Organic acid secretion as a mechanism of aluminium resistance: a model incorporating the root cortex, epidermis, and the external unstirred layer

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Abstract

The resistance of some plants to Al (aluminium or aluminum) has been attributed to the secretion of $\text{Al}^{3+}$-binding organic acid (OA) anions from the Al-sensitive root tips. Evidence for the ‘OA secretion hypothesis’ of resistance is substantial, but the mode of action remains unknown because the OA secretion appears to be too small to reduce adequately the activity of $\text{Al}^{3+}$ at the root surface. In this study a mechanism for the reduction of $\text{Al}^{3+}$ at the root surface and just beneath the epidermis by complexation with secreted OA$^-$ is considered. According to our computations, $\text{Al}^{3+}$ activity is insufficiently reduced at the surface of the root tips to account for the Al resistance of Triticum aestivum L. cv. Atlas 66, a malate-secreting wheat. Experimental treatments to decrease the thickness of the unstirred layer (increased aeration and removal of root-tip mucilage) failed to enhance sensitivity to $\text{Al}^{3+}$. On the basis of additional modelling, the observed spatial distribution of Al in roots, and the anatomical responses to Al, it is proposed that the epidermis is an essential component of the diffusion pathway for both OA and Al. We suggest that $\text{Al}^{3+}$ in the cortex must be reduced to small concentrations in order substantially to alleviate the inhibition of root elongation and so that the outer surface of the epidermis can tolerate relatively large concentrations of $\text{Al}^{3+}$. If OA secretion is required for reducing $\text{Al}^{3+}$ mainly beneath the root surface, rather than in the rhizosphere, then the metabolic cost to plants will be greatly reduced.

Key words: Aluminium, aluminum, diffusion, haematoxylin, malate, organic acid, toxicity, wheat.

Introduction

Many investigators appear to have concluded that the resistance of some plant roots to Al results from the secretion, into the rhizosphere, of $\text{Al}^{3+}$-binding organic acid (OA) anions from the Al-sensitive regions of the root tips (Ojima et al., 1987; Miyasaka et al., 1991; Ryan et al., 2001; Kochian et al., 2004). The correlative evidence for the OA secretion hypothesis of Al resistance is substantial, and now an Al resistance gene from wheat, ALTM1, has been cloned and expressed in other genotypes (Sasaki et al., 2004; Delhaize et al., 2004). Nevertheless, the hypothesis is problematical, principally because the estimated OA concentrations at the root surface appear to be too small to reduce adequately the root-surface activity of $\text{Al}^{3+}$ ($a_{\text{Al}^{3+},\text{root surf}}$) (Ryan et al., 1995b; Parker and Pedler, 1998; Parker et al., 2005).

Current investigations of Al resistance appear to concentrate upon the genetics and controls of OA secretion (Kochian et al., 2004) rather than upon a mechanism by which resistance may occur (but see the recent physiological study by Piñeros et al., 2005). The authors are not aware of any direct experimental evidence for a detailed mechanism, nor of any attempt to model such a mechanism quantitatively. According to our understanding of the literature, OA secretion is considered to confer resistance principally by reducing $a_{\text{Al}^{3+},\text{root surf}}$, but there does not seem...
to have been any consideration of the possibility that reduction of $a_{\text{Al}^{3+}, \text{root surf}^1}$ by itself, may be inadequate, and that a further significant reduction of $a_{\text{Al}^{3+}}$ across the epidermis may be a crucial component of the mechanism. That is, reduction of $a_{\text{Al}^{3+}}$ in the cortex may be more important than reduction at the root surface.

The mechanism to be evaluated is embodied in the ‘biphasic diffusion hypothesis’. It states that as OA diffuses through the epidermis (the first phase) and then away from the root surface through an unstirred layer (the second phase) it encounters Al. In each phase the divalent anion (OA$^{2-}$) binds Al$^{3+}$ according to the equilibrium $d_{\text{OAAl}^{+}} = a_{\text{Al}^{3+}}d_{\text{OA}^{2-}}K_{\text{OAAl}^{+}}$, which relates chemical activities to an equilibrium constant, $K_{\text{OAAl}^{+}}$ (Table 1). Binding to OA$^{2-}$ will deplete $a_{\text{Al}^{3+}}$ at the root surface and in the cortical free space, reduce toxicity, and establish a concentration gradient resulting in a flux of Al$^{3+}$ toward the root surface and into the root. The influx of Al$^{3+}$ (and some other species) will be countered by an efflux of OAAl$^{+}$ (and some other species).

This study has two objectives. The first is to compute the rate of total OA secretion 1 nmol apex $\frac{1}{C_{255}}$ and the second to determine whether increasing the agitation of the culture medium will deplete mucilage was removed.

Root elongation was sometimes expressed as relative root length computed as

$$RRL = 100(R_{L,C} - R_{L,A}) / (R_{L,C} - R_{L,0})$$

where $R_{L,A}$ is the root length in an Al-containing solution, $R_{L,C}$ is the root length in a solution containing enough Al to minimize elongation, and $R_{L,0}$ is the root length in an Al-free solution. Growth in response to a toxicant, such as Al$^{3+}$, may be expressed as

$$RRL = 100 / \exp([p\text{Al}^{3+}, \text{medium}, q])$$

which generates a downward exponential curve when $q=1$, a sigmoidal curve when $q>1$, and a curve with a steep initial decline when $0<q<1$. $p$ is a strength coefficient. When $d_{\text{Al}^{3+}, \text{medium}} = 1/p$, $RRL = 36.8\%$, irrespective of the value of $q$. Equation 2 may be expanded to incorporate other toxicants or ameliorants (Kinraide, 2003b).

### Materials and methods

#### Growth experiments

The biphasic diffusion hypothesis, for which a quantitative model will be presented, inspired four lines of experimentation: the first to determine whether increasing the agitation of the culture medium (thereby reducing the thickness of the unstirred layer at the root surface) increases the sensitivity to Al$^{3+}$ (i.e. to a given $d_{\text{Al}^{3+}, \text{medium}}$), the second to determine whether reducing the thickness of the apical layer of seedlings cultured in solution in order to evaluate the plausibility of the ‘biphasic diffusion hypothesis’. The second is to test components of the hypothesis experimentally.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root radius (mm)</td>
<td>2</td>
<td>Puthota et al., 1991</td>
</tr>
<tr>
<td>Rate of total OA secretion</td>
<td>$J_{\text{OA}}$</td>
<td>Ryan et al., 1995a; Pellet et al., 1996; Papernik et al., 2001</td>
</tr>
<tr>
<td>OA flux (mol m$^{-2}$ s$^{-1}$)</td>
<td>$J_{\text{OA}}$</td>
<td>Computed from an apical surface area of 3.14 mm$^2$</td>
</tr>
<tr>
<td>Rate of Al accumulation</td>
<td>$U_{\text{AL}}$</td>
<td>Degenhardt et al., 1998; in Ryan et al. (1992) $J_{\text{Al}} = 300$</td>
</tr>
<tr>
<td>Thickness of the external diffusion pathway</td>
<td>$UL$</td>
<td>Assigned</td>
</tr>
<tr>
<td>Diffusion coefficient</td>
<td>$D$</td>
<td>Puthota et al., 1991; Wolterbeek, 1987</td>
</tr>
<tr>
<td>$d_{\text{OAAl}^{+}}(d_{\text{Al}^{3+}, \text{OA}^{2-}})$</td>
<td>$K_{\text{OAAl}^{+}}$</td>
<td>Weast, 1990</td>
</tr>
<tr>
<td>$d_{\text{AAC}^{2+}}(d_{\text{Al}^{3+}, \text{AAC}^{2-}})$</td>
<td>$K_{\text{AAC}^{2+}}$</td>
<td>Degenhardt et al., 1998</td>
</tr>
<tr>
<td>$d_{\text{BAA}^{2+}}(d_{\text{Al}^{3+}, \text{BAA}^{2-}})$</td>
<td>$K_{\text{BAA}^{2+}}$</td>
<td>Ryan et al., 2001</td>
</tr>
<tr>
<td>$d_{\text{ASSO}^{4+}}(d_{\text{Al}^{3+}, \text{ASSO}^{4-}})$</td>
<td>$K_{\text{ASSO}^{4+}}$</td>
<td>Nordstrom and May, 1989</td>
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<td>$c_{\text{Al}^{3+}, \text{medium}}$</td>
<td>$C_{\text{Al}^{3+}}$</td>
<td>Ryan et al., 2001</td>
</tr>
<tr>
<td>Ionic strength of medium</td>
<td>$I$</td>
<td>Assigned</td>
</tr>
</tbody>
</table>

#### Table 1. Some parameter values for the standard diffusion model (OA = malate)

### Haematoxylin staining for Al

Scout and Atlas wheat seedlings were cultured in 1 mM CaCl$_2$ at pH 4.5 with variable concentrations of AlCl$_3$. Seedlings were withdrawn from solutions in which they had been cultured for 1 or 2 d, rinsed by dipping a few times in one then another large volume of distilled water, placed in stain for periods of 5-60 min, then rinsed again as just described. The roots were excised, mounted, and photographed; the stain consisted of 2 g haematoxylin and 0.2 g NaIO$_3$ in 255.
A model for the solute fluxes and concentrations

Anatomical considerations and diffusion exterior to the root surface: Readers may consult Fig. 3 in Degenhardt et al. (1998) for a depiction of an Arabidopsis root, its shape, and its principal region of H+ influx. Readers may also consult Fig. 1 in Sivaguru and Horst (1998) for a depiction of a maize root, its shape, and its principal region of Al sensitivity. These roots are smaller and larger, respectively, than the Atlas wheat roots considered here. Photographs of the latter are presented in Fig. 1A in Puthota et al. (1991), and a scale drawing from the photographs is presented in this paper. It illustrates that the root is about 0.5 mm in diameter 2 mm from the tip and that the apical 2 mm is mostly enclosed in a drop of mucilage. The greatest diameter of root plus mucilage is about 1.36 mm. The region 1–2 mm from the tip is considered to be the region of principal Al sensitivity, alkalization, and OA efflux, but fluxes are assumed to occur approximately from 0.5–2.5 mm from the tip.

It is assumed here that the mean diameter of the root over its sensitive region is approximately 0.5 mm and that the length of the external diffusion pathway is 400 μm. This length will be determined by mucilage and the unstirred layer near the surface of the root. OA diffusing away from the root surface will pass through imaginary cylindrical surfaces that are proportional to the radii of the cylinders. Thus the area through which the solute passes will be 2.6 times larger at 400 μm from the root surface (corresponding to a radius of 0.65 mm) than at the surface (corresponding to a radius of 0.25 mm).

The flux of solute \( j \), at the root surface, is designated \( J \), and fluxes away from the root are assigned negative values. The flux of \( j \) at position \( x \) external to the root is

\[
\frac{J}{R_x} = \frac{J}{x}
\]

where \( R \) is the root radius. As illustrated in Fig. 1, \( x \) increases from 0 at the centre of the root to \( R \) at the surface, to \( UL \) at the outer edge of the unstirred layer. The term \( R_x/x \) adjusts the flux for the changes in the areas of the cylindrical surfaces through which the solute passes.

The flux of total OA (TOA, the sum of all OA species), total Al (TAI, the sum of all Al species), and \( H^+ \) at the root surface are designated \( J_{TOA} \), \( J_{TAI} \), and \( J_{H^+} \), respectively. Table 1 gives the values assumed for the standard model. \( J_{TOA} \) is not constant. OA secretion is small in the absence of Al and increases as Al in the medium increases until it achieves a maximum at concentrations above 100 μM Al (see references in Table 1). For that reason, \( J_{TOA} \) should be adjusted whenever a treatment causes a change in Al sensitivity. Such treatments include those that change the length of the diffusion pathway. However, such adjustments cause small effects in the cases considered here (as will be seen), so \( J_{TOA} \) has been taken to be constant when \( A_{Al^3+} \), medium is constant. \( J_{TAI} \) is an Al accumulation term and is taken to be zero for the standard run, but is given positive values for other runs. \( J_{H^+} \) is large relative to other fluxes and is therefore not considered to be much affected by changes in proton consuming or releasing reactions (e.g. \( OA^2^- + H^+ = HOA^- \)). To express the flux of any of these solutes at position \( x \), the flux at the root surface (designated by upper-case \( J \)) must be multiplied by \( R/\sigma \) as in Equation 3.

Diffusion interior to the root surface: Diffusion does not stop at the outer surface of the root. OA, Al3+, H+, and other solutes diffuse through, or around, the cells of the epidermis. Equation 3 describes flux through the diffusion pathway external to the surface of the root, so now it is necessary to describe flux through the interior of the root. In the case of OA, and some other solutes, it is necessary to account for the fact that these solutes are synthesized or consumed within the root. For OA, the assumption is made that synthesis is uniform throughout the volume of the sensitive region of the root. A term, \( S_{TOA} \), is therefore added to the rate of synthesis of OA per unit volume. This will be \( 2.78 \times 10^{-13} \) mol m⁻³ s⁻¹ (3.93 × 10⁻¹⁰ m⁻³ s⁻¹) = 0.000707 mol m⁻³ s⁻¹. The flux at any position \( x \) within the root will equal the amount of OA synthesized within the cylinder, having \( x \) as its radius, divided by the area of the cylinder. The volume of the cylinder (\( V(x) \)) is \( \pi x^2 \), the area (\( A(x) \)) is \( 2\pi x \), and the length of the sensitive region (\( l \)) is 0.002 m. Thus,

\[
J_{TOA}(x) = -S_{TOA}V(x)/A(x) = -S_{TOA}x/2
\]

Equations for concentration based upon fluxes: Equations for concentration at position \( x \) external to the root may be derived from equations for the fluxes according to Fick’s First Law of Diffusion,

\[
j_x = -D_xc_x/d_x
\]

which states that the flux of solute \( j \) along the axis \( x \) is proportional to the concentration gradient (Nobel, 1991). The proportionality constant \( D_x \) is the diffusion coefficient, and the negative sign signifies that flux is in the direction of decreasing concentration. The concentration of solute \( j \) may be obtained by integrating and rearranging Equation 5. In these equations \( c_j \) must be expressed in mol m⁻³ when \( D \) is expressed in m² s⁻¹.

\[
c_j(x) = -1/D_x \int j_x dx + C_{j,UL}
\]

\( C_{j,UL} \) is the constant of integration which in this study’s treatment will be the concentration of solute \( j \) at \( x = UL \), that is, at the outer edge of the unstirred layer, and equal to the concentration in the external, bulk-phase medium. For cylindrical diffusion,

\[
j_x(x) = -1/D_x \int j_x (R/x) dx + C_{j,UL}
\]

which, when integrated from \( x = x \) to \( x = UL \), becomes

\[
c_j(x) = -J/R/D_x [\ln(UL) - \ln(x)] + C_{j,UL}
\]
When Equation 4, which pertains to diffusion within the root tissue, is rearranged and integrated from \( x = x \) to \( x = R \) then
\[
c_{TOA}(x) = s_{TOA}/(4D_{TOA,tissue})(R^2 - x^2) + c_{TOA,R}
\]
is obtained, where \( C_{TOA,R} \) is the constant of integration equal to the concentration of total OA at the root surface. It is assumed that \( D_{TOA,tissue} \) is greatly different from \( D \), especially through the epidermis whose cells fit together tightly. If fluxes are channelled between the cells, the area through which the fluxes occur will be reduced. This will increase fluxes and concentration gradients (\( \Delta c/\Delta x \)) through the channels. These effects can be simulated by making changes in the diffusion coefficient. In any case, the apparent diffusion coefficient will decrease whether fluxes are mainly channelled between the cells or through the cells: the latter possibility being unlikely because of the large electrochemical gradient against OA entry into the symplasm from the apoplasm. Fluxes and concentrations of \( H^+ \) within the root can be treated similarly.

Speciation: The equations presented thus far are sufficient for the computation of \( c_{TOA}(x) \), \( c_{TAl}(x) \), and \( c_{TH}(x) \). To compute the concentrations of species such as \( c_{Al3+}(x) \) or \( c_{TOAAl3+}(x) \), values for \( c_{TOA}(x) \), \( c_{TAl}(x) \), and \( c_{TH}(x) \) may be linked to a chemical speciation programme. Other inputs to the speciation programme will include other solutes that do not change significantly with \( x \) or that do not react with Al or OA. These would include Cl\(^-\) and Na\(^+\). Solutes, such as SO\(_4^{2-}\), that do react with Al, but whose concentrations are much greater than \( Al^{3+} \), may be considered invariant with \( x \) external to the root, but these solutes will generate species such as \( ABO_5^{2-} \), whose concentrations do vary with \( x \).

The following rooting medium was assumed for the standard run: 23.85 \( \mu \text{M AlCl}_3 \), 1 mM CaCl\(_2\), and 2638 \( \mu \text{M NaCl} \) adjusted to pH 4.3 with HCl. For this solution \( c_{Al3+} = 20.75 \mu \text{M}, a_{Al3+} = 10.00 \mu \text{M}, I = 5825 \mu \text{M}, \) and \( \gamma_{Al3+} = 0.482 \) (where \( \gamma_{Al3+} \) is an activity coefficient). This solution is moderately toxic to Atlas wheat and very toxic to Scotch wheat (Parker and Pedler, 1998; this study), which does not secrete appreciable amounts of OA (Pellet et al., 1996; Nian et al., 2002). For some other solutions, AlCl\(_3\) and NaCl were adjusted so that neither \( a_{Al3+} \) nor \( I \) changed. In a solution containing Na\(_2\)SO\(_4\), for example, the input concentrations were AlCl\(_3\) = 35.54 \( \mu \text{M} \), 1 mM CaCl\(_2\), 0 \( \mu \text{M} \) NaCl, and 1 mM Na\(_2\)SO\(_4\) adjusted to pH 4.3 with HCl so that \( a_{SO4^{2-}} = 10.25 \mu \text{M} \) and \( a_{Al3+} = 10.00 \mu \text{M} \). The concentration of total OA at the root surface. It is assumed that 4.90 \( \mu \text{M} a_{Al3+} \), medium is very intoxicating to sensitive genotypes. Furthermore, the computed concentration of 21.13 \( \mu \text{M} \) total OA (malate) at the root surface is about 10-fold less than that required to alleviate significantly the \( Al^{3+} \) intoxication (Delhaize et al., 1993b; Parker and Pedler, 1998).

The influence of the length of the diffusion pathway: Figure 3 presents runs of the standard model except that the length of the external diffusion pathway (UL) was varied. When the path length is short \( a_{Al3+} \), declines steeply as the root surface is approached, but does not achieve values as small as when the path length is long. One might assume, therefore, that a long path length would reduce \( Al^{3+} \) intoxication, but a more detailed analysis indicates that intoxication may be quite independent of diffusion path length unless \( J_{TOA} \) is much greater than the \( -88.4 \text{ nmol m}^{-2} \text{s}^{-1} \) of the standard run.

Figure 4A presents \( a_{Al3+} \), root surf as a function of the diffusion path length, and Fig. 4B presents pH root surf. Ions at the ‘root surface’ are still in the bathing medium; they are not at the plasma membrane (PM) surface; but it is well established that ion uptake, intoxication, or both, correlate well with ion activities at the PM surface and often correlate poorly with ion activities in the bathing medium (Gimmler et al., 2001; Zhang et al., 2001; Kinraide, 2003a, b).

Ion activities at the PM surface are computed by the Nernst Equation,
\[
a_j \text{ PM} = a_j \text{ root surf} \exp[-Z_j \psi_{PM}/25.7]
\]
where \( Z_j \) is the charge of ion \( j \), and 25.7 is a collection of constants appropriate when the PM surface electrical potential (\( \psi_{PM} \)) is expressed in mV at 25 °C. \( \psi_{PM} \) is computed with a Gouy–Chapman–Stern model (Yermiyahu et al., 1997; Kinraide, 2003b). Ions that bind strongly to the PM, such as \( Al^{3+} \) and \( H^+ \), reduce the negativity of the PM surface and thereby reduce the activity of cations at the surface.

Because \( a_{Al3+} \), root surf and \( a_{H^+} \), root surf increase with decreasing diffusion path length, \( \psi_{PM} \) becomes less negative with decreasing path length (Fig. 4C). Consequently, cations become attracted less strongly, and Fig. 4D presents \( a_{Al3+} \), PM as a function of path length. One can see that \( a_{Al3+} \), PM is quite insensitive to path length under the conditions of the standard run, but if \( J_{TOA} \) is increased 10-fold (enough to elevate malate to 211 \( \mu \text{M} \) at the root surface when the unstirred layer is 400 \( \mu \text{M} \) thick) then \( a_{Al3+} \), PM is sensitive to path length. Because root elongation is...
a function of $a_{\text{Al}^{3+}, \text{PM}}$, rather than $a_{\text{Al}^{3+}, \text{root surf}}$. Root elongation may be expected to be insensitive to path length under conditions of the standard run.

The influence of pH: The value for $J_{\text{H}^+} (= 1000 \text{ nmol m}^{-2} \text{ s}^{-1})$ may be somewhat large for wheat roots (Table 1). In the standard run, pH approaches 4.58 as the root surface is approached (Fig. 4B). If the rooting medium were buffered, or if the proton flux were reduced, then the pH would not change as much. Figure 5A illustrates that the effects of reducing $J_{\text{H}^+}$ to zero are to reduce pH$_{\text{root surf}}$ to 4.30 and to reduce the decline of $a_{\text{Al}^{3+}}$ toward the surface of the root. By contrast, the effects of increasing $J_{\text{H}^+}$ 1.5-fold are to increase pH$_{\text{root surf}}$ to 4.83 and to increase the decline in $a_{\text{Al}^{3+}}$. However, $a_{\text{Al}^{3+}, \text{PM}}$ actually increases as pH$_{\text{root surf}}$ increases and $a_{\text{Al}^{3+}, \text{root surf}}$ declines (see the values for $a_{\text{Al}^{3+}, \text{PM}}$ written in Fig. 5A).

The influence of Al accumulation: For the standard run it is assumed that $J_{\text{TAI}} = 0$, but some Al accumulation must occur in roots that elongate in Al solutions. This accumulation may be apoplastic, symplastic, or both. Figure 5B presents the case where $J_{\text{TAI}} = -J_{\text{TOA}}/2 = 44.2 \text{ nmol m}^{-2} \text{ s}^{-1}$, rather than zero as in the case of the standard run. The effect is to reduce $a_{\text{Al}^{3+}, \text{root surf}}$, but it is estimated that the assumed accumulation rate of 44.2 nmol m$^{-2}$ s$^{-1}$ is unreasonably large and would lead to a concentration of Al of about 2.55 mmol l$^{-1}$ root volume or about 688 $\mu$g Al g$^{-1}$ root DW, a concentration that would be very inhibitory to Atlas wheat (Samuels et al., 1997).

Fig. 2. Computed concentrations and fluxes of several solutes external to the root tip of Atlas wheat. Subscript TMA refers to the sum of all OA species (essentially, malate$^-$, Al-malate$^+$, H-malate$^-$, and H$_2$-malate$^0$). The sum of all concentrations of Al species (essentially, Al$^{3+}$, Al-malate$^+$, AlOH$^2+$, and Al(OH)$_2^+$) is constant at 23.85 $\mu$M, and the flux of total Al is zero in the standard run of the model.
The influence of the parameters $J_{\text{TOA}}$, $D$, $K_{\text{OAAl}^+}$, and $K_{\text{HOA}^+}$: Changing some of the parameters of the model will affect $a_{\text{Al}^{3+}}$, root surf. Increasing $J_{\text{TOA}}$ 2-fold and decreasing $D$ 2-fold have identical effects; both reduce $a_{\text{Al}^{3+}}$, root surf to 2.95 $\mu$M (Fig. 5C). The standard run incorporates malate as the OA, but the citrate and oxalate secreted by some species have larger association constants (Ryan et al., 2001). The effect of increasing the association constant, $K_{\text{OAAl}^+}$, to $10^7$ M$^{-1}$ reduces $a_{\text{Al}^{3+}}$, root surf to 2.35 $\mu$M (Fig. 5C).

The influence of buffering $\text{Al}^{3+}$: Just as $H^+$ may be buffered, so too may $\text{Al}^{3+}$. Figure 5D presents a run in which $\text{Na}_2\text{SO}_4 = 1$ mM. NaCl was reduced to maintain constant $I$, and $\text{AlCl}_3$ was adjusted so that $a_{\text{Al}^{3+}}$, medium $= 10$ $\mu$M (see speciation section above). The effect was to increase the $\text{Al}^{3+}$ buffering capacity from 2.40 for the standard run to 3.55 in the presence of $\text{Na}_2\text{SO}_4$ (because of the reaction $\text{Al}^{3+} + \text{SO}_4^{2-} \rightarrow \text{AlSO}_4^{3+}$). The buffering capacity was computed as $\Delta$ added $\text{AlCl}_3)/\Delta a_{\text{Al}^{3+}}$, medium. As expected, the buffered solutions resisted declines in $a_{\text{Al}^{3+}}$, root surf.

Modelling for internal activities of $\text{Al}^{3+}$

Figure 6 illustrates the profile of $a_{\text{Al}^{3+}}$ through the external diffusion pathway and 30 $\mu$m into the root tissue. The middle curve from distance 0–400 $\mu$m is the curve of the standard run presented in Fig. 5. The continuation of the curve into the tissue (distance <0) uses Equation 9 and related equations for $\text{Al}^{3+}$ and $H^+$. To use Equation 9, values must be assigned to $D_{\text{TOA, tissue}}$, $D_{\text{Al}^{3+, tissue}}$, and $D_{\text{H+, tissue}}$. For the initial assignments, $D$ and $D_{\text{H+}}$ were similarly reduced so that $D_{\text{TOA, tissue}} = D_{\text{Al}^{3+, tissue}} = D/9$ and $D_{\text{H+, tissue}} = D_{\text{H+}}/9$. With these changes, $a_{\text{Al}^{3+}} = 0.278$ $\mu$M and $pH = 5.83$, 30 $\mu$m into the root.
These new values for $a_{Al^3+}$ and pH would alleviate inhibition, but a problem arises. The reduction of $a_{Al^3+}$ to 0.278 $\mu$M is almost entirely the consequence of the elevated pH because $c_{TOA,\,tissue}$ is only 43.5 $\mu$M. If it is assumed that $J_{TOA} = 0$, as is the case for Scout wheat, then the pH increase alone is enough to reduce $a_{Al^3+}$ to 0.415 $\mu$M. These results indicate that Scout would be only 1.4-fold more sensitive than Atlas, rather than the 16-fold more sensitive that is known (Kinraide, 1997; Parker and Pedler, 1998).

An additional problem is that a 9-fold reduction of $D$ to obtain $D_{TOA,\,tissue}$ is probably much too small. If OA diffuses around, rather than through, the epidermal cells, then the area through which OA must pass will be closer to 1% of the total surface area. If $c_{TOA}$ is to rise to the estimated required values (Delhaize et al., 1993b; Parker and Pedler, 1998), $D_{TOA,\,tissue}$ must be a similarly small fraction of $D$. Furthermore, apoplastic pH just beneath the epidermis may not rise to 5.82. If these adjustments are made, $D_{TOA,\,tissue} = D_{Al^3+,\,tissue} = D/72$ and $D_{H,\,tissue} = D_{H}/6.7$, then $a_{Al^3+,\,tissue} = 0.290 \mu$M, pH = 5.10, and $c_{TOA,\,tissue} = 201 \mu$M, 30 $\mu$m into the root of Atlas wheat.

What would be the subepidermal conditions in Scout exposed to the same medium as Atlas or to a medium containing 1/16 the AI (and therefore intoxicated similarly to Atlas)? In order to estimate that, a similar pH profile, but no secretion of OA, was assumed. Under those conditions $a_{Al^3+} = 4.56 \mu$M (Fig. 6, top curve) or 0.286 $\mu$M (Fig. 6, bottom curve), 30 $\mu$m into the root of Scout wheat.

Finally, it was considered whether the outer cell walls of the epidermis could themselves provide a substantial protective barrier. If they could, then it would not be necessary to propose, as we shall, that the epidermal cells are either more resistant to $Al^3+$ than other tissues or that the epidermis plays a minor role in whatever events, essential for growth, are disrupted by $Al^3+$. According to these calculations, a 2 $\mu$m thick cell wall would need diffusion coefficients for OA and $Al^3+$ that were nearly 1000 times smaller than those in water, if the pH at the inner face of the cell walls were allowed to rise to 5.5 (i.e. $D_{H}$ 119-fold smaller than its value in water). Nobel (1991), in his calculations of diffusion across cell walls assumed a thickness of 1 $\mu$m and a 5-fold decrease in the value of diffusion coefficients.

**Experimental**

**Variable agitation:** The results of the modelling inspired experiments in which Atlas wheat seedlings were cultured so as to reduce the length of the diffusion pathway. The reduced path length will increase the $a_{Al^3+,\,root\,surf}$ (Fig. 4A).
but not necessarily increase the sensitivity to $a_{Al^{3+}, medium}$ (Fig. 4D). In five experiments the concentrations of AlCl$_3$ ranged from 0 to 200 µM in 1 mM CaCl$_2$ and 5320 µM NaCl at pH 4.3 or 4.5. Aeration treatments were no aeration, gentle aeration, and vigorous aeration and were assigned the categorical values 0, 1, or 2. Root lengths for the pooled data conformed to this equation.

$$RL = RL_S + \left(37.3 / (1 + 0.265 \text{Aeration})\right) / \exp\left(0.0385 a_{Al^{3+}, medium}^{0.802}\right)$$  \hspace{1cm} (11)

where $a_{Al^{3+}, medium}$ is expressed in µM, and $RL_S$, defined in the Materials and methods, is specific for each experiment, and the expression $(37.3/(1+0.265 \text{Aeration}))$ is equivalent to $RL_C$, the Al-free control. Thus aeration was moderately inhibitory to root elongation in the absence of Al so that the 2-d elongation was 37.3 mm in still solution and 24.4 mm in vigorously aerated solution. However, aeration had no effect on Al$^{3+}$ sensitivity. That is, the strength coefficient, 0.0385, was unaffected by aeration.

Figure 7A presents relative root lengths in response to $a_{Al^{3+}, medium}$ for the pooled data. The drawn curve conforms to Equation 2 for which $p = 0.0416$ and $q = 0.734$. Aeration could not be incorporated into this equation in a statistically significant way, and Fig. 7B illustrates the absence of an effect upon Al$^{3+}$-sensitive elongation.

For readers who fear that something may be hidden by the use of $RRL_S$, or question the incorporation of aeration into the computation of $RL_C$, unadjusted RL versus $a_{Al^{3+}, medium}$ was analysed over the interval of 0–10 µM, where the relationship is linear. For the equation

$$RL = a + a_{Al^{3+}, medium} (b + c \text{Aeration}) + d \text{Aeration}$$  \hspace{1cm} (12)

$a_{Al^{3+}, medium}$ is expressed in µM, and the coefficient $a$ was positive and significant and coefficients $b$ and $d$ were negative and significant for the pooled data and for each experiment individually. The coefficient $c$ was never significantly negative, thereby indicating no aeration-enhanced sensitivity to Al$^{3+}$.

To confirm that aeration does reduce the unstirred layer, and presumably the length of the diffusion pathway, two simple experiments were performed. Strips of dye-soaked paper were suspended in unaerated and aerated water. The
dye washed out of the strips in the aerated water much more rapidly than in the unaerated water. In addition, a cube of ice melted much faster in the aerated water.

Removal of mucilage: If the length of the external diffusion pathway is determined by mucilage, then the model predicts that removal of mucilage will cause an increase in $d_{Al^{3+}, \text{root surf}}$, but not necessarily an increase in sensitivity to $d_{Al^{3+}, \text{medium}}$ as noted above. To investigate that proposition, three experiments were performed. Culture solutions contained variable concentrations of AlCl$_3$ and other solutes. Within each experiment the solutions were prepared in duplicate so that the roots for half the solutions could be wiped free of mucilage, under magnification, three times daily.

Datum points in Fig. 8 are scattered. All data are presented in the interests of objectivity, but the reason for the five low values represented by the open circles across the bottom of Fig. 8A is now known. They refer to the first experiment in which mucilage was removed. In that experiment, and to a lesser degree in the second experiment, removal of mucilage appeared to injure the root tips generally. Eventually mucilage was removed so as to avoid root injury, as described in the Materials and methods.

With or without general injury, removal of mucilage failed to enhance sensitivity to Al$^{3+}$. Whether the experiments were analysed individually or collectively, whether the data were analysed over the entire range of $RL$ or $RRL$, roots with intact mucilage were just as sensitive to Al$^{3+}$ as the mucilage-free roots. This equation was applied to the pooled data.

$$RL = RL_S + \left(\frac{a}{(1 + f \text{Mucilage})}\right) / \exp\left[\frac{p}{(1 + g \text{Mucilage})d_{Al^{3+}, \text{medium}}}\right]$$

where $RL_S$ was specific for each experiment, and Mucilage was assigned the value 0 (not removed) or 1 (removed). The coefficients $a$, $f$, $p$, and $g$ were positive and significant, indicating that removal of mucilage reduced root elongation (at least in two of three experiments); and $g$ was non-significant, indicating that removal of mucilage did not enhance Al$^{3+}$ sensitivity. Previously published results regarding the protective effect of mucilage are mixed (Horst et al., 1982; Li et al., 2000). Removal of the mucilage-secreting root cap of maize had no effect upon sensitivity (Ryan et al., 1993).

Al$^{3+}$ buffering

Experiments were performed to test the effects of Al$^{3+}$ buffering capacity upon Atlas seedlings. Two experiments included variable concentrations of AlCl$_3$, Na$_2$SO$_4$ (0 or 2000 μM), and NaCl (5320 or 0 μM) in a background of 1 mM CaCl$_2$ at pH 4.3. Two additional experiments included variable concentrations of AlCl$_3$, succinic acid (0–4000 μM), and NaCl (approximately 4000–0 μM) in a background of 1 mM CaCl$_2$ at pH 4.5. Succinate was used because the possible formation of jurbanite (AlSO$_4$OH.5H$_2$O) imposes a limit to the combined activities of Al$^{3+}$ and SO$_4^{2-}$ (Nordstrom, 1982), but the use of succinate is problematic as well because of the pH buffering that will reduce root surface pH and thereby reduce the effects of increased Al$^{3+}$ as noted in the text relating to Figs 4 and 5A.

Figure 9A presents relative root lengths in response to Al$^{3+}$ activity and the removal of mucilage.

![Fig. 8. Relative root length of Atlas wheat in response to Al$^{3+}$ activity and the removal of mucilage.](https://academic.oup.com/jxb/article-abstract/56/417/1853/484301)
Co-ordinated growth and staining experiments

A 50% inhibition of root elongation was observed in Atlas in a medium containing 50 µM AlCl₃ in 1 mM CaCl₂ at pH 4.5 (\(a_{Al^{3+}}, \text{medium} = 22.3\) µM), but Scout was inhibited 50% in 3 µM AlCl₃ (\(a_{Al^{3+}}, \text{medium} = 1.36\) µM). According to our computations, the \(a_{Al^{3+}}, \text{root surf}\) was 6.88 µM for Atlas and 0.609 µM for Scout. It was considered that these greatly differing values for \(a_{Al^{3+}}, \text{root surf}\) would be observable using the stain haematoxylin. Specifically, it was considered that the root surface of Atlas would stain darkly relative to Scout and thereby confirm the modelled inability of Atlas (inhibited 50%) to reduce \(a_{Al^{3+}}, \text{root surf}\) to the activities that inhibit Scout 50%.

Figure 10 illustrates that these expectations were met. For Scout, neither the root surface, nor the root cap, nor the epidermal cells stained appreciably. The cell walls of the interior tissues did stain with a particularly abrupt transition from unstained to stained just behind the root cap and at the outer cortex, but nowhere were nuclei seen to stain. For Atlas, the cortex stained weakly, but the root surface and the root cap stained much more intensely. Staining was particularly intense in the nuclei of the border cells of the root cap and surface cells in the region of transition between cap and main root. Hand-cut longitudinal sections through the tip confirmed the surface staining in Atlas and the sub-surface staining in Scout. At Al concentrations sufficient...
to inhibit Atlas more than 50% but less than 100% (e.g. 100 μM) some cortical staining was observed, but the surface staining was always more intense. When Scout was very severely inhibited (Al >6 μM) staining of the surface and of nuclei does occur, but the cortex was always stained more intensely than the epidermis.

For Atlas and Scout the anatomical responses in the epidermis to 50% inhibition were quantitatively different. Whether Al intoxicated or not, the surface of the first 2–3 mm of the root was smooth and the cells were small and tightly packed. In non-intoxicated roots the cells farther back than 2–3 mm from the tip enlarged in a typical elongated pattern, and the surface remained smooth except for the developing hairs. In roots expressing 50% inhibition (50 μM AlCl₃ for Atlas, 3 μM AlCl₃ for Scout), the cells farther than 2–3 mm from the tip became swollen, especially in the epidermis, and these effects were greater for Atlas than for Scout. These anatomical effects, together with the results of staining, indicate greater $a_{Al^{3+}, \text{root surf}}$ for Atlas than for Scout at 50% inhibition.

**Discussion**

The authors accept that Al resistance is almost certainly related to OA secretion, but the idea that OA secretion reduces $a_{Al^{3+}, \text{root surf}}$ to very small values (comparable to the <1 μM values tolerated by the non-secreting sensitive genotypes) is probably wrong. Neither modelling nor experimentation supports that view. Apparently, the resistance mechanism requires a component other than (the apparently inadequate) reduction of $a_{Al^{3+}, \text{root surf}}$. We propose that the additional component is a substantial reduction in $a_{Al^{3+}}$ across the epidermis, caused by a resistance to OA diffusion through the epidermis that is much greater than the resistance through the unstirred layer. This increased resistance allows the OA concentration to increase to the needed values (about 200 μM). Thus the $a_{Al^{3+}}$ in the free space of the cortex will be small (<1 μM) for both resistant and sensitive genotypes when each is incubated in solutions causing equal and modest inhibitions of growth (see Fig. 6).

Our ‘biphasic diffusion hypothesis’ for Al resistance embodies several implications about the nature of Al intoxication. First is the implication that the site of Al action lies principally in the cortex, or root interior, and not in the epidermis. Readers are referred to a growing body of literature supporting the hypothesis that Al³⁺ acts in the transition zone of the root (lying between the meristematic zone and the elongation zone) to inhibit elongation in the elongation zone (Ryan et al., 1993; Hasenstein and Evans, 1988; Sivaguru and Horst, 1998; Kollmeier et al., 2000, and reviewed in Baluška et al., 2001). The elongation zone, when it alone was exposed to Al³⁺, continues to elongate. A candidate for the Al-initiated effect in the transition zone is the inhibited translocation of auxin through the cortex into the elongation zone from the meristematic zone into which the auxin had been transported through the phloem from the shoot. It strikes the authors as more than likely that exposure of the cortex to Al³⁺ is more important to the inhibition of auxin transport through the cortex than is exposure of the outer surface of the epidermis. An intriguing observation may relate to this proposition: the outermost cortical cells, rather than the epidermal cells, are the most sensitive to disruption of microtubules by Al³⁺ (Sivaguru et al., 1999).

There is no direct evidence that epidermal cells are not involved in the train of events leading to inhibited root elongation, but in many respects these cells, and others, appear to be individually resistant to Al³⁺. $a_{Al^{3+}}$ sufficient to inhibit elongation causes epidermal cells behind the transition zone to swell, whether applied to whole roots or to the zone of elongation alone, in which case elongation continues but swelling occurs (Ryan et al., 1993). These swollen cells exhibit vigorous cytoplasmic streaming and typical negative transmembrane potential differences that hyperpolarize normally in response to stimulated proton extrusion (Kinrade, 1988). That is, these cells appear to be healthy in many respects and capable of enlargement, albeit disordered, to the lengths they would have achieved in the absence of Al. Thus the anatomical changes (e.g. swelling and altered microtubule orientation) may be local effects and may not be related to the inhibition of root elongation. Furthermore, the fact that Atlas (inhibited 50%) exhibits greater anatomical lesions in the epidermis than Scout (inhibited 50%) may be indicative of a small role for Al intoxication of the epidermis in the inhibition of root elongation.

Why, at 50% inhibition of root elongation, does the interior of Scout roots stain while the surface does not? Before providing an interpretation here, readers are directed to several previous studies of Al localization in roots that indicate a greater accumulation of Al in the cortex than in the epidermis of sensitive genotypes incubated in moderate to low concentrations of Al. Compare Fig. 8A to Fig. 8B and Fig. 9A to Fig. 9B in Delhaize et al. (1993a). Tracing the root surface in the A figures onto clear plastic and superimposing them onto the B figures is helpful. See also Fig. 6 in Kataoka et al. (1997); Figs 3 and 6 in Silva et al. (2000); Fig. 3 in Silva et al. (2001); Fig. 5d in Tice et al. (1992); and compare Fig. 3C and 3D with Figs 3C’ and 3D’ in Ahn et al. (2002). It is concluded that the immature cortex has a greater capacity to accumulate Al than other regions of the root. Perhaps the cell walls there contain more binding sites for Al³⁺ or that Al³⁺ enters the cell interiors more readily. It is proposed here that it is the cortex, rather than the root surface, that must be protected from Al³⁺.

A second implication of the ‘biphasic diffusion hypothesis’ is that resistant and sensitive genotypes (at least in
wheat) have similar intrinsic sensitivities to Al\(^{3+}\). That is, both are similarly intoxicated by a given \(d_{\text{Al}^{3+}}\) in the sensitive region of the root. If the sensitive region is the root surface, then \(d_{\text{Al}^{3+}, \text{root surf}}\) must be similarly small (<1 \(\mu M\)) for Scout and Atlas, but if the sensitive region is the root interior, then \(d_{\text{Al}^{3+}, \text{root surf}}\) may be different for the two cultivars.

Some support for similar intrinsic sensitivities is provided by Samuels et al. (1997) who measured growth responses and Al uptake by Al-resistant Atlas and Al-sensitive Tam 105 cultivars of wheat. For the former, 105 \(\mu M\) AlCl\(_3\) in the medium inhibited growth 50% and caused a tissue accumulation of 377 \(\mu g\) Al g\(^{-1}\) root DW. For the latter, 11 \(\mu M\) AlCl\(_3\) in the medium inhibited growth 50% and caused a tissue accumulation of 546 \(\mu g\) Al g\(^{-1}\) DW. These values (377 and 546) may indicate that Atlas actually has a greater intrinsic sensitivity than Tam. Further support is presented by Tice et al. (1992) from whose data one may see that equal root-tip content of Al (in \(\mu mol g^{-1} FW\)) results in equal inhibition in the resistant Yecora Rojo and the sensitive Tyler cultivars of wheat.

A third implication of the ‘biphasic diffusion hypothesis’ is that the metabolic cost of Al resistance is small relative to the popularly assumed alternative. Only one-tenth the amount of OA need be synthesized to maintain adequate concentrations of OA beneath the epidermis, compared with the amount needed to maintain similar concentrations at the root surface.

How may the ‘biphasic diffusion hypothesis’ be tested further? It is acknowledged that much of the argument is based upon modelling. Therefore, it is hoped that readers will examine the model critically, including this study’s selection of parameter values and the extent to which they have been altered for variations of the standard run. The absence of direct measurements of root-surface OA and Al\(^{3+}\) concentrations is also acknowledged. Eventually, these measurements must be obtained. It is thought that the experiments with removal of mucilage, agitation of the medium, and Al\(^{3+}\) buffering all provided results consistent with the biphasic diffusion hypothesis, but it is acknowledged that the complex interaction of Al\(^{3+}\), H\(^{+}\), and cell-surface electrical charges makes an interpretation of those experiments difficult (refer to Fig. 4 and related text). In the authors’ view, the haematoxylin staining experiments (as well as the observations of anatomical effects) provide the best experimental complement to the modelling because they are a rough, qualitative measure of \(d_{\text{Al}^{3+}, \text{root surf}}\). Nevertheless, further anatomical studies and studies to localize tissue Al, especially at root surfaces, may be productive. Finally, the sufficiency of the outer cell walls of the epidermis (together with closely adhering mucilage not easily removed by wiping) as an effective barrier cannot be entirely ruled out, although it is considered to be an unlikely prospect.

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