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# Stem hypertrophic lenticels and secondary aerenchyma enable oxygen transport to roots of soybean in flooded soil

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• *Background and Aims* Aerenchyma provides a low-resistance $O_2$ transport pathway that enhances plant survival during soil flooding. When in flooded soil, soybean produces aerenchyma and hypertrophic stem lenticels. The aims of this study were to investigate $O_2$ dynamics in stem aerenchyma and evaluate $O_2$ supply via stem lenticels to the roots of soybean during soil flooding.
• *Methods* Oxygen dynamics in aerenchymatous stems were investigated using Clark-type $O_2$ microelectrodes, and $O_2$ transport to roots was evaluated using stable-isotope $^{18}O_2$ as a tracer, for plants with shoots in air and roots in flooded sand or soil. Short-term experiments also assessed venting of $CO_2$ via the stem lenticels.
• *Key Results* The radial distribution of the $O_2$ partial pressure ($pO_2$) was stable at 17 kPa in the stem aerenchyma 15 mm below the water level, but rapidly declined to 8 kPa at 200–300 μm inside the stele. Complete submergence of the hypertrophic lenticels at the stem base, with the remainder of the shoot still in air, resulted in gradual declines in $pO_2$ in stem aerenchyma from 17·5 to 7·6 kPa at 13 mm below the water level, and from 14·7 to 6·1 kPa at 51 mm below the water level. Subsequently, re-exposure of the lenticels to air caused $pO_2$ to increase again to 14–17 kPa at both positions within 10 min. After introducing $^{18}O_2$ gas via the stem lenticels, significant $^{18}O_2$ enrichment in water extracted from roots after 3 h was confirmed, suggesting that transported $O_2$ sustained root respiration. In contrast, slight $^{18}O_2$ enrichment was detected 3 h after treatment of stems that lacked aerenchyma and lenticels. Moreover, aerenchyma accelerated venting of $CO_2$ from submerged tissues to the atmosphere.
• *Conclusions* Hypertrophic lenticels on the stem of soybean, just above the water surface, are entry points for $O_2$, and these connect to aerenchyma and enable $O_2$ transport into roots in flooded soil. Stems that develop aerenchyma thus serve as a 'snorkel' that enables $O_2$ movement from air to the submerged roots.

**Key words:** Aerenchyma, oxygen transport, soybean (*Glycine max*), flooding, root aeration, hypertrophic lenticels, soil waterlogging.

## INTRODUCTION

In flooded soils, the root environment supplies insufficient $O_2$ because the diffusion of gases in water is approx. 10 000 times slower than in air (Armstrong, 1979) and diffusion of atmospheric $O_2$ to the roots is largely prevented. Many crop plants exhibit poor root growth, injury, and death when exposed to saturated soil. On the other hand, wetland plants are well-adapted and can survive prolonged flooding. Enlarged intercellular gas-filled spaces, called 'aerenchyma', are present throughout most of the body of most wetland plants. Therefore, aerenchyma provides a low-resistance pathway for transport of $O_2$ from the shoot to the roots, and enables plant roots to maintain respiration. For example, some *Rumex* species that inhabit frequently flooded environments produce high volumes of aerenchyma in their root cortex and have high flood tolerance, whereas species that seldom inhabit flooded environments develop less aerenchyma and have low flood tolerance (Laan *et al.*, 1989). These anatomical differences have been observed in many plants (Smirnoff and Crawford, 1983; Justin and Armstrong, 1987), suggesting that aerenchyma is an important anatomical characteristic that helps plants to survive soil flooding.

Many field crops, including soybean (*Glycine max*), are very sensitive to flooding stress. Soybean suffers from such stress during its vegetative stages, leading to decreased yield (Sojka, 1985; Griffin and Saxton, 1988; Scott *et al.*, 1989; Linkemer *et al.*, 1998). According to Shimamura *et al.* (2003), however, stems, roots and root nodules of soybean develop aerenchyma within a few weeks under flooded conditions. This aerenchyma arises from successive cell division by the phellogen, and is composed of white and porous (spongy) tissues that are referred to as 'secondary aerenchyma' (Arber, 1920; Fraser, 1931; Williams and Barber, 1961; Jackson and Armstrong, 1999). This type of aerenchyma is the tissue of secondary origin, and morphologically and anatomically different from cortical (primary) aerenchyma (i.e. lysigenous and schizogenous aerenchyma), which can be distinguished by their process of formation. Lysigenous aerenchyma is created through cell disintegration (death), and schizogenous aerenchyma is created by cell separation (Evans, 2003; Visser and Voesenek, 2004). Both aerenchymas

occur in the primary cortex of roots and shoots, whereas secondary aerenchyma occurs not in the primary cortex but in the phellogen region and is homologous with cork tissue. In addition, hypertrophy of secondary aerenchyma enhances formation of large cracks (i.e. hypertrophic lenticels) on the surface of stems and roots, and the aerenchyma is exposed to the atmosphere through the lenticels (e.g. for *Sesbania javanica* see fig. 1 in Jackson *et al.*, 2009), which may facilitate $O_2$ entry into the aerenchyma. Flood-tolerant leguminous plants, such as *Sesbania aculeata* (Scott and Wager 1888), *Sesbania rostrata* (Saraswati *et al.*, 1992; Shiba and Daimon, 2003), *Neptunia oleracea* (Metcalfe, 1931), and *Viminaria juncea* (Walker *et al.*, 1983), produce secondary aerenchyma in their stems, roots and root nodules. Thomas *et al.* (2005) reported that when soybean plants are flooded, $N_2$ fixation decreases quickly in their root nodules but recovers when aerenchyma formation begins. Similarly, when both petroleum jelly and film are applied to the soybean hypocotyl surface to prevent the entry of atmospheric $O_2$ into the secondary aerenchyma through hypertrophic lenticels under flooding, root growth is sharply inhibited compared with shoot growth (Shimamura *et al.*, 2003). At the same time, the activity of root nodules is significantly restricted (Shimamura *et al.*, 2002). These reports indicate that formation of hypertrophic lenticels at the stem base and development of aerenchyma are important acclimations enabling $O_2$ supply into roots and root nodules of soybean in flooded soil.

Internal $O_2$ movement from shoot to roots via the aerenchyma has been demonstrated in various ways. Drew *et al.* (1985) found a higher adenylate energy charge [defined as $(ATP + \frac{1}{2}ADP)/(ATP + ADP + AMP)$] in adventitious roots of *Zea mays* under hypoxic conditions that developed aerenchyma, compared with roots in which formation of aerenchyma was blocked by the application of silver ions to inhibit ethylene action. These results indicate that aerenchyma supplies $O_2$ to hypoxic roots in *Z. mays*. One drawback of the approach using adenylate energy charge assessments is that it requires destructive samplings, and thus cannot measure changes over time within a single plant. As an alternative, the $O_2$ concentration in aerenchyma can be directly measured in intact plants using a Clark-type $O_2$ microelectrode (Armstrong *et al.*, 2000). In completely submerged plants, photosynthetic $O_2$ is transported from shoots to roots in *Halosarcia pergranulata* (Pederson *et al.*, 2006) and rice (*Oryza sativa*); Colmer and Pedersen, 2008), because $O_2$ in aerenchymatous roots decreases sharply when photosynthetic tissues are placed in darkness. Use of $O_2$ isotopes is another technique that can be used to study plant aeration. For example, Dacey and Klug (1982) showed exposure of leaves of the yellow waterlily (*Nuphar luteum*) to the stable-isotope $^{18}O_2$ (used as a tracer) confirmed movement of $^{18}O_2$ via aerenchyma to the rhizomes. In the present paper, the $O_2$ dynamics of aerenchymatous soybean stems were investigated using Clark-type $O_2$ microelectrodes after preventing the entry of atmospheric $O_2$ into secondary aerenchyma through hypertrophic stem lenticels. Also stable-isotope $^{18}O_2$ was used as a tracer to confirm $O_2$ transport from stem to the roots via aerenchyma and demonstrate that this $O_2$ was used to sustain root aerobic respiration.

## MATERIALS AND METHODS

### *Experiment 1: $O_2$ dynamics in aerenchymatous stems*

*Plant material and experimental set-up.* Seeds of soybean (*Glycine max* 'Asoaogari') were sown in 200 mL of silica sand (passed through an 18–26 mesh) without chemical fertilizer in 400-mL plastic pots (one seed per pot). The plants were grown in a growth cabinet under artificial light (25 °C, 14 h light and 10 h dark, 590 μmol $m^{-2}$ $s^{-1}$ PAR). Ten days after sowing, the pots were placed inside tanks 11 cm in diameter × 14 cm in height (one pot per tank) and the plants were grown under continuously flooded conditions, with the water level maintained at 6–7 cm above the sand surface. Approximately 14 d after flooding, plants that developed secondary aerenchyma from the bottom of the stems to just above the water level were used for the experiment (Table 1).

The plant was placed in a tank in the same growth cabinet, and the water level was subsequently maintained at 7 mm below the top of the aerenchymatous stem so that hypertrophic lenticels were exposed to the atmosphere. A Clark-type $O_2$ microelectrode with a guard cathode and a tip diameter of 25 μm (OX-25; Unisense A/S, Aarhus, Denmark) was used. The microelectrode was connected to a pA meter (PA2000; Unisense A/S) and the output was logged at 1 s intervals on a computer using an analogue-to-digital converter (ADC-16; Pico Technology, St Neots, Cambridgeshire, UK). The electrode was calibrated immediately before measurement in air and in $O_2$-free $N_2$. The $O_2$ concentration of the water was 228 μmol $L^{-1}$ on average at 3 cm below the water surface.

*Radial $O_2$ profile across an aerenchymatous stem of intact soybean plants.* The microelectrode tip was positioned at the surface of the aerenchymatous stem 15 mm below the water level using a micromanipulator (MM5; Marzhauser, Wetzlar, Germany). The microelectrode was set to move toward the stele in 100-μm steps and set at a frequency of a step every 10 s. After obtaining measurements, a transverse section of the fresh stem was cut on a plant microtome (MTH-1; Nippon Medical & Chemical Instruments, Osaka, Japan), and photographed using a microscope.

*$O_2$ movement in the stem aerenchyma.* The microelectrodes were inserted into aerenchymatous stem tissue at 13 and 51 mm below the water level using the micromanipulator, and the tips were positioned within the aerenchyma approx. 1 mm from the stem surface. After confirming the presence of stable signals from both sensors, the hypertrophic stem lenticels were completely submerged by raising the water level to 3 mm above the uppermost lenticels. After 2 h, the water level was reduced to the level at the start of the experiment to re-expose the hypertrophic lenticels to air.

### *Experiment 2: $O_2$ transport from stem lenticels to roots via the aerenchyma*

*Plant material and growth conditions.* Three seeds of 'Asoaogari' were sown in low-humic andosols containing 4 g of inorganic fertilizer (3 % N, 10 % $P_2O_5$, 10 % $K_2O$), 1·4 g of dolomite and 3 g of fused magnesium phosphate in a plastic pot (one of two sizes: either 11·5 or 14 cm in height and 11 cm in diameter). The plants were grown in a

TABLE 1. *Plant growth and development of secondary aerenchyma and lenticels after approx. 14 d flooding: the area of stele (vascular cylinder), secondary aerenchyma and stem in cross-sections of stems at the water surface*

| | |
|---|---|
| Shoot dry weight (g) | 0·29 ± 0·03 |
| Root dry weight (g) | 0·15 ± 0·01 |
| Stele area ($mm^2$) | 2·91 ± 0·15 |
| Secondary aerenchyma area ($mm^2$) | 8·37 ± 1·45 |
| Stem area ($mm^2$) | 12·7 ± 1·48 |
| Percentage of aerenchyma area in stems (%) | 63·8 ± 4·74 |
| Lenticel height at stems above water surface (mm) | 6·60 ± 0·51 |

Aerenchyma area indicates the area not of gas spaces but of aerenchymatous tissues in cross-sections. Percentage of aerenchyma area indicates not porosity but the percentage of stem area occupied by aerenchymatous tissue area in cross-sections.

Digital images of stem cross-sections were photographed using a microscope and analysed with ImageJ 1·38x software (National Institutes of Health, USA) to measure each tissue area.

Values are the mean (± s.e.) of five plants.

growth cabinet under artificial light (25 °C, 14 h light and 10 h dark, approx. 800 μmol $m^{-2}$ $s^{-1}$ PAR). After germination, seedlings were thinned to one plant per pot. When the primary leaves had fully expanded, some pots were placed inside 3·7-L tanks (one pot per tank) and the plants were grown under continuously flooded soil conditions, with the water level maintained 3·5 cm above the soil surface. The remaining plants were used as non-flooded (i.e. drained) controls. Approximately 5 weeks after flooding, plants that had developed secondary aerenchyma in the stems, roots and root nodules were used for the experiment, and hypertrophic lenticels and aerenchyma in the stems were formed just above the water level (Fig. 1 and Table 2). The $O_2$ concentration of the flood water was 175 μmol $L^{-1}$ on average at 3 cm below the water surface just before $^{18}O_2$ treatment.

FIG. 1. Development of secondary aerenchyma and hypertrophic lenticels in soybean plants after approx. 5 weeks of flooding. The broken line on the stem indicates the water level at 3·5 cm above the soil surface. Secondary aerenchyma (white tissue) was developed in the stem and the adventitious roots, and hypertrophic lenticels were observed just above the water surface. Therefore, when water level was raised from 3·5 cm to 6 cm, the hypertrophic lenticels were completely submerged. Scale bar = 20 mm.

TABLE 2. *Plant growth and development of secondary aerenchyma after approx. 5 weeks flooding: the area of stele, secondary aerenchyma and stem in cross-sections of stems at 3·5 cm above the soil surface (control plants) or at the water surface (flooded plants)*

| | Control | Flooded |
|---|---|---|
| Shoot dry weight (g) | 9·10 ± 0·38 | 5·58 ± 0·72 |
| Root dry weight (g) | 3·93 ± 0·20 | 3·73 ± 0·37 |
| Stele area ($mm^2$) | No data | 12·6 ± 1·71 |
| Secondary aerenchyma area ($mm^2$) | 0* | 43·2 ± 8·73 |
| Stem area ($mm^2$) | No data | 62·0 ± 11·2 |
| Percentage of aerenchyma area in stems (%) | 0* | 68·8 ± 2·38 |

Aerenchyma area indicates the area not of gas spaces but of aerenchymatous tissues in cross-sections. Percentage of aerenchyma area indicates not porosity but the percentage of stem area occupied by aerenchymatous tissue area in cross-sections. The development of secondary aerenchyma was not observed in control plants, so the percentage of aerenchyma area in the stems was zero (*).

Digital images of stem cross-sections were photographed using a microscope and analysed with ImageJ 1·38x software (National Institutes of Health, USA) to measure each tissue area.

Values are the mean (± s.e.) of six plants.

*Tracing $^{18}O_2$ transport via the aerenchyma.* In aerobic respiration with $^{18}O$ in plants, carbohydrates are metabolized as follows:

$$C_6H_{12}O_6 + 6{}^{18}O_2 + 6H_2O \rightarrow 6CO_2 + 12H_2{}^{18}O$$

Thus, to measure the uptake of $^{18}O_2$ by root metabolism, the $^{18}O$ content of the water produced by root respiration was measured (Yoshida and Eguchi, 1994).

The control pots (14 cm in height) were placed inside 3·7-L tanks (one pot per tank), and were embedded in the soil. The surface was covered with paraffin to prevent $O_2$ diffusion into soils. After covering the tank with an acrylic board (with the space where the plant stem passed through the board sealed with putty), the gas space (a gas layer about 7 mm thick with a volume of about 150 mL) was filled between the paraffin layer and the acrylic board with approx. 100 % $O_2$ gas (95 atom % excess $^{18}O$) to supply $^{18}O_2$ to the basal region of the stem that lacked both aerenchyma and lenticels (Fig. 2). Similarly, flooded plants were treated with $^{18}O_2$ gas. In the flooded taller pots (14 cm in height), the water level was maintained at 3·5 cm above the soil surface, so aerenchyma and lenticels just above the water surface could be exposed to $^{18}O_2$ gas layer (Flood Ae-Open conditions). In contrast, in the flooded pots of small size (diameter = 11 cm; height = 11·5 cm), water level was raised from 3·5 cm to 6 cm above the soil surface to cover both aerenchyma and lenticels on the stem, which was 2·5 cm higher than the level of pre-flooded conditions. Therefore, aerenchyma and lenticels of stem base were completely submerged. The basal region of the stem, but above the region that contained aerenchyma and lenticels, was exposed to $^{18}O_2$ gas layer (Flood Ae-Submerged conditions).

Plants were harvested before exposure to the $^{18}O_2$ gas, and at 0·5, 1·0 and 3·0 h after exposure to the $^{18}O_2$. They were then separated into shoots and roots (including nodules). The fresh weights (FW) of the samples were measured, and they were then placed in a cryogenic vacuum distillation apparatus for several hours to extract water from the tissues. The dry weights

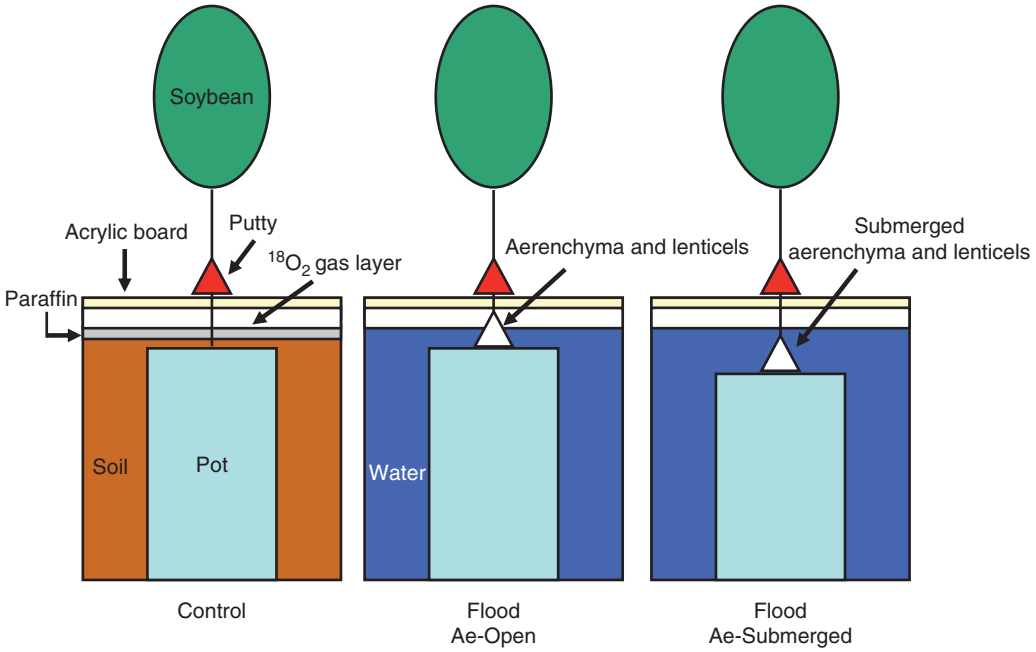


FIG. 2. The experimental system used to supply $^{18}O_2$ gas to the base of the stems of soybean plants. Control: $^{18}O_2$ was supplied to the basal region of the stem of irrigated control plants that lacked both aerenchyma and hypertrophic lenticels. Flood Ae-Open (Ae, aerenchyma): $^{18}O_2$ was supplied to the basal region of the stem aerenchyma and hypertrophic lenticels of flooded plants. Water level was maintained at 3·5 cm that was the same level as in pre-flooded conditions, so aerenchyma and lenticels just above the water surface could be exposed to $^{18}O_2$ gas layer. Flood Ae-Submerged: $^{18}O_2$ was supplied to the basal region of the stem, but above the region that contained aerenchyma and lenticels in flooded plants. Aerenchyma and hypertrophic lenticels were well-developed in the basal stem, but the water level was raised to cover them. Water level was maintained at 6 cm that was 2·5 cm higher than the level of pre-flooded conditions, so aerenchyma and lenticels of the stem base were completely submerged.

(DW) of the shoots and roots were then measured after oven-drying at 80 °C for 48 h. The water samples extracted from the shoots and roots were analysed to determine their $O_2$ isotope ratios using an isotope-ratio mass spectrometer (Delta plus LX; Thermo Fisher Scientific, Waltham, MA, USA) with an automated $CO_2$–$O_2$ equilibration unit. All $O_2$ isotope ratios were expressed as the $\delta^{18}O$ values (‰) relative to the Vienna-standard mean ocean water (V-SMOW) values:

$$\delta_{\text{sample}} = \left[\left(R_{\text{sample}} - R_{\text{standard}}\right)/R_{\text{standard}}\right] \times 1000$$

where $R$ = the $^{18}O/^{16}O$ ratio for the sample and the standard (respectively).

The weight of plant water (=FW−DW) and $O_2$ isotope ratios of the extracted water were used to estimate the volume of $^{18}O_2$ gas transported to each tissue over the 3-h period:

$$^{18}O_2 \text{ gas volume transported} = (O_{2\text{total}} \times {}^{18}\text{O atom \% excess})/100,$$

where $O_{2\text{total}}$ is the total amount of $O_2$ (mol) in the plant water after 3 h of exposure, and $^{18}O$ atom % excess is the increase in the $^{18}O$ atom % after 3 h in the extracted water.

### *Experiment 3: Gas exchange through the aerenchyma*

The plant materials and experimental conditions were the same as in expt 2 (Fig. 2), and non-planted conditions were used as blank controls. However, the gas layer in each pot was supplied with air or approx. 100 % $O_2$ gas. The concentrations of $O_2$ and $CO_2$ in the gas layer were then measured in each treatment. Gas samples (0·2 mL) were extracted from the initial gas layer and the gas layer 3 h after exposure and were analysed using a gas chromatograph (GC-8APT; Shimadzu, Kyoto, Japan) with 3 mm (i.d.) × 2 m column of 60/80 mesh Molecular Sieve 5A for $O_2$ and 3 mm (i.d.) × 2 m column of 50/80 mesh Porapak N for $CO_2$, a column temperature of 80 °C, and helium as the carrier gas.

## RESULTS

### *Experiment 1: $O_2$ dynamics in aerenchymatous stems*

*Radial distribution of $O_2$ in aerenchymatous stems.* The radial distribution of $O_2$ in an aerenchymatous stem at 15 mm below the water level is shown in Fig. 3. At the stem surface, $pO_2$ was 18 kPa. As the microelectrode began moving into the tissues at 100 μm per step, the internal $pO_2$ in the aerenchymatous layers (approx. 2500 μm in thickness) between the stem surface and the stele remained relatively stable at about 17 kPa, but $pO_2$ gradually decreased to between 15 and 16 kPa at the boundary of the stele. As the electrode tip entered the stele, $pO_2$ decreased rapidly, reaching 8 kPa at a distance of 200–300 μm inside the stele.

*$O_2$ movement in the stem aerenchyma.* Two microelectrode tips were positioned within the aerenchyma approx. 1 mm from the stem surface, and internal $pO_2$ values of 17·5 kPa measured at a depth of 13 mm below the water level and 14·7 kPa at 51 mm. The $pO_2$ values had remained stable for >10 min

before the start of the experiment (Fig. 4). After raising the water level enough to submerge the hypertrophic stem lenticels completely, $pO_2$ gradually declined for the first 60 min, then slowly decreased for the next 60 min, and finally reached 7·6 kPa (13 mm) and 6·1 kPa (51 mm), representing decreases to about 40 % of the values at the start of experiment. When the water level was reduced to the same level as at the start of experiment, thereby re-exposing the hypertrophic stem lenticels to the air, internal $pO_2$ values increased dramatically and reached approximately the same levels as at start of the experiment after only 10 min. The $pO_2$ profiles remained stable thereafter.

### *Experiment 2: $O_2$ supply from the stem to the roots via the aerenchyma*

After the non-aerenchymatous stems of the control plants were supplied with $^{18}O_2$ for 3 h, the volume of $^{18}O_2$ transported was about 1 μmol g $DW^{-1}$ $h^{-1}$ in both the shoots and the roots of the control plants (Fig. 5). Similarly, in plants continuously flooded for about 5 weeks, after exposure of the basal stem without aerenchyma or lenticels to $^{18}O_2$ for 3 h (Flood Ae-Submerged conditions), the volume of $^{18}O_2$ transported in the roots and shoots of plants in this treatment was not significantly different from the value of about 1 μmol g $DW^{-1}$ $h^{-1}$ in the control plants (Fig. 5). In contrast, when the basal region of the flooded stem with the lenticels was exposed to $^{18}O_2$ for 3 h (Flood Ae-Open conditions), the volume of $^{18}O_2$ transported (5·0 μmol g $DW^{-1}$ $h^{-1}$ in shoots and 7·2 μmol g $DW^{-1}$ $h^{-1}$ in roots) was significantly higher than in the control and the Flood Ae-Submerged plants (Fig. 5). Moreover, the $\delta^{18}O$ values in the roots from 0 to 3 h increased steadily over time, with significant ($P < 0.05$) $^{18}O_2$ enrichment detected after only 0·5 h of exposure to the $^{18}O_2$ (Fig. 6).

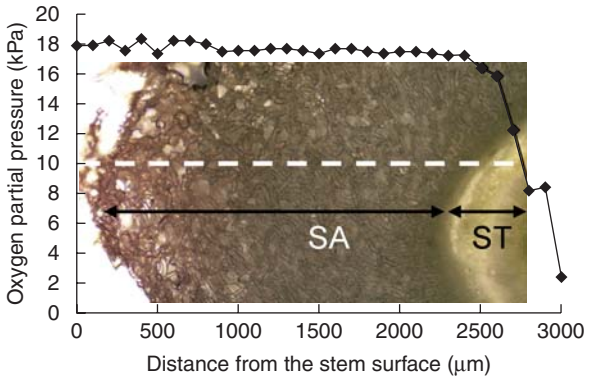


FIG. 3. Radial $O_2$ profile across an aerenchymatous stem of an intact soybean plant at a position 15 mm below the water level. The white broken line represents the path of the $O_2$ microelectrode inserted from the stem surface through the secondary aerenchyma and into the stele. The experiment was repeated twice (not shown). SA, Secondary aerenchyma; ST, stele (vascular cylinder).

### *Experiment 3: Gas exchange through the aerenchyma*

Table 3 shows the rates of decrease in $O_2$ and increase in $CO_2$ in the gas layer in each treatment. The rate of decrease in $O_2$ (56·6 μmol g $DW_{Root}^{-1}$ $h^{-1}$ in air and 191 μmol g $DW_{Root}^{-1}$ $h^{-1}$ in $O_2$ treatments) in the Flood Ae-Open plants was significantly higher than in the control and the Flood Ae-Submerged plants. Similarly, the rate of increase in $CO_2$ (22·2 μmol g $DW_{Root}^{-1}$ $h^{-1}$ in air and 33·4 μmol g $DW_{Root}^{-1}$ $h^{-1}$ in $O_2$ treatments) in the Flood Ae-Open plants was significantly higher than in the other conditions. Aerenchyma and lenticels at the base of the flooded stem enhanced exchange of these gases between flooded tissues and gas layer.

## DISCUSSION

### *Radial $O_2$ profile in stem aerenchyma*

In the distribution of $O_2$ within aerenchymatous tissues, it has been reported that radial $O_2$ profile was studied for cortical aerenchyma in several plants (Armstrong *et al.*, 2000; Darwent *et al.*, 2003; De Simone *et al.*, 2003), but there is no information about the profile of secondary aerenchyma. The $O_2$ profile of soybean stems in the present study remained stable

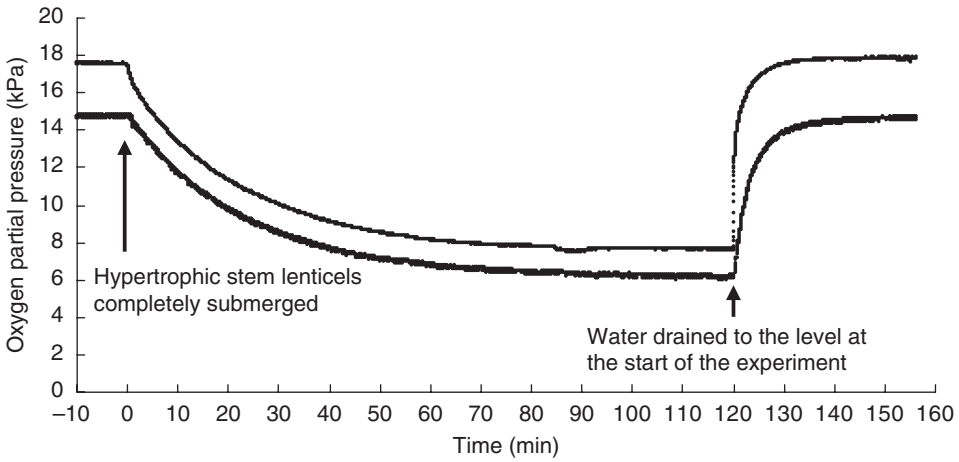


FIG. 4. The effect of complete submergence of hypertrophic stem lenticels on $pO_2$ values in aerenchyma in the lower stem of soybean. The upper and lower lines represent the $pO_2$ values in the stem aerenchyma at 13 and 51 mm below the water level, respectively. The experiment was repeated twice (not shown)

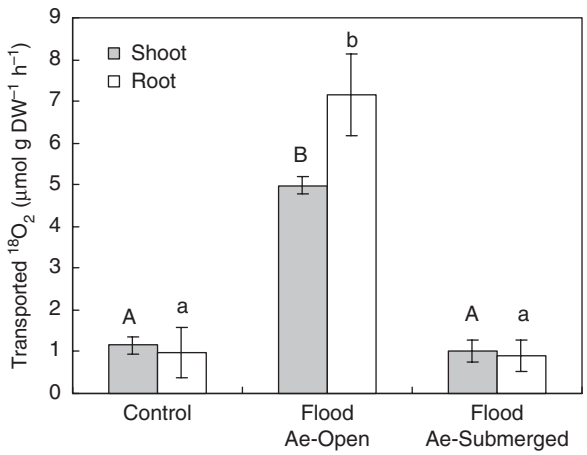


FIG. 5. Volume of $^{18}O_2$ transported from the stem to the roots with or without exposure of the aerenchyma to the $^{18}O_2$ gas in each treatment. See Fig. 2 for an illustration of the treatment conditions. Flood Ae-Open, aerenchyma and lenticels above the water; Flood Ae-Submerged, submerged aerenchyma and lenticels. Values are the mean (±s.e.) of four or five plants. Means in the shoot followed by the same upper-case letter and means in the root followed by the same lower-case letter do not differ significantly ($P < 0{\cdot}01$, Tukey–Kramer's test).

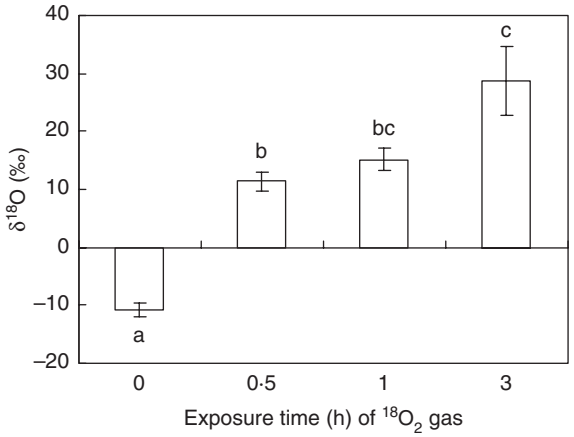


FIG. 6. $\delta^{18}O$ enrichment (‰) of water extracted from flooded soybean root tissues during 3 h of exposure of aerenchyma in the basal stem to $^{18}O_2$ gas. Values are the mean (±s.e.) of three plants. Bars labelled with the same letter do not differ significantly ($P < 0{\cdot}05$, Tukey's test).

at $pO_2$ values between 15 and 17 kPa in the aerenchymatous layers (secondary aerenchyma), but decreased rapidly to about 2 kPa in the stele (Fig. 3). This profile was similar to that of root with cortical aerenchyma; e.g. Armstrong *et al.* (2000) demonstrated that $pO_2$ remained stable at approx. 15 kPa in the aerenchymatous cortex of *Phragmites australis* at 100 mm from the root apex, but decreased to 12 kPa in the stele at that position. Similarly, the $O_2$ levels were higher in cortical lysigenous aerenchyma than in the stele at different positions along a *Z. mays* root (Darwent *et al.*, 2003). Therefore, the $O_2$ levels were stable and high from the outer to the inner aerenchyma (both cortical and secondary); in contrast, $O_2$ levels decreased at all positions as the microelectrode penetrated into the stele.

TABLE 3. *Rates of decrease in $O_2$ and increase in $CO_2$ in the gas space above the water in each treatment*

| Gas treatment in gas space | Plant conditions | Decreased $O_2$ (μmol g$^{-1}$ $DW_{Root}$ h$^{-1}$) | Increased $CO_2$ (μmol g$^{-1}$ $DW_{Root}$ h$^{-1}$) |
|---|---|---|---|
| Air | Control | $1{\cdot}72 \pm 0{\cdot}56^{a}$ | $0{\cdot}98 \pm 0{\cdot}10^{a}$ |
| | Flood Ae-Open | $56{\cdot}6 \pm 3{\cdot}49^{b}$ | $22{\cdot}2 \pm 0{\cdot}27^{c}$ |
| | Flood Ae-Submerged | $12{\cdot}2 \pm 0{\cdot}98^{a}$ | $7{\cdot}49 \pm 0{\cdot}82^{b}$ |
| Oxygen | Control | $15{\cdot}8 \pm 6{\cdot}84^{a}$ | $0{\cdot}82 \pm 0{\cdot}22^{a}$ |
| | Flood Ae-Open | $191 \pm 18{\cdot}3^{b}$ | $33{\cdot}4 \pm 1{\cdot}68^{c}$ |
| | Flood Ae-Submerged | $35{\cdot}2 \pm 13{\cdot}4^{a}$ | $8{\cdot}12 \pm 0{\cdot}34^{b}$ |

See Fig. 2 for an illustration of the treatment conditions.
Flood Ae-Open, Aerenchyma and lenticels above the water; Flood Ae-Submerged, submerged aerenchyma and lenticels.
Values are the mean (±s.e.) of three plants.
Means followed by the same letter within each column for the treatments do not differ significantly ($P < 0{\cdot}01$, Tukey's test).

### *$O_2$ transport through lenticels and aerenchyma*

The swelling of the stem and the hypertrophic lenticels in submerged portions of the lower stem appear to be the first entry points for $O_2$. These structures facilitate $O_2$ entry into the aerenchyma of nearby adventitious roots in various herbaceous dicots and woody species (Jackson and Ricard, 2003). Stevens *et al.* (2002) reported that artificial disruption of the continuity of the aerenchymatous tissues that develop in submerged portions of the lower shoot in *Lythrum salicaria* caused a significant reduction in root $O_2$ levels, indicating inhibition of $O_2$ transport from the atmosphere into the roots. *Salix viminalis* (Jackson and Attwood, 1996) and some woody plants (Armstrong, 1968) show inhibition of $O_2$ transport to the roots and reduced plant growth when hypertrophic lenticels at the base of the stem are blocked.

In soybean, hypertrophic stem lenticels also appear to be the first entry points of $O_2$ into the aerenchyma because experimental blocking of these lenticels inhibits plant growth and root nodule activity (Shimamura *et al.*, 2002, 2003). This was confirmed by the present experiments, in which $pO_2$ gradually decreased for 120 min within aerenchyma in the lower stem when the hypertrophic stem lenticels were completely submerged (Fig. 4). The decrease in $pO_2$ in the aerenchyma would result from both $O_2$ diffusion into the surrounding water such as radial $O_2$ loss and from $O_2$ consumption by living cells of the stem, roots and aerenchyma. A similar reaction was reported in *Alnus glutinosa*: the $O_2$ concentration in the rhizosphere decreased from 100 μmol $L^{-1}$ to almost 0 μmol $L^{-1}$ within a few minutes when $N_2$ gas was applied to the base of stems with well-developed lenticels under flooded conditions (Dittert *et al.*, 2006). In the present study, internal $pO_2$ in the aerenchyma of the submerged stems increased steeply when lenticels in the upper stem were exposed to the air for a few minutes (Fig. 4), and there was little difference in the response time for the increased influx of atmospheric $O_2$ between the two sensor positions, even though the distance between the sensors was 38 mm. These results indicate high gas diffusibility in the aerenchyma and that aerenchyma development in the stem provides a 'snorkel' that supplies flooded tissues with $O_2$.

The $O_2$ isotope experiments also indicated that the aerenchyma provides $O_2$ transport into flooded roots. When the basal region of a stem that contained lenticels and aerenchyma was exposed to $^{18}O_2$, significant enrichment of $\delta^{18}O$ in water extracted from the root system was confirmed within 0·5 h (Fig. 6), and 7·2 μmol g $DW^{-1}$ $h^{-1}$ of $^{18}O_2$ was transported into the roots (Fig. 5). In addition, the volume of $^{18}O_2$ transported to the shoots was 5·0 μmol g $DW^{-1}$ $h^{-1}$ under the same conditions. On the other hand, slight enrichment of $^{18}O_2$ in the roots and the shoot was observed after exposure of non-aerenchymatous stem tissue to $^{18}O_2$ under control and flooded conditions (Fig. 5), indicating that the non-aerenchymatous stem has a low ability to transport $O_2$ to the roots, and that little water was produced by respiration using $^{18}O_2$ in the stem. So, it seems that the volume of $^{18}O_2$ in the shoot under flooded Ae-Open conditions, which was 5 times the value observed under the other two experimental conditions (Fig. 5), was due to greater production of water by root respiration and its movement into the shoots via the xylem. Dittert *et al.* (2006) reported that the $O_2$ transport rate into the root system of 2-year-old *A. glutinosa* averaged 0·12 mmol $h^{-1}$ $plant^{-1}$ in a flooded stem with well-developed lenticels, versus 0·01 mmol $h^{-1}$ $plant^{-1}$ in a non-flooded stem with poorly developed lenticels. By the way, the rate of decrease in $O_2$ in the gas layer was somewhat higher in the Flood Ae-Submerged plants than in the control plants (Table 3), which might mean the difference in $O_2$ diffusivity between paraffin and water, i.e. $O_2$ was easily dissolved in water. However, these results did not agreed with the $^{18}O_2$ experiment (Fig. 5) which did not show the difference in the volume of $^{18}O_2$ in roots between the Flood Ae-Submerged plants and the control plants. So an effect of $O_2$ diffusion from the gas layer to water in order to supply flooded tissues was not confirmed in the present studies.

In the present experiments, $^{18}O_2$ gas transport was calculated equal to 7·2 μmol g $DW^{-1}$ $h^{-1}$ based on the enrichment of $\delta^{18}O$ in the water of root tissues after 3 h of supplying $^{18}O_2$ gas to the lenticels in the basal region of the stem (Fig. 5). However, this volume might have been underestimated because the water produced by root respiration is available for cellular metabolism and for leaf transpiration. In addition, the rate of decrease in $O_2$ in the gas layer (Fig. 2) was 191 μmol g $DW_{Root}^{-1}$ $h^{-1}$ under the same conditions (Table 3).

The diffusion of $CO_2$, produced in the roots by respiration and in the soil by micro-organisms under flooding, to the atmosphere is very slow, and a high $CO_2$ level in the root zone inhibits growth of soybean under flooding and anoxia (Boru *et al.*, 2003; Araki, 2006). However, aerenchyma and lenticels at the base of the flooded stem accelerated venting of $CO_2$ (Table 3), so secondary aerenchyma may reduce the likelihood of $CO_2$ accumulating in the flooded soils and root tissues.

### *Conclusions*

The results showed that hypertrophic lenticels in the lower stem of soybean, just above the water surface, facilitate $O_2$ entry into the aerenchyma, after which the aerenchyma enhance $O_2$ transport into roots in the flooded soil. Although soybean plants can produce aerenchyma in their root systems, they are nonetheless very sensitive to flooding stress (Table 2). The development of an aerenchymatous network in stems, adventitious roots and root nodules requires a few weeks after flooding begins, so the diffusion of atmospheric $O_2$ into flooded tissues is limited to $O_2$ that penetrates the non-aerenchymatous stem and $O_2$ absorbed at the water surface. Thus, soybean plants suffer from flooding stress until they develop a sufficient aerenchymatous network. In contrast, many wetland plants possess aerenchyma in their roots even under aerobic conditions (Justin and Armstrong, 1987), and are thus able to adapt rapidly to sudden flooding. Therefore, to improve the flood tolerance of soybean, it will be necessary to investigate the potential for improvement of aerenchyma characteristics, such as diffusibility, porosity, amount, and rate of formation. Further research will also be needed to elucidate the regulation of aerenchyma formation to accelerate aerenchyma formation under aerobic conditions.

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