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KINETICS OF ALUMINUM UPTAKE BY CELL SUSPENSIONS OF AN ALUMINUM-RESISTANT AND AN ALUMINUM-SENSITIVE CULTIVAR OF *Phaseolus vulgaris* L.

BY

JULIE LYNNE MCDONALD

A thesis submitted to the faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE.

IN

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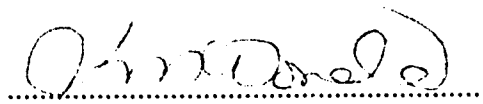

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled KINETICS OF ALUMINUM UPTAKE BY CELL SUSPENSIONS OF AN ALUMINUM-RESISTANT AND AN ALUMINUM SENSITIVE CULTIVAR OF *Phaseolus vulgaris* L. submitted by JULIE LYNNE MCDONALD in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in PLANT PHYSIOLOGY.

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Date Aug 27, 1993

Abstract

The kinetics of aluminum (Al) uptake by cell suspensions derived from an Al-resistant (Dade) and an Al-sensitive (Romano) cultivar of *Phaseolus vulgaris* L. were investigated. Uptake of Al from low volume (3 mL) uptake solutions containing 75 μM $AlCl_3$ was rapid for 20 minutes with little additional absorption occurring over the remainder of the 180 minute experimental period. In contrast, excised roots showed a longer rapid phase of uptake (30 min) which was followed by a linear phase of uptake. The rate, extent, and saturable nature of uptake in this cell system suggested that the lack of a linear phase of uptake could reflect depletion of Al from uptake solutions. However, when the concentration of Al in uptake solutions was increased up to 1000 μM, total accumulation of Al increased while the general pattern of uptake was not affected. Increasing the concentration of Al in uptake solutions led to concerns about precipitation of Al. Thus, experiments with high volume (100 mL), low concentration (75 μM $AlCl_3$) uptake solutions were also performed. In these experiments, a biphasic pattern of uptake was observed, with a rapid initial phase of uptake (20 minutes) followed by a linear phase of uptake over the remainder of the 180 minute uptake period. The linear phase of uptake was isolated after desorption in 9.0 mM citric acid. These studies suggest that patterns of short-term Al uptake observed in excised roots reflect transport events occurring at the cellular level.

The feasibility of using kinetic studies at the cellular level to evaluate the role that exclusion plays in resistance was also investigated. In previous studies with excised roots of *Triticum aestivum*, a DNP-induced stimulation of uptake of Al by Al-resistant cultivars and the lack of an effect of gramicidin were consistent with the operation of an exclusion mechanism. However, these studies provided only indirect evidence for exclusion due to complexities arising from the presence of a cell wall. If

it could be demonstrated that exclusion occurs at the cellular level, more direct information about the role exclusion plays in resistance could be obtained by comparing the kinetics of Al uptake between protoplast and cell suspensions. In the present study the uptake of Al by excised roots of an Al-resistant cultivar of *P. vulgaris* increased in the presence of DNP. In contrast, DNP had no effect on Al uptake in cell suspensions derived from either Al-resistant or Al-sensitive cultivars. Uptake of Al was unaffected by the presence of gramicidin in both the Al-resistant and the Al-sensitive cultivar in both excised roots and cell suspensions. These results suggest that exclusion is not occurring at the cellular level in *P. vulgaris* and that gramicidin does not facilitate the transmembrane transport of the membrane mobile species of Al in *P. vulgaris*. Although exclusion may be a cellular phenomenon, its expression may be limited to a level of organization found in whole plant tissues.

It appears that the role exclusion plays in resistance cannot be evaluated by kinetic analysis of Al uptake at the cellular level in *P. vulgaris*. Nevertheless, cell suspension systems may be useful for investigating transport of Al across the plasma membrane.

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Table of Contents

List of Tables

List of Figures

1 General Introduction

Soil acidity is a major factor limiting growth of plants in vast regions of the world (Foy, 1983). Recent estimates suggest that 40% of the world's arable soils are acidic (Haug, 1984). Growth of plants on these soils is affected by a number of factors, including manganese toxicity, mineral deficiencies and drought intolerance (Haug, 1984). However, the major growth-limiting factor on most acid soils is aluminum (Al) toxicity (Foy, 1983). Aluminum toxicity is most prominent in soils where the pH is below 5.0, but may also occur in soils with a pH up to 5.5 (Foy *et al.*, 1978). It is a problem in naturally acidic soils as well as in soils where acidity has been increased by industrial or agricultural activities (Jackson *et al.*, 1990). Acid rain and the use of nitrogen fertilizers are two important factors contributing to the acid soil problem (Haug, 1984).

Despite a large amount of research in this area, our comprehension of the phytotoxic effects of Al remains limited (Taylor, 1988; Jackson *et al.*, 1990). Taylor (1988) suggested this was the result of a number of factors, including the complex chemistry of Al, the lack of an appropriate radioisotope, inadequate techniques and equipment to detect low levels of Al, and contamination problems due to the ubiquitous nature of Al. Although these problems still exist, advances have been made to reduce their magnitude. For example, improvements in atomic absorption spectrophotometry have made it possible to detect nanogram quantities of Al. Contamination problems can be minimized by subjecting experimental materials to rigorous cleaning procedures and by the use of high grade reagents. With respect to the complex chemistry of Al, the identity of the primary toxic species remains uncertain (Jackson *et al.*, 1990; Kinraide, 1991), but studies have implicated Al^{3+} as the primary phytotoxic monomeric species (Parker *et al.*, 1987). Recently it has been

suggested that an Al_{13} polynuclear species is also phytotoxic, but there are few indications as to exactly which form of polymeric Al is responsible for toxicity (Parker *et al.*, 1989; Hunter and Ross, 1991). In contrast, Al sulphate, Al fluoride, and Al organic complexes are generally non-toxic (Cameron *et al.*, 1986; Hue *et al.*, 1986; Parker *et al.*, 1987). With the information currently available, experiments can now be designed to provide better control over the variety of species of Al present in solution (Kinraide, 1991).

1.1 Aluminum Toxicity and Potential Aluminum Resistance Mechanisms

While there is no consensus on the mechanisms of Al toxicity in higher plants, it appears that Al acts on a number of critical physiological processes (Taylor, 1988; Jackson *et al.*, 1990). Aluminum induces membrane leakiness either by affecting membrane lipids or membrane carriers (Zhao *et al.*, 1987). Aluminum also inhibits mitosis (Minocha *et al.*, 1992), either directly by inhibiting DNA replication during interphase or indirectly by affecting processes associated with cell division (Clarkson, 1965). Furthermore Al inhibits cell elongation, possibly by reducing the rate of cell wall biosynthesis (Klimashevskii and Dedov, 1975). Many authors argue that mineral deficiencies form the basis of Al phytotoxicity, but this idea has been criticized on the basis that Al has been shown to have rapid toxic effects which cannot be explained by induced mineral deficiencies. Nevertheless, studies support the idea that Al interferes with the uptake and metabolism of phosphate, calcium, magnesium and a number of other mineral nutrients (Taylor, 1988). Foy *et al.* (1978) suggested that the physiological mechanisms of Al toxicity might differ in different plant species and cultivars.

Just as there is a lack of a clear understanding of the phytotoxic effects of Al, our comprehension of resistance mechanisms is also incomplete. Aluminum resistance could be achieved either by internal or by external resistance mechanisms. External resistance mechanisms work by preventing accumulation of Al in the symplast and by protecting sensitive extracellular sites from the toxic effects of Al, while internal resistance mechanisms involve the detoxification of Al once it has crossed the plasma membrane and entered into the symplast. Studies have suggested that low concentrations of Al in the symplast may be toxic to plants. If Al does enter the symplast, then internal resistance mechanisms must be present for plants to grow in the face of stress (Taylor, 1991). Detoxification of Al in the symplast may occur by chelation of Al by organic ligands in the cytoplasm. Internal resistance could also be achieved by accumulating Al in insensitive sites within the cytoplasm, such as the vacuole. Evolution of Al-resistant enzymes (isozymes) or production of Al-binding proteins could also confer resistance. Another internal resistance mechanism could involve an increased level of enzyme synthesis, so activity can be maintained in the face of Al stress. These hypotheses are explained in more detail by Taylor (1991).

Several hypotheses have been put forward to explain how resistance could be achieved by external resistance mechanisms. Aluminum could be immobilized in the cell wall. If Al accumulated in the cell wall, or if a low cation exchange capacity (CEC) existed in the cell wall, then uptake of Al across the plasma membrane might be reduced. Plants might also achieve resistance by maintaining a high pH in their rhizosphere or apoplast. The plasma membrane could maintain its integrity under Al stress and behave as a selective barrier restricting the uptake of Al into the symplast. Exudation of chelate ligands could also protect the plasma membrane from Al stress and act as an external resistance mechanism. Finally resistance might also be

accomplished by the exudation of phosphate or active efflux of Al from the symplast (Taylor, 1991).

1.2 The Role that Exclusion May Play in Aluminum Resistance

External resistance has been used as an encompassing term to describe resistance mechanisms which limit accumulation of Al in the symplast (exclusion) and/or protect sensitive extracellular sites from Al-induced injury. In the remainder of this thesis my focus will be on exclusion mechanisms. Exclusion mechanisms are external resistance mechanisms in that they operate within the apoplast. By definition they would limit the rate of Al transport across the plasma membrane and they may or may not provide protection to sensitive extracellular sites.

The idea that resistance to Al may be achieved through exclusion is slowly gaining acceptance in the scientific community, but experimental support for such an idea is still limited (Taylor, 1991). In the past, a number of studies have looked at accumulation of Al at the whole plant level (see for example Wallace *et al.*, 1982; Aniol, 1983). However, to evaluate potential resistance mechanisms which rely on exclusion of Al from the symplast, it is necessary to differentiate between uptake into the apoplasmic and symplasmic compartments. In this regard gross tissue analyses will be of little help, since decreased accumulation of Al in the symplast could be accompanied by increased accumulation in the apoplast. This could mask differences between Al-resistant and Al-sensitive cultivars if whole plants or tissues are used (Taylor, 1991).

Numerous studies have employed microscopy as a means to determine sites of Al accumulation (Naidoo *et al.*, 1978; Cuenca *et al.*, 1991; Hodson and Wilkins,

1991). These studies do not always quantify the amount of Al in different cellular compartments. They have also been criticized because severe chemical fixation and dehydration steps used in preparing tissues for microscopy may cause redistribution or loss of Al. These problems prevent accurate determination of sites of accumulation. Other studies have involved measuring Al in different compartments by separating cell parts following exposure to Al (Niedziela and Aniol, 1983; Aniol, 1984; Zhang and Taylor, 1990). This approach may also lead to redistribution of Al during homogenization, a commonly employed step in fractionation experiments. In an attempt to overcome this problem, uptake into cell constituents has been measured after they are isolated (Wagatsuma, 1983b; Zhang and Taylor, 1990). However, binding of Al to isolated cell parts may not accurately reflect binding which occurs in a highly organized intact cell or root. For example, disruption of cells could create additional binding sites for Al by exposing surfaces which may not ordinarily be available to Al for binding.

Attempts have been made to differentiate and quantify uptake of Al into the apoplasmic and symplasmic compartments, but reports of the sites of Al accumulation and amounts of Al accumulated are conflicting. In roots of *Pisum sativum*, Al accumulated primarily in the nuclei and cell walls (Matsumoto *et al.*, 1976). Naidoo *et al.* (1978) found the greatest concentration of Al in the nuclei in roots of *Gossypium hirsutum* and *Phaseolus vulgaris*. Studies with roots of *Picea abies* revealed that most of the Al was associated with cell walls and little was observed in the nuclei and cytoplasm (Hodson and Wilkins, 1991). Quantitative studies have revealed equally conflicting data. In roots of *Hordeum vulgare*, *Brassica oleracea*, *Lactuca sativa*, and *Pennisetum clandestinium*, uptake experiments suggested that 75-95% of the Al taken up by roots was associated with the cell wall (Clarkson, 1967; Huett and Menary, 1979), while other root uptake studies in *Triticum aestivum* reported that 45 to 75%

of Al was associated with the apoplast (Zhang and Taylor, 1990). In yet another study, where sequential washing steps and staining techniques were used to distinguish between uptake into apoplasmic and symplasmic compartments, only 30 to 40% of Al taken up by roots of *T. aestivum* was found to be located in the apoplasmic compartment (Tice *et al.*, 1992).

Conflicting reports concerning the quantification and localization of Al may be the result of numerous factors. As mentioned previously, microscopic examinations suffer from potential redistribution or loss of Al during preparation for examination. Uptake experiments have also been conducted with different species under different experimental conditions including pH, temperature, duration of exposure, and Al concentrations, all of which have been shown to influence patterns of Al uptake. For example, Clarkson (1967) measured uptake by whole roots from a complete nutrient solution at a pH which was not indicated. The use of complex nutrient solutions makes it difficult to predict Al speciation and to relate root responses with rhizotoxicity (Tice *et al.*, 1992). Huett and Menary (1979) and Zhang and Taylor (1990) used simple $CaSO_4$ solutions with Al supplied as $Al_2(SO_4)_3$ and $AlK(SO_4)_2$ respectively. Test solutions containing ligands such as phosphate or sulfate may be prone to precipitation or formation of complexes which could lead to an overestimation of Al in the apoplast (Tice *et al.*, 1992). These problems can be minimized by keeping test solutions simple, generally containing $CaCl_2$, $AlCl_3$, and HCl as the only inputs (Kinraide and Parker, 1989; Kinraide, 1991).

1.3 Contribution of Kinetic Studies

Many studies have looked at sites of accumulation as a means of determining potential sites of Al action. If this type of study is to provide experimental support for

exclusion mechanisms and increase our understanding of Al resistance, different sites of accumulation must be correlated with different degrees of resistance. Several kinetic studies involving the characterization of Al uptake over time have provided this kind of data. Zhang and Taylor (1989) characterized uptake of Al into excised roots of Al-resistant and Al-sensitive cultivars of *T. aestivum*. Their results showed a rapid, non-linear initial phase of uptake superimposed over a second phase in which accumulation of Al was slower and linear with time. Similar patterns of uptake were also observed in roots of *Hordeum vulgare* (Clarkson, 1967), *Brassica oleracea*, *Lactuca sativa*, *Pennisetum clandestinum* (Huett and Menary 1979) and *Cucumis sativus* (Wagatsuma, 1983a), but in these studies plants were exposed to high concentrations of Al which makes drawing conclusions about potential resistance mechanisms speculative (Zhang and Taylor, 1989). The rapid initial phase of uptake has traditionally been interpreted as representing uptake into the apoplast and the slower linear phase representing uptake across the plasma membrane (Taylor, 1991). Zhang and Taylor (1989) were able to isolate the linear phase of uptake by using citric acid to remove the loosely bound Al from the apoplast, although a slight deviation from linearity was found during the first 30 minutes of uptake. Extrapolation of the linear phase to time zero indicated that some non exchangeable Al still remained in the apoplasmic compartment following desorption (Zhang and Taylor, 1989). Subsequently, studies with isolated cell wall material led Zhang and Taylor (1989,1990) to question the precise identity of the linear phase. They showed that *in vivo* uptake of Al into cell wall material showed a linear phase that could not be desorbed with citric acid. These results suggest that the linear phase of Al uptake may also include a cell wall component (Zhang and Taylor, 1990).

If exclusion mechanisms do exist, then it should be possible to demonstrate that net uptake of Al across the membrane occurs to a greater extent in sensitive

organisms than in resistant organisms. When the kinetics of Al uptake in Al-resistant and Al-sensitive cultivars of *T. aestivum* were studied by Pettersson and Strid (1989) and Zhang and Taylor (1989), no differences in uptake of Al were observed between the sensitive and resistant cultivars. Pettersson and Strid (1989) suggested that this indicated that Al resistance was not linked to initial uptake of Al. However, when Zhang and Taylor (1989) treated roots with 2,4-dinitrophenol (DNP), a general metabolic inhibitor, rates of uptake increased in Al-resistant cultivars while rates of uptake in Al-sensitive cultivars were relatively unaffected. A similar effect of DNP was also observed in several species by Huett and Menary (1979) and Wagatsuma (1983a), who suggested that the effect of DNP was due to an increase in membrane permeability to Al. However, since DNP is reported to uncouple oxidative phosphorylation, reduce the proton gradient across the plasma membrane, and affect membrane structure and permeability, specific conclusions cannot be drawn on how DNP affects uptake of Al (Zhang and Taylor, 1989). Nevertheless, Zhang and Taylor (1989) offered an alternative explanation for the effects of DNP. They suggested that increased uptake of Al in resistant cultivars in the presence of DNP may represent disruption of an exclusion mechanism which operates under normal metabolic conditions. If the linear phase of uptake does include an apoplasmic component, then differences between cultivars under normal metabolic conditions might be masked by differences in accumulation of Al in the apoplast (Zhang and Taylor, 1989). Under altered metabolic conditions, differences in the rate of Al uptake in Al-resistant and Al-sensitive cultivars may become apparent.

Studies using gramicidin, a monovalent cation channel forming ionophore, also supported the idea of an active exclusion mechanism. In Al-sensitive cultivars of *T. aestivum*, gramicidin stimulated uptake of Al into excised roots. In Al-resistant cultivars, the rate of Al uptake was unaffected in the presence of gramicidin (Zhang

and Taylor, 1989, 1991). Zhang and Taylor (1991) suggested that the apparent lack of a gramicidin effect in resistant cultivars may have been observed if these cultivars possessed an active exclusion mechanism. For example, an Al efflux pump or active efflux of chelate ligands or phosphate could prevent or mask the effect of gramicidin. This idea is consistent with the increased uptake of Al which was observed in Al-resistant cultivars when DNP and gramicidin were supplied simultaneously (Zhang and Taylor, 1991).

Due to the complex nature of the linear phase of uptake, differences in the effect of DNP and gramicidin which were observed between cultivars (Zhang and Taylor, 1991) could be the result of either differential binding of Al in the apoplast or differential uptake across the membrane. However, results from several experiments were inconsistent with the idea that the effects of DNP and gramicidin could be explained by increased uptake of Al into the cell wall fraction (Zhang and Taylor, 1991). An increase in uptake of Al into cell wall fractions occurred in the presence of DNP, but the extent of stimulation was the same in both Al-resistant and Al-sensitive cultivars (Zhang and Taylor, 1991). Furthermore, gramicidin had no effect on uptake of Al into cell wall fractions in either cultivar, suggesting that the increased uptake of Al observed in excised roots was due to uptake of Al across the plasma membrane (Zhang and Taylor, 1991).

Although the effects of DNP and gramicidin suggest metabolic exclusion may be occurring in resistant cultivars, these results are not conclusive. Furthermore, little can be said about the precise nature of the putative exclusion mechanism, if exclusion does indeed exist. Thus, further studies are needed to distinguish between uptake into the apoplast and uptake across the plasma membrane. Studies comparing the kinetics of Al uptake between protoplast and cell suspensions of Al-resistant and Al-sensitive

cultivars could provide more definite information about the distribution of Al in apoplasmic and symplasmic compartments during the linear phase of uptake.

1.4 An Alternate Kinetic Approach

A modification of the kinetic approach has recently been used to distinguish between uptake into apoplasmic and symplasmic compartments (Tice *et al.*, 1992). This approach has provided results which are consistent with an exclusion mechanism being involved in resistance to Al. Aluminum accumulated by intact roots of an Al-resistant and an Al-sensitive cultivar of *T. aestivum* was divided into an operationally defined apoplasmic fraction using six sequential washes with $CaCl_2$, while the soluble symplasmic fraction was determined by four sequential washes with $CaCl_2$ following disruption of membranes by freezing excised roots. A residual fraction was then collected and allocated to the symplasmic compartment on the basis of staining root tips with the fluorophore morin (Tice *et al.*, 1992). In the presence of Al at concentrations which caused a 50% reduction in growth, differences in the amount or distribution of Al were not observed between the Al-sensitive and the Al-resistant cultivar. However, when plants were grown in the presence of equal concentrations of Al, the Al-sensitive cultivar contained a greater concentration of Al in the symplast than the Al-resistant cultivar (Tice *et al.*, 1992).

At all concentrations of Al examined in the study of Tice *et al.*'s (1992) the majority of non-exchangeable Al appeared to accumulate in the symplast in both cultivars. However, Al associated with the symplasmic compartment could have been overestimated if the stain used to identify the location of the residual fraction was unable to form a complex with tightly bound, non-exchangeable Al in the apoplast. To verify the existence or nonexistence of a non exchangeable apoplasmic fraction, Tice et

al. (1992) suggested that further experiments are needed to test the ability of morin to stain nonlabile forms of Al (Tice *et al.*, 1992). Dan Archambault (personal communication) has performed experiments with excised roots of *T. aestivum* which were similar to those of Tice *et al.* (1992). When examining *in vivo* uptake of Al by cell wall material, he found that $CaCl_2$ was less effective than citric acid in removing Al from the apoplast. These results suggest that morin may not have the ability to stain nonlabile forms of Al in the apoplast.

In recent years, advances have been made with respect to differentiating between uptake into the apoplasmic and symplasmic compartments, a distinction which is necessary if we are to evaluate the possible role of exclusion in Al resistance. The most successful approaches providing support for the existence of an exclusion mechanism are studies looking at the kinetics of Al uptake (Zhang and Taylor, 1989, 1990, 1991) and the sequential washing technique of Tice *et al.* (1992). However, if the linear phase of Al uptake observed in kinetic experiments does contain a cell wall component, or if the morin stain used by Tice *et al.* (1992) cannot identify non-exchangeable Al in the apoplast, we could still be miscalculating the amount of Al associated with the apoplasmic and symplasmic compartments. In these studies, an unidentifiable apoplasmic Al component could still mask differences in Al uptake between Al-sensitive and Al-resistant cultivars.

If it could be demonstrated that kinetics of Al uptake were similar in cell suspension systems and intact or excised roots, this could provide us with an ideal system to further investigate the possible role of exclusion as a mechanism of Al resistance in plants. Cell suspensions could provide us with a system where manipulation of the cell wall is possible. By comparing the kinetics of Al uptake in protoplast and cell suspensions of an Al-sensitive and an Al-resistant cultivar, more

direct information regarding uptake into the apoplast and symplast could be obtained and the role that the plasma membrane and cell wall may play in exclusion could be evaluated.

1.5 Studies on Aluminum Toxicity and Resistance Using Cell Culture Systems

Aluminum toxicity and most of the postulated resistance mechanisms to Al appear to have a fundamental cellular basis (Foy *et al.*, 1978; Haug, 1984). Therefore, Al resistance may be an important agricultural trait that can be selected for in cell culture (Conner and Meredith, 1985a). Not surprisingly, a number of studies using cell culture systems have focused on selection and characterization of Al-resistant variants isolated from callus cultures (Conner and Meredith, 1985a; Ojima and Ohira 1982 and 1983). Although a number of authors have claimed to have isolated Al-resistant variants, close scrutiny of their experimental work leads one to question the actual resistance of their selected lines. To clearly demonstrate that a putative Al-resistant cell line has been isolated, the line must be selected under conditions which allows separation of the direct toxic effects of Al, EDTA-induced mineral deficiencies, and reduced phosphate availability. This observation has also been made at the whole plant level (Munns, 1965).

Ojima and Ohira (1983) selected a putative Al-resistant cell line from *Daucus carota*. Characterization of this cell line suggested that resistance was due to the cell's ability to release citric acid into the culture medium (Ojima and Ohira, 1985). Citric acid is known to chelate Al and render it less toxic to plants (Taylor, 1991). Although Ojima and Ohira (1985) assumed that inhibition of growth of the Al-sensitive, wild-type cells was due to the toxic effects of ionic Al, they also acknowledged that

inhibition of growth may have been a result of Al-induced phosphate deficiency. Further characterization of the selected cell line indicated that citric acid was excreted into the medium when phosphorus was supplied as either insoluble Al-phosphate or iron-phosphate. Furthermore, when precautions were taken to increase the solubility of Al in their medium, inhibition of growth was greater in selected cells than in wild type cells (Koyama *et al.*, 1988). Thus, the release of citric acid into the medium appeared to be a response to the low solubility of phosphate in the medium rather than toxic concentrations of Al.

For this and several other reasons a number of modifications to standard culture media are required to simulate Al toxic conditions when working with cell culture systems (Conner and Meredith, 1985b). These include decreasing the phosphate concentration into the range of 10 µM and lowering the pH to 4.0 to prevent the precipitation of Al, and using unchelated iron to prevent the formation of Al-EDTA complexes. Calcium (Ca) concentrations should also be lowered to around 100 µM because Ca has been shown to partially alleviate Al toxicity. Employing the modifications suggested by Conner and Meredith (1985b), several successful selections for Al-resistant cell lines have been documented. Conner and Meredith (1985a) selected cell lines from diploid callus cultures of *Nicotiana plumbaginifolia* that appeared to be resistant to Al. Fertile plants were regenerated from these selected cell lines and all plants transmitted Al-resistance to their progeny (Conner and Meredith, 1985a).

Ojima *et al.* (1989) also isolated an Al-resistant cell line from *N. tabacum* in a culture medium containing a reduced concentration of phosphate. Analysis of the culture medium following centrifugation suggested that all of the Al was present in the supernatant and therefore in soluble form. The authors concluded from these results

that all of the Al present in their experimental medium was in the toxic ionic form. This conclusion could be premature, because the technique employed to measure Al did not distinguish between ionic and chelated forms of Al. Thus, Ojima *et al.*(1989) may have overestimated the toxicity of Al in their selection medium. Lack of information about culture conditions, pH control, and speciation calculations also generates doubt about the actual toxicity of their experimental medium. Aluminum-resistant cell lines were also selected from cell cultures of *D. carota* (Arihara *et al.*, 1991). The same criticisms regarding lack of information about culture conditions can be applied to this study. However, in this case, whole plants were regenerated from their resistant cell lines. Using root elongation in the absence of phosphate as a measure of Al toxicity, four out of six Al-resistant cell lines maintained their resistance at the whole plant level. This suggests that selection of the cell lines was conducted under Al-toxic conditions (Arihara *et al.*, 1991). The studies of Conner and Meredith (1985a), Ojima *et al.* (1989) and Arihara *et al.* (1991) suggest that cell culture systems can be used to select Al-resistant variants, but it appears this selection needs to be confirmed by regenerating plants and conducting Al toxicity tests at the whole plant level.

Although a number of studies at the cellular level have used cell culture systems to select Al-resistant cell lines and regenerate plants with improved resistance, few studies have attempted to demonstrate that Al resistance at the whole plant level is retained at the cellular level. A correlation between resistance to Al at the whole plant and cellular level must be demonstrated if we are to use cell culture systems to gain information about Al toxicity and resistance in plants. Callus cultures derived from four Al-sensitive cultivars of *Sorghum bicolor*, all showed sensitivity to Al at the cellular level. However, sensitivity was measured in a full strength medium containing Al as Al-EDTA (Smith *et al.*, 1983). Use of Al-EDTA has been questioned on the

basis that chelated forms of Al are non-toxic to plants (Conner and Meredith, 1985b). The inhibition of growth observed in the presence of Al-EDTA could be due to EDTA induced mineral deficiencies resulting from the formation of EDTA-micronutrient complexes rather than to toxicity of Al (Taylor, 1993). Ojima and Ohira (1983) reached a similar conclusion in studies with *D. carota* when Al was supplied as Al-EDTA. In another study, Parrot and Bouton (1990) were able to demonstrate resistance at the cellular level in callus cultures derived from acid Al-resistant and acid Al-sensitive germplasm of *Medicago sativa*. These authors used the experimental medium designed by Conner and Meredith (1985b) containing low phosphate and Ca concentrations, unchelated iron, and $AlCl_3$ (pH 4.00) thus, their results appear credible. Unfortunately Parrot and Boutin (1990) did not evaluate their cell lines over a range of Al doses, making it unclear whether resistance was fully expressed at the cellular level (Taylor, 1993). Other work has shown that callus derived from a zinc and copper resistant clone of *Agrostis stolonifera* retained its resistance in tissue culture as well as in plants regenerated from callus (Wu and Antonovics, 1978). Callus cultures of *Anthoxanthum odoratum* also showed the same response to zinc and lead as the whole plants from which the cultures were derived (Qureshi *et al.*, 1981). Thus, whole plant responses to metals appear to be similar to those at the cellular level. However, more substantive verification that the same conclusion applies to Al is needed before we can be sure that the toxic effects of Al and mechanisms of Al-resistance have a fundamental cellular basis.

1.6 Uptake of Aluminum at the Cellular Level

Although numerous studies have looked at accumulation and distribution of Al in animals and plants, few studies have looked at uptake and distribution at the cellular level. This information is important for understanding mechanisms of Al toxicity (Shi

and Haug, 1990). In rat hepatocytes, uptake of Al increased in a time and concentration-dependent manner. Most of the Al was found to be associated with the mitochondrial (45%) and endoplasmic reticulum-cytoplasmic fraction (45%), while only minimal amounts (5%) were found to be associated with the nuclei and cellular debris (Muller and Wilhelm, 1987). Uptake of Al into *Anabaena cylindrica* also increased with increasing time and concentration. X-ray microanalysis revealed that Al was associated with polyphosphate granules and cell walls, but was not detected in the cytoplasm (Pettersson *et al.*, 1985). Increased uptake of Al was observed after treatment with carbonyl cyanide m-chlorophenyl hydrazone, an uncoupler of oxidative phosphorylation, leading the authors to conclude that uptake of Al into cells occurred via passive diffusion (Pettersson *et al.*, 1986). Concentration and time dependent uptake was also observed in murine neuroblastoma cells. In this system, uptake of Al was dependent on pH, reaching a maximum at pH 6.0, thus, this experimental system may have little relevance to plant systems. In contrast to work in plant systems (Zhang and Taylor, 1989, 1991), the metabolic inhibitor DNP, had no effect on the rate or pattern of uptake by murine neuroblastoma cells, suggesting that uptake of Al occurred via a passive mechanism (Shi and Haug, 1990).

Studies with multilayered phospholipid liposomes provide an experimental system which may be of more relevance to plant systems. Shi and Haug (1988) analyzed the kinetics of Al uptake in liposomes composed of dimyristol phosphatidylcholine over a six hour time period. Uptake studies were conducted with an initial Al concentration of 10 µM, but pH was high (6.50). A citric acid wash was used to delineate between superficially bound Al and Al which had accumulated in the interior of the liposome. Initial association of Al with liposomes was found to be rapid, reaching saturation within a few minutes. However, uptake of Al into the liposomes, occurred at a much slower rate and still continued to rise even after 6

hours. Time course experiments were then conducted at temperatures below and above the gel to liquid crystalline phase transition point. At temperatures above the phase transition point, initial association and uptake occurred at a greater rate than at temperatures below the phase transition point. After 3 hours of uptake, no differences were observed in total amount of Al taken up by the liposome, regardless of incubation temperature. The authors concluded that membrane fluidity influences initial rate of Al uptake but does not affect its distribution once equilibrium is reached. Although Shi and Haug (1988) assumed that a major portion of the Al measured after the citric acid wash was Al which had accumulated in the interior of the liposome, they acknowledged that some Al may have been trapped on the surface by phospholipids and inaccessible to citric acid. Therefore, uptake of Al across the lipid bilayer may have been overestimated (Shi and Haug, 1988).

As a whole, these cell studies have not provided a clear and consistent view of Al uptake at the cellular level. Nevertheless, they do indicate that the kinetics of Al uptake can be measured in cell systems. As of yet, studies of Al uptake by higher plant cell systems have not been performed.

1.7 Summary

Aluminum toxicity is a major agricultural problem contributing to poor plant growth on acid soils (Foy, 1983). A better understanding of how resistance is achieved would help in efforts to overcome the low productivity associated with acid soils. A number of studies, including those investigating the kinetics of Al uptake have made important contributions in distinguishing between uptake into apoplasmic and symplasmic compartments. These studies have provided tentative support for the existence of exclusion mechanisms. However, further experiments are required to

characterize metabolism-dependent exclusion more fully (if it even exists). It would also be useful to demonstrate that Al resistance has a fundamental cellular basis. In an attempt to more fully characterize the potential role of exclusion in mediating Al resistance at the cellular level, the objectives of my research were (1) to develop techniques to investigate short-term kinetics of Al uptake in cell cultures of *Phaseolus vulgaris* L., (2) to characterize the kinetics of Al uptake in cell suspensions derived from an Al-sensitive and an Al-resistant cultivar of *P. vulgaris*, (3) to determine if cell suspensions of *P. vulgaris* show the same pattern of uptake that is observed in excised roots of *T. aestivum*, and (4) to determine if uptake of Al by cell suspensions responds to specific inhibitors (e.g. DNP, gramicidin) in a similar manner to excised roots. If we can demonstrate that patterns of Al uptake are similar to those previously observed in whole or excised roots, we may have an ideal system to further characterize the role that exclusion may play in Al-resistance.

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2 Development of an Experimental System to Investigate the Kinetics of Aluminum Uptake at the Cellular Level

2.1 Introduction

Our current understanding of the physiological and biochemical basis of aluminum (Al) toxicity and resistance in plants is limited by the lack of reliable information concerning the dynamics of Al movement into plant cells. Research on the entry of Al into cells is required, not only to provide information about the primary toxic effects of Al (Haug, 1984), but also in evaluating potential resistance mechanisms which may rely on exclusion of Al from the symplast (Taylor, 1991). To a large extent, our lack of knowledge concerning the uptake of Al into the symplast of plant cells is a direct result of the lack of a suitable radioisotope for Al and complexities arising from the presence of a cell wall. Such barriers, however, need not be viewed as insurmountable. Several short-term kinetic studies, involving the characterization of Al uptake over time have been reported. (Clarkson, 1967; Huett and Menary, 1979; Pettersson and Strid, 1989; Zhang and Taylor, 1989, 1990, 1991). These studies represent a first step towards measuring trans-membrane transport of Al.

In *Triticum aestivum*, the kinetics of short-term uptake of Al by excised roots are characterized by a rapid, non-linear, initial phase of uptake which is superimposed over a second phase where accumulation is slower and linear with time (Zhang and Taylor, 1989). These two phases have traditionally been interpreted as representing

passive accumulation into the apoplast and uptake across the plasma membrane respectively. Zhang and Taylor (1989) were able to isolate the linear phase of uptake by desorbing excised roots in citric acid to remove readily exchangeable Al from the putative apoplasmic compartment. However, subsequent studies with isolated cell wall material led Zhang and Taylor (1990) to question the precise identity of the linear phase. They showed that uptake of Al into purified cell wall material showed a metabolism-dependent linear phase which could not be desorbed with citric acid, and concluded that the linear phase of uptake may also include a cell wall component (Zhang and Taylor, 1990). While Tice *et al.* (1992) argued that a linear rate of Al accumulation in the cell wall may reflect experimental conditions conducive to formation of solid phase Al in the apoplast, the potentially complex nature of the linear phase makes it difficult to distinguish between uptake in the apoplast and symplast. Thus, it appears that techniques for quantifying Al uptake across the plasma membrane are still lacking.

Studies such as those described above suggest that accurate measurement of Al movement across the plasma membrane will ultimately require an experimental system which permits manipulation of the cell wall. For example, studies comparing the kinetics of Al uptake in protoplast and cell suspensions of an Al-resistant and an Al-sensitive cultivar could provide direct information about the role of the cell wall and plasma membrane in mediating exclusion of Al from the symplast. Such studies would also provide an opportunity to evaluate the extent to which short-term patterns of Al uptake in excised roots provide a view of Al uptake at the cellular level. Recent interpretations of kinetic data reflect what are believed to be events occurring at the cellular level, but this has not been experimentally tested. Furthermore, a growing body of published work using cell culture systems indicates that a degree of caution is

warranted. Conner and Meredith (1985) found that a number of modifications to standard culture media were required to prevent precipitation of Al from solution. Problems with precipitation made it difficult to determine if reduced cell growth was due to Al-induced nutrient deficiencies, or due to the toxicity of Al itself (Conner and Meredith, 1985). Similarly, in *Daucus carota*, a cell line originally selected as Al-resistant was later found to be more sensitive to ionic Al than wild type cells (Ojima and Ohira, 1983). The culture medium used in these selection studies contained inorganic phosphate which made it difficult to distinguish between the toxic effect of Al and an Al-induced phosphate deficiency (Koyama *et al.*, 1988). Another problem encountered in using cell suspension systems is the difficulty involved in controlling pH of culture media under aseptic conditions (Conner and Meredith, 1985). Since the speciation of Al is highly dependent on pH (Kinraide, 1991), strict control of pH is required to express Al toxicity and resistance in cell suspensions. Once again, these barriers need not be viewed as insurmountable, but further research is still required to overcome these problems.

As part of an ongoing effort to develop techniques for measuring trans-membrane transport of Al, the first objective of this study was to develop a reliable system to study the uptake of Al in cell suspensions and to overcome technical problems associated with working with Al in cell systems. The second objective was to investigate the kinetics of Al uptake in cell suspensions of an Al-resistant and an Al-sensitive cultivar of *Phaseolus vulgaris* L. to determine if cell suspensions show a pattern of Al uptake which is similar to patterns previously observed in excised roots (Clarkson, 1967; Huett and Menary, 1979; Pettersson and Strid, 1989; Zhang and Taylor, 1989, 1990, 1991).

2.2 Materials and Methods

2.2.1 Preparation of Plant Material

Seeds of *Phaseolus vulgaris* L. cvs. Dade and Romano (Musser Seed Co. Inc., Twin Falls, Idaho) were germinated aseptically and 3-mm discs excised from 5 day old hypocotyls were used for initiation of callus. Callus was maintained on a modified MS (Murashige and Skoog, 1962) medium containing (g L^{-1}) 8.0 Agar (purified, inhibitor free, BDH Inc.), 2.0 Casein (Hy-Case SF, from bovine milk, Sigma Chemical Co.); (mM) 100 Sucrose, 40 NH_4NO_3, 20 KNO_3, 2.5 $CaCl_2 \cdot 2H_2O$, 1.25 KH_2PO_4, 1.0 $MgSO_4 \cdot 7H_2O$, 0.1 $MnSO_4 \cdot 1H_2O$, 0.1 H_3BO_3, 0.1 FeEDTA, 0.03 $ZnSO_4 \cdot 7H_2O$; and (μM) 5.0 KI, 0.99 $Na_2MoO_4 \cdot 2H_2O$, 0.11 $CoCl_2 \cdot 6H_2O$, 0.1 $CuSO_4 \cdot 5H_2O$ (pH 5.00). This basic medium was supplemented with the following growth regulators and vitamins: (mM) 0.6 Myo-inositol (Sigma Chemical Co.); and (μM) 37.5 Nicotinic acid (Aldrich Chemical Co.), 10.0 Picloram (Aldrich Chemical Co.), 6.0 Ascorbic acid (Fisher Scientific Co.), 5.0 Thiamine HCl (Aldrich Chemical Co.), 2.5 Pyridoxin (Aldrich Chemical Co.), and 1.0 Kinetin (Aldrich Chemical Co.). Cultures were maintained by subculturing to fresh media every 30 days. Cell suspensions were initiated from callus and maintained on a gyratory shaker (New Brunswick Scientific) at 150 rpm in a liquid media of the same composition, but without agar. Suspensions were subcultured at two week intervals by transferring 10 mL of cells to 40 mL fresh media. All callus and suspension cultures were incubated at 25°C in the dark. Eight days prior to experiments cell suspensions were filtered through a Buchner funnel with a perforated plate (pore size 2 mm), providing a fine suspension of cells. Cell suspensions were filtered again immediately before uptake experiments were initiated.

2.2.2 Preparation of Cell Suspensions for Aluminum Uptake Experiments

Methods used in this study were similar to those used by Zhang and Taylor (1989) to investigate the kinetics of Al uptake by excised roots of *Triticum aestivum*. Cell suspensions derived from an Al-resistant cultivar (Dade) and an Al-sensitive cultivar (Romano) of *P. vulgaris* were used to investigate the kinetics of Al uptake in a cell suspension system. To initiate experiments (which were repeated at least twice), filtered cell suspensions of *P. vulgaris* were collected by centrifugation (60 sec, 250 x g) in a Centaur 2 centrifuge (Johns Scientific Inc.) and washed once with 1.0 mM $CaCl_2$ (certified ACS grade, Fisher Scientific Co.). The cells were then resuspended in 1.0 mM $CaCl_2$ and stirred on a magnetic stir plate for 60 minutes. During this period, pH was readjusted to 4.50 with 1.0 N HCl at 15 min intervals. Concomitantly, dry weights were determined from each of 3 2-mL aliquots of cell suspensions and then suspensions for each cultivar were adjusted to equal densities with 1.0 mM $CaCl_2$.

2.2.3 Uptake of Aluminum by Cell Suspensions From Low Volume Solutions

Following the pH stabilization period, low volume uptake experiments were initiated by dispensing 3-mL aliquots of cell suspensions (approximately 10 mg dry weight) into each of 72 12x75-mm polystyrene tubes. Cells were pelleted by centrifugation (60 sec, 325 x g), spent $CaCl_2$ was removed, and cells were resuspended in 1.5 mL of 1.0 mM $CaCl_2$. Following a 30 minute equilibration period, experiments were initiated by adding 1.5 mL of 1.0 mM $CaCl_2$ containing 150 µM $AlCl_3$ (certified grade, Fisher Scientific Co.) to produce a final Al concentration of 75 µM. Throughout the $CaCl_2$ equilibration and Al uptake periods cells were rotated on an orbital mixer (Clay Adams, model #1105) in order to keep the cells in suspension. Four replicate tubes were removed after 0, 5, 10, 20, 30, 60, 90, 120, and 180 min of

exposure to Al. Cells were immediately centrifuged to remove the absorption solution, and washed three times with 1.0 mM $CaCl_2$ and once with deionized H_2O (resistance > 18 megohm cm^{-1}; Milli-Q H_2O System, Millipore Corp.). Cells were transferred to borosilicate tubes and prepared for determination of Al.

2.2.4 Uptake of Aluminum by Cell Suspensions From High Volume Solutions

Following the pH stabilization period, high volume uptake experiments were initiated by dispensing 3 mL aliquots of cell suspensions (approximately 10 mg dry weight) into each of 72 125-mL Erlenmeyer flasks containing 47 mL of 1.0 mM $CaCl_2$. Following a 30 min equilibration period in $CaCl_2$, experiments were initiated by adding 50 mL of 1.0 mM $CaCl_2$ containing 150 µM $AlCl_3$ to produce a final Al concentration of 75µM. Throughout the $CaCl_2$ equilibration and Al uptake periods flasks were rotated on a gyratory shaker (New Brunswick Scientific) at 150 rpm. Four replicate flasks were removed after 0, 5, 15, 25, 40, 60, 90, 120, and 180 min of exposure to Al. Cells were immediately collected by filtration through nylon mesh (mesh opening 70 µm), washed with 50 mL of 1.0 mM $CaCl_2$, followed by 50 mL deionized H_2O. Cells were transferred to borosilicate tubes and prepared for determination of Al.

2.2.5 Desorption Treatments

In experiments which included a desorption treatment, cells were collected by centrifugation (low volume experiments) or macrofiltration (high volume experiments). Cells were then rinsed with 1.0 mM $CaCl_2$ (4°C), resuspended in 3 or 15 mL citric acid (0.5-9.0 mM, 0°C; certified ACS grade, Fisher Scientific Co.) for low and high volume experiments respectively, and rotated on an orbital mixer for 30

min to allow for desorption of readily exchangeable Al from the apoplast. Following desorption, cells were rinsed with 1.0 mM $CaCl_2$, followed with deionized water, transferred to borosilicate tubes, and prepared for determination of Al.

2.2.6 Determination of Aluminum

Cell material was dried at 55°C under a stream of air and then ashed at 500°C. The resultant ash was dissolved in 10 mL deionized H_2O containing 0.1% HNO_3 (v/v; 65%, general reagent grade, less than 0.000005% Al, E. Merck, West Germany), and 0.1% H_2O_2 (v/v; certified grade, Fisher Scientific Co.). Aluminum concentrations were determined with a Perkin-Elmer 3030 atomic absorption spectrophotometer equipped with a HGA-500 graphite furnace attachment using a protocol initially adopted from Zhang and Taylor (1989). Twenty µL of diluted sample (0.2 mL: 1.8 mL deionized H_2O) was mixed with 20 µL of 17.5 mM $MgSO_4$ (certified ACS grade, Fisher Scientific Co.) as a matrix modifier, dried at 150°C for 60 s, pretreated at 1700°C for 45 s, and atomized at 2500°C for 5 s on a L'vov platform in a pyrolytically coated graphite tube. Integration of peak area was used to determine Al concentrations, which were expressed as micrograms Al per gram dry weight. The standards used for GFAAS were prepared using an Al reference solution (certified atomic absorption standard, Fisher Scientific Co.). Except for the ashing procedure samples and standards were prepared and stored in polypropylene containers which were soaked for one week in dilute HNO_3 (4% v/v) and rinsed in deionized water. The borosilicate tubes used for ashing were also soaked for one week in dilute HNO_3 (4% v/v) and rinsed with deionized water.

2.2.7 Modifications to the Basic Protocol Used to Study Uptake of Aluminum by Excised Roots

Several modifications to the basic protocol of Zhang and Taylor (1989) were essential to the success of my technique. Since the speciation and phytotoxicity of Al is dependent on pH (Kinraide, 1991), it is important to control the pH during uptake experiments. In initial experiments, the pH of uptake solutions inoculated with cell suspensions rose rapidly, an effect which was not consistent between cultivars. In order to maintain a pH of 4.50 or lower during uptake, it was necessary to incubate cells in a solution of 1.0 mM $CaCl_2$ open to the air for a period of 60 minutes prior to the initiation of experiments. During this period, the pH was readjusted to 4.50 at 15 minute intervals.

A requirement for rapid pelleting of cells and a minimum biomass for dry weight measurements constrained us to using high cell densities in low volume uptake experiments. This led to problems because the dry weight of cell material per tube was similar to that used in Zhang and Taylor's (1989) excised root experiments (approximately 10 mg), while the volume of uptake solution used in these experiments was 17 times smaller. This appears to have caused problems with respect to depletion of Al in uptake solutions. With this in mind, it is perhaps not surprising that I found it necessary to control the initial density of cells. Experiments with varying initial cell densities showed that uptake of Al on a dry weight basis decreased with increasing density (see Appendix, Table 5.1). It was therefore necessary to standardize the initial densities of cell suspensions. This was achieved by determining cell density by dry weight followed by adjustment of density with 1.0 mM $CaCl_2$. Although depletion of Al from uptake solutions appeared to be prevented in high volume uptake experiments, the initial density of cells was still controlled in these experiments.

Modifications were also required in the ashing procedure that was used. Initial uptake experiments showed high error, which I determined was attributable to sporadic contamination by Al liberated from ashing tubes during dissolution of ash with concentrated HNO_3. Because all glassware was soaked in dilute HNO_3 (4% v/v) for one week and rinsed in deionized water, the source of this contamination was likely leaching of Al from the glassware. Eliminating glassware in the ashing step of sample preparation was not possible, and HNO_3 could not be eliminated from the sample preparation step as little Al was recovered in the absence of HNO_3 (see Appendix, Table 5.2). By dissolving ash in a mixture of HNO_3, H_2O_2, and deionized water as opposed to adding the reagents separately (Zhang and Taylor, 1989), a reduction in error was observed with no significant loss in the amount of Al recovered (see Appendix, Table 5.3). Further reductions in error were observed by reducing the final concentration of acid from 2.0% to 0.1% (v/v) (see Appendix, Table 5.2). While I cannot be sure about the precise source of contamination in early experiments, it appears that most problems arose from release of Al from borosilicate tubes as a result of devitrification which arises after continual use of tubes for ashing. While changing procedures for solubilization of ash does not appear to affect the rate of devitrification, it does appear to eliminate contamination problems.

2.3 Results and Discussion

2.3.1 Uptake of Al from Low Volume Solutions

When cell suspensions of the Al-resistant cultivar, Dade and the Al-sensitive cultivar, Romano were exposed to low volume uptake solutions containing 75 µM $AlCl_3$ (pH 4.50, 22°C), uptake of Al was rapid for 20 min, with little additional

absorption occurring over the remainder of the experimental period (Fig. 2.1A). This pattern of uptake differed from previous reports of Al uptake in both intact and excised roots, which have typically shown a biphasic pattern of Al uptake consisting of a rapid non-linear initial phase superimposed over a second phase where accumulation of Al is slower and linear with time (Clarkson, 1967; Huett and Menary, 1979; Pettersson and Strid, 1989; Zhang and Taylor, 1989). In experiments with excised roots (Zhang and Taylor, 1989), the rapid initial phase of uptake has been interpreted as representing accumulation of loosely bound Al into the apoplast, and the slower linear phase as representing both uptake across the plasma membrane and metabolism-dependent accumulation in the cell wall (Zhang and Taylor, 1990). Applying this interpretation to the results presented in Figure 2.1A would lead to the conclusion that uptake in cell systems is dominated by accumulation of loosely bound Al in the apoplast. This passive accumulation is quantitatively greater in the cell system (2.25 times), and saturates in 20 minutes, 10 minutes earlier than in excised roots (Zhang and Taylor, 1989).

Differences in Al uptake between excised roots and cell suspensions could reflect real differences in uptake between root and cell suspension systems, although previous kinetic studies have shown that patterns of ion transport observed at the cellular level are similar to that observed at the whole plant level (Mettler and Leonard, 1979; Kochian and Lucas, 1983). Differences may also have reflected the unique characteristics of my cell suspension system. The finely dispersed nature of cells in suspension appears to have facilitated more rapid and extensive binding of Al in the apoplast, which might have effectively masked linear phase accumulation. If uptake into the apoplast was masking uptake across the plasma membrane, then removing Al from the apoplast should have allowed me to observe the linear phase of uptake. In an attempt to isolate the linear phase, cell suspensions were exposed to 3

mL 75 μM $AlCl_3$ (pH 4.50, 22°C). Each period of uptake (0-180 min) was then followed by a 30 min desorption period in 3 mL 0.5 mM citric acid (pH 4.50, 0°C). Desorption with 0.5 mM citric acid has been shown to be effective in removing readily exchangeable Al from the putative apoplasmic compartment (Zhang and Taylor 1989). In my cell system, however, desorption with 0.5 mM citric acid was only partially effective in removing the rapid initial phase of uptake and a clear linear phase of uptake over the 180 min uptake period was not observed (Fig. 2.1B).

To determine if the concentration of citric acid (0.5 mM) used in desorption treatments was sufficient to remove the large amount of Al absorbed into the apoplast, experiments were performed using higher concentrations of citric acid in desorption solutions. Cell suspensions of cvs. Dade and Romano were exposed to 75 μM $AlCl_3$ for varying periods of uptake (0-180 min) followed by a 30 min desorption in 3.0 mM citric acid, a concentration which provides at least 75 times more citric acid than total Al absorbed by cells. Desorption with 3.0 mM citric acid was more effective in removing the rapid initial phase of uptake. However, a clear linear phase of uptake over the 180 min uptake period was still not observed (Fig. 2.1C).

I hypothesized that my failure to observe a linear phase of uptake in these experiments was due to depletion of Al from uptake solutions, an observation which was consistent with the sensitivity of uptake to initial cell density (see Material and Methods). Furthermore, accumulation of Al by cell suspensions was quantitatively greater (2.25 times) than the extent of uptake by excised roots observed by Zhang and Taylor (1989). Because of the small volume of uptake solutions used in these experiments, 46% of the total Al supplied was absorbed by cells during the first 20 minutes of absorption. In an effort to reduce the potential impact of depletion, a series of experiments were performed in which cell suspensions of cv. Dade were exposed to

75, 500, and 1000 μM $AlCl_3$ (pH 4.50, 22°C) without a desorption period. Increasing the concentration of $AlCl_3$ to 500 and 1000 μM increased the total amount of accumulation, but did not affect the general pattern of uptake. In each experiment the rapid phase of accumulation was complete within 20 min and a linear phase was not observed (Fig. 2.2A). It was possible that the linear phase of uptake would continue to be masked until uptake of Al in the apoplast was saturated. Thus, experiments were conducted with increasing concentrations of Al from 0 to 5.0 mM. Following a 30 min period of uptake (without desorption), the amount of Al accumulated by cells increased linearly with increasing concentrations of Al, with no sign of saturation (Fig. 2.3).

In these high concentration experiments, the large amounts of Al accumulated by cells may have reduced the efficiency of desorption treatments. To determine if desorption treatments were effective at higher concentrations of added $AlCl_3$, cell suspensions of cv. Dade were exposed to 75, 500 and 1000 μM $AlCl_3$ in $CaCl_2$ (pH 4.50, 22°C). Following each uptake period (0-180 min) the cell suspensions were exposed to a 30 min desorption period in citric acid (pH 4.50, 0°C) at a concentration roughly 75 times the total amount of Al absorbed by the cells. At all three concentrations of $AlCl_3$, desorption with citric acid was effective in removing a major portion of the rapidly accumulated Al, but a clear linear phase of uptake was still not observed (Fig. 2.2B). From these results it appears that the concentration of citric acid in desorption treatments was not affecting my ability to isolate the linear phase of uptake. To provide further support for this conclusion, cell suspensions of cv. Dade were exposed to 75 μM $AlCl_3$ for 120 min. Following uptake, the cell suspensions were exposed to 0.5, 1.5, or 3.0 mM citric acid (pH 4.50, 0°C). After 0, 10, 20, 30, 60, 90, 120, and 180 min of desorption, 3 replicate tubes were removed from each citric acid concentration. At 1.5 and 3.0 mM citric acid desorption of Al was rapid for

30 min, with little additional desorption over the remainder of the experimental period (Fig. 2.4). At 0.5 mM citric acid, desorption was slower, taking 180 min to remove an equivalent amount of Al to that removed in 30 min at higher concentrations of citric acid (Fig. 2.4). Although these experiments suggest that initial desorption treatments may not have been completely effective, the concentration of desorption agent did not have a major effect on the pattern of Al desorption from the putative apoplasmic compartment. The increased desorption observed at the higher concentrations of citric acid was not substantive enough to account for the lack of an observable linear phase of uptake.

Increasing the concentration of Al in uptake solutions to concentrations of 500 μM or higher is problematic due to the very real possibility of precipitation of Al. Speciation calculations using version 2.0 of GEOCHEM-PC indicated that the formation of solid phase Al species was a possibility in all of the uptake solutions. This led me to question what I was actually measuring in these experiments. Was it apoplasmic Al or solid phase Al that was inadvertently collected with cell pellets? If solid phase Al was present in uptake solutions, this could account for the linear increase in Al uptake observed with increasing concentrations of $AlCl_3$ (Fig. 2.3). To determine if I was collecting solid phase Al, a mock uptake experiment was conducted in the absence of cells with concentrations of Al in uptake solutions ranging from 0 to 1000 μM. After a 2 hour mock uptake period, tubes were centrifuged (325 x g) to mimic cell removal. An aliquot (0.5 mL) of the low speed supernatant was sampled and analyzed directly for Al content by GFAAS. The bottom 1.0 mL of solution in the tubes, which was used to represent cell pellets was washed three times with $CaCl_2$, and then analyzed directly for Al content by GFAAS. With 0 to 1000 μM $AlCl_3$ in solution between 68 and 86% of the Al was recovered in the first low speed supernatants (Table 2.1). The amount of Al measured in the bottom 1.0 mL of

solution was less than 17% of the original Al in solution in all cases, suggesting that little solid phase Al was collected using my protocol (Table 2.1). The washing of the cell pellet fraction with $CaCl_2$ could account for the lack of a full recovery of Al observed in the supernatant and cell pellet fractions (Table 2.1). To determine if low speed centrifugation was insufficient to pellet out Al, similar experiments were done using higher speed centrifugation (2330 x g). Increasing the speed of centrifugation had little effect on the amount of Al recovered in the supernatant fraction (Table 2.1). Despite these results I cannot rule out the possibility of the existence of solid phase Al in uptake solutions. It is possible that cells may act as nucleation sites, which in turn could encourage the formation of solid phase Al (David R. Parker, personal communication). To ease my concerns regarding the potential for precipitation of Al in uptake solutions, experiments with high volume (100 mL), low concentration (75 µM $AlCl_3$) uptake solutions were performed.

2.3.2 Uptake of Al From High Volume Solutions

When cell suspensions of the Al-resistant cultivar, Dade and the Al-sensitive cultivar, Romano were exposed to 75µM $AlCl_3$ in a high volume system (100 mL), accumulation of Al was rapid for the first 20 min and linear over the remainder of the experimental period (Fig. 2.5A). These results are in contrast to results from low volume uptake experiments where the initial rapid phase of Al uptake was followed by little additional uptake over the remainder of the experimental period (Fig. 2.1A). Accumulation of Al was also more extensive when higher volume uptake solutions were used, suggesting that depletion of Al from uptake solutions could have accounted for my inability to observe a biphasic pattern of Al uptake in previous experiments. Increasing the volume of uptake solutions to 100 mL, while maintaining the same mass of cells, was an effective way of increasing the amount of Al available

for uptake while minimizing the potential for precipitation of Al. The observed biphasic pattern of uptake, where initial accumulation of Al was rapid followed by a linear phase was similar to the pattern of uptake previously reported for experiments with intact or excised roots (Clarkson, 1967; Huett and Menary, 1979; Pettersson and Strid, 1989; Zhang and Taylor, 1989, 1990, 1991). These results suggest that kinetic data obtained with intact or excised roots reflect events occurring at the cellular level.

A more dramatic difference between low volume and high volume uptake experiments was observed after desorption with citric acid. In an attempt to isolate the linear phase of uptake, cell suspensions from high volume experiments were exposed to 100 mL 75 μM $AlCl_3$. Each period of uptake was then followed by a 30 min desorption period in 15 mL of 9 mM citric acid, a concentration which is roughly 75 times the concentration of Al accumulated by the cells over a 180 min uptake period. For both cultivars, accumulation of Al was nearly linear with time (Fig. 2.5B). This is in striking contrast to low volume uptake experiments where desorption with citric acid was only partially effective in isolating a linear phase of uptake. These results provide further support for my conclusion that the inability to isolate a linear phase of uptake in low volume uptake experiments was due to insufficient amounts of Al available for uptake across the plasma membrane.

A linear phase of uptake has also been observed in excised roots of *T. aestivum* when uptake of Al (0-180 min) was followed by a 30 min desorption in citric acid (Zhang and Taylor, 1989). Classical interpretation of kinetic data suggests that the linear phase of uptake represents uptake across the plasma membrane. However, recent studies suggest that the linear phase of uptake may also include a cell wall component (Zhang and Taylor, 1990). In the present study I have not investigated the nature of the linear phase so conclusions regarding the components of linear phase

must await further studies. Although our current comprehension of the physiological and biochemical basis of Al toxicity and resistance continues to be limited by the lack of techniques for measuring the uptake of Al across the plasma membrane, this cell system could provide us with an experimental system in which the cell wall could be manipulated, in turn providing us with more direct information regarding movement of Al across the membrane.

Table 2.1 Aluminum recovered in the supernatant and the cell pellet fractions from mock uptake experiments. Values are expressed as % of original Al in solution that was recovered in supernatant following centrifugation at low speed (325 x g) and at high speed (2330 x g). Values represent means of 3 replicates ± S.E.

	Aluminum concentration (μM)					
Fraction	100	200	300	500	750	1000
low speed supernatant	85.9±9.3	68.2±3.5	79.0±3.1	84.7±4.2	69.6±9.1	84.9±8.2
high speed supernatant	47.8±2.2	74.4±9.8	66.1±4.9	76.7±3.6	80.5±4.6	86.7±4.0
low speed cell pellet	16.0±8.6	6.5±3.7	3.9±2.7	1.7±1.5	1.1±0.4	1.0±1.6
high speed cell pellet	10.3±6.0	4.9±3.4	4.9±4.0	1.6±1.1	1.8±0.7	1.4±0.5

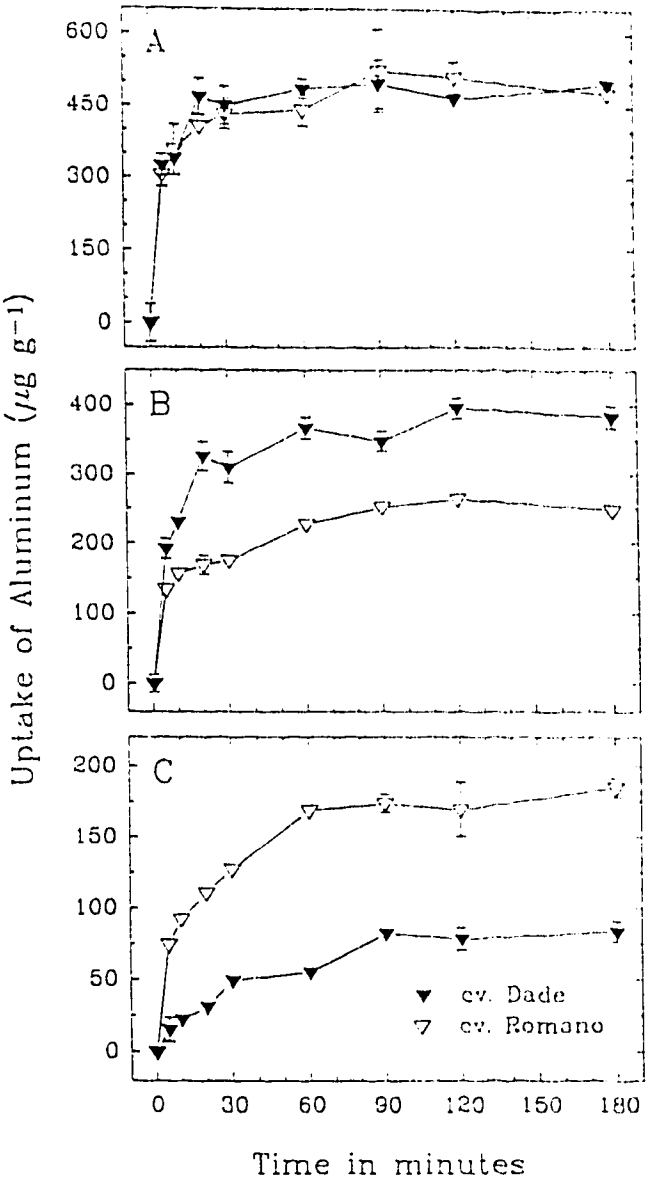

Figure 2.1 Uptake of Al from low volume (3 mL) uptake solutions by cell suspensions derived from an Al-resistant (Dade) and an Al-sensitive (Romano) cultivar of *P. vulgaris*. A. Uptake solutions contained 75 μM $AlCl_3$ in 1.0 mM $CaCl_2$ (pH 4.50, 22°C). B. Uptake in a solution of 75 μM $AlCl_3$ and 1.0 mM $CaCl_2$ (pH 4.50, 22°C) was followed by a 30 min desorption period in 0.5 mM citric acid (pH 4.50, 0°C). C. Uptake in a solution of 75 μM $AlCl_3$ and 1.0 mM $CaCl_2$ (pH 4.50, 22°C) was followed by a 30 min desorption period in 3.0 mM citric acid (pH 4.50, 0°C). Values represent means of 4 replicates ± S. E.

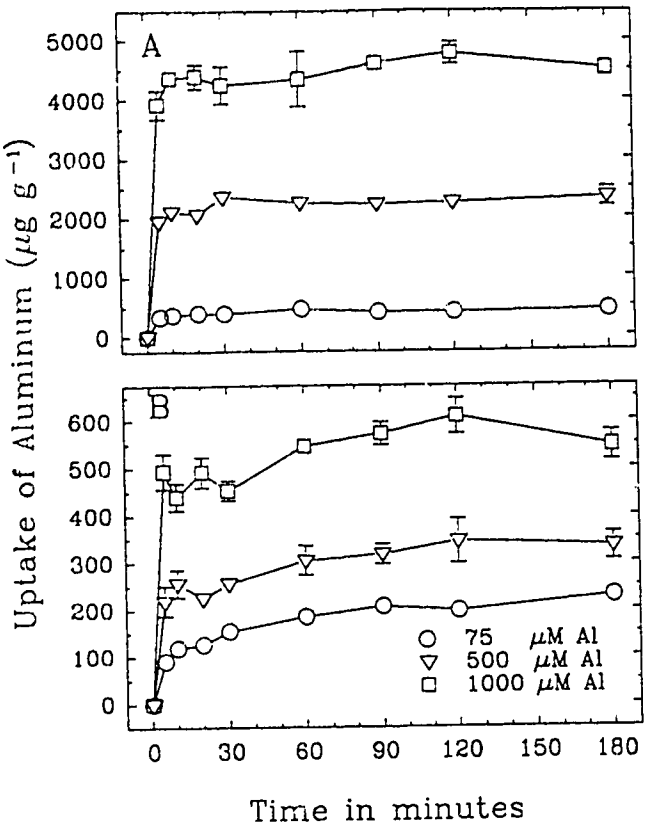

Figure 2.2 Uptake of Al by cell suspensions derived from an Al-resistant (Dade) cultivar of *P. vulgaris* with increasing concentrations of Al in solution. A. Uptake solutions contained 75, 500, or 1000 µM $AlCl_3$ in 1.0 mM $CaCl_2$ (pH 4.50, 22°C). B. Uptake in solutions of 75, 500, 1000 µM $AlCl_3$ in 1.0 mM $CaCl_2$ (pH 4.50, 22°C) was followed by a 30 min desorption period in 3.0, 10, or 45 mM citric acid (pH 4.50, 0°C) respectively. Concentrations of citric acid in desorption solutions were at least 75 times the total amount of Al absorbed by the cells during uptake. Values represent means of 4 replicates ± S. E.

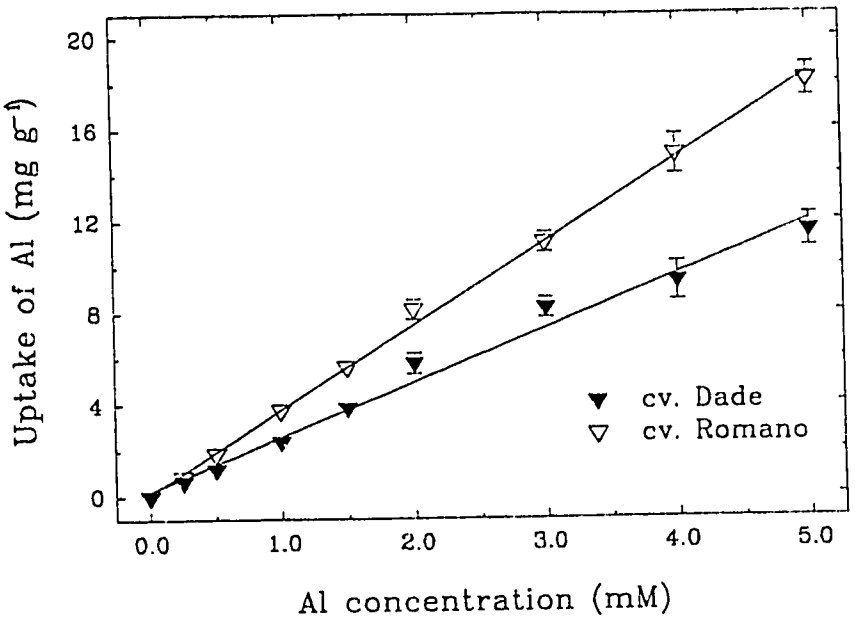

Figure 2.3 Uptake of Al by cell suspensions derived from an Al-resistant (Dade) and an Al-sensitive (Romano) cultivar of *P. vulgaris* at increasing concentrations of Al. Uptake for 30 min was in solutions containing 0, 0.25, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, or 5.0 mM $AlCl_3$ in 1.0 mM $CaCl_2$ (pH 4.50, 22°C). Values represent means of 4 replicates ± S. E.

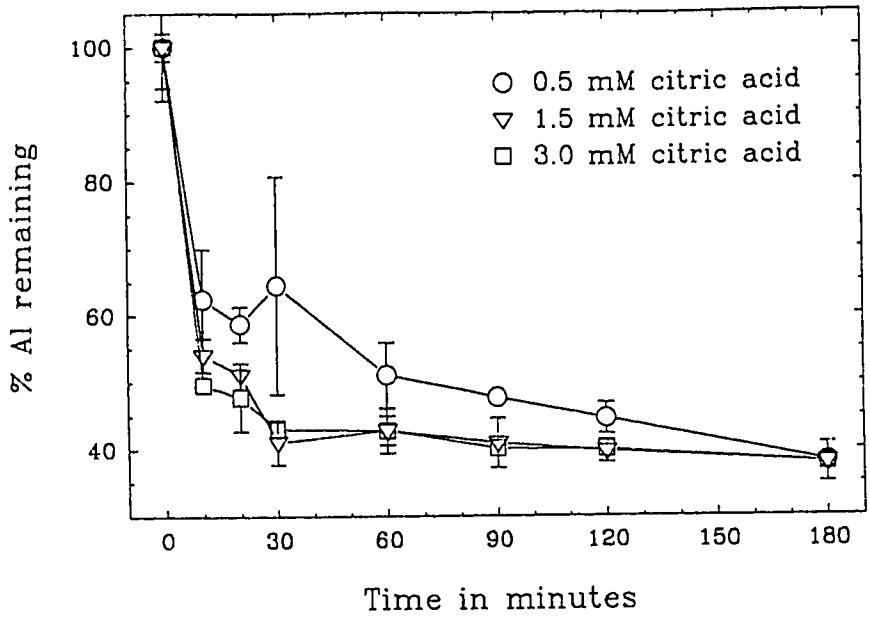

Figure 2.4 Desorption of Al from cell suspensions derived from an Al-resistant cultivar (Dade) of *P. vulgaris*. Uptake for 2 h in 75 μM $AlCl_3$ and 1.0 mM $CaCl_2$ (pH 4.50, 22°C) was followed by desorption in 0.5, 1.5, and 3.0 mM citric acid (pH 4.50, 0°C). Values represent means of 3 replicates ± S. E.

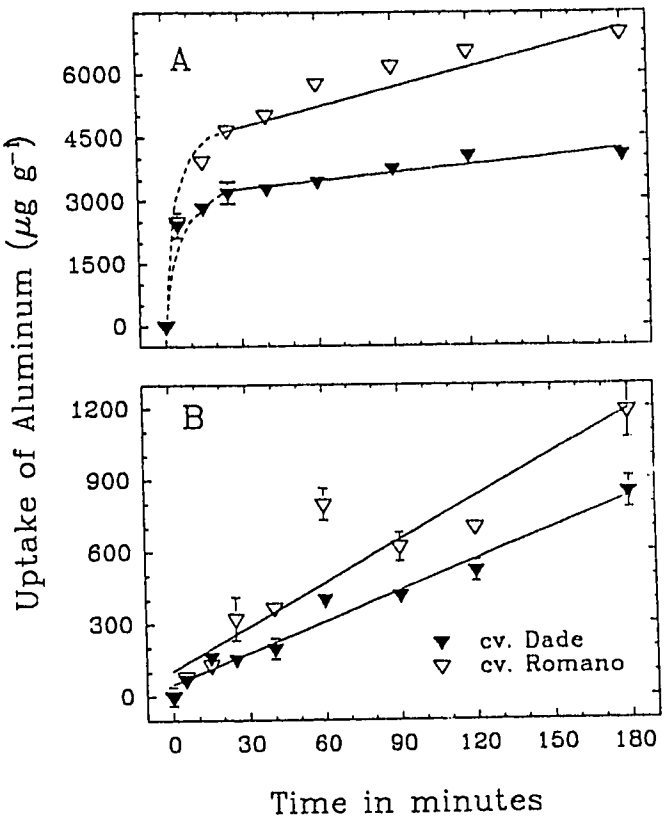

Figure 2.5 Uptake of Al from high volume (100 mL) uptake solutions by cell suspensions derived from an Al-resistant (Dade) and an Al-sensitive (Romano) cultivar of *P. vulgaris*. A. Uptake solutions contained 75 μM $AlCl_3$ in 1.0 mM $CaCl_2$ (pH 4.50, 22°C) B. Uptake in a solution of 75 μM $AlCl_3$ in 1.0 mM $CaCl_2$ (pH 4.50, 22°C) was followed by a 30 min desorption period in 9.0 mM citric acid (pH 4.50, 0°C). Solid lines represent regression lines drawn for the linear phase of uptake and dotted lines, which were drawn by hand, represent the rapid initial phase of uptake. Values represent means of 4 replicates ± S. E.

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3 The Feasibility of Using Kinetic Studies at the Cellular Level to Evaluate the Role that Exclusion Plays in Aluminum Resistance.

3.1 Introduction

Plants may resist the phytotoxic effects of aluminum (Al) either by external resistance mechanisms which detoxify or immobilize Al in the apoplast or by internal resistance mechanisms which immobilize, compartmentalize, or detoxify Al once it has crossed the plasma membrane and entered into the symplast. Postulated external resistance mechanisms include immobilization of Al in the cell wall, selective permeability of the plasma membrane, creation of a pH barrier in the apoplast or rhizosphere, exudation of chelate ligands or phosphate, or active efflux of Al. Internal resistance mechanisms may include chelation of Al in the symplast, sequestration of Al in the vacuole, evolution of Al-resistant enzymes, or enhanced synthesis of enzymes (Taylor, 1991).

The idea that resistance to Al may be achieved through external resistance mechanisms which exclude Al from the symplast has recently been gaining support in the scientific community (Taylor, 1991). Regardless of the particular mechanisms involved, if resistance is achieved by exclusion the rate of Al uptake across the plasma membrane should be greater in Al-sensitive cultivars than in Al-resistant cultivars. To distinguish between uptake of Al into apoplasmic and symplasmic compartments, several short-term kinetic studies characterizing the uptake of Al over time have been performed (Clarkson, 1967; Huett and Menary, 1979; Pettersson and Strid, 1989; Zhang and Taylor, 1989). In all of these studies the pattern of Al uptake consisted of a rapid initial phase followed by a linear phase over the remainder of the experimental

period. Traditional interpretation of this biphasic pattern of uptake suggests that the initial phase represents uptake into the apoplast while the linear phase represents uptake across the plasma membrane (Korner *et al.*, 1986).

When the kinetics of Al uptake were investigated in roots of *Triticum aestivum* by Pettersson and Strid (1989) and Zhang and Taylor (1989), differences in uptake of Al were not observed between Al-sensitive and Al-resistant cultivars. Thus Pettersson and Strid (1989) suggested that Al resistance was not linked to initial uptake of Al. The linear phase of uptake, however, appears to include a cell wall component which could mask differences in uptake of Al across the plasma membrane by differential accumulation of Al in the cell walls of Al-sensitive and Al-resistant cultivars (Zhang and Taylor, 1990). In the presence of 2,4-dinitrophenol (DNP), a general metabolic inhibitor, Zhang and Taylor (1990) found an increase in the rate of uptake in Al-resistant cultivars while no change was observed in the rate of Al uptake in Al-sensitive cultivars. Several other studies have also reported an increase in Al uptake in the presence of DNP and the authors suggested that DNP increased membrane permeability to Al (Huett and Menary, 1979; Wagatsuma, 1983).

Since DNP is reported to uncouple oxidative phosphorylation, modify membrane permeability, and diminish the proton gradient across the plasma membrane (Humphries, 1975; Jackson, 1982) it is hard to determine exactly how DNP affects uptake of Al. Nevertheless, Zhang and Taylor (1989) offered an alternate explanation for the DNP-induced increase in uptake of Al in Al-resistant cultivars. They suggested DNP disrupts an exclusion mechanism which operates under normal metabolic conditions. Studies using gramicidin, a channel forming ionophore, provided further data consistent with the existence of an exclusion mechanism. In *T. aestivum*, increased uptake of Al by excised roots was observed in Al-sensitive cultivars in the

presence of gramicidin, while in Al-resistant cultivars uptake of Al was relatively unaffected. The lack of an observable gramicidin effect in Al-resistant cultivars could have been due to the continued operation of an exclusion mechanism. In support of this idea, increased uptake of Al was observed in Al-resistant cultivars when DNP and gramicidin were applied in combination (Zhang and Taylor, 1991). Although these kinetic studies have provided support for the existence of exclusion mechanisms, this evidence is indirect due to the potential for non-specific effects of DNP and gramicidin and complexities arising from the presence of a cell wall.

If the putative exclusion mechanism of Zhang and Taylor (1991) operates at the cellular level, the cell suspension system used for my studies may provide an opportunity to further characterize the role that exclusion plays in resistance. I have demonstrated that cell suspensions derived from Al-sensitive and Al-resistant cultivars of *Phaseolus vulgaris* show similar patterns of Al uptake to those previously observed in whole or excised roots, suggesting that kinetic studies at the whole plant level reflect events occurring at the cellular level. Direct information about uptake of Al into the apoplast and symplast could then be obtained by comparing the kinetics of Al uptake by protoplasts and intact cells. This type of study could also provide information about the role that the plasma membrane and cell wall may play in Al resistance.

To evaluate the feasibility of using cell suspensions to study the potential role that exclusion plays in Al resistance, this study (1) investigated the effects of DNP, an uncoupler of oxidative phosphorylation, and gramicidin, a channel forming ionophore, on the kinetics of Al uptake by cell suspensions derived from an Al-resistant and an Al-sensitive cultivar of *P. vulgaris*, (2) compared the effects of DNP and gramicidin on uptake of Al by cell suspensions of *P. vulgaris* to those previously reported for

excised roots of *T. aestivum* (Zhang and Taylor, 1991) and (3) investigated the kinetics of Al uptake by excised roots of an Al-resistant and an Al-sensitive cultivar of *P. vulgaris* to determine if differences between species could account for the dissimilarities observed between cell suspensions of *P. vulgaris* and excised roots of *T. aestivum.*

3.2 Materials and Methods

3.2.1 Preparation of Cell Suspension Material

Cell suspensions derived from an Al-sensitive (Romano) and an Al-resistant (Dade) cultivar of *Phaseolus vulgaris* L. were initiated from callus cultures derived from 5 d old hypocotyls. Cell suspensions were grown in a modified Murashige and Skoog (1962) liquid medium as described previously (see Materials and Methods, Chapter 2). Suspension cultures were incubated at 25°C, in the dark on a gyratory shaker (150 rpm) and subcultured by transferring 10 mL of cells to 40 mL of fresh medium every 14 days. Eight days prior to experiments cell suspensions were filtered through a Buchner funnel with a perforated plate (pore size 2 mm), providing a fine suspension of cells. Cell suspensions were filtered again immediately before uptake experiments were initiated.

3.2.2 Preparation of Whole Plant Material

Seeds of an Al-sensitive (Romano) and an Al-resistant (Dade) cultivar of *P. vulgaris* L. were nicked with a razor blade and rolled in paper towels moistened with 1.0 mM $CaCl_2$ (pH 4.50). The paper towel rolls were then placed upright in plastic buckets with 3 L of 1.0 mM $CaCl_2$ (pH 4.50) containing 5.0 mg L^{-1} Vitavax to limit

the growth of fungi. After 3 d of growth in the dark at 25°C, seedlings were removed from the paper towels and prepared for uptake experiments.

3.2.3 Uptake of Aluminum by Cell Suspensions

For all experiments, $CaCl_2$ and uptake solutions were initially adjusted to pH 4.50. Cell suspensions were collected by centrifugation (60 sec, 250 x g) and rinsed once with 1.0 mM $CaCl_2$. The cells were resuspended in 1.0 mM $CaCl_2$ and stirred on a magnetic stirrer for 60 min. At this time, pH was readjusted to 4.50 at 15 min intervals with 1.0 N HCl. Dry weights (mg mL^{-1}) were determined from each of 3 2-mL aliquots of cell suspensions and suspensions for each cultivar were adjusted to equal densities with $CaCl_2$. Uptake experiments were initiated by dispensing 3 mL-aliquots of cell suspensions for each cultivar into 47 mL of 1.0 mM $CaCl_2$. Following a 30 min equilibration period 50 mL of 150 µM $AlCl_3$ in $CaCl_2$ with or without inhibitors (2.0 mM 2,4-dinitrophenol, Eastman Kodak Co. and/or 10 µM gramicidin D from *Bacillus brevis*, Sigma Chemical Co.) was added to each flask to bring the final concentration of Al to 75 µM and that of DNP and gramicidin to 1.0 mM and 5 µM respectively. Solutions of DNP and gramicidin were prepared by initially dissolving inhibitors in 95% ethanol (25 mL L^{-1}). A similar amount of ethanol was added to control solutions. Flasks were rotated on a gyratory shaker (150 rpm) throughout the $CaCl_2$ equilibration and uptake periods. After 0, 15, 25, 40, 60, 120, and 180 min, 3 replicate flasks were removed and the cells were collected by macrofiltration (mesh opening 70 µM). Cells were rinsed with 50 mL of cold $CaCl_2$ (4°C), then transferred to 15 mL of 9.0 mM citric acid (4°C) and rotated on an orbital mixer for 30 min to allow desorption of the readily exchangeable Al from the apoplast. Following desorption, cells were collected, rinsed first with 50 mL $CaCl_2$ and then with deionized water and transferred to borosilicate tubes for determination of Al

content by graphite furnace atomic absorption spectrophotometry (GFAAS) as described previously (see Materials and Methods, Chapter 2).

3.2.4 Uptake of Aluminum by Excised Roots

For all experiments $CaCl_2$ and uptake solutions were initially adjusted to pH 4.50. Twenty root tips (1 cm) were excised and placed in each of 32 to 50 "absorption tubes" as described by Zhang and Taylor (1989). During excision, absorption tubes containing excised roots were placed in an aerated solution of 1.0 mM $CaCl_2$. When excision was complete (within 60 min), the tubes were left in the $CaCl_2$ solution to equilibrate for an additional 30 min. 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 $AlCl_3$ in 1.0 mM $CaCl_2$ with or without inhibitors (1.0 mM DNP and or 10 µM gramicidin) in a water bath at 23°C. After each period of uptake 5 replicate tubes were removed from absorption solutions, rinsed with $CaCl_2$ (22°C) and transferred to 0.5 mM citric acid (0°C) for 30 min to remove loosely bound Al from the apoplast. Following desorption the roots were rinsed with 300 mL deionized water and prepared for determination of Al content by GFAAS. Although this work is described as part of my thesis, experiments with excised roots were performed by Guichang Zhang.

3.2.5 Determination of Aluminum in Cell Suspensions

Cells were dried overnight at 55°C, under a stream of air. Dry weights were determined and cells were ashed at 500°C for 12 h. The resultant ash was dissolved in 10 mL of a dilute HNO_3 and H_2O_2 solution. Aluminum concentrations were

determined by GFAAS as previously described previously (See Materials and Methods, Chapter 2).

3.2.6 Determination of Aluminum in Excised Roots

Roots were dried overnight at 55°C, weighed, transferred to 50 mL borosilicate tubes, and ashed at 500°C for 12 h. The resultant ash was dissolved in 40 mL deionized water. Aluminum concentrations were determined by GFAAS as described by Zhang and Taylor (1989).

3.2.7 Statistical Analysis of Data

Data were analyzed by simple linear regression using SAS Release 6.06. Due to deviations from linearity during the initial uptake times (a result of incomplete desorption of Al from the symplast), regressions were calculated from t = 15 min for cell suspensions and t = 60 min for excised roots. Differences between regression coefficients, in the presence and absence of inhibitors (DNP, gramicidin, or DNP and gramicidin in combination) were tested for with a parametric *t*-test (Zar, 1984).

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

where b_1= regression coefficient of regression line one, b_2 = regression coefficient of regression line two, s_1 = standard error of b_1, s_2 = standard error of b_2. 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 = 15, and m = 2. Significance was defined as $P < 0.05$.

3.3 Results

The pattern of Al uptake in cell suspensions derived from an Al-resistant and an Al-sensitive cultivar of *P. vulgaris* was unaffected by the presence of 1.0 mM DNP ($P < 0.05$; Fig. 3.1). When cells were exposed to 75 µM $AlCl_3$, rates of Al uptake with and without DNP were $24.66 \pm 0.1.82$ and 26.77 ± 0.74 µg g^{-1} min^{-1} for Dade and 5.03 ± 0.47 and 3.90 ± 0.47 µg g^{-1} min^{-1} for Romano. Quantitatively the uptake of Al was much greater in the Al-resistant cultivar (Dade) than in the Al-sensitive cultivar (Romano). Although the following hypothesis was not tested, I suspect that the lower amount of uptake observed in the Al-sensitive cultivar was a result of a higher degree of clumping in suspensions of this cultivar.

No significant effect on the rate of Al uptake was observed when cell suspensions were exposed to 0.50 µM gramicidin ($P < 0.05$). The rates of Al uptake were 2.74 ± 0.39 and 2.71 ± 0.69 µg g^{-1} min^{-1} in the Al-resistant cultivar, Dade, and 3.70 ± 0.30 and 4.53 ± 0.71 µg g^{-1} min^{-1} in the Al-sensitive cultivar, Romano (Fig. 3.2). In previous studies with *T. aestivum* when DNP and gramicidin were supplied in combination, the stimulation of Al uptake observed in Al-sensitive cultivars was similar to that expected on the basis of treatment with the inhibitors in isolation, while the stimulation of uptake in Al-resistant cultivars was greater than expected (Zhang and Taylor, 1991). To determine the combined effects of inhibitors on the uptake of Al, cell suspensions derived from Al-sensitive and Al-resistant cultivars of *P. vulgaris* were exposed to Al, DNP, and gramicidin. As expected from the lack of an effect when inhibitors were supplied in isolation, no significant differences in Al uptake were observed in either cultivar when the inhibitors were supplied concomitantly ($P < 0.05$).

Rates of Al uptake with or without inhibitors were 4.69 ± 0.44 and 5.72 ± 0.48 μg g^{-1} min^{-1} for Dade and 3.05 ± 0.13 and 2.62 ± 0.51 μg g^{-1} min^{-1} for Romano (Fig. 3.3).

Most previous kinetic work with Al used *T. aestivum* as an experimental species (Pettersson and Strid, 1989; Zhang and Taylor, 1989, 1990, and 1991). This species could not be used in this study because of problems with recalcitrance in cell suspensions (Cutler *et al.*, 1989). To determine whether dissimilarities between cell suspensions of *P. vulgaris* and excised roots of *T. aestivum* reflected differences between cells and excised roots or differences between species, the kinetics of Al uptake were investigated in excised roots of an Al-resistant (Dade) and an Al-sensitive cultivar (Romano) of *P. vulgaris*. In the presence of 50 μM $AlCl_3$, uptake of Al by an Al-resistant and an Al-sensitive cultivar of *P. vulgaris* was biphasic, consisting of a rapid phase in the first 20 min followed by a linear phase up to 180 min (Fig. 3.4A). This pattern was similar to that observed in cell suspensions of *P. vulgaris* and in excised roots of *T. aestivum*. Quantitatively, however, uptake from cell suspensions of *P. vulgaris* was greater than uptake from roots. The rate of the linear phase of uptake was also greater in cell suspensions (see Chapter 2, Fig. 2.5). The linear phase of uptake for excised roots of *P. vulgaris* was isolated by following each uptake period with a 30 min desorption in 0.5 mM citric acid (Fig. 3.4B).

When roots of *P. vulgaris* were exposed to 50 μM $AlCl_3$ in the presence of DNP, rates of Al uptake by the Al-resistant cultivar, Dade increased by 99.1% (from 2.96 ± 0.77 to 5.88 ± 0.79 μg g^{-1} min^{-1}) compared to control (Fig. 3.5A). Although the rates of Al uptake were 37.9% higher (2.93 ± 0.17 to 4.04 ± 0.56 μg g^{-1} min^{-1}) in the Al-sensitive cultivar, Romano in the presence of DNP (Fig. 3.5B), this increase was not statistically significant ($P < 0.05$). The rate of Al uptake by excised roots of

both cultivars was unaffected by gramicidin. When roots were exposed to 50 μM $AlCl_3$ the rates of Al uptake were 2.42 ± 0.40 and 1.85 ± 0.45μg g^{-1} min^{-1} for Dade and 2.27 ± 0.27 and 1.96 ± 0.27 μg g^{-1} min^{-1} for Romano in the presence and absence of gramicidin respectively (Fig. 3.6). When roots were exposed to DNP and gramicidin in combination, the rate of Al uptake increased in both cultivars. In the presence of Al, DNP and gramicidin, uptake increased by 94.9% (from 3.00 ± 0.41 to 5.83 ± 0.91 μg g^{-1} min^{-1}) in the Al-resistant cultivar and by 63.5% (from 2.11 ± 0.31 to 3.45 ± 0.40 μg g^{-1} min^{-1}) in the Al-sensitive cultivar ($P < 0.05$; Fig. 3.7).

3.4 Discussion

Although experimental support for external resistance mechanisms which involve exclusion of Al from the symplast is indirect, studies characterizing the effects of DNP and gramicidin on kinetics of Al uptake by excised roots of Al-resistant and Al-sensitive cultivars of *T. aestivum* have provided results consistent with the operation of an exclusion mechanism in Al-resistant cultivars (Zhang and Taylor, 1989, 1990, 1991). If Al resistance is a cellular phenomenon, I would expect uptake of Al by cell suspensions to respond to these inhibitors in a manner similar to that of excised roots. However, in contrast to Zhang and Taylor's studies (1991) with excised roots, this study using cell suspensions of *P. vulgaris* showed no effect of DNP or gramicidin on the kinetics of Al uptake (Figs. 3.1, 3.2, and 3.3). Therefore, I have not found evidence consistent with the operation of an exclusion mechanism at the cellular level in *P. vulgaris*.

Most postulated resistance mechanisms appear to have a fundamental cellular basis (Haug, 1984; Taylor, 1993), thus resistance mechanisms should be expressed in cell suspension systems. Parrot and Bouton (1990) demonstrated that Al resistance at

the whole plant level in *Medicago sativa* was retained at the cellular level. As of yet however, such a correlation has not been demonstrated for *P. vulgaris*. Numerous attempts have been made in our laboratory to develop techniques which can consistently express differential resistance in cell suspensions derived from Al-resistant and Al-sensitive cultivars of *P. vulgaris*. These attempts have been confounded by difficulties involved in controlling the speciation of Al in the culture medium. Control of the species of Al present in solution requires that concentrations of phosphate and other inorganic ions are kept low. However, conditions of low ionic strength are not conducive to growth of cell suspensions. Since the speciation of Al is highly dependent on pH (Kinraide, 1991), strict control of pH is also required to express Al toxicity and resistance in cell culture and this has been difficult to accomplish with cell suspensions of *P. vulgaris*. Further difficulties have arisen as a result of the sensitivity of cell suspensions derived from an Al-sensitive cultivar to low pH. This pH sensitivity makes it difficult to distinguish between sensitivity to Al and pH. Unfortunately, my inability to demonstrate differential resistance to Al in cell suspensions of *P. vulgaris* means I cannot rule out the possibility that resistance may be primarily a whole plant phenomenon in *P. vulgaris*.

Another problem arises from difficulties with the seed source of the Al-sensitive cultivar, Romano. Some of the seeds appear to give rise to seedlings which are resistant to Al. Therefore some cell lines may not be sensitive to Al. This cannot account for my inability to reproduce the effects of DNP and gramicidin observed in excised roots of Al-resistant cultivars of *T. aestivum* (Zhang and Taylor, 1991), but it could account for the lack of observable differences between cell suspensions derived from an Al-resistant and an Al-sensitive cultivar of *P. vulgaris*. This hypothesis will be tested in the future by repeating the above experiments with cell suspensions of

Romano generated from seedlings which will be maintained until maturity and examined for Al-sensitivity at the whole plant level.

Differences observed in the effects of DNP and gramicidin on uptake of Al between cell suspensions of *P. vulgaris* and excised roots of *T. aestivum* may also have resulted from the use of different experimental species. The possibility that resistance in cell suspensions may be occurring via an internal resistance mechanism or an exclusion mechanism which does not rely on metabolism cannot be ignored since different species may possess different Al-resistance mechanisms. In fact, Taylor (1991) argued that the range of resistance observed within and between species is likely mediated by a suite of resistance mechanisms. To determine the effect of using a different experimental species, the kinetics of Al uptake in the presence and absence of inhibitors were investigated in excised roots of *P. vulgaris*. Although this work is described as part of my thesis, experiments were performed by Guichang Zhang using techniques he had developed for *T. aestivum* (Zhang and Taylor, 1989, 1991). In both the Al-resistant (Dade) and the Al-sensitive (Romano) cultivar, uptake of Al by excised roots was biphasic (Fig. 3.4A), a pattern which was similar to that previously observed in cell suspensions of *P. vulgaris* and excised roots of *T. aestivum* (Fig. 2.5A and B, Chapter 2; Zhang and Taylor, 1989). The linear phase of uptake was isolated by following each period of uptake with a 30 min wash in 0.5 mM citric acid (Fig. 3.4B).

In excised roots of *P. vulgaris*, treatment with DNP increased the rate of Al uptake in the Al-resistant cultivar, Dade, but not in the Al-sensitive cultivar, Romano (Fig. 3.5). The exact nature of how DNP affects plants is not well established. However, increased uptake of Al by the Al-resistant cultivar is consistent with results from previous studies, suggesting that a metabolism-dependent exclusion mechanism

may be operating under normal metabolic conditions (Zhang and Taylor, 1989). Because the linear phase of uptake may include a cell wall component, increased uptake of Al in the presence of DNP could reflect increased accumulation in the apoplast or an increased uptake across the plasma membrane. Experiments investigating the effects of DNP on the kinetics of Al uptake into purified cell wall material have not been performed with *P. vulgaris*, but results with excised roots of *T. aestivum*, suggest that the DNP-induced stimulation in Al uptake cannot be explained by a cell wall component (Zhang and Taylor, 1991).

Previous studies at the whole plant level with *P. vulgaris* cvs. Dade and Romano suggested that resistance may be achieved via an exclusion mechanism (Miyasaka *et al.*, 1991). In the presence of Al, the Al-resistant cultivar (Dade) exuded 70 times more citric acid into the rooting medium than in the absence of Al and 10 times more than that of the Al-sensitive cultivar (Romano) in the presence or absence of Al. Unfortunately, no attempt was made to determine if the citric acid was being released in response to Al stress or in response to Al-induced phosphate deficiency (Miyasaka *et al.*, 1991). Furthermore they did not look at uptake of Al making it unclear whether the exudation of citric acid was an effective exclusion mechanism. If exudation of citric acid is involved in Al resistance in *P. vulgaris* then resistance should be observed in cell suspensions. Nevertheless, the lack of a DNP effect in cell suspensions of *P. vulgaris* (Fig. 3.1) is inconsistent with the concept of metabolism-dependent exclusion (Miyasaka *et al.*, 1991; Zhang and Taylor, 1991).

Unfortunately, I cannot however, rule out the possibility that the dose of DNP and duration of exposure may have been insufficient to exert inhibitory effects on kinetics of Al uptake in this cell suspension system. The concentration of DNP chosen for this study was based on concentrations used in previous kinetic studies with

excised roots of *T. aestivum* (Zhang and Taylor, 1989). In root cortical cells of *Beta vulgaris*, maximum depolarization of the potential across the plasma membrane was observed at a concentration of 0.1 mM DNP (Lindberg *et al.*, 1991), a concentration well below the one used in this study (1.0 mM). If anything, I would expect that less DNP would be required to exert an effect in my cell suspension system due to the intimate contact between individual cells and the experimental solution. Cell suspensions of *Nicotiana plumbaginifolia* showed a higher sensitivity to Al than do plated cells, callus cultures, or whole plants and this was attributed to the close contact between cells and the toxic medium (Conner and Meredith, 1985). To determine if the concentration or duration of exposure of DNP affected my results, experiments investigating the effects of different concentrations of DNP and different times of exposure on the uptake of Al in conjunction with measurements of other physiological properties including membrane potential, membrane permeability and rates of oxidative phosphorylation could be performed.

In excised roots of *P. vulgaris*, gramicidin had no effect on uptake of Al in both the Al-resistant and the Al-sensitive cultivars (Fig. 3.6). This observation was consistent with the effect of gramicidin on uptake of Al by cell suspensions of *P. vulgaris* (Fig. 3.2), but it was in contrast to results obtained by Zhang and Taylor (1991) with excised roots of *T. aestivum*. The lack of a gramicidin effect in *P. vulgaris* suggests that transport of the membrane mobile species of Al is not facilitated by the presence of gramicidin channels. Gramicidin is a non-specific channel forming ionophore which facilitates the transport of numerous monovalent cations across the plasma membrane (Hodges *et al.*, 1971; Riedell and Schmid, 1986). Thus, uptake of Al might not be affected if the dominant membrane mobile species in *P. vulgaris* is Al^{3+}. The strong charge density of this ion, may prevent transport through monovalent channels (Zhang and Taylor, 1991). The different effects of gramicidin on

uptake of Al by excised roots of *P. vulgaris* and *T. aestivum* might suggest that the dominant membrane mobile species of Al is different in these two species. Kinraide and Parker (1990) suggested that the primary toxic species of Al responsible for Al-induced injury may differ in monocots and dicots and this may depend on the form of Al more readily taken up by the plants. However, Kinraide and Parker's (1990) conclusions on which species of Al is toxic to monocots and which species is toxic to dicots is inconsistent with the interpretation I have provided for the different effects of gramicidin on the uptake of Al between *P. vulgaris* and *T. aestivum.* Gramicidin could have effects on other cellular processes, such as the activities of proton ATPases (Zhang and Taylor, 1991) or rates of membrane turnover which in turn could affect the uptake of Al across the plasma membrane. If the effects of gramicidin on these cellular processes was different in different species this could also account for the inconsistent effects of gramicidin on uptake of Al between *P. vulgaris* and *T. aestivum.* Unfortunately my experiments have not provided any insight into these different hypotheses.

In previous studies with *T. aestivum*, when DNP and gramicidin were supplied in combination, the stimulation in Al uptake in Al-sensitive cultivars was similar to that expected on the basis of treatment with the inhibitors in isolation, while the stimulation in uptake in Al-resistant cultivars was greater than expected (Zhang and Taylor, 1991). In the present study, no additional effect on the uptake of Al by cell suspensions was observed when DNP and gramicidin were supplied in combination providing further support for the hypothesis that gramicidin does not facilitate transport of the membrane mobile species in *P. vulgaris* (Fig. 3.3). In excised roots of both the Al-resistant and the Al-sensitive cultivar, a significant increase in the uptake of Al was observed when the inhibitors were supplied in combination (Fig. 3.7). This increase in Al uptake by the Al-sensitive cultivar is inconsistent with the lack of a significant effect

on the uptake of Al observed when inhibitors were supplied in isolation. Further experiments are required to confirm this significant effect of inhibitors observed in the Al-sensitive cultivar.

Results of experiments with excised roots of *P. vulgaris* suggest that differences between the effects of inhibitors on the uptake of Al by cell suspensions of *P. vulgaris* and excised roots of *T. aestivum* are a combination of differences between species and between cells and whole roots. Although the use of different experimental species can account for the unique gramicidin effects observed in the cell suspension system used in this study, it cannot account for the lack of a DNP effect. The DNP-induced stimulation of Al-uptake observed in excised roots of *P. vulgaris* is consistent with the operation of a metabolism dependent exclusion mechanism in the Al-resistant cultivar, Dade. Although I have found no evidence that exclusion is occurring in cell suspensions derived from this Al-resistant cultivar of *P. vulgaris*, this does not rule out the possibility that exclusion does occur at the whole plant level. While, exclusion should be a cellular phenomenon, it may reside at a level of organization in whole plant tissues that is not expressed in cell suspensions. The overall architecture of a plant root may facilitate the expression of exclusion by creating a microenvironment in the root apoplast. Exudation of organic ligands, such as citric acid (Miyasaka, 1991) or the active efflux of phosphate (Lindberg, 1990) or Al (Zhang and Taylor, 1991), could all lead to the precipitation or polymerization of Al in the cell wall or intracellular spaces which could in turn reduce the uptake of Al across the plasma membrane. In a cell suspension system, each individual cell is in immediate contact with the experimental solution which makes it difficult to maintain a microenvironment different from that of the bulk solution. Although exudation of organic ligands or phosphate into the media should effectively reduce the toxicity of Al by chelating Al in the bulk solution this may not be observed in short-term uptake studies.

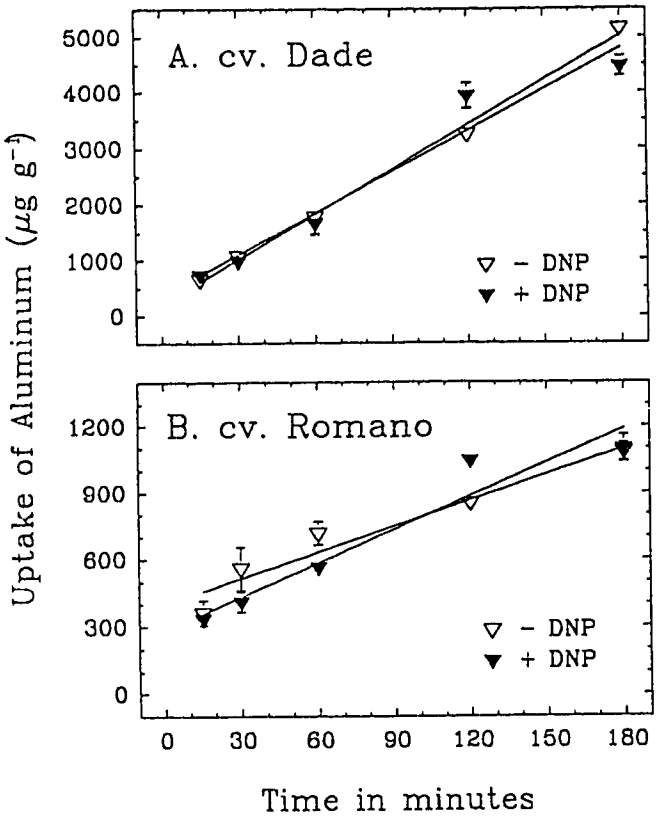

Figure 3.1 The effect of DNP on uptake of Al by cell suspensions of *P. vulgaris*. Uptake solutions contained 75 μM $AlCl_3$ in 1.0 mM $CaCl_2$ (22°C, pH 4.50) with or without 0.1 mM DNP. Each period of uptake was followed by a 30 min desorption in 9.0 mM citric acid (4°C, pH 4.50). A. Uptake of Al by an Al-resistant cultivar (Dade). B. Uptake of Al by an Al-sensitive cultivar (Romano). Values represent means of 3 replicates ± S.E.

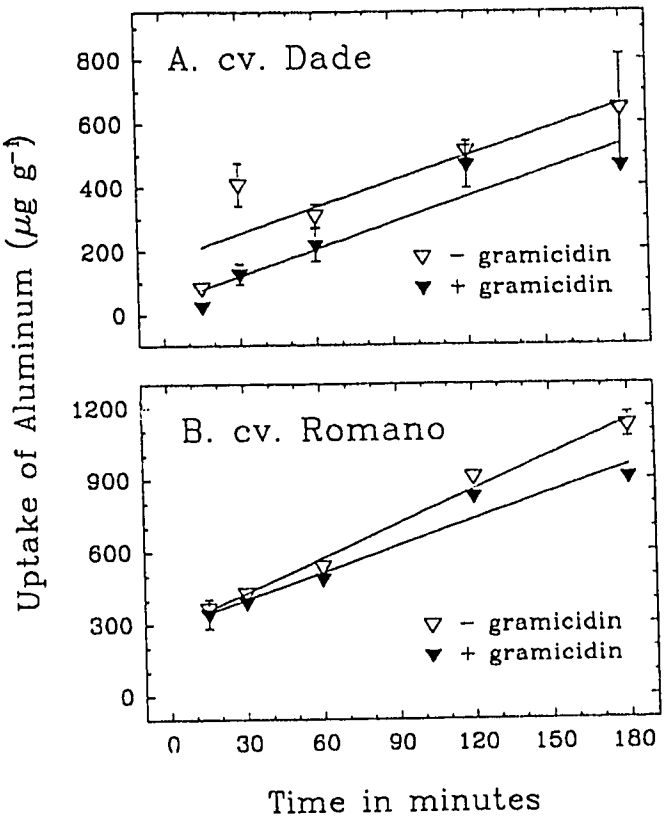

Figure 3.2 The effect of gramicidin on uptake of Al by cell suspensions of *P. vulgaris*. Uptake solutions contained 75 μM $AlCl_3$ in 1.0 mM $CaCl_2$ (22°C, pH 4.50) with or without 5 μM gramicidin. Each period of uptake was followed by a 30 min desorption period in 9.0 mM citric acid (4°C, pH 4.50). A. Uptake of Al by an Al-resistant cultivar (Dade). B. Uptake of Al by an Al-sensitive cultivar (Romano). Values represent means of 3 replicates ± S.E.

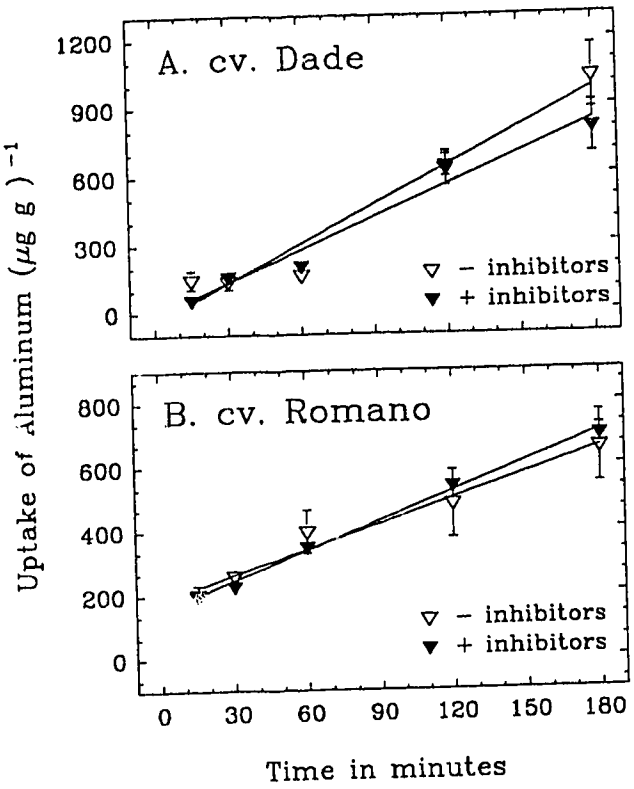

Figure 3.3 Effect of DNP and gramicidin in combination on uptake of Al by cell suspensions of *P. vulgaris*. Uptake solutions contained 75 μM $AlCl_3$ in 1.0 mM $CaCl_2$ (22°C, pH 4.50) with or without 0.1 mM DNP and 5 μM gramicidin. Each period of uptake was followed by a 30 min desorption in 9.0 mM citric acid (4°C, pH 4.50). A. Uptake of Al by an Al-resistant cultivar (Dade). B. Uptake of Al by an Al-sensitive cultivar (Romano). Values represent means of 3 replicates ± S.E.

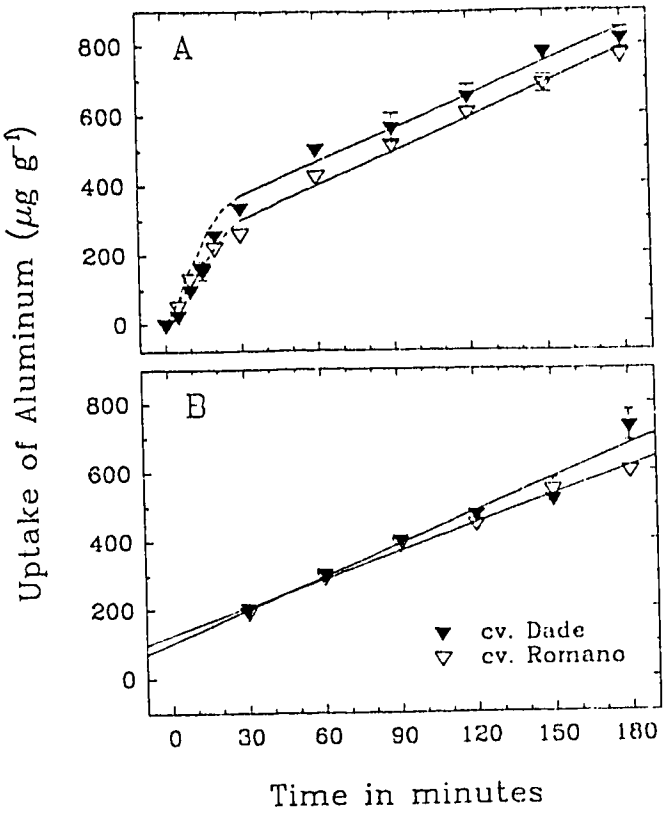

Figure 3.4 Uptake of Al by excised roots of an Al-resistant (Dade) and an Al-sensitive (Romano) cultivar of *P. vulgaris*. A. Uptake solutions contained 50 μM $AlCl_3$ in 1.0 mM $CaCl_2$ (22°C, pH 4.50). B. Uptake in a solution of 50 μM $AlCl_3$ was followed by a 30 min desorption period in 0.5 mM citric acid (0°C, pH 4.50). Solid lines represent linear regression lines drawn for the linear phase of uptake and dotted lines which were drawn by hand represent the rapid initial phase of uptake. Values represent means of 5 replicates ± S.E.

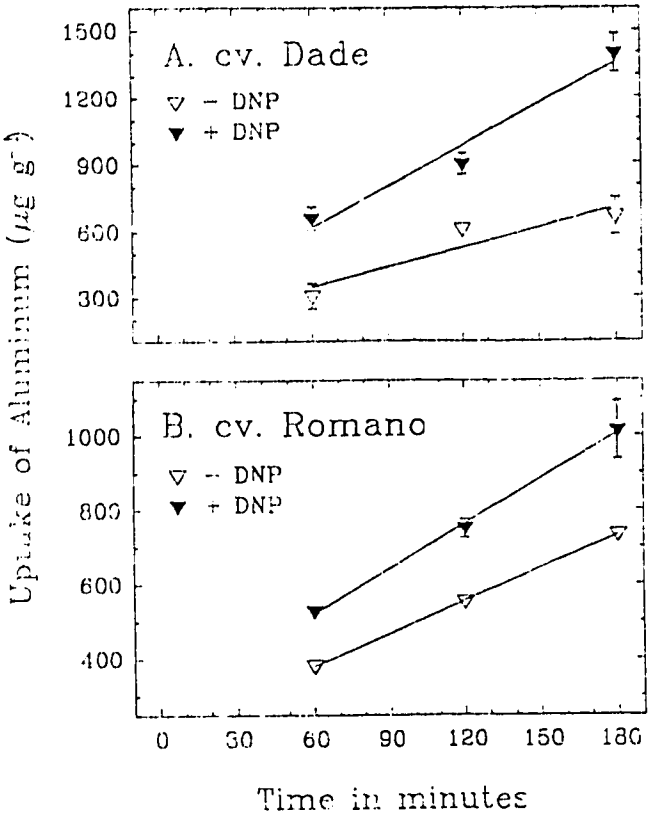

Figure 3.5 The effect of DNP on uptake of Al by excised roots of *P. vulgaris*. Uptake solutions contained 50 μM $AlCl_3$ in 1.0 mM $CaCl_2$ (22°C, pH 4.50) with or without 0.1 mM DNP. Each period of uptake was followed by a 30 min desorption period in 0.5 mM citric acid (0°C, pH 4.50). A. Uptake of Al by excised roots of an Al-resistant cultivar (Dade). B. Uptake of Al by excised roots of an Al-sensitive cultivar (Romano). Values represent means of 5 replicates ± S.E.

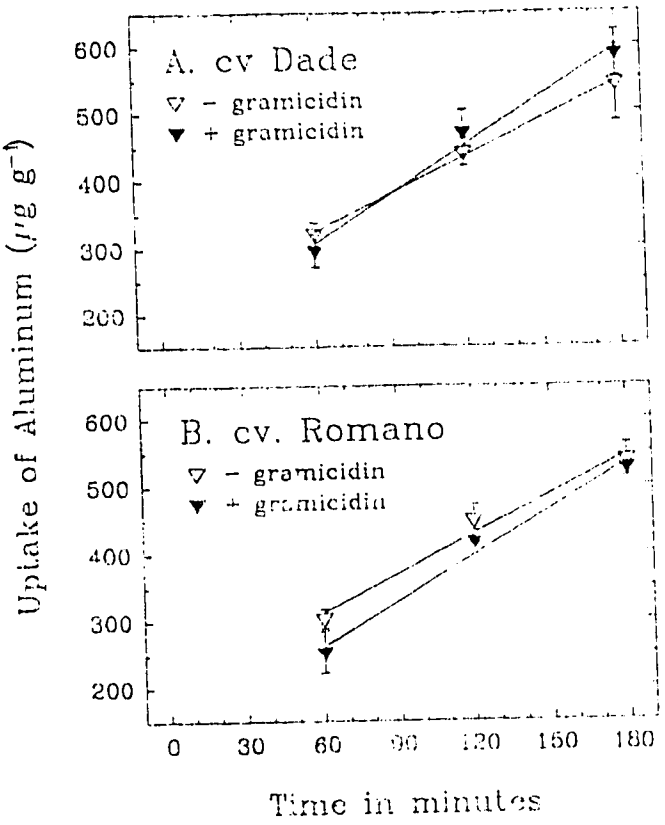

Figure 3.6 The effect of gramicidin on uptake of Al by excised roots of *P. vulgaris*. Uptake solutions contained 50 μM $AlCl_3$ in 1.0 mM $CaCl_2$ (22°C, pH 4.50) with or without 10 μM gramicidin. Each period of uptake was followed by a 30 min desorption period in 0.5 mM citric acid (0°C, pH 4.50). A. Uptake of Al by excised roots of an Al-resistant cultivar (Dade). B. Uptake of Al by excised roots of an Al-sensitive cultivar (Romano). Values represent means of 5 replicates ± S.E.

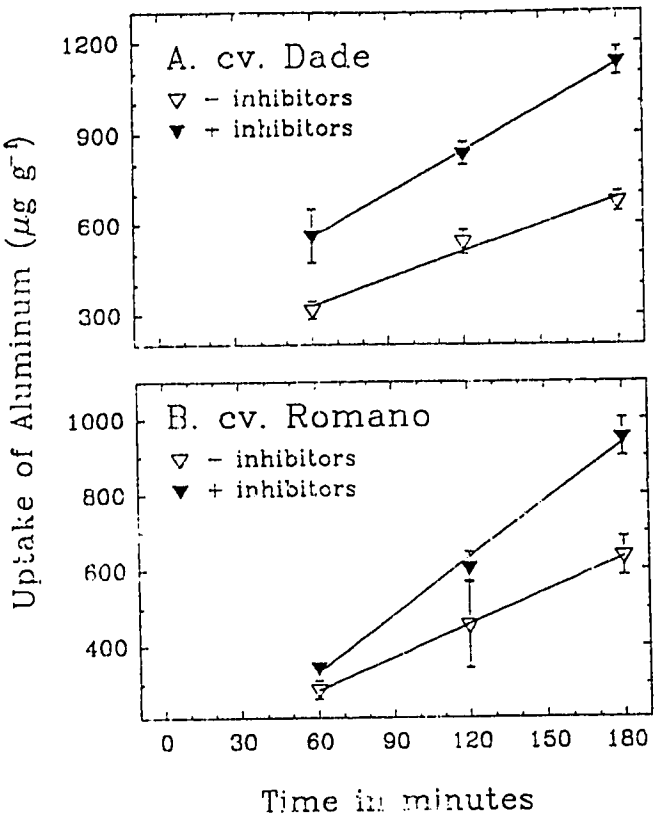

Figure 3.7 Effect of DNP and gramicidin in combination on uptake of Al by excised roots of *P. vulgaris*. Uptake solutions contained 50 μM $AlCl_3$ in 1.0 mM $CaCl_2$ (22°C, pH 4.50) with or without 0.1 mM DNP and 10 μM gramicidin. Each period of uptake was followed by a 30 min desorption period in 0.5 mM citric acid (0°C, pH 4.50). A. Uptake of Al by excised roots of an Al-resistant cultivar (Dade). B. Uptake of Al by excised roots of an Al-sensitive cultivar (Romano). Values represent means of 5 replicates ± S.E.

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4 Concluding Discussion

When I began this research, our understanding of the physiological and biochemical basis of Al resistance in plants was limited by a lack of information about the movement of Al across the plasma membrane. A number of short term kinetic studies had made important advances in distinguishing between uptake of Al into the apoplasmic and symplasmic compartments and providing support for the possible involvement of a metabolism-dependent exclusion mechanism in Al resistance. These studies, however, were largely indirect, a result of complexities arising from the presence of a cell wall. As a result they may have resulted in inaccurate estimates of the amount of Al associated with the apoplasmic and symplasmic compartments making possible differences in Al uptake between Al-sensitive and Al-resistant cultivars difficult to distinguish. In an attempt to move towards providing direct information regarding transmembrane transport of Al and to evaluate the involvement of exclusion in resistance, the overall objectives of this study were (1) to determine if the kinetics of Al uptake could be studied at the cellular level and (2) to assess the feasibility of using a cell suspension system to evaluate the role that exclusion may play in resistance. This research was conducted with hopes of developing a system in which the cell wall could be manipulated. By comparing the kinetics of Al uptake between protoplast and cell suspensions, more direct information about the uptake of Al into the apoplasmic and symplasmic compartments could be obtained.

To investigate the kinetics of Al uptake in cell suspensions of *Phaseolus vulgaris*, a number of technical difficulties associated with working at the cellular level had to be overcome. Cell suspensions had to be exposed to a pH stabilization period prior to initiating uptake experiments to prevent an inconsistent rise in pH between

cultivars during uptake experiments. Since the speciation and phytotoxicity of Al are highly dependent on pH (Kinraide, 1991), control of pH was essential during uptake experiments. When initial cell density was varied, uptake of Al on a dry weight basis decreased with increasing density. Therefore dry weights of cells had to be determined and initial cell densities had to be standardized prior to initiation of uptake experiments. Modifications to the ashing procedure adopted from Zhang and Taylor (1989) were also necessary. Initial experiments showed high experimental error which I attributed to sporadic contamination by Al liberated in the ash dissolution step. This contamination most likely arose as a result of the devitrification of the borosilicate tubes I used in ashing. Reducing the amount of HNO_3 used in the ashing procedure and dissolving ash in a dilute solution of HNO_3, as opposed to adding it in isolation, appeared to be an effective means of reducing the previously observed contamination by Al. With these modifications incorporated into my protocol I proceeded to investigate the kinetics of Al uptake in cell suspensions of *P. vulgaris*.

In low volume (3 mL) experiments, uptake of Al was rapid for the first 20 min with little additional accumulation occurring over the remainder of the experimental period. This was in contrast to previous studies with whole or excised roots where the rapid initial phase of uptake was followed by a linear phase (Clarkson, 1967; Huett and Menary, 1979; Pettersson and Strid, 1989; Zhang and Taylor, 1989). Uptake of Al by cell suspensions was also more rapid and more extensive than by excised roots (Zhang and Taylor, 1989). Applying traditional interpretation of kinetic data, where the rapid initial phase of uptake represents uptake into the apoplast, uptake by cell suspensions appeared to be dominated by loosely bound Al in the apoplast. The finely dispersed nature of the cell suspensions used appeared to facilitate rapid and extensive accumulation of Al in the apoplast and this may have effectively masked linear phase accumulation of Al. However, if this had been the case, then removing Al from the

apoplast should have allowed us to observe a linear phase of uptake. Desorption with citric acid was only partially effective in removing the rapid initial phase of uptake and a linear phase of uptake was still not observed. These results suggested that uptake into the apoplast was not masking the linear phase of uptake.

The rate, extent, and saturable nature of uptake by cell suspensions of *P. vulgaris* in conjunction with my observation that uptake of Al on a dry weight basis decreased dramatically with increasing cell density suggested that depletion of Al from uptake solutions could also have accounted for my inability to isolate an observable linear phase. To overcome the potential impact of depletion, low volume uptake experiments were performed using high concentrations of Al (500 and 1000 µM). Increasing the concentration of Al in uptake solutions increased total uptake, but the rapid initial phase of uptake was still followed by little additional absorption over the remainder of the uptake period. Desorption with citric acid was effective in removing a large amount of the rapidly absorbed Al, but a linear phase of uptake was still not observed.

Increasing concentrations of Al in uptake solutions beyond 75 µM led to concerns about the formation of solid phase Al. If precipitation was occurring in uptake solutions, I may have been measuring solid phase Al that was inadvertently collecting with cell pellets rather than Al which was associated with the apoplast. Although mock uptake experiments suggested that little solid phase Al was being collected with cell pellets, I still had reservations because of the possibility that cells may have been acting as nucleation sites and encouraged formation of solid phase Al.

At this point I looked for an alternate means of increasing the amount of Al available for uptake and minimizing the potential for precipitation of Al. This was

when I began studying the uptake of Al from high volume (100 mL) uptake solutions with the same cell masses used in previous low volume uptake solutions. In high volume uptake experiments, accumulation of Al was rapid for the first 20 min and linear over the remainder of the experimental period. The rapid initial phase of uptake was much more extensive than uptake from low volume uptake experiments supporting the hypothesis that depletion of Al from uptake solutions may have accounted for my inability to isolate an observable linear phase of uptake. This biphasic pattern of Al uptake observed with cell suspensions of *P. vulgaris* was similar to patterns previously reported for intact or excised roots (Clarkson, 1967; Huett and Menary, 1979; Pettersson and Strid, 1989; Zhang and Taylor, 1989). In contrast to uptake experiments, I was able to isolate the linear phase of uptake by cells in citric acid following each uptake period. These results suggested that kinetic data obtained at the intact or excised root level reflected events occurring at the cellular level.

Throughout these studies, the comparisons I have made between cell suspensions derived from the Al-resistant cultivar, Dade, and the Al-sensitive cultivar, Romano, have been strictly qualitative. Quantitative comparisons were not made between rates and extents of Al uptake by cell suspensions because of inconsistencies with respect to absolute amounts of Al accumulated by cell suspensions from one experiment to the next. Although the following hypothesis was not tested, I suspect that the differences among experiments were a result of different degrees of cell clumping that I observed in the cell suspensions used for each experiment. Although I attempted to control for clumping by sieving cells prior to initiating experiments, I still observed a difference in the fineness of cell suspensions between experiments.

Another reason I did not quantitatively compare uptake of Al between the two cultivars is because I made no attempt to identify the nature of the linear phase of uptake by cell suspensions of *P. vulgaris*. Previous studies with excised roots of *Triticum aestivum* suggest that the linear phase of uptake may be complex, consisting of both uptake across the plasma membrane and uptake into cell wall material. More recent studies, however, which have measured uptake of Al by cell wall material from excised roots of *T. aestivum* suggest that when $AlCl_3$ and $CaCl_2$ are used in uptake experiments the linear phase of uptake may not include a cell wall component (Archambault, personal communication). In the future, the nature of the linear phase of uptake in cell suspension of *P. vulgaris* should be determined by investigating the kinetics of *in vivo* and *in vitro* uptake of Al by cell wall material.

In previous kinetic studies with excised roots of *T. aestivum*, patterns of Al uptake which distinguished between Al-resistant and Al-sensitive cultivars were observed in the presence of 2,4-dinitrophenol (DNP) and gramicidin. The effects of these inhibitors on the uptake of Al provided preliminary support for the existence of a metabolism-dependent exclusion mechanism operating in Al-resistant cultivars (Zhang and Taylor, 1991). When I conducted similar studies, I found no differences in the effect of DNP and/or gramicidin on the uptake of Al by cell suspensions derived from an Al-resistant and Al-sensitive cultivar of *P. vulgaris*. The lack of a DNP and gramicidin effect was inconsistent with the operation of an exclusion mechanism at the cellular level. Because most postulated resistance mechanisms appear to have a fundamental cellular basis (Haug, 1984; Taylor, 1993), I expected resistance to be expressed at the cellular level. Unfortunately this has not yet been demonstrated for the cultivars of *P. vulgaris* with which I worked in this study. Thus, I cannot rule out the possibility that resistance in *P. vulgaris* may primarily be a whole plant phenomenon.

Prior to investigating this hypothesis any further I decided to investigate the effects of DNP and gramicidin on the uptake of Al by excised roots of *P. vulgaris* cvs. Dade and Romano (these experiments were kindly performed by Guichang Zhang). The different effects of DNP and gramicidin on uptake of Al between cell suspensions of *P. vulgaris* and excised roots of *T. aestivum* could have resulted from the use of different experimental species which possess different resistance mechanisms. In the presence of DNP, the rate of Al uptake increased in the Al-resistant cultivar, but was relatively unaffected in the Al-sensitive cultivar. Although the exact nature of the DNP effect in plants is not well established, these results were consistent with previous results from studies with *T. aestivum*, which were interpreted to represent the operation of an exclusion mechanism in the Al-resistant cultivar. Other studies at the whole plant level with *P. vulgaris* cvs. Dade and Romano have also suggested that resistance may be achieved via an exclusion mechanism, possibly involving the exudation of chelate ligands (Miyasaka, 1991).

Results from experiments using excised roots suggested that the lack of an effect of DNP on uptake of Al by cell suspensions was a result of using a different system rather than using a different experimental species. Although the lack of a DNP effect in my cell suspension system places doubt on the concept of metabolism dependent exclusion (Miyasaka, 1991; Zhang and Taylor, 1991), I cannot rule out the possibility that it was a result of my experimental conditions. Although I consider this unlikely, both the dose of DNP and time of exposure may have been insufficient to exert inhibitory effects. This hypothesis will be tested in the future by performing DNP dose and time response experiments with my cell suspension system.

In contrast to the results of my DNP experiments, the different effects of gramicidin on the uptake of Al observed between cell suspensions of *P. vulgaris* and excised roots of *T. aestivum* can be explained by the use of a different experimental species. Gramicidin had no effect on the uptake of Al by excised roots or cell suspensions of either cultivar suggesting that gramicidin does not facilitate the transport of the membrane mobile species in *P. vulgaris*. The different effects of gramicidin on uptake of Al by *P. vulgaris* and *T. aestivum* suggest that the dominant membrane mobile species may be different in the two species. Alternately, gramicidin could have affected other cellular processes which indirectly encouraged the uptake of Al by excised roots of *T. aestivum*, but had no effect on the uptake of Al by *P. vulgaris*. My data do not provide any insight into these two possibilities.

Although I have not found evidence for the operation of exclusion mechanism in cell suspensions derived from an Al resistant cultivar of *P. vulgaris*, results from studies with excised roots suggest that exclusion may be a whole plant phenomenon. The DNP-induced stimulation in Al uptake observed with excised roots of *P. vulgaris* is consistent with the operation of a metabolism dependent exclusion mechanism. The overall architecture of a plant root may be important for exclusion to be manifest, by facilitating the creation of an unique microenvironment in the immediate vicinity of the individual cells of the root. The finely dispersed nature of cells in suspension may prevent cells from maintaining a microenvironment that is different from the bulk solution. This microenvironment may be essential in limiting the uptake of toxic forms of Al. Approaches other than investigating the short-term kinetics of Al uptake may have to be taken to provide support for the existence of metabolism-dependent exclusion mechanisms in cell suspensions.

Throughout my discussions I have suggested a number of ways to answer questions that my research has generated. I have certain reservations, however, about continuing research with the cell suspension system of *P. vulgaris* that I used for this study. If research is to continue in this direction, I suggest that a new experimental species be identified which can be easily grown in cell suspension. This species should demonstrate a wider range of resistance between the resistant and sensitive cultivars than is observed in *P. vulgaris*. Although Dade is clearly more resistant to Al than Romano at the whole plant level (Lee and Foy, 1986), the degree of differential resistance does not appear to be as dramatic as the difference observed in other well studied species, for example *T. aestivum*. The degree of differential resistance could affect the ability to detect differences between Al-resistant and Al-sensitive cultivars. I would expect this potential problem to be exaggerated when working with cell suspensions because of their increased sensitivity to Al over that of whole plants (Conner and Meredith, 1985). In addition sensitivity to low pH should not be a characteristic of the Al-sensitive cultivar, since this makes seperation of Al-toxic responses from responses to acid sensitivity difficult. Most importantly, resistance must be unequivocally demonstrated at the cellular level before the system is used to further characterize mechanisms which may be involved in resistance.

Although results of this study suggest that the role of exclusion in resistance to Al cannot be studied at the cellular level using short-term kinetics studies, I believe I have developed an ideal system for characterizing uptake of Al by plant cells. There is currently a lack of information regarding the transport of Al across the plasma membrane. A number of questions need to be addressed, including what species of Al is membrane mobile, what type of transport mechanisms are involved in uptake, whether there are any limiting reactions for the transport of Al, and what role does the physical state of the membrane play in uptake? The lack of direct information about

the uptake of Al by plants is in part a result of complications arising from the presence of a cell wall. The magnitude of this problem could be reduced by using a p toplast system to investigate the kinetics of Al uptake.

By comparing kinetics of Al uptake between protoplast and cell suspensions, I believe direct information regarding uptake of Al into the apoplast and symplast could be obtained. However, to achieve this the clumping of cells in suspension would have to be eliminated. Otherwise uptake of Al between these two systems may not be comparable since the protoplasts will exist as single units in suspension. Kinetic studies investigating the effects of temperature on uptake of Al could be performed to assess the role that the membrane's physical state may play in uptake. Although these types of studies have previously been performed with algal cells, liposomes, and a number of animal cell systems (Pettersson *et al.*, 1985; Muller and Wilhelm, 1987; Shi and Haug, 1988), to my knowledge they have not been investigated in higher plant cells. The effects of inhibitors including ionophores and channel formers, such as A23187, and channel blockers, such as nifedipine and verapamil could be examined to assess the role that channels may play in uptake. Studies investigating the effects of inhibitors of oxidative phosphorylation such as carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone or m-chlorocarbonylcyanidephenylhydrazone could also be performed. Inhibitors of plasma membrane ATPases could also be examined to provide insight into how Al crosses the plasma membrane. To provide a clearer picture on how inhibitors may be affecting the uptake of Al, kinetic studies should be performed in conjunction with measurements of other physiological processes, for example, membrane potential, permeability, and levels of oxidative phosphorylation. Examining the effects of external pH on the uptake of Al by protoplasts could provide information about the species of Al which enters the

symplast. The use of some or all of these types of studies could provide valuable information about uptake of Al by plant cells.

All of this lies in the future, but I believe that my research has provided information that will be required to undertake such experiments. My research has (1) provided a reliable system to study the kinetics of Al uptake in cell suspensions of *P. vulgaris*, (2) characterized the kinetics of Al uptake at the cellular level, (3) provided support for the hypothesis that kinetic studies at the whole or excised root level reflect transport events occurring at the cellular level, (4) suggested expression of Al resistance by exclusion may rely on the integrity of the whole root, and (5) developed a system which can be used for further investigation of uptake of Al at the cellular level in plants.

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5 Appendix

Table 5.1 Uptake of Aluminum by Cell Suspension as a Function of Cell Dry Weight. The large cell densities that were used in my uptake experiments appeared to have caused problems with respect to rapid depletion of Al from uptake solutions. This experiment with varying initial cell densities showed that uptake of Al on a dry weight basis decreased with increasing cell density thereby making it necessary to standardize the initial density of cells in suspension (see Chapter 2). Values represent means of 4 replicates ± S. E.

Dade		Romano	
Dry weight (mg)	Aluminum uptake (µg g-1)	Dry weight (mg)	Aluminum uptake (µg g-1)
3.2 ± 0.2	724.0 ± 12.2	3.7 ± 0.2	628.3 ± 52.5
4.5 ± 0.1	586.5 ± 21.3	5.4 ± 0.2	501.2 ± 25.4
7.0 ± 0.2	468.2 ± 16.8	8.4 ± 0.2	442.0 ± 18.9
9.5 ± 0.2	417.4 ± 14.5	10.7 ± 0.1	384.3 ± 14.4
11.0 ± 0.4	383.0 ± 17.0	11.9 ± 0.2	374.3 ± 32.8
11.8 ± 0.8	363.5 ± 31.6	13.5 ± 0.3	418.0 ± 39.9
12.7 ± 0.6	381.4 ± 14.0	17.1 ± 0.2	324.7 ± 43.4

Table 5.2 Recovery of Aluminum as a Function of HNO_3 Used in the Ash Dissolution Step. Initial uptake experiments showed high error and this was attributed to sporadic contamination by Al liberated from the borosilicate tubes used for ashing during the ash dissolution step. These results show that HNO_3 could not be eliminated from the ash dissolution step as little Al was recovered in the absence of HNO_3. However, when the concentration of HNO_3 used to dissolve the ash was reduced from 2.0% to 0.1% (v/v) a reduction in error was observed (see Chapter 2). Values represent the means of 4 replicates ± S. E.

H_2O (ml)	HNO_3 (µl)	H_2O_2 (µl)	Al concentration (µg g^{-1})	
			Romano	Dade
10.0	0.00	0.00	63.10 ± 8.4	140.8 ± 12.2
9.90	0.00	100	77.20 ± 10.7	260.0 ± 43.1
9.98	10.0	10.0	521.1 ± 15.6	467.3 ± 11.1
9.96	20.0	20.0	497.7 ± 21.2	487.7 ± 13.8
9.94	30.0	30.0	508.8 ± 29.7	499.8 ± 9.6
9.92	40.0	40.0	520.6 ± 34.0	460.1± 12.6
9.90	50.0	50.0	560.7 ± 22.1	499.0 ±18.0
9.84	80.0	80.0	585.5 ± 35.1	525.5 ± 8.6
9.80	100	100	462.9 ± 14.8	522.8 ± 15.4

Table 5.3 Recovery of Aluminum Using Different Ash Dissolution Methods. In an attempt to reduce the standard error observed in initial uptake experiments, which appeared to result from the sporadic contamination by Al liberated from the borosilicate tubes used in the sample preparation step, various ash dissolution methods were investigated. These results show that a reduction in error was observed without any significant loss in the amount of Al recovered when the ash was dissolved in a mixture of HNO_3, H_2O_2, and deionized H_2O as opposed to adding the reagents separately (see Chapter 2).

Treatment	Replicate	Al Concentration ($\mu g\ g^{-1}$ dry weight)	Mean (n=3) ± S. E.
Reagents added separately	1	1034	
	2	1309	1218 ± 92.2
	3	1312	
Reagents added as a mixture	1	1172	
	2	1076	1123 ± 28
	3	1122	