Aerenchyma Formation and Recovery from Hypoxia of the Flooded Root System of Nodulated Soybean

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INTRODUCTION

The lack of O2 in the root system of soybean (Glycine max), besides inhibiting symbiotic N2 fixation, also inhibits the uptake of N, leading to diminished root growth and nodulation (Sallam and Scott, 1987a, b; Sung, 1993). The stress brought on by low O2 concentrations in flooded soils leads to the formation of aerenchyma in tolerant plant species, a process that can occur in roots, nodules, rhizomes, stems and submerged leaves (Pankhurst and Sprent, 1975; Kawase and Whitmoyer, 1980; Justin and Armstrong, 1987; Armstrong et al., 1994; Loureiro et al., 1995; Drew, 1997; Jackson and Armstrong, 1999; Drew et al., 2000; Schussler and Longstreth, 2000; Gibberd et al., 2001). In the case of nodulated legumes, a large number of studies have shown the beneficial effects of aerenchyma towards N2 fixation under flooded conditions (Minchin and Summerfield, 1976; Walker et al., 1983; Loureiro et al., 1995; James and Crawford, 1998; Bacanamwo and Purcell, 1999b; James and Sprent, 1999; Shimamura et al., 2002), by providing a pathway for the diffusion of gases to the submerged nodules. Indeed, nodules of soybean grown at low P02 produced morphological changes that were similar to those grown under waterlogged conditions and were responsible for maintaining N2-fixing activity under these conditions (Parsons and Day, 1990).

Aerenchyma can be of two types: cortical and secondary. Secondary aerenchyma arises from successive cell division, induced by hypoxia, giving rise to spongy parenchymous tissue (Justin and Armstrong, 1987). Cortical aerenchyma may be formed by a specific process of separation and differential expansion of mature cells (schizogenous aerenchyma) or by death and dissolution of such cells (lysigenous aerenchyma). Nevertheless, both types of aerenchyma can occur in the same plant and schizogenous aerenchyma may precede the occurrence of lysigenous aerenchyma in the same organ (Justin and Armstrong, 1987; Armstrong et al., 1994; Schussler and Longstreth, 1996; Jackson and Armstrong, 1999; Drew et al., 2000).

Waterlogged soybean plants form aerenchyma in the base of the stem, adventitious roots (Becanamwo and Purcell, 1999a; Pires et al., 2002; Shimamura et al., 2003), taproot (Shimamura et al., 2003), lateral roots (Bacanamwo and Purcell, 1999a) and in nodules (Pankhurst and Sprent, 1975; Shimamura et al., 2003). Shimamura et al. (2003) demonstrated that the origin of secondary aerenchyma in the hypocotyl, taproot, adventitious roots and submerged nodules was the phellogen.

Some of the principal changes in N metabolism that have been reported to occur in the soybean plant subjected to waterlogging are reduced N2 fixation (Bacanamwo and...
Purcell, 1999b) and the accumulation of alanine (Sousa and Sodek, 2003). The consequences of such changes are readily seen in the transport of N compounds via xylem, where elevated levels of alanine are found (Puiatti and Sodek, 1999; Sousa and Sodek, 2003) together with strongly elevated levels of alanine are found (Puiatti and Sodek, 2003). The consequences of such changes associated with the waterlogged root system of nodulated soybean plants in order to better understand the influence of anatomical changes on N metabolism under these conditions.

MATERIALS AND METHODS

Experiment 1: anatomical study of submerged organs

Soybean plants (Glycine max L.), ‘FT-Abyara’ (determinate growth habit), inoculated on sowing with Bradyrhizobium elkanii, strain SEMIA 5019, were grown in a glasshouse under natural light and temperature (average maximum, 38°C, average minimum, 19°C) conditions. Plants were cultivated in 2-L pots (two plants per pot) in vermiculite and irrigated twice a week with 200 mL of N-free nutrient solution (Hoagland and Arnon, 1950) to the V6-7 stage (plants with five or six expanded trifoliate leaves, as defined by Fehr et al., 1971).

When the V6-7 stage was reached, the 2-L pots were placed inside 3-L pots (non-perforated) and the whole root system flooded to the base of the stem with N-free nutrient solution at one-third strength. The water level was maintained 2–3 cm above the surface of the vermiculite (giving a total volume of 1.8 L) by the daily addition of nutrient solution. The duration of the flooding treatment was 21 d.

Non-flooded controls were also run simultaneously where 200 mL of N-free nutrient solution was supplied twice a week and then increased to three times a week when the flooding treatments were initiated such that an equivalent amount of nutrients was made available (i.e. 600 mL of undiluted solution equivalent to 1.8 L of nutrient solution diluted one to three). The controls also received water as necessary.

Segments of 1 cm were taken from the waterlogged and control plants, first at daily intervals from days 1–7, and then at days 14 and 21, with three replicates per treatment. For the waterlogged plants, the segments were taken from the submerged part of the stem, the transition region between the submerged stem and the root, the taproot (at 4–5 cm below the substrate level), the lateral roots (situated about 1–2 cm below the substrate and 1 cm from the taproot) and the adventitious roots (1 cm from the root–shoot junction). As a control, plants segments were taken only from the stem, taproot and lateral roots. Nodules located on lateral roots (at 1–3 cm below the substrate surface) were taken from both control and waterlogged plants. The segments were fixed in neutral buffered formalin (100 mL of formol, 4.9 g of NaH2PO4·H2O and 6.5 g of anhydrous Na2HPO4 in 1 L of distilled water). Dehydration was carried out by treating segments sequentially with 30%, 50% and 70% ethanal, for 12 h at each concentration. The segments were recut in the form of a wedge of 3 mm or less in height, width and depth, for infiltration in glycol methacrylate, according to Gerrits and Smid (1983). The polymerized resin blocks were then glued onto wooden blocks with plastic adhesive and 12-μm-thick sections cut on a rotary microscope (American Optical, model 820).

The sections were mounted on glass slides (2.5 cm × 7.5 cm), heated to 65°C, followed by staining for 3 min in 0.05% toluidine blue in 0.1 M acetate buffer pH 4.7 and washing in running water for 5 min (O’Brien et al., 1964). After drying the slides at room temperature, the sections were viewed and photographed with a light microscope (Olympus, model BX40).

The root system and nodules (as shown in Fig. 1) were photographed with a stereoscopic microscope (Olympus, model SZX9).

Experiment 2: formation of aerenchyma and its effect on metabolism of the root system under hypoxia

Soybean plants were cultivated as for expt 1 up to flowering (10–12 expanded trifoliate leaves), when the root systems were flooded for a period of 10 d.

The levels of O2 in the nutrient solution were measured by a portable Jenway dissolved oxygen meter, model 9071, with the electrode placed in the solution between the inner and outer pots.

Gas volume of tissues (i.e. porosity) was determined for nodules and taproot according to Jensen et al. (1969). Nodules were harvested from roots to a depth of 3 cm, since below a depth of 5 cm there was practically no formation of aerenchyma. The segment of taproot studied was restricted to the top 8 cm due to the fact that in preliminary studies this region presented the most rapid development of aerenchyma, visible to the naked eye within 2 d of flooding.

Sap bleeding from the xylem was collected between 1100 and 1300 h during a 30- to 60-min period, according to McClure and Israel (1979). The separation and analysis of the free amino acid composition of the sap was carried out by reverse-phase HPLC of their OPA derivatives using a column of Spherisorb ODS-2, 4.6 mm × 250 mm, based on Jarret et al. (1986). The amino acids were eluted from the column by a gradient formed from two solvents, buffer A (50 mM NaAc and Na2HPO4, adjusted to pH 7.25 with HAc, plus 20 mL methanol and 20 mL tetrahydrofuran in 1 L of MilliQ water) and solvent B (65% methanol in MilliQ water), such that the proportion of solvent B increased linearly from 20% to 28% between 0 and 5 min, 28% to 58% between 5 and 35 min, 58 to 75% between 35 and 40 min, 75 to 95% between 40 and 56 min and finally 95 to 100% between 56 and 61 min. Standards (Sigma AA-S-18 plus Gln, Asn and GABA) were used at 250 nmol mL−1. Total amino acid concentration was calculated as the sum of the
amino acids recovered after chromatographic separation and quantification. Uricides were determined in the xylem sap by the method of Vogels and Van Der Drift (1970) using allantoin as standard.

Data are expressed as the means of three replicates where each replicate consisted of one pot containing two plants (material pooled). Where appropriate, the data were subjected to an analysis of variance and when $F$ was significant the means compared by Duncan’s multiple range test at a 5% probability level.

RESULTS

Experiment 1: anatomical study of submerged organs

The $O_2$ concentration of the nutrient solution in the pots on flooding was approx. 0.42 mmol dm$^{-3}$, but rapidly fell to between 0.06 to 0.03 mmol dm$^{-3}$ at day 1. The low levels persisted until the end of the experiment on day 21 (data not shown), demonstrating that hypoxic conditions existed throughout the experiment.

The morphological changes that occurred in the submerged segments began to appear after the first day of flooding. Adventitious root formation was apparent near the junction between shoot and root. Aerenchyma was formed in the root–shoot junction, adventitious roots, taproot and nodules, presumably establishing a connection of aerenchyma between these tissues (Fig. 1). Aerenchyma may be seen in tissue sections of the waterlogged material (Figs 2–4) contrasting its almost absence in the non-flooded controls. Details of anatomical changes in the different tissues were as follows.

Submerged stem portion and adventitious roots. After the first day of flooding, hypertrophy of lenticels was apparent on the stem–root segment. By the second day, hypertrophy of the lenticels increased while adventitious roots appeared. Examination of sections under the microscope revealed signs of initial breakdown of the epidermis with the formation of aerenchyma involving cells arising by division of the pericycle (Fig. 4A and B). On day 3 the adventitious roots were 2–5 mm long. By days 4 and 5 the formation of aerenchyma in the stem–root segment became evident while the adventitious roots had grown to a length of 1 cm or more. After 21 d of flooding, the secondary aerenchyma of the stem–root segment was very prominent (Fig. 4B).

The adventitious roots of the submerged stem, originating from the pericycle, formed schizogenous aerenchyma in the cortex (Fig. 2A; day 7). As the flooding treatment became more prolonged, lysigenous aerenchyma formed in the cortex (Fig. 2B; day 14) and by day 21 aerenchyma was also seen to arise from cell divisions of the pericycle (Fig. 2C). The aerenchyma produced by the pericycle displaced the endodermis and the cortex radially, causing the epidermis to break away from the tissue.

Taproot and lateral roots. Formation of aerenchyma on the upper 8 cm of the taproot was only slight up to day 4 of flooding, but increased rapidly thereafter. Here, too, secondary aerenchyma was formed from cell divisions of the pericycle (Fig. 3C). The actual sequence of events is shown in Fig. 3. During the first 7 d of flooding lysigenous aerenchyma in the cortex became quite pronounced (Fig. 3B). Subsequently, tissue formed by division of the pericycle displaced the endodermis radially and produced secondary aerenchyma. By day 21 (Fig. 3C), this radial displacement caused some of the epidermis to break away, resulting in the cortical aerenchyma being largely substituted by
the secondary aerenchyma. Displacement of the endodermis by cell division of the pericycle was not seen in the non-flooded material (Fig. 3A). Such controls revealed the presence of only occasional lysigenous aerenchyma in the cortex.

In the lateral roots, aerenchyma appeared from days 5 or 6 after flooding, first in the cortex and subsequently (by day 14) as secondary aerenchyma produced from cell divisions of the pericycle. By day 21 (Fig. 4D) the secondary aerenchyma had displaced the endodermis radially causing much of the epidermis to break away leaving a very reduced amount of cortical tissue. The sequence of events was in fact quite similar to that observed for the taproot shown in Fig. 3. In the non-flooded lateral roots there was no evidence for the presence of any large gas-filled spaces in the cortex (Fig. 4C).

**Nodules.** Formation of aerenchyma in nodules resulted in a profound change in their external appearance, with the proliferation of white friable aerenchymatous tissue (Fig. 1B) contrasting with the normally firm, light-brown and beige striped nodule surface. A transverse section of a nodule at 21 d after flooding (Fig. 1C) shows such nodules apparently viable in view of the intense colouring of the central infected zone due to leghaemoglobin. The presence...
of aerenchyma at this time was very prominent. Aerenchyma formation in waterlogged nodules arose from cells produced by the phellogen (Fig. 4F). However, for nodules situated below a depth of 7–8 cm, there was practically no development of aerenchyma. Even nodules of plants not subjected to flooding formed some aerenchyma through hypertrophy of lenticels (Fig. 4E), again originating from cell division of the phellogen.

Experiment 2: formation of aerenchyma and products of N metabolism in the xylem of the root system under hypoxia. The concentration of dissolved O₂ in the nutrient solution (Table 1) followed a similar pattern to that of the first experiment, falling rapidly on the first day of flooding and thereafter it remained at very low levels (0–0.7 mg of O₂ L⁻¹) throughout the 10 d of the experiment.

Aerenchyma formation was followed by measuring the increase in tissue porosity (Table 1). Thus, the porosity of nodules increased from 0.4 to 1.9% after the first day of waterlogging and remained close to this value throughout the experiment, whereas the porosity of the taproot increased progressively, from 4.7% on the 4th day of waterlogging to its maximum value of 22% on the 7th day.

The levels of xylem sap ureides and glutamine fell sharply to low values over the first day of flooding (Table 1). These low levels persisted for several days but started increasing at day 7 following the large increase in root porosity and returned to near pre-flooded levels by day 10. In contrast, there was a very steep rise in alanine over the first day of flooding (from 0.4 to 34%) followed by a gradual fall to more moderate levels over the next 4 d. Levels continued falling towards the end of the experiment but initial levels were not recovered. Total amino acid levels also fell to less than half their initial value over the first 5 d of flooding but most of the fall was seen on the first day. In parallel with ureides, total amino acid levels largely recovered towards the end of the experiment, again coinciding with the large increase in root aerenchyma.

DISCUSSION

The observed morphological modifications concerning the formation of adventitious roots and aerenchyma that occur
in the submerged root system of soybean were similar to those reported by Bacanamwo and Purcell (1999a), Pires et al. (2002) and Shimamura et al. (2003). However, the present data provide additional information on the development of aerenchyma in lateral, adventitious and taproots, as well as providing a clearer picture of the sequence of events. The present data demonstrate (Fig. 4B) that the origin of the secondary aerenchyma at the root–shoot junction is the pericycle, since it is from the pericycle that cells of the parenchyma are produced that dislocate the endodermis, cortex and epidermis. Shimamura et al. (2003) reported that aerenchyma in this tissue originates from the external part of the interfascicular cambium. Nevertheless, for some authors the interfascicular tissue is in fact the pericycle, as shown for stems of Ricinus communis, Wedelia paludosa and Aristolochia sp. (Angyalossy-Alfonso and Marcati, 2003).

The appearance of adventitious roots over the first few days of flooding, together with the development of aerenchyma, demonstrates that adventitious roots constitute an adaptation of soybean to flooding. Schizogenous aerenchyma (Fig. 2A) was formed in the cortex of adventitious roots during the first week of waterlogging, followed by lysigenous aerenchyma (Fig. 2B), while the formation of secondary aerenchyma arising from the pericycle (Fig. 2C) after more prolonged waterlogging led to the gradual substitution of the cortical aerenchyma. The presence of lysigenous aerenchyma in the cortex of adventitious roots was described by Bacanamwo and Purcell (1999a) and Pires et al. (2002), whereas the presence of schizogenous aerenchyma in the cortex of adventitious roots had not been reported previously, although it is known that schizogenous aerenchyma can precede lysigenous aerenchyma in the same organ in other species (Jackson and Armstrong, 1999). Similarly, the taproot and lateral roots initially formed lysigenous aerenchyma in the cortex, followed by substitution with secondary aerenchyma formed from the pericycle (referred to as phellogen by Shimamura et al., 2003). The substitution of cortical by secondary aerenchyma in taproot and adventitious roots was also reported for soybean by Shimamura et al. (2003).

The origin of secondary aerenchyma in the nodule was the phellogen (Fig. 4E and F) and its formation was very prominent in waterlogged nodules (Figs 1C and 4F), yet on occasions it also appeared in nodules of control plants (Fig. 4E), possibly due to the high capacity for water retention of the vermiculite (about 400 mL of water per 2 L of vermiculite). The present observations for aerenchyma formation in the nodules are similar to those reported by Pankhurst and Sprent (1975) and Shimamura et al. (2003).

Porosity of the taproot increased substantially between days 4 and 7 of flooding, reflecting the formation of aerenchyma. The substantial changes in certain xylem sap nitrogenous metabolites provoked by flooding were restored to or near initial levels coincident with aerenchyma formation in the taproot. Changes in nitrogenous components of the xylem sap are known to reflect important changes in root N metabolism. Xylem sap ureides are closely associated with N2 fixation (McClure et al., 1980; Herridge and Peoples, 1990), and more recently glutamine has been shown to hold a similar relationship for soybean (Amarante and Sodek, 2005). Alanine is strongly associated with N2 fixation (McClure et al., 1980; Herridge and Peoples, 1990), and more recently glutamine has been shown to hold a similar relationship for soybean (Amarante and Sodek, 2005). Alanine is strongly associated with hypoxia of the root system, being transported in large amounts in the xylem of waterlogged soybean plants (Sousa and Sodek, 2003), while N deficiency can result in profound changes in aspartate and asparagine (Lima and Sodek, 2003). The sharp drop in ureides and glutamine on the first day of flooding apparently reflects, therefore, a strong inhibition of N2 fixation imposed by this treatment. N2 fixation is known to be highly sensitive to oxygen deficiency and, even under ideal growth conditions, oxygen appears to be limiting for nitrogenase (Hunt et al., 1989; Layzell et al., 1990). The recuperation of xylem sap glutamine and ureides after aerenchyma formation, together with the timing of these events, suggests some recovery of N2 fixation, consistent with the restoration of a more adequate oxygen supply to the nodules as a result of the observed morphological adaptations (i.e. formation of adventitious

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**Table 1.** \( \text{O}_2 \) levels in the nutrient solution, porosity of nodules and taproot, xylem sap alanine, glutamine, ureides and total amino acids in soybean plants, during 10 d of waterlogging with N-free nutrient solution at the flowering stage

<table>
<thead>
<tr>
<th>Days</th>
<th>( \text{O}_2 ) levels (mg L(^{-1}))</th>
<th>Porosity (gas/tissue %v/v)</th>
<th>Xylem sap (mol%)</th>
<th>Xylem sap (μmol mL(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nodules</td>
<td>Root*</td>
<td>Alanine</td>
<td>Glutamine</td>
</tr>
<tr>
<td>Flooded</td>
<td>0</td>
<td>6.6</td>
<td>0.4(^{b,1})</td>
<td>0.3(^{c})</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.9</td>
<td>1.9(^{a})</td>
<td>1.6(^{c})</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.7</td>
<td>1.9(^{a})</td>
<td>2.5(^{c})</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.6</td>
<td>1.9(^{a})</td>
<td>2.6(^{c})</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.8</td>
<td>1.7(^{a})</td>
<td>4.7(^{c})</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.6</td>
<td>1.8(^{a})</td>
<td>13.4(^{b})</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.6</td>
<td>1.8(^{a})</td>
<td>17.3(^{b})</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.6</td>
<td>2.1(^{a})</td>
<td>22.0(^{a})</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.7</td>
<td>1.7(^{a})</td>
<td>21.7(^{a})</td>
</tr>
<tr>
<td>Non-flooded</td>
<td>5</td>
<td>6.7</td>
<td>0.5(^{b})</td>
<td>0.3(^{c})</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>6.7</td>
<td>0.5(^{b})</td>
<td>0.4(^{c})</td>
</tr>
</tbody>
</table>

* Upper 8 cm portion of the taproot.
1 Means followed by different letters, within columns, are statistically different by the multiple range test of Duncan \((P \leq 0.05)\).
roots and aerenchyma) in the roots to flooding. The data of Bacanamwo and Purcell (1999a) also suggest that such morphological adaptations of soybean plants to flooding lead to recovery of N2 fixation, since the rate of biomass and N accumulation returned to control levels between days 14 and 21 of flooding. Shimamura et al. (2002) reached a similar conclusion when a sharp decline in xylem ureide levels and nitrogenase activity was observed in flooded soybean plants following blockage of the hypocotyl aerenchyma with vaseline.

The changes in xylem sap alanine are also consistent with the ability of aerenchyma to supply the roots with an adequate supply of oxygen. The high levels recorded during the first few days of waterlogging would result from the prevailing hypoxic conditions, while the subsequent fall after aerenchyma formation surely is indicative of the greater availability of oxygen to the root system. The fact that alanine did not return fully to pre-hypoxic levels might be explained by the fact that aerenchyma does not penetrate to deeper-lying regions of the root which would presumably continue with anaerobic metabolism.

In conclusion, the present data are consistent with the view that a more adequate supply of oxygen to the root system associated with aerenchyma formation leads to at least partial recovery of N metabolism of the nodulated root system of soybean during a prolonged period of flooding. Furthermore, the measurement of exported N compounds in the xylem sap as a means of assessing N2 fixation activity and anaerobic metabolism of the roots, despite being indirect and therefore more qualitative than quantitative, appears to have the merit of enabling estimates of in situ activity without disturbing the waterlogged root system.

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LITERATURE CITED


