University of Alberta

Aluminum uptake and distribution in roots of *Triticum aestivum* L.

by

Daniel Jean-Paul Joseph Archambault

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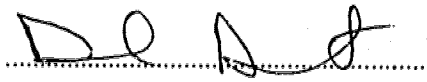

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Aluminum uptake and distribution in roots of *Triticum aestivum* L." submitted by Daniel Jean-Paul Joseph Archambault in partial fulfillment of the requirements for the degree of *Doctor of Philosophy* in *Physiology and Cell Biology.*

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Abstract

A kinetic protocol was developed to characterize the accumulation and distribution of aluminum (Al) in various cellular compartments in roots of wheat (*Triticum aestivum* L.). The kinetics of uptake and desorption were similar when Al was supplied as $AlK(SO_4)_2$ or as $AlCl_3$. When both salts were supplied at low concentration (50 μM), Al bound to a purified cell wall fraction could be reduced to less than 10 - 20% of non-exchangeable Al, if roots were washed for 30 min in citric acid following exposure. In contrast, when $AlK(SO_4)_2$ was supplied at a high concentration (200 μM), a strong linear phase of uptake into cell wall material was observed, which accounted for approximately 48% of non-exchangeable Al in roots. A series of multiple-desorption experiments confirmed that citric acid was effective in removing Al from the cell wall compartment of roots exposed to Al for short periods (3h). However, long exposures (48h) appeared to create conditions conducive to the accumulation of non-exchangeable Al in the cell wall.

While the use of low concentrations of Al in simple salt solutions was effective in limiting accumulation of non-exchangeable Al in the cell wall during short exposure experiments, the rapid phase of uptake was not totally eliminated by desorption in citric acid. This led me to postulate the presence of other non-exchangeable pools of Al in the apoplasm. Because the mucilage is an important sink for cations, a revised kinetic protocol was developed which included removal of root mucilage. By comparing the Al content of root tips with and without the removal of the mucilage, it was estimated that mucilage-bound Al accounted for up to 35% non-exchangeable Al (Al remaining after

desorption in citric acid). Short-term experiments (3 h) showed that Al uptake into mucilage was also biphasic.

By incorporating a step for removal of mucilage into a revised kinetic protocol, a linear phase of uptake, with only slight deviation from linearity in the first five minutes, could be isolated. Using this protocol, the kinetics of Al uptake into various root segments was investigated and uptake in cultivars differing in their ability to resist Al was compared. While the time course of Al accumulation in each portion of the root was biphasic, accumulation of non-exchangeable Al was 33 to 37% greater in root tips than in the mature root regions. The rate of accumulation of non-exchangeable Al in 20 mm root tips did not differ between Al-resistant and Al-sensitive cultivars. However, the rate of Al accumulation in 5 mm root tips was nearly twice as high in an Al-sensitive cultivar than in an Al-resistant cultivar. Furthermore, the rate of Al uptake in the resistant cultivar increased nearly 2-fold in the presence of DNP, while DNP had little effect on uptake in the sensitive cultivar. The effect of DNP was also specific to the root tip tissue. No differences between treatments were observed in mature root portions. While unambiguous interpretation of kinetic data is not possible, these results support the hypothesis that DNP inhibits an Al exclusion mechanism operating at the root tip in Al-resistant cultivars.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. GENERAL INTRODUCTION

1.1 General

Aluminum (Al) toxicity is a major cause of reductions in plant growth and crop productivity on acid soils (Foy *et al.*, 1978). Fortunately, plants differ widely in their resistance to Al, and many plant species or cultivars grow well in the face of Al stress (Foy *et al.*, 1965, 1967; Armiger *et al.*, 1968; Reid *et al.*, 1969; Taylor and Foy, 1985 a,b; Keltjens and van Ulden, 1987; Baligar *et al.*, 1988). Although these plants are clearly resistant to Al, the mechanisms involved in mediating resistance are not well understood. The current literature contains a host of hypotheses about potential Al resistance mechanisms and a distinction has been made between two types of mechanisms based upon the site of Al detoxification or immobilization. Internal resistance mechanisms are defined as cases where metals are detoxified in the cytosol. External resistance mechanisms are those that detoxify metals in the apoplasm or limit the rate of uptake across the plasma membrane (Foy, 1983 a,b; Taylor, 1987, 1988 a,b, 1991). External mechanisms which limit the accumulation of metals in the symplasm have been called exclusion. Exclusion mechanisms might include a pH barrier at the plasma membrane (Taylor and Foy, 1985 a,b), selective permeability of the plasma membrane (Huett and Menary, 1979; Wagatsuma 1983; Zhang and Taylor, 1989), exudation of chelator ligands (Ojima *et al.*, 1984; Delhaize *et al.*, 1993a; Basu *et al.* a,b; Pellet *et al.*, 1995), immobilization of Al in the cell wall (Mugwira and Elgawhary, 1979) or efflux of Al from the symplasm (Zhang and Taylor, 1989, 1991; Lindberg, 1991). If exclusion mechanisms play a role in mediating resistance to Al, the rate of Al accumulation in the symplasm of resistant plants should be measurably lower than in sensitive plants. Clearly, accurate measurements of the distribution of Al within plant tissues and cellular compartments will be essential if we are to gain evidence for exclusion. In a historical perspective, several advancements have been made in the measurement of Al in plant

tissues. The following section summarizes advancements which, in my opinion, represent significant contributions in this endeavor.

1.2 Improvements in the localization and measurement of aluminum

Stains have commonly been used to quantify Al in root tissues. For example, Mugwira *et al.* (1976) and Mugwira and Elgawhary (1979) used Cyanine R to detect Al in roots exposed to Al. Similarly, hematoxylin has been used to stain Al (Wallace *et al.*, 1982). While such studies may yield reasonable measurements of total Al in root tissue, they cannot provide information regarding the localization of Al at the subcellular level, and more importantly between symplastic and apoplastic compartments. Other staining techniques such as hematoxylin, aluminon and morin have been combined with microscopic observations to localize Al within cellular compartments, but do not provide quantitative estimates of Al levels (Galsomies *et al.*, 1992; Tice *et al.*, 1992).

For many elements, radioisotopes can be used in efflux experiments to estimate the relative size of pools of the element of interest. Typically, this is accomplished by exposing plants to the radioisotope and washing them in a highly concentrated solution of the non-radioactive element. As the isotope is replaced by the non-labeled element over a period of several hours, the pattern of isotope efflux is monitored. A semilog plot of the isotope content of plant tissue or the elution solution as a function of time reveals the components of the elution profile, which could be attributed to specific cellular compartments. Estimates of the accumulation of the element in various cellular compartments can thus be computed. However, the lack of a suitable radioisotope of Al which can be purchased and detected at a reasonable cost, has limited the utility of this technique. Fortunately, kinetic influx analysis has the potential to provide an accurate estimate of the rate of Al uptake into the symplasm.

A number of kinetic studies characterizing the uptake of Al over time have provided fundamental information upon which future studies can be based. Zhang and Taylor (1989) showed that Al uptake into roots of Al-resistant and Al-sensitive cultivars of *T. aestivum* consisted of two phases. A rapid, non-linear, initial phase of uptake superimposed over a slower linear phase. Similar uptake patterns were observed by Clarkson (1967) in roots of *Hordeum vulgare*, by Huett and Menary (1979) in *Brassica oleracea*, *Lactuca sativa* and *Pennisetum clandestinum*, by Wagatsuma (1983) in *Cucumis sativus* and by Petterson and Strid (1989) in *T. aestivum*, all using substantially higher concentrations of Al in uptake solutions. While the general patterns of Al uptake were well documented, the identity of the different phases was not clearly ascertained. The rapid phase of uptake has traditionally been interpreted as representing metal uptake into the apoplasm, while the slower linear phase representing uptake into the symplasm (Taylor, 1991), however until recently this interpretation had not been tested for trivalent ions such as Al^{3+}.

Zhang and Taylor (1989) attempted to identify the specific cellular pools which contribute to the two phases of Al uptake. This and subsequent studies (Zhang and Taylor, 1990) demonstrated that neither phase was simply defined. Zhang and Taylor (1989) showed that part of the rapid, non-linear phase could be partially eliminated with a 30 min wash in citric acid to remove loosely bound Al in the apoplasm. This demonstrated the existence of a readily-exchangeable and a non-exchangeable component, both of which were presumably located in the apoplasm. Extrapolation of the linear phase of uptake following desorption to time zero, showed that the non-exchangeable component could account for up to 24% of total remaining Al. Subsequently, Zhang and Taylor (1990) verified that a portion of the Al accumulated in the cell wall *in vivo* could not be desorbed using citric acid. However, this pool of non-exchangeable Al accumulated at a constant rate with time and thus contributed to the

linear phase of uptake. They suggested that the linear phase of uptake included two distinct pools of Al which might reflect uptake across the plasma membrane and a slow, metabolism-dependent binding in the apoplasm.

While the amount of Al contributing to the rapid and linear phases of uptake is relatively easy to ascertain, quantification of Al in specific cellular compartments has been more problematic. While Zhang and Taylor (1990) suggested that 45 to 75 percent of total Al taken up by roots was located within the apoplasm, previous studies by Huett and Menary (1979) and Clarkson (1967) reported much greater values, between 75 and 95 percent. More recently, Hodson and Sangster (1993) used X-ray micro-analysis to observe the accumulation of Al in roots of plants that had been exposed to Al for 6 to 8 days. They noted that Al was virtually absent from the cytosol. Several factors could account for these discrepancies. Differences in the cation exchange capacity of the cell wall may differ between species. Protocols for detecting Al in the apoplasm and symplasm may also differ in sensitivity and reliability. Another possibility is that different experimental conditions such as the concentration of Al and the complexity of uptake solutions could lead to differences in Al speciation and thus, to differences in the adsorption of Al in the apoplasm.

It has recently been suggested that the use of chemically complex uptake solutions might produce conditions conducive to the production of polynuclear or precipitated hydroxy-Al species that could accumulate in the apoplasm (Tice *et al.*, 1992). Given the lack of techniques for speciation of Al at this level of spatial resolution, the effect of experimental conditions on the formation of polynuclear or solid phase Al is largely speculative and unexplored. Nonetheless, several interesting observations can be made regarding the effects of bulk solution speciation on Al uptake. Experiments by Huett and Menary (1979) and Clarkson (1967) were conducted using chemically complex uptake

solutions. Thus, experimental conditions almost certainly favored Al precipitation. Zhang and Taylor (1990) used relatively simple solutions, but supplied Al in the form of $AlK(SO_4)_2$. Formation of polynuclear or precipitated hydroxy-Al species and may have still contributed to the linear phase of uptake that they observed in cell wall material (Tice *et al.*, 1992). Recent experiments using cell suspensions isolated from Al-tolerant and Al-sensitive cultivars of *Phaseolus vulgaris* have demonstrated that experimental conditions can have both quantitative and qualitative impacts on Al uptake (McDonald-Stephens and Taylor, 1995). These authors reported that total accumulation of Al increased with increasing concentrations of Al (up to 1000 μM) in low-volume uptake solutions, but in all cases uptake was dominated by a saturable phase. In contrast, when experiments were performed using large volumes of low concentrations of Al (75 μM), the pattern of uptake became biphasic, suggesting that the pattern of uptake is strongly dependent on Al speciation and that high concentrations of Al lead to high levels of non-exchangeable Al in the cell wall. In order to simplify test solutions to control speciation and the formation of solid phase or polynuclear Al species, it has been suggested that low concentrations of $AlCl_3$ should be used so that experimental solutions would contain high proportions of Al^{3+}, a species reportedly responsible for rhizotoxicity in wheat (Tice *et al.* 1992).

Tice *et al.* (1992) developed a kinetic protocol to operationally define apoplastic and symplastic Al in root tips of *T. aestivum*. This protocol made use of dilute, simple salt solutions containing only $AlCl_3$ (18 and 55 μM) and $CaCl_2$ (1 mM). After exposing roots to 55 μM Al for 2 days, a series of washes in $CaCl_2$ were used to desorb readily exchangeable Al from apoplastic and symplastic compartments. Aluminum remaining in roots after the lengthy desorption treatments (residual Al) was assigned to the symplasm on the basis of a spectrofluorometric analysis with the fluorophore morin. In interpreting their results, Tice *et al.* (1992) suggested that these experimental conditions and

techniques yielded very low levels of Al in the apoplasm. However, they assumed that all Al desorbed prior to cell rupture was part of the apoplastic pool. Zhang and Taylor (1989) suggested that slow desorption of Al (the linear phase of desorption) was a result of elution from the symplasm. Furthermore, it remains possible that Al tightly bound to cell wall material may not form a fluorescent complex with morin, leading Tice *et al.* to underestimate the extent of Al accumulation in the cell wall.

The recent development of highly sensitive secondary ion mass spectrometry (SIMS) techniques has great promise in the localization and quantification of Al within cellular compartments. Lazof *et al.* (1994a), investigated the entry of Al into root sections of *Glycine max.* They found measurable quantities of Al in root tip cells within a 30 min exposure of intact roots to Al, a time frame consistent with the rapid toxic effects of Al. Furthermore, they claimed that most of the Al was found in the cell symplasm when uptake was followed by a 30 min wash in potassium citrate. It should be noted however, that the efficacy of potassium citrate in removing Al from the apoplasm has not been tested in their experimental system, which includes the exposure of roots to complex uptake solutions. Removal of loosely bound Al may not be sufficient to eliminate the possibility of smearing or redistribution of Al during tissue preparation. Furthermore, while the degree of spatial resolution provided by SIMS is exceptional, unequivocal localization of Al in the symplasm has yet to be offered. With this in mind, the secondary ion images which show areas of Al accumulation only in damaged edges of the cryosection provided by Lazof *et al.* are less than convincing.

Given the lack of unambiguous estimates of Al uptake and distribution at the cellular and subcellular levels, evidence for exclusion as the basis for Al resistance is meager. Nonetheless, a few studies have provided indirect, preliminary evidence of such

mechanisms. In the following section, I have provided a summary of evidence for exclusion which I view as valuable contributions to the field.

1.3 Evidence of exclusion

Crude measurements of Al tissue contents have shown that, above-ground tissues of Al-resistant plants of *Medicago sativa* contained less Al than those of Al-sensitive plants (Ouellette and Dessureaux, 1958). While this might suggest that exclusion is occurring in Al-resistant plants, it can also be interpreted as being a difference in translocation from roots to shoots. This pattern, however, has proven to be far from universal. For example, studies with *Glycine max* (Foy *et al.*, 1969), *Hordeum vulgare* (MacLean and Chiasson, 1966; Foy *et al.*, 1967) *Phaseolus vulgaris* (Foy *et al.*, 1974) and *Triticum aestivum* (Foy *et al.*, 1967; Foy *et al.*, 1974; Foy *et al.*, 1982) found no differences between Al-resistant and Al-sensitive plants in the accumulation of Al in above-ground tissues. This lack of a correlation might suggest that Al levels in above-ground tissue may not reflect exclusion at the root level. Alternatively, it might suggest that translocation is not important in Al resistance. Because roots are in direct contact with soluble Al at the root-soil interface, it is more likely that exclusion would occur at this level. Thus, it is more likely that studies which include the measurement of Al levels in roots will yield evidence of exclusion.

Using the Cyanine R method to detect Al, Mugwira *et al.* (1976) and Mugwira and Elgawhary (1979) showed that roots of Al-resistant cultivars of x. Triticosecale and *T. aestivum* accumulated less Al than Al-sensitive cultivars in both short and long exposure periods. Similarly, when hematoxylin was used to stain Al, Wallace *et al.* (1982) found qualitative differences between an Al-sensitive and an Al-resistant cultivar of *T. aestivum*. While 5 mm root apices of sensitive plants stained more intensely than

resistant plants, quantitative colourimetric analyses failed to support these results. Furthermore, recent studies have suggested that selective hematoxylin staining in Al-sensitive wheat cultivars was the result of damage by Al to root cells, leading to the leakage of phosphorus into the apoplasm and the subsequent formation of Al-P-hematoxylin complexes (Ownby, 1993). While such staining techniques are useful in the localization of Al at the tissue level, they are incapable of differentiating between apoplastic and symplastic Al, and therefore provide little evidence of exclusion.

While kinetic studies have the potential for accurate measurement of ion uptake, conflicting results have been reported regarding differences in Al uptake in roots of wheat genotypes that differ in their resistance to Al. Differences between studies may be due to the portion of the root tested. Alternatively, in experiments where total Al levels are measured, contradictory results may arise as a result of differences in the distribution of Al between apoplastic and symplastic compartments. Most commonly, differences in Al content of roots between Al-resistant and Al-sensitive plants have been found in root tips. Rincon and Gonzales (1992) showed major differences in concentrations of Al in roots of Al-sensitive and Al-resistant cultivars. Aluminum concentrations were 9 times greater in the 0-2 mm root portions of an Al-sensitive cultivar than in an Al-resistant cultivar. Also working with short root portions, Delhaize *et al.*(1993b) reported that Al-sensitive genotypes accumulated more Al in 2-3 mm root apices than did Al-resistant genotypes after a 4 h exposure to 100 μM Al in nutrient solution. These differences became more marked with time (up to 16 h). In both studies, total Al was measured and no distinction was made between apoplastic and symplastic pools. In the case of Delhaize *et al.*, their results also indicated that the sensitive genotype grew less than the resistant genotype in the experimental period. It is perhaps not surprising that in long exposure experiments, a root that grows little takes up more Al in the root tip than does a root that grows

considerably more. Root growth rates should be taken into account in studies where significant growth might occur.

Experimental support for exclusion also comes from studies which have provided putative estimates of symplastic Al levels. Tice *et al.* (1992) showed that an Al-resistant cultivar accumulated less Al in their putative symplastic fraction than an Al-sensitive cultivar. As mentioned in the previous section, however, differences between genotypes hinge on the putative assignment of Al in the "residual" fraction to the symplasm. Using SIMS, Lazof *et al.* (1994b) also demonstrated greater Al accumulation in all regions of roots of an Al-sensitive cultivar of *Glycine max* than in an Al-resistant cultivar, although the validity of their symplastic assignment of Al also remains in doubt.

Taken together, the results of the studies described above provide preliminary evidence of the operation of Al exclusion mechanisms in resistant plants. Additional support for the existence of such mechanisms comes from studies using metabolic inhibitors. Zhang and Taylor (1991) showed that the biological inhibitor 2,4-dinitrophenol (DNP; which uncouples oxidative phosphorylation and acts as a protonophore), increased the rate of Al uptake in roots of Al-resistant cultivars of *T. aestivum*, but had little effect on uptake in sensitive cultivars. They suggested that DNP might disrupt a metabolism-dependent exclusion mechanism. While the effects of DNP suggest that metabolic exclusion may be occurring in resistant cultivars, this has not been unambiguously demonstrated. The effects of DNP could reflect changes in Al binding in the apoplasm. Zhang and Taylor (1991) showed that DNP caused an increase in Al binding in the cell wall fraction, although the extent of increase was the same in both Al-resistant and Al-sensitive cultivars. These results suggest that the observed effects of DNP reflected the effects of DNP on uptake across the plasma membrane. Rincon and Gonzales (1992) demonstrated that cyanide m-chlorophenylhydrazone (CCCP), an

inhibitor of oxidative phosphorylation, and cycloheximide, a protein synthesis inhibitor, increased Al-uptake by intact roots of an Al-resistant cultivar of *T. aestivum*. They suggested that CCCP induced both Al binding to the cell wall, and Al uptake across the membrane due to depolarization of the membrane leading to the opening of channels. They also showed that cycloheximide inhibited the release of Al from root tips exposed to nutrient solution, but not from mature root regions. This suggests that exclusion may be active only at the root apex. If the effects of DNP reported by Zhang and Taylor (1991) reflect the operation of exclusion mechanisms, the effects of DNP should be more pronounced at the root apex.

To obtain further evidence for exclusion, we require accurate estimates of the rate of Al uptake into the symplasm. I believe that such measurements can be achieved through improvements in existing kinetic protocols which eliminate conditions which are conducive to the accumulation of non-exchangeable Al in the apoplasm and which make use of regions of the root tip that are most likely to exhibit differences in Al uptake between Al-resistant and Al-sensitive cultivars. In order to achieve this goal, specific objectives listed in the following section must be met.

1.4 Objectives

While the idea that exclusion mechanisms may be responsible for Al resistance has gained acceptance throughout the scientific community, experimental evidence to support this idea is far from complete. Barriers such as the lack of accurate measurements of the rate of uptake of Al into the symplasm and contradictory evidence observed in the literature have molded the objectives of this study. Specific objectives were to:

1) Compare the patterns of Al uptake and distribution using different Al salts.

2) Compare the efficiency of $CaCl_2$ and citric acid as desorbing agents.

3) Determine if the kinetics of Al uptake differ between young meristematic tissue and more mature tissue.

4) Measure Al uptake into the mucilage, to quantify the contribution of mucilage-bound Al to total uptake and to eliminate the mucilage as an apoplastic pool of Al.

5) Examine whether differences in the rates of Al uptake into the symplasm occur between plants that differ in their abilities to resist Al.

6) Use metabolism modifiers to characterize putative uptake and exclusion mechanisms.

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2. A comparison of the kinetics of aluminum (Al) uptake and distribution in roots of wheat (*Triticum aestivum* L.) using different aluminum sources. A revision of the operational definition of symplastic Al.

2.1 Introduction

Our understanding of the physiological and biochemical bases of aluminum (Al) tolerance and toxicity is currently limited by a lack of information about the dynamics of Al uptake in plants and its distribution within various cellular compartments. The lack of information about the movement of Al across the plasma membrane presents a significant barrier to the elucidation of tolerance mechanisms that may involve exclusion of Al from the symplast (Taylor, 1995). It has been proposed that Al crosses the plasma membrane with phospholipids serving as negatively charged carriers in the process of endocytosis, with organic chelators as neutral carriers, or through hydrophilic pores or protein channels (Haug, 1984). Such proposed transport mechanisms can only be viewed as speculative. In fact, transmembrane transport of Al itself has yet to be unequivocally demonstrated. While Al has tentatively been located within the symplasm using fluorescence microscopy (Tice *et al.*, 1992) and secondary ion mass spectrometry (Lazof *et al.*, 1994), such studies require physical sectioning of plant tissues and fail to provide estimates of the rate of membrane transport. Short-term kinetic studies involving the characterization of Al uptake over time have demonstrated the existence of a linear phase of uptake, which may include uptake of Al across the plasma membrane (Zhang and Taylor, 1989). However, the precise identity of this phase has not been ascertained and contributions from sources such as the cell wall, have been proposed (Zhang and Taylor, 1990).

The current literature contains conflicting reports regarding the localization of Al within root cells following exposure to Al. Discrepancies could reflect inherent problems in methods used. For example, Zhang and Taylor (1990), demonstrated that apoplastic Al accounted for less than 50% of total uptake in various cultivars of *Triticum aestivum*, a value well below the 75 to 95% of total Al reported to be associated with the cell wall in roots of *Hordeum vulgare, Brassica oleracea, Lactuca sativum, and Pennisetum clandestinium* (Clarkson, 1967; Huett and Menary, 1979). Quantitative differences between these experiments could reflect concentrations of Al in uptake solutions (μM versus mM), the precipitation of Al phosphate compounds, or the formation of insoluble polynuclear Al species in the apoplasm when Al is supplied at ion activity ratios ($\{Al^{3+}\}/\{H^{+}\}^3$) in excess of $10^{8.8}$, a value which appears to represent the threshold for onset of polynucleation and/or precipitation reactions in bulk solutions (Kinraide and Parker, 1989). Even in Zhang and Taylor's (1990) experiments, where Al was supplied as 75 μM $AlK(SO_4)_2$ at pH 4.5 ($\{Al^{3+}\}/\{H^{+}\}^3 = 10^{8.76}$, marginally lower than the critical value), precipitation or polymerization of Al at the plasma membrane-cell wall interface could be responsible for an apparent metabolism-dependent uptake in the cell wall (Tice *et al.*, 1992).

Recent experiments using cell suspensions isolated from Al-tolerant and Al-sensitive cultivars of *Phaseolus vulgaris* have demonstrated that experimental conditions can have both quantitative and qualitative impacts on Al uptake (McDonald-Stephens and Taylor, 1995). These authors reported that total accumulation of Al increased with increasing concentrations of Al (up to 1000 μM) in low-volume uptake solutions, but in all cases uptake was dominated by a saturable phase. In contrast, when experiments were performed using large volumes of low concentrations of Al (75 μM), the pattern of uptake became biphasic, suggesting that the pattern of uptake is strongly dependent on Al

speciation. Careful control of experimental conditions would appear to be essential for accurate estimates of transport across the plasma membrane.

In an attempt to operationally define apoplastic and symplastic Al in root tips of *T. aestivum*, Tice *et al.* (1992) developed a protocol making use of dilute, simple salt solutions containing only $AlCl_3$ and $CaCl_2$. These authors argued that use of $AlCl_3$ in uptake solutions containing 1.5 mM $CaCl_2$ at pH 4.3 (where $\{Al^{3+}\}/\{H^+\}^3 = 10^{7.78}$ and $10^{8.26}$ for 18 μM and 55 μM respectively) reduces the possibility of producing conditions at the plasma membrane which are conducive to the precipitation of solid phase Al. After exposing roots to 18, or 55 μM Al for 2 days, a series of washes in 1.5 mM $CaCl_2$ were used to desorb readily exchangeable Al from apoplastic and symplastic compartments. Aluminum remaining in roots after the lengthy desorption treatments (residual Al) accounted for approximately 40 to 60 % of total Al, and was assigned to the symplasm on the basis of spectrofluorometric analysis with the fluorophore morin. At first sight, these results contradict the results reported by Zhang and Taylor (1990), who observed substantive, non-exchangeable, metabolism-dependent accumulation of Al in the cell wall. Unfortunately, information about the specificity of morin for Al complexes with organic ligands and more specifically to cell wall functional groups is lacking. It remains possible that Al tightly bound to cell wall material may not form a fluorescent complex with the fluorophore morin, leading Tice *et al.* to underestimate the extent of Al accumulation within the cell wall.

A review of the current literature might lead to the conclusion that the distribution of Al within the cell apoplasm and symplasm is largely dependent on the uptake solutions used; with dilute, simple salt solutions allowing for greater accumulation of Al in the symplasm while facilitating the desorption process. More specifically, Tice *et al.* (1992) hypothesized that the accumulation of non-exchangeable Al in the apoplasm might be

attributable to the Al-salt used. It also follows that differences in the concentration and/or activity of Al in uptake solutions could affect the distribution of Al between cellular compartments. In order to test these hypotheses the objectives of the present study were (1) to determine whether the patterns of Al uptake observed by Zhang and Taylor (1989) are dependent on the Al salt used (Tice *et al.*, 1992) or on concentration; (2) to determine whether metabolism-dependent accumulation of Al in the cell wall can be minimized or eliminated by changes in experimental conditions; (3) to test the efficiency of $CaCl_2$ and citric acid as desorption agents using graphite furnace atomic absorption spectrophotometry (GFAAS) to directly measure Al accumulation in the cell wall; and (4) provide more information about the identity of the linear phase of Al uptake.

2.2 Materials and methods

2.2.1 Plant Material

To prepare plants for experimentation, seeds of an Al-tolerant cultivar (PT 741) and/or an Al-sensitive cultivar (Neepawa) were surface sterilized for 20 min in 1.2% sodium hypochlorite and germinated for 24 h in Vitavax (0.005 g/L). Seedlings were placed on nylon mesh in aquaria containing a full nutrient solution (Zhang and Taylor, 1989), and grown for 6 d in a growth chamber with 16 h of light (20°C, 68% relative humidity) and 8 h of darkness (16°C, 85% relative humidity). The photosynthetic photon flux density (PPFD) was 335 $\pm$ 12 μmol m^{-2} s^{-1} at plant base level.

2.2.2 Uptake experiments

Short-term uptake experiments were performed to examine the possible effect of different sources of Al ($AlCl_3 \cdot 6H_2O$, $AlK(SO_4)_2 \cdot 12H_2O$) on the kinetics of Al uptake. To prepare for individual experiments, thirty root tips (2 cm) were excised, placed into each of 55 absorption tubes, and submerged in an aerated nutrient solution until excision was complete (<60 min). Roots were then allowed to equilibrate for 30 min in 1.0 mM $CaCl_2$ (pH 4.5 at room temperature). Uptake experiments were initiated by transferring absorption tubes into uptake solutions containing 50 μM $AlCl_3$ in 1.0 mM $CaCl_2$, 50 μM $AlK(SO_4)_2$ in 1.0 mM $CaSO_4$, or 200 μM $AlK(SO_4)_2$ in 1.0 mM $CaSO_4$. All experiments were conducted at pH 4.5 and 23°C. In some experiments, uptake was followed by a 30 min desorption in 0.5 mM citric acid at pH 4.5 and 0°C. Roots were then dried in an oven at 55°C, weighed, ashed in a muffle furnace at 500°C, dissolved in concentrated HNO_3, diluted in deionized, distilled water and analyzed for Al using GFAAS as described by Zhang and Taylor (1989).

2.2.3 Desorption experiments

Short-term desorption experiments were performed to examine the possible effect of different sources of Al ($AlCl_3 \cdot 6H_2O$, $AlK(SO_4)_2 \cdot 12H_2O$) on the kinetic pattern of Al desorption. Roots were prepared as described above and subsequently exposed to uptake solutions containing either 50 μM $AlCl_3$ in 1.0 mM $CaCl_2$, 50 μM $AlK(SO_4)_2$ in 1.0 mM $CaSO_4$, or 200 μM $AlK(SO_4)_2$ in 1.0 mM $CaSO_4$, both at pH 4.5 and 23°C for 3h. Following uptake, roots were rinsed with deionized, distilled water and placed in 0.5 mM citric acid at pH 4.5 and 0°C for time periods ranging from 0 to 180 min. Following desorption, roots were rinsed with deionozed, distilled water and prepared for Al analysis as described above.

2.2.4 Isolation of purified cell wall material and microsomal membrane fraction

In several experiments, purified cell wall material was isolated according to the methods described by Zhang and Taylor (1990). Following uptake and desorption treatments, excised roots were blotted, weighed, cut into 1 mm sections, placed in a Hepes-Mes buffer at pH 7.8, and stored on ice. Roots were then homogenized for 10 sec using a Brinkmann PT 10/35 Homogenizer with a PTA 10S generator, and placed in a Parr Cell Disruption Bomb (4639) under nitrogen pressure (110 kg/cm^2) for 10 min. After extrusion to atmospheric pressure, the homogenate was sonicated for 7 min in an ice bath at 60% output control on a 25 W ultrasonic homogenizer (Cole-Parmer 4710). The homogenate was then passed through a 20 μm mesh on which the cell wall material was retained. Cell wall material was rinsed 4 times with 10 ml of Milli-Q water before preparation for GFAAS as described above. Cell wall material isolated according to this protocol is virtually free of cytosolic contamination (Zhang and Taylor, 1990).

In some experiments, a microsomal membrane fraction was also isolated with all isolation procedures conducted at 4°C. The filtrate remaining after removal of large cell wall fragments was centrifuged at 10,000 g for 20 min to remove cellular debris and organelles. The high speed supernatant was then centrifuged at 100,000 g for 60 min. The resulting pellet was collected as the microsomal membrane fraction and the supernatant was collected to represent the remainder of the root (vacuole and cytoplasm). Samples were prepared for Al analysis using GFAAS as above.

2.2.5 Multiple-desorption experiments

A series of multiple-desorption experiments similar to those of Tice *et al.* (1992) were conducted in order to compare the desorption patterns and Al content of residual fractions of roots using uptake and desorption conditions employed by Tice *et al.* (1992) and those presently employed in our laboratory. Seedlings of cv. PT 741 were prepared for experimentation as described above and then transferred to an aquarium containing either (a) 15 L of 55 μM $AlCl_3$ in 1.5 mM $CaCl_2$ at pH 4.3 (Tice *et al.*, 1992); or (b)15 L of 50 μM $AlCl_3$ in 1.0 mM $CaCl_2$ at pH 4.5 (our conditions) for either 3 or 48 h. In the case of 48 h exposures fresh solutions were supplied after 24 h. At the end of the uptake period, 30 root tips (2cm) from each absorption treatment were excised and placed into each of five replicate desorption tubes (20 total). The roots were subjected to six sequential 30 min desorption treatments, a -75°C freezing treatment for 30 min, and four more desorption treatments. Two different desorption solutions were compared. Roots exposed to uptake conditions described in (a) above were desorbed using 5.0 mM $CaCl_2$ (pH 4.3 at 23°C) as used by Tice *et al.* (1992). Roots exposed to the uptake conditions described in (b) above were desorbed using 0.5 mM citric acid (pH 4.5 at 0°C) as presently used in our laboratory and by Zhang and Taylor (1989, 1990). Desorption solutions were analyzed immediately without further processing using GFAAS. Purified

cell wall material, and remaining filtrates were prepared for Al analysis using GFAAS as described above.

2.2.6 Measurement of monomeric aluminum in uptake solution

The amount of soluble monomeric Al present in uptake solutions employed in this study was estimated using a modified version of the pyrocatechol violet (PCV) colourimetric method described by Menzies *et al.* (1992). Aluminum standards were prepared by dilution of a 100 mg L^{-1} reference solution (Fisher Scientific) in 1.0 mM HCl. Assays were performed by pipetting 3.0 ml of standard or sample and 0.5 ml of an iron interference reagent containing 1,10-phenanthroline (5 mM) and ascorbic acid (28 mM) into 10 ml polypropylene vials. Following 3 seconds of vortexing and 1 min reaction time, 0.2 ml of the PCV reagent (2.8 mM) and 1.0 ml of an imidazole buffer (1M, pH 5.6) were added. The resulting solution was mixed and the color was allowed to develop for 20 min. Absorbance was read at 578 nm. All equipment used was washed with dilute HNO_3 and rinsed with distilled, deionized water prior to experimentation. (See Appendix I for a detailed protocol)

2.2.7 Statistical analysis

Where required, data points from the rapid phase of uptake and desorption were joined visually based on earlier, more detailed kinetic data whereas the data points from the linear phase of uptake were fitted to a linear regression using Sigmaplot version 3.02 Values for r^2 were never below 0.92.

2.3 Results

When 2 cm excised roots were exposed to 50 μM $AlCl_3$ or $AlK(SO_4)_2$ with no subsequent desorption treatment, no fundamental differences in the pattern of Al uptake over time were observed (Fig. 2.1 A). In each case, uptake of Al was biphasic with a rapid, non-linear phase for the first 30 min, and a linear phase for the remainder of the uptake period. The magnitude of uptake varied between Al sources in both phases. When $AlCl_3$ was used as the Al source, uptake during the first 30 min (175 μg g^{-1}) and the rate of linear phase uptake (1.9 μg g^{-1} min^{-1}) were greater than when $AlK(SO_4)_2$ was used as the Al source (140 μg g^{-1}; 1.2 μg g^{-1} min^{-1}). This is perhaps not surprising since the speciation of Al was different in the two solutions. Speciation analysis using GEOCHEM-PC version 2.0, predicted that the free activity of Al^{3+} was nearly 3 times greater in $AlCl_3$ solutions than in $AlK(SO_4)_2$ solutions (Table 2.1) and 34% of the Al wa in solid form with SO_4 in $AlK(SO_4)_2$ solutions, while 11% of the Al was in solid form ir $AlCl_3$ solutions. Furthermore, analysis of monomeric Al in uptake solutions using the PCV technique revealed that 100% of Al in $AlCl_3$ solutions was in monomeric form, compared to 72.6% in $AlK(SO_4)_2$ solutions (Table 2.1). While the PCV method is unlikely to detect complexation of Al with a weak ligand such as SO_4, it should distinguish monomeric Al from polymerized and precipitated forms. Thus, precipitation of Al sulfates could account for the lower recovery of monomeric Al in both SO_4 solutions (72.6% for 50 μM; 31.8% for 200 μM; Table 2.2). It is important to recognize, however, that both the GEOCHEM-PC and PCV methods, which are useful in predicting speciation of Al in bulk solutions, cannot predict or measure the effects of the rhizospher or apoplasm/free space on Al speciation. Experiments examining the kinetics of desorption using citric acid also showed that the basic pattern of Al desorption over time did not differ between Al sources. Desorption occurred rapidly in the first 30 min and became linear for the remainder of the 180 min desorption period (Fig. 2.1 B).

Although the basic pattern of Al uptake versus time did not differ between Al sources, this does not mean that Al accumulated equally in different cellular compartments. It was therefore important to determine whether accumulation of Al in the cell wall contributed to the linear phase of uptake as reported by Zhang and Taylor (1990). In experiments comparing the Al content of purified cell wall material and the resulting filtrate (presumed to contain the symplasmic fraction of Al), less than 10% of the non-exchangeable (using citric acid) Al was found in the cell wall when Al was supplied as $AlCl_3$ (Fig. 2.2). Although uptake into the cell wall fraction was small, it did contribute slightly (approximately 8%) to the overall linear phase of uptake (the total non-exchangeable pool). In subsequent experiments where Al was supplied in the form of $AlK(SO_4)_2$, a linear phase of accumulation in the cell wall fraction was once again observed (Fig. 2.3 A and B). At low concentrations of Al (50 μM) uptake into cell wall material was again minor (Fig. 2.3 A), contributing approximately 20% to the overall linear phase of uptake. However at high concentrations of Al (200 μM), substantial amounts of Al accumulated in the cell wall fraction. Under these conditions, Al in the cell wall fraction accounted for approximately 48% of the overall linear phase of uptake (Fig. 2.3 B).

Aluminum accumulating in the cell wall could reflect Al which became tightly bound to the cell wall during *in vivo* exposure, or loosely bound Al arising from redistribution during the process of fractionation. While we would be more likely to view the cell wall as a source of Al for redistribution, these alternative hypotheses were nevertheless tested by applying a second desorption treatment to cell wall material which had been previously isolated from roots exposed to low concentrations of $AlCl_3$. This second desorption treatment was effective in removing 50% of remaining cell wall Al (Table 2.3).

Quantitative differences in the extent of Al accumulation in the cell wall between the various absorption protocols raised the possibility of further optimizing kinetic protocols for the measurement of uptake across the plasma membrane. Thus, we decided to investigate the effectiveness of alternative desorption treatments in removing exchangeable Al from the apoplasm. More specifically, multiple desorption experiments adapted from Tice *et al.* (1992) were used to test the effectiveness of $CaCl_2$ and citric acid as desorption agents. In these experiments, intact roots were exposed to Al (either 55 μM $AlCl_3$ in 1.5 mM $CaCl_2$ at pH 4.3 as used by Tice *et al.* (1992) or 50 μM $AlCl_3$ in 1.0 mM $CaCl_2$ at pH 4.5 as presently used in our laboratory) and subsequently subjected to 6 sequential 30 min washes in desorption solution, freeze ruptured, and washed 4 more times in the desorption solutions. Aluminum desorbed by sequential washes was measured in order to estimate exchangeable Al in the apoplasm and symplasm. According to Tice *et al.* (1992) Al removed in the first 6 washes represents the apoplastic pool whereas Al removed following cell rupture can be assigned to be part of the symplastic pool. Following desorption, we separated the Al remaining in roots after desorption (the residual, non-exchangeable fraction) into Al associated with purified cell wall material and Al associated with the remaining filtrate.

In long exposure experiments (48h), both protocols yielded results which were qualitatively similar to those of Tice *et al.* (1992) (Fig. 2.4 A and B). Major elution peaks were found for the first and the seventh washes. When $CaCl_2$ (5.0 mM, pH 4.3 at 23°C) was used as a desorption agent (Fig. 2.4 A), very little Al was removed from roots in subsequent washes. In contrast, a more substantial and sustained desorption occurred in washes 2 through 6 when citric acid (0.5 mM, pH 4.5 at 0°C) was used as a desorption agent, consistent with our previous findings that desorption occurred in a biphasic pattern (Fig. 1B). Similar results were found in short exposure (3h) experiments (Fig. 2.5 A and

B). Using both protocols 50-60% of total Al was found in the residual fraction after 48 h of exposure and greater than 40% of the residual Al was located in the cell wall fraction (Fig. 2.4 A and B). Similarly, greater than 50% of total Al was found to be associated with the residual fraction when $CaCl_2$ was used as desorption agent in 3h exposure experiments and nearly 50% of residual Al was associated with the cell wall fraction (Fig. 2.5 A). In contrast, less than 40% of total Al was associated with the residual fraction when citric acid was used as desorption agent in short exposure experiments, and approximately 30% of the residual Al was associated with the cell wall fraction. Thus, after a short exposure (3 h), Al associated with cell wall material was approximately 50% lower when citric acid was used than that when $CaCl_2$ was used. These results are consistent with those shown in Fig. 2 where short exposure to Al followed by a citric acid wash yielded cell wall Al levels of less than 10% of total.

The finding that a substantial portion of residual Al is associated with cell wall material is in striking contrast to the results reported by Tice *et al.* (1992) who, on the basis of results from staining experiments with the fluorophore morin, assigned the residual Al to the symplastic fraction. In experiments with long term exposure (48 h) and using $CaCl_2$ as a desorption agent (similar to Tice *et al.*, 1992), approximately 42% of residual and 28% of total Al could be attributed to the cell wall. Our results support Tice *et al.*'s concern that tightly bound, non-exchangeable (using $CaCl_2$)forms of Al on the cell wall may not form a stable complex with morin, and hence might not be detected using fluorescence microscopy.

Inasmuch as Shi and Haug (1988) showed that citric acid is also effective in desorbing Al bound to the outer surface of the plasma membrane, we chose to estimate the contribution of membrane bound Al to total uptake. In experiments where a microsomal membrane fraction was isolated from roots which were exposed to 50 μM

$AlCl_3$ and 1.0 mM $CaCl_2$ (pH 4.5, 23°C) for 2 h and desorbed in citric acid (pH 4.5, 0°C) for 30 min, relatively small quantities of Al (<4% of total non-exchangeable Al) were found to be associated with the microsomal fraction (Fig. 2.6). Since the plasma membrane accounts for only a portion of the microsomal fraction, this estimate of Al bound to the plasma membrane is likely high. In these experiments, we compared Al distribution in the microsomal membranes, cell wall and supernatant (residual) fractions between an Al-resistant (PT 741) and an Al-sensitive (Neepawa) cultivar. Unlike studies by Rincon and Gonzales (1992), Tice *et al.* (1992), Delhaize *et al.* (1993) and others, we found no significant difference between cultivars in either of the fractions.

2.4 Discussion

Zhang and Taylor (1989) demonstrated that uptake of Al into roots of wheat exhibited a biphasic character, with a rapid, non-linear phase occurring in the first 30 min superimposed over a linear phase over the remainder of the 3 h exposure to Al. This pattern of uptake with time is a common characteristic of kinetic studies with Al (Huett and Menary 1979, Petterson and Strid 1989). While the rapid phase of uptake has traditionally been interpreted as representing passive, saturable accumulation into the apoplast, the linear phase of metal uptake has been suggested to represent uptake into the symplasm (Korner *et al.*, 1986; Petterson and Strid, 1989). Zhang and Taylor (1989) were able to decrease the magnitude of the rapid phase by desorbing roots with citric acid to remove readily exchangeable Al from the putative apoplastic compartment. However, in a subsequent paper (1990), these authors reported that the linear phase of Al uptake included metabolism-dependent accumulation of Al in the cell wall fractions. While these experiments were conducted using relatively simple uptake solutions containing $AlK(SO_4)_2$ and $CaSO_4$, Tice *et al.* (1992) expressed concern about the possibility that metabolism-dependent accumulation of Al in the cell wall may reflect experimental conditions which are conducive to the formation of solid phase Al. They argued that uptake of Al into various cellular compartments could be strongly salt-dependent and proposed the use of uptake solutions containing only $AlCl_3$ and $CaCl_2$ to reduce the possibility of the formation of Al complexes and solid phase Al which would hinder attempts to measure Al uptake across the plasma membrane.

In order to test this hypothesis, we compared the kinetics of Al uptake and desorption using the uptake solutions employed by Zhang and Taylor (1989,1990,1991) and Tice *et al.* (1992). While small quantitative differences in the rapid phase of uptake and desorption were noted, qualitatively the results were similar (Fig. 2.1 A and B). A

biphasic pattern of uptake was observed in experiments using $AlCl_3$ and $AlK(SO_4)_2$ as the Al source. Although the patterns of uptake were similar, we could not preclude the possibility that the extent of Al accumulation in various cellular compartments differed between the two Al sources. For this reason we decided to measure the accumulation of Al in purified cell wall and microsomal membrane fractions.

When $AlCl_3$ was used as the source of Al in uptake solutions, we found that citric acid was very effective in removing Al from the cell wall fraction. The linear phase of Al uptake in this fraction was negligible, accounting for less than 10% of total non-exchangeable Al (Fig. 2.2). However, when we measured Al levels in cell wall material isolated from roots that had been exposed to $AlK(SO_4)_2$, the contribution of the cell wall to the linear phase of uptake was more substantive (greater than 20% of non-exchangeable Al; Fig. 2.3 A). Furthermore, at high concentrations of $AlK(SO_4)_2$ (200 μM), accumulation of Al in the cell wall fraction accounted for approximately 48% of non-exchangeable Al (Fig. 2.3 B). These results were perhaps not surprising. Speciation analyses using GEOCHEM-PC (Version 2.0) demonstrated that a greater portion of total Al was present as Al^{3+} in solutions containing $AlCl_3$ compared to solutions containing $AlK(SO_4)_2$ (Table 2.1). Furthermore, our PCV analysis revealed striking differences in monomeric Al in uptake solutions. Whereas all of the Al present in $AlCl_3$ solutions was in monomeric form, incomplete recovery (27% - 68%) of monomeric Al was observed in solutions containing $AlK(SO_4)_2$. These data suggest a possible inverse relationship between free activity of Al in uptake solutions and accumulation of slowly-exchangeable Al in the cell wall fraction. As suggested by Tice *et al.* (1992), experimental conditions conducive to the formation of insoluble Al species may be responsible for the accumulation of slowly-exchangeable Al in the cell wall as measured in this study and in those of Zhang and Taylor (1990).

While we have identified a rapidly exchageable and a more tightly bound (slowly exchangeable) pool of Al in the apoplasm, the nature of Al binding in the cell wall remains to be elucidated. Several desorption agents have been shown to remove Al bound in the cell apoplast (Zhang and Taylor, 1989; Tice *et al.*, 1992) but these desorption agents are not equally effective in removing tightly bound Al. Tice *et al.* (1992) suggested that $CaCl_2$ was effective in removing Al from the cell wall. Despite close scrutiny, they could not identify any wall-associated Al in root tips that had been subjected to 6 sequential washes in $CaCl_2$ and subsequently stained with the fluorophore morin. This is in striking contrast to the results reported here. Using $CaCl_2$ as a desorption agent in both short (3h) and long (48h) exposure studies, we found that the residual fraction of roots exposed to the extensive desorption procedures designed by Tice *et al.* could be separated into a cell wall and filtrate fraction each accounting for nearly 50% of the residual (Figs. 2.4 and 2.5). The same was also true for long exposure studies when citric acid was used as a desorption agent (Fig. 2.4 B). In contrast, when citric acid was used as a desorption agent following a 3h exposure to Al, approximately 30% of the residual Al was associated with cell wall (Fig. 2.5 B). This supports the earlier report of Zhang and Taylor (1989) that calcium was not as effective as citric acid in desorbing Al from the apoplasmin short term experiments. Furthermore, it raises an interesting paradox. It would appear that the fluorophore morin (Tice *et al.* 1992) is not capable of detecting Al which is tightly bound to cell wall material. Yet the lack of fluorescent staining of the cell wall was what led Tice *et al.* (1992) to conclude that Al remaining after their lengthy desorption treatment was located in the symplasm. Our results suggest that Tice *et al.* may have overestimated the magnitude of the symplastic fraction by as much as 25%, this potential source of error is significant, since the residual fraction in their experiments accounted for 40 to 60% of total Al in roots.

With our experimental protocols, Al bound exofacially to the plasma membrane could be measured as being part of the symplastic pool of Al. For this reason it was necessary for us to estimate the maximum contribution of this pool of Al to total uptake. We have done this by measuring total Al in the microsomal fraction of roots after desorption in citric acid. We found relatively small quantities of Al (less than 4% of total non-exchangeable Al), to be associated with the microsomal membrane fraction (Fig. 2.6). Since only a portion of this fraction would constitute plasma membrane, this estimate of plasma membrane bound Al is high. We therefore conclude that residual Al bound to the plasma membrane does not make a major contribution to the linear phase of uptake and should not hinder attempts to estimate the uptake of Al into the root symplast. This conclusion is consistent with the results of Shi and Haug (1988), who showed that citric acid was effective in removing Al bound to the outer surface of membrane vesicles.

Using our new protocols, we have compared uptake into a putative symplastic fraction in Al-resistant (PT 741) and Al-sensitive (Neepawa) cultivars and found no differences. While these results contrast with those of other researchers (Rincon and Gonzales,1992; Tice *et al.*, 1992; Delhaize *et al.*, 1993) differences between experimental protocols could explain this discrepancy. For example, differences between cultivars may only exist in certain pools of Al. Inasmuch as different authors utilized different experimental techniques which measured differnt pools of Al, we might expect discrepancies in the results obtained. Alternatively, because Al toxicity symptoms are mostly expressed at the root tip, it is likely that exclusion mechanisms, if they exist, would also be localized at the root tip. In our experiments, we have used 2 cm root tips and perhaps differences between cultivars were masked by having a greater portion of the sample from regions of the root that do not exhibit cultivar-specific differences in Al accumulation. Experiments underway in our lab suggest that this is a likely scenario.

In conclusion, our results support the idea that the accumulation of Al in the cell wall is concentration-, salt- and time-dependent. However, in short exposure experiments (3h) where Al has been supplied at low concentrations in simple salt solutions (50 μM $AlCl_3$ and 1.0 mM $CaCl_2$), Al in the cell wall can be effectively desorbed using citric acid. Thus, Al remaining after desorption could largely reflect accumulation in the symplastic compartment (Fig. 2.6). These results are in accordance with those of Lazof *et al.* (1994) who used secondary ion mass spectrometry to tentatively localize Al in the symplasm of soybean roots after desorption with citric acid. Nevertheless, it is important to recognize that our experiments cannot eliminate the possibility that other apoplastic pools contribute to the linear phase of uptake. Aluminum has been reported to accumulate in the root mucilage (Horst *et al.*, 1982). We are now studying the contribution of mucilage-bound Al to both phases of Al uptake, and the possibility of removing the mucilage to improve our ability to estimate symplastic Al.

Table 2.1 Speciation analysis (GEOCHEM-PC Version 2.0) of major monomeric species in uptake solutions*. Solutions were adjusted to pH 4.5 using 1 M HCl.

Composition of uptake solution	Activity of Al species (M)					Sum of activities of
	Al^{3+}	$Al(OH)^{2+}$	$Al(OH)_2^+$	$AlSO_4^{2+}$	$Al(SO_4)_2^-$	monomeric Al
50 µM $AlCl_3$ in 1.0 mM $CaCl_2$	2.0×10^{-5}	6.3×10^{-6}	1.6×10^{-6}	n.a.	n.a.	27.6 µM
50 µM $AlK(SO_4)_2$ in 1.0 mM $CaSO_4$	6.8×10^{-6}	2.1×10^{-6}	5.4×10^{-7}	1.6×10^{-5}	3.7×10^{-7}	25.8 µM
200 µM $AlK(SO_4)_2$ in 1.0 mM $CaSO_4$	6.0×10^{-6}	1.9×10^{-6}	4.8×10^{-7}	1.6×10^{-5}	4.1×10^{-7}	24.8 µM

*The log K values of thermodynamic constants used were -8.80 for gibbsite ($Al(OH)_3$; Kinraide and Parker, 1989) and -5.0, -10.1, -16.8, and -22.7 for the hydrolysis of Al (Nordstrom and May, 1989). (n.a. = not applicable)

Table 2.2 Analysis of monomeric aluminum in uptake solutions using the pyrocatechol violet (PCV) colourimetric method. All solutions were adjusted to pH 4.5 using 1 M HCl.

Composition of uptake solution	Concentration of monomeric Al	Monomeric Al (% of total)
50 μM $AlCl_3$ in 1.0 mM $CaCl_2$	50.0 ± 0.3 μM	100.0 ± 0.6
50 μM $AlK(SO_4)_2$ in 1.0 mM $CaSO_4$	36.4 ± 0.2 μM	72.6 ± 0.4
200 μM $AlK(SO_4)_2$ in 1.0 mM $CaSO_4$	63.7 ± 0.1 μM	31.8 ± 0.1

Table 2.3 Uptake of Al (μg g root fresh weight^{-1}) into purified cell wall material from the Al-tolerant cultivar PT 741, with and without a second desorption. Roots were pretreated with 50 μM $AlCl_3$ in 1.0 mM $CaCl_2$ (pH 4.5, 23°C) followed by desorption in 0.5 mM citrate (pH 4.5, 0°C) for 30 min. Cell wall material was then isolated and half the samples received a second desorption treatment. Values represent means of 5 replicates ± S.E.

Time (min.)	No desorption	Desorption
30	2.1 ± 0.3	0.9 ± 0.2
60	1.7 ± 0.2	1.0 ± 0.1
120	2.3 ± 0.2	1.0 ± 0.2
180	2.1 ± 0.3	0.9 ± 0.3

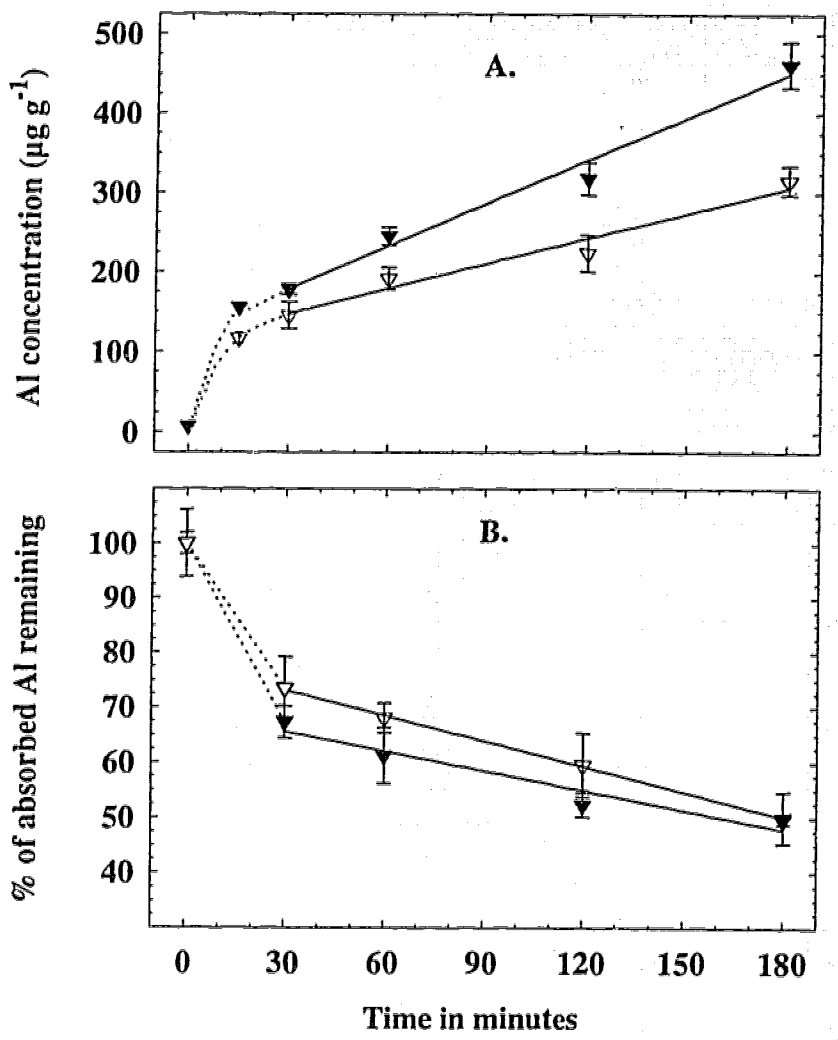


Figure 2.1 Kinetics of aluminum uptake and desorption in excised roots of the Al-resistant cultivar PT 741. Roots were exposed to 50 µM Al in solutions containing either $AlCl_3$ (closed triangles) or $AlK(SO_4)_2$ (open triangles). In experiments using desorption protocols, roots were desorbed using 0.5 mM citric acid. (A) Total uptake of Al versus time. (B) Desorption of Al versus time. Values represent means of 5 replicates $\pm$ S.E.

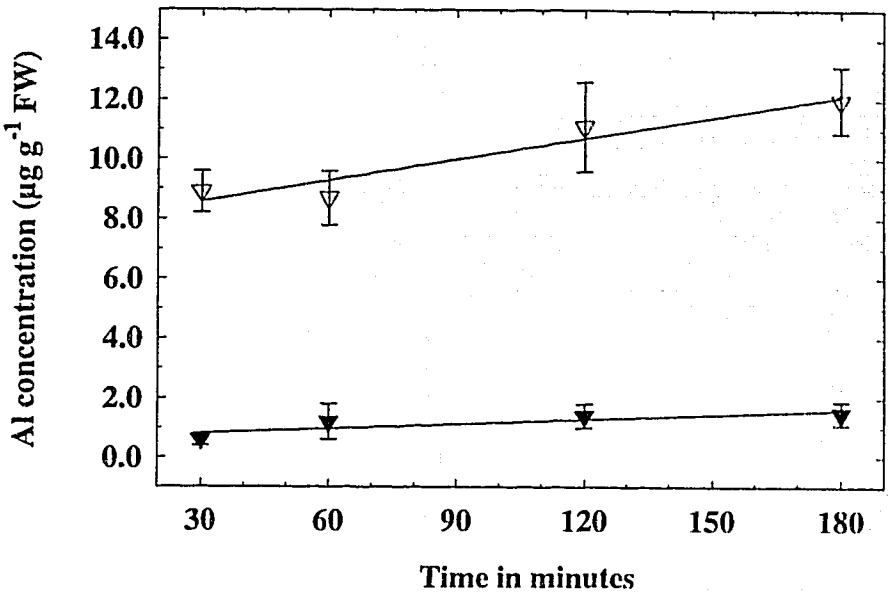


Figure 2.2 Uptake of Al (µg g root fresh weight^{-1}) into purified cell wall material (closed triangles) and the remaining filtrate (open triangles) of the Al-resistant cultivar PT 741. Excised roots were pretreated with 50 µM $AlCl_3$ in 1.0 mM $CaCl_2$ (pH 4.5, 23°C) followed by desorption in 0.5 mM citrate (pH 4.5, 0°C) for 30 min. Values represent means of 5 replicates $\pm$ S.E.

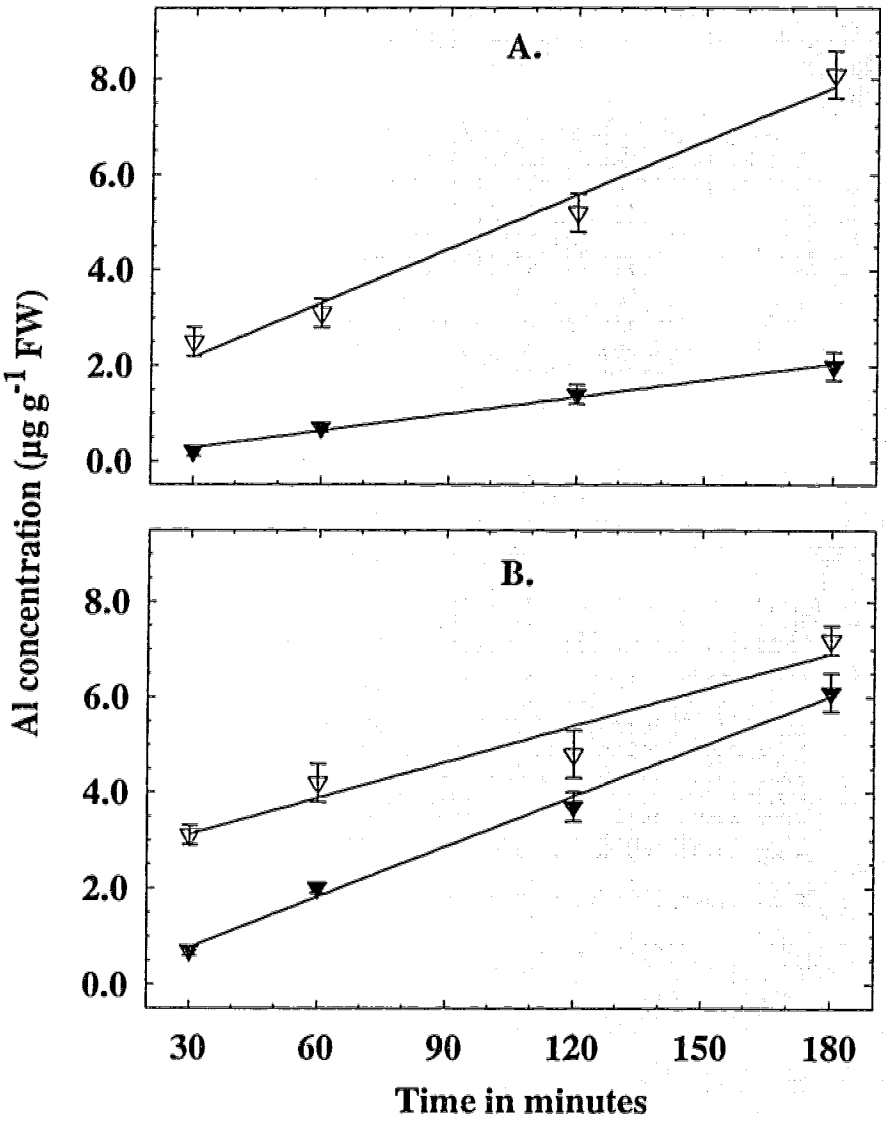


Figure 2.3 Uptake of Al (µg g root fresh weight^{-1}) into purified cell wall material (closed triangles) and the remaining filtrate (open triangles) of the Al-resistant cultivar PT 741. Excised roots were pretreated with (A) 50 µM $AlK(SO_4)_2$ in 1.0 mM $CaSO_4$; or (B) 200 µM $AlK(SO_4)_2$ in 1.0 mM $CaSO_4$ (both at pH 4.5, 23°C) followed by desorption in 0.5 mM citrate (pH 4.5, 0°C) for 30 min. Values represent means of 5 replicates ± S.E.

Figure 2.4 Al recovered (μg g root fresh weight^{-1}) from desorbing solution (washes), purified cell wall, and remaining filtrates of roots of the Al-resistant cultivar PT 741 exposed to Al for 48 h. (A) Roots were pretreated with 55 μM $AlCl_3$ in 1.5 mM $CaCl_2$ (pH 4.3, 23°C) for 48 h, excised, and desorbed using 5.0 mM $CaCl_2$ (pH 4.3, 23°C) for 6 sequential 30 min periods, frozen at -75°C for 30 min, followed by 4 more washes in the desorbing solution (Tice *et al.* 1992). Excised roots (0.5 cm) were then fractionated into a cell wall and filtrate. (B) Roots were pretreated with 50 μM $AlCl_3$ in 1.0 mM $CaCl_2$ (pH 4.5, 23°C) for 48 h and treated as above except 0.5 mM citric acid (pH 4.5, 0°C) was used as the desorption agent. Values represent means of 5 replicates $\pm$ S.E.

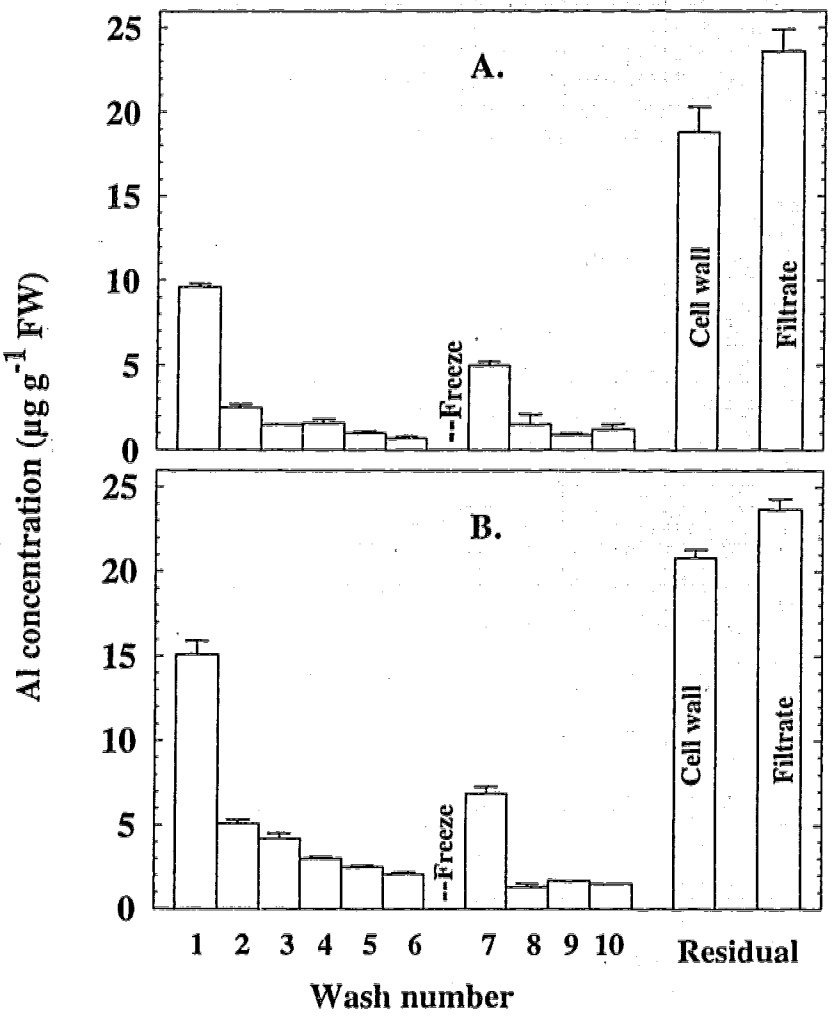
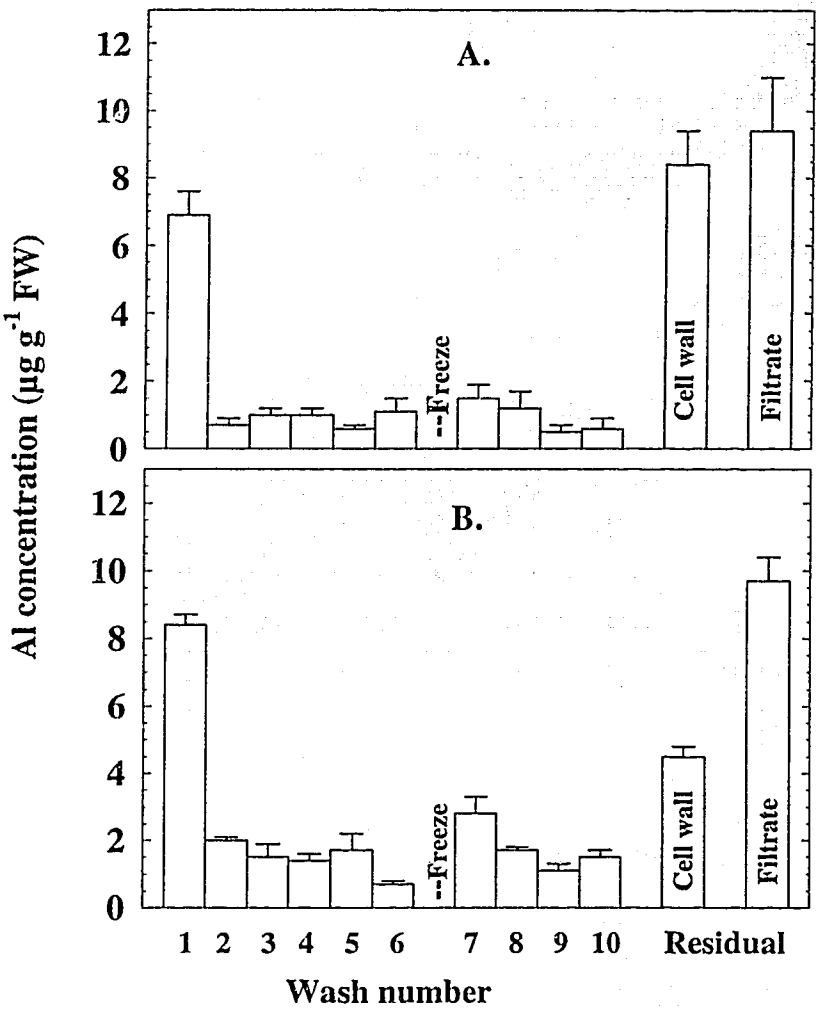

A.
B.
Al concentration (µg g-1 FW)
25
20
15
10
5
0
--Freeze
Cell wall
Filtrate
1 2 3 4 5 6 7 8 9 10
Residual
Wash number

Figure 2.5 Al recovered (μg g root fresh weight^{-1}) from desorbing solution (washes), purified cell wall, and remaining filtrates of roots of the Al-resistant cultivar PT 741 exposed to Al for 3 h. (A) Roots were pretreated with 55 μM $AlCl_3$ in 1.5 mM $CaCl_2$ (pH 4.3, 23°C) for 3 h, excised, and desorbed using 5.0 mM $CaCl_2$ (pH 4.3, 23°C) for 6 sequential 30 min periods, frozen at -75°C for 30 min, followed by 4 more washes in the desorbing solution (Tice *et al.* 1992). Excised roots (0.5 cm) were then fractionated into a cell wall and filtrate. (B) Roots were pretreated with 50 μM $AlCl_3$ in 1.0 mM $CaCl_2$ (pH 4.5, 23°C) for 3 h and treated as above except 0.5 mM citric acid (pH 4.5, 0°C) was used as the desorption agent. Values represent means of 5 replicates ± S.E.


A.
B.
Al concentration (µg g-1 FW)
0
2
4
6
8
10
12
--Freeze
Cell wall
Filtrate
1 2 3 4 5 6 7 8 9 10 Residual
Wash number

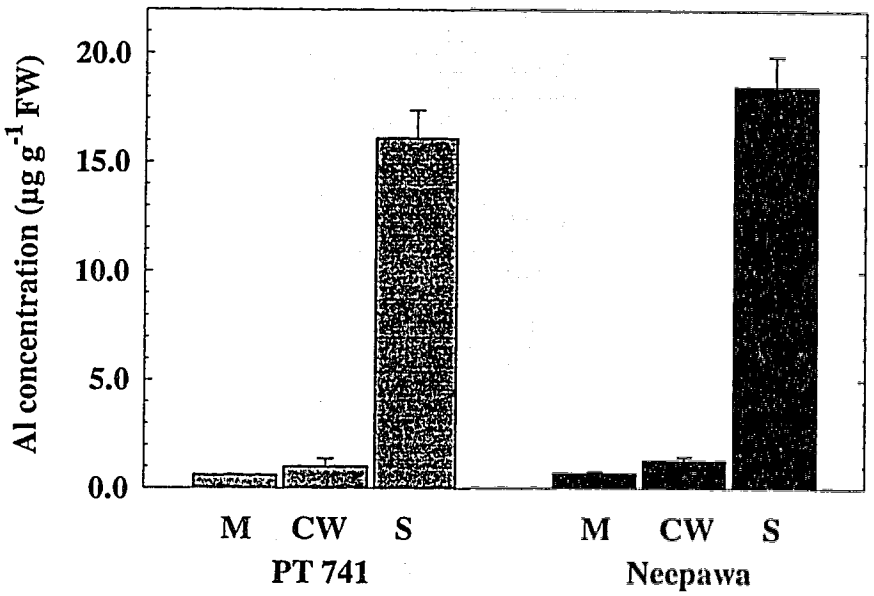


Figure 2.6 Aluminum remaining (μg g root fresh weight^{-1}) in a microsomal membrane fraction (M), cell wall material (CW) and the putative symplastic fraction (S). Roots of the Al-resistant cultivar PT 741 and the Al-sensitive cultivar Neepawa were pretreated with 50 μM $AlCl_3$ in 1.0 mM $CaCl_2$ (pH 4.5, 23°C) for 2h followed with a 30 min desorption in 0.5 mM citric acid (pH 4.5, 0°C). Values represent means of 5 replicates ± S.E.

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3. Accumulation of aluminum in root mucilage of an Al-resistant and an Al-sensitive cultivar of wheat (*Triticum aestivum* L.).

3.1 Introduction

A number of recent studies have emphasized the importance of the root tip in the expression of aluminum (Al) toxicity and resistance in plants. This was perhaps most elegantly demonstrated by Ryan *et al.* (1993) who showed that Al must be supplied to the terminal 2-3 mm of the root apex of *Zea mays* in order for symptoms of Al toxicity to be expressed. This observation is consistent with an array of less direct evidence which also supports the role of the root tip as the primary site of Al related lesions. For example, in *Allium cepa* and *Vignia unguiculata,* decreased rates of mitosis have been associated with accumulation of Al in the root apex (Clarkson, 1965; Horst *et al.*, 1982, 1983). Aluminum has also been shown to bind to cell nuclei in root tips of *Zea mays* (Galsomies *et al.*, 1992) and, more specifically, to DNA in roots of *Pisum sativum* and *Allium cepa* (Matsumoto *et al.*, 1976; Morimura *et al.*, 1978). If the root tip is indeed the site where toxicity is most clearly expressed, we would expect potential resistance mechanisms to be most clearly expressed in this region as well. While mechanisms of Al resistance are poorly understood, Delhaize *et al.* (1993) demonstrated that the terminal 3-5 mm of root tips of an Al-resistant cultivar of *Triticum aestivum* were the primary source of Al-induced malic acid excretion. Similarly, Basu *et al.* (1994) provided evidence that an Al-induced membrane protein was most abundant in the terminal 5 mm of roots of an Al-resistant cultivar of *T. aestivum*.

For Al to reach sensitive meristematic regions, it must first penetrate and cross the root mucilage. Because the root tip region is the site of most intensive mucilage production (Paull and Jones, 1975), immobilization of Al in this layer may constitute an

important mechanism that protects the meristem from Al injury (Horst *et al.*, 1982) through exclusion of Al from the cell symplasm (Taylor, 1988). Chelate ligands present in the mucilage may bind Al and thereby present a physical or chemical barrier to the inward movement of Al (Henderson and Ownby, 1991). Enhanced exudation of malate (Delhaize *et al.*, 1993; Basu *et al.*, 1994b; Ryan *et al.*, 1995 a,b) and citric acid (Miyasaka *et al.*, 1991; Pellet *et al.*, 1995) have been reported in Al-resistant cultivars of *T. aestivum*, *Phaseolus vulgaris* and *Zea mays*. Furthermore, Horst *et al.* (1982) showed that 50% of total Al in 5 mm root tips of *Vignia unguiculata* was bound to mucilage. Removal of the mucilage prior to treatment with Al facilitated the entry of Al into root tissue and rendered roots more sensitive to Al (Horst *et al.*, 1982).

If mucilage plays a role in mediating exclusion of Al, it is expected that sensitive plants would accumulate Al in the symplasm more rapidly than resistant plants, and these differences would be more pronounced at the root tip. Rincon and Gonzales (1992) found that in root tips of *T. aestivum*, Al-sensitive plants absorbed more Al than did those of Al-resistant plants. Similarly, Delhaize *et al.* (1993) found that root apices of Al-sensitive lines of *T. aestivum* accumulated more Al than did Al-resistant lines. Unfortunately, their protocols did not allow them to differentiate between apoplastic and symplastic Al, and thus these studies cannot provide direct evidence of exclusion mechanisms operating at the plasma membrane.

To determine if exclusion mechanisms play a role in resistance, Al uptake into the symplasm of root tips must be measured independently of apoplastic uptake. Progress in this regard has been hindered by the lack of a suitable radioisotope which can be purchased and detected at reasonable cost, and the lack of analytical techniques capable of measuring minute quantities of Al internalized by plant cells. Nonetheless, several important obstacles have been overcome. Perhaps most importantly, we have shown that

it is possible to virtually eliminate metabolism-dependent accumulation of Al in the apoplasm (Zhang and Taylor, 1990) by using low concentrations of Al in simple uptake solutions (50 μM $AlCl_3$ and 1.0 mM $CaCl_2$) with a subsequent wash in 0.5 mM citric acid (Archambault *et al.*, 1996). Under these conditions concerns about contamination of the symplasm during fractionation are minimized, and binding of Al to membranes contributes only 4% of total non-exchangeable uptake. However, identifying the remaining linear phase as uptake into a putative symplastic compartment remains speculative. The kinetics of Al uptake into mucilage remain to be studied and uptake into this apoplastic compartment could contribute to both the rapid, non-linear phase and the linear phase of Al uptake.

In this study, we have investigated the contribution of mucilage-bound Al to total uptake and the possibility of removing the mucilage in order to isolate the linear phase of uptake in roots of *T. aestivum*. Our studies demonstrate that the mucilage represents an important apoplastic pool for Al which can be removed with a 10 min wash in NH_4Cl. A revised kinetic protocol is proposed which may provide a more accurate estimate of symplastic levels of Al.

3.2 Methods and Materials

3.2.1 Preparation of plant material

To prepare plants for experimentation, seeds of an Al-resistant cultivar (PT 741) and an Al-sensitive cultivar (Neepawa) of *T. aestivum* were surface sterilized in 1.2% sodium hypochlorite for 20 min, and germinated for 24 h in a solution of Vitavax (0.005 g/L) to prevent fungal growth. Seedlings were grown on nylon mesh suspended in aquaria containing a full nutrient solution (Zhang and Taylor, 1989) for 4 to 7 days. In experiments requiring excised roots, thirty 2 cm root tips were excised and placed into each of 50 - 55 replicate absorption tubes. The tubes were then placed in a full nutrient solution until excision was complete (<60 min). Following a 30 min equilibration period in 1.0 mM $CaCl_2$ (pH 4.5, 23°C), the tubes were transferred to uptake solutions.

3.2.2 Visualization of mucilage

Plants were prepared for experimentation as described above. Roots of 5 day old seedlings were observed and photographed at 100x magnification to reveal the presence of a droplet of substance at the root apex. In order to verify whether the droplet was indeed mucilage, the roots were immersed in 25 ml of 100 μM Ruthenium Red, a stain for pectins, and rinsed with deionized, distilled water (> 18 MΩ). Visual observations showed that the droplet stained an intense red and could not be removed by rinsing with water. Based on these observations we concluded that the droplets consisted of mucilage and proceeded to test protocols that might allow us to remove this layer.

3.2.3 Removal of mucilage

The potential role of mucilage as an apoplastic pool for Al was evaluated in a series of experiments in which mucilage was removed using a 10 min wash in 1 M NH_4Cl (Brams, 1969). To test the efficacy of this treatment, microscope studies were undertaken to visually observe the root tip-mucilage region of the Al-resistant cultivar PT 741. We also compared this treatment to two other chloride salts, namely 1M KCl and 1M $CaCl_2$, as well as the sulfate salts of NH_4^+, K^+, and Ca^{2+}, to determine which part of the ion pair would be responsible for the observed effects. Plants were prepared for experimentation as described above. Roots of 5 day old seedlings were a) left untreated (control), or washed for 10 min in 50 ml of a 1M solution (pH 4.5, 23°C) of either chloride or sulfate salts of: b) NH_4^+; c) K^+; or d) Ca^{2+}. Following washes roots were rinsed with deionized water. Roots were photographed under a dissecting microscope at a 100x magnification.

3.2.4 Kinetics of Al desorption from roots

Excised roots of the Al-resistant cultivar (PT 741) were prepared for experimentation as described above and transferred to uptake solutions containing 50 μM $AlCl_3$ in 1.0 mM $CaCl_2$ at pH 4.5 and 23°C. Following 3 h exposure to Al, half of the samples were subjected to a 10 min wash in 1M NH_4Cl at pH 4.5 and 23°C, the remainder received no NH_4Cl wash. Roots from both treatments were then desorbed in 0.5 mM citric acid at pH 4.5 and 0°C for 0, 30, 60, 120 or 180 min. Upon completion of desorption, roots were rinsed with deionized distilled water, dried in an oven at 55°C, weighed, ashed in a muffle furnace at 500°C, solubilized in 200μL nitric acid, and the volume adjusted using distilled, deionized water. Solutions were analyzed for Al using

graphite furnace atomic absorption spectrophotometry (GFAAS) as described by Zhang and Taylor (1989).

3.2.5 Contribution of mucilage-bound Al to uptake

The amount of Al tightly bound to mucilage was estimated by quantitative analysis of the Al content of excised roots from Al-resistant (PT 741) and Al-sensitive (Neepawa) cultivars following a series of washing procedures. Excised roots were prepared as described above and loaded with Al in solutions containing 50 μM $AlCl_3$ in 1.0 mM $CaCl_2$ (pH 4.5, 23°C) for two hours. Five replicate tubes containing roots of each genotype were (a) harvested immediately for determination of total Al; (b) washed for 30 min in 0.5 mM citric acid (pH 4.5, 0°C); (c) washed for 30 min in 0.5 mM citric acid (pH 4.5, 0°C) and 10 min with 1 M NH_4Cl (pH 4.5, 23°C); (d) washed for 30 min in 0.5 mM citric acid (pH 4.5, 0°C) and 10 min with 1 M KCl (pH 4.5, 23°C); (e) washed for 30 min in 0.5 mM citric acid (pH 4.5, 0°C) and 10 min with 1 M $CaCl_2$ (pH 4.5, 23°C); (f) washed for 10 min with 1 M NH_4Cl (pH 4.5, 23°C) and 30 min in 0.5 mM citric acid (pH 4.5, 0°C); or (g) washed for 30 min in 0.5 mM citric acid (pH 4.5, 0°C), 10 min with 1 M NH_4Cl (pH 4.5, 23°C) and 30 min in 0.5 mM citric acid (pH 4.5, 0°C). Roots were prepared for Al analysis as described above.

3.2.6 Patterns of Al uptake in mucilage

Measurements of total Al remaining after desorption in citric acid, NH_4Cl, and KCl suggested that the mucilage represents a significant apoplastic pool of Al. Thus, kinetic experiments were conducted to determine the time course of Al accumulation in this pool. These experiments allowed us to investigate the possibility that differences in the pattern of Al accumulation in mucilage might exist between Al-resistant and Al-

sensitive cultivars. We looked at the kinetics of Al uptake into mucilage of PT 741 and Neepawa in both short- (3 h) and long- (6 h) term exposure studies. For short term studies, excised roots were prepared for experimentation as described above and transferred to uptake solutions containing 50 μM $AlCl_3$ in 1.0 mM $CaCl_2$ (pH 4.5, 23°C). After 0, 10, 30, 60, 120 and 180 min, five replicate tubes for each genotype were removed from uptake solutions and roots were desorbed for 30 min in 0.5 mM citric acid (pH 4.5, 0°C) and washed for 10 min with 1 M NH_4Cl (pH 4.5, 23°C) (Brams, 1969). Aliquots (2 mL) of the NH_4Cl wash were analyzed for Al using GFAAS without further preparation. Roots were then prepared for Al analysis as described above.

For long term studies, whole plants were prepared for experimentation as described above. After 7 days of growth, plants growing on nylon mesh were removed from aquaria containing nutrient solution, rinsed with distilled water, and placed in aquaria containing 50 μM $AlCl_3$ and 1.0 mM $CaCl_2$ (pH 4.5 and 23°C) for 0, 2, 4 and 6 h. Following exposure to Al, roots were rinsed with distilled, deionized water, excised 2 cm from the root tip and subjected to a desorption treatment and removal of the mucilage as described above. The NH_4Cl solution was then assayed directly in order to estimate the Al content of the root mucilage.

3.2.7 Kinetics of Al uptake using new protocol

Experiments were performed to compare patterns of Al uptake using the protocol described previously by Archambault *et al.* (1996) and a new protocol that includes removal of mucilage. Excised roots of the cultivar PT 741 were prepared as described above and transferred to uptake solutions containing 50 μM $AlCl_3$ in 1.0 mM $CaCl_2$ (pH 4.5 and 23°C). Following 0, 5, 10, 15, 20, 30, 60, 90, 120, 150 and 180 min of uptake, 5 replicate tubes were removed from uptake solutions and the roots were desorbed in 0.5

remaining roots were placed in a 1 M solution of NH_4Cl (pH 4.5, 23°C) for 10 min to remove the mucilage, rinsed with deionized, distilled water and prepared for Al analysis as described above.

3.2.8 Statistical analysis

Where required, data points from the rapid phase of uptake and desorption were joined visually based on earlier, more detailed kinetic data whereas the data points from the linear phase of uptake were fitted to a linear regression using Sigmaplot version 3.02 Values for r^2 were never below 0.92.

3.3 Results

Staining of excised roots with Ruthenium Red, a stain for pectins, suggested that droplets of substance at the root apex were mucilage (Fig. 3.1 A and B). Older portions of the roots stained less intensely (Fig. 3.1 B), indicating that pectins were found along the entire length of the root, but at lower concentrations than at the root tip. Loss of small portions of the mucilage were commonly observed if roots were subjected to extensive manipulations. Care in preparation of roots for analysis completely overcame this problem. Treatment of roots with NH_4Cl and KCl for 10 min appeared to completely remove the mucilage (Fig. 3.2 B and C). While $CaCl_2$ had little effect (Fig. 3.2 D) on the mucilage, the layer seemed smaller than in the control. The same effects were observed when sulfate salts were used (data not shown). The finding that $CaCl_2$ did not eliminate the mucilage also suggests that the effect of NH_4Cl and KCl on the mucilage was not a simple dehydration caused by high ionic strength (the ionic strength of the $CaCl_2$ solution was greater than that of the NH_4Cl and KCl solutions). Our results indicate that high concentrations of monovalent cations (NH_4^+ and K^+) were responsible for the removal of the mucilage and that the Cl^- and SO_4^- anions had little or no effect. These data suggest (as demonstrated by Brams (1969)) that root mucilage is successfully removed using an NH_4Cl wash.

Horst *et al.* (1982) demonstrated that the root mucilage represents a significant apoplastic sink for Al. Thus, desorption of Al from the mucilage or complete removal of the mucilage itself will be required in order to accurately estimate uptake of Al into the root symplasm using a kinetic approach. The kinetic protocol developed in our laboratory (Zhang and Taylor, 1989; Archambault *et al.*, 1996) includes a desorption step using citric acid, which has been shown to remove Al from the cell wall. In previous studies (Archambault *et al.*, 1996), we have shown that the rapid phase of uptake in excised roots

could not be completely desorbed using a citric acid wash, suggesting the existence of non-exchangeable pools of apoplastic Al. We speculated that citric acid may not be fully effective in desorbing Al from the mucilage and we wanted to test whether the linear phase of desorption previously observed (Zhang and Taylor, 1989; Archambault *et al.*, 1996) might also represent a slow exchange process from the mucilage. To test these hypotheses, we studied the effects of removal of the mucilage on the pattern of desorption using citric acid. Our results show that the removal of the mucilage prior to desorption in citric acid had little effect on the slope of the linear phase (Fig. 3.3). These results suggest that slow, linear desorption from roots does not reflect desorption of Al from the mucilage or that desorption occurs very slowly.

In order to further quantify Al tightly bound in the mucilage we designed experiments to compare the effects of various desorption solutions on the Al content of root tips. We compared the amount of Al remaining in roots after a desorption in citric acid with that remaining after desorption in citric acid and removal of the mucilage using a 10 min wash in 1M NH_4Cl. After desorption with citric acid, root tips of the Al-resistant cultivar PT 741 retained 301 $\pm$ 11 $\mu g\ g^{-1}$ of Al, while root tips of the Al-sensitive cultivar Neepawa retained 368 $\pm$ 5 $\mu g\ g^{-1}$ of Al. When mucilage was subsequently removed from root tips using NH_4Cl, the amount of Al remaining was approximately 25 to 35% lower than when the mucilage was left intact (Fig. 3.4 A and B). A similar effect was observed when NH_4Cl was substituted with KCl (Fig. 3.4). However, such was not the case when $CaCl_2$ was employed. Aluminum levels were the same when the citric acid wash was followed by a $CaCl_2$ wash as when citric acid was used singly (Fig. 3.4). This observation is consistent with the results of our microscopic work, which suggested that Ca is not effective in removing the mucilage and further demonstrates that Ca is incapable of removing Al remaining in the apoplasm after a citric acid wash. While citric acid may be capable of desorbing a portion of the Al present in

the mucilage, our results suggest that it is not completely effective in desorbing this apoplastic pool.

The effectiveness of NH_4Cl in displacing Al from root tips after a citric acid wash may be a direct result of the removal of the mucilage itself, removing a significant pool of tightly-bound Al which cannot be desorbed using citric acid alone. Alternatively, intact mucilage might protect underlying apoplastic binding sites from desorption, and removal of the mucilage facilitates desorption of Al from these sites. It is also possible that NH_4^+ itself acts as a powerful desorption agent which is capable of removing Al not previously desorbed with citric acid. We cannot reject the latter hypothesis on the grounds that another monovalent cation, K^+, was also effective in removing a significant pool of Al. However, Ca^{2+} was an ineffective desorption agent. Inasmuch as divalent cations should be more effective than monovalent cations in desorbing Al, this argues against a direct role for these cations in direct desorption of Al. We have attempted to differentiate between the remaining alternative hypotheses by varying the order of the wash treatments (NH_4Cl followed by citric acid) and by including a second wash in citric acid (citric acid, followed by NH_4Cl, and a second wash in citric acid). If removal of the mucilage with NH_4Cl exposes underlying sites to the effect of a desorption agent, citric acid should be a more effective desorption agent when used after the mucilage has been removed. In both the Al-resistant PT 741 and the Al-sensitive Neepawa, changing the order of the NH_4^+ and citric acid washes, or adding a second wash in citric acid following mucilage removal did not desorb additional Al from the roots (Fig. 3.4). Thus, we are inclined to believe that NH_4Cl effectively removes a significant apoplastic pool of Al which cannot be removed by citric acid alone.

Having ascertained that the mucilage represents a significant pool of apoplastic Al, we then focused our attention on the time course of Al accumulation in this pool. Is

binding of Al to mucilage a rapid, saturable process, or can it contribute to the linear phase of uptake with time? Short-term (3 h) exposure experiments showed that patterns of Al uptake into root mucilage were bi-phasic for both PT 741 and Neepawa (Fig. 3.5 A and B). A rapid phase of Al uptake was observed in the first 30 min of exposure, followed by a linear phase of uptake occurring over the remainder of the 180 min experimental period. Despite the qualitative similarities, there were some quantitative differences. Extrapolation of the linear phase of Al accumulation back to time zero indicated that rapid phase accumulation was approximately 5 times greater in the Al-sensitive Neepawa (140 $\mu g\ g^{-1}$) than in the Al-resistant PT 741 (27 $\mu g\ g^{-1}$). Furthermore, while the linear phase of Al uptake was substantive in PT 741 (0.67 $\mu g\ g^{-1}\ min^{-1}$), it was weak in Neepawa (0.27 $\mu g\ g^{-1}\ min^{-1}$). Long-term studies (6 h) showed that in both cultivars, the linear phase of Al uptake into the mucilage persisted throughout the experimental period with no sign of saturation (Table 3.1). Thus, in both cultivars, accumulation of Al into the mucilage has the potential to make a significant contribution to the rapid saturable phase of uptake and to the linear phase of uptake which has been observed in excised roots (Zhang and Taylor, 1989; Archambault *et al.*, 1996).

Given the importance of mucilage as a sink for apoplastic Al, we have incorporated a step for removal of mucilage into our kinetic protocol. This step provides a significant improvement in our ability to isolate the linear phase of Al uptake (putatively uptake into the symplasm). Comparison of the kinetics of Al uptake into roots subjected to a simple desorption in 0.5 mM citric acid (pH 4.5, 0°C for 30 min) to that of roots washed in citric acid followed by a 10 min wash in 1M NH_4Cl (pH 4.5, 23°C), demonstrated that removal of the mucilage effectively eliminated most of the rapid phase of uptake and also reduced the magnitude of the linear phase. This left a linear phase of uptake which deviated only slightly from linearity during the first 5 to 10 min of uptake (Fig. 3.6).

3.4 Discussion

The experiments reported here represent an ongoing effort to improve techniques which provide quantitative estimates of Al accumulation in the symplasm of plant roots. Given the lack of a suitable radioisotope for Al which can be purchased and detected at reasonable cost, direct, unambiguous measurement of the rate of Al accumulation within the symplasm has been problematic. Nonetheless, we still believe that a kinetic approach has the potential to provide an accurate estimate of the rate of symplastic uptake. The accuracy of this type of approach clearly depends on the validity of our operational definition of symplastic uptake. Two factors are particularly important in this regard. First, an efficient desorption protocol must be developed to effectively desorb Al which accumulates within the apoplasm. Ideally, this would allow experimental isolation of the linear phase of uptake which might putatively be designated as uptake into the symplasm. If success in this endeavor is to be achieved, efforts will also be required to identify all possible apoplastic pools of Al which might contribute to the linear phase of uptake *in vivo* (and perhaps *in vitro* as a result of contamination arising from experimental perturbation).

In previous work, considerable progress has been made towards achieving these goals. Zhang and Taylor (1989) demonstrated that the kinetics of Al uptake in excised roots were bi-phasic with a rapid phase of uptake superimposed over a linear phase of uptake with time. Subsequently, they showed that the linear phase of Al uptake may include metabolism-dependent uptake into the cell wall (Zhang and Taylor, 1990). The potential contribution of metabolism-dependent uptake into the cell wall presents a barrier to measuring Al accumulation within the symplasm. However, we have since discovered that this metabolism-dependent binding can be virtually eliminated by the use of experimental conditions which are less conducive to the formation of solid phase Al in

the apoplasm (Tice *et al.*, 1992; Archambault *et al.*, 1996). Under these conditions, citric acid effectively desorbed Al in cell wall material, and binding of Al to membrane components represented less than 4% of non-exchangeable Al (Archambault *et al.*, 1996). Despite these advances, previous experiments cannot eliminate the possibility that other apoplastic pools contribute to the linear phase of uptake. In this paper, we have tested the hypothesis that tight binding of Al to mucilage may prevent a complete desorption of Al from the apoplasm. If this is the case, elimination of mucilage-bound Al as a pool of apoplastic Al will be required to obtain accurate estimates of symplastic Al and rates of trans-membrane transport of Al.

Brams (1969) used a 1 min wash in 1 M NH_4Cl to remove the mucilaginous layer surrounding the roots of citrus plants. We decided to test the effectiveness of this method in removing the mucilage from roots of *Triticum aestivum*. When roots were washed in 1M NH_4Cl (pH 4.5, 23°C), visual and microscopic examination confirmed that the mucilage was removed (Fig 3.2 B). The ability to remove the mucilage provided us with a means of testing whether the linear phase of desorption from excised roots might represent a slow exchange of Al from the mucilage. Zhang and Taylor (1989) and Archambault *et al.* (1996) hypothesized that the linear phase of desorption from excised root tips represents slow desorption of Al from the symplasm, however, an alternative hypothesis is that citric acid is desorbing Al from the mucilage at a slow, but constant rate (linear desorption with time). Comparison of the kinetics of citric acid desorption between roots with the mucilage intact (desorption in citric acid only) and roots with the mucilage removed (desorption in NH_4Cl followed by a wash in citric acid) demonstrated that removal of the mucilage had a major effect on the rapid phase of desorption, but little or no effect on the linear phase (Fig. 3.3). Thus, these results suggest that the slow, linear phase of desorption from excised roots either does not reflect desorption of Al from mucilage or that the rate of desorption is extremely slow.

Experiments with a variety of desorption agents demonstrated that desorption in citric acid alone is not sufficient to remove Al from the mucilage and that Al tightly bound to the mucilage can only be removed by removal of the mucilage itself. This would appear to be an important part of kinetic protocols, since Al in the mucilage accounted for up to 35% of non-exchangeable Al (Fig. 3.4). This value is consistent with that reported by Horst *et al.* (1982), who used physical removal of mucilage to estimate the amount of Al in this compartment. Examination of the pattern of Al uptake into the mucilage of Al-resistant and Al-sensitive plants, demonstrated that accumulation of Al in the mucilage was rapid for the first 30 min and linear throughout the remainder of the experimental period (Figs. 3.5 A and B). Long term experiments showed that the linear phase of Al uptake persisted up to 6 h (Table 3.1). This is also consistent with the results of Horst *et al.* (1982) who demonstrated that Al accumulation in *Vignia unguiculata* was time-dependent with no sign of saturation occurring even after 48 h of exposure.

Quantification of mucilage weight or volume was not possible using our technique, thus results for the Al-resistant and the Al-sensitive cultivars cannot be quantitatively compared on a mass of mucilage or volume of mucilage basis. Nonetheless, Al concentrations were calculated on a root dry weight basis, and interesting observations were made. The general (biphasic) pattern of Al uptake into the mucilage did not differ between PT 741 and Neepawa in short exposure (3 h) studies (Fig. 3.5), although quantitatively the relative importance of the linear phase was greater in PT 741. The reasons for quantitative differences in linear phase accumulation of Al in the mucilage are not clear. We must recognize, however, that production of mucilage itself has been shown to be inhibited by Al (Horst *et al.*, 1982). If inhibition of mucilage production is more pronounced in sensitive plants, this might limit the extent of linear phase accumulation and the degree of protection afforded to underlying tissues of the root meristem. Saturation of binding sites within the mucilage would subsequently lead to a

greater exposure to toxic Al ions. Higher Al sensitivity has been related to higher Al contents in root tips (Horst *et al.*, 1982). These authors have also reported that root elongation can be considerably more inhibited when the mucilage is removed. In Al-resistant plants, continued synthesis of mucilage in the face of Al stress could serve to maintain the binding capacity of the mucilage providing ongoing protection for the growing region. Knowledge of such dynamic aspects of mucilage excretion and Al binding may be required for a complete understanding of the role of mucilage in mediating resistance.

The observation that citric acid is not capable of desorbing Al from the mucilage, suggests that this layer binds Al tenaciously or that the mucilage matrix does not allow for rapid diffusion of citric acid throuhout the bulk of the mucilage during the experimental period observed. Henderson and Ownby (1991) suggested that the mucilage layer, by nature, only allows for slow diffusion of substances, thus creating an area of high organic acid concentration. If these organic acids are relatively immobile, they could decrease the activity of Al^{3+} in the apoplasm and hence the rate at which Al crosses the plasma membrane. This hypothesis is consistent with the results of McCormick and Borden (1974), who found localized accumulations of an Al-phosphate precipitate in mucilaginous material at the surface of root tips in *Hordeum vulgare*, and those of Horst *et al.* (1982) who showed that the mucilage of 5 mm root tips of *Vignia unguiculata* contained approximately 10 times more Al than the root tissue proper after a 6 h exposure to Al. Investigation of the dynamics of complex formation, however, have not yet been explored.

Mucilage represents a significant pool of apoplastic Al accounting for as much as 35% of non-exchangeable Al. This substantial pool of Al in the apoplasm complicates the interpretation of previous kinetic work. Results from this study suggest that the

mucilage must be removed in order to obtain an accurate estimate of symplastic Al levels. Having incorporated a step for removal of the mucilage into our kinetic protocol, we reviewed the kinetics of Al uptake using an Al-resistant cultivar (PT 741). We found that it was possible to isolate the linear phase of uptake with deviation from linearity observed only during the first 5 min of uptake (Fig. 3.6). While kinetic studies such as this cannot provide an unambiguous definition of symplastic Al, we believe this linear phase provides the best available estimate of the rate of Al uptake into the symplasm. We are now in a position to compare rates of Al accumulation in the symplasm of plants that differ in their resistance to Al.

Table 3.1 Long term uptake of Al into the mucilage of an Al-resistant cultivar (PT 741) and an Al-sensitive cultivar (Neepawa) of *T. aestivum* L. Roots were exposed to 50 μM $AlCl_3$ in 1.0 mM $CaCl_2$ (pH 4.5, 23°C) for 2, 4 and 6 h, desorbed for 30 min in citric acid (pH 4.5, 0°C), and washed in 1M NH_4Cl for 10 min (pH 4.5, 23°C). A 2 ml sample of the NH_4Cl was taken and analyzed directly for Al content. Values represent means of 3 replicates ± S.E.

Time in hours	Al concentration (μg g^{-1})	
	PT 741	Neepawa
2	91 ± 19	84 ± 1
4	130 ± 12	100 ± 12
6	146 ± 19	118 ± 9
Rate of Al uptake (μg g^{-1} min^{-1})	0.23	0.14

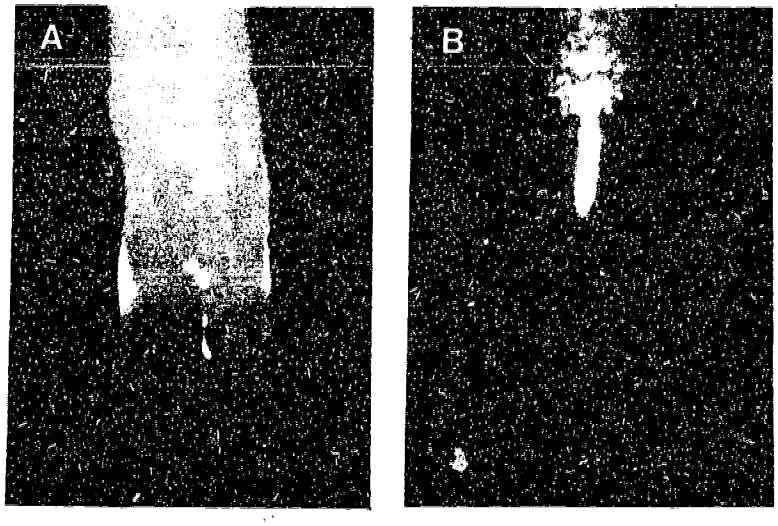


Figure 3.1 Roots of *T. aestivum* L. cultivar PT 741 which were (A) untreated (B) stained with Ruthenium Red. Photographs were taken using a 35 mm camera mounted on a dissecting microscope using 100x magnification.

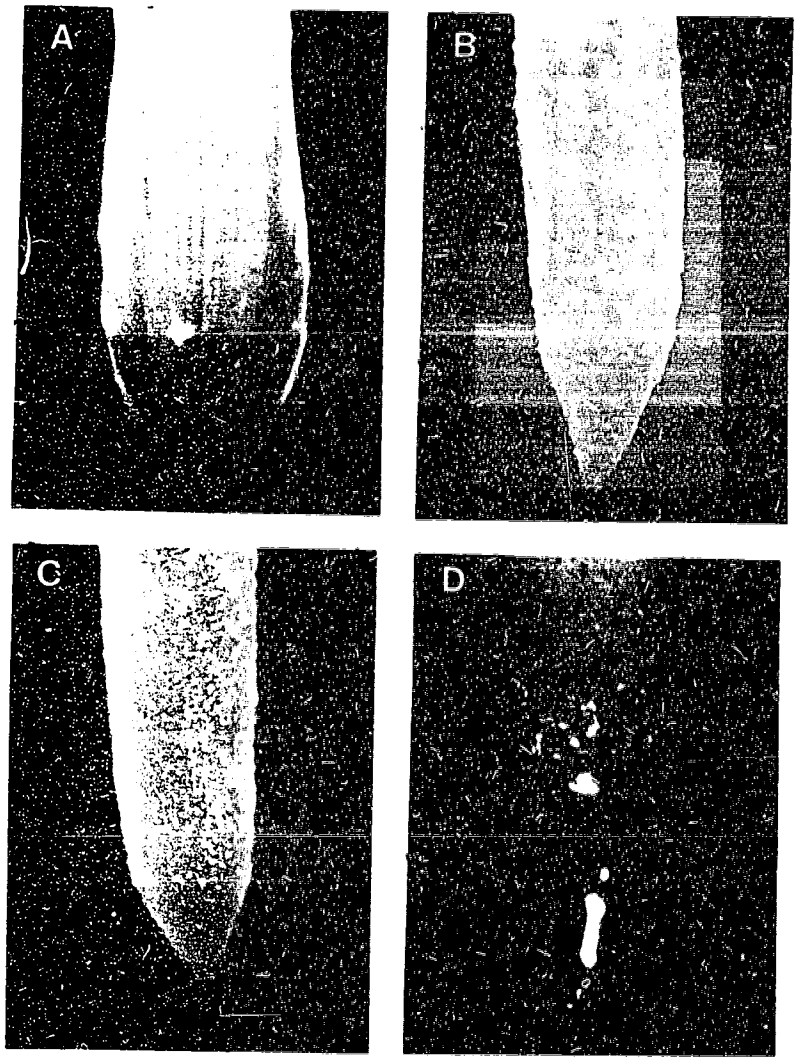


Figure 3.2 Roots of *T. aestivum* L. cultivar PT 741 which were (A) untreated; (B) washed in 1M NH_4Cl (pH 4.5, 23°C); (C) washed in 1M KCl (pH 4.5, 23°C); (D) washed in 1 M $CaCl_2$ (pH 4.5, 23°C). Photographs were taken using a 35 mm camera mounted on a dissecting microscope using a 100x magnification.

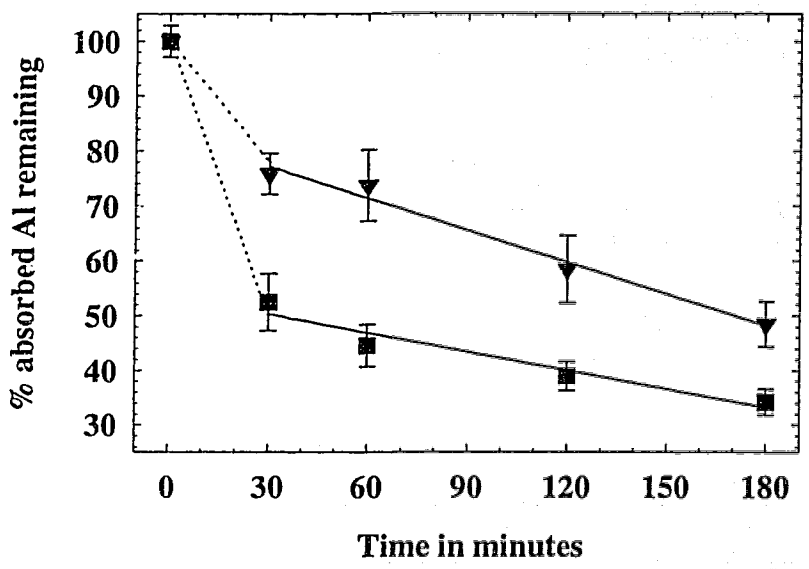
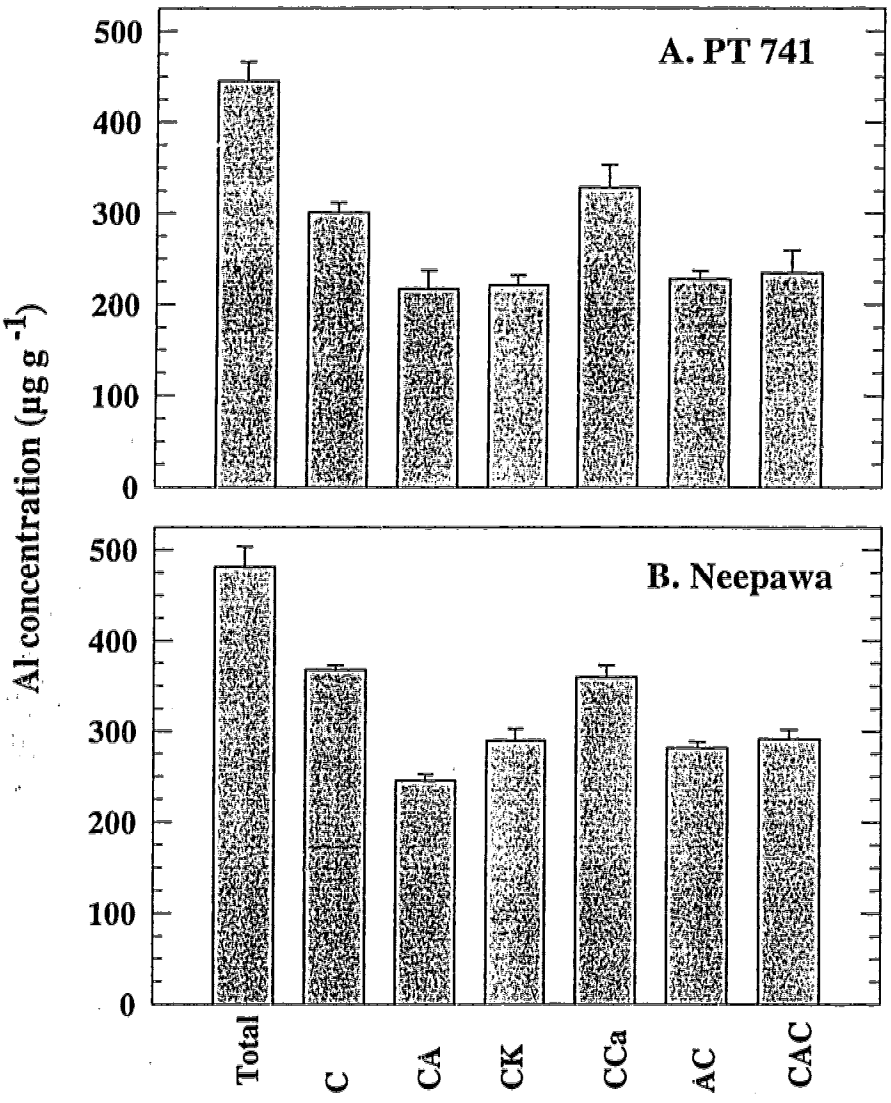


Figure 3.3 Kinetics of Al desorption from roots of an Al-resistant cultivar PT 741 of *T. aestivum* L. exposed to 50 μM $AlCl_3$ in 1.0 mM $CaCl_2$ (pH 4.5, 23°C) for 2 h with the mucilage intact (triangles) or with the mucilage removed (squares) following desorption using a 10 min wash in 1M NH_4Cl (pH 4.5, 23°C). Desorption was carried out in 0.5 mM citric acid (pH 4.5, 0°C). Values represent means of 5 replicates ± S.E.

Figure 3.4 Aluminum remaining in pretreated roots (2 cm) of *T. aestivum* L. after various desorption protocols. Roots were exposed to 50 μM $AlCl_3$ in 1.0 mM $CaCl_2$ (pH 4.5, 23°C) for 2 h and subjected to: a 30 min desorption in 0.5 mM citric acid (pH 4.5, 0°C) (C); a 10 min wash in 1M NH_4Cl (pH 4.5, 23°C) followed by a 30 min desorption in 0.5 mM citric acid (pH 4.5, 0°C) (AC); a 30 min desorption in 0.5 mM citric acid (pH 4.5, 0°C) followed by a 10 min wash in 1M NH_4Cl (pH 4.5, 23°C) (CA); a 30 min desorption in 0.5 mM citric acid (pH 4.5, 0°C) followed by a 10 min wash in 1M KCl (pH 4.5, 23°C) (CK); a 30 min desorption in 0.5 mM citric acid (pH 4.5, 0°C) followed by a 10 min wash in 1M $CaCl_2$ (pH 4.5, 23°C) (CCa); a 30 min desorption in 0.5 mM citric acid (pH 4.5, 0°C) followed by a 10 min wash in 1M NH_4Cl (pH 4.5, 23°C) and a second citric acid treatment (CAC). Values represent means of 5 replicates ± S.E.


A. PT 741
B. Neepawa
Al concentration (µg g-1)
500
400
300
200
100
0
Total
C
CA
CK
CCa
AC
CAC

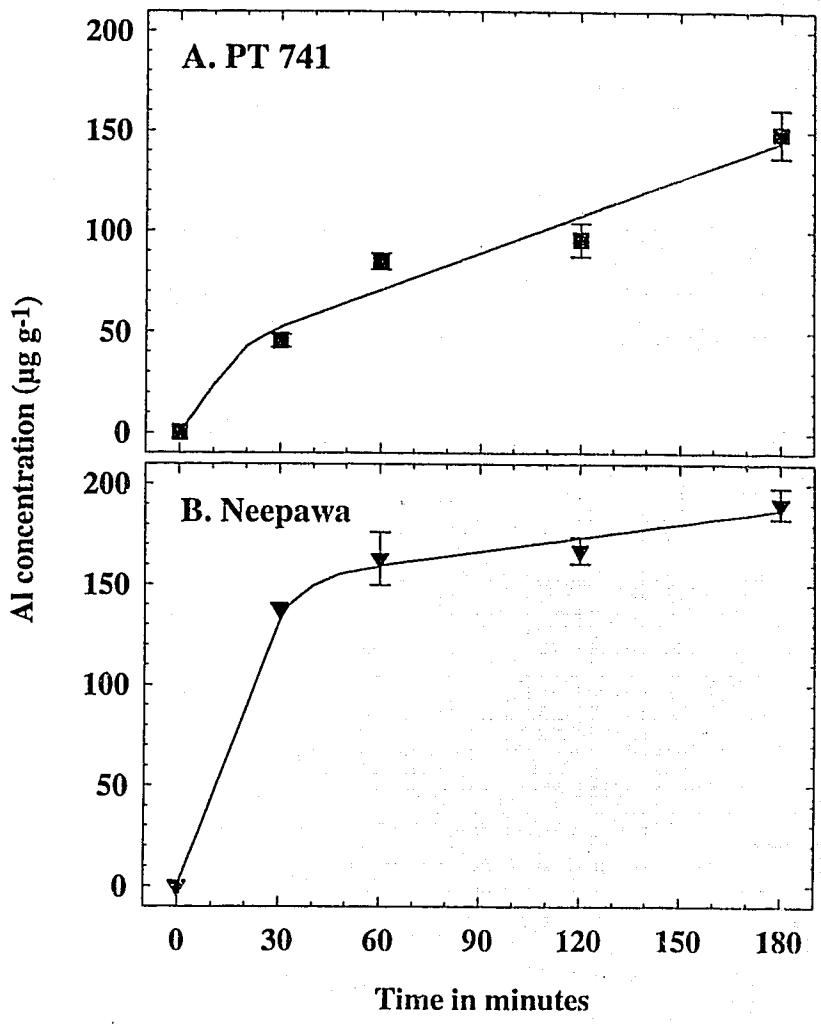


Figure 3.5 Kinetics of Al uptake into the mucilage of (A) an Al-resistant cultivar PT 741 and (B) an Al-sensitive cultivar Neepawa of *T. aestivum* L. Roots were exposed to 50 µM $AlCl_3$ in 1.0 mM $CaCl_2$ (pH 4.5, 23°C), desorbed for 30 min in 0.5 mM citric acid (pH 4.5, 0°C), and washed in 1 M NH_4Cl for 10 min (pH 4.5, 23°C). A sample of the NH_4Cl was taken and analyzed for Al content without further preparation. Values represent means of 5 replicates ± S.E.

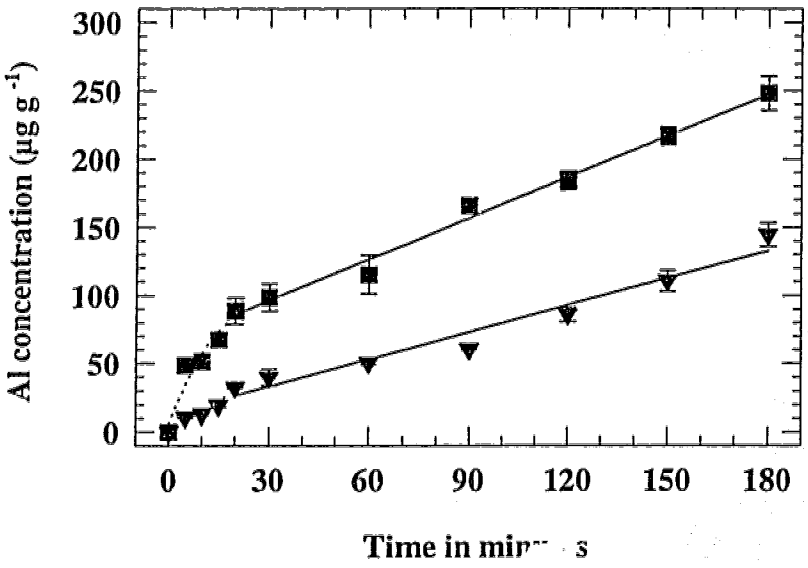


Figure 3.6 Kinetics of Al uptake in excised roots (2 cm) of the Al-resistant cultivar PT 741 of *T. aestivum* L. Roots were exposed to 50 µM $AlCl_3$ in 1.0 mM $CaCl_2$ (pH 4.5, 23°C) for 0, 5, 10, 15, 20, 30, 60, 90, 120, 150 and 180 min, followed by either a 30 min desorption in 0.5 mM citric acid at pH 4.5, 0°C (squares) or a 30 min desorption in 0.5 mM citric acid at pH 4.5, 0°C and a 10 min wash in 1M NH_4Cl at pH 4.5, 23°C (triangles). Values represent means of 5 replicates ± S.E.

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4. Spatial variation in the kinetics of aluminum (Al) uptake in roots of wheat (*Triticum aestivum* L.) exhibiting differential resistance to Al. Further evidence for metabolism-dependent exclusion of Al.

4.1 Introduction

One of the most rapid and dramatic effects of aluminum (Al) on plants is inhibition of root growth in Al-sensitive genotypes (Taylor, 1988). Typically, the main axis of roots is inhibited and the roots become stubby, thickened, brown, brittle and occasionally necrotic. At the cellular level, one of the first signs of Al stress is a disorganization of the plasma membrane and disruption of normal functioning of the nucleus (Bennet *et al.*, 1985), where mitosis is arrested (Rengel, 1992). Aluminum is also thought to inhibit cell elongation by decreasing cell wall elasticity (Klimashevskii and Dedov, 1975), or by inhibiting cell wall synthesis (Huck, 1972). Ryan *et al.* (1993) recently demonstrated that the primary lesions responsible for the Al-induced inhibition of root growth are located at the root apex. Selective exposure of the first 10 to 15 mm of the root tip to Al resulted in ihibition of growth while application of Al to more mature areas of the root had little or no effect. The unique sensitivity of the root apex may reflect the intense metabolic acitivity associated with actively dividing and expanding cells. Decreased rates of mitosis have been shown to be associated with the accumulation of Al in the root apex (Clarkson, 1965; Horst *et al.*, 1982, 1983). As of yet, however, a direct link between accumulation of Al in the symplasm and expression of Al toxicity has not been established.

It is now well established that plant species, cultivars and ecotypes differ in their sensitivity to Al (Taylor, 1988), and kinetic analysis of Al uptake in Al-resistant and Al-sensitive cultivars of *Triticum aestivum* suggests that resistance may be mediated by

exclusion of Al at the plasma membrane (Zhang and Taylor, 1990). If exclusion does play a role in mediating resistance to Al, this phenomenon should be most clearly expressed at the root apex. Several recent studies have provided experimental support for this hypothesis. Rincon and Gonzales (1992) used a kinetic approach to show that an Al-sensitive cultivar of *Triticum aestivum* accumulated more Al (total Al) in 2 mm root tips than did an Al-resistant cultivar. Near-isogenic lines of *T. aestivum* differing in resistance to Al also showed differences in total Al uptake after exposure of 2-3 mm root tips to Al for 4h, and differences became greater over the remainder of a 16 h experimental period (Delhaize *et al.*, 1994). While these studies suggest that Al-resistant genotypes may be capable of limiting Al uptake into the sensitive root apex, the protocols employed did not allow differentiation between apoplastic and symplastic Al. By virtue of the experimental design, these studies can not provide conclusive evidence for exclusion mechanisms that may operate at the plasma membrane.

While direct measurement of Al uptake in the symplasm of complex tissue is still problematic, recent advances in kinetic protocols provide an operational definition of symplastic Al that appears to be virtually free of contamination from the cell wall, plasma membrane and the mucilage. Archambault *et al.* (1996b) provided evidence that the presence of non-exchangeable Al in the cell wall is salt-, concentration- and time-dependent. They demonstrated that use of simple uptake solutions containing 50 μM $AlCl_3$ and 1.0 mM $CaCl_2$ followed by a 30 min desorption in 0.5 mM citric acid effectively eliminated accumulation of non-exchangeable Al in the apoplasm. Under these conditions, tight binding of Al to the microsomal membrane fraction accounted for less than 4% of the putative symplastic compartment. In a subsequent paper, Archambault *et al.* (1996a) demonstrated that a 10 min wash in NH_4Cl effectively removed the mucilage eliminating another important pool of extracytosolic Al. We have made use of this new kinetic protocol to measure the spatial variation of Al uptake in

roots of an Al-resistant cultivar (PT 741) and an Al-sensitive cultivar (Neepawa), and examined the effects of the metabolic inhibitor and protonophore, DNP, on Al uptake in these two wheat genotypes. Our results suggest that resistance may be mediated by metabolism-dependent exclusion mechanisms operating in cells of the meristematic region of the root.

4.2 Methods and Materials

4.2.1 Preparation of plant material

Seeds of an Al-resistant (PT 741) and an Al-sensitive (Neepawa) cultivar of *Triticum aestivum* L. were surface sterilized in 1.2 % sodium hypochlorite for 20 min and germinated for 48 h in 0.005 g/L Vitavax to limit fungal growth. Following germination, seedlings were transferred to nylon mesh floated in aquaria containing 15 L a nutrient solution (pH 4.5) containing (mM) 3.30 NO_3^--N, 0.30 NH_4^+-N, 0.10 P, 0.80 K, 1.00 Ca, 0.30 Mg, 0.10 S; and (mM) 34 Cl, 60 Na, 10 Fe, 6 B, 2 Mn, 0.15 Cu, 0.5 Zn, and 0.1 Mo. Plants were grown for 6 days on a lab bench in constant light at room temperature (~23°C).

4.2.2 Short-term uptake experiments

Thirty root tips (20 mm) were excised, placed into absorption tubes, and submerged in an aerated nutrient solution until excision was complete (<60 min). Roots were transferred to 1.0 mM $CaCl_2$ (pH 4.5, 23°C) and allowed to equilibrate for 30 min. Uptake experiments were initiated by transferring the tubes to 80 ml glass jars containing 50 μM $AlCl_3$ in 1.0 mM $CaCl_2$ (pH 4.5, 23°C) for various exposure periods. Following exposure to Al, roots were rinsed thoroughly with deionized, distilled water. In some experiments, this was followed by a 30 min desorption treatment in 0.5 mM citric acid at pH 4.5 and 0°C to remove exchangeable Al from the apoplasm and a 10 min wash in 1 M NH_4Cl at pH 4.5 and 23°C to remove the mucilage. Roots were rinsed with distilled, deionized water, further segmented if necessary (0 - 5 mm, 5 - 10 mm, 10 - 20 mm, or 5 - 20 mm segments), dried in an oven at 55°C, and weighed. To prepare samples for analysis, roots were ashed in a muffle furnace at 500°C, solubilized in concentrated

HNO_3, diluted using deionized, distilled water and analyzed for Al using graphite furnace atomic absorption spectrophotometry (GFAAS) using techniques described by Zhang and Taylor (1989).

4.2.3 Long-term uptake experiments

Plants were prepared for experimentation as described above. After 7 days of growth, plants growing on nylon mesh were removed from aquaria containing nutrient solution, rinsed with deionized, distilled water, and placed in aquaria containing 50 μM $AlCl_3$ and 1.0 mM $CaCl_2$ (pH 4.5 and 23°C) for 0, 2, 4 and 6 hours. Following exposure to Al, roots were rinsed with deionized, distilled water and excised 20 mm from the root tip. For each exposure period, 30 root tips were placed into 5 replicate absorption tubes and subjected to desorption (30 min in citric acid) and removal of the mucilage (10 min in NH_4Cl) as described above. The 5 mm root tips were excised from the roots and prepared for Al analysis as described above.

4.2.4 Effects of DNP on aluminum uptake

Excised roots were prepared for experimentation as described in the short-term experiments described above. Uptake experiments were initiated by transferring absorption tubes containing roots to 80 ml glass jars containing 50 ml of an aerated solution of 50 μM Al as $AlCl_3$ in 1.0 mM $CaCl_2$ (pH 4.5, 23°C), with or without 0.1 mM DNP. Five replicate tubes were removed from absorption solutions after 0, 10, 30, 60, 120, and 180 min of uptake and rinsed briefly with deionized, distilled water. Roots were then subjected to a 10 min wash in NH_4Cl (pH 4.5 and 23°C) and a 30 min desorption treatment in 0.5 mM citric acid (pH 4.5 and 0°C). Roots were rinsed with deionized,

distilled water, further segmented as necessary, and prepared for Al analysis as described above.

4.2.5 Statistical analysis

Data points from the linear phase of Al uptake were fitted to a linear regression using SAS release 6.10. Regressions were calculated from t = 10 min to t = 180 min for comparisons between rates of Al uptake between cultivars and for individual cultivars in the presence or absence of DNP. Values for r^2 were never below 0.94. Differences between regressions were tested using a parametric t-test (Zar, 1984), using the following formula;

$$t = b_1 - b_2 / (s_1^2 + s_2^2)^{1/2}$$

where b_1 and b_2 are the regression coefficients (slopes) of regression lines and s_1 and s_2 are the standard errors of b_1 and b_2 respectively. The calculated t values were compared to the t distribution with $v = (n_1 - m) + (n_2 - m)$ degrees of freedom, where n is the number of values for each regression, and m is the number of parameters for each regression (Zar, 1984). For all regressions n = 25 and m = 2, degrees of freedom = 46. Significance was defined as $P \leq 0.001$. Where required, data points from the rapid phase of uptake were joined visually based on earlier, more detailed kinetic data.

4.3 Results

Total uptake of Al in the Al-resistant cultivar, PT 741, did not differ between root tips (0 - 5 mm) and segments from more mature root regions (5 - 10 mm and 10 - 20 mm) after 60 and 180 min exposure to Al (Fig. 4.1). However, when measurements were repeated on roots that had been desorbed in 0.5 mM citric acid (pH 4.5 and 0°C for 30 min) to remove Al from the apoplasm and 1M NH_4Cl (pH 4.5 and 23°C for 10 min) to remove the mucilage (Archambault *et al.*, 1996a), non-exchangeable Al was 33 to 37% greater in root tips than in the 5 - 10 mm and 10 - 20 mm root segments (Fig. 4.1 A and B). Having observed quantitative differences in Al uptake between various root segments, we decided to investigate the possibility that spatial variations in the kinetic patterns of accumulation of non-exchangeable Al also existed. Because quantitative differences between 5 - 10 mm and 10 - 20 mm root segments were small, we compared the kinetic patterns of 5 mm root tips to those of 5 - 20 mm segments, again using the Al-resistant cultivar PT 741. In both root segments, Al remaining after desorption in citric acid and NH_4Cl was nearly linear with time, with a slight deviation from linearity observed during the first 5 min of uptake (Fig. 4.2). In this experiment, the rate of linear phase accumulation of Al in root tips (2.82 $\mu g\ g^{-1}\ min^{-1}$) was more than twice (245%) that of 5 - 20 mm sections (1.15 $\mu g\ g^{-1}\ min^{-1}$).

Several studies have suggested that exclusion may play a role in mediating resistance to Al (see for example: Zhang and Taylor, 1990; Rincon and Gonzales, 1992 and Delhaize *et al.* 1993), although interpretation of data in these studies was complicated by the presence of tightly bound Al in the cell wall, particularly in studies which failed to make use of desorption agents (Rincon and Gonzales, 1992; Delhaize *et al.* 1993). If exclusion does indeed occur, it should be possible to measure quantitative differences in the accumulation of Al between Al-resistant and Al-sensitive cultivars using new

techniques which minimize metabolism-dependent binding of Al in the cell wall (Archambault *et al.* 1996b). Furthermore, because the primary toxic lesions of Al have been shown to be concentrated at the root tip (Ryan *et al.*, 1993), these quantitative differences should be most pronounced in that region of the root. Thus, we compared the kinetics of uptake of non-exchangeable Al in various root regions between the Al-resistant cultivar, PT 741, and the Al-sensitive cultivar, Neepawa. The rate of Al accumulation in 0 - 20 mm root tips did not differ between the two cultivars throughout a 180 min experimental period (Fig. 4.3 A). Similarly, differences between cultivars were not observed in the accumulation of Al in mature root regions (5 - 20 mm segments) (Fig. 4.3 B; Table 4.1). In contrast, the Al-sensitive cultivar, Neepawa, showed nearly a 2-fold greater rate of Al uptake in 5 mm root tips (3.94 $\mu g\ g^{-1}\ min^{-1}$) than the Al-resistant cultivar, PT 741 (2.09 $\mu g\ g^{-1}\ min^{-1}$)(Fig. 4.3 C; Table 4.1). Long exposure studies also showed that differences in Al uptake between the two genotypes continued to increase throughout the 6 h exposure period, with no sign of saturation to the linear phase (Fig. 4.4).

In order to test whether this putative exclusion was linked to metabolism, we amended our Al uptake solutions with 0.1 mM DNP, an inhibitor of oxidative phosphorylation which also acts as a protonophore. In 0 - 20 mm excised roots, the linear phase of uptake in the sensitive cultivar, Neepawa, was not affected by DNP (the 21% decrease was not statistically significant), whereas the rate of Al uptake in the resistant PT 741 increased significantly (86%) (Fig. 4.4 A and B; Table 4.2). Because differences between cultivars in Al uptake were only observed in the root tip region, we decided to determine whether the effect of DNP was also specific to this region of the root. Dinitrophenol had no significant effect on the rate of Al uptake in mature root tissue (5 - 20 mm) in both cultivars (Figs. 4.4 C and D; Table 4.2), while the effect of DNP was accentuated in 0 - 5 mm root tips of the resistant line. Dinitriphenol had no

effect in any root segment in the Al-sensitive Neepawa (Figs. 4.4 E and F). In the Al-resistant, PT 741, DNP increased the rate of Al uptake by 142%, bringing the rate of Al uptake to 4.20 μg g^{-1} min^{-1}, a value 49% greater than that observed in the Al-sensitive Neepawa (2.81 μg g^{-1} min^{-1}) (Table 4.2).

4.4 Discussion

In contrast to the results of Polle *et al.* (1978) and Wallace *et al.* (1982) which showed that Al levels were greater in the apical region (0 - 5 mm) of the root, than in mature regions, we found no such differences when measuring total Al (Fig 4.1 A and B). However, when roots were desorbed using a 30 min wash in 0.5 mM citric acid (pH 4.5 and 0°C) to remove exchangeable Al from the apoplasm and subsequently washed in 1 M NH_4Cl (pH 4.5 and 23°C) for 10 min to remove the mucilage, quantitative differences in Al uptake between root segments did become evident, with the root tips accumulating up to 37% more Al than the more mature root regions. As previously described for longer root segments (20 mm; Zhang and Taylor 1989, Archambault *et al.* 1996a,b), uptake in root tips (0 - 5 mm) and mature tissue (5 - 20 mm) was biphasic, with a rapid phase (occurring in the first 5 min) superimposed over a linear phase occurring throughout the 180 min experimental period (Fig. 4.2). Quantitative differences in the accumulation of Al in regions of different maturity and possible spatial differences in accumulation of Al between cultivars might explain the fact that the current literature does not universally support the hypothesis that Al resistance may be achieved via the exclusion of Al from the actively growing regions of the root. In order to gain evidence for exclusion, comparisons between sensitive and resistant genotypes should be concentrated on the root tip.

Previous studies by Polle *et al.* (1978) and Wallace *et al.* (1982) found that hematoxylin (a stain used for the localization of Al in plant tissue), stained root apices of Al-sensitive wheat genotypes more intensely than those of Al-resistant genotypes. While Ownby (1993) presented evidence that selective hematoxylin staining of Al-sensitive wheat cultivars likely results from direct damage by Al to roots, leading to leakage of phosphorous into the cell wall region and the subsequent complexation of hematoxylin

with Al-phosphorus groups, the results of Polle *et al.* (1978) and Wallace *et al*, (1982) suggested that resistance to Al might be a root apex phenomenon. More recently, Rincon and Gonzales (1992) also showed that root tips (0 - 2 mm segments) of an Al-sensitive cultivar of wheat accumulated more Al than those of an Al-resistant cultivar. Using a differential staining procedure, Delhaize *et al.* (1994) also demonstrated isoline-specific differences in long exposure studies. While the above-mentioned studies do provide evidence of resistance, the techniques used in these studies did not differentiate between Al in the apoplastic and symplastic pools and thus, are of limited value in evaluating the role of Al exclusion mechanisms that may operate at the plasma membrane.

Using a protocol that allowed for an operational definition of apoplastic and symplastic Al, Tice *et al.* (1992) showed that an Al-resistant cultivar accumulated less Al in the putative symplastic compartment than did an Al-sensitive cultivar, when both were supplied with the same Al concentration. However, subsequent experiments have demonstrated that Tice *et al.*'s use of $CaCl_2$ as a desorption agent may not have been effective in removing Al from the cell wall (Archambault *et al.* 1996a) which may have led to overestimations of Al in the putative symplastic compartment. Furthermore, because of the long term nature of their studies (48 h), differences in root growth between Al-resistant and Al-sensitive cultivars may have contributed to dilution of Al in root tissue of the Al-resistant cultivar and might account for observed differences in putative symplastic Al levels. To minimize the problems associated with differential root growth during experimentation, we employed short-term experiments (3 h) in which root growth is negligible. We also used a protocol developed in our laboratory, which provides an estimate of non-exchageable Al with little contamination from apoplastic sources. When we compared rates of Al uptake in various root segments between genotypes differing in their ability to tolerate Al, we found that accumulation of non-exchangeable Al in 5 mm root tips was approximately 2-fold more rapid in the Al-sensitive cultivar than in the Al-

resistant cultivar (Fig. 3C; Table 4.1). No differences were observed in the mature root regions (Fig. 4.3 B; Table 4.1). In agreement with the results of Delhaize *et al.* (1993), we found that differences in Al accumulation between genotypes in the root tip region continued to increase for up to 6 h with no sign of saturation (Fig. 4.4). Our experiments also help to explain why Zhang and Taylor's (1989) studies failed to provide evidence for differences between genotypes. Use of conditions conducive to the accumulation of Al in the apoplasm (as demonstrated by Archambault *et al.*, 1996a) and the use of relatively long root segments may have prevented Zhang and Taylor from measuring differences in Al uptake between wheat cultivars under normal metabolic conditions, differences which only became apparent in the presence of DNP (Zhang and Taylor, 1991).

Our results suggest that the Al-resistant cultivar, PT 741, has the ability to limit the accumulation of non-exchangeable Al in the highly sensitive meristematic root tip region. Such differences in Al uptake between genotypes could account for resistance. While some researchers have suggested that exclusion is not important in conveying resistance to Al (Haug and Caldwell, 1985; Roy *et al.*, 1988), our results suggest otherwise. In order to investigate the possibility that Al exclusion may be metabolism-dependent, we examined the effects of DNP, on the linear phase of Al uptake. As previously described by Zhang and Taylor (1989), the effects of DNP were specific to the resistant plants (Fig 4.5). Moreover, we found the effect of DNP to be localized at the root tip. Dinitrophenol prompted an 86% increase in the rate of Al uptake in 2 cm root tips of the Al-resistant PT 741. This effect however, was not a whole root phenomenon. Treatment with DNP had no effect on Al uptake in the mature regions of the root, but had a profound effect (143%) at the root tip (0 - 5 mm segment) where it increased Al uptake to a level 24% higher than the Al-sensitive cultivar (Fig. 4.5 E). While the effects of DNP are not fully understood, our results do suggest that a DNP-sensitive exclusion mechanism operates at the root tip of this Al-resistant cultivar. At this point, we cannot

explain why DNP would increase uptake in PT 741 to a level in excess of that observed in Neepawa, although it remains possible that other factors differentially affect the rate of Al accumulation in these cultivars.

The precise identity of mechanisms that would allow resistant genotypes to limit the entry of Al into the symplasm remains to be elucidated although current data suggests that exclusion may be achieved by the exudation of chelators such as malate (Basu *et al.*, 1994a; Delhaize *et al.*, 1994) citrate (Miyasaka *et al.*, 1991; Pellet *et al.*, 1995) or low molecular weight polypeptides (Basu *et al.*, 1994b).

Table 4.1 Rates of Al uptake (μg Al g^{-1} min^{-1}) in various segments of excised roots of an Al-resistant (PT 741) and an Al-sensitive (Neepawa) cultivar of *Triticum aestivum* L.

Root segment	Cultivar	Rate of Al uptake (μg g^{-1} min^{-1})
0 -20 mm	PT 741	1.12
	Neepawa	1.37
	% difference	12.2
5 - 20 mm	PT 741	1.04
	Neepawa	1.07
	% difference	2.9
0 - 5 mm	PT 741	2.09
	Neepawa	3.94
	% difference	88.5***

*** Indicating a significant difference in the rate of Al uptake between the cultivars at $P \leq 0.001$.

Table 4.2 Rates of Al uptake (μg Al g^{-1} min^{-1}) by excised roots of an Al-resistant (PT 741) and an Al-sensitive (Neepawa) cultivar of *Triticum aestivum* L. from absorption solutions in the presence or absence of 0.1 mM DNP.

Root segment		Al-resistant cultivar PT 741	Al-sensitive cultivar Neepawa
0 -20 mm	Control	0.79	1.41
	DNP treatment	1.47	1.11
	% increase	86.1***	-21.3
5 - 20 mm	Control	0.57	0.88
	DNP treatment	0.72	1.07
	% increase	26.3	21.6
0 - 5 mm	Control	1.73	2.87
	DNP treatment	4.20	2.81
	% increase	142.8***	-2.1

*** Indicating a significant difference in the rate of Al uptake between the treatment and the control at $P \leq 0.001$.

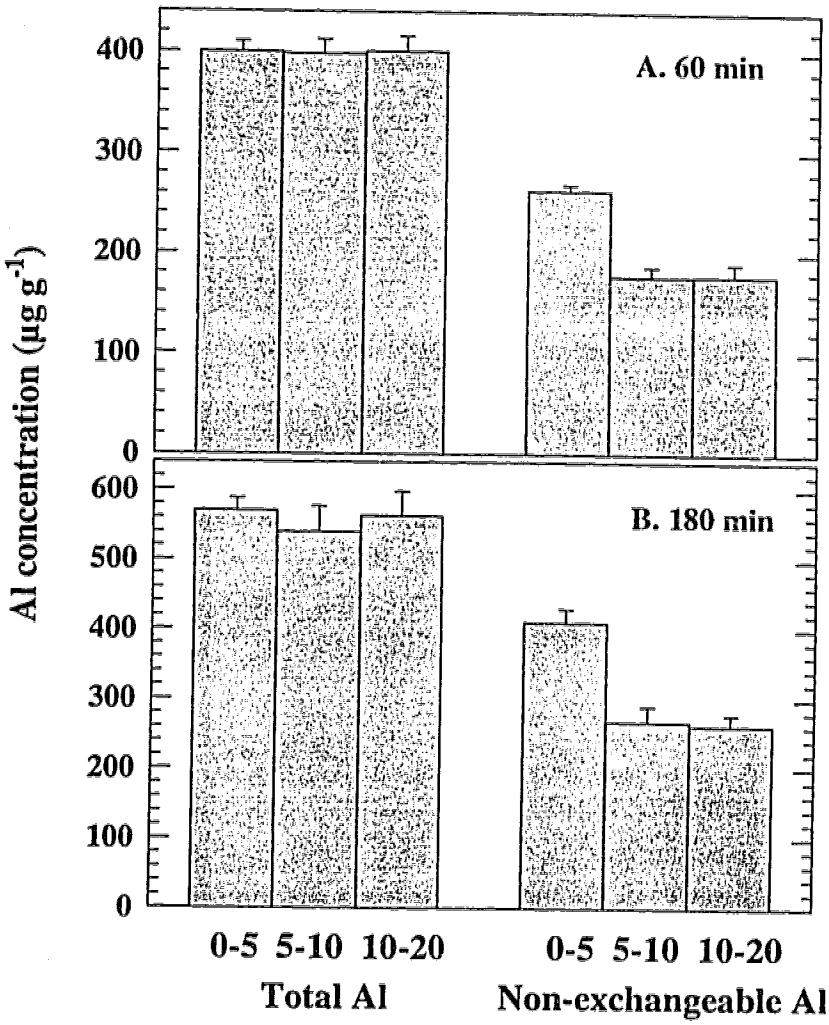


Figure 4.1 Accumulation of total and non-exchangeable Al in (a) 0-5 mm root segments, (b) 5 - 10 mm segments, and (c) 10 - 20 mm segments of the Al-resistant cultivar PT 741. Two cm root tips were exposed to 50 µM $AlCl_3$ in 1.0 mM $CaCl_2$ (pH 4.5, 23°C) for (A) 60 min or (B) 180 min with (non-exchageable Al) or without (total Al) desorption in 0.5 mM citric acid (pH 4.5, 0°C) for 30 min and a 10 min wash in 1M NH_4Cl (pH 4.5, 23°C) and subsequently excised into segments. Values represent means of 5 replicates ± S.E.

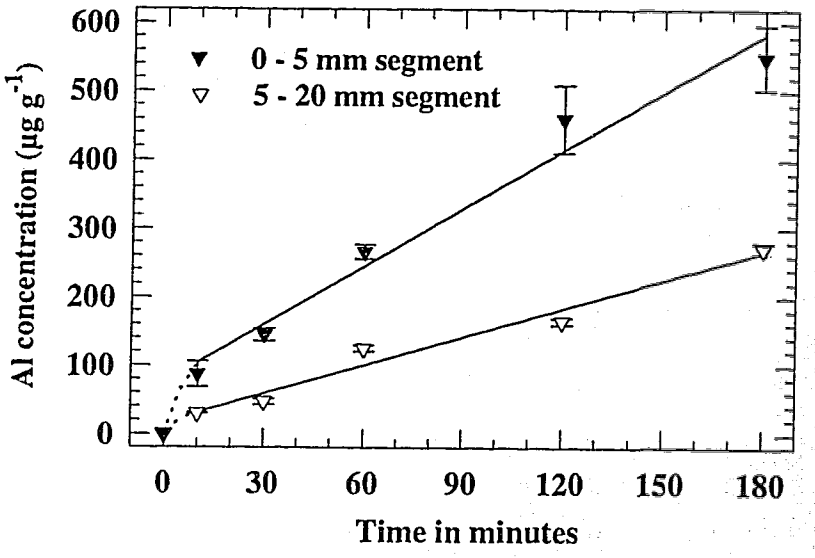
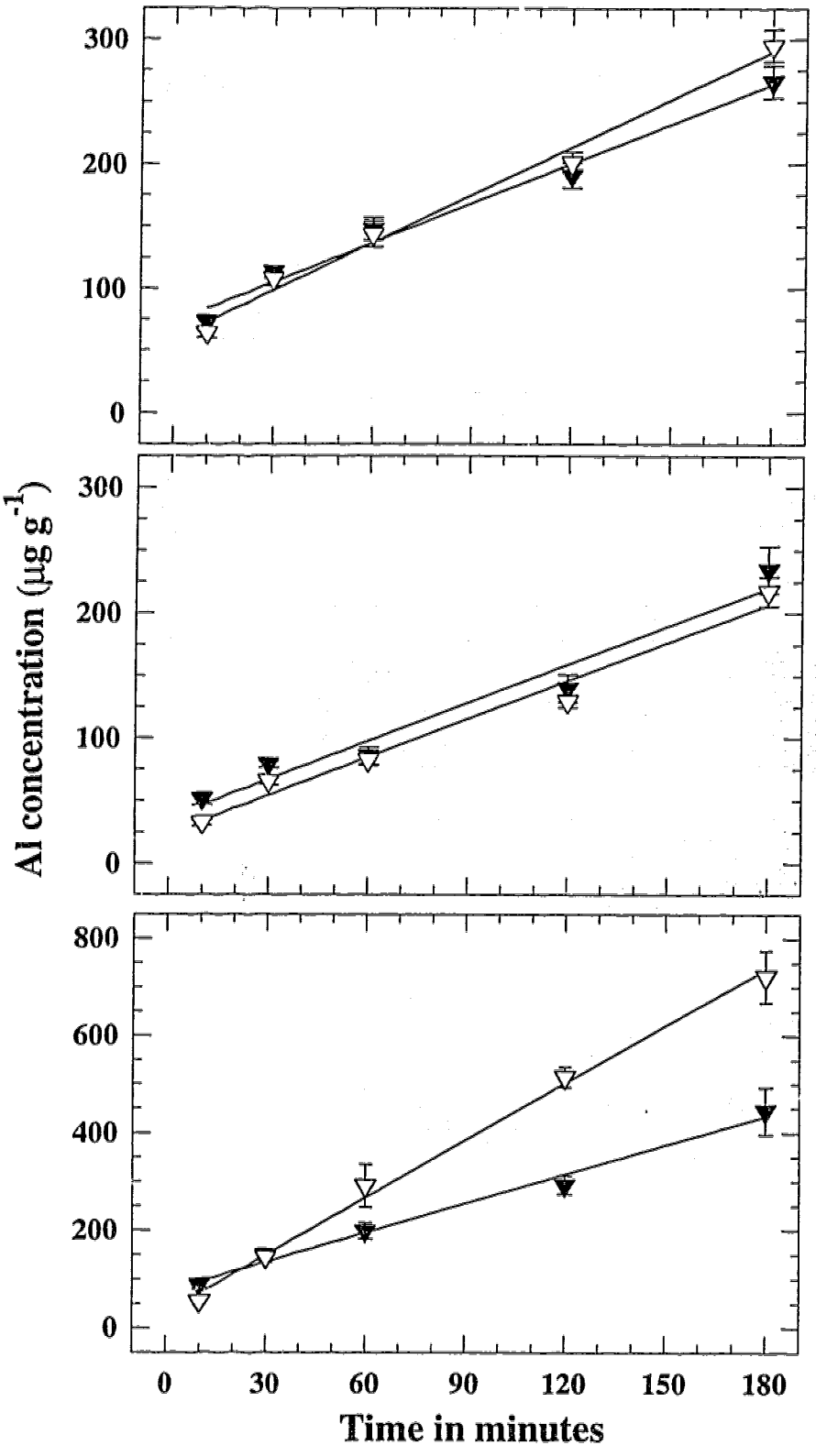


Figure 4.2 Kinetics of Al uptake into 0 - 5 mm root segments and 5 - 20 mm segments of roots of the Al-resistant cultivar PT 741. Excised roots were exposed to 50 µM $AlCl_3$ in 1.0 mM $CaCl_2$ (pH 4.5, 23°C) for 0, 10, 30, 60, 120 and 180 min followed by a 30 min desorption in 0.5 mM citric acid (pH 4.5, 0°C) and a 10 min wash in 1M NH_4Cl (pH 4.5, 23°C). Values represent means of 5 replicates ± S.E.

Figure 4.3 Kinetics of Al uptake into (A) 0 - 20 mm root tips, (B) 5 - 20 mm root segments and (C) 0 - 5 mm root tips of the Al-resistant cultivar PT 741 and the Al-sensitive cultivar Neepawa. Roots were exposed to 50 μM $AlCl_3$ in 1.0 mM $CaCl_2$ (pH 4.5, 23°C) for 0, 10, 30, 60, 120 and 180 min followed by a 30 min desorption in 0.5 mM citric acid (pH 4.5, 0°C) and a 10 min wash in 1M NH_4Cl (pH 4.5, 23°C). Values represent means of 5 replicates $\pm$ S.E.


Al concentration (μg g-1)
300
200
100
0
300
200
100
0
800
600
400
200
0
0
30
60
90
120
150
180
Time in minutes

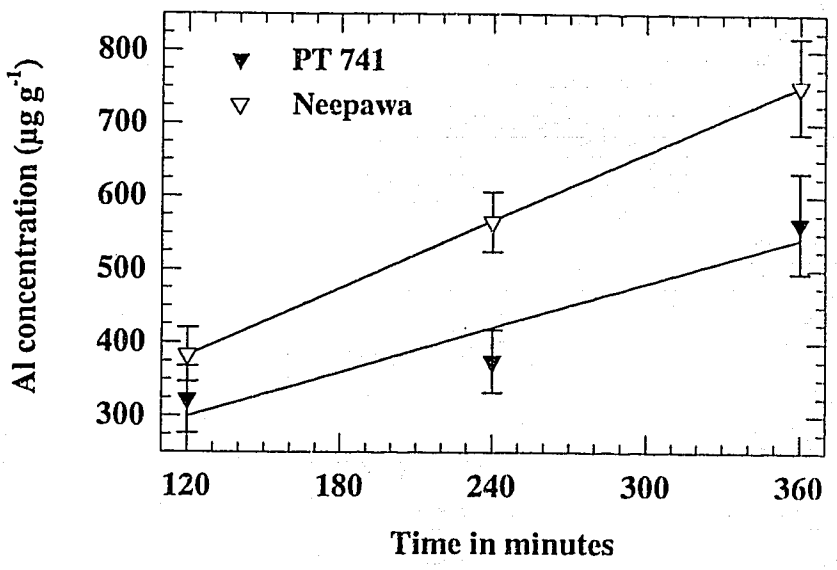
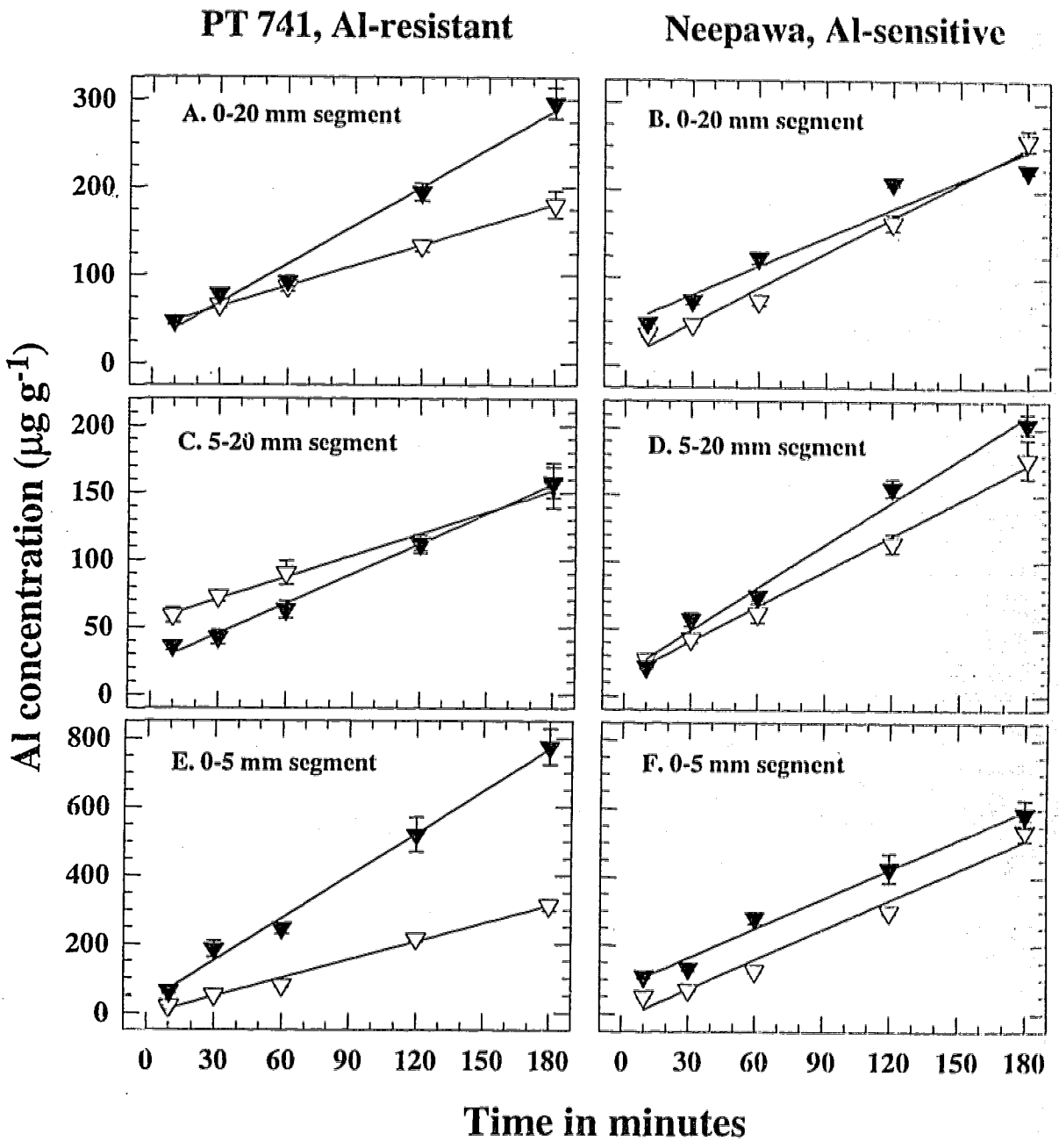


Figure 4.4 Kinetics of Al uptake into 0 - 5 mm segments of excised roots of the Al-resistant cultivar PT 741 and the Al-sensitive cultivar Neepawa. Roots were exposed to 50 µM $AlCl_3$ in 1.0 mM $CaCl_2$ (pH 4.5, 23°C) for 0, 120, 240 and 360 min followed by a 30 min desorption in 0.5 mM citric acid (pH 4.5, 0°C) and a 10 min wash in 1M NH_4Cl (pH 4.5, 23°C). Values represent means of 5 replicates $\pm$ S.E.

Figure 4.5 The effect of DNP on the linear phase of uptake in various root segments of an Al-resistant cultivar PT 741 and an Al-sensitive cultivar Neepawa. Uptake in 0 - 20 mm root tips of PT 741 (A) and Neepawa (B). Uptake in a 5 - 20 mm root portion of PT 741 (C) and Neepawa (D). Uptake in root tips (0 - 5 mm) of PT 741 (E) and Neepawa (F). Roots were exposed to 50 μM $AlCl_3$ in 1.0 mM $CaCl_2$ (pH 4.5, 23°C) with or without 0.1 mM DNP for 10, 30, 60, 120 and 180 min, followed by a 30 min desorption in 0.5 mM citric acid (pH 4.5, 0°C) and a 10 min wash in 1 M NH_4Cl (pH 4.5, 23°C). Rates of uptake are compared in Table 4.1. Values represent means of 5 replicates $\pm$ S.E.


PT 741, Al-resistant
Neepawa, Al-sensitive
A. 0-20 mm segment
B. 0-20 mm segment
C. 5-20 mm segment
D. 5-20 mm segment
E. 0-5 mm segment
F. 0-5 mm segment
Al concentration (μg g-1)
Time in minutes
0
30
60
90
120
150
180
0
100
200
300
0
50
100
150
200
0
200
400
600
800

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5. General Discussion and Conclusions

5.1 Kinetic studies

5.1.1 Uptake and desorption in roots and cell wall material

In the present study, short-term experiments were used to characterize the kinetics of Al uptake and desorption in roots of *Triticum aestivum* L. exposed to two different Al salts namely, $AlK(SO_4)_2$ and $AlCl_3$. With both salts the kinetics of Al uptake by excised roots was biphasic, with a rapid phase in the first 30 minutes followed by a linear phase occurring throughout the remainder of the experimental period (180 min). The time course of desorption using citric acid, a ligand capable of forming stable complexes with Al, was also biphasic in both cases. While a major portion of the rapid phase of uptake could be removed through desorption, the fact that the rapid phase could not be totally eliminated suggested that Al remained in the apoplasm or that a rapidly accumulating non-exchangeable pool of Al existed in the symplasm. Thus, further experiments were undertaken to better characterize the components of these phases of uptake.

Although the linear phase is classically interpreted as representing the symplastic compartment, the precise identity of the linear phase has previously been questioned (Zhang and Taylor, 1990). The possibility that the linear phase included both an apoplastic and symplastic compartment was first suggested by Zhang and Taylor (1989), who noted differences in the estimated size of the apoplastic compartment as determined by extrapolation of the linear phase of uptake and the linear phase of desorption to time zero. Also, the reduced rate, but non-saturable nature of uptake at 0°C suggested a non-metabolic component of the linear phase of Al uptake. This was later confirmed with kinetic and fractionation studies. These studies demonstrated the existence of a linear

phase of adsorption in the purified cell wall fraction which was not removable by desorption in citric acid. In the present study, I examined the possibility of developing a kinetic protocol which would allow for a more complete desorption of Al from the apoplasm and hence, a more accurate estimation of uptake of Al into the symplasm. My results showed that by using low concentrations of Al in simple salt solutions, citric acid as a desorption agent, and short exposures, it is possible to limit the contribution of non-exchangeable Al in the cell wall to total non-exchangeable Al, which I have putatively assigned to the symplastic fraction.

I have been careful throughout this thesis to refer to the non-exchangeable fraction in these new kinetic studies as the "putative symplastic fraction". This is because unambiguous assignment of non-exchangeable Al to a specific cellular pool will not be possible using a kinetic approach with complex tissues. Because thermodynamic considerations predict that Al should bind tenaciously to negatively charged sites within the cell wall, it is reasonable to suggest that complete chemical desorption of this Al would be difficult. This is a concern, since the cell wall has traditionally been viewed as the dominant pool of Al in roots. If desorption is incomplete and the wall remains the dominant pool of non-exchangeable Al, it is possible that redistribution of even a small portion of this pool during fractionation could contaminate the smaller pool in the cytoplasm. This would result in an overestimation of Al in the symplastic fraction.

While I cannot discount this possibility, my experiments with purified cell wall material suggest that a 30 min wash in citrate is effective in removing Al from the cell wall in short term experiments using my revised kinetic protocols. Less than 10% of non-exchangeable Al was present in purified cell wall material; the dominant pool of Al was in the putative symplastic fraction. This observation is inconsistent with a massive pool of Al in the cell wall contaminating the smaller cytoplasmic fraction during fractionation.

Given the relative size of the cell wall and putative symplastic fractions observed in my studies, a major redistribution of non-exchangeable Al would be required.

In response to this, it is possible to argue that I have underestimated the true amount of non-exchangeable Al in the cell wall. Dr. Leon Kochian (personal communication) has suggested that the techniques I have used to isolate cell wall material (desorption, homogenization, cell disruption, sonication, and filtration) are harsh and thus, could lead to mechanical removal of Al from the cell wall, Al which might normally be present in the wall after desorption in citrate. This view arises from experiments on uptake of zinc (Zn) into giant algal cells of *Chara corallina*. Using a similar cell wall isolation technique as I have used, they found that Zn in the cell wall was not desorbed by citrate, but was lost from cell wall material during cell wall isolation (Dr. Leon Kochian, personal communication). However, comparisons between experiments using different cations, conditions, test organs, and species are difficult and can only be seen as speculative. Furthermore, my cell wall isolation techniques were not effective in removing non-exchangeable Al from the cell wall in long term experiments, in experiments using high concentrations ($\geq$ 75 μM) of Al, and in experiments conducted with *Chara corallina* (unpublished results).

Interestingly, the possibility that Al present in the cytoplasm might contaminate the cell wall during the process of fractionation has also been raised. If this were true, my revised kinetic protocols would underestimate the amount of Al in the putative symplastic fraction. This possibility was discounted by Zhang and Taylor (1990), but is attractive because it might be used to reconcile differences between my experiments and those of Tice *et al.* (1992). My experiments showed that Al in purified cell wall material was a significant component of non-exchangeable Al in studies using $CaCl_2$ as a desorption agent. In contrast, microscopic observations of desorbed roots stained with the

fluorophore morin led Tice *et al.* (1992) to conclude that a lengthy desorption in $CaCl_2$ was sufficient to remove Al from cell wall material. In chapter 2, I argued that the fluorophore morin may not be capable of forming a fluorescent complex with Al tightly bound to the cell wall, and measurement of Al in purified cell wall material is a more direct measure of non-exchangeable Al in this subcellular fraction.

While I cannot prove that the non-exchangeable fraction isolated using my new kinetic protocol is in fact an accurate estimate of Al in the symplasm, results from several of my subsequent experiments are consistent with that interpretation.

5.1.2 Characterization, measurement and removal of Al from root mucilage

While the results from studies described in the first experimental chapter suggest that my new kinetic protocol was effective in limiting the accumulation of tightly-bound Al in the cell wall, the persistence of a rapid phase of uptake in excised roots following desorption using citric acid, suggested the existence of other non-exchangeable pools of apoplastic Al. I speculated that citric acid may not be fully effective in desorbing Al from the mucilage. In order to test this hypothesis, I investigated the contribution of mucilage-bound Al to the non-exchangeable pool and the possibility of removing the mucilage in order to isolate the linear phase of uptake in roots. I found that the root mucilage was a significant pool of apoplastic Al which had the potential to contribute to both the rapid phase and the linear phase of uptake. Using a wash in NH_4Cl, it was possible to eliminate this pool of Al, thus allowing for the isolation of the linear phase of Al uptake with only slight deviation from linearity occurring in the first 5 minutes.

5.1.3 A new kinetic protocol for the comparison of Al uptake in Al-resistant and Al-sensitive cultivars

Clearly the classical interpretation of the identity of the linear phase must be applied with caution in studies with Al. Under certain conditions, the linear phase of uptake is composed of Al from both symplastic and apoplastic compartments. However, my newly developed kinetic protocol minimizes the accumulation of tightly-bound Al in apoplastic pools and provides a putative estimate of symplastic Al. With these new techniques at hand, I decided to compare the rates of Al uptake in Al-resistant and Al-sensitive cultivars.

Initially, these comparisons were made on 2 cm root tips as previously used by Zhang and Taylor (1989, 1990, 1991). Much to my disappointment, I found no differences between the two cultivars. It was at that time that studies by Rincon and Gonzales (1992) reported that cultivars which differed in their ability to resist Al showed differences in total Al uptake in short root portions (2 mm). A year later, Ryan *et al.* (1993) demonstrated that the root tip was the only part of the root which was sensitive to Al. These two studies prompted me to investigate uptake of Al in root tips using my new protocol. When 10 mm root tips of the Al-sensitive (Neepawa) and the Al-resistant (PT 741) cultivars were used, I found a greater rate of Al uptake in the sensitive cultivar than in the resistant cultivar. Encouraged by these results, I then decided to use even shorter root segments to see whether the effect would be magnified as I approached the root tip. Using 0.5 cm root tips, I found that the rate of Al uptake was approximately twice as great in the sensitive cultivar as that of the resistant cultivar. Furthermore, no differences were observed in the more mature root tissue. While these results suggest that the resistant cultivar is capable of limiting the rate of accumulation of Al in the symplasm of

cells in the meristematic region, I was also interested in determining whether this phenomenon was metabolism-dependent.

5.2 The use of 2,4-dinitrophenol

In previous studies, increased uptake of Al in roots treated with DNP has been reported in *T. aestivum* L. (Pettersson and Strid, 1989; Zhang and Taylor, 1989), *Beta vulgaris* (Lindberg, 1990), and in several other species (Huett and Menary, 1979). Zhang and Taylor (1990) found that DNP increased Al uptake in Al-resistant cultivars of *T. aestivum* L., but had no effect on sensitive cultivars. They suggested that DNP might inactivate metabolism-dependent exclusion mechanisms, which could be important components of an integrated Al resistance strategy. However, because Zhang and Taylor's protocol was not completely effective in limiting accumulation of non-exchangeable Al in the apoplasm, they could not imply that the effects of DNP were indeed due to inhibition of an exclusion mechanism operating at the plasma membrane. In the present study, similar effects of DNP were found using my improved kinetic protocol, to measure Al in the putative symplastic fraction. In addition, I found that the effects of DNP on the resistant cultivar were most pronounced at the root tip. This is consistent with the implication that the root tip is the region most sensitive to Al stress, and hence the region most likely to exhibit active resistance mechanisms. Since DNP has multiple effects on plant metabolism, the way which metabolism-dependent accumulation of Al relates to the effect of DNP is not clear. Nevertheless, several proposed exclusion mechanisms may be consistent with the above observations.

5.3 Possible metabolism-dependent exclusion mechanisms

In the present study, differences in the rate of Al uptake at the root tip between cultivars were observable within minutes of exposure to uptake solutions. It would therefore appear that the exclusion mechanisms employed do not require induction, or that induction is rapid. Perhaps efflux of metal cations described in bacterial systems (Tynecka *et al.*, 1981; Sensfuss and Schlegel, 1986; Nies and Silver, 1989; Nies *et al.*, 1989; Nucifora *et al.*, 1989) would fit this description. These efflux systems are driven by ATP or by a transmembrane gradient, and are inhibited by low temperature and DNP (Nies and Silver, 1989). However, Kochian (1995) argued that an Al^{3+}-ATPase would be an unlikely candidate for a transport system because of the large inwardly directed electrochemical gradient for the transport of Al^{3+} across the plasma membrane meaning that the energy released by the hydrolysis of ATP would not be sufficient to drive Al^{3+} efflux. This of course is based on the assumption that Al^{3+} is both the species which is taken up across the plasma membrane and the species involved in efflux out of the symplasm. This assumption remains to be tested.

Immobilization of Al in the cell wall itself could also result in reduced uptake into the symplasm. Zhang and Taylor (1989) and the results of the present study have shown that under certain conditions even the most rigorous of desorption treatments is ineffective in removing tightly-bound Al from cell wall material. The cell wall could conceivably act as a substantial sink for Al, particularly in Al-resistant plants where cell wall synthesis may not be arrested by Al stress, and hence would continuously produce apoplastic binding sites in the sensitive root tip region. However, the results of Zhang and Taylor (1989) and this study suggest that binding of Al to the cell wall cannot explain the effects of DNP on Al uptake in resistant cultivars.

The plasma membrane may also act as a selective barrier to the uptake of Al. While far from conclusive, support for this hypothesis comes from studies using metabolic inhibitors and anaerobiosis. Excised roots of a number of species have shown increased uptake of Al in the presence of various metabolic inhibitors (Huett and Menary, 1979). Also, species-specific differences in membrane resistance to anaerobiosis have been paralleled by differences in resistance to Al (Wagatsuma, 1983).

Finally, metabolism dependent exclusion of Al might also be mediated by efflux of chelator ligands, which has been described in a number of species in response to phosphate deficiency (Gardner *et al.*, 1981, 1983; Gardner and Parbery, 1982, 1983; Koyama *et al.*, 1988) and Fe deficiency (Ohfune *et al.*, 1981; Sugiura *et al.*, 1981; Ripperger *et al.*, 1982; Mino *et al.*, 1983; Takagi *et al.*, 1984). An increased concentration of chelator ligands in the apoplast, in the mucilage, or in bulk solution (soil or hydroponic) could lead to decreased Al^{3+} activity at the plasma membrane surface thereby decreasing entry of Al into the symplasm. Supporting evidence for a putative chelator efflux system has recently surfaced. Enhanced exudation of citrate (Myasaka *et al.*, 1991; Pellet *et al.*, 1995), malate (Basu *et al.*, 1994a; Delhaize *et al.*, 1993; Ryan *et al.*, 1995 a,b) and low-molecular weight polypeptides (Basu *et al.*, 1994b) by resistant plants might play a role in resistance. While evidence of cultivar-specific differences in exudation of chelators and the ameliorative effects of the addition of exogenous chelators on Al toxicity is convincing, direct evidence of decreased activity of Al^{3+} at the plasma membrane surface and decreased uptake of Al into the symplasm as a result of Al-induced exudation of chelators is lacking. It therefore remains difficult to distinguish between the possibilities that enhanced exudation of organic acids and polypeptides results from toxic lesions produced by Al stress or that it is part of an Al-resistance mechanism.

5.4 Future studies

5.4.1 Characterization of Al uptake mechanisms

Investigation of several other aspects of Al uptake would be useful to further characterize putative exclusion mechanisms. Further support for the putative assignment of the linear phase as uptake into the symplasm could be provided by characterizing the effects of inhibitors such as vanadate and low temperature treatments on the uptake of Al. Further characterization of the mechanisms involved in the transport of Al to the symplasm might be achieved by testing the effects of ionophores or channel formers, such as A-23187 which facilitates divalent cation permeability, and channel blockers such as nifedipine and verapamil which decrease uptake by inactivating cation channels. Such studies may provide information on the role of ion channels in Al uptake. Inhibitors of plasma membrane ATPases and acid phosphatases, as well as calmodulin antagonists might also be used to investigate mechanisms of uptake of Al. While secondary effects of these chemical modifiers are of concern when attempting to interpret kinetic data, these experiments could be performed in conjunction with close monitoring of physiological processes such as membrane permeability, membrane potential, and levels of oxidative phosphorylation. The elucidation of Al uptake mechanisms and their possible species-specificity could shed light on the mechanisms involved in Al resistance.

5.4.2 Alternative systems

More direct evidence of exclusion could also be obtained by kinetic studies with protoplasts. Use of protoplasts would eliminate problems associated with Al binding in the cell wall and the contribution of the cell wall to the linear phase of uptake. As an

alternative, *in vitro* uptake studies with plasma membrane vesicles devoid of cell walls could also be employed. Vesicles with inside-out and right-side out orientations might be used to simplify experimental procedures and to characterize the operation of membrane proteins. Newly developed phase-partitioning techniques (Johansson *et al.*, 1995) which yield vesicle populations which are largely inside out (80-90%) provide a good opportunity to investigate the possible existence of Al efflux mechanisms analogous to ATPases identified in bacterial systems. The above-mentioned systems could also be used to evaluate the permeability of the plasma membrane to various species of Al by altering the pH of uptake solutions which causes shifts in the speciation of Al.

Another possible system involves the use of ^{26}Al isotope. Unfortunately, the world supply of this isotope (< 5 μCi) is inadequate for detection using conventional gamma counting. However, the recent development of uptake systems using giant algal cells of *Chara*, combined with the extremely sensitive technique of accelerator mass spectrometry which can detect mass differences between ^{26}Al and ^{27}Al, may allow for the use of minute quantities of radioisotope, thus experiments with ^{26}Al are now feasible. In fact this technique is presently being employed in our laboratory with success. A combination of some or all of these techniques may provide more information on Al uptake mechanisms and exclusion of Al from the symplasm.

5.5 Conclusions

In conclusion, this research has: **1)** demonstrated that the accumulation of non-exchangeable Al within the symplasm is salt-, concentration-, and time-dependent; **2)** developed a kinetic protocol which minimizes accumulation of tightly-bound Al in the cell apoplasm, giving us a putative estimate of Al uptake in the symplasm; **3)** provided information about the accumulation of Al in root mucilage and a method of removing the

mucilage, thus eliminating the contribution of Al in this compartment to both the rapid and the linear phases of uptake; 4) demonstrated differences between Al-resistant and Al-sensitive cultivars in the rate of Al accumulation in the putative symplastic fraction, with differences being most pronounced at the root tip; and 5) provided further support for putative metabolism-dependent Al exclusion mechanisms using DNP.

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APPENDIX I

Pyrocatechol Violet Colourimetric Method for Determination of Soluble, Inorganic Aluminum.

A) Precautions

1) Contamination

All reagents, standards, and samples were stored in polypropylene containers. All containers were frequently cleaned to avoid contamination. All equipment to be used was first washed with conventional dishwashing detergent and after this initial wash, soaked overnight in dilute HNO_3 and rinsed with distilled, deionized water. Only analytical grade reagents were used.

B) Preparation of Reagents

1) Pyrocatechol Violet.

The concentration of PCV solution was adjusted according to the estimated concentration of soluble Al. For low concentrations of Al (below 25 μM), 38.5 mg / 100 ml was used. For concentrations between 25 μM and 50 μM Al, 75 mg / 100 mL was used. For high concentrations (up to 75 μM), 110 mg / 100 ml was used. These concentrations ensured that the PCV was in ample supply for the reaction to occur.

The appropriate amount of PCV (Sigma) was dissolved in distilled, deionized water and diluted to 100 mL. This solution has been shown to be stable for up to three months if refrigerated.

2) Imidazole buffer.

A 1 M buffer was prepared and adjusted to pH 5.6 using HCl. Imidazole (17.02 g; Sigma) was dissolved in 170 mL of distilled, deionized water by stirring for approximately 30 minutes. The pH was adjusted to 5.6 by slowly adding 11 M HCl while stirring. Approximately 17 mL was required. The buffer was then diluted to 250 mL with distilled, deionized water.

3) Iron interference reagent.

This solution is not stable and therefore was prepared fresh each day prior to experimentation. 50 mg of 1,10 phenanthroline (Sigma) and 250 mg of L-ascorbic acid (Sigma) were dissolved in 50 mL of distilled, deionized water by stirring for approximately 30 min.

4) Aluminum stock solution.

A stock solution was prepared from which a series of Al standards were made. A 1000 µM stock solution was prepared by pipetting 1 mL of 1 M HCl into a 1 L volumetric flask and adding 26.982 ml of a 1000 ppm reference solution (Fisher) and diluted down to 1 L using distilled, deionized water. The stock solution was in 1 mM HCl.

5) Aluminum standards.

Aluminum standards were prepared in a range of concentrations from 0 to 75 µM. In 250 mL volumetric flasks, 500 µL of 1 M HCl were pipetted, the appropriate volume of Al stock solution (See table 1) was added, and the solution was diluted to volume using distilled deionized water. Standards were in 1 mM HCl.

Table 1: Volume of stock solution added to produce standards of various concentrations.

Final Al concentration	Volume of stock solution added (mL)
0	0
0.1	0.025
0.5	0.125
1	0.250
2	0.500
5	1.25
10	2.50
20	5.00
25	6.25
30	7.50
40	10.00
50	12.50
75	18.75

C) Methods.

Three ml of standard solutions were pipetted into 10 mL vials. Iron interference reagent (0.5 mL) was added and the solution was vortexed for approximately 3 seconds. Following a reaction time of at least 1 minute, 0.2 mL of the PCV reagent and 1.0 mL of the imidazole buffer were added and shaken lightly. The colour was allowed to develop for 20 minutes. The absorbance was measured on a spectrophotometer at a wavelength of 578 nm.