Carbon (C) metabolism and N₂ fixation in legume nodules is limited by O₂ supply. Using a nonaqueous technique to quantify adenylate pools in intracellular fractions, Kuzma et al. (1999) found that the adenylate energy charge (AEC = [ATP + 0.5 ADP]/[ATP + ADP + AMP]) was 0.65 ± 0.04 in the plant fraction of active, N₂-fixing nodules, whereas bacteroids had an AEC of 0.76 ± 0.09. However, when nodule metabolism was more severely O₂ limited by exposure to 10% (v/v) O₂ for 3 min, AEC in the plant cell compartment was not affected but in the bacteroids decreased from 0.76 ± 0.09 to 0.56 ± 0.06. The decrease in bacteroid AEC accounted for the decrease in whole-nodule AEC (from 0.70–0.61, Kuzma et al., 1999) after low O₂ treatment. Other treatments known to reduce infected cell pO₂ (e.g. NO₃⁻ fertilization and stem girdling) also reduced the AEC of whole nodules (de Lima et al., 1994), presumably because of a decrease in bacteroid rather than plant AEC.

These data (de Lima et al., 1994; Kuzma et al., 1999) are consistent with the bacteroids, not the plant fraction, being the site of O₂ limitation in legume nodules. This conclusion was supported by models of infected cells in nodules (Thumfort et al., 1994, 1999) that simulated diffusion and reactions involving O₂. The models predicted steep gradients in O₂ concentration from the cytosol adjacent to the intercellular space of infected cells where mitochondria and plastids are located to the center of the cell where symbiosomes containing bacteroids can be found. Therefore, mitochondria are likely to experience pO₂ well in excess of the K_m(O₂) for their terminal oxidases (50–100 mm; Bergersen, 1994; Thumfort et al., 1994), and, as long as sufficient oxidizable substrate is available, ATP synthesis is unlikely to be limited by O₂ supply.

Nevertheless, at 0.65 ± 0.04, AEC of the plant fraction is lower than that normally associated with hypoxic tissues (approximately 0.75 or higher; Pradet and Raymond, 1983). To explain this fact, Kuzma et al. (1999) proposed that a steep ATP gradient may be present across the infected cells, from the mitochondrial zone (MZ) near the intercellular spaces (and the site of ATP synthesis) to the cytosol-surrounding symbiosomes where the majority of plant-derived ATP would be used.

To develop a framework for an experimental test of this hypothesis, the present study modified the model of Thumfort et al. (1994) by incorporating a separate MZ adjacent to the intercellular gas spaces (as in Thumfort et al., 1999) and by including diffusion of adenylates between sites of ATP synthesis in the MZ and sites of ATP utilization (and ADP and AMP production) in the bacteroid zone (BZ), i.e. within the cytosol surrounding the symbiosomes. The main ATP demand in the cytosol would be associated with Gln synthetase (GS) activity for the assimilation of fixed nitrogen (N) and the ATP cost of transporting C₄ acids across the peribacteroid membrane (PBM) to fuel bacteroid respiration. Separate models
are developed for nodules that produce amides (Asn) or ureides (allantoin and allantoic acid) as predominant end products of assimilation of fixed N.

The simulations were also used to explore the effect of Ar:O2 exposure on ATP demand and the resulting cytosolic adenylate and AEC gradients across the cell. In Ar:O2, N2 fixation ceases, but nitrogenase activity continues, and we predicted that there should be less of an ATP gradient in Ar:O2-treated nodules. If true, this would help account for the results of de Lima et al. (1994) who observed that Ar:O2 treatment (1 h) had no effect on whole-nodule AEC, despite the fact that nodule permeability declines and infected cell O2 concentration decreases.

RESULTS

Developing the Models

The infected cell was assumed to be a three-dimensional (3D) rhombic dodecahedron, which was reduced to a one-dimensional (1D) representation and divided into 400 layers. Layers 400 to 365 were considered to be within the MZ adjacent to the gas space interface, whereas layers 364 to 1 (ureide nodules) or 364 to 181 (amide nodules) were identified as the BZ. In the amide nodules, layers 180 to 1 constituted a central vacuole zone (VZ). The O2 was assumed to diffuse from the gas space interface (i.e. layer 400) into the innermost layer of the BZ (i.e. layer 1 in ureide nodules or layer 181 in amide nodules).

Mathematical models of O2 and adenylate diffusion were developed in two phases. In the first, rates of ATP demand and synthesis were calculated in the plant cytosol within both the BZ and MZ of infected cell. This was done by coupling the C, N, and energy demands of 400 cell layers to the modeled O2 concentration gradients that are thought to exist between the intercellular spaces and the center of the cell. The second phase of the models used these values for ATP demand to generate gradients of cytosolic ATP, ADP, and AMP across the infected cell.

For ureide- and amide-based models, the O2 concentration gradient was anchored by setting an O2 concentration at the innermost layer to achieve a volume-weighted average fractional oxygenation of leghemoglobin (Lb; FOLavg) of 0.4. The ATP gradient was anchored by setting the cytosolic ATP concentration at the innermost layer such that the AEC in the cytosol adjacent to the gas space (AEC400) was 0.82.

The amide model differed from the ureide model in two biochemical parameters: (a) A demand of three ATPs per N (versus one per N for ureide nodules) would be required for NH3 assimilation in cytosol of BZ, and (b) a value of 78 nm for O2 concentration at which 50% of Lb is oxygenated (Kslb, versus 48 nm for ureide model).

Figure 1. Model predictions of the gradients in free O2 concentration (A), fractional oxygen of Lb (FOL; B), and O2 consumption in infected cells of ureide (solid lines) and amide (dotted lines) nodules under N2:O2 treatment (C), with values for the innermost O2 concentration (O2n = 1 or O2n = 181 for ureide and amide nodules, respectively) to give an average FOL (FOLavg) of the cell = 0.4. The x axis shows the 400 layers along the diffusion path, from layer 400 (0-μm distance) to layer 1 (31.2 μm). The shaded areas show MZ (layers 400–365); BZ included layers 364 to 1 in the ureide-based nodule model and layers 364–181 in the amide model. Layers 180 to 1 in the amide model are occupied by central vacuole.

O2, LbO2 Gradient, and O2 Consumption in the Infected Cell

In the ureide model, innermost O2 concentration (O2n) was fixed at 13 nm to generate a FOLavg of 0.4. The resultant O2 concentration adjacent to the space (O2400) was 400 nm, and the volume-weighted O2 concentration (O2avg) was predicted to be 38 nm (Fig. 1A, solid line). As a consequence, the O2avg values were slightly higher than the average O2 concentration calculated from the average fractional oxygenation of Lb [i.e. O2avg(FOL) = 32 nm; O2avg(FOL) = FOL × Kslb/(1 – FOL), Kslb = 48 nm], an observation consistent with previous reports (Thumfort et al., 1994, 1999).
A steep O2 gradient was predicted to exist from the gas space interface to the center of the BZ (Fig. 1A). The decline in O2 concentration across the MZ, from 400 to 70 nm over a diffusion path of 2.8 μm, was much steeper than that in the BZ, where O2 declined from 70 to 13 nm over a path of 28.4 μm (Fig. 1A). A steep gradient in FOL was also predicted, declining from 0.90 to 0.22 across the infected cell (Fig. 1B).

The volume-specific respiration rates in the MZ (0.35–0.23 mol O2 m–3 s–1) were predicted to be considerably higher than those in the BZ (0.07–0.04 mol O2 m–3 s–1; Fig. 1C) as a result of the different kinetic constants for O2 uptake [i.e. Vmax and Km(O2)] were chosen for the two regions. The sharp discontinuity in respiration rate between the two zones (Fig. 1C) would be dampened if a thin, mixed zone of mitochondria and bacteroids was inserted between MZ and BZ, without altering the respiration rate in most of the layers in MZ and BZ. Total O2 consumption was 2.5 × 10–15 mol O2 s–1 cell–1 in the MZ and 5.7 × 10–15 mol O2 s–1 cell–1 in the BZ.

The amide model predicted profiles for infected cell O2 concentration, FOL, and tissue respiration that were similar to those generated by the ureide model (Fig. 1A–C, dotted lines). The innermost O2 concentration (O2inner) was fixed at 32 nm to generate a FOLavg of 0.4. The resultant O2 concentration adjacent to the space (O2space) was 600 nm. The volume-weighted O2 concentration (O2avg) was predicted to be 63 nm (Fig. 1A, dotted lines), and this value was slightly higher than the calculated O2avg/FOL avg (52 nm) based on a KsLavg value of 78 nm. In any layer between layer 400 and 181, the modeled O2 concentration was slightly higher in amide than in ureide nodules. By running a series of simulations, these differences were attributed to the higher value chosen for KsLavg. Total O2 consumption was 2.7 × 10–15 mol O2 s–1 cell–1 in the MZ and 5.8 × 10–15 mol O2 s–1 cell–1 in the BZ.

Nitrogenase Activity and NH3 Production in Bacteroids

The models predicted a rate of NH3 production within the BZ (NH3) that ranged from 0.030 to 0.014 mol NH3 m–3 s–1 in ureide nodules and from 0.03 to 0.024 mol NH3 m–3 s–1 in amide nodules (Fig. 2). Integrated over the entire infected cell, NH3 production was calculated to be 2.3 × 10–15 and 2.4 × 10–15 mol NH3 s–1 cell–1 in ureide and amide nodules, respectively.

Adenylate Demand in the Plant Fraction of the BZ

Large differences were observed in predicted ATP metabolic demand (P ATP) in the plant cytosol, with values for amide nodules being approximately twice that for ureide nodules (Fig. 3A), despite the fact that the models predicted similar rates of NH3 assimilation in the two nodule types (Fig. 2). These differences were attributed to a 3-fold higher ATP cost per NH3 assimilated for amide than ureide nodules.

In the ureide nodule, total metabolic ATP demand [Σinnermost P ATP(metab)] in the plant fraction of BZ (i.e., the sum of P ATP(metab) from the innermost layer of n = 3) for the entire cell was 5.4 × 10–15 mol ATP s–1.
Adenylate Demand in the MZ

The total metabolic ATP demand in the plant fraction of BZ for the entire cell was assumed to be met by oxidative phosphorylation within the MZ. The volume-specific metabolic ATP “demand” in layer “n” of the MZ \( P_{ATP(\text{metab})} \), moles ATP per cell, was calculated to be 1.8 \( \times 10^{-15} \) mol ATP \( \text{s}^{-1} \) cell\(^{-1}\) in the ureide nodule and 3.7 \( \times 10^{-15} \) mol ATP \( \text{s}^{-1} \) cell\(^{-1}\) in the amide nodule. Dividing these values by the volume-specific metabolic ATP demand in the plant fraction of BZ (described previously), it was calculated that 33\% (ureide) or 36\% (amide) of the total ATP metabolic requirement was regenerated through AK activity.

Figure 4. Model predictions for the diffusive flux rates of ATP (A and D), ADP (B and E), and AMP (C and F) in the plant fraction of infected cell layers from the more centrally located layers of the BZ (see below) was sufficient to counter the activity of \( P_{ATP(\text{metab})} \) and reverse the AK reaction.

Diffusive Flux of Adenylates in Plant Fraction

Based on metabolic ATP demand and AK activity in maintaining adenylate equilibrium, the transfer of adenylates into layer \( n \) from layer \( n+1 \) (moles per second) was solved at each layer \( n \). The predicted rates of adenylate transfer were converted into fluxes (nanno meters per meter squared per second) by dividing by the surface area of each layer. The resultant values, shown in Figure 4, A to C, provide a comparison of adenylate diffusion at various distances from the gas space interface (layer 400) to the innermost
layer. The flux was positive for ATP but negative for ADP and AMP because ATP diffused from layer 400 to the innermost layer, whereas ADP and AMP diffused in the opposite direction.

ATP flux between layers 399 and 364 of the MZ was predicted to increase from 39 to 716 nmol m\(^{-2}\) s\(^{-1}\) in the ureide nodule and from 69 to 1,330 nmol m\(^{-2}\) s\(^{-1}\) in the amide nodule (ATP flux was 0 at layer 400 according to the definition). In contrast, ATP flux between layers 364 and the innermost layer of BZ was predicted to decrease from 730 to 2 nmol m\(^{-2}\) s\(^{-1}\) in the ureide nodule and 1,350 to 4 nmol m\(^{-2}\) s\(^{-1}\) in the amide nodule. The highest ATP flux was predicted in layer 364, i.e. at the MZ:BZ interface for both ureide and amide nodules (Fig. 4A).

The fluxes of ADP and AMP showed a similar trend but in the opposite diffusion direction and are, therefore, negative in Figure 4, B and C. Similarly, the largest absolute value for ADP and AMP flux was observed at the MZ:BZ interface at layer 364 but with a lower magnitude than that predicted for ATP. At any layer within the infected cell, the sum of cytosolic ATP, ADP, and AMP fluxes equaled zero, reflecting the assumption of models that the total cytosolic adenylate concentration was constant throughout the infected cell.

Cytosolic Adenylate Gradients in Plant Fraction

The simultaneous equations also solved for concentrations of cytosolic ATP, ADP, and AMP in each layer of the infected cell. To ensure that the models predicted an AEC of 0.82 for AEC\(^{400}\), the innermost ATP (ATP\(^{1}\)) had to be set to 1.04 mM in the ureide model and 0.79 mM in the amide model. The cytosolic ATP, ADP, and AMP gradients across the infected cells were predicted to be greater in amide than in ureide nodules (Fig. 5, A–C).

These cytosolic adenylate gradients resulted in predictions of large gradients in AEC (Fig. 6A) or ATP to ADP ratio (Fig. 6B) for both nodule types but much steeper gradients in amide than in ureide nodules. Although model parameters were chosen to give an AEC of 0.82 (ATP:ADP = 3.1) at the gas space interface (n = 400), the AEC predicted to exist in the cytosol at the innermost layer was 0.62 (ATP:ADP = 1.4) in the ureide nodule and 0.52 (ATP:ADP = 1.0) in the amide nodule. The average AEC (AEC\(^{Avg}\)) of the entire plant fraction of the cell (BZ and MZ) was predicted to be 0.61 in the amide nodules and 0.70 in the ureide nodules. The latter values were similar to the experimentally determined value for soybean...
(Glycine max) nodules (0.65 ± 0.04; Kuzma et al., 1999).

Effect of Ar:O₂ Treatment on Predictions of Models

The models were also used to simulate the effect of exposing ureide- or amide-forming nodules to an Ar:O₂ atmosphere, a treatment that prevents N₂ fixation and NH₃ assimilation without immediate effects on nitrogenase activity or nodule carbohydrate metabolism. Extended exposure to Ar:O₂ is known to decrease the nodule’s permeability to O₂ diffusion (Hunt and Layzell, 1993), but the simulations carried out here were focused on the physiological status of the nodule after a few minutes of Ar:O₂ exposure (5–7 min), when the rate of NH₃ assimilation was reduced to zero but before the diffusion barrier responds to reduce the incoming flux of O₂ (King and Layzell, 1991).

Cessation of NH₃ production in bacteroids would reduce ATP demand for NH₃ assimilation in the plant fraction. Therefore, the models predicted much lower ATP demand \([P_{ATP(\text{metab})}\n]\) in Ar:O₂-treated (Fig. 3D) than in N₂:O₂-treated nodules (Fig. 3A). As a consequence, the AK activity indicated by its ATP demand \([P_{ATPIAK}\n]\) was also predicted to be lower in Ar:O₂-treated nodules (Fig. 3E).

As a result of lower adenylate demand, diffusive fluxes of adenylate were predicted to be much lower in Ar:O₂ (Fig. 4, D–F) than in N₂:O₂ (Fig. 4, A–C). Although parameters of models were chosen to give an AEC of 0.82 (ATP:ADP = 3.1) in layer 400, the simulation showed that the cytosolic adenylate gradients predicted during steady-rate N₂ fixation in N₂:O₂ were significantly reduced after the switch to Ar:O₂ (compare Fig. 5, D to F with A to C). The average AEC in the plant fraction of an infected cell was 0.77 in both ureide and amide nodules treated with Ar:O₂ (Fig. 6C). Thus, the ureide model predicted a rise of average plant AEC from 0.70 in N₂:O₂ to 0.77, whereas the amide model predicted a rise from 0.61 in N₂:O₂ to 0.77.

**DISCUSSION**

Cytosolic Adenylate Gradients in Legume Nodules

The models predicted that substantial gradients in adenylate concentration could occur in the plant cytosol of the infected cell, even in the absence of membrane barriers. Both adenylate and AEC gradients would be reduced dramatically after short-term exposure of nodules to an Ar:O₂ atmosphere, a treatment that stops N₂ fixation and NH₃ assimilation but not nitrogenase activity.

The predictions from these models are the first of their kind, to our knowledge, and may account for a number of physiological observations that to date have defied easy explanation. For example, Oresnik and Layzell (1994) have reported low values for AEC (i.e. less than 0.8), even in metabolically active, N₂-fixing nodules. Using a nonaqueous fractionation technique, Kuzma et al. (1999) showed values for the plant AEC of soybean nodules as low as 0.65 ± 0.04, but when nodules were exposed to a severe O₂ limitation, the plant AEC was unaffected, whereas bacteroid AEC declined sharply from 0.76 to 0.56. These findings indicated that bacteroids were the site of O₂ limitation but did not provide an explanation for the low AEC in the plant compartment. A low AEC value of 0.70 was also observed by Sa and Israel (1991) in plant fraction of soybean nodules, whereas the AEC values in bacteroids and whole nodules were measured to be 0.84 and 0.75, respectively. Results of the current study readily account for these earlier observations by predicting steep AEC gradients across the infected cell. Even when the AEC at the interface with the gas-filled spaces was set at 0.82, the average AEC within the plant compartment of the infected cell was predicted to be 0.70, a value consistent with that measured in soybean nodules (0.70, Sa and Israel, 1991; 0.65 ± 0.04, Kuzma et al., 1999).

Results of this study may also account for the observation of de Lima et al. (1994) that extended exposure to an Ar:O₂ atmosphere differs from all other treatments known to reduce infected cell O₂ concentration (e.g. low O₂ concentration and NO₃⁻ treatment and carbohydrate deprivation) in that it was not correlated with a decrease in whole-nodule AEC. The predictions of models from the current study indicate that, unlike these other treatments, Ar:O₂ exposure would dramatically reduce the adenylate gradient across the infected cell and, in effect, increase AEC in the plant compartment. This may offset a decrease in bacteroid AEC that would be expected when the O₂ permeability of these nodules decreases and the infected cell becomes severely limited by O₂ supply.

Validity of the Predicted Rates of Respiration and Nitrogenase Activity

To test the validity of models, the predicted rates of respiration and NH₃ assimilation in the infected cell were converted and then compared with measured values.

The models predicted a rate of mitochondrial respiration of 2.5 \(\times 10^{-15}\) to 2.7 \(\times 10^{-15}\) mol O₂ s⁻¹ cell⁻¹ in ureide and amide nodules. Assuming 12,472 mitochondria per cell and 0.25 pg of protein per mitochondrion (Table IV of Millar et al., 1995), the mitochondrial respiration rate was equivalent to 48 to 51 nmol O₂ min⁻¹ mg⁻¹ protein, which was comparable with reported data for isolated nodule mitochondria (35–140 nmol O₂ min⁻¹ mg⁻¹ protein for ureide-producing soybean nodules, Millar et al., 1995; 25 nmol O₂ min⁻¹ mg⁻¹ protein for amide-producing lupin nodules, Tomaszewska et al., 1991). The predicted rate of NH₃ production was 2.3 \(\times\)
10^{-15} to 2.4 \times 10^{-15} \text{ mol NH}_3 \text{ s}^{-1} \text{ cell}^{-1} in ureide and amide nodules in N_2:O_2 (apparent nitrogenase activity). When this value was scaled to a whole nodule using an electron allocation coefficient of 0.67, the total nitrogenase activity was estimated to be equivalent to 310 and 340 \mu\text{mol H}_2 \text{ g}^{-1} \text{ dry weight [nod]} \text{ h}^{-1} for ureide and amide nodules [nod], respectively. These values were similar to those observed in intact, undisturbed nodules (i.e. 200-350 \mu\text{mol H}_2 \text{ g}^{-1} \text{ dry weight [nod]} \text{ h}^{-1} for ureide nodules, Hunt and Layzell, 1993; 367 \pm 31 \mu\text{mol H}_2 \text{ g}^{-1} \text{ dry weight [nod]} \text{ h}^{-1} for amide-forming lupin nodules, Diaz del Castillo et al., 1992). The above conversions were based on: (a) for ureide nodules, 1.8 \times 10^5 \text{ cells} and 9.5 \times 10^{-3} \text{ g dry weight per soybean nodule} (Bergersen, 1994); and (b) for amide nodules, 1.1 \times 10^5 \text{ cells per nodule} (calculated from a nodule volume of 2.2 \times 10^{-8} \text{ m}^3, Fernandez-Pascual et al., 1992; assuming 55\% of nodule volume is occupied by infected cell of a legume nodule. To address the concern that 1D models differed by less than 15\%, Thus, the predicted values for the 1D models (Thumfort et al., 1994; Thumfort, 1996) that retained the essential features of a polyhedron cell with respect to O_2 diffusion from gas-filled intercellular spaces. To address the concern that 1D models of O_2 diffusion may be oversimplified and, therefore, their predictions compromised, Thumfort (1996) and Thumfort et al. (2000) constructed full 3D models of O_2 diffusion and compared their predictions with those of the 1D models. The predicted values for the two simulations differed by less than 15\%. Thus, the 1D modeling approach proved to be a simple yet powerful and valid tool for simulating O_2 diffusion into the infected cell of a legume nodule.

For simplicity, the 1D models developed in this study did not consider O_2 diffusion into and out of the adjacent uninfected cell. Based on a two-cell model prediction, the amount of O_2 entering the infected cell from an adjoining uninfected cell accounted for only 15\% of the total amount of O_2 entering when average FOL was from 0.4 to 0.5 (Thumfort et al., 1999).

### Validity of 1D Models Compared with 3D Models

The methods used in this study was largely based on 1D models (Thumfort et al., 1994; Thumfort, 1996), that retained the essential features of a polyhedron cell with respect to O_2 diffusion from gas-filled intercellular spaces. To address the concern that 1D models of O_2 diffusion may be oversimplified and, therefore, their predictions compromised, Thumfort (1996) and Thumfort et al. (2000) constructed full 3D models of O_2 diffusion and compared their predictions with those of the 1D models. The predicted values for the two simulations differed by less than 15\%. Thus, the 1D modeling approach proved to be a simple yet powerful and valid tool for simulating O_2 diffusion into the infected cell of a legume nodule.

### Assumptions for Anchoring the Gradients of O_2 and Adenylates

The O_2 concentration gradient in the models was anchored by setting an O_2 concentration at the innermost layer to achieve an average FOL of 0.4, a value measured by nodule oximetry and thought to exist in active, undisturbed ureide and amide nodules (Table I, item 1). Increasing the average FOL from 0.4 to 0.6 would significantly increase O_2\text{400} from 400 to 7,600 nm (Table II, item 1, a and b); this range of O_2 concentrations was still in agreement with that in a two-cell model of Thumfort et al. (1999). The resultant plant AEC gradients would become steeper (Table II, item 1, a and b). Increasing the average FOL from 0.4 to 0.6 had a similar impact on amide nodules (data not shown).

The cytosolic ATP gradient in the models was anchored by choosing the cytosolic ATP concentration in the innermost layer such that the AEC\text{400} was 0.82, a value similar to that measured in fully aerobic nodule cortex tissue (Oresnik and Layzell, 1994). To test the effect of this assumption, AEC at the intercellular space was set at a range of 0.72 to 0.92. This had no effect on the magnitude of the predicted

<table>
<thead>
<tr>
<th>Parameter (abbreviation)</th>
<th>Value and Units</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. FOL in ureide- and amide-forming nodules</td>
<td>0.4</td>
<td>King and Layzell (1991); Kuzma et al. (1993); Shimada et al. (1997)</td>
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<tr>
<td>2. Diffusion coefficient for Lb and LbO_2 in cytosol</td>
<td>$5 \times 10^{-11} \text{ m}^2 \text{ s}^{-1}$</td>
<td>Bergersen (1993)</td>
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<td>3. Diffusion coefficient for O_2 in cells</td>
<td>$7.4 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$</td>
<td>Denison (1992)</td>
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<td>4. Diffusion coefficient for adenylate in cytosol</td>
<td>$1.0 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$</td>
<td>Kushmerick and Podolsky (1969)</td>
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<td>5. Tortuosity factor for diffusion of Lb and adenylate</td>
<td>2</td>
<td>Satterfield and Sherwood (1963)</td>
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<tr>
<td>6. P:O ratio for bacteroid oxidative phosphorylation</td>
<td>2</td>
<td>Wittersberg et al. (1974); p. 4065</td>
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<tr>
<td>7. Ks(O_2) for Lb oxygenation (KsLb)</td>
<td>$4.8 \times 10^{-5} \text{ mol} \text{ m}^{-3}$ in ureide nodules; $7.8 \times 10^{-5} \text{ mol} \text{ m}^{-3}$ in amide nodules</td>
<td>Gibson et al. (1989)</td>
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<td>8. Apparent equilibrium constant of adenylate kinase in plant fraction (K_eK)</td>
<td>0.9 (unitless)</td>
<td>Calculated from Kuzma et al. (1999)</td>
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<td>9. Total cytosolic adenylate concentration (Aden)</td>
<td>2.29 mol m^{-3}</td>
<td>Calculated from Oresnik and Layzell (1994) and Kuzma et al. (1999)</td>
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<td>10. Vmax for respiration</td>
<td>0.40 mol m^{-3} s^{-1} in MZ; 0.096 mol m^{-3} s^{-1} in BZ</td>
<td>Bergersen (1994); Thumfort et al. (1999)</td>
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<td>11. Ks(O_2) for respiration</td>
<td>5.3 \times 10^{-5} \text{ mol} \text{ m}^{-3} in MZ; 2.0 \times 10^{-5} \text{ mol} \text{ m}^{-3} in BZ</td>
<td>Bergersen (1994); Kuzma et al. (1993); Bergersen and Turner (1993)</td>
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<td>12. Total cytosolic Lb concentration</td>
<td>3.9 mol m^{-3}</td>
<td>Bergersen (1993)</td>
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Table II. Effect of changing input parameters for the models on the predicted values for O₂ concentrations at the gas space interface (O₂<sub>400</sub>) and innermost layer (O₂<sub>min</sub>), average O₂ concentration of cell (O₂<sub>Avg</sub>), FOL at the gas space interface (FOL<sub>400</sub>), average FOL of cell (FOL<sub>Avg</sub>), O₂ respiration in mitochondria and its percentage in the whole cell’s respiration, cytosolic AEC at the gas space interface (AEC<sub>400</sub>) and innermost layer (AEC<sub>min</sub>), and average cytosolic AEC of cell (AEC<sub>Avg</sub>)

All simulations assume an AEC<sub>400</sub> of 0.82.

<table>
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<th>Item No. and Parameter Changed</th>
<th>O₂&lt;sub&gt;400&lt;/sub&gt;</th>
<th>O₂&lt;sub&gt;Avg&lt;/sub&gt;</th>
<th>FOL&lt;sub&gt;400&lt;/sub&gt;</th>
<th>FOL&lt;sub&gt;Avg&lt;/sub&gt;</th>
<th>Mito Respiration&lt;sup&gt;a&lt;/sup&gt;</th>
<th>AEC&lt;sub&gt;400&lt;/sub&gt;</th>
<th>AEC&lt;sub&gt;min&lt;/sub&gt;</th>
<th>AEC&lt;sub&gt;Avg&lt;/sub&gt;</th>
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<td>1. Ureide nodule model</td>
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<tr>
<td>(a) Standard parameters and predictions&lt;sup&gt;b&lt;/sup&gt;</td>
<td>400 13 39 0.90 0.40 2.5 (31%) 0.82 0.62 0.70</td>
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<td>(b) Increased average FOL to 0.6</td>
<td>7,600 26 205 0.99 0.60 3.5 (32%) 0.82 0.56 0.66</td>
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<td>(c) Decreased P:O ratio from 2 to 1.3</td>
<td>400 13 39 0.90 0.40 2.5 (31%) 0.82 0.66 0.72</td>
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<td>2. Amide nodule model</td>
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<tr>
<td>(a) Standard parameters and predictions&lt;sup&gt;b&lt;/sup&gt;</td>
<td>600 32 63 0.88 0.40 2.9 (32%) 0.82 0.52 0.61</td>
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<td>(b) Decreased P:O ratio from 2 to 1.3</td>
<td>600 32 63 0.88 0.40 2.9 (32%) 0.82 0.60 0.67</td>
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<td>(c) Increased Ks&lt;sub&gt;Lb&lt;/sub&gt; from 78 to 127 nM</td>
<td>1,300 46 103 0.91 0.40 3.2 (33%) 0.82 0.48 0.58</td>
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<td>(d) Decreased Ks&lt;sub&gt;Lb&lt;/sub&gt; from 78 to 48 nM</td>
<td>210 21 36 0.81 0.40 2.3 (31%) 0.82 0.57 0.64</td>
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<td>(e) Increased Aden from 2.29 to 3.29 mM</td>
<td>600 32 63 0.88 0.40 2.9 (32%) 0.82 0.61 0.67</td>
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<tr>
<td>(f) Decreased Aden from 2.29 to 1.29 mM</td>
<td>600 32 63 0.88 0.40 2.9 (32%) 0.82 0.29 0.44</td>
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<sup>a</sup> The value in parentheses (31%–33%) indicates the percentage of O₂ respiration in mitochondria accounting for O₂ respiration of whole cell, which is lower than that predicted value of 56% in Bergersen’s model (Table 5 of Bergersen, 1996). The latter value may reflect an overestimation of mitochondrial respiration (Thumfort, 1996).

<sup>b</sup> Standard parameters and predictions as presented in Figures 1 to 6.

Validity of Physiological Parameters

The diffusive path for O₂ was defined in both the cytosol and symbiosome of the infected cell, whereas the diffusive paths for LbO₂, and plant ATP, ADP, and AMP were restricted to the cytosol. Diffusion coefficients (D) of LbO₂, O₂, and ATP in cell (Table I, items 2–4) used in the simulation were expected to have an impact on the predicted features of the models. The reported D values for LbO₂, O₂, and ATP in water were 12.5 × 10⁻¹¹, 17 × 10⁻¹⁰, and 2.70 × 10⁻¹⁰ m² s⁻¹ (Kushmerick and Podolsky, 1969; Bergersen, 1993), respectively. The calculated D<sub>Water:D_Cell</sub> ratio for these three molecules was 2.11, 2.30, and 2.45, respectively. Assuming the effective radius of a diffusing molecule does not vary between pure water and the cytosol, D<sub>Water:D_Cell</sub> would reflect the relative viscosity of cytosol, resulting in similar values for different diffusing molecules (Kushmerick and Podolsky, 1969). Because D of LbO₂ (Table I, item 2) was measured in the cytosol of an infected cell of a nodule, the similarity in D<sub>Water:D_Cell</sub> (2.11–2.45) for LbO₂, O₂, and ATP permits confidence in the D values used in the models. Simulations in which D values were varied by ±30% only slightly changed the predictions in both ureide and amide models (data not shown).

The models used a P:O ratio of 2 (Table I, item 6) to couple bacteroid O₂ consumption rate to the production of ATP in bacteroids of BZ, which was then used to generate the rate of nitrogenase activity. Wittenberg et al. (1974) suggested that the P:O ratio in bacteroids might vary in the range of 2 to 1 with an average of 1.3. Simulations with a P:O of 1.3 would reduce the rate of nitrogenase activity to 1.4 × 10⁻¹⁵ and 1.5 × 10⁻¹⁵ mol NH₃ s⁻¹ cell⁻¹ (apparent nitrogenase activity) in ureide and amide nodules, respectively. The equivalent rates of total nitrogenase activity would be 190 to 200 μmol H₂ g⁻¹ dry weight (nod) h⁻¹, values at the lower limit but still comparable with those reported for nodules in vivo (Hunt and Layzell, 1993). Adopting a P:O of 1.3 the predicted cytosolic adenylate gradients were reduced in magnitude but were still generated (Table II, items 1c and 2b).

It is noteworthy that the P to O ratio was not used to calculate the ATP production in mitochondria in MZ (layers 400–365). Instead, the metabolic ATP “demand” (i.e. production) in each layer of MZ was assumed to be proportional to the contribution of that layer to the total O₂ uptake within the MZ.

Impact of Changes in Vacuole Volume, Ks<sub>Lb</sub>, K<sub>Aden</sub>, and Aden and in the Amide Model

The amide model assumed a large central vacuole in the infected cells, accounting for 12% of whole cell volume (i.e. layers 180–181 of the cell). Increasing VZ volume to 20% shortened the diffusion pathway in the BZ but did not significantly reduce the adenylate gradients (data not shown). Pugh et al. (1995) reported that the fraction of VZ volume was increased from 20% to 74% in infected cells of white clover (Trifolium repens) nodules by waterlogging. A larger central vacuole may make O₂ in the intercellular space more accessible to the bacteroids, thus increasing the nodule’s tolerance to waterlogging. A simulation of an infected cell in an amide nodule with a fraction of VZ as high as 74% essentially abolished...
both the O₂ and adenylate gradients (data not shown).

A $K_{SLb}$ value (the O₂ concentration at which 50% of Lb is oxygenated) of 78 nM (Table I, item 7) was used for the amide model. This value was originally determined for lupin Lb II and was between the reported $K_{SLb}$ for soybean Lb (48 nM) and pea (Pisum sativum) Lb IV (127 nM, Kawashima et al., 2001). Changing $K_{SLb}$ over this range (i.e. 48–127 nM) still predicted a substantial gradient of plant AEC (Table II, item 2, a, c, and d).

The apparent equilibrium constant of cytosolic AK ($K_{AK} = 0.90$; Table I, item 8) was based on measurements from ureide-producing nodules. The reported values of $K_{AK}$ in plant tissues were in the range of 0.3 to 1.5 (Igamberdiev and Kleczkowski, 2001). For the amide model, increasing $K_{AK}$ to 1.5 would slightly change the ATP gradient to 1.65 mM in layer 400 and 0.86 mM in layer 181. In contrast, decreasing $K_{AK}$ to 0.3 would predict a steeper ATP gradient ranging from 1.55 mM in layer 400 to 0.64 mM in layer 181.

The value for total cytosolic adenylate concentration ($Aden$, 2.29 mM; Table I, item 9) was based on measurements from ureide-producing nodules. For amide model, increasing the value for $Aden$ would reduce the magnitude of adenylate gradients (Table II, item 2, a and e). In contrast, decreasing the value for $Aden$ would cause the adenylate gradients to become steeper (Table II, item 2, a and f), consistent with the infected cell requiring steeper adenylate gradients to meet the ATP demand in the inner part of cell.

Ammonia Assimilation Pathways in Nodules and Implications for the Models

The central assumptions about assimilation of fixed N in the present models was that NH$_4^+$ was the sole N solute transferred from bacteroids to the plant cytosol and that it was incorporated exclusively into the amide group of Gln through cytosolic GS. Further, the ultimate products of N$_2$ fixation were in one and Asn in the other type of nodule. There have been a number of proposals that amino compounds may be exported from bacteroids (for review, see Day et al., 2001), and recently, evidence has been presented (Waters et al., 1998) for a significant flux of fixed-N as Ala from soybean bacteroids. Even if this were to be the case in vivo, Asn and ureide synthesis depend on the amide group of Gln, and Ala-N would need to be transferred, most likely through a cytosolic pool of NH$_4^+$, to the amide; thus, the ATP demand by GS would be unchanged. $^{15}$N$_2$ labeling of cowpea (Vigna unguiculata) nodules (a ureide-based symbiosis) indicates a flow of N to Ala, but the labeling kinetics are not consistent with this N being a precursor for the amide group of Gln (Atkins and Thumford, 2002).

If the amidotransferases of either purine (phosphoribosyl pyro-phosphate or 5-phosphoribosyl-N-formylglycinamide amidotransferases) or AS could utilize NH$_4^+$ directly in nodules, and then ATP demand by GS would be reduced. Although Vance (2000) reported that AS from alfalfa (Medicago sativa) nodules could use NH$_4^+$ in vitro, unreasonably high concentrations were needed, and similar observations were made for de novo purine synthesis by cowpea nodule extracts (Atkins et al., 1982). Thus, it is reasonable to assume that the amide-N of Gln, formed by cytosolic GS, is the substrate in both cases. However, the sites at which Gln-N is used are different in the two types of nodule. In the case of Asn synthesis, AS is soluble in the cytosol of the infected cell (Shelp and Atkins, 1984) and, like GS, is assumed to be functional in the BZ. Purines, on the other hand, are formed in both plastids and mitochondria in the MZ (Smith and Atkins, 2002).

ATP is used in the MZ for purine synthesis, but this is unlikely to significantly affect the simulated adenylate gradients generated by the ureide model. Plastids are distributed together with mitochondria adjacent to the intercellular space. These organelles are assumed to be distributed evenly across the MZ and that in each layer, the ATP demand in plastids would be met by ATP produced by the mitochondria located in the same layer. Therefore, the extra ATP use in plastids would not affect the adenylate gradients generated by diffusion from mitochondria toward the inner cytosol.

ATP Gradients and the Regulation of Biochemical Activities in Infected Cells

It is known that cytosolic ATP level can regulate the activity of specific glycolytic enzymes, including phosphofructokinase, pyruvate kinase, hexokinase, enolase, and phosphoenolpyruvate carboxylase in plants (Plaxton, 1996). For example, in chickpea (Cicer arietinum) nodules, cytosolic phosphofructokinase was disaggregated in the presence of high ATP, resulting in a lower affinity for its substrate, Fru-6-phosphate (Lee and Copeland, 1996). Large gradients across an infected cell would create the potential for very dynamic and complex interactions in metabolic regulation.

ATP “demand” by AK [$P_{ATP(AK)^{−}}$] was negative in most layers of BZ (Fig. 3B), indicating the AK reaction was in the direction 2ADP → ATP + AMP, thereby regenerating 33% to 36% of the total ATP metabolic demand in the plant fraction. Such activity of AK could be a strategy for the cell to reduce the ATP diffusive flux from mitochondria to the inner cytosol. In contrast, the predicted ATP “demand” by AS [$P_{ATP(AK)^{−}}$] in MZ was positive, indicating that the AK reaction was in the opposite direction, thereby helping provide ADP for mitochondrial oxidative phosphorylation.

The pyrophosphate (PPI) cleavage of ATP by AS raises one further possibility that might alter the ATP
dynamics assumed in the models. If PPi is substrate for an H⁺-pyrophosphatase (PPase) in the PBM, then its activity could generate sufficient proton motive force for malate transport, eliminating the need for ATP to drive an alternate H⁺ pump. However, in PBM preparations from soybean, there is no evidence for a PBM-localized PPase (D. Day, personal communication). Thus, the models assume that PPi generated by AS is cleaved by a PPase such that there are no consequences for the adenylate dynamics of the BZ.

A Possible Experimental Test of the Models

The simulations with Ar:O₂ indicate a rationale to test the models experimentally. Both amide and ureide models predict that the cytosolic adenylate gradients will decrease in Ar:O₂, resulting in large increases in the average AEC of the plant fraction as soon as the N pools are depleted. This could be tested experimentally using a nonaqueous fractionation technique (Kuzma et al., 1999) to separate and analyze adenylate pools in plant and bacteroid fractions before and after Ar:O₂ exposure. If the predictions of the models are realistic, brief (<8 min), exposure to Ar:O₂ should increase AEC in the plant fraction with minimal effect on bacteroid AEC. Then, as the nodule’s O₂ gaseous permeability is decreased, there should be a large decline in AEC of the bacteroid fraction but only a minor effect on AEC in the plant fraction, resulting in little change from the AEC of whole nodules treated with air.

MATERIALS AND METHODS

Cell and Tissue Geometry

The central, bacteria-infected zone of nodules is assumed to consist only of tightly packed infected cells (the actual ratio of infected cell:nodules) with a volume of 0.0156 m³ (Dakora and Atkins, 1990). The edges of each plane of the cell were modified by removal of an “arc” representing one-third of a circle so as to create a network of cylindrical gas-filled spaces (total volume = 0.98% of cell) surrounding each cell. This resulted in a gas space interface of 1719 μm² cell⁻¹ and a ratio of gas space interface:infected cell volume of 0.0156 μm² μm⁻³ (Dakora, 1989). These parameters were similar to those used previously (Thumfort et al., 1994, 1999, 2000).

To model the adenylate diffusion within the infected cell, the method of Thumfort et al. (1994, 2000) and Thumfort (1996) was used to derive a 1D representation, i.e. the “surface area versus distance profile” of a 3D cell assumed to be a rhombic dodecahedron (Fig. 7A) with a side length of 33 μm and a volume of 1.1 × 10⁻¹³ m³ (Dakora and Atkins, 1990). The edges of each plane of the cell were modified by removal of an “arc” representing one-third of a circle so as to create a network of cylindrical gas-filled spaces (total volume = 0.98% of cell) surrounding each cell. This resulted in a gas space interface of 1719 μm² cell⁻¹ and a ratio of gas space interface:infected cell volume of 0.0156 μm² μm⁻³ (Dakora, 1989). These parameters were similar to those used previously (Thumfort et al., 1994, 1999, 2000).

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The 31.2-μm distance from the gas space interface to the innermost point in the cell was divided into 400 layers, each with an identical depth (0.078 μm) and with the relative surface area shown in Figure 7B. Layers 400 to 365, occupying a depth of 2.8 μm, were considered to be within the MZ adjacent to the gas space interface, whereas layers 364 to 1 (1 ureide nodule) or 364 to 181 (amide nodule) were identified as the BZ. In the amide nodules, layers 180 to 1 constitututed a central VZ. This partitioning resulted in the volume ratio of MZ:BZ = 8.5:91.5% in the infected cell of ureide nodules (Bergersen, 1994; Thumfort et al., 1999) or the volume ratio of MZ:BZ:VZ = 8.5:79.5:12% in that of amide nodules.

A five-step process (Fig. 8) was used to model cytosolic adenylate gradients in the infected cells of ureide and amide nodules. A detailed description of the methods is described in the supplemental material or from the authors.

Step 1. O₂ Diffusion and Consumption across the Infected Cell

The modeling of O₂ diffusion (Fig. 8, item 1) into the cells was similar to that described by Thumfort et al. (1994). However, the infected cells were assumed to have two metabolically active zones (MZ and BZ), each with separate kinetic constants for O₂ uptake (i.e. Vmax and K₅⁵⁵(O₂); Table I, items 10 and 11). The ureide and amide models were also assigned different half-saturation constants for Lb oxygenation (Table I, item 7).

Equations were derived that described Lb equilibrium with free O₂, the diffusion of O₂ and LbO₂ between layers in the infected cell, and the respiratory consumption of O₂ by the bacteroids or mitochondria within each layer (Eq. 1-4 in Thumfort et al., 1994). These equations were combined to derive a quadratic equation that permitted the calculation of O₂ concentration in layer n + 1 (O₂(n+1)), moles per meter cubed) from parameters obtained for layers n and n - 1.

The model was built in Excel 98 (Microsoft, Redmond, WA) by first selecting an O₂ concentration for the innermost layer (O₂(n), n = 1 for ureide nodules, n = 181 for amide nodules, moles per meter cubed; layers 180 to 1 in amide nodules were occupied by vacuole) and then calculating the corresponding oxygenated Lb concentration and O₂ consumption rate for.
ATP Diffusion in Legume Nodules

Step 3. ATP Demand for NH₃ Assimilation and Malate Transport in Plant Cytosol of BZ

The NH₃ produced by the bacteroids was assumed to diffuse into the plant cytosol, where it was assimilated into amino acids by GS and Glu synthase, in ureide nodules or by GS, Glu synthase, and ASN in amide nodules. The ATP requirements for GS (one ATP/NH₃) and AS (two ATP/NH₃) were assumed to be synthesized by the mitochondria in the MZ, and diffuse through the cytoplasm to the layers in which NH₃ was produced. The ATP demand along the diffusion pathway included that for the transport of malate into the symbiosomes (Fig. 8, item 6) and the plant growth and maintenance (Fig. 8, item 7), in addition to the cost of NH₃ assimilation. The ATP demand for malate transport across the symbiosome membrane (Fig. 8, item 6) was calculated assuming one ATP per malate transported. To calculate malate transported into the symbiosomes, values were generated to account for: (a) the C requirement for bacterial growth (relative growth rate = 6.49 × 10⁻⁷ g dry weight g⁻¹ dry weight s⁻¹; Bouma et al., 1997), and (b) the requirement for reducing power (12 e⁻/malate) to satisfy the demands for bacteroid O₂ consumption (4e⁻/O₂) and nitrogenase activity (4.5 e⁻/NH₃) calculated above.

Step 4. Adenylate Diffusion Gradients in Plant Cytosol of BZ

Estimates of the ATP demand within the plant fraction of each layer [P_ATPmetabol] of the BZ were used to generate the diffusion gradients of adenylates across the cells. The total cytosolic adenylate pool (Aden; Table I, item 9) was assumed to be constant for each layer, and within each layer, an AK was assumed to maintain a balance among the adenylate pools according to the equation of ATP × AMP٤١٨٢١ = KAK (× (ADP٤١٨٢٩)², where KAK is the apparent equilibrium constant of AK (Table I, item 8). The ATP demand for AK activity [P_ATPmetabol] was generally negative because the AK was needed primarily to build the AMP pool, which involves the production of ATP from 2ADP.

The calculations of the cytosolic adenylate gradient were begun by first choosing a value for the cytosolic ATP concentration for the innermost layer (range of 0.01–2.29 mol m⁻³, determined according to the value of Aden; Table I, item 9). Then, a series of equations were used to calculate the diffusion of cytosolic ATP, ADP, and AMP (Fig. 8, item 11, a–c) between adjacent layers while ensuring that the adenylate pools were in equilibrium via the AK and assuming each layer met its respective demand for ATP.

Step 5. Continuity of Adenylate Diffusion Gradients in Plant Cytosol of MZ

Calculation of the cytosolic ATP gradients across the MZ assumed that this zone was responsible for providing the ATP needs of the plant fraction in the entire BZ. In the MZ, as in the BZ, cytosolic AK was assumed to maintain a balance among the adenylate pools. The contribution of each MZ layer to the total ATP demand of plant fraction of BZ was assumed to be proportional to the contribution of that layer to the total O₂ uptake within the MZ. This permitted the calculation of the volume-specific metabolic ATP “demand” in each layer of the MZ [P_ATPmetabol]; Fig. 8, item 15].

Although formation of ureides relies on purine synthesis, which itself requires ATP (Smith and Atkins, 2002), this demand was considered to be met within the plastids and mitochondria and, therefore, to have no impact on the demand for ATP within the cytosol.

Given these values, a series of equations were used to calculate the diffusion of cytosolic ATP, ADP, and AMP between adjacent layers while ensuring that the adenylate pools were in equilibrium. Finally, the AEC ([AEc] = ([ATP] + 0.5 × [ADP])/([ATP] + [ADP] + [AMP])) was calculated for each layer, and these values were used to calculate the volume-weighted average AEC for the plant cytosol of entire cell.

For both ureide and amide models, the cytosolic ATP concentration for the innermost layer (initialized in Step 4) was set at a value that resulted in an AEC at the gas space (AECgas) of 0.82, a value representing fully aerobic tissue (Oresnik and Layzell, 1994).
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