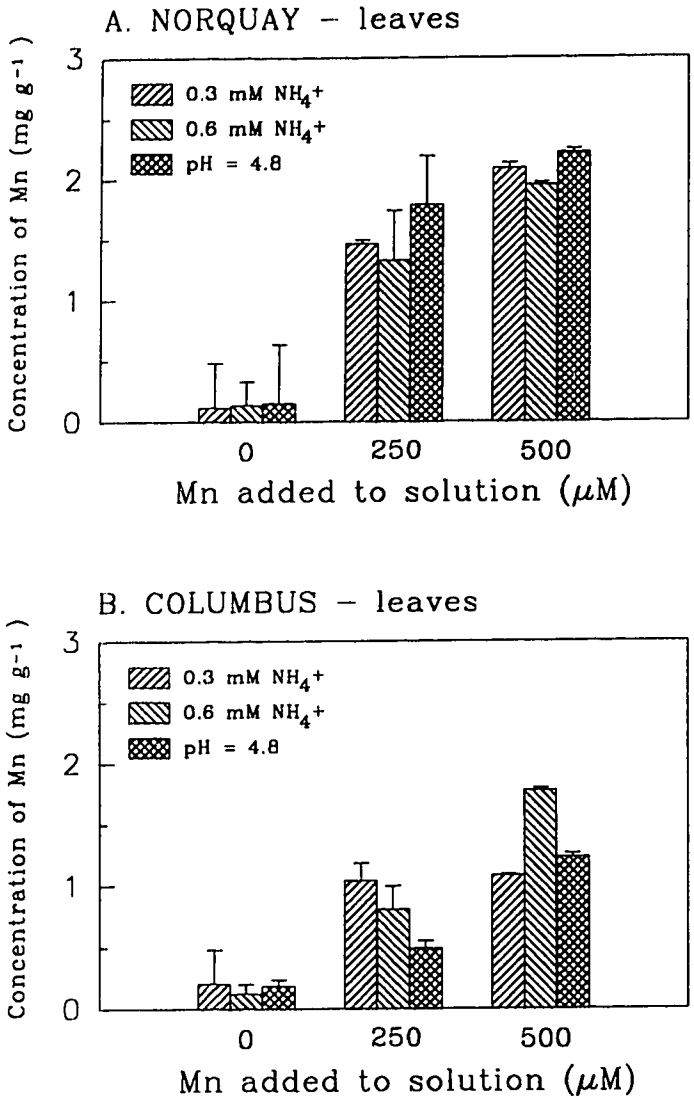

Figure 3.3 Mean concentrations of Mn ± s.e. in leaves of (A) Mn-tolerant 'Norquay' and (B) Mn-sensitive 'Columbus'. Plants were grown in solutions with three concentrations of Mn and three treatments to control pH change.

Table 3.1. Dry weights (g) ± s.e. of root and leaf tissues for Mn-tolerant 'Norquay' and Mn-sensitive 'Columbus' grown in solutions with three concentrations of Mn and either 0.3 mM NH_4^+, 0.6 mM NH_4^+ or pH held constant at 4.8.

		Manganese added (μM)		
		0	200	500
	pH treatment		LEAVES	
NORQUAY	0.3 mM NH_4^+	3.55 ± 0.26	3.45 ± 0.04	3.39 ± 0.15
	0.6 mM NH_4^+	2.50 ± 1.30	3.05 ± 0.05	2.42 ± 0.09
	pH = 4.8	3.27 ± 0.04	3.01 ± 0.06	3.01 ± 0.20
COLUMBUS	0.3 mM NH_4^+	3.58 ± 0.01	3.58 ± 0.13	1.92 ± 0.002
	0.6 mM NH_4^+	3.28 ± 0.02	2.39 ± 0.07	1.78 ± 0.04
	pH = 4.8	3.87 ± 0.10	3.22 ± 0.08	1.79 ± 0.01
			ROOTS	
NORQUAY	0.3 mM NH_4^+	1.18 ± 0.07	1.26 ± 0.04	1.08 ± 0.02
	0.6 mM NH_4^+	1.84 ± 0.76	0.98 ± 0.003	0.63 ± 0.01
	pH = 4.8	1.12 ± 0.01	1.21 ± 0.01	1.03 ± 0.07
COLUMBUS	0.3 mM NH_4^+	2.02 ± 0.03	0.70 ± 0.01	0.16 ± 0.001
	0.6 mM NH_4^+	1.49 ± 0.002	0.45 ± 0.01	0.11 ± 0.001
	pH = 4.8	1.95 ± 0.04	0.56 ± 0.01	0.20 ± 0.001

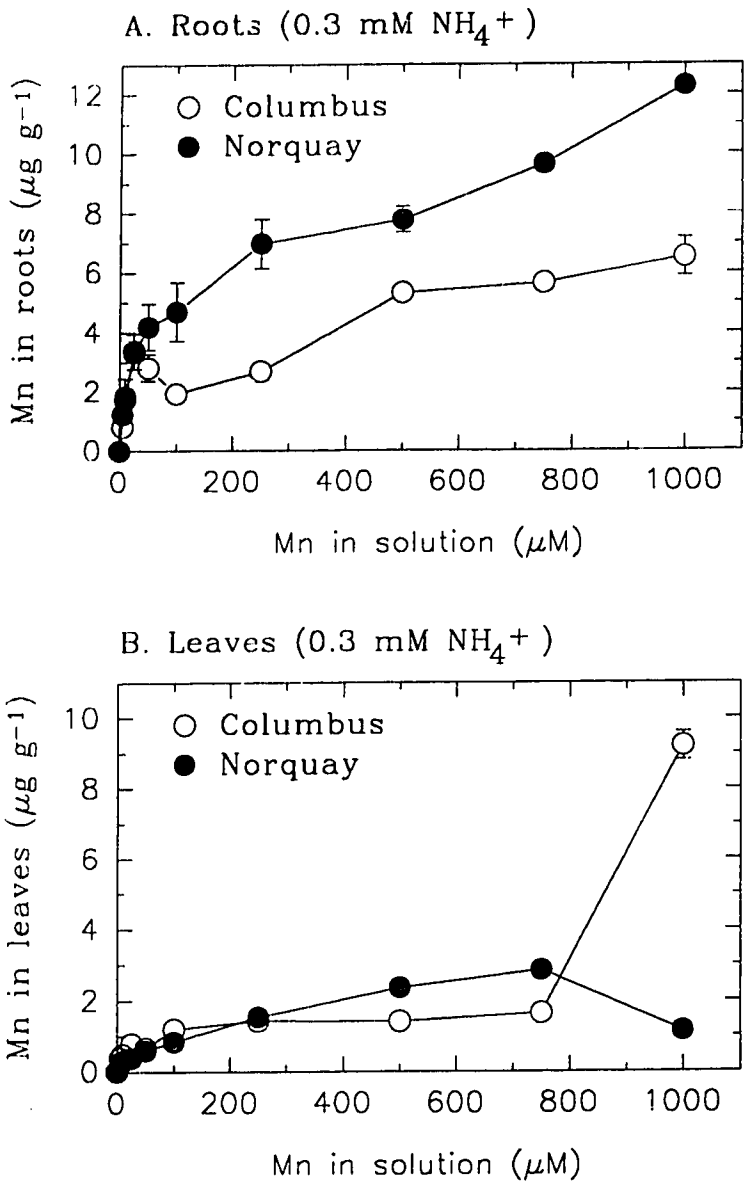

Figure 3.4. Mean concentrations of Mn ± s.e. in (A) roots and (B) leaves of 'Norquay' and 'Columbus'. Plants were grown in solutions with 0.3 mM NH_4^+.

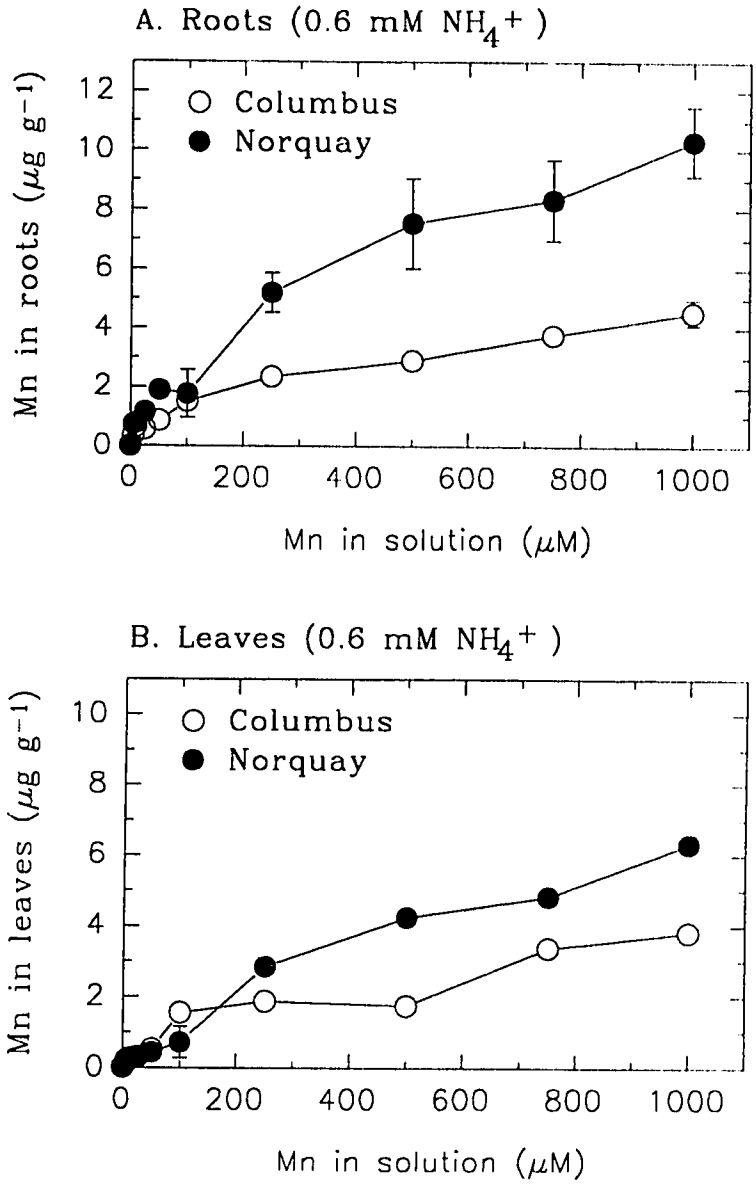

Figure 3.5. Mean concentrations of Mn ± s.e. in (A) roots and (B) leaves of 'Norquay' and 'Columbus'. Plants were grown in solutions with 0.6 mM NH_4^+.

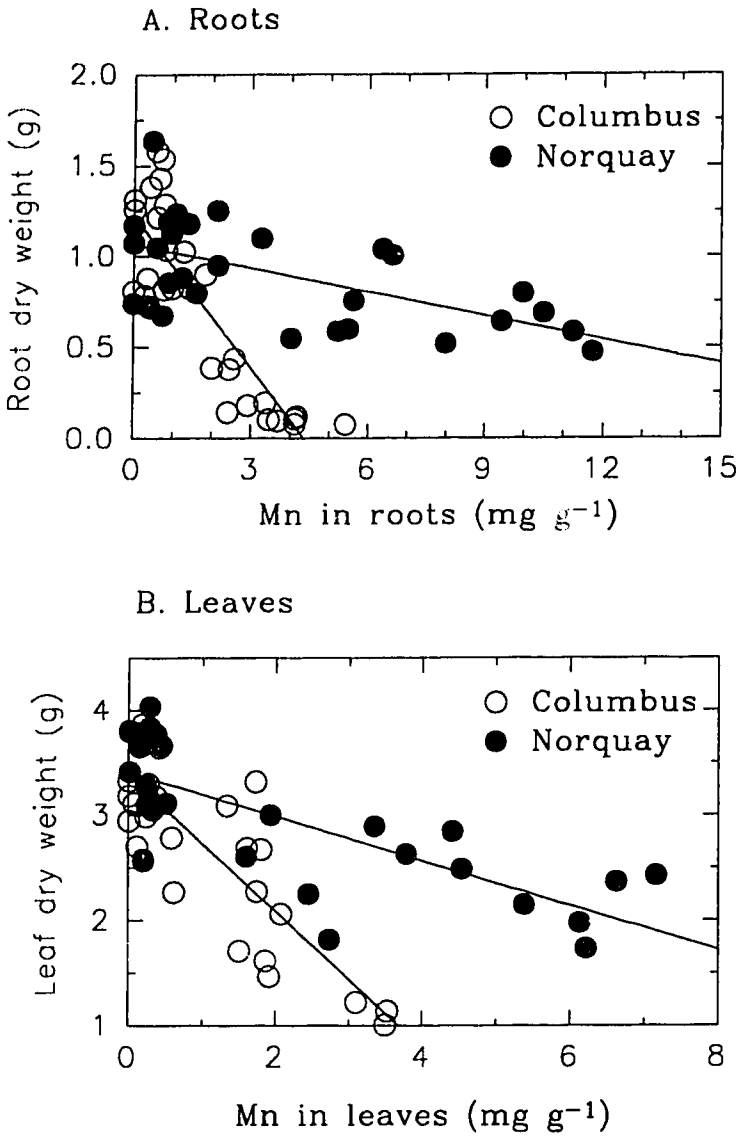

Figure 3.6. Correlation between (A) root dry weight and (B) leaf dry weight and tissue concentration of Mn for 'Norquay' and 'Columbus'.

3.4 Discussion

The patterns of pH change reported in this study were similar to those observed in aluminum (Al)-tolerant and Al-sensitive cultivars of wheat (Taylor and Foy 1985b, 1985c, Foy and Fleming 1982). Foy and Fleming (1982) and Kang and Fox (1980) suggested that acid soil, Al-tolerant cultivars may be more efficient at nitrogen uptake than sensitive cultivars. These authors argued that metal-tolerant cultivars are more efficient at extracting nitrate from solution, thereby inducing a higher pH of nutrient solution by the end of the experimental period. High rhizosphere pH may protect plants from metal toxicity by decreasing the chemical activity of metals in the rhizosphere; hence, less metal would be available to the plant at higher solution pH. However, differential tolerance has been observed prior to depletion of NH_4^+ from solutions (Taylor 1988b), and Taylor (1988a) demonstrated that Al-tolerance of wheat was not a result of the plant's ability to raise the pH of a solution with mixed sources of nitrogen. In Mn-tolerant and Mn-sensitive cultivars of *Phaseolus vulgaris*, differential patterns of pH were not observed (Kohno *et al.* 1984), indicating that Mn-tolerance was not related to plant-induced changes in pH of solution in that species.

An alternate hypothesis is that differential uptake of nitrogen and differences in plant-induced pH are a result, not a primary mechanism, of metal tolerance (Taylor 1988b). In this study, the failure of Mn-sensitive plants to induce a rise in pH of Mn-toxic solutions may reflect the toxic effect of Mn on nitrogen assimilation rather than the basis of differential tolerance of Mn. Reduced plant growth in solutions with excess Mn may have resulted in less uptake of both ammonium and nitrate and a less marked change in pH of solution. When Mn toxicity was not a factor, the patterns of pH change for 'Columbus' grown in control solutions were similar to those of 'Norquay'. With toxic but sublethal doses of Mn it might be expected that a slow pH decline followed by a rise (as observed for tolerant cultivars) would also be observed for sensitive cultivars, but over a longer time period; and that the extent of lag in pH change would be positively correlated with the concentration of Mn in solution.. The patterns of pH change would reflect the sensitivity (or tolerance) of the cultivar to Mn, rather than efficiency of nitrogen uptake.

In this study, plant-induced changes in pH of solutions appeared to control precipitation of Mn on root surfaces. With excess Mn, roots of Mn-tolerant 'Norquay' showed brown discolouration when pH of solution rose above 5.5. Addition of extra NH_4^+ to solutions (0.6 mM total) delayed the pH rise and prevented deposition of Mn on root surfaces. In contrast, roots of Mn-sensitive 'Columbus' grown in excess Mn remained white, and pH of solutions remained below 4.8. Precipitation of Mn might have occurred on these roots if pH of solutions rose above 5.5, but pH rise

was inhibited with excess Mn in solution (Fig. 3.1B). Although control plants of both cultivars induced a similar rise in pH, low concentrations of Mn in these solutions did not permit visual discolouration of roots. The control of precipitation of Mn on roots may be similar to that for precipitation of Fe. In natural wetlands, precipitation of Fe on roots of *Typha latifolia* was positively correlated with pH of soil (Macfie and Crowder 1987). Plants with the most Fe on their roots grew in soils with pH near 7.0. In a field study, oxidation of Fe on root surfaces was also influenced by amount of extractable Fe in the soil, and percentage of inorganic and organic carbon (Macfie and Crowder 1987). In solution culture, oxidation of Fe on roots of *T. latifolia* was influenced by concentration of Fe, Fe source, and pH of solution (Taylor *et al.* 1984).

Precipitation of Mn on root surfaces affected the distribution of Mn in root and leaf tissues. Precipitation of Mn was reflected by higher concentrations of Mn associated with roots. A concomitant decrease in concentration of Mn in leaves may reflect a reduction in the mobility of Mn through roots. It is interesting; however, that both cultivars showed similar patterns (Figs. 3.4, 3.5) even though roots of 'Norquay' grown with 0.3 mM NH_4^+ had dark brown discolouration whereas roots of 'Columbus' remained white. While the pH of solutions for 'Columbus' did not rise above 5.5, it is possible that some Mn had precipitated onto root surfaces; such precipitates may have been sufficient to alter Mn distribution, but insufficient to cause discolouration of roots. The increase in concentration of Mn in leaves of 'Columbus' grown with 1000 µM Mn may reflect an influx of Mn into the plant caused by severe Mn toxicity.

It has been suggested that immobilization of metals at the root surface may contribute to tolerance in some species (Horiguchi 1987). In these species, tolerant plants (with the precipitate) might be expected to show lower concentrations of the metals in aerial parts than sensitive plants. This pattern of metal distribution was observed in this study, and preventing precipitation of Mn on roots resulted in decreased concentrations of Mn in root tissues and increased concentrations of Mn in leaf tissues. Nevertheless, Mn-tolerant 'Norquay' accumulated greater amounts of Mn in roots and leaves than Mn-sensitive 'Columbus' both with and without precipitation of Mn on roots. In many species, however, Mn-tolerant cultivars are capable of sustained growth despite high internal concentrations of Mn (Ohki 1984, Foy *et al.* 1973, White 1970). For example, in two populations of *Vaccinium vitis-idaea*, which showed no visual or physiological symptoms of Mn toxicity, tissue concentrations of Mn ranged from 6000 to 15000 µg g^{-1} dry weight in one population and from 18 to 158 µg g^{-1} dry weight in the other (Miller 1987). In *Phaseolus vulgaris*, symptoms of Mn toxicity were observed in Mn-sensitive plants when concentrations of Mn in leaves rose above 90 µg g^{-1} dry weight, while Mn-tolerant plants showed toxicity symptoms only above 348 µg g^{-1} dry weight

(Kohno and Foy 1983a). Thus, tolerance of Mn in 'Norquay' may not be unusual despite higher concentrations of Mn in tissues relative to 'Columbus'.

In contrast with previous reports (Aoba 1977, Foy 1984), sustained growth of Mn-tolerant 'Norquay' despite precipitation of Mn on roots, and reduced growth of Mn sensitive 'Columbus' in the absence of a precipitate of Mn indicate that brown discolouration of roots is not a symptom of Mn toxicity. Precipitation of Mn on root surfaces of wheat was correlated with a plant-induced rise in solution pH above 5.5 and affected the distribution of Mn within the plant. Such changes in distribution, however, did not confer tolerance of Mn. Greater biomass production of 'Norquay' over 'Columbus' in both the presence and absence of Mn precipitation further indicated that tolerance of Mn was unrelated to oxidation of Mn at the root surface.

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4. The effects of excess manganese on photosynthetic rate and concentration of chlorophyll in *Triticum aestivum* grown in solution culture[3]

4.1 Introduction

A typical symptom of manganese (Mn) toxicity in vascular plants is interveinal chlorosis resembling iron (Fe) deficiency (Foy 1984, Ohki 1984, Campbell and Nable 1988). Nable *et al.* (1988), however, have determined that this chlorosis is not related to Fe deficiency. For this symptom to be expressed, Mn must have a direct effect on either chlorophyll synthesis or degradation. It is known that Mn plays a role in the biosynthesis of chlorophyll, and several enzymes along the isoprenoid biosynthetic pathway (which produces plant pigments such as chlorophyll) are sensitive to both manganese deficiency and toxicity (Wilkinson and Ohki 1988). In *Nicotiana tabacum* callus, chlorophyll synthesis was inhibited by 10-100 mM Mn (Clairmount *et al.* 1986). In the blue-green alga *Anancystis nidulans*, Mn toxicity interrupts the insertion of Mg into protoporphyrin (Csatorday *et al.* 1984) resulting in reduced synthesis of chlorophyll. Chlorophyll degradation may also be affected by Mn toxicity. At normal concentrations, Mn plays a role in protecting chlorophyll from photooxidation, however, it is possible that oxidized Mn in the leaf causes oxidation of either chlorophyll or chloroplasts (Horiguchi 1988).

Another effect of Mn toxicity is a decline in photosynthetic rate in plants such as wheat (*Triticum aestivum*, Ohki 1985), *Nicotiana tabacum* (Nable *et al.* 1988) and *Glycine max* (Ohki 1981). In addition to the effects of Mn on chlorophyll synthesis and degradation, toxic concentrations of metals such as Mn may inhibit photosynthesis at a variety of physiological levels (Clijsters and Van Assche 1985). In contrast to metals such as copper (Cu) and cadmium (Cd), Mn does not appear to be involved in the stomatal mechanism (Ohki 1985). However, Mn is functionally associated with proteins on chloroplast thylakoid membranes which are involved with the water splitting apparatus (Cheniae and Martin 1968, Dismukes *et al.* 1983) and functions as a structural component in the lamellar membrane of the chloroplast (Weiland *et al.* 1975). Manganese also seems to act as an electron transfer agent in photosystem II (Wilkinson and Ohki 1988), however, Hill activity was unaffected by Mn toxicity in *Nicotiana tabacum* (Houtz *et al.* 1988). It is clear that Mn deficiency

[3] A version of this chapter has been submitted for publication. Macfie, S.M. and Taylor, G.J. The effects of excess manganese on photosynthetic rate and concentration of chlorophyll in *Triticum aestivum* grown in solution culture. Submitted to Physiol. Plant.

will affect photosynthesis. It is also reasonable, however, to suggest that Mn toxicity will also have an adverse effect. Addition of 5 μM Cu, Cd and Zn (borderline metals which have ligand binding preferences similar to Mn, Nieboer and Richardson 1980) to a membrane preparation from *Anacystis nidulans* was inhibitory to Hill activity and O_2 evolution (Singh and Singh 1987). In *Phaseolus vulgaris* seedlings treated with excess Zn, ribulose-1,5-bisphosphate carboxylase (rubisco) activity (Van Assche and Clijsters 1986b), electron transport and photophosphorylation (Van Assche and Clijsters 1986a) were also inhibited. In contrast, in *Nicotiana tabacum*, Mn toxicity affected rubisco activity, but not electron transport (Houtz *et al.* 1988).

Ohki (1985) grew wheat in solution culture under Mn-toxic and Mn-deficient conditions and measured photosynthetic rate and total plant chlorophyll in relation to the concentration of Mn in the leaf tissue. As the concentration of Mn in the leaf increased above optimal levels, both photosynthetic rate and concentration of chlorophyll declined. While these data clearly indicate that both photosynthesis and chlorophyll content are affected by excess Mn, it was not clear whether the reduction in photosynthetic rate was a direct result of Mn toxicity or whether it simply reflected a reduction in chlorophyll content. In addition, Ohki's experiments used a single cultivar of wheat thus there was no indication of a relationship between differential tolerance of excess Mn and the plant's ability to maintain photosynthesis and chlorophyll content in the face of Mn stress. In a preliminary study with wheat, the photosynthetic rate for a Mn-tolerant cultivar grown with 500 μM Mn in solution remained unaffected up to 4 days of exposure to Mn. After this time, the rate declined to approximately 60% of control. In a Mn-sensitive cultivar, the photosynthetic rate declined to near 20% of control by the end of day 1. The question arises as to whether the greater effect of Mn on photosynthetic rate in the Mn-sensitive cultivar reflected a higher concentration of Mn in the leaf, a larger decline in chlorophyll content, or a loss of photosynthetic activity per unit chlorophyll. The objectives of this study were; (i) to determine the effects of excess Mn on photosynthetic rate and concentration of chlorophyll, (ii) to relate photosynthetic rate to concentrations of Mn and chlorophyll in a single leaf and (iii) to identify the relationship between photosynthetic rate, chlorophyll content and differential tolerance of Mn.

4.2 Methods

Two cultivars of wheat (*Triticum aestivum*), Mn-tolerant 'Norquay' and Mn-sensitive 'Columbus' (Macfie *et al.* 1989) were selected for experimentation. Two hundred seeds of each cultivar were surface sterilized in 1.2% sodium hypochlorite for 20 min and germinated overnight

immersed in an aerated solution of 0.005 g L^{-1} Vitavax to prevent fungal growth. Seedlings were placed on nylon mesh suspended over 10 L of nutrient solution and grown for 3 days in a solution containing (μM): Ca, 1000; Mg, 300; NO_3, 2900; NH_4, 300; then for 5 days in a complete nutrient solution containing (μM): Ca, 1000; Mg, 300; K, 800; NO_3, 3300; NH_4, 300; PO_4, 100; SO_4, 101; Cl, 34; Na, 20; Fe, 10; B, 6; Mn, 2; Zn, 0.5; Cu, 0.15; Mo, 0.1. Iron was supplied as Fe-EDTA prepared from equimolar amounts of $FeCl_3$ and NaEDTA. All growth solutions were acidified to pH 4.8 with HCl.

Nine-day old seedlings were mounted on Plexiglas frames which covered each of 36 polyethylene containers of 10 L capacity. Each frame supported eight plants in four groups of two, and shielded growth solutions from light to inhibit algal growth. Plants were grown in a controlled environment room with temperature maintained between 23 and 26°C during a 16-h light period and between 17 and 19°C during darkness. Relative humidity was maintained between 56 and 62% during the light period and between 68 and 70% during darkness. Solution temperatures were maintained between 20 and 23°C by immersing all containers in a common waterbath. The growth room was illuminated by 12 HID mercury halide (400W) and 4 HID high pressure sodium (400W) lamps located 1.3 m above the plant bases. The photosynthetic photon flux density (PPFD) was 230 ± 14 μmol m^{-2} s^{-1} at plant base level.

A randomized, block factorial design with 2 cultivars, 6 concentrations of Mn (0, 100, 250, 500, 750, 1000 μM) and 3 replicates was used (totalling 36 containers). For the Mn treatment, Mn supplied as $MnCl_2 \cdot H_2O$, was superimposed over the basal nutrient solution. Due to sampling restraints, threefold replication was achieved in time such that germination, planting and sampling dates were staggered by a day interval. The pH of aerated nutrient solutions were adjusted initially to 4.8 with HCl or KOH and were recorded every third day. Nutrient solutions were adjusted periodically to 10 L with distilled water to compensate for water loss by evaporation and transpiration.

Plants were harvested on days 0, 5, 8, and 12. For each harvest, net photosynthetic (Ps) and respiration (Rs) rates were measured for blade 1 (the most recently matured leaf with developed ligule) from one plant in each container. These same leaves were then cut from the plants and split in two lengthwise, one half for chlorophyll analysis, the other half for determination of concentration of Mn. On subsequent harvest days corresponding leaves from different plants in each container were used. Wilkinson and Ohki (1988) determined that blade 1 is a good physiological indicator of the Mn

status of wheat grown in solution culture. On day 12, the roots from all plants in each container were collected, dried at 60°C and weighed. A root dry weight index (RWI) was used as a measure of relative tolerance of excess Mn (root dry weight of plants grown in excess Mn divided by root dry weight of control plants).

Rates of net Ps and dark Rs were measured using an ADH portable infrared gas analyser (IRGA) set on differential mode, with a flow rate of 180-190 ml min^{-1}, a PPFD of 240-250 µmol $m^{-2}s^{-1}$ and an air temperature of 23°C. Chlorophyll content was determined following the methods of Hiscox and Israelstam (1979). Approximately 0.1 g of fresh leaf tissue (cut in 5 mm strips) was heated at 65°C for 2 hours in 7 ml dimethyl sulphoxide (DMSO). The leaf tissue was removed and the liquid brought up to 10 ml with DMSO. Chlorophyll absorbance was read at 645 and 663 nm on a Milton Roy Spectronic 1201 spectrophotometer. To determine concentration of Mn, half leaves (approximately 0.125 g) were ashed at 500°C for 48 h. The resulting ash was dissolved in 0.1 ml HNO_3, oxidized with 0.1 ml H_2O_2 and diluted to 5 ml with distilled water. Concentrations of Mn were determined using a Perkin-Elmer atomic absorption spectrophotometer (AAS). Data were analysed using analysis of variance (ANOVA) with significance defined at α=0.05.

4.3 Results

The two cultivars differed in tolerance of Mn based on the RWI (Fig. 4.1A). Root weight of the Mn-sensitive cultivar 'Columbus' declined to 41% of control with 100 µM Mn in solution and to 10% of control at 250 µM Mn. Little additional inhibition was observed at higher concentrations. In contrast, root weight of the Mn-tolerant cultivar 'Norquay' remained greater than 42% of control with 1000 µM Mn in solution.

Results of the various ANOVAs performed for this experiment are tabulated in appendix 1 to enable simpler comparisons. Detailed descriptions of the results follow. An ANOVA for concentration of Mn in leaves indicated significant main effects due to cultivar and concentration of Mn in solution for harvest days 5, 8 and 12. The cultivar by concentration of Mn interaction was significant for days 5 and 8 and only marginally significant on day 12 (p=0.0568). There were no differences between the cultivars on day 0. Data from day 5 were selected for illustrative purposes (Fig. 4.1B). For all concentrations of Mn in solution above control, 'Norquay' contained higher concentrations of Mn in leaves than 'Columbus', accounting for the cultivar effect. For both cultivars, the concentration of Mn in leaves increased with increasing Mn in solution, accounting for the

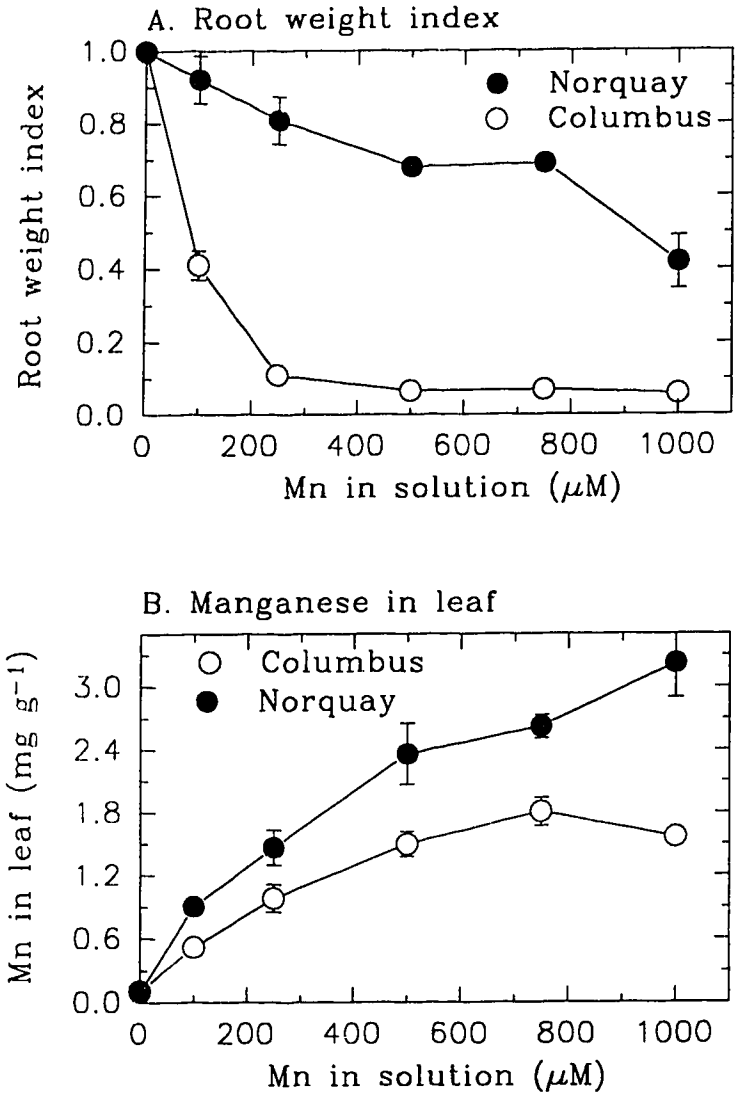

Figure 4.1. Root weight index ± s.e. (RWI; A) and concentration of Mn ± s.e. in the leaf (B) for Mn-tolerant 'Norquay' and Mn-sensitive 'Columbus' grown in a range of concentrations of Mn in solution. Data for concentration of Mn in the leaf are from harvest day 5 only.

treatment effect. The significant interaction effect was a result of 'Norquay' accumulating proportionately more Mn as the concentration of Mn in solution increased.

An ANOVA for respiration (Rs) rate indicated significant main effects for cultivar on day 5 only and significant main effects for concentration of Mn in solution on days 5, 8 and 12. The cultivar by Mn treatment interaction was a significant on day 5 only. On day 5, the Rs rate for 'Norquay' increased to 170% of control with up to 750 µM Mn in solution, above which Rs rate declined to 114% of control (Fig. 4.2A). For 'Columbus', the Rs rate increased to 183% of control with 100 µM Mn in solution, but fell back to 100-80% of control with 250-1000 µM Mn. On days 8 and 12, the Rs rate for both cultivars declined with increasing Mn in solution (Appendix 2).

An ANOVA for photosynthetic (Ps) rate indicated significant main effects for cultivar and concentration of Mn in solution, as well as a significant cultivar by Mn treatment interaction for harvest days 5, 8 and 12. Once again, there were no differences between cultivars on day 0. The Ps rate declined with increasing Mn in solution for both cultivars, but more so in 'Columbus' (Table 4.1). The Ps rate for 'Norquay' grown in 1000 µM Mn fell to 57% of control by day 12 whereas the respective rate for 'Columbus' dropped to 6% of control. Comparing sequential harvest days demonstrates that Ps rate declined over time (even in control plants); however, the extent of this decline was greater with higher concentrations of Mn in solution. The Ps data for day 5, expressed as a percent of control (Fig. 4.2B) are representative of data from days 8 and 12. The Ps rate of 'Columbus' declined to 69% of control with 250 µM Mn in solution and to 27% of control with 1000 µM Mn. In contrast, the Ps rate for 'Norquay' declined from 100 to 75% of control with 1000 µM Mn in solution.

Analyses of variance for concentrations of chlorophyll (chl) a and b indicated significant main effects due to cultivar and concentration of Mn in solution for days 5, 8 and 12. The cultivar by Mn treatment interaction was a significant on days 5 and 12 for chl a and day 12 for chl b. Again, data for day 5 were chosen as representative, and are presented in Fig. 4.3. 'Norquay' maintained higher concentrations of chl a and b across all concentrations of Mn accounting for the significant effect due to cultivar. For 'Norquay', the concentration of chl a rose to 138% of control with 100 µMn in solution (Fig. 4.3A). The concentration then declined gradually to 97% of control with increasing Mn. The concentration of chl b in 'Norquay' increased to 109% of control with 250 µM Mn (Fig. 4.3B). At higher concentrations of Mn, the concentration of chl b declined rapidly to approximately 50% of control. For 'Columbus', the concentrations of both chl a and b declined with increasing Mn

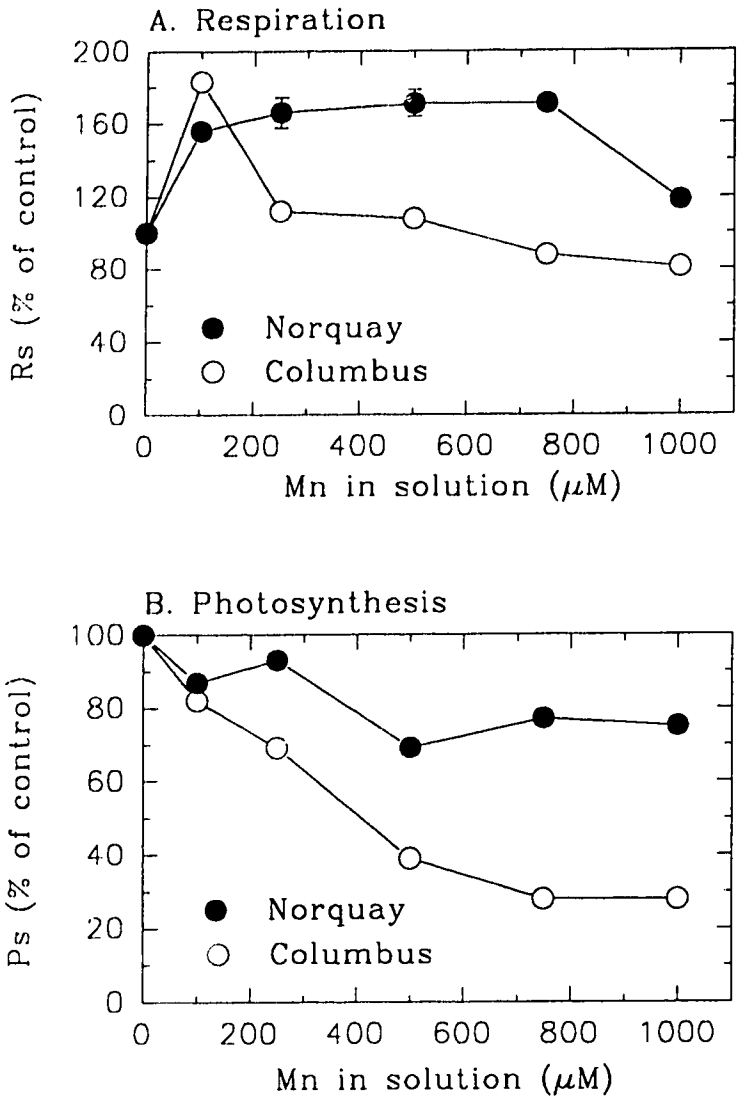

Figure 4.2. Mean relative rates of respiration (Rs, A) and photosynthesis (Ps, B) ± s.e. for Mn-tolerant 'Norquay' and Mn-sensitive 'Columbus' grown in a range of concentrations of Mn in solution on day 5. Control rates of Rs were 1.2 ± 0.1 and 1.2 ± 0.1 mg CO_2 gFW^{-1} h^{-1} for Norquay and Columbus respectively. Control rates of Ps were 10.8 ± 1.0 and 11.2 ± 1.0 mg CO_2 gFW^{-1} h^{-1} for Norquay and Columbus respectively.

Table 4.1. Mean photosynthetic rate ± s.e. for Mn-tolerant 'Norquay' and Mn-sensitive 'Columbus' on days 0, 5, 8 and 12 after exposure to a range of concentrations of Mn in solution.

		Mean photosynthetic rate (mg CO_2 gFW^{-1} h^{-1})			
		Days after exposure to Mn			
Cultivar	[Mn]	0	5	8	12
Norquay	0	12.4±0.4	11.2±1.0	9.8±1.0	11.8±1.4
	100	12.8±0.6	9.7±0.9	11.0±0.3	9.8±0.5
	250	13.0±0.3	10.4±0.6	8.2±0.6	7.5±1.0
	500	12.7±0.2	7.7±0.3	7.9±0.7	4.5±1.2
	750	13.1±0.3	8.6±0.6	5.1±1.6	8.1±0.7
	1000	12.9±0.4	8.4±0.3	2.7±1.1	6.7±2.5
Columbus	0	13.4±0.8	10.8±1.0	10.5±0.5	10.9±1.2
	100	12.8±0.4	8.9±1.3	8.2±0.6	4.7±0.4
	250	12.7±0.7	7.5±1.4	4.1±0.2	3.5±0.6
	500	13.0±0.6	4.2±0.2	3.0±1.0	1.9±0.3
	750	13.1±0.3	3.0±0.2	2.0±0.3	1.7±0.2
	1000	12.6±0.5	3.0±0.5	1.7±0.7	0.6±0.2

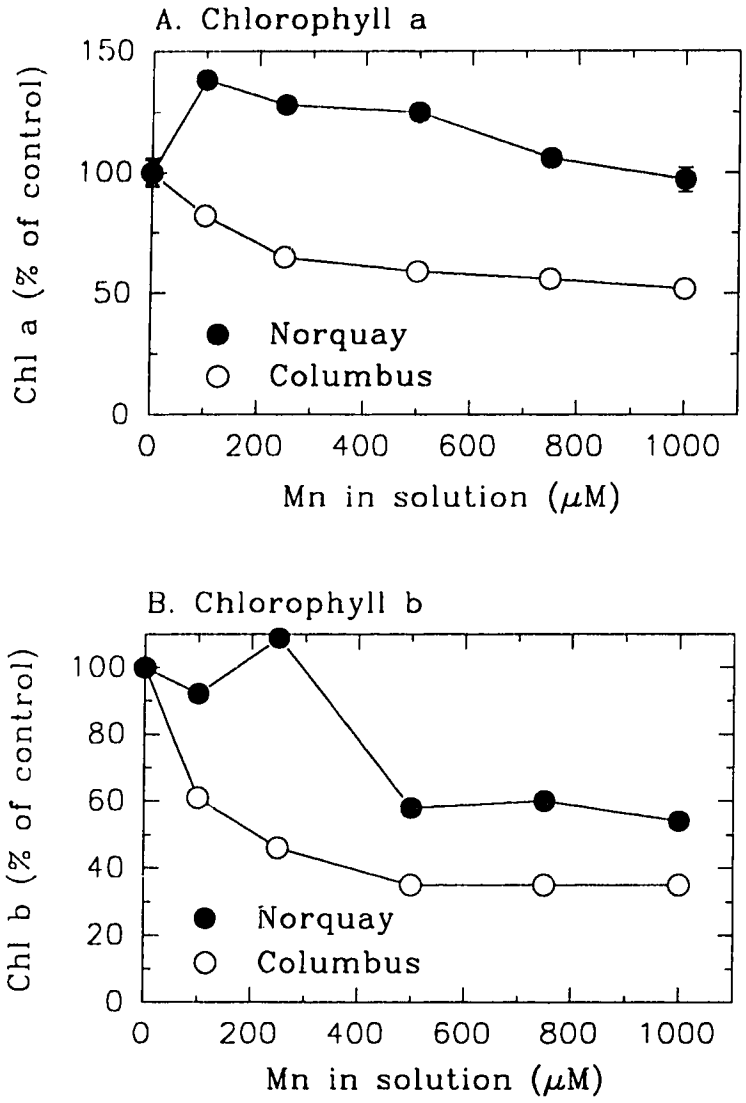

Figure 4.3. Mean concentrations of chlorophyll a (A) and b (B) expressed as a percent of control ± s.e. for Mn-tolerant 'Norquay' and Mn-sensitive 'Columbus' grown in a range of concentrations of Mn in solution on day 5.

in solution (Fig. 4.3B). With 1000 µM Mn, the concentration of chl a fell to 50% of control and the concentration of chl b fell to 36% of control. In general, the ratio of chl a:b remained consistent across the range of concentrations of Mn in solution for each cultivar on a given harvest day (Table 4.2). An ANOVA for the ratio of chl a to b indicated a significant main effect due to cultivar on days 0, 5 and 12. On each of these harvest days, 'Norquay' had a higher chl a:b ratio than did 'Columbus' across the range of concentrations of Mn in solution. There was also a significant main effect due to concentration of Mn in solution on day 5 at which time the ratio of chl a:b increased with increasing Mn in solution. The cultivar by Mn treatment interactions were not significant.

For both cultivars, the Ps rate declined with increasing Mn in leaf tissues (Fig. 4.4A); however, 'Norquay' maintained a higher Ps rate with high concentrations (greater than 1000 µg g^{-1}) in the leaf. Similarly, the concentration of chl declined with increasing Mn in leaf tissue (Fig. 4.4B). Again, 'Norquay' maintained a higher concentration of chl than 'Columbus' with increasing Mn in the leaf. The Ps rates of both cultivars increased with increasing chl in leaf tissues with no apparent difference between the cultivars (data not shown). The Ps rate per unit chl decreased in 'Columbus' as concentration of Mn in solution increased for each sampling day (Fig. 4.5A). In contrast, the Ps per unit chl remained constant across the range of concentrations of Mn for 'Norquay' on days 5 and 8 and increased for plants grown in 750 and 1000 µM Mn on day 12 (Fig. 4.5B).

4.4 Discussion

The use of a RWI to measure the relative tolerance of wheat to excess Mn is appropriate since wheat roots are more sensitive to Mn toxicity than leaves based on dry weight (Ohki 1984). This index confirms the tolerance of 'Norquay' and the sensitivity of 'Columbus' to excess Mn over a broad range of concentrations of Mn in solution (Fig. 4.1A, Chapt. 2, Macfie *et al.* 1989). Furthermore, the extent of differential tolerance of Mn is comparable to the more widely documented differences in tolerance of Al (Briggs *et al.* 1989, Zhang and Taylor 1988), indicating that Mn toxicity may be as important as Al toxicity for plants growing on acidic soils. The patterns for concentrations of Mn in leaves reported here (Fig. 4.1B) are also consistent with previous reports (Chapt. 3, Macfie and Taylor 1989, Ohki 1984) in that concentrations of Mn in leaves were proportional to concentrations of Mn in solution. Ohki (1984) found that concentrations of Mn increased progressively with age in leaves of wheat, but the slope of the relationship between internal and external Mn did not vary with age. It is therefore appropriate to use blade 1 as an indicator of the

Table 4.2. Mean ratio of concentration of chlorophyll a:b ± s.d. for Mn-tolerant 'Norquay' and Mn-sensitive 'Columbus' on days 0, 5, 8 and 12 after exposure to a range of concentrations of Mn in solution.

		Mean ratio of chlorophyll a:b			
		Days after exposure to Mn			
Cultivar	[Mn]	0	5	8	12
Norquay	0	2.89±0.46	5.09±0.10	4.49±0.90	6.37±1.12
	100	3.23±0.06	5.52±0.70	4.59±1.40	6.73±0.14
	250	3.46±0.08	4.72±1.79	4.54±1.79	6.71±0.13
	500	3.91±0.21	7.59±0.10	4.12±0.55	5.88±0.004
	750	3.99±0.29	6.26±0.14	5.21±1.96	5.86±0.05
	1000	3.66±0.22	6.33±0.09	5.31±0.23	5.47±0.08
Columbus	0	4.85±0.90	4.07±2.08	4.54±0.48	5.92±0.21
	100	3.98±0.21	4.62±0.27	4.34±0.60	6.35±0.09
	250	4.01±0.07	5.18±0.22	5.23±2.06	5.27±0.07
	500	4.54±0.06	6.44±1.32	4.21±0.05	4.96±0.11
	750	4.50±0.39	5.57±0.03	4.46±0.28	4.78±0.10
	1000	4.00±0.04	5.13±0.27	4.90±0.16	5.74±1.67

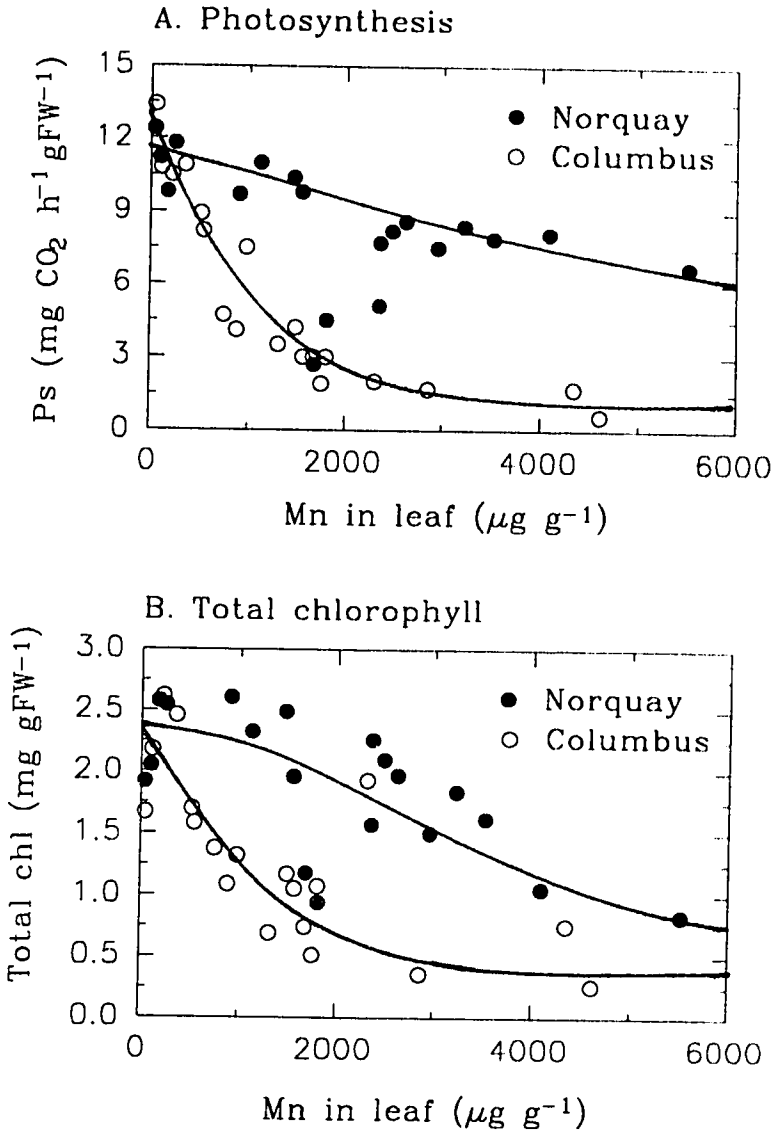

Figure 4.4. Rate of photosynthesis ± s.e. (Ps, A) and concentration of chlorophyll ± s.e. (chl, B) against concentration of Mn in the leaf for Mn-tolerant 'Norquay' and Mn-sensitive 'Columbus'. Data are pooled from all harvest days. Lines are hand-drawn and are intended for illustrative purposes only; I do not wish to suggest that they are accurate representations of the stress response.

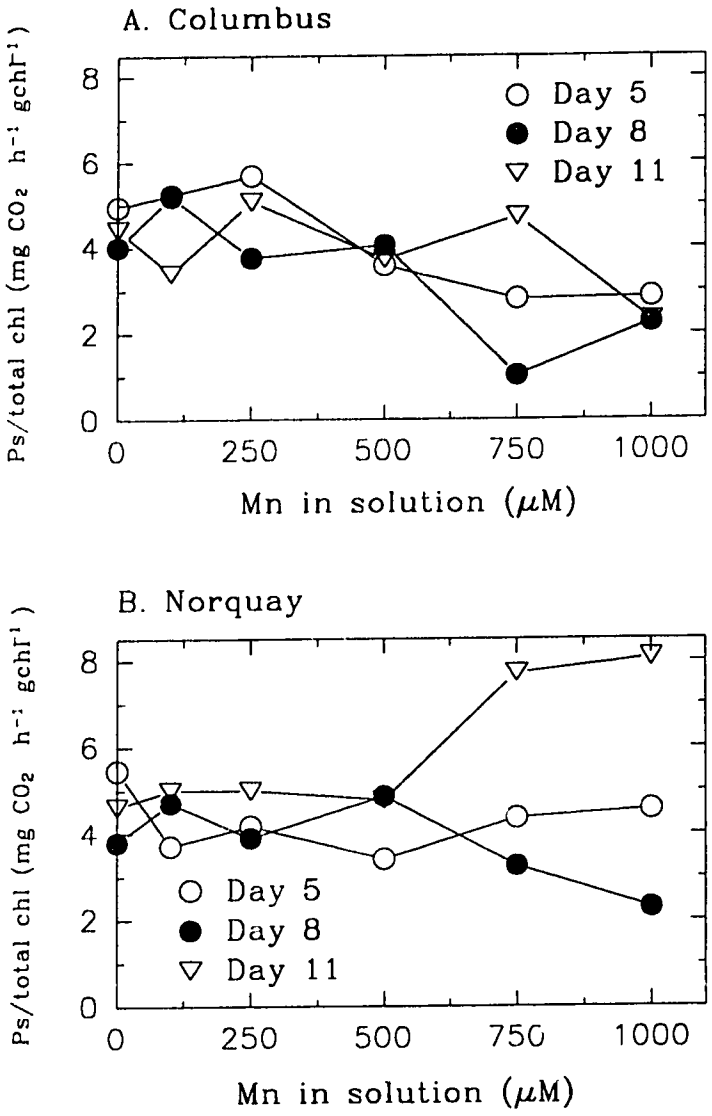

Figure 4.5. Mean photosynthetic rate per unit chlorophyll against concentration of Mn in solution ± s.e. for Mn-tolerant 'Norquay' (A) and Mn-sensitive 'Columbus' (B) for days 5, 8 and 12.

plant's Mn status. Comparison of the RWI and data on the concentration of Mn in leaves indicate that 'Norquay' is more tolerant of excess Mn than 'Columbus', despite increased concentrations of Mn in aerial tissues. The chemical form and distribution of Mn within the leaf was not investigated here. In *Vigna unguiculata*, the concentration of Mn in the leaves of a Mn-tolerant cultivar was uniformly distributed and in an easily extractable form, whereas in a Mn-sensitive cultivar, Mn was localized and not as easily extractable (Horst 1980).

Relative rates of respiration for both cultivars declined with time, however, the rates for 'Norquay' were higher than those of 'Columbus' over the range of concentrations of Mn supplied (Fig. 4.2A). Campbell and Nable (1988) suggested that a decline in respiration after a period of Mn deficiency reflects a secondary effect related to the impairment of photosynthesis and the supply of assimilates. It is possible that the same explanation holds for Mn toxicity. However, Mn is also involved in several enzyme-catalysed reactions (Burnell *et al.* 1988) thus, it is possible that Mn has a primary toxic effect on respiration as well. In this study, relative rates of photosynthesis also declined with increasing Mn in solution (Fig. 4.2B). It is common for metal toxicity to cause a decline in photosynthetic rate. This has been reported for Mn toxicity of *Nicotiana tabacum* (Houtz *et al.* 1988, Nable *et al.* 1988), Cd toxicity of *Lycopersicon esculentum* (Baszynski *et al.* 1980), Al toxicity of *Oryza sativa* (Sarkunan *et al.* 1984), and for Cu, Cd, Pb and Zn toxicity of both *Hordeum vulgare* and *Zea mays* (Stiborova *et al.* 1987). In a similar study with wheat, Ohki (1985) measured a decline in photosynthetic rate with increasing Mn in solution, but took only a single measurement at the end of a 12-day exposure to excess Mn. A decline in photosynthetic rate with time was also found in this study. In *Glycine max*, photosynthetic rates declined both with age of leaf and time in plants grown under Mn deficiency (Cooper and Girton 1963). It is perhaps not surprising that Mn toxicity had a similar effect. It is apparent, however, that Mn-tolerant 'Norquay' was able to maintain higher rates of photosynthesis in the face of Mn toxicity. Wilkinson and Ohki (1988) suggested that reductions in net photosynthetic rate and growth are a direct result of reduced pigment content, although they did not present data to eliminate the possible direct effects of excess Mn on photosynthesis. Houtz *et al.* (1988) determined that Mn-induced reduction in photosynthesis in *Nicotiana tabacum* was a direct result of altered carboxylase/oxygenase activity of rubisco. Perhaps the differences between cultivars observed in this study reflect differential sensitivity of rubisco to excess Mn.

Although concentrations of chlorophyll a and b declined with increasing Mn in solution and with time, 'Norquay' maintained higher concentrations than did 'Columbus' (Fig. 4.3). Once again, it is common for metal toxicity to induce a decline in chlorophyll concentration. A reduction in total

chlorophyll was observed after exposure to excess Mn in *Phaseolus vulgaris, Zea mays* (Horiguchi 1988), *Glycine max* (Ohki 1981) and wheat (Moroni 1991). In *Hordeum vulgare* and *Zea mays*, total chlorophyll concentrations declined with increasing concentrations of Cd, Cu and Pb in nutrient solution (Stiborova *et al.*1987). A decrease in total chlorophyll content was also reported for Cd toxicity of *Lycopersicon esculentum* (Baszynski *et al.* 1980) and *Hordeum vulgare* (Stobart *et al.* 1985) and for Al toxicity of wheat and *Sorghum bicolor* (Ohki 1986). The ratio of chlorophyll a to b was also sensitive to Mn toxicity (Table 4.2). These findings were consistent with those of Wilkinson and Ohki (1988) and Kriedmann *et al.* (1985) who determined that the ratio of chl a to b increased as Mn in solution increased despite a decrease in the concentrations of chlorophylls a and b. In contrast, Moroni (1991) found no change in the chl a:b ratio for wheat grown in 1000 µM Mn. Interestingly, Baszynski *et al.* (1980) reported that Cd toxicity caused a decrease in the chl a:b ratio in *Lycopersicon esculentum* and Sarkunan *et al.* (1984) reported a similar decline in *Oryza sativa* grown with excess Al. It would seem that the specific effect of a metal on chlorophyll content and the chl a:b ratio depends on the nature of the toxic response.

In this study, rates of photosynthesis declined with increasing concentrations of Mn in the leaf, however, Mn-tolerant 'Norquay' maintained higher rates of photosynthesis over a broader range of concentrations of Mn (Fig. 4.4A). Once again, a number of studies have demonstrated that photosynthetic rates decline as tissue concentrations of a toxic metal increase. In wheat and *Sorghum bicolor*, rates of photosynthesis declined with increasing Al (Ohki 1986) and Mn (Ohki 1985) in leaf tissues. Similarly, the concentrations of chlorophyll decreased as tissue concentrations of Mn increased (Fig. 4.4B). Wilkinson and Ohki (1988) also found that the molar ratio of chlorophyll to Mn in leaves of wheat decreased as concentration of Mn in solution increased. In this study, it is unclear whether Mn toxicity is first reflected in chlorophyll content or photosynthesis, since both parameters had declined by the first sampling day. In *Nicotiana tabacum* grown with excess Mn in solution culture, a decline in net photosynthesis preceded any changes in chlorophyll abundance by at least 2-3 days (Nable *et al.*1988).

Not surprisingly, there was a positive relationship between photosynthetic rates and concentrations of chlorophyll in the leaf for both 'Norquay' and 'Columbus'. Interestingly, photosynthesis per unit chlorophyll declined with increasing Mn in solution for the sensitive cultivar, yet remained constant or increased for the tolerant cultivar (Fig. 4.5). Nable *et al.*(1988) determined that photosynthesis per unit chlorophyll declined with time for *Nicotiana tabaccum* grown in excess Mn, but not in control plants. If photosynthesis per unit chlorophyll remained constant, it would

indicate that the observed decline in photosynthetic rate was a direct result of reduced chlorophyll content. A decline in photosynthesis per unit chlorophyll indicates that the toxic element acted directly on photosynthesis. A rise in photosynthetic rate relative to concentration of chlorophyll has been interpreted to reflect a toxic effect on antennae chlorophyll (Baszynski *et al.*1980). The response of the sensitive cultivar in this study could therefore indicate that Mn exerted its toxic effect on photosynthesis and chlorophyll content independently. The rapid decline in photosynthetic rate was a function of reduced chlorophyll as well as inhibition of photosynthesis. In contrast, the response of the tolerant cultivar could indicate that the primary toxic effect of Mn was on the concentration of chlorophyll in the leaf, reduction in photosynthetic rate reflecting a secondary response. The increase in photosynthesis per unit chlorophyll observed at high concentrations of Mn in solution on day 12 could reflect increased concentrations of Mn in leaves, possibly accounting for reduced concentrations of antennae chlorophyll. This hypothesis has not been tested here.

The results reported here clearly indicated differences between 'Norquay' and 'Columbus' in the physiology of the Mn stress response. In Mn-sensitive 'Columbus', concentrations of Mn in the leaf above 500 $\mu g\ g^{-1}$ coincided with decreased chlorophyll content and photosynthetic rate. In Mn-tolerant 'Norquay', similar decreases were not observed until concentrations of Mn in leaves exceeded 1200 $\mu g\ g^{-1}$. The cultivars also differed with respect to photosynthetic rate per unit chlorophyll indicating that the primary targets(s) of Mn toxicity differ between the cultivars. More insight into the physiological mechanism(s) of this differential tolerance requires research into the compartmentation of Mn within the leaf tissue and potential differential sensitivity of rubisco isozymes to Mn. It would also be interesting to test whether the primary effect of Mn on chlorophyll acts with respect to synthesis or degradation and whether the cultivars vary in this respect.

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5. Production of organic acids in *Triticum aestivum* grown in excess manganese[4]

5.1 Introduction

Metal tolerance is not necessarily dependent upon exclusion of the toxic metal from the plant. Indeed, it is common for tissues of tolerant ecotypes to have concentrations of a toxic metal which exceed concentrations which are injurious to non-tolerant ecotypes (Thurman 1983, Mathys 1980). Several mechanisms may operate to enable higher plants to tolerate high internal concentrations of toxic metals;(i) evolution of tolerant enzymes, (ii) elevated enzyme activity, (iii) complexation with metal-binding proteins (the phytochelatins), (iv) chelation by ligands in the cytosol and (v) compartmentation in the vacuole or other locations removed from metabolic activity (Taylor 1991). The role of carboxylate anions as metal chelators in the cytosol has received much attention in recent literature. They have also been implicated in the compartmentation of metals in the vacuole.

Jones (1961) was the first to suggest that carboxylate anions in the cytosol may chelate toxic metals, thereby reducing their phytotoxicity. He identified aluminum (Al)-citrate and Al-oxalate complexes in leaves of Al-tolerant *Hordeum vulgare* and roots of Al-tolerant *Pisum sativum*. In 1980, Mathys proposed a model for zinc (Zn) tolerance based on data which implicated malate and oxalate as chelators of Zn. With 0.4 mM Zn in solution, leaves of Zn-tolerant *Silene cucubalis* and *Rumex acetosa* produced increased concentrations of oxalate, whereas oxalate production was inhibited in Zn-sensitive cultivars. Mathys (1980) also found that leaves of Zn-tolerant ecotypes of *Silene cucubalis*, *Rumex acetosa*, *Agrostis tenuis* and *Thlaspi alpestre* contained higher concentrations of malate than their Zn-sensitive counterparts. Mathys (1980) suggested that Zn is chelated by malate in the cytosol and transported to the vacuole where, after dissociation from malate, it forms a stable complex with oxalate. In Zn-tolerant *Deschampsia caespitosa*, greater concentrations of malate were also found and Zn seemed to be transported into the vacuole (Thurman and Rankin 1982), although in the same species, Godbold *et al* .(1984) suggested that vacuolar Zn (based on analysis of root sap) was complexed with citrate, not oxalate.

[4] A version of this chapter is being prepared for publication. Macfie, S.M. , Cossins, E.A. and Taylor, G.J. Production of organic acids in *Triticum aestivum* grown in excess manganese. Being prepared for Plant Physiol.

Since Mathys' model was published, many researchers have demonstrated positive correlations between organic acid content and tolerance to toxic metals. Unfortunately, the bulk of this literature is of a purely descriptive nature. Concentrations of citrate and/or malate increased with Al stress in Al-tolerant cultivars of *Sorghum bicolor* (Cambraia *et al.* 1983) and *Zea mays* (Suhayda and Haug 1986). Lee *et al.* (1978) identified 17 nickel (Ni)-tolerant species which responded to Ni stress with increased concentrations of citrate in leaves. Similarly, increased concentrations of citrate and malate correlated with increased tolerance of excess copper (Cu; Ernst 1976) and Zn (Thurman and Rankin 1982). In *Hordeum vulgare*, *Pisum sativum* and *Triticum aestivum* (wheat), the absolute concentrations of citrate and malate declined under Al-stress; however, tolerant cultivars were able to maintain higher tissue concentrations than sensitive cultivars (Klimashevski and Chernesheva 1980). A notable exception to this trend was reported for wheat grown in excess Mn, where concentrations of *trans*-aconitate (Scott *et al.* 1987, Burke *et al.* 1990, Foy *et al.* 1990), citrate and malate (Scott *et al.* 1987, Burke *et al.* 1990) were higher in the sensitive cultivar. While there is obviously a correlation in many species between maintenance of high internal concentrations of carboxylate anions (especially citrate and malate) and superior tolerance to metal stress, causal relationships cannot be inferred from these data.

The relationship between tolerance to toxic metals and organic acid content has also been studied *in vitro*. For example, the activities of two membrane-associated ATPases in *Pisum sativum*, which had been inhibited by addition of $AlCl_3$, were restored by adding citrate, glutamate or malate to the assay (Matsumoto and Yamaya 1986). Similarly, an Al-induced reduction in ATPase activity in roots of *Zea mays* was ameliorated by pretreatment with citrate (Suhayda and Haug 1986). Interestingly, growth of roots of wheat, which had been inhibited by Al, was restored by the addition of citrate to the growth medium (Ownby and Popham 1989). While addition of various organic acids to metal-stressed plants ameliorated metal toxicity, Krotz *et al.* (1989) found that neither Cd nor Zn stimulated citrate or malate biosynthesis in *Nicotiana tabacum* suspension cells. There is little doubt that the presence of carboxylate anions such as citrate and malate at sufficient concentrations can reduce or prevent the toxic effects of metals at the cellular level (Suhayda and Haug 1986), but it is not clear that this process acts as a primary metal tolerance mechanism.

Most researchers in this field have not attempted to explain the mechanism of differential organic acid production, although some have suggested that it is a result of specific enzyme activity. For example, Thurman and Rankin (1982) looked for differential stimulation or inhibition of citric acid cycle enzymes, but found no differences in the sensitivity of malic dehydrogenase, aconitase, pyruvate kinase, PEP carboxylase or citric acid synthase in Zn-tolerant and sensitive cultivars of

Deschampsia caespitosa. Brookes *et al.* (1981) suggested that Ni inactivates malic dehydrogenase in the citric acid cycle, leading to build-up of malate and Cambraia *et al.* (1983) ascribed increased concentrations of aconitate in a tolerant cultivar to inhibition of aconitase and isocitrate dehydrogenase. Burke *et al.* (1990) also suggested that inhibition of citric acid cycle enzymes, either directly or indirectly, may result in build up of citric acid cycle intermediates. The studies of Brookes *et al.* (1981) and Cambraia *et al.* (1983); however, did not test enzyme sensitivity to the various metal stresses. In many ways, it seems counterintuitive to expect a mechanism of metal tolerance to involve selective sensitivity of certain enzymes to the metal stress.

The objectives of this study were (i) to determine the effects of excess Mn on concentrations of organic acids in Mn-tolerant and Mn-sensitive cultivars of wheat and (ii) to determine the specific radioactivity of these organic acids in leaves of these cultivars incubated with [2-^{14}C]sodium acetate. This radiolabel was chosen in order to test the role of the citric acid cycle in any observed changes in organic acid content. If a specific enzyme (or enzymes) was inhibited, I would expect to see a concomitant increase in the radioactivity of one or more organic acid intermediates. In a previous study with wheat (Chapter 4, Macfie and Taylor Submitted), I found that Mn toxicity affected both photosynthetic and respiratory rates. In this study I will also report on altered photosynthetic and respiratory rates in relation to observed changes in organic acid content and specific radioactivity.

5.2 Methods

5.2.1 *Concentrations of organic acids in root and leaf tissues from intact plants*

Two cultivars of wheat (*Triticum aestivum*), Mn-tolerant 'Norquay' and Mn-sensitive 'Columbus' (Macfie *et al.* 1989) were selected for experimentation. Four hundred seeds of each cultivar were surface sterilized in 1.2% sodium hypochlorite for 20 min and germinated overnight immersed in an aerated solution of 0.005 g L^{-1} Vitavax to prevent fungal growth. Seedlings were placed on nylon mesh suspended over 10 L of nutrient solution and grown for 3 days in a solution containing (μM): Ca, 1000; Mg, 300; NO_3, 2900; NH_4, 300; then for 5 days in a complete nutrient solution containing (μM): Ca, 1000; Mg, 300; K, 800; NO_3, 3300; NH_4, 300; PO_4, 100; SO_4, 101; Cl, 34; Na, 20; Fe, 10; B, 6; Mn, 2; Zn, 0.5; Cu, 0.15; Mo, 0.1. Iron was supplied as Fe-EDTA prepared from equimolar amounts of $FeCl_3$ and NaEDTA. All growth solutions were acidified to pH 4.8 with HCl.

Nine-day old seedlings were mounted on Plexiglas frames which covered each of 18 polyethylene containers of 10 L capacity (experimental day 0). Each frame supported eight plants in four groups of two, and shielded growth solutions from light to inhibit algal growth. Plants were grown in a controlled environment room with temperature maintained between 26 and 27°C during a 16-h light period and between 18 and 19°C during darkness. Relative humidity was maintained between 63 and 68% during the light period and between 73 and 81% during darkness. Solution temperatures were maintained between 18 and 19°C by immersing all containers in a common waterbath. The growth room was illuminated by 12 HID mercury halide (400W) and 4 HID high pressure sodium (400W) lamps located 1.3 m above the plant bases. The photosynthetic photon flux density (PPFD) was 296 ± 22 μmol m^{-2} s^{-1} at plant base level.

A randomized block factorial design with 2 cultivars ('Norquay' and 'Columbus'), 3 concentrations of Mn (0, 200, 500 μM) and 3 replicates was used (totalling 18 containers). For the Mn treatment, Mn supplied as Mn $Cl_2 \cdot H_2O$ was superimposed over the basal nutrient solution. The pH of aerated nutrient solutions were adjusted initially to 4.8 with HCl or KOH and were recorded every day. On day 7, 5 ml of 600 mM NH_4NO_3 was added to each container to keep the pH of solution below 4.8. Nutrient solutions were adjusted periodically to 10 L with distilled water to compensate for water loss by evaporation and transpiration.

Plants were harvested on days 5, 7, 9 and 11. One plant from each container was harvested on each sampling day. Plants were rinsed three times in deionized distilled water, blotted dry with filter paper and divided into root and leaf tissues after which fresh weights were recorded. An additional plant from each container was harvested on day 12 and used for fresh weight information only. To determine organic acid content, approximately 1g fresh weight of each tissue for each sample was frozen with liquid nitrogen and ground using a pre-frozen (-70°C) mortar and pestle. Samples were homogenized for 3 min at high speed in 5 ml 0.01 N H_3PO_4 then centrifuged at 18,000 RPM for 20 min. The supernatant was purified using reverse phase octadecyl (C_{18}) columns. The first ml of sample was allowed to pass through the column, the next 2 ml were collected and passed through a 0.45 μm millipore filter. One hundred μl aliquots were injected into a Waters high performance liquid chromatography (HPLC) system. Organic acids were separated on an Aminex HPX-87H reverse-phase ion exchange column. Separation was performed isocratically at a flow rate of 0.5 ml min^{-1} with 0.01 N H_3PO_4 as the mobile phase. Authentic organic acid standards were obtained from Sigma. Data were analyzed using analysis of variance (ANOVA) with significance defined at $\alpha = 0.05$.

5.2.2 *Radiolabelling of leaf tissue*

The same two cultivars of wheat, Mn-tolerant 'Norquay' and Mn-sensitive 'Columbus', were selected for experimentation. Four hundred seeds of each cultivar were sterilized, germinated and grown for eight days as described in the first experiment. Nine-day old seedlings were mounted on each of 12 polyethylene containers (experimental day 0). The growth conditions were the same as described above except for the following differences; the temperature was maintained between 22 and 23°C during the light period and between 18 and 19°C during darkness, relative humidity was maintained between 60 and 65%, the PPFD was 253 ± 29 µmol m^{-2} s^{-1} and the containers were not immersed in a waterbath.

A randomized block factorial design with 2 cultivars, 2 concentrations of Mn (0, 500 µM) and 3 replicates was used (totalling 12 containers). One plant from each container was harvested on day 11. Leaves were rinsed three times in deionized distilled water, blotted dry and chopped into 2mm slices. Approximately 0.7g fresh weight of each sample was placed in a 100 ml beaker covered with Parafilm then pre-incubated in 5ml of 1mM KPO_4 plus 1% sucrose (w/v) at pH 6.0 in a shaking water bath for 3h at 25°C (Mansfield and Key 1987). Samples were then incubated with 5 µCi of [2-^{14}C]sodium acetate (specific activity 27.2 µCi µmol^{-1}, 1006.4 Bq nmol^{-1}) in 100 µL distilled water for 1h in the dark in a shaking water bath at 25°C. A second set of leaves from of control plants were harvested, pre-incubated and radiolabelled as above, but with 500 µM Mn added to the labelling solution. After 1h incubation, 15ml boiling EtOH (95%) was added to each sample which was then boiled for an additional 3 min. Samples were ground for 5 min in a mortar with approximately 0.5 g sea sand. Extracts were removed by centrifugation at 6000 RPM for 15 min. After two extractions, the supernatants were dried using a flash evaporator then stored at 4°C.

Plant extracts were separated into four fractions, lipids, sugars, amino acids and organic acids following the resin exchange procedures of Canvin and Beevers (1961). Pigments and lipids were extracted with 15 ml diethyl ether. The water soluble fraction was dissolved in 15 ml distilled water and passed through a cation exchange resin (Dowex-50W, 200-400 mesh, hydrogen form). Sugars and organic acids were eluted with 40 ml distilled water, amino acids were displaced from the column with 50 ml 6N HCl. The sugar and organic acid fraction was passed through an anion exchange resin (Dowex-2, 100-200 mesh size, chloride form). Sugars were eluted with 60 ml distilled water, organic acids were displaced from the column with 30 ml 4 N formic acid followed by 50 ml 8 N formic acid. Radioactivity of each fraction was determined by scintillation counting (Beckman LS 6000TA)

using 0.1 to 0.5 ml aliquots brought to 4 ml volume with Bray's solution (4 g 2,5-diphenyloxazol (PPO), 0.2 g 1,4 di[2-(5-phenyloxazolyl)] benzene (POPOP), 60 g napthalene, 100 ml methanol, 20 ml ethyleneglycol and dimenthoyethane to make 1 L; Bray 1960). Concentrations of organic acids were determined from 100 µl aliquots using the same HPLC system described above. The radioactivity of each organic acid detected was determined by collecting 0.5 ml fractions from the HPLC system. Glutamate and aspartate were separated from the neutral and basic amino acids using Dowex-2 anion exchange resin (acetate form) eluted with 0.5 N acetic acid (Hirs *et al.* 1954).

On day 12, another plant was harvested from each container (2 from control solutions) and the same procedures as above were followed except that leaf slices were incubated in ^{14}C-sodium acetate under a 250W mercury lamp (453.3 µmol $m^{-2}s^{-1}$). Data were analyzed using analysis of variance (ANOVA) with significance defined at α=0.05. To achieve homogeneity of variance, the ANOVA of data expressed as a percent of total label was performed on the arcsine square root transformation of the data.

5.2.3 *Rates of photosynthesis and respiration*

The same two cultivars of wheat, Mn-tolerant 'Norquay' and Mn-sensitive 'Columbus' were selected for experimentation. Fifty seeds of each cultivar were sterilized, germinated and grown for eight days as described above. Nine-day old seedlings were mounted on 12 containers (experimental day 0) as described in the experiment above with the following exceptions; the temperature was maintained between 26 and 27°C during the light period and between 20 and 21°C during darkness, relative humidity was maintained between 61 and 70% during the light period and between 75 and 85% during darkness and the growth room was illuminated by 6 mercury halide (400W) and 6 sodium (400W) lamps located 0.8 m above the plant bases, with a PPFD of 613 $\pm$ 21 µmol m^{-2} s^{-1} at plant base level.

A randomized block factorial design with 2 cultivars, 2 concentrations of Mn (0, 500 µM) and 3 replicates was used (totalling 12 containers). Photosynthetic and respiratory rates were measured using a portable infrared gas analyzer (IRGA) set on differential mode, with a flow rate of 180 ml min^{-1}, a PPFD of 620 µmol $m^{-2}s^{-1}$ and an air temperature of 25°C. Measurements were taken immediately prior to adding the plants to the Mn treatments, after 6 hours of exposure to excess Mn, then daily for a total of 9 measurements.

5.3 Results

5.3.1 *Effects of manganese on growth, photosynthetic rate and respiratory rate*

Fresh weights of leaves and roots of Mn-tolerant 'Norquay' increased with time and were unaffected by concentration of Mn in solution (Fig. 5.1A,C), although both root and leaf weight may have started to decline on day 12. In contrast, leaves and roots of Mn-sensitive 'Columbus' were clearly affected by excess Mn by day 11 (Fig. 5.1B,D). Roots of 'Columbus' were more severely affected than leaves. On day 12, leaf weights of plants grown with 250 and 500 µM Mn fell to 81 and 70% of control respectively and root weights decreased to 68 and 37% of control.

The photosynthetic rate also declined in the Mn-sensitive 'Columbus' immediately after exposure to excess Mn. After 6 h, the rate fell to 60% of control (Fig. 5.2A). Between 1 and 7 days of growth in 500 µM Mn, photosynthetic rates remained approximately 20% of control. In contrast, the photosynthetic rates of Mn-tolerant 'Norquay' stayed greater than or equal to control rates for up to 3 days after exposure to excess Mn. By day 6, the rate declined to 65% of control. Respiratory rates for both cultivars increased up to 4 days after growth in 500 µM Mn, after which they decreased (Fig. 5.2B). In 'Columbus', respiratory rates increased to near 400% of control by day 4, on day 7, they declined to 65% of control. In contrast, respiratory rates in 'Norquay' increased to 310% of control on day 4, and fell to 110% of control on day 7.

5.3.2 *Concentrations of organic acids in root and leaf tissues*

Six carboxylate anions were detected by HPLC analysis in root and leaf tissues; aconitate (*cis*- and *trans*-aconitate combined), α-ketoglutarate, citrate, malate, succinate and an unknown. Identification of the carboxylate anions was based on comparison to retention times of known carboxylate anions (Fig. 5.3). Slight shifts in retention times were found for the root and leaf extracts, therefore identification was confirmed by use of internal standards. The small unidentified peaks at 7.8 and 22.8 min represent products leached from the plastic HPLC inserts used for sample injection. These peaks increased with time but were never greater than shown in Fig. 5.3. In attempting to identify the unknown peak fumarate, glycolate, glyoxalate, lactate, malonate, oxalacetate, oxalate, oxalsuccinate and tartarate were eliminated based on differences in retention times (retention times for these were; 18.51, 15.20, 11.56, 19.08, 12.12, 10.68, 9.50, 9.61, 10.46 respectively). Using either H_3PO_4 or H_2SO_4 as the mobile phase, the unknown organic acid co-eluted with acetate. Use of an internal acetate standard also indicated that the unknown acid was acetate.

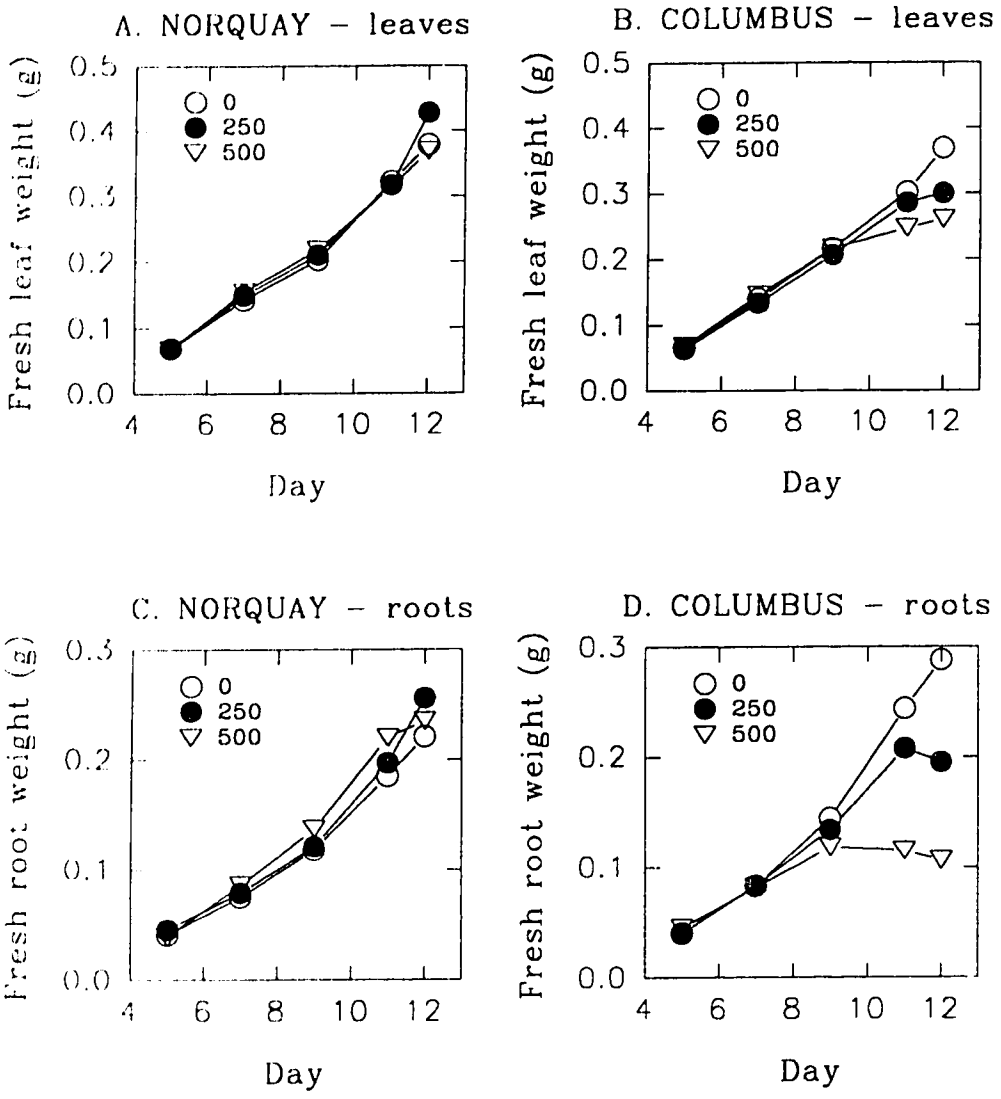

Figure 5.1. Mean fresh leaf weights ± s.e. of (A) Mn-tolerant 'Norquay' and (B) Mn-sensitive 'Columbus' and mean fresh root weights ± s.e. of (C) 'Norquay' and (D) 'Columbus' grown with 0, 250 or 500 µM Mn added to solution culture.

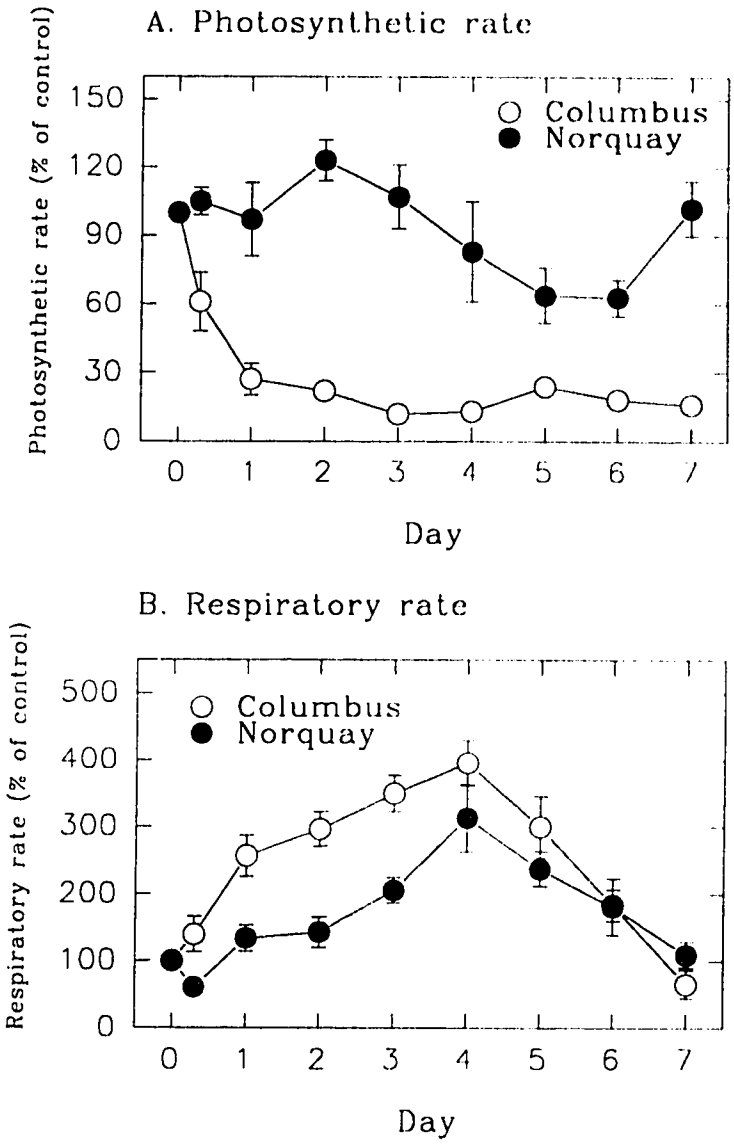

Figure 5. 2. (A) mean photosynthetic rate and (B) mean respiratory rate (expressed as a percent of control ± s.e.) for Mn-tolerant 'Norquay' and Mn-sensitive 'Columbus' grown with 500 µM Mn in solution culture.

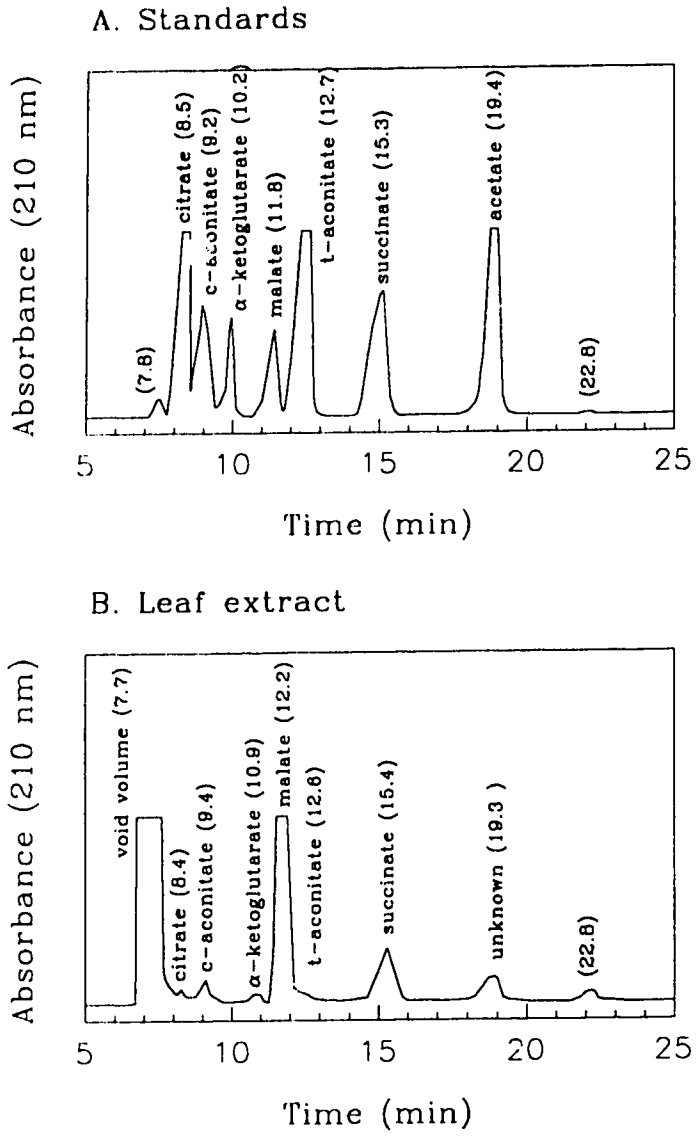

Figure 5.3. HPLC separation of organic acids in (A) a mixture of standards and (B) a typical extract of Mn-tolerant 'Norquay' leaves grown in control solution culture. Retention times are indicated in parentheses.

However, evaporation of leaf extracts to dryness reduced amounts of the unknown organic acid by 92%, whereas the acetate peak was eliminated from a mixture of standards which had been dried. In addition, if the unknown acid was acetate, I would have expected a specific radioactivity approximately 100 times higher than was observed (assuming that the radiolabelled acetate was in equilibrium with the plant's pool of acetate). I have calculated the concentration of the unknown acid as if it were acetate for comparative purposes, but until an independent analysis confirms its identity I prefer to refer to it as an unknown. It is interesting to note that Krotz *et al.* (1989) found an organic acid which co-eluted with acetate in *Nicotiana tabacum* and that both Scott *et al.* (1991) and Foy *et al.* (1990) reported high concentrations of acetate in roots and leaves of wheat, perhaps reflecting a compound similar to that reported here.

Concentrations of each of the organic acids detected responded differently to concentrations of Mn in the growth solutions and to length of time grown with excess Mn. Different responses were also observed between leaves and roots and between cultivars. An ANOVA for concentrations of organic acids in roots indicated a significant main effect of cultivar for the unknown acid and significant main effects of Mn treatment and time for α-ketoglutarate and succinate. Cultivar by manganese, and cultivar by time interaction effects were not significant. Concentrations of the unknown acid were higher in roots of Mn-tolerant 'Norquay' at all concentrations of Mn in solution, accounting for the significant cultivar effect (Fig. 5.4D). Concentrations of aconitate and the unknown acid did not vary with concentration of Mn in solution or with time (Fig. 5.4A and D). Concentrations of α-ketoglutarate and succinate did not vary with cultivar, but did decline with time and concentration of Mn in solution (Fig. 5.4B and C). Concentrations of malate did not vary with cultivar, time or treatment, concentrations of citrate were at or below the detection limit of 5 μg $10\mu l^{-1}$ (data not shown).

An ANOVA for concentrations of organic acids in leaves indicated a significant main effect of cultivar for aconitate and succinate, a significant main effect of Mn treatment for aconitate, α-ketoglutarate and succinate, and a significant main effect of time for aconitate, α-ketoglutarate, succinate and the unknown. The cultivar by Mn treatment and cultivar by time interactions were significant for aconitate, α-ketoglutarate and succinate. Concentrations of both aconitate and α-ketoglutarate increased with increasing Mn in solution in Mn-sensitive 'Columbus', but remained constant with time and treatment in Mn-tolerant 'Norquay' (Fig. 5.5A,B). Concentrations of succinate declined in the tolerant cultivar with time but increased with increasing concentration of Mn in solution in the sensitive cultivar (Fig. 5.5C). Concentrations of the unknown acid in leaves declined

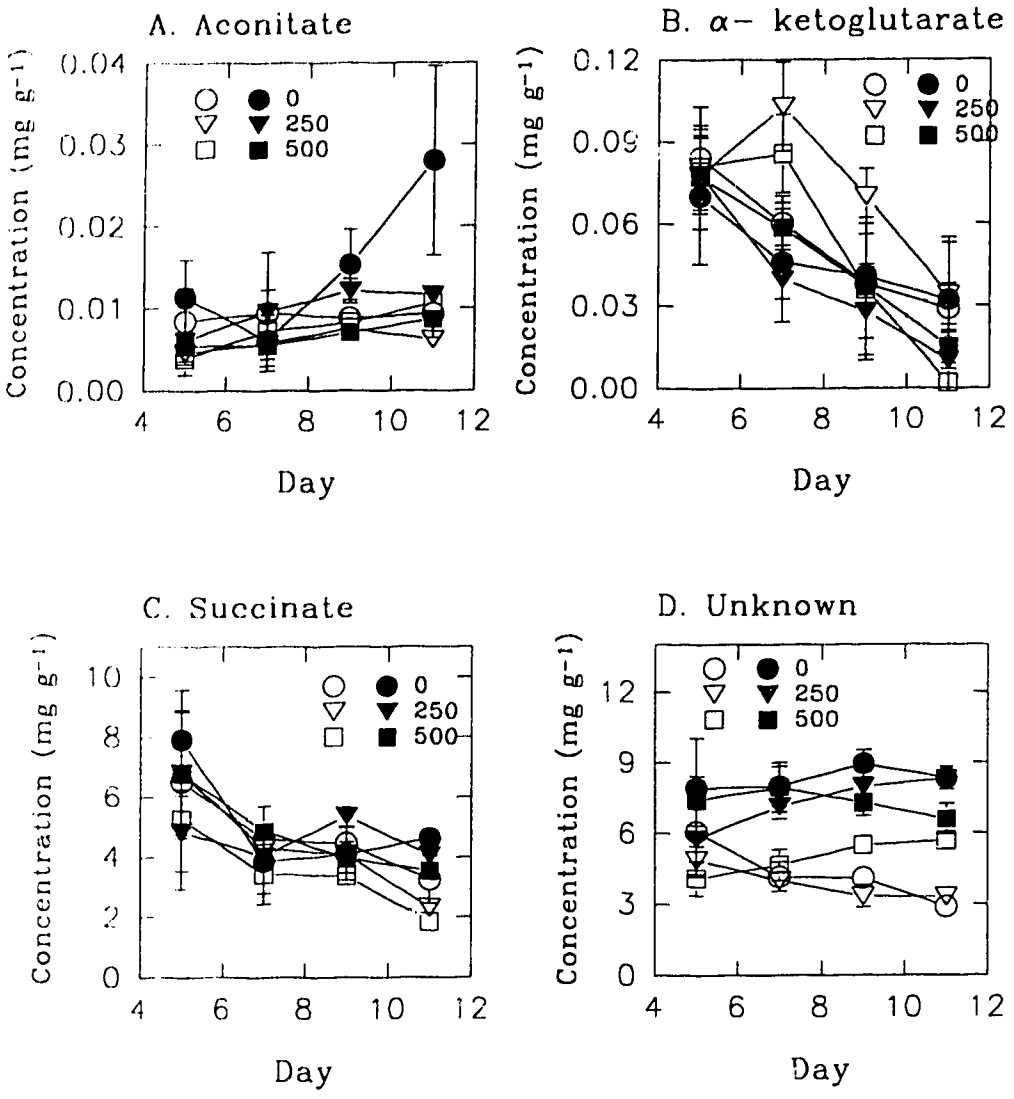

Figure 5.4. Mean concentrations ± s.e. of (A) aconitate, (B) α-ketoglutarate, (C) succinate and (D) unknown organic acid in roots of a Mn-tolerant (filled symbol) and a Mn-sensitive (open symbol) cultivar of *Triticum aestivum* grown with 0, 250 or 500 μM Mn added to solution culture.

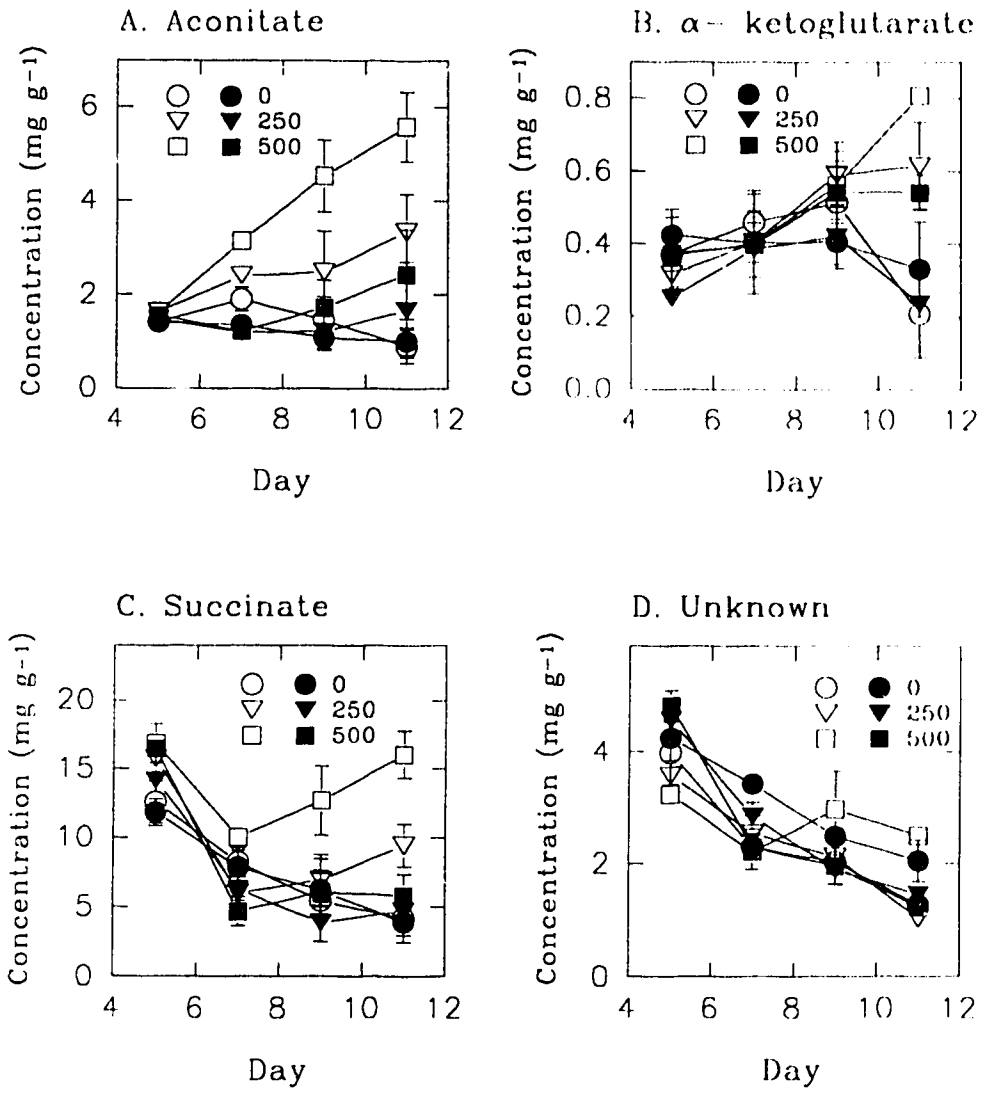

Figure 5.5. Mean concentrations ± s.e. of (A) aconitate, (B) α-ketoglutarate, (C) succinate and (D) unknown organic acid in leaves of a Mn-tolerant (filled symbol) and a Mn-sensitive (open symbol) cultivar of *Triticum aestivum* grown with 0, 250 or 500 μM Mn added to solution culture.

with time in both cultivars and were unaffected by concentration of Mn in solution (Fig. 5.5D). Concentrations of malate did not vary with cultivar, time or treatment, concentrations of citrate were at or below the detection limit of 5 µg 10 µl^{-1} (data not shown).

5.3.3 *Radiolabelling leaf tissue*

Fractionation of ^{14}C-labelled leaf slices into lipids, amino acids, sugars and organic acids indicated some differences between cultivars, Mn treatment and dark/light treatment (Table 5.1). An ANOVA of the proportion of ^{14}C incorporated into the four metabolic pools indicated significant main effects due to cultivar, dark/light treatment and Mn treatment for lipids, amino acids and organic acids. For sugars, main effects due to cultivar and dark/light treatment were significant. The dark/light treatment by Mn treatment interaction was also significant for percent of total ^{14}C in lipids and amino acids. For both cultivars, more ^{14}C was incorporated into lipids than into the other three pools (37-83% of total label, Table 5.1). In all cases, the least amount of radioactivity was incorporated into sugars (0.8-3.8%).

A smaller proportion of ^{14}C was incorporated into lipids when leaf slices were incubated in the dark, with leaves of 'Norquay' incorporating proportionately more ^{14}C in all Mn treatments. This pattern accounted for the significant main effects due to dark/light treatment and cultivar. When incubated in the dark, more ^{14}C was incorporated into lipids of leaves which had either been pretreated with Mn, or had been incubated in the presence of Mn accounting for the significant main effect due to Mn treatment. When incubated in the light, Mn treatment did not affect incorporation of ^{14}C into lipids for either cultivar (Table 5.1). The proportion of ^{14}C incorporated into amino acids declined in leaves incubated in the dark for both Mn treatments ('Norquay' 37.9-7.6%, 'Columbus' 39.8-11.5%), with Mn-treated 'Columbus' having a larger proportion of ^{14}C in the amino acid pool compared to 'Norquay', accounting for the significant main effects (Table 5.1). In addition, the proportion of ^{14}C incorporated into glutamate and aspartate decreased in leaves incubated with excess Mn (data not shown). The proportion of ^{14}C incorporated into sugars did not vary with Mn treatment; however, it was higher for leaves incubated in the light and was higher in 'Columbus' than in 'Norquay'. The proportion of ^{14}C incorporated into organic acids was higher for 'Columbus' leaves incubated in the dark. Treatment with Mn resulted in less incorporation of ^{14}C into organic acids in leaves of 'Norquay' incubated in the light.

The organic acids measured in leaf slices, in order of increasing abundance, were succinate, citrate, and malate (Table 5.2). If the unknown is acetate, it would rank third in abundance.

Table 5.1. Incorporation of ^{14}C (percent of total ± s.e.) into four metabolic pools for Mn-tolerant 'Norquay' and Mn-sensitive 'Columbus' incubated in the dark or the light.

Dark	Cultivar	Treatment	Lipids	Amino acids	Sugars	Organic acids
	Norquay	control[a]	47.3±4.5	37.9±3.3	1.2±0.3	13.6±1.9
		500 µM Mn[b]	72.5±8.2	7.8±1.7	0.8±0.06	19.0±6.5
		control + Mn[c]	82.9±2.0	7.6±0.9	1.7±0.01	12.0±1.0
	Columbus	control	36.7±7.5	39.8±5.0	1.8±0.3	21.8±2.5
		500 µM Mn	56.6±1.6	14.7±1.6	2.1±0.8	26.6±3.6
		control + Mn	61.8±6.2	11.5±2.8	1.1±0.3	25.5±3.0
Light	Norquay	control	71.9±3.2	7.8±2.6	3.3±0.9	17.1±1.6
		500 µM Mn	78.7±3.7	7.8±1.4	2.0±0.4	11.5±2.0
		control + Mn	76.1±3.3	10.9±1.0	1.5±0.2	11.5±2.1
	Columbus	control	68.9±0.9	12.9±1.1	3.8±0.2	14.4±0.4
		500 µM Mn	72.0±6.9	10.8±1.1	3.8±1.0	13.5±4.8
		control + Mn	69.6±2.6	14.8±1.9	2.3±0.2	13.4±1.0

[a] plants grown in control solution, incubated in control solution

[b] plants grown with 500 µM Mn, incubated in control solution

[c] plants grown in control solution, incubated with 500 µM Mn

Table 5.2. Mean concentration (μg g^{-1} fresh weight) ± s.e. of four organic acids in leaves of Mn-tolerant 'Norquay' and Mn-sensitive 'Norquay' incubated in the dark or the light.

Dark	Cultivar	Treatment	Citrate	Malate	Succinate	Unknown
	Norquay	control[a]	23.2±0.5	829.7±21.8	0.015±0.001	524.5±20.5
		500 μM Mn[b]	20.0±2.7	989.2± 3.7	0.029±0.009	276±12.6
		control + Mn[c]	21.2±6.4	779 .4	0.012±0.002	209.0±7.5
	Columbus	control	35.2±1.5	.1±12.3	0.019±0.001	826.5±85.9
		500 μM Mn	30.9±6.4	1722±59.6	0.053±0.025	112.8±69.9
		control + Mn	35.4±10.4	816.4±92.6	0.024±0.009	138.9±22.5
Light	Norquay	control	42.5±8.2	670.1±15.6	0.020±0.006	372.9±44.2
		500 μM Mn	53.8±10.7	1015.2±88.8	0.045±0.004	127.5±17.0
		control + Mn	42.8±5.4	635.9±80.4	0.017±0.006	88.3±25.5
	Columbus	control	44.0±14.7	938.4±38.7	0.027±0.008	120.4±5.8
		500 μM Mn	45.5±9.6	1554.9±67.3	0.056±0.015	120.5±23.4
		control + Mn	33.7±6.3	880.2±14.5	0.025±0.007	107.8±8.6

[a] plants grown in control solution, incubated in control solution

[b] plants grown with 500 μM Mn, incubated in control solution

[c] plants grown in control solution, incubated with 500 μM Mn

Concentrations of citrate were unaffected by Mn treatment, but were higher in leaves of 'Norquay' incubated in the light. Higher concentrations of malate and succinate were found in leaves which had been pretreated with Mn (119-191% of control), but concentrations in leaves incubated in Mn did not differ from control. Concentrations of the unknown acid declined with Mn treatment for leaves of 'Norquay' under both light conditions (53- 34% of control), and for leaves of 'Columbus' incubated in the dark (14% of control, Table 5.2).

The specific radioactivities of citrate, malate and succinate declined with Mn treatment for both cultivars incubated in the light and the dark (Table 5.3). The specific radioactivities of citrate and malate were higher for 'Norquay' when incubated in the light. The specific radioactivity of succinate was higher for 'Columbus' when incubated in the light. In contrast, the specific radioactivity for the unknown acid increased in response to Mn treatment for 'Norquay' under both light conditions and for 'Columbus' incubated in the dark.

5.4 Discussion

Based on fresh tissue weight (Fig. 5.1) and rates of photosynthesis and respiration (Fig. 5.2), 'Norquay' was demonstrated to be Mn-tolerant and 'Columbus' was demonstrated to be Mn-sensitive over the course of the experimental period. The patterns for increase in fresh weight with time reported here are consistent with previous reports (Macfie and Taylor 1989) in that root weights of Mn-tolerant 'Norquay' grown in excess Mn did not vary from control values until after 11 days of exposure to excess Mn, whereas tissue weights of 'Columbus' responded more quickly to Mn stress. In addition, the tolerant cultivar maintained higher rates of photosynthesis under conditions of Mn stress over the experimental period. This result is consistent with an earlier report (Chapt. 4, Macfie and Taylor Submitted) in which 'Norquay' maintained higher rates of photosynthesis across a range of concentrations of Mn in solution. In this study, the rates of respiration increased in response to Mn stress (Fig. 5.2) confirming the results of Horiguchi and Fukumoto (1987) who reported that respiration rate increased up to 196% of control in *Oryza sativa*, *Zea mays*, *Hordeum vulvare*, *Medicago sativa* and *Phaseolus vulgaris* after 14 days exposure to 583 µM in solution. Manganese is involved in several enzyme-catalyzed reactions and therefore can be expected to affect processes such as respiration (Burnell 1988).

To determine the relationship between tolerance of Mn and organic acid content, concentrations of organic acids in roots and leaves were measured using HPLC techniques. In both cultivars, concentrations of aconitate, α-ketoglutarate and succinate were lower in root tissues than in

Table 5.3. Mean specific activity (Bq nmol^{-1}) ± s.e. of four organic acids in leaves of Mn-tolerant 'Norquay' and Mn-sensitive 'Norquay' incubated in the dark or the light.

Dark	Cultivar	Treatment	Citrate	Malate	Succinate	Unknown
	Norquay	control[a]	10.15±0.20	0.58±0.01	3848.2±56.9	0.015±0.003
		500 µM Mn[b]	4.19±0.03	0.31±0.04	1898.9±67.2	0.070±0.012
		control + Mn[c]	2.32±0.42	0.36±0.08	1305.4±11.6	0.181±0.05
	Columbus	control	18.98±0.36	0.56±0.05	3927.3±130.2	0.053±0.012
		500 µM Mn	12.85±0.22	0.42±0.02	1833.6±57.9	0.200±0.060
		control + Mn	6.61±0.44	0.51±0.04	917.0±110.2	0.265±0.043
Light	Norquay	control	17.24±0.20	1.59±0.02	3763.9±200.4	0.071±0.006
		500 µM Mn	9.17±0.44	0.63±0.01	1958.5±163.2	0.342±0.020
		control + Mn	10.45±0.50	0.77±0.10	2364.0±129.2	0.448±0.011
	Columbus	control	24.44±1.70	1.62±0.11	5955.1±167.3	0.259±0.044
		500 µM Mn	13.66±0.81	0.42±0.03	2393.3±106.9	0.261±0.021
		control + Mn	1.70±0.32	0.39±0.08	1468.5±245.9	0.230±0.041

[a] plants grown in control solution, incubated in control solution

[b] plants grown with 500 µM Mn, incubated in control solution

[c] plants grown in control solution, incubated with 500 µM Mn

leaves (Figs. 5.4 and 5.5). Foy *et al.* (1990) also found lower concentrations of *trans*-aconitate and succinate in roots and Burke *et al.* (1990) found that concentrations of organic acids were, on average, six times lower in roots than in leaves. In my study, concentrations of aconitate in roots were approximately 10 times lower than in leaves. This is in contrast to the results reported by Cambraia *et al.* (1983) who found 3.5 times more *trans*-aconitate in roots of Al-tolerant *Sorghum bicolor*. In this study, only concentrations of α-ketoglutarate and succinate varied in response to concentration of Mn in solution, concentrations of the other organic acids did not respond to Mn treatment. Burke *et al.* (1990) found that concentrations of organic acids in roots of *Triticum aestivum* were unrelated to concentration of Mn in solution. Similarly, up to 300 μM Zn had no effect on concentrations of citrate and malate in roots of Zn-sensitive clones of *Deschampsia caespitosa* (Thurman and Rankin 1982). While both Zn and Cd stimulated citrate and malate levels in roots of Zn-Cd-tolerant *Deschampsia caespitosa*, Co, Cu, Hg and Ni had no effect on organic acid concentrations (Thurman and Rankin 1982).

In leaves, concentrations of aconitate, α-ketoglutarate and succinate increased in Mn-sensitive 'Columbus' grown in excess Mn by the end of the experimental period (Fig. 5.5). In Mn-tolerant 'Norquay', concentrations of only aconitate and α-ketoglutarate increased in response to Mn stress. In contrast to many previous reports on different plant species, higher concentrations of organic acids in the Mn-sensitive cultivar were measured in this study. In comparing Al-sensitive and Al-tolerant cultivars of *Zea mays*, Suhayda and Haug (1986) measured 1.5 times higher concentrations of malate and *trans*-aconitate in the tolerant cultivar when under Al stress. Similarly, Cambraia *et al.* (1983) found a 55% increase in *trans*-aconitate and malate in Al-tolerant *Sorghum bicolor* in response to Al. Foy and Lee (1987) found that concentrations of malate increased with concentration of Al in solution in Al-sensitive and Al-tolerant cultivars of *Hordeum vulgare*, whereas concentrations of citrate and succinate declined in the sensitive cultivar but were unchanged in the tolerant cultivar. Interestingly, my results agree with other reports of the effects of metal stress on organic acid concentrations in wheat. Burke *et al.* (1990) found that concentrations of aconitate, malate and citrate increased (14, 2.8 and 3.4 fold respectively) with increasing Mn in solution in Mn-sensitive cultivars of wheat but did not change in Mn-tolerant cultivars. Similarly, Scott *et al.* (1991) found that concentrations of *cis*-aconitate, malate, succinate and acetate increased more in an Al-sensitive cultivar than in an Al-tolerant cultivar when grown in excess Al.

The observed increases in concentrations of organic acids in Mn-sensitive cultivars, coupled with lesser or no increases in Mn-tolerant cultivars, indicates that the accumulation of organic acids is not a primary mechanism of Mn-tolerance in wheat. A similar conclusion has been reached by other

workers. For example, Thurman and Rankin (1982) suggested that organic acid metabolism does not play a role in Zn-tolerance *Deschampsia caespitosa*. Similarly, Foy *et al.* (1990) concluded that differential tolerance of Al in wheat was not correlated with changes in concentrations of organic acids in either roots or leaves. They suggested that observed differences in organic acid concentrations are a result, not a cause, of differential tolerance of Al. If this is the case, it could explain discrepancies between studies with respect to which organic acids increase in tolerant cultivars as well as the observations in wheat in which higher concentrations are found in sensitive cultivars.

The relative proportion of ^{14}C incorporated into lipids, sugars, amino acids and organic acids should give some indication of the metabolic activity of the leaf slices with and without Mn stress and aid in characterization of the Mn stress response. In this study, proportionately more ^{14}C was incorporated into lipids and proportionately less ^{14}C was incorporated into amino acids (specifically glutamate and aspartate) in leaf slices incubated in the dark with [2-^{14}C]sodium acetate and excess Mn (Table 5.1). This indicates that the normal synthesis of amino acids *via* α-ketoglutarate in the citric acid cycle was probably inhibited by Mn toxicity, and that more ^{14}C was diverted to lipid synthesis. Incubation in the light lessened these responses to Mn stress, possibly indicating that sufficient sugars were available to the citric acid cycle when photosynthesis was operating. Use of percent incorporation of ^{14}C into the various metabolic pools provides information on synthesis of the constituents only, it would also be interesting to look at the absolute concentrations of these components in response to Mn stress in order to detect changes in response to the breakdown or degradation of various metabolic products. In roots of *Sorghum bicolor*, concentrations of sugars, amino acids and organic acids increased after treatment with Al (Cambraia *et al.* 1983). Similarly, Rao and Gupta (1979) reported that Mn toxicity in *Saccharum officiale* resulted in higher concentrations of amino acids. Unfortunately, concentrations of lipids, sugars and amino acids were not measured in this study. The observed decreases in photosynthetic rates and increases in respiration rates (Fig. 5.2), however, would indicate that concentrations of sugars in leaves should have decreased. The observed increases in respiration rates (Fig. 2) indicate that the citric acid cycle was functioning. Indeed, the concentrations of malate, succinate and the unknown organic acid increased (Table 5.2). Interestingly, however, the specific radioactivities of citrate, malate and succinate decreased (Table 5.3), indicating that organic acid synthesis was occurring, but not *via* acetyl CoA feeding into the citric acid cycle. In addition, the relative distribution of radiolabel among citrate, malate, succinate and the unknown did not change with dark/light or Mn treatment, further indicating that C was being diverted into these pools during Mn stress. Again, it is possible that Mn

toxicity resulted in the breakdown of amino acids into glutamate and aspartate which could feed into the citric acid cycle.

Another interesting observation is that Mn treatment affected concentrations of malate and succinate in both cultivars under both light treatments (Table 5.2). In all cases, increases in concentrations of malate and succinate were only observed in leaves from plants which had grown in 500 μM Mn for 11 days. Leaves from plants grown in control solutions which were subsequently radiolabelled in the presence of excess Mn did not show increases in concentrations of malate or succinate. These results indicate that changes in organic acid concentrations are a whole-plant phenomenon, or that there is a time lag between exposure to excess Mn and changes in organic acid content. If the latter is true, then this is further evidence that changes in organic acid content reflect a secondary response to Mn toxicity.

An increase in the cellular concentration of any metabolite can be the result of either increased production, or decreased utilization by enzymatic reactions for which it is a substrate or product. While both explanations are possible, Burke *et al.* (1990) suggested that metabolite accumulation is almost always due to decreased activity of a single enzyme for which the metabolite serves as a substrate. Based on my results, it seems unlikely that the activity of a single citric acid cycle enzyme was affected by Mn resulting in increased organic acid synthesis. Had this been the case, the specific radioactivity of one or more of the organic acids would have increased. The increase in specific radioactivity for the unknown organic acid may indicate that it alone was synthesized in response to Mn stress. Interestingly, Krotz *et al.* (1989) reported biosynthesis of an organic acid which co-eluted with acetate in *Nicotiana tabacum* suspension cells grown with excess Cd and Zn. Further work is needed to positively identify this constituent.

Based on the results of this study, it is clear that concentrations of some organic acids increase in response to Mn stress; however, the increases were greater for the Mn-sensitive cultivar making it unlikely that organic acid synthesis acts as a mechanism of Mn-tolerance. Furthermore, increases in concentrations of organic acids did not occur *via* the normal functioning of the citric acid cycle (*eg.* not *via* acetyl CoA) since the specific radioactivities of these intermediates decreased. One possible interpretation is that sugars were not available for degradation in Mn-stressed plants as a result of inhibition of photosynthetic rate. While the derivation of stress-induced organic acids were not tested in this study, the data are consistent with the breakdown of amino acids providing α-ketoglutarate which could feed into the citric acid cycle.

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6. General discussion

When I started my investigations, information on differential tolerance of Mn in Canadian cultivars of wheat was lacking, and studies on the physiology of Mn toxicity were few. The results presented in this dissertation provide valuable information with respect to Mn toxicity in wheat. In addition, my experiments have generated several pathways for future research.

Reflecting the lack of published information on Mn, my first task was to identify the range of tolerance of Mn within Canadian wheat germplasm. Thirty cultivars of wheat were screened for tolerance of Mn using conventional solution culture techniques (Chapter 2, Macfie *et al.* 1989). While the Canadian germplasm was predominantly sensitive of Mn, the range of tolerance measured was as broad as that reported for tolerance of Al (Briggs *et al.* 1989). This indicates that Mn toxicity may be as important as Al toxicity for plants growing on acid soils. Furthermore, the selection of cultivars which had been previously tested for tolerance of Al permitted investigation of the relationship between tolerances of the two metals. Contrary to previous reports, a positive but weak correlation between tolerance of Mn and tolerance of Al was found. It is important to consider tolerance of both metals when breeding for tolerance of acid soils since both Al and Mn toxicities are related to soil acidity. Identification of Al/Mn-tolerant germplasm should provide plant breeders with important information for use in developing lines better suited to areas with acid soil.

Based on the results of Chapter 2 (Macfie *et al.* 1989), two cultivars were chosen to investigate the physiology of differential tolerance of Mn; Mn-tolerant 'Norquay' and Mn-sensitive 'Columbus'. Ideally, investigations into differential tolerance should involve cultivars which are very closely related, differing only in the tolerance gene(s). By using such isolines, physiological responses to specific stresses can be more clearly attributed to tolerance mechanisms. A backcross program designed to introduce tolerance of Mn into Canadian Western Red Spring (CWRS) cultivars is now underway in the Department of Plant Science at the University of Alberta (Dr. Keith Briggs, pers. comm.). Unfortunately, isogenic germplasm is not yet available. One avenue for future work would be to continue studies on differential tolerance on these isolines once they become available. As a follow-up to my screening experiment, Moroni (1991) investigated the probable origin of tolerance of Mn in Canadian wheat germplasm. His results are interesting because it would appear that tolerance of Mn can be traced back to two Brazilian land races, 'Polyssu' and/or 'Alfredo Chavez 6.21'.

In looking for potential mechanisms of tolerance of Mn, I started with an investigation into exclusion of Mn through plant-induced changes in the rhizosphere (Chapter 3, Macfie and Taylor 1989). It would appear that precipitation of Mn in the apoplasm or cell wall did occur, and that plant-induced changes in pH of nutrient solution controlled precipitation of Mn onto root surfaces. Such precipitation; however, did not confer tolerance of Mn. Based on these results, and the fact that Mn readily crosses the plasma membrane (Graham 1979), it would seem that internal mechanisms are more important than external mechanisms in determining tolerance of Mn in wheat. Indeed, concentrations of Mn in tissues of Mn-tolerant cultivars are often higher than those of Mn-sensitive cultivars (Scott and Fisher 1989, Foy *et al.* 1988). The relationships which I observed between concentrations of Mn in leaves, photosynthetic rates and concentrations of chlorophyll supported this view, and indicated important differences between Mn-tolerant 'Norquay' and Mn-sensitive 'Columbus' (Chapter 4, Macfie and Taylor Submitted). In both cultivars, photosynthetic rates and concentrations of chlorophyll declined in response to Mn toxicity; however, the tolerant cultivar maintained higher photosynthetic rates and concentrations of chlorophyll than the sensitive cultivar. Examination of photosynthetic rate per unit chlorophyll indicated that the cultivars differed in their response to Mn stress. The primary toxic effect of Mn in the tolerant cultivar appeared to be a reduction in chlorophyll content which, in turn, resulted in a lowered rate of photosynthesis. In contrast, Mn seemed to exert its toxic effect in the sensitive cultivar on photosynthesis and chlorophyll content independently. While this information does not identify the Mn-tolerance mechanism, some interesting questions arise from these results. It is worth investigating the effects of Mn on photosynthesis in more detail to test (i) whether the activities of photosynthetic enzymes in the tolerant cultivar increase in response to Mn and (ii) whether Mn is compartmentalized in a location removed from photosynthetic activity. The response of chlorophyll to Mn is [illegible]. Manganese is known to affect both chlorophyll synthesis (Wilkinson and Ohki 1988) and degradation (Horiguchi 1988). It would be interesting to know whether Mn affects chlorophyll synthesis and/or degradation differently in the two cultivars. In my opinion, closer examination of the effects of Mn on photosynthesis and chlorophyll content provides the most promising avenue for identification of the mechanism(s) of Mn-tolerance.

The dramatic effects of excess Mn on photosynthetic and respiratory rates led me to investigate the relationship between organic acid content and tolerance of excess Mn (Chapter 5). In roots, concentrations of aconitate, α-ketoglutarate, citrate, malate and succinate did not differ between Mn-tolerant and Mn-sensitive cultivars, with or without excess Mn. In leaves, growth in excess Mn resulted in increases in concentrations of aconitate, α-ketoglutarate, and succinate in the Mn-sensitive cultivar only. Increased concentrations of organic acids has been associated with

tolerance of certain metals (for example Al and Zn) in many plant species. One must be careful, however, in ascribing such changes in concentrations of organic acids to a metal tolerance mechanism. they may simply reflect a secondary response to metal toxicity. Indeed, if changes in organic acid content reflect toxicity rather than tolerance, it would explain the diversity of responses seen in organic acid concentrations in different plant species exposed to a variety of metal stresses (see for example, Burke *et al.* 1990, Foy *et al.* 1990, Scott *et al.* 1987, Klimashevski and Chernesheva 1980, Mathys 1980, Jones 1961). To my knowledge, this is only the second study to use radiolabelled material to test whether organic acids are actually synthesized in response to metal stress. Krotz *et al.* (1988) found that neither Cd nor Zn stimulated citrate or malate biosynthesis in *Nicotiana tabacum* suspension cells. In this study, the specific radioactivities of citrate, malate and succinate declined in response to excess Mn yet remained in equilibrium with each other, indicating that these organic acids were not derived from acetyl CoA feeding into the citric acid cycle. Interestingly, both Krotz *et al.* (1988) and I reported an increase in the specific radioactivity of a compound which coelutes with acetate (using HPLC) in response to metal stress. An obvious follow-up experiment would be to use a technique other than HPLC (such as nuclear magnetic resonance, gas chromatography or mass spectrometry) to identify this compound.

The results presented in Chapter 5 indicated that biosynthesis of organic acids is not a primary mechanism of Mn-tolerance in wheat, indeed synthesis of organic acids through the citric acid cycle is probably inhibited by Mn toxicity. Observed increases in concentrations of organic acids could have arisen from the breakdown of amino acids, providing α-ketoglutarate which would feed into the citric acid cycle. Clearly, organic acid content is altered by metal stress, but the biochemistry of this response remains unclear. An interesting direction for future research would be to use a radioactive label to quantify the responses of specific amino acids (such as aspartate and glutamate) to Mn toxicity.

In closing, I would like to acknowledge that the studies reported in this dissertation involve a single phytotoxic metal and a single species, and that the results may not necessarily be extrapolated to the toxicity of other metals or plant species. Nevertheless, I have made progress with respect to potential mechanisms of Mn-tolerance in wheat. The majority of evidence indicates that tolerance of excess Mn occurs within the cytosol and my results indicate that differential tolerance is related to the toxic effects of Mn on chlorophyll content and photosynthetic processes. In addition, it would seem that observed changes in organic acid content are likely a result of Mn toxicity rather than differential tolerance of Mn. These results have generated some interesting ideas for future research which should help to clarify the physiology and biochemistry of the Mn toxic response.

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7. APPENDIX 1. Summary of ANOVA results in Chapter 4.

Variable	Source of variation	Significant at p=0.05?			
		Day 0	Day 5	Day 8	Day 12
Mn in leaf	cultivar	no	yes	yes	yes
	Mn in solution	no	yes	yes	yes
	cultivar x Mn in solution	no	yes	yes	no
Respiration	cultivar	no	yes	no	no
	Mn in solution	no	yes	yes	yes
	cultivar x Mn in solution	no	yes	no	no
Photosynthesis	cultivar	no	yes	yes	yes
	Mn in solution	no	yes	yes	yes
	cultivar x Mn in solution	no	yes	yes	yes
Chlorophyll a	cultivar	no	yes	yes	yes
	Mn in solution	no	yes	yes	yes
	cultivar x Mn in solution	no	yes	no	yes
Chlorophyll b	cultivar	no	yes	yes	yes
	Mn in solution	no	yes	yes	yes
	cultivar x Mn in solution	no	no	no	yes
Chl a:Chl b	cultivar	yes	yes	no	yes
	Mn in solution	no	yes	no	no
	cultivar x Mn in solution	no	no	no	no

8. APPENDIX 2. Mean rates of respiration ± s.e. for Mn-tolerant 'Norquay' and Mn-sensitive 'Columbus' on days 8 and 12 after exposure to a range of concentrations of Mn in solution.

Mean respiratory rate (mg CO_2 gFW^{-1} h^{-1})			
		Days after exposure to Mn	
Cultivar	[Mn]	8	12
Norquay	0	3.7±0.4	2.9±0.4
	100	3.9±0.3	2.5±0.5
	250	2.6±0.6	2.0±0.1
	500	2.9±0.7	2.1±1.2
	750	2.4±0.6	2.0±0.7
	1000	2.2±0.1	2.0±0.5
Columbus	0	3.6±0.5	3.9±1.2
	100	3.2±0.2	3.0±0.4
	250	2.7±0.2	2.9±0.4
	500	2.6±0.1	1.8±0.3
	750	1.6±0.3	1.7±0.2
	1000	1.9±0.7	1.0±0.2