Transport of oxygen in roots of rice (*Oryza sativa* L.) and of water in developing grape berries (*Vitis vinifera* L.)

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Printed articles:

1. **Kotula L, Steudle E. 2009.** Measurements of oxygen permeability coefficients of rice (*Oryza sativa* L.) roots using a new perfusion technique. *Journal of Experimental Botany* **60,** 567-580. **(Publications - Chapter 1)**

2. **Kotula L, Ranathunge K, Schreiber L, Steudle E. 2009.** Functional and chemical comparison of apoplastic barriers to radial oxygen loss in roots of rice (*Oryza sativa* L.) grown in aerated or deoxygenated solution. *Journal of Experimental Botany* **60**, 2155-2167. **(Publications - Chapter 2)**

3. **Tyerman SD, Tilbrook J, Pardo C, Kotula L, Sullivan W, Steudle E. 2004.** Direct measurements of hydraulic properties in developing berries of *Vitis vinifera* L. cv Shiraz and Chardonay. *Australian Journal of Grape Wine Research* **10,** 170-181. **(Publications - Chapter 4)**

Article in press:

1. **Kotula L, Ranathunge K, Steudle E. 2009.** Apoplastic barriers effectively block oxygen permeability across outer cell layers of rice roots under deoxygenated conditions: roles of apoplastic pores and of respiration. *New Phytologist*, submitted. **(Publications - Chapter 3)**

Other printed article which is not part of this dissertation:

1. **Ranathunge K, Kotula L, Steudle E, Lafitte R. 2004.** Water permeability and reflection coefficient of the outer part of young rice roots are differently affected by closure of water channels (aquaporins) or blockage of apoplastic pores. *Journal of Experimental Botany* **55,** 433-447.

(this article was part of my master thesis completed at the University of Silesia, Katowice, Poland)

Declaration of the self-contribution of research articles

This thesis is completed with four research articles, which include different research work. Most of the research work in was carried out by myself independently at the Department of Plant Ecology, University of Bayreuth under the supervision of Prof. Dr. E. Steudle.

In **Chapter 1**, all experimental work and writing of the manuscript was conducted by myself.

In **Chapter 2**, most of the experiments were conducted by myself. The biochemical analysis of rice roots were carried out in co-operation with Dr. Kosala Ranathunge and Prof. Dr. Lukas Schreiber at the University of Bonn. As a guest in Prof. Schreiber's laboratory, I participated in the analysis. I drafted the manuscript, which was completed after discussion with the co-authors.

In **Chapter 3**, all experimental work was conducted by myself. I drafted the manuscript, which was completed after discussion with co-authors.

In **Chapter 4**, I have participated in experiments on hydraulic conductance of grape-berry pedicel during my stay in the laboratory of Prof. Dr. Stephen Tyerman at the School of Agriculture and Wine, University of Adelaide, Australia.

All published articles can be downloaded from the web-side:
http://www.old.uni-bayreuth.de/departments/planta/research/steudle/index.html

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I

Detailed Summary

1. General Introduction

All higher plants require water for their survival, however, excess water can be harmful or even lethal for land plants. Excess water can arise from heavy rain or irrigation on poorly drained land, from surface floodwaters, or from the rise of water table (van't Voudt and Hagan, 1957; Kozlowski, 1984). Excess water fills the large soil pores that normally remain filled by gas (Drew and Stolzy, 1996). Because of the slow diffusion of gasses in water compared with air, there is a major threat resulting from an inadequate supply of oxygen to submerged tissues. Oxygen starvation in waterlogged soils may occur within a few hours. Since roots and rhizomes are essentially aerobic organs, the consequences can be fatal (Visser *et al*., 2003). In addition to the threat of oxygen deficiency, excess water leads to physico-chemical changes in the soil that affect plants e.g. accumulation of products of anaerobic metabolism of soil micro-organisms (Mn^{2+}; Fe^{2+}, S^{2-}, H_2S; Ponnanperuma, 1984; Drew and Lynch, 1980). Growth and development of the majority of plants is impeded in these conditions. However, wetland species have evolved a wide range of responses to reduce the impact of the stress. One of the most characteristic features of wetland plants is the development of aerenchyma (Jackson and Armstrong, 1999). This tissue of interconnected gas-filled spaces provides a low resistance internal network for the diffusion of oxygen from well-aerated aerial shoots to submerged organs. The effectiveness of aerenchyma can be enhanced by the formation of tight barriers in the peripheral cell layers of roots that inhibit radial losses of oxygen to the surrounding soil (Armstrong, 1979; Colmer, 2003b).

Most rice (*Oryza sativa* L.) crops are produced on flooded, anaerobic soils that are chemically reduced (Ponnamperuma, 1984). Rice is the only cereal that can survive or even thrive in these soils (Jackson and Pearce, 1991). A number of adaptive mechanisms enable rice to complete its life cycle in an environment, which excludes most other species. It is able to germinate without oxygen, and rapidly extend its vegetative organs enabling shoots and leaves to gain access to the air (Jackson and Pearce, 1991; Crawford and Braendle, 1996). Root tips are provided with oxygen delivered by the constitutively present aerenchyma. Besides the internal aeration, roots of rice acquire increased resistance to radial oxygen loss, which improves the efficiency of the axial movement of oxygen to the root apex (Colmer *et al*., 1998; Colmer, 2003a).

The formation of a barrier to ROL in the cell layers exterior to aerenchyma is regarded as an adaptive significance of wetland species, however, it may also have drawbacks for some root functions. For example, it may impede water and nutrient uptake (Armstrong, 1979; Koncalova, 1990). The hydraulic conductivity of rice roots was shown to be low in comparison with other species (Miyamoto *et al*., 2001; Ranathunge *et al*., 2003). This may result in problems for roots to meet traspirational demands by shoot (Miyamoto *et al*., 2001). It has been observed that rice suffers water shortage during the day, even when plants are growing under waterlogged conditions (Hirasawa *et al*., 1992). As a consequence, the productivity and yield may be reduced (Hirasawa *et al*., 1992, 1996). To understand the mechanisms that help rice plants to cope with submergence, it is of particular importance to know the relationship between the water and oxygen transport across the outer cell layers of the rice root.

As an 'Erasmus' student from the University of Silesia (Katowice, Poland) I joined the project at the University of Bayreuth in 2002, after attending Prof. Steudle's courses at my home university. The project was dealing with water transport across rice roots and, at that time, it was run by Kosala Ranathunge in co-operation with International Rice Research Institute (IRRI) in Manila. The project aimed: (i) to find out the location of major apoplastic barriers in rice roots and their relative resistances to the overall root water uptake and (ii) to quantify the relative contribution of apoplastic barriers and cell-to-cell paths to the overall radial water flow across the outer part of the root (OPR). The results revealed that the OPR had a hydraulic conductivity that was larger by a factor of 30 than that of the whole roots (Ranathunge *et al*., 2003). Despite the presence of apoplastic barriers, such as suberized exodermis and liginfied sclerenchyma, the apoplastic water flow contributed much more to the overall water flow across the OPR than the transmembrane component (Kotula, 2003; Ranathunge *et al*., 2004). Comparison between bulk and diffusional water flow across the OPR showed that during water uptake by roots bulk water transport rather that diffusional dominates. This suggested that rice has the ability to minimize oxygen losses from roots in the presence of high water uptake. It could be achieved by differences in transport mechanism – bulk water *vs*. diffusional oxygen flow. At that time, however, there were no data of the oxygen permeability coefficient across the OPR to compare with permeabilities of water. **Thus, my aim was to develop a technique to measure oxygen diffusion across the OPR and apply the technique to rice roots grown under aerated or**

deoxygenated conditions. This was the main goal of my PhD thesis. However, before I have started to work on oxygen transport, I joined a project in co-operation with Prof. Stephen Tyerman (University of Adelaide), dealing with water flows in the developing grape berries. I did this to further improve my knowledge in plant water relations and in the use of Bayreuth pressure probe.

An intruding aspect of the water relations of some sweet, fleshy fruits like grape is that during their development they become hydraulically isolated from the rest of the plant. Water and sugars continue to arrive in the phloem, but little water enters through the xylem (Coombe and McCarthy, 2000). At this time, little back flow of water occurs from the fruit to the stem through the xylem when the gradient in water potential favours it. This hydraulic isolation allows the fruit to accumulate high concentration of sugars. In grape, the change from hydraulic connection to hydraulic isolation occurs during veraison, when rapid growth and colour change in the fruit begin. This hydraulic isolation has been ascribed to discontinuities in the tracheids of the xylem (Düring *et al*. 1987, Findlay *et al*., 1987). Alternatively, the xylem parenchyma cells that surround the berry xylem vessels become less water permeable during development. In that case, aquaporins might play an important role. Another possible reason why water flow ceased after veraison is that the pericarp cell membranes lose osmotic selectivity (reflection coefficient goes to zero).

Berries of *Vitis vinifera* L. *cv*. Shiraz can undergo weight losses during later stages of ripening. It may have impacts on sugar metabolism and flavour development, mineral nutrition concentration of berries, and the practical estimation of final yields by viticulturists (McCarthy, 1999; Coombe and McCarthy, 2000; Rogiers *et al*., 2000). Most of the weight loss in Shiraz was accounted for loss of water (McCarthy and Coombe, 1999). **The aim of this study was to estimate the role of the xylem in berry weight loss and the way in which the hydraulic conductance and selectivity of the xylem-berry interface changes during development.**

2. Background

2.1. Transport of oxygen in rice roots: effects of anaerobic conditions

2.1.1. Rice: importance, cultivation and morphology

Importance

Rice (*Oryza sativa* L.) is the most important staple food crop in Asia where it provides 35-80% of total calorie uptake. It is estimated that half of the world's population subsists wholly or partially on rice. The demand for rice is projected to increase by 70% over the next 30 years, primarily due to rapid population growth.

Cultivation

Rice is highly adaptable and can be grown in diverse environments. Current cultivation of *O. sativa* is worldwide extending from latitude 35°S (Argentina) to 50°N (North China). It is grown from sea level up to 3 000 m in both tropical and temperate climates. It can be grown upon variety of water regimes including upland or dryland rice (13% of rice planted area in the world, and 4% of total rice production), deepwater or flood-prone rice (water depth may exceed 5 m), rainfed lowland rice (lack irrigation, experiences discontinuous waterlogging; 25% of the total rice area, 17% of total production) and irrigated lowland rice (soil is waterlogged during entire season; 55% of the world rice area and 75% of world production).

Irrigated rice is consequently the most important agricultural ecosystem in Asia, and the present and future food security depends on it. It is grown in paddy fields with assured water supply (Fig. 1).

Fig. 1. Terraced paddy-fields in Japan (taken by Kazuko Tomiyama; www.asiarice.org)

Taxonomy and morphology

The genus *Oryza* belongs to the tribe *Oryzeae* of the grass family *Poaceae.* It contains approximately 22 annual and perennial species of which 20 are wild and only two *O. sativa* (Asian rice) and (African rice) are cultivated (Vaughan, 1994).

Oryza sativa is a typical annual grass forming a fibrous root system bearing erect culms and developing long, slender leaves (Fig. 2). The round culm is made up of a number of nodes and hollow internodes that increase in length and decrease in diameter from the lower to the upper part of the stem. The leaves consisting of a sheath and a flat leave blade are born on the culm in two ranks, one at each node. Mature plant has a main stem and several tillers (or side branches), with primary tillers arising form lowermost nodes and giving rise to secondary tillers, which may in turn generate tertiary tillers. Each tiller consists of culm and leaves and although remains attached to the plant, at later stages it is independent because it produces its own roots. Some tillers (productive tillers) terminate with panicle bearing flowers in spikelets, which develop into grains. The fibrous roots posse rootlets and root hairs. Seminal roots persist only for a short time after germination. Secondary adventitious roots are produced from underground nodes of the young culms. As the plant grows, adventitious prop roots often form from the nodes above the ground. The height of the plant varies with the variety and environmental conditions, ranging from around 0.4 m to more than 5 m in deepwater rice varieties.

Fig. 2. Sketch of rice plant (taken from www.nature.com).

2.1.2. Waterlogging

Waterlogging occurs when the input of water from rainfall, flooding or irrigation exceeds rate of subsurface drainage and evapotranspiration, leading to saturation of the soil. The frequency and duration of waterlogging depends on the soil properties, location in the landscape, and climate. Waterlogging of ecosystems may be permanent, or may occur in a regular daily or seasonal pattern (Ernst, 1990). The main ecological change for plant growth by waterlogging is the reduction of oxygen available to the root. In well-structured soils, the presence of pores of large diameters that drain under gravity ensures that the bulk of the soil receives sufficient molecular oxygen for the respiration of microorganisms and roots. During the growing season, aerobic respiration of plant roots and soil microorganisms under temperate conditions consumes typically between 5 and 24 g oxygen m^{-2} day^{-1} (Russell, 1973; Jackson and Drew, 1984). This is equivalent to a daily net flux of oxygen through the soil surface of about 3.5 to 17 liters m^{-2} (Jackson and Drew, 1984). In waterlogged soils, the free exchange of gases between the atmosphere and the soil is much restricted. Water blocks the soil pore spaces that are normally available for oxygen diffusion, the diffusion coefficient of oxygen in water is about 10 000 times lower than in air (Fig. 3; Drew and Lynch, 1980; Drew, 1992). Thus, oxygen dissolved in the soil water and in any entrapped air is soon consumed by aerobic microorganisms and roots (Drew, 1992; Focht, 1992; Jackson, 2004). Depletion of oxygen may require only a few hours (Meek *et al*., 1983) to several days (Blackwell and Ayling, 1981; Blackwell, 1983), depending on soil temperature, the soil respiring biomass (roots and microorganisms) that is consuming oxygen, and the frequency and duration of saturation soil with water (Drew and Stolzy, 1996).

In addition to the threat of oxygen deficiency, waterlogging also impedes diffusive escape of gases such as ethylene or carbon dioxide (Fig. 3). The hormone ethylene was attributed to slow root extension, shoot growth, leaf chlorosis (Drew *et al*., 1979; El-Beltagy and Hall, 1974; Drew and Lynch, 1980; Jackson, 1985; Visser *et al*., 1997). The concentration of CO_2 in the flooded soil may reach up to 50% (v/v) of the total dissolved gasses, and can be toxic to plants (Ponnamperuna, 1972; Boru *et al*., 2003).

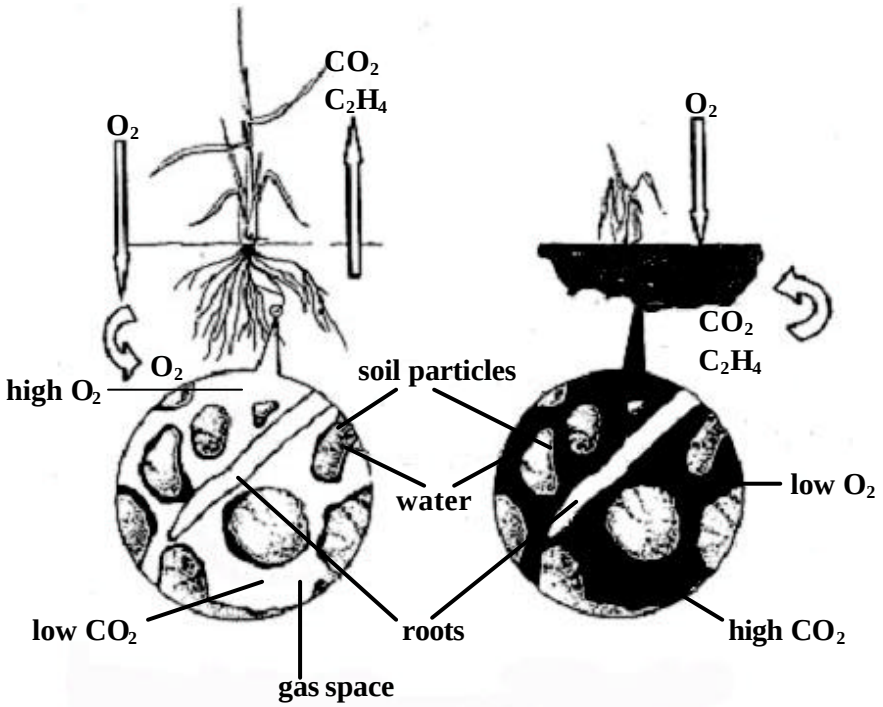

Fig. 3. Effects of flooding on gas exchange between roots, soil, and atmosphere (modified after Jackson, 2004).

The absence of O_2 triggers a sequence of changes in the physico-chemical properties of the soil (Ponnamperuma, 1984). Many of these changes can adversely affect plant growth. After the soil microorganisms have used up the oxygen present in the soil, facultative and obligate anaerobes utilise inorganic ions as alternative electron acceptors during respiration. As a consequence, the redox potential of the flooded soil gradually declines from 0.8 to 0.3V in aerobic to 0.2 to –0.4V in anaerobic soils (Ponnamperuma, 1984; Drew and Lynch, 1980). The first anaerobic process to occur after oxygen depletion is reduction of nitrate (NO_3^-) to nitrous oxide (N_2O) and nitrogen gas (N_2) rendering nitrate unavailable to roots (denitrification). When the nitrate present in the soil is consumed, then anaerobic bacteria use Mn^{4+}, Fe^{3+} as electron acceptors and convert them to Mn^{2+}, Fe^{2+}. High concentrations of these highly soluble ions may be toxic (Drew and Lynch, 1980; Ernst, 1990). When flooding is prolonged and the redox potential of the soil decreased to –0.20V, sulphate (SO_4^{2-}) is reduced to hydrogen sulphide (H_2S). This compound inhibits respiration and poison cytochrome oxidase in rice roots (Allan and Hollis, 1972; Drew and Lynch, 1980). In the most severely reducing soils, methogenic bacteria reduce carbon dioxide to methane (CH_4). Although

the gas is harmless to plants, it is one of the most important greenhouse gases. Rice paddy fields are globally significant methane sources.

Anaerobic metabolism within O_2-deficient roots may also give rise to products (acetaldehyde, ethanol) that are potentially injurious to metabolism if they accumulate to abnormally large concentrations. Also microbial metabolites may accumulate to concentrations that are injurious to roots (Drew and Lynch; 1980; Jackson and Drew, 1984; Ponnamperuma, 1984).

2.1.3. Adaptations to waterlogging and aeration of roots

The most widespread cause of waterlogging injury is due to lack of O_2 to drive respiratory ATP synthesis. This may cause energy deficits resulting in the inhibition of processes to maintain cell functions and growth (Armstrong, 1979; Garthwaite, 2005). The main adaptations to avoid O_2 deficiency include (i) the internal aeration by forming aerenchyma, and (ii) induction of strong barriers in the root peripheral layers external to aerenchyma to impede radial oxygen loss (ROL; Armstrong, 1979; Colmer, 2003b).

Aerenchyma

Aerenchyma is a tissue containing enlarged gas-filled spaces exceeding those commonly found as intercellular spaces (Fig. 4; Jackson and Armstrong, 1999; Evans, 2003). The gas space network connects shoots with roots providing a low resistance internal pathway for long distance gas transport along the plant (Armstrong, 1979; Colmer, 2003b; Voesenek *et al.*, 2006). It delivers O_2 to organs of plants submerged in flooded environments and to the rhizosphere, and removes gases from roots and soil (CO_2, ethylene, methane; Visser *et al.*, 1996; Colmer, 2003b; Evans, 2003).

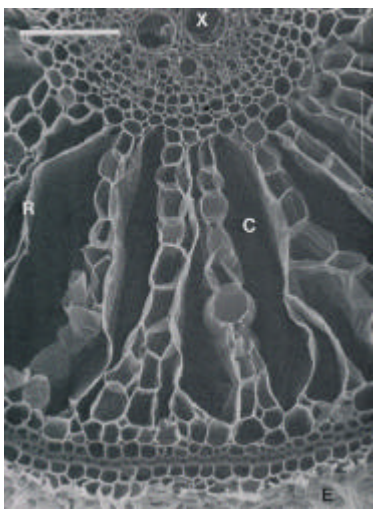

Fig. 4. Aerenchyma in adventitious roots of rice. X, Xylem; R, residues of radial-cell walls of lysed cells; C, cortical gas space; E, epidermis. Bar = 100µm. (taken from Jackson and Drew, 1984)

In addition, formation of aerenchyma decreases O_2 demand due to removal of some cells in the cortex (Jackson and Armstrong, 1999). Two basic forms of aerenchyma have been recognised (Sachs, 1882; Evans, 2003): (i) schizogenous aerenchyma, which develops by cell separation at middle lamellae (*Rumex*), and (ii) lysigenous aerenchyma, formed by death and collapse of cells (barley, wheat, maize and rice; Arikado and Adachi, 1955; Trought and Drew, 1980; Justin and Armstrong, 1991; He *et al*., 1996). In roots, aerenchyma usually forms in the cortex, whereas in stems it can occur in the cortex and pith cavity (Armstrong, 1979; Voesenek *et al*., 2006). In rice roots, cortical cells start to collapse at 10-15 mm from the apex, and aerenchyma gradually develops along the root (Fig. 5). Fully developed aerenchyma observed at a distance of around 100 mm from the apex separates the inner stele from the outer part of the root (OPR; Ranathunge *et al*., 2003). Strands of remaining cells and cell walls separate gas lacunae in the cortex, which resembles a 'spoked wheel' arrangement in cross-sections. Survival of some cells in the structure is important for the structural integrity of the root and for both apoplastic and symplastic transport of nutrients (Drew and Fouracy, 1986; Evans, 2003).

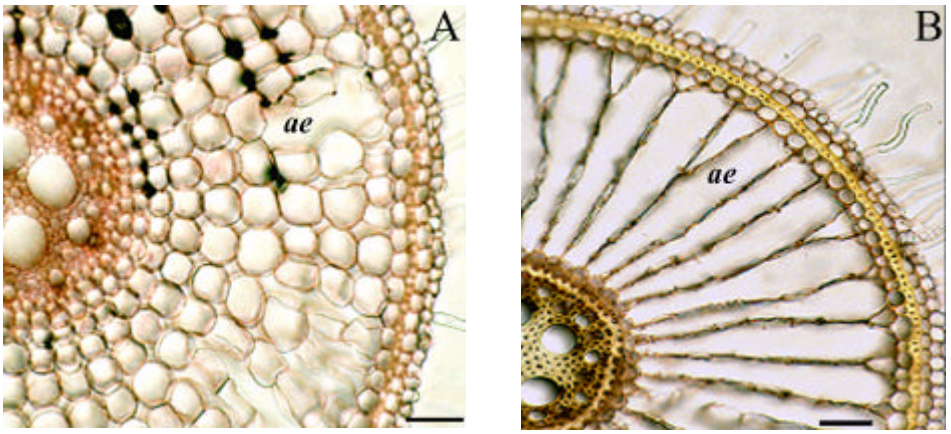

Fig. 5. Development of aerenchyma along the root of rice. Cross-sections taken at (A) 20 mm and (B) 100 mm from the apex. *ae* = aerenchyma. Bar = 100 µm (taken from Ranathunge *et al*., 2004)

In mesophytes such as maize, wheat, barley, and oat, aerenchyma is absent but may be induced by poor aeration (McDonald *et al*., 2002). In many hygrophytes, such as rice, aerenchyma is formed constitutively. Its development may be enhanced in response to waterlogging. The porosity of roots due to constitutive aerenchyma of most wetland species usually ranges from 5-30%. It may double or triple after exposure to flooding

(Justin and Armstrong, 1987; Visser *et al*., 2000; McDonald *et al*., 2002). In rice, gas spaces occupy around 20-30% of the total volume of the root when grown in aerated conditions. Growth in deoxygenated solution increases root porosity to around 40-50% (Armstrong, 1971; Colmer *et al*., 1998; McDonald *et al*., 2002; Colmer, 2003a).

Diffusion of O_2 in roots

Diffusion is the mechanisms, which moves oxygen (and other gases) along roots (Armstrong, 1979; Colmer, 2003b). The effectiveness of the oxygen transport depends on:

- the root anatomy: (i) cross-sectional area of aerenchyma, (ii) cuboidal packing of cortical cells, (iii) large cortical as compared to small stelar volume (according to McDonald *et al*. (2002), in adventitious roots of rice the stele occupies 5% of the root cross-sectional area, whereas in *Sorghum bicolor* it occupies 24-36%), and (iv) resistance to radial oxygen loss (see below; Armstrong, 1979; Kawase, 1981),
- morphology: thicker roots and small number of laterals (Armstrong, 1979, Armstrong *et al*., 1983),
- physiology: lower demand for O_2 consumption in respiration, due to removal of some cells in the cortex (Armstrong, 1979),
- environmental conditions: temperature – cooler conditions reduce O_2 consumption along the diffusion path by slowing respiration in root tissues (temperature declines cause diffusivity being slowed down much less than respiration rates; Armstrong, 1979; Colmer, 2003b).

Largely, oxygen diffusivity of cortical tissue is governed by the amount of intercellular gas-space and degree of its interconnections. The oxygen diffusion coefficient in the cortex can be estimated from the expression (Armstrong and Beckett, 1987):

$$D_e = D_o ET \qquad (1)$$

where, D_o is the diffusion coefficient for oxygen in air (2.0×10^{-5} m^2 s^{-1} at 20 °C; Armstrong, 1979), E is fractional porosity, and T is a tortuosity factor (in most cases is likely to be close to unity; Armstrong, 1979). Assuming the porosity to be 20%, this gives the value of oxygen diffusion in cortical tissues (D_e) = 0.4×10^{-5} m^2 s^{-1}. This is smaller by a factor of five than oxygen diffusion in air but larger by three orders of

magnitude when compared with oxygen diffusion in water at the same temperature (2.1×10^{-9} m^2 s^{-1}; Armstrong, 1979).

Radial Oxygen Loss (ROL)

Oxygen molecules diffusing longitudinally through the roots may be either consumed by cells in adjacent tissues or diffuse radially to the rhizosphere and be consumed in the soil (Armstrong, 1979; Colmer, 2003b). The flux of O_2 to the soil is called radial oxygen loss (ROL), and is determined by:

- the concentration difference between the aerenchyma and the rhizosphere,
- the consumption of O_2 by cells along the radial diffusion path,
- the physical resistance to O_2 diffusion in radial direction (permeability coefficient of the outer part of the root).

Radial oxygen loss can protect the plants against ingress of phytotoxins and support nitrification in the rhizosphere. However, it reduces the supply of oxygen to the root apex and, therefore, decreases the length of roots in anaerobic soil (Armstrong, 1979; Jackson and Armstrong, 1999; Colmer *et al*., 1998; Colmer, 2003b).

Many wetland species prevent radial diffusion of O_2 to rhizosphere by forming a barrier in the root peripherial cell layers external to aerenchyma. This adaptive feature, in combination with extensive volume of aerenchyma, enhances longitudinal movement of O_2 within the root towards the apex (Armstrong, 1979). Oxygen microelectrode studies with adventitious roots of *Phragmites australis* showed the significant role of the barrier on O_2 transport along the root (Armstrong *et al*., 2000). Despite the increasing oxygen concentration in the cortex towards the root base, the O_2 concentrations at the root surface decreased, suggesting high resistance to oxygen diffusion across outer cell layers in basal part of the root (Fig. 6; Armstrong *et al*., 2000).

Roots of some wetland species, such as *Juncus effesus*, *Eleocharis acuta, Schoenoplectus validus* have a constitutively present barrier to ROL (Visser *et al*., 2000; McDonald *et al*., 2002). Regardless of growth conditions, these plants develop ‘tight’ barrier to ROL in mature root zones (very low rates of ROL). In other plants, barrier is induced during growth in anaerobic conditions (the basal zones remained permeable, when grown in aerated conditions) e.g. *Oryza sativa, Hordeum marinum, Lolium multiflorum,* and *Caltha palustris* (Colmer *et al*., 1998; Visser *et al*., 2000; McDonald *et al.,* 2001; McDonald *et al.,* 2002; Colmer, 2003a). However, not all wetland plants

develop ‘tight’ barrier to ROL. Roots of *Phalaris aquatica*, *Ranunculus sceleratus* and *Rumex palustris* showed substantial rates of ROL from basal zones, even when grown under anaerobic conditions (Visser *et al*., 2000; McDonald *et al*., 2002). Different patterns of ROL among wetland species may be related to their (i) root anatomy and morphology – importance of a barrier to ROL diminishes as the as the volume of aerenchyma and root diameters become large (Armstrong, 1979; Colmer, 2003b), (ii) habitat – species from not highly reduced substrates may have more permeable roots (Smits *et al*., 1990).

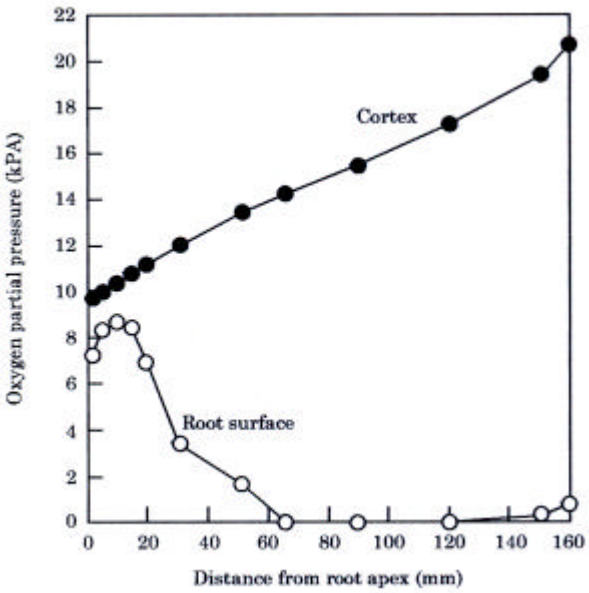

Fig. 6. O_2 partial pressure within the cortex and at the root surface of an intact 110 mm long root of *Phragmites australis*, measured using O_2 microelectrodes. The root was depleted in O_2 free agar solution and shoots were in the air. The cortical O_2 concentration increases towards the base, while O_2 concentration at the root surface is highest near the root apex (taken from Armstrong *et al*., 2000).

Anatomical and chemical nature of the barrier to ROL

Usually, the barrier to ROL has been related to suberization and/or lignification of cell walls of root peripheral layers (Colmer *et al*., 1998; Armstrong *et al*., 2000; De Simone *et al*., 2003; Soukup *et al*., 2007). In all hygrophytes, the exodermis is well developed even in species with weak or irregular aerenchyma development (e.g. *Caltha palustris*; Seago *et al*., 2000), and may have one or more additional layers of thickened cells interior to it (Hose *et al*., 2001). In *Phragmites australis* the suberization and lignification of the exodermis preceded even those of endodermis (Soukup *et al*., 2002). There are two subsequent constitutive developmental stages of the exodermis: (i) the formation of Casparian bands in radial and transverse walls impregnating liphophilic and aromatic substances and (ii) the deposition of suberin lamellae to the inner surface of anticlinal and tangential cell walls (Peterson, 1989). Chemical analysis of roots of nine different species revealed the occurrence of suberin, lignin, carbohydrates and cell

wall proteins in the rhizo- and hypo-dermal cell walls (RHCW; Schreiber *et al*., 1999; Zeier *et al*., 1999). Among these substances, biopolymers lignin and suberin deposited within the cell wall matrix are considered to form the apoplastic barrier (Schreiber *et al*., 1999).

In rice, the well-developed OPR consists of four cell layers: rhizodermis, exodermis, sclerenchyma cells and one layer of lignified cortical cells (Fig. 7; Clark and Harris, 1981; Ranathunge *et al*., 2004). The barrier to ROL may result from heavily lignified sclerenchyma cells and/or suberized exodermis (Casparian bands and suberin lamellae). The presence of these layers is a constitutive feature (Morita and Abe, 1999). The exodermis forms a tight juncture with the epidermis and a layer of sclerenchyma (Clark and Harris, 1981). In hydroponically grown rice, exodermal suberin lamellae was observed at about 30 mm from the apex and matured at about 50 mm (Ranathunge *et al*., 2003, 2004). Well developed Casparian bands were found at about 50 mm from the apex (Ranathunge *et al*., 2003).

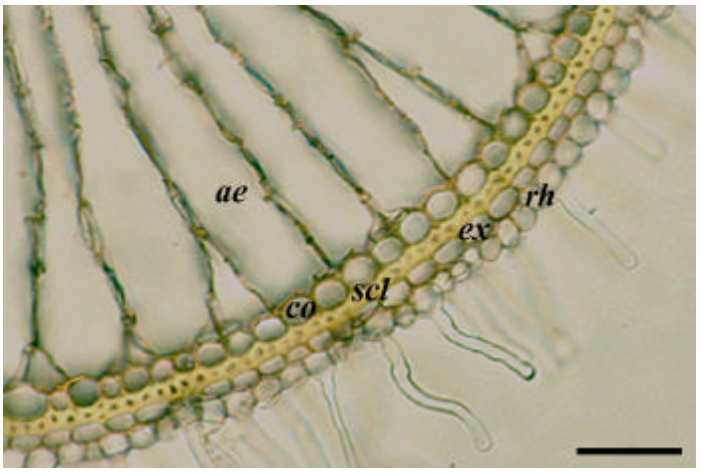

Fig. 7. Well-developed outer part of the root of rice. It consists of four cell layers: the outer most rhizodermis (rh), exodermis (ex), sclerenchyma cells (scl), and innermost cortical cell layer (co). ae, aerenchyma. Bar = 100 µm. (By courtesy of Kosala Ranathunge).

In addition to suberization and lignification, the barrier to ROL is usually linked to dense arrangement of cells such as in the hypodermal cylinder of *Halophila ovalis* (Connell *et al*., 1999). In *Phragmites australis* and *Eleocharis sphacelata,* a combination of densely packed cells, suberin deposits and lignification in the outer cell layers as well as O_2 consumption by cell layers exterior to aerenchyma may form the barrier to ROL (Sorrell, 1994; Armstrong *et al*., 2000). In rice, the ROL from the basal

region increased, when respiration was inhibited by cooling the root medium to 3°C. However, the barrier to ROL remained ‘tight’ compared to ROL rates near the root apex, indicating that both cell respiration (the ‘metabolic resistance’) and the physical impedance were active in reducing ROL from roots (Armstrong, 1971).

In all the research mentioned so far, there was a lack of data of the permeability coefficient for oxygen diffusion across outer cell layers. This limited an understanding of the mechanisms controlling ROL and prevented quantitative comparison of the radial oxygen permeabilities in the outer cell layers of roots of different species, or of a given species grown under various conditions (Colmer, 2003). In the present thesis, a new technique for measuring oxygen permeability coefficient across the OPR was developed. It was applied to roots of rice grown under aerated or deoxygenated conditions.

2.1.4. The barrier to ROL *vs.* root water uptake

As outlined above, the barrier to ROL is usually thought to be an ameliorative, adaptive feature of wetland plants to waterlogging, but it was suggested that the structures forming the barrier to ROL in the outer cell layers may also impede water and nutrient uptake (Armstrong, 1979; Koncalova, 1990). Water can flow into the root through three parallel pathways: (i) the apoplastic path via cell walls and intercellular spaces, (ii) symplastic path through plasmodesmata, and (iii) transcellular path across plasma membranes, which is dominated by aquaporins (AQPs; water channels; Steudle and Peterson, 1998). The combined symplastic and transcellular pathways are considered as a cell-to-cell pathway. In plants, water flow is hydraulic in nature and depends on the force (osmotic or hydrostatic pressure gradients), which drives water across roots and on the hydraulic conductivity (water permeability) of the roots. In the presence of hydrostatic water potential gradients as generated during transpiration, both pathways will be used. In the presence of osmotic gradients, the cell-to-cell transport will dominate. Water uptake by roots has been shown to be variable for several reasons. In short terms, water flow can be affected by a switching between cellular and apoplastic pathways or by a gating of AQPs. In longer terms (days, weeks), the hydraulic conductivity of roots is related to changes in root growth, morphology and anatomy (e.g. suberization and lignification; Steudle and Peterson, 1998). The formation of an exodermis with its Casparian bands and suberin lamellae may add to the overall radial

resistance to water flow from the rhizosphere to the xylem vessels. For example, the development of an exodermis in roots of *Zea mays* caused a 4-fold reduction in hydraulic conductivity (Zimmermann and Steudle, 1998). According to Melchior and Steudle (1993), the hydraulic conductivity of *Allium cepa* roots decreased substantially in basal zones as Casparian bands in the exodermis, and suberin lamellae in the exodermis and endodermis developed. Aliphatic suberin was identified to be a major component, which reduced the permeability in roots of *Zea mays* (Zimmermann *et al*., 2000).

The effects of barrier to ROL on water uptake by rice roots

For rice roots, water shortage may occur even when plants are growing in paddy fields where water supply should be no problem. This may be due to the limitations of water uptake by rice roots, which lack the ability to adjust the hydraulic conductivity according to demand from the shoot (Miyamoto *et al*., 2001). The hydraulic conductivity of adventitious roots of rice was found to be around 6×10^{-8} m s^{-1} MPa^{-1} (Miyamotoet *et al*., 2001). This was substantially lower when compared with roots of other herbaceous plants, even when they posses exodermis (e.g. maize 4×10^{-7} m s^{-1} MPa^{-1}; Hose *et al*., 2000). The low hydraulic conductivity of rice roots has been related to a high resistance to water flow at the strongly developed endodermis and/or outer part of the root with exodermis and sclerenchyma (Miyamoto *et al*., 2001). However, despite the existence of apoplastic barriers, the outer part of the root represented surprisingly low resistance to water flow (Ranathunge *et al*., 2003). Hydraulic conductivity of the external layers was 30-fold higher than the overall hydraulic conductivity, indicating that the apoplastic barriers in the OPR of rice do not limit water uptake. Hence, it was concluded that the highest resistance was located in the endodermis (Ranathunge *et al*., 2003).

Rice roots living in anaerobic media should posses the ability to retain oxygen in the aerenchyma, in the presence of relatively high water permeability. This may be achieved by differences in the transport mechanisms – diffusional oxygen versus bulk water flow (Ranathunge *et al*., 2004). However, they were to date no data of the permeability coefficient of oxygen across the OPR, which could be compared with that of water. For the first time, they are presented in the present thesis.

2.2. Transport of water in grape berries

2.2.1. Structure of grape berry

The grape berry is a simple fleshy fruit, consisting of two seed cavitates, surrounded by an ovary wall (pericarp; Hardie *et al*., 1996; Fig. 8).

The pericarp, is composed of three anatomically-distinct tissues:

(1) the *exocarp* (the dermal system or 'skin') consisting of a cuticle-covered epidermis and several layers of underlying thick-walled cells (hypodermis),

(2) the *mesocarp* consisting of thin-walled parenchyma (flesh, at maturity makes up about 64% of berry`s final volume),

(3) the *endocarp* constituted by thin layer of cells in contact with the seed.

The vascular system of the berry includes dorsal (peripheral), ventral (central), and ovular vascular bundles. The vascular bundles contain the xylem and phloem tissues through which water, sugars, and other substances are supplied to the berry.

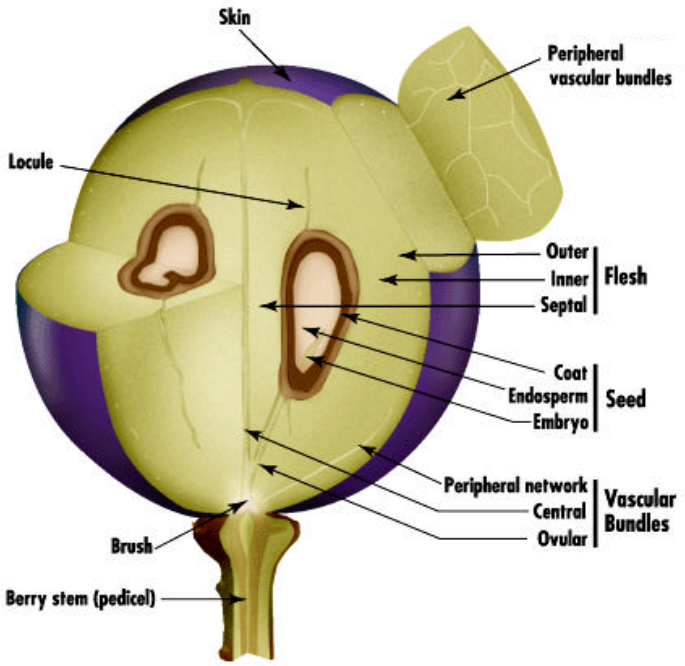

Fig. 8. Structure of a ripe grape berry partially sectiond on the long and central axis to show internal parts (taken from Kennedy, 2002).

2.2.2. Grape berry development

The growth of grape berry consists of two successive sigmoidal cycles separated by a lag phase (Fig. 9; Coombe and McCarthy, 2000).

(1) The first period – berry formation- last from bloom to approximately 60 days afterward. During this time, cell division and enlargement occurs. At the end of this stage, the berry is hard, green and slowly growing. The sugar content of the berry remains low, while organic acids accumulate (mostly malic acid).

(2) The second cycles begins with the onset of sugar accumulation, berry softening and colouring and renewed size increase. These events constitute what is termed 'veraison' i.e. the begging of the ripening process. Ripening is characterised by the accumulation of hexose sugars in the flesh and skin and of potassium and phenolics in the skin (Coombe, 1987). Flavour compounds accumulate late in the ripening stage. The berry approximately doubles in size between the beginning of the second period and harvest.

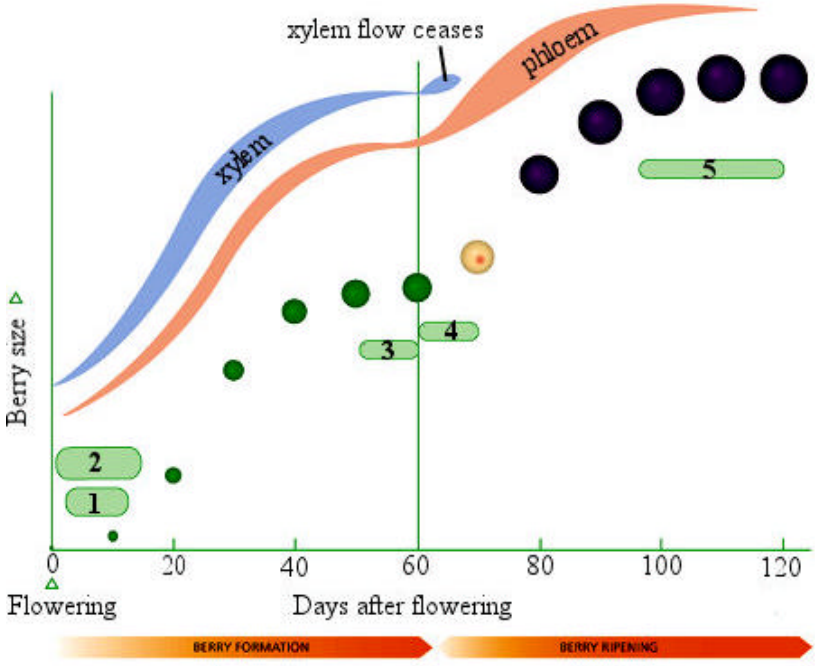

Fig. 9. Diagram showing relative size and colour of berries at 10-day intervals after flowering. Rounded green boxes indicate major developmental events: (1) setting, (2) pericarp cell division, (3) lag phase, (4) veraison, (5) engustment (late period of ripening). Also shown, estimates of rates of vascular flow into berries, partitioned between xylem and phloem (modified from Kennedy, 2002).

2.2.3. Transport of water into the grape berry

Grape berries require a significant amount of water for growth and development, and water typically contributes 70 to 80% of berry fresh weight at harvest. The amount of water that accumulates each day is the sum of water moved into the berry from xylem and phloem sap, and general absorption of through dermal tissues, minus movement out of the berry by transpirational losses and movement back through the xylem (Coombe, 1992).

The berry is supplied through the berry stem or pedicel by a vascular system composed of xylem and phloem. Current evidence indicates that xylem is functional in grape berries early in development (up to veraison). Afterwards its function is reduced or even eliminated (Findlay *et al*., 1987; Greenspan *et al*., 1994). Düring et al. (1987) found that breaks developed throughout the peripheral bundle system after berries had started to expand during the ripening cycle. Findlay *et al*. (1987) showed that after veraison, xylem flow into flesh was blocked due to stretched and broken segments of tracheids, principally those at the top of the pedicel. Disrupted xylem within the berry probably contributes to a reduced conductance to the parent plant (Greenspan *et al*., 1994). After veraison phloem became the primary source of berry water, contributing more than 80% of the total inflow under well-watered conditions (Greenspan et al., 1994). It may result from the fact that after veraison there is an increased sucrose translocation to the fruit, and this must be associated with an increased transport of solvent through the phloem. By contrast, phloem has reduced function early in the berry development.

The partial disruption of xylem function, thus limiting water export, combined with increased phloem inflow may contribute to the resumption of growth in the ripening berry (Greenspan *et al*., 1994).

3. Aims of Research

(I) Even though radial oxygen loss has been extensively studied in the past, neither the oxygen permeability coefficient could be determined nor the precise nature of the barrier to ROL in rice roots was known. Hence, the present thesis aimed:

(1) To develop a technique for measuring oxygen permeability coefficient across outer part of the root at different distances form the root apex.

There are many data in the literature reporting different aspects of radial oxygen loss (ROL) from plant roots. These methods provide quantitative data of ROL. However, as the force driving the ROL could not be quantified, none of them enabled to work out oxygen permeability coefficient of roots. In this study, a new perfusion technique was developed, which was based on the perfusion of aerenchyma of excised root segments with O_2/N_2 gas mixtures and measuring at the same time radial losses of oxygen using a root-sleeving platinum electrode (Armstrong, 1967; Armstrong and Wright, 1975). The first measurements of oxygen permeability coefficient across the OPR were applied to rice grown under aerated conditions, and compared with the permeability of water.

(2) To determine the physical barrier to ROL in rice roots grown in either aerated or deoxygenated conditions.

In previous reports, losses of oxygen from rice roots crossing the peripheral cell layers have been extensively investigated, but the potential barriers to ROL have not yet been determined. In these studies, the focus was on the function (ROL) rather than on detailed structural basis. Combining root histochemistry and biochemistry, the present study for the first time examined the effects of different growth conditions on changes of ROL in relation to the formation of apoplastic barriers in the OPR.

(3) To quantify the oxygen permeability coefficient of the OPR of rice roots grown in either aerated or deoxygenated conditions, and to determine the relative contribution of the apoplast and living cells for the overall movement of oxygen across root peripheral layers.

A gas perfusion technique was used to measure oxygen permeability coefficients of the outer cell layers of roots (P_{OPR}) of rice grown in either aerated or stagnant, deoxygenated conditions. In addition, blocking of apoplastic pores with copper ferrocyanide precipitates (as used in Pfeffer cells; Pfeffer, 1921) and killing the cells of the OPR with 0.1 N HCl enabled to estimate the relative contribution of apoplast and living cells for the overall oxygen flow across the OPR. This is the first quantitative comparison of the radial permeability of peripheral layers to O_2 in roots and how this changes, when the OPR was modified.

(II) Weight loss of berries *cv*. Shiraz during later stages of ripening may have impact on sugar metabolism and flavour development, and consequently on the quality of the vintage. The shrinkage of the berry might be due to some xylem back flow of water from the berry to the parent plant. Despite the importance of the water relations of the berry during entire development, there are neither quantitative measurements of hydraulic conductance of the berry, nor the elucidation of where changes in hydraulic conductance might occur within the berry or the pedicel. The aim of the study was:

(1) To measure the water flow into the berry of two grape varieties Shiraz and Chardonnay as they develop, using a pressure probe.

(2) To determine the location of the highest resistance to water flow into the berry by systematic excision of tissue segments of the berry and pedicel.

This research work can be divided into following sub-sections to investigate above aims:

I. **Measurements of oxygen permeability coefficient of rice (*Oryza sativa* L.) roots using a new perfusion technique (Kotula and Steudle, 2009)**

II. **Functional and chemical comparison of apoplastic barriers to radial oxygen loss in roots of rice (*Oryza sativa* L.) grown in aerated or deoxygenated solution (Kotula *et al*., 2009)**

III. **Apoplastic barriers effectively block oxygen permeability across outer cell layers of rice roots under deoxygenated conditions: roles of apoplastic pores and of respiration. (Kotula *et al*., 2009)**

IV. **Direct measurements of hydraulic properties in developing berries of *Vitis vinifera* L. *cv.* Shiraz and Chardonay (Tyerman *et al*., 2004)**

4. Materials and Methods

4.1. Transport of oxygen in rice roots: effects of anaerobic conditions

4.1.1. Plant material and growth conditions

The upland rice cultivar Azucena (International Rice Research Institute, Manila, Philippines) was used throughout. This was grown in a climatic chamber either in aerated hydroponics or stagnant deoxygenated 0.1% agar nutrient solution (for details see Kotula and Steudle, 2009; Kotula *et al.*, 2009). This concentration has been shown to prevent convection in the nutrient solution, thus simulating the impeded gas movements in waterlogged soil (Wiengweera *et al*., 1997). Aerated and agar nutrient solution were renewed weekly. Plants used in experiments were 30-40 days old.

4.1.2. Measurements of root porosity

The porosity expressed as v/v percent of gas space of tissue was measured on adventitious roots using the microbalance method (Visser and Bögemann, 2003). Ten millimetre long root segments were cut with a razor blade from roots at distances of 5-15, 15-25, 25-35, 35-45, 45-55, 55-65 and 95-105 mm from the apex. The weights of segments (w_1) were measured after gently blotting them on tissue papers to remove adherent water. Then root segments were infiltrated with water under vacuum, blotted, and weighted again (w_2). The porosity was calculated from:

$$Porosity = 100 \times (w_2 - w_1) \times \frac{SW}{w_2} (\%; v/v) \quad , \qquad (2)$$

where SW is specific weight of water (=1.00 g ml^{-1}).

4.1.3. Histochemical detection of apoplastic barriers in the OPR

Adventitious roots were cross-sectioned at different distances from the root apex and stained using: (i) berberine aniline blue to detect Casparian bands (Brundrett *et al*., 1988), (ii) fluorol yellow 088 to detect suberin lamellae (Brundrett *et al*., 1991), and (iii) phloroglucinol/hydrochloride to detect lignin (Jensen, 1962).

4.1.4. Biochemical analyses of apoplastic barriers in the OPR

Root segments of a length of 10 mm were excised from adventitious roots at different distances from to roots apex. Cell walls of the roots segments were digested by incubation in enzymatic solutions containing 1% (v/v) cellulase and 1% (v/v) pectinase (for details see Kotula *et al*., 2009). After this period, the walls, which were not modified by apoplastic barriers such as suberin or lignin, were digested away. For chemical analyses, OPR sleeves were manually separated splitting roots segments longitudinally using a fine-tipped forcep and pulling the central cylinders away from the segments. The amounts of suberin and lignin were estimated quantitatively by gas chromatography and mass spectrometry following the procedures of depolymerization of cell walls. Amounts suberin and lignin were referred to root unit surface area.

4.1.5. Measurements of radial oxygen loss from roots of intact plants

Rates of radial oxygen loss from adventitious roots of intact plants were measured using root-sleeving platinum O_2 electrodes of the Clark type (Clark *et al*., 1953) following the method of Armstrong (1967), and Armstrong and Wright (1975). Root systems of plants were immersed in a chamber containing deoxygenated solution of composition: 0.1% (w/v) agar, 5 mol m^{-3} KCl and 0.5 mol m^{-3} $CaSO_4$ (Colmer, 2003a). The shoot base was fixed on the top of the chamber so that shoot was in air and roots in the O_2-free medium. For each plant, one adventitious root was inserted through the cylindrical platinum electrode (inner diameter: 2.25 mm, height: 5mm). ROL was measured along the root with the centre of the electrode at positions of 5, 10, 20, 30, 40, 50 and 60 mm from the apex. Measurements were taken at 28°C in the climatic chamber, where plants had been previously grown.

4.1.6. Responses of ROL to an increased O_2 concentration around the shoot

The roots of intact plants grown in aerated solution were immersed in a Perspex chamber in the O_2-free medium and shoots were left in the air, as described above. Then, the chamber with the plant was placed in a 12-L-glass container flushed with humidified air. ROLs were measured at positions of 10, 30 or 60 mm from the root apex. When ROL at a given position reached a steady value, the O_2 concentration in the container was changed to 38.7 and subsequently to 58.1, and 96.8 %, and the responses

to ROL were measured. Given values of O_2 concentration were corrected for humidification effects, they were equivalent to 21, 40, 60 and 100% O_2 concentration in dry gas mixtures.

4.1.7. Measurements of oxygen permeability coefficient of rice root segments

The technique was based on the perfusion of aerenchyma of the root segments with O_2/N_2 gas mixtures and measuring at the same time radial losses of oxygen. Root segments excised from adventitious roots were inserted through the cylindrical Pt electrode, fixed to glass capillaries and firmly secured in the Perspex chamber. The chamber was filled with deoxygenated 0.1% (w/v) agar solution containing 5 mol m^{-3} KCl and 0.5 mol m^{-3} $CaSO_4$. The basal side of the root segment was connected to tanks with mixtures of compressed O_2/N_2 of known oxygen concentrations (Fig. 10). Segments were perfused with moistened gas mixtures at oxygen concentrations of 10%, 21%, 40%, 60% and 100% (equivalent to 9.7%, 20.3%, 38.7%, 58.1% and 96.8% corrected for the water vapour concentration) at an overpressure of 20 kPa (0.02 bar). Rates of radial oxygen flow (J_{O2} in nmol m^{-2} s^{-1}) were measured along the segments at distances of 30, 40, 50 and 60 mm from the apex (since the term ROL was always used to describe oxygen loss from intact plants, the oxygen loss from root segments is denoted here as radial oxygen flow J_{O2}). Permeability coefficients were calculated from the slopes of J_{O2}/C_i curves where C_i (mol m^{-3}) is the concentration of oxygen in the liquid phase of aerenchyma at the inner surface of the OPR. Due to polarization effects at the platinum electrode, responses to J_{O2} to increasing O_2 concentration were not linear (see Results). Hence, the permeability coefficient was calculated only from the initial slopes of the flow/concentration curves (C_i = 0-38.7% O_2).

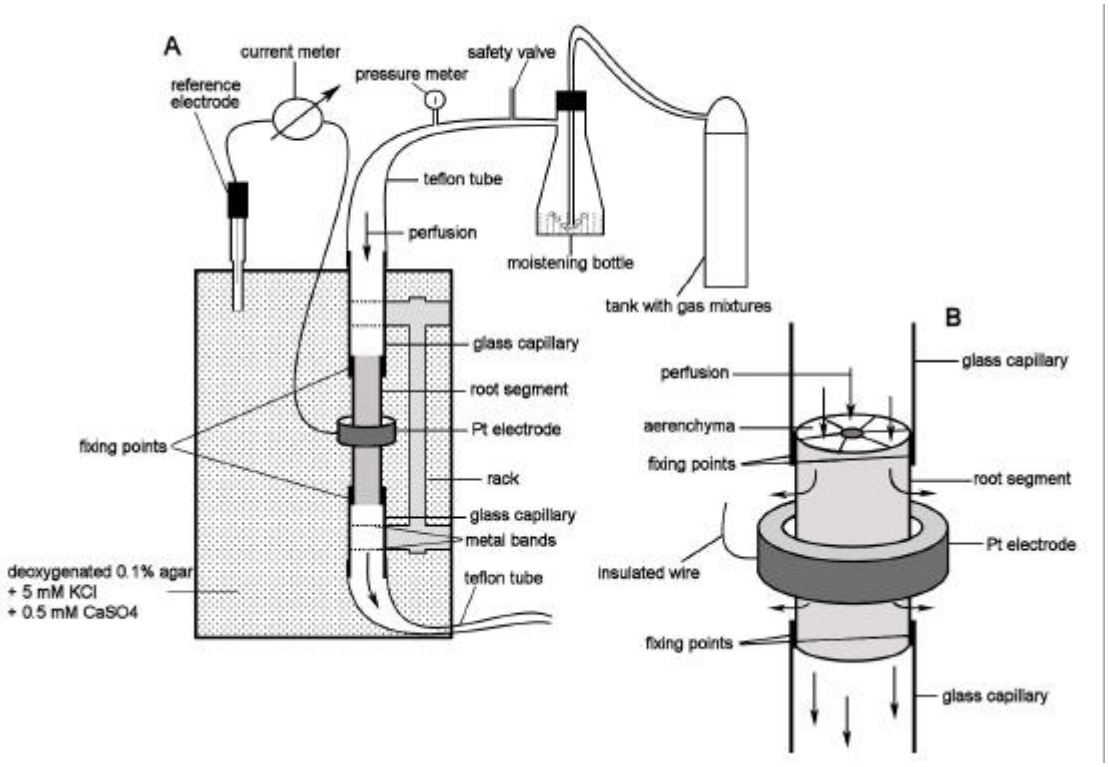

Fig. 10. (A) Schematic diagram of the experimental set-up used to measure radial oxygen flow (J_{O2}) across the outer part of rice roots (OPR). The root segment was inserted through a cylindrical platinum electrode and its ends were glued to glass capillaries. Perfusion of the aerenchyma with the moist gas mixtures at overpressures of 20 kPa (reference: atmospheric pressure) was performed from the inlet side of the segment connected to tanks with different mixtures of compressed O_2/N_2 of known oxygen concentration. Radial oxygen flow (J_{O2}) was measured as an electrical current. (B) Schematic diagram to show radial oxygen flow across the outer part of the root segment during perfusion.

Measured values of oxygen permeability coefficients (Pd_{O2}) obtained from perfusion experiments incorporated diffusion of O_2 across the shell of 0.1% of agar (P_{ag}) between the root and the electrode surface, which acted as an unstirred layer (USL). These effects may be substantial when the overall Pd_{O2} was relatively large. Since a diffusion coefficient of oxygen in the 0.1% of agar solution was known (2.3×10^{-9} m^2 s^{-1} at 25°C; see Kotula and Steudle, 2009), and the diffusional resistances of the OPR and of the agar solution were in series, the oxygen permeability coefficient of just the OPR (P_{OPR}) was worked out by:

$$\frac{1}{Pd_{O2}} = \frac{1}{P_{OPR}} + \frac{1}{P_{agar}}, \qquad (3)$$

where P_{agar} is the oxygen permeability coefficient in the shell of agar.

4.1.8. Blockage of apoplastic pores in the OPR with inorganic salt precipitates

The apoplastic pores in the OPR of rice roots were blocked by insoluble salt precipitates. Root segments, previously used to measure permeability coefficients, were connected to the perfusion apparatus, and aerenchyma was perfused with potassium ferrocynide $\{K_4[Fe(CN)_6]\}$ while the root segment was bathed in copper sulphate $\{CuSO_4\}$. Salt diffused across the barrier and formed a dense, brown precipitates of copper ferrocyanide $\{Cu[CuFe(CN)_6]\}$ in the apoplast. Following the treatment, root segments were fixed to the gas perfusion set up, all solution in the aerenchyma was exchanged by perfusion and P_{OPR} re-measured.

4.1.9. Treatment of root segments with 0.1 N HCl (pH = 1) to kill living cells

Root segments previously used to measure oxygen permeability coefficients were attached to a syringe, and HCl was gently injected into the aerenchyma to displace the air by the solution. Then the entire root segments were immersed in HCl solution for 30 min. Subsequently, root segments were carefully washed and perfused with distilled water to remove HCl from aerenchyma. After fixing HCl-treated segments to the perfusion set up, P_{OPR} was re-measured.

4.2. Transport of water in developing grape berries

4.2.1. Fruit material

Hydraulic measurements were conducted on pre-veraison and post-veraison berries collected from *Vitis vinifera cv*. Shiraz and Chardonnay growing on own roots in the vineyard of the University of Adelaide. Whole shoots were cut from the vine, the cut stem placed in the water and transported to the laboratory. Berries were cut from the bunch with the pedicel under water immediately before experiments.

4.2.2. Pressure probe measurements

The root pressure probe was used to measure the water relations of whole berries and berries cut back at different positions. The probe was connected to the pedicel of single berries assuming that the xylem provided hydraulic connection between the probe and the berry (Fig. 11).

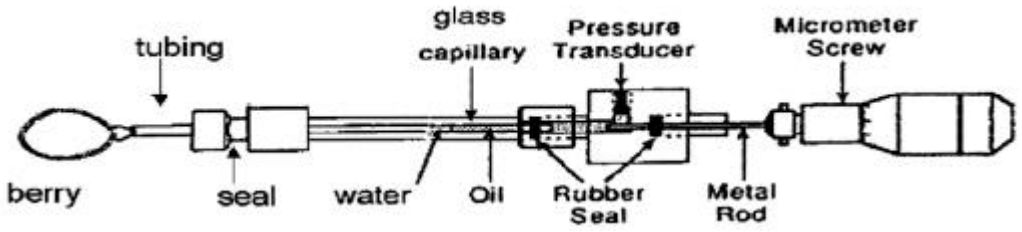

Fig. 11. Diagram of the "berry" pressure probe illustrating the method of attaching the berry pedicel to a piece of plastic tubing that is sealed into the probe. The oil/water meniscus can be used to measure volume flow into or out of the berry. The pressure transducer is connected to a computer and the micrometer screw is used to inject or withdraw water from the pedicel-attached berry system.

When a steady state pressure was achieved, the hydraulic conductance was determined by pressure clamp procedure (volume relaxation). In these experiments, the pressure was elevated by a certain recorded amount (between 0.01 and 0.05 MPa) and maintained while recording the rate of volume flow into the berry, when this was steady or nearly so. To determine the position of major resistance to water flow into the berry, a series of cuts through the fruit and pedicel were made, and the hydraulic conductance was measured following each cut. Positions of cuts were at the base of the seeds, base of the berry and base of the receptacle (torus; Fig. 12).

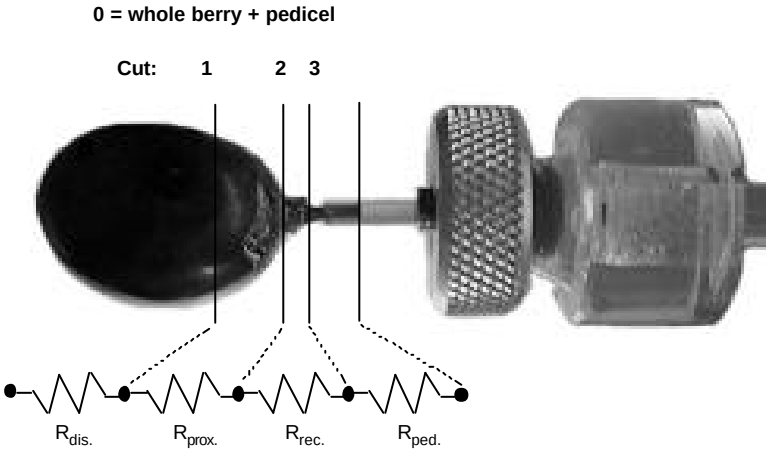

Fig. 12. Diagram of the cut positions along the berry, position 0 corresponds to the intact whole berry.

5. Results and Discussion

5.1. Transport of oxygen in rice roots: effects of anaerobic conditions

5.1.1. Growth of rice plants in aerated or deoxygenated solution

Growth in the stagnant solution resulted in fewer tillers and slower leaf and root development (Fig. 13). In the aerated solution, the average length of the longest adventitious roots was between 530 and 580 mm. When grown in the stagnant solution, the longest adventitious roots were only about 45% of the length of those grown in aerated solution (300 to 320 mm shorter). Adventitious roots of 30 d old plants in aerated and stagnant solutions grew at rates of 31±2 mm d^{-1} and 19±1 mm d^{-1}, respectively (SE; n = 18 to 40 plants).

Fig. 13. 30-d-old rice plants grown under either aerated (left) or deoxygenated (right) conditions. Compared with stagnant solution, plants from aerated culture displayed longer shoots and roots.

5.1.2. Porosity of adventitious roots of rice plants grown in aerated or deoxygenated solution

Both, aerated and stagnant conditions resulted in increases of root porosity along adventitious roots ($F_{6,48}$ = 186.0; $P < 0.001$; repeated measures analysis of variance - ANOVAR). For plants grown in aerated solution, root porosity increased by a factor of 4.6 from 9.6 ± 0.7% at 10 mm to 44.0 ± 2.7% at 100 mm from the apex. During stagnant treatment, porosity increased 5.2-fold, from 10 ± 1% at 10 mm to 54 ± 4% at 100 mm. At all distances, the stagnant treatment resulted in a somewhat greater root porosity when compared with roots of plants grown in aerated solution ($F_{1,8}$ = 14.7; P =

0.005; ANOVAR). However, there were no significant differences at distances of 10 and 100 mm (two-sample t-test at $P \leq 0.05$).

Increased root porosity in plants grown in deoxygenated conditions not only enhance axial transport of oxygen towards root apex, but also enable venting of other gasses such as ethylene, CO_2, and methane (Visser *et al*., 1997; Colmer, 2003b).

5.1.3. Histochemical detection of apoplastic barriers in the OPR of rice grown in aerated or deoxygenated conditions

Deoxygenated conditions induced early development of exodermal Casparian bands (CBs) and suberin lamellae at positions closer to root apex, where it did not develop in roots grown in aerated solution (Fig. 14). Depositions of Casparian bands in plants grown in stagnant solution were observed already at 10 mm from the apex, while in plants grown in aerated solution CBs started to develop at 20 mm from the apex.

Suberin lamellae in hypodermal cell walls were absent at 20 mm in roots grown in aerated, but were already discernible in roots grown in stagnant solution. In the latter, well-developed suberin lamellae were observed at the rest of the distances along roots. In roots grown in aerated solution, suberin lamellae started to develop at around 30 mm from the apex. At this distance, depositions of suberin were observed only in few cells indicating a patchy development. Fully developed suberin lamellae were present at 60 mm. In addition to suberization, early lignification of walls of densely packed sclerenchyma cells was found closer to root apex in plants grown in stagnant solution. It is generally accepted that the presence of apoplastic barriers such as Casparian bands and suberin/lignin depositions impede water loss from the root and control solute transport through non selective apoplastic path (Steudle and Peterson, 1998). However, these apoplastic barriers should restrict gas diffusion as well. It has been shown that early development of suberized/lignified barriers in the outer cell layers of *Phragmites* acted as a barrier to gas diffusion reducing ROL (Armstrong *et al*., 2000; Soukup *et al*., 2007). Our results clearly indicated that there was a strong correlation between oxygen diffusion from aerenchyma to the outer medium and early suberization and lignification of the OPR in stagnantly grown rice roots (see below).

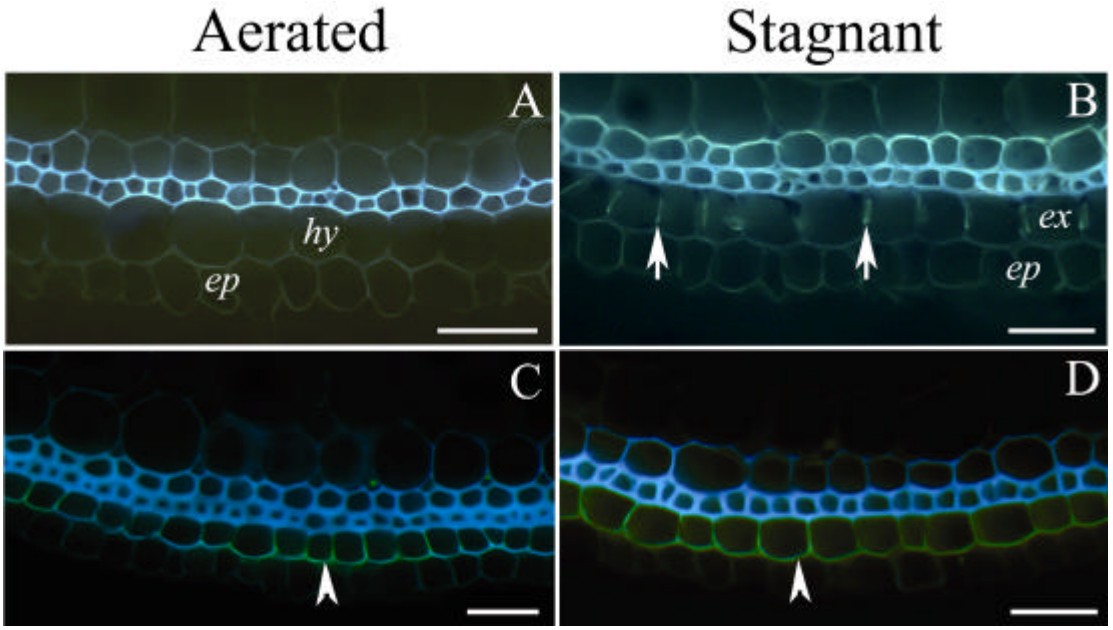

Fig. 14. Comparison of development of Casparian bands (A, B; 10 mm from the apex) and suberin lamellae (C, D; 40 mm from the apex) in the exodermis of roots grown in either aerated (left column) or deoxygenated (right column) solution. The presence of Casparian bands (CBs) was indicated by faint yellow fluorescence with berberine-aniline blue (see arrows in B). At 10 mm there were no CBs observed in walls of plants grown in aerated solution, but they were deposited in plants grown in stagnant solution. The presence of suberin lamellae was indicated by yellow-green fluorescence with Flourol Yellow 088 (see white arrowheads in C and D). At 40 mm from the apex only about 50% of exodermal cells deposited suberin lamellae in aerated roots (C), fully developed suberin lamellae was observed in exodermis of roots grown in stagnant solution.

5.1.4. Amounts of suberin and lignin in the OPR of rice grown in aerated or deoxygenated conditions

In both growth conditions, total amounts of aliphatic and aromatic suberin, and lignin in the OPR increased along the root as it matured. However, when grown in stagnant agar medium, the densities of these substances of corresponding root regions were significantly higher than those obtained from aerated hydroponics (Table 1). The data showed that both the eleveted levels of lignin in sclerenchyma cell walls and suberin levels in the exodermis correlated with patterns of radial oxygen loss from rice roots grown in stagnant conditions (see below). It is obvious that one or both of these substances was/were responsible for the formation of a ‘tight’ barrier to oxygen loss. However, relative contribution of these two substances is not clear. In addition to elevated levels of these substances in the OPR of roots grown in stagnant condition, the

microstructure of the barrier i.e., how these substances deposited into the cell walls, may contribute to the tightness of the barrier.

Distance from apex (mm)	Aliphatic suberin (µg cm^{-2})		Aromatic suberin (µg cm^{-2})		Lignin (µg cm^{-2})	
	Aerated	Stagnant	Aerated	Stagnant	Aerated	Stagnant
10	0.3±0.01	***0.8±0.1	0.2±0.02	**0.9±0.1	6.7±0.9	*11.7±0.6
20	0.3±0.01	**1.5±0.2	0.8±0.08	**3.4±0.3	20.6±1.5	*30.2±2.8
30	0.6±0.02	***2.4±0.1	1.9±0.1	**5.7±0.5	22.2±0.6	*31.8±2.0
40	2.0±0.09	**3.6±0.2	7.6±0.1	*9.0±0.5	38.4±3.7	*57.2±5.7
50	2.2±0.2	**3.6±0.2	9.9±0.3	*12.0±0.8	45.3±2.9	*68.5±3.9
60	2.5±0.2	**3.6±0.1	12.5±0.5	*15.0±0.9	66.6±7.4	*115±5.5

P £ 0.001*; P £ 0.01**; P £ 0.05* (two-sample t-test)**

Table 1. Total amounts of aliphatic and aromatic suberin, and lignin released from OPR sleeves of different zones along the roots of plants grown in either aerated or stagnant solution. Regardless of growth conditions, the amounts of both aromatic and aliphatic suberin, as well as lignin increased along the roots from apex to base. In all root zones, OPR sleeves from plants grown in stagnant solution had significantly greater amounts of these polymers than sleeves from plants grown in aerated solution. Stars indicate significant difference between growth conditions within certain polymers (two-sample t-test). Data are means ±SE (n = 3 plants).

5.1.5. Radial O_2 loss (ROL) from adventitious roots of intact rice plants grown in aerated or stagnant solution

The profiles of ROL along adventitious roots of plants grown in either aerated or stagnant solutions differed considerably ($F_{6,90}$ = 83.7; P < 0.001; ANOVAR; Fig. 15). ROL from adventitious roots of plants grown in aerated solution was the highest at 30 mm from the apex. Lower rates of ROL at positions near the root apex indicated a lower oxygen concentration within the aerenchyma due to losses of oxygen in more basal positions (but not at the very base), and/or consumption of oxygen by living cortical cells. Lower rates of ROL near the basal zones indicated a formation of apoplastic barrier to radial O_2 diffusion. By contrast, adventitious roots of plants grown in stagnant solution showed the highest rates of ROL just behind the apex (5 and 10 mm from the

apex). When the O_2 electrode was moved towards the base, ROL significantly declined to almost zero already at 40 mm.

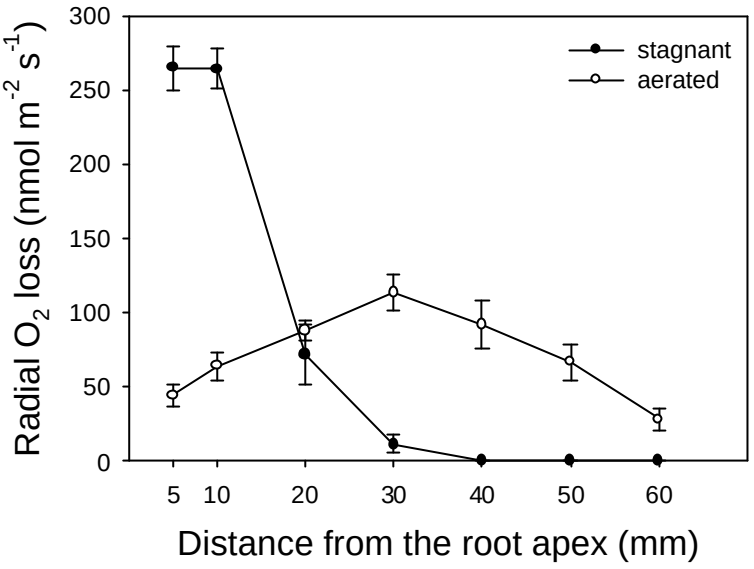

Fig. 15. Rates of radial O_2 loss (ROL) along adventitious roots of intact rice plants raised in either aerated (open symbols) or stagnant (closed symbols) solutions for 30 to 40 days. Measurements were taken in a growth chamber at 27°C with roots in O_2-free medium and shoots in the air. Lengths of the roots used in measurements were 90 to 130 mm. The figure shows that the ROL from roots grown under stagnant conditions substantially decreased along the roots towards the base, most likely due to suberization of the OPR. Data are means ± SE (n = 9 to 8 plants).

The results showed that deoxygenated conditions induced a strong barrier to ROL at the basal positions of rice roots. This agreed with earlier data for diverse rice genotypes and many other wetland species (Colmer *et al*., 1998; Visser *et al*., 2000; McDonald *et al.*, 2001; McDonald *et al.*, 2002; Colmer, 2003a). Strong barrier in the root peripheral layers impede ROL to the rhizosphere enhancing longitudinal movement of O_2 within the root towards the apex (Armstrong, 1979).

5.1.6. Responses of ROL to increased O_2 concentration around the shoot

Oxygen concentrations around the shoot of plants grown in aerated solution were manipulated to test whether or not ROL increased with increasing oxygen concentration in the atmosphere as one would expect. Results showed that different from the perfusion experiment with root segments (see Fig. 17), the increase of O_2 concentration caused linear increase in rates of ROL at all tested distances (Fig. 16). It may result from the

fact that the absolute values of ROL were relatively small when compared with those of J_{O2} (see below). According to Figure 16, the increases of ROL were linear, but in all cases, the regression lines did not pass through the origin. This may be explained by the fact that, at low O_2 concentrations in the cortex, most of the oxygen will be used by respiration. Extrapolating the regressions to intercept x-axis provide a concentration values when the use of oxygen in the plant just compensate axial diffusional transport along aerenchyma. The compensation points would be at 11, 7 and 14% for 10, 30 and 60 mm from the apex, respectively.

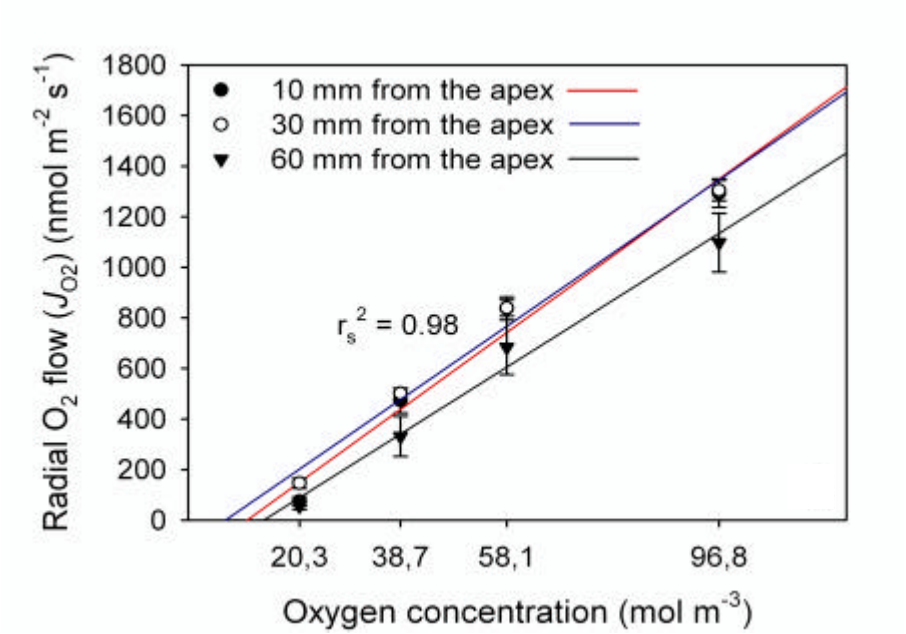

Fig. 16. Effect of increasing oxygen concentration around the shoot of intact rice plants grown in aerated solution on radial oxygen loss (ROL) at distances of 10, 30 and 60 mm from the apex, when roots were in O_2-free agar solution. Measurements were taken in a growth chamber at 27°C for roots of 90 - 120 mm in length. Increases of ROL with increasing oxygen concentration were linear. Extrapolating the regressions to intercept *x*-axis provided concentration values when the use of oxygen in the plant just compensated axial diffusional transport along aerenchyma. Red, blue and black lines represents regressions for 10, 30 and 60 mm, respectively. Data are means ± SE (n = 5 - 7).

5.1.7. Oxygen permeability coefficient of the OPR of rice plants grown in aerated and stagnant solution

The new method to measure O_2 permeability coefficient across outer root cell layers was based on perfusion of aerenchyma with gas mixtures of know oxygen concentration while measuring at the same time rates of radial oxygen loss. The data showed that the increase of oxygen concentration in the perfusing gas did not result in linear increase in

radial oxygen flow (J_{O2}; Fig. 17). It may have resulted from the fact that at high rates of J_{O2} there may be processes tending to underestimate the actual value of radial flow of oxygen (e.g. incomplete reduction of O_2 at electrode surface or other polarizing effects). Thus, some care has to be taken when using root-sleeving platinum electrode in the presence of high rates of J_{O2} (such as for root segments of plants from aerated solution). Because of polarizing effects, the O_2 permeability was calculated only from the initial slopes of J_{O2}/C_i curve in Fig. 17 (C_i = 0 to 40% O_2).

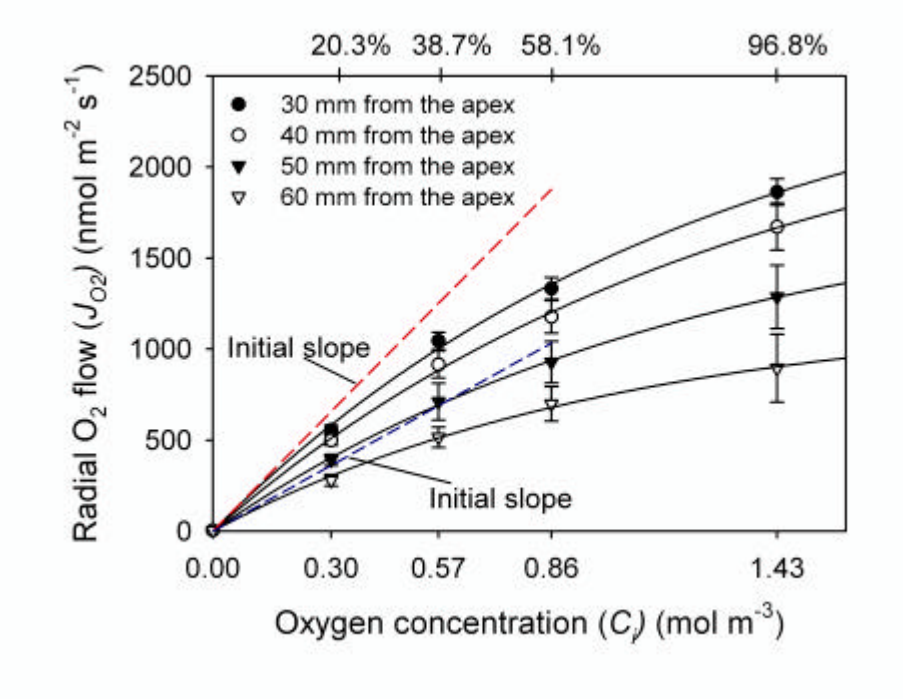

Fig. 17. Rates of radial oxygen flow (J_{O2}) along root segments plotted against the internal oxygen concentration (C_i). According to the Henry`s law, 20.3, 38.7, 58.1 and 96.8% of oxygen (at the mean overpressures of 10 kPa) referred to 0.3, 0.57, 0.85 and 1.43 mol m^{-3} at the inner surface of OPR, respectively. The radial O_2 flows (J_{O2}) increased with increasing O_2 concentrations, but increases of J_{O2} were not linear, which was due to limitations of the technique. Red and blue dashed lines indicate the initial slopes (0% of O_2) of J_{O2}/C_i curves for 30 and 60 mm, respectively. Data given are means ± SD (n=6 - 25 root segments).

The O_2 permeability coefficients (P_{OPR}s) of rice grown in either aerated or stagnant solutions, corrected for the diffusional resistance of the shell of agar between the root and electrode surface, were the highest at 30 mm from the apex and decreased along the root axis reaching the lowest values at 60 mm (Fig. 18). For plants grown in aerated solution, decreases were always significantly different (paired t-test at $P \leq 0.05$), and the mean value of P_{OPR} at 60 mm was lower by a factor of 3.8 than that at 30 mm. During

stagnant treatment, the P_{OPR} decreased by a factor of 5.7 from 30 to 60 mm, however, the significant decrease was not observed between distances of 50 and 60 mm (paired t-test at $P \leq 0.05$). Apparently, the "tight barrier" to the radial oxygen loss was already present at these positions. When compared with roots grown in aerated solution, stagnant treatment resulted in several fold lower oxygen permeability coefficient across the OPR at all distances ($F_{1,47} = 204.1$, $P < 0.001$; ANOVAR).

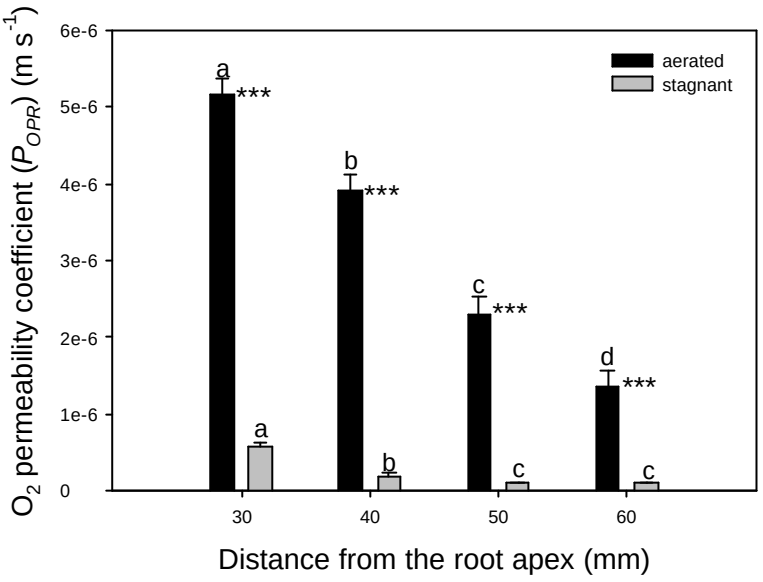

Fig. 18. Oxygen permeability coefficients (P_{OPR}) of the outer part of roots of rice grown in either aerated (black bars) or deoxygenated (grey bars) nutrient solution for 30 to 40 days. Measurements of P_{OPR} were performed at different distances along the root segments. Values of P_{OPR} were corrected for the resistance of the shell of agar between the root and the electrode surface. In both growth conditions, P_{OPR} decreased along the root (significance level of $P \leq 0.001$ denoted by ***, two sample t-test). However, at all distances, P_{OPR} was significantly lower for plants grown in stagnant solution. Different letters indicate significant difference between distances within a one growth condition (paired t-test). Data are means ± SE (n = 19-30).

The lower P_{OPR} in plants grown in stagnant medium as well as the reduction of P_{OPR} along roots of plants grown in both conditions strongly correlated with the development of the apoplastic barriers in the OPR. Apparently, the apoplastic barriers in rice roots impede gas diffusion across the OPR. Suberin, in particular, is known to offer high resistance to O_2 diffusion (De Simone *et al.*, 2003; Soukup *et al.*, 2007).

When compared with water, the oxygen permeability across the OPR of plants grown in aerated solution was higher by an order of magnitude. However, it was shown that for plants grown in aerated solution the bulk water permeability as derived from the hydraulic conductivity of the OPR (Lp_{OPR}) was greater by a factor of 600 (20-50 mm from the apex) and 1400 (50-100 from the apex) than the diffusional (Pd_{OPR}; high Lp_{OPR}/Pd_{OPR} ratios; Ranathunge *et al*., 2004). Hence, during water uptake by roots, bulk (hydraulic) water transport across apoplast rather than diffusional water flow dominated. Overall, the OPR of rice allows a rather high water flow in the presence of a relatively high resistance to oxygen. It is achieved by differences in transport mechanisms: there is bulk flow of water, but the flow of oxygen is diffusional in nature (Kotula and Steudle, 2009). However, to date, the above conclusion was drawn only for rice grown in aerated conditions. When grown in stagnant, deoxygenated solution, which mimics natural paddy-field condition, roots induced several folds greater amounts of suberin and lignin in the OPR and drastically reduced P_{OPR}. These apoplastic barriers could also affect flow of water, however, to date, there are no data of water permeability coefficient across the OPR. These values are badly needed.

5.1.8. Oxygen permeability coefficient of the OPR after blocking the apoplast with precipitated salt crystals

Development of salt precipitates in cell wall pores was observed only in roots grown in aerated conditions. Surface of these roots turned brown after treatment with 1 mM $K_4[Fe(CN)_6]$ and 0.5 mM $CuSO_4$. In stagnant roots, no brown precipitates appeared even after exposing them for 30 min to 0.1 N of HCl. This strongly indicated that well-developed apoplastic barriers in the exodermis such as Casparian bands and suberin lamellae, as well as lignified fibre cells blocked off ion movement across the OPR.

Due to lack of precipitates in roots of stagnantly grown plants, the reduction of P_{OPR} was observed only in roots of plants grown in aerated solution. In these roots, the P_{OPR} decreased by 20, 16, 11 and 5% at distances of 30, 40, 50 and 60 mm, respectively ($F_{1,5}$ = 25.7; P= 0.004; ANOVAR). Blockage of the apoplastu caused similar reduction of the diffusional water permeability (about 20%; Ranathunge *et al*., 2005). By contrast, it caused a massive 3- to 4-fold reduction of hydraulic conductivity. It indicated that treatment with $K_4[Fe(CN)_6]/CuSO_4$ affected bulk flow of water much more than diffusive flows of water and oxygen. This is further evidence that the OPR of rice

allows a rather high water flow in the presence of relatively high resistance to oxygen. As mentioned above it has to be verified for plants grown in deoxygenated solution.

5.1.9. Oxygen permeability coefficient of the OPR after killing root segments with 0.1 N HCl

When compared with control, treatment with HCl caused a significant increase of P_{OPR} at all distances along root segments of plants grown in either aerated or stagnant conditions ($F_{1,5}$ = 66.9, P < 0.001 for aerated plants and $F_{1,5}$ = 46.5, P = 0.001 for stagnantly grown plants; repeated measures analysis of variance - ANOVAR). For plants grown in aerated solution, P_{OPR} increased approximately by 20-30%. When grown in stagnant solution, P_{OPR} of HCl-treated root segments was approximately 40-55% higher.

The increases may result from the fact that killing of cells switched off respiration in the OPR. In addition to respiratory activity, the acid treatment may affect the "tightness" of the apoplastic barrier by some disintegration of the suberin polymers allowing higher oxygen diffusion. It is remarkable that the oxygen permeability coefficient of HCl-killed roots grown in deoxygenated solution was still significantly smaller than that of untreated roots grown in aerated solution. Overall, the physical resistance plays a dominant role in impeding O_2 loss from rice roots, however, respiratory consumption of O_2 may contribute in the presence of low rates of radial oxygen loss. These results support the previous study on roots of *Phragmites australis* and *Hordeum marinum* (Armstrong *et al*., 2000; Garthwaite *et al*., 2008).

5.2. Transport of water in developing grape berries

5.2.1. Hydraulic conductance of berries of *Vitis vinifera* L. *cv.* Shiraz and Chardonnay

Pedicel equilibrium pressures were initially negative at veraison in both Shiraz and Chardonnay, and gradually increased to slightly positive values of up to 0.025 MPa. For both Shiraz and Chardonnay the pedicel equilibrium pressure reached zero about 80-90 days after flowering (DAF). Berry hydraulic conductance (L_o) decreased during and after veraison for both varieties. It indicated that the hydraulic conductance did not go to near zero right after veraison. Rather, there is a continuos decline during ripening.

Shiraz appeared to have higher hydraulic conductance than Chardonnay. The hydraulic conductances 85 DAF were 1.6×10^{-11} m^3 MPa^{-1} s^{-1} for Shiraz and 3.1×10^{-12} m^3 MPa^{-1} s^{-1} for Chardonnay. The large differences in L_o might correlate with berry weight loss in Shiraz. Cutting back experiment showed that during development the hydraulic resistance increased in the proximal (brush) region and distal part of the berry of both varieties but not in the pedicel or in pedicel/fruit junction. However, in Chardonnay the increase of the resistance was about twice of that in Shiraz.

Usually, the reduction in hydraulic conductance was attributed to anatomical changes in xylem vessels. However, it can not be excluded that xylem parenchyma cells that surround the berry xylem vessels become less permeable to water during development. The reduction of hydraulic conductance could be due to combination of both: reduced aquaporin activity in the xylem parenchyma and restrictions in the xylem vessels.

6. General conclusion

6.1. Transport of oxygen in rice roots: effects of anaerobic conditions

The oxygen permeability coefficient of the outer part of the roots (P_{OPR}) of rice was measured using a newly developed perfusion technique. The technique was based on the perfusion of aerenchyma of the root segments with gas mixtures of known oxygen concentration and at the same time measuring radial flows of oxygen using root-sleeving platinum electrode. Non-linear responses of rates of radial oxygen flow (J_{O2}) to increased oxygen concentration in the perfusing gas mixtures indicated that some care has to be taken when using root-sleeving platinum electrodes. At high rates of J_{O2} there may be processes tending to underestimate the actual value of radial flow of oxygen (e.g. incomplete reduction of O_2 at the electrode surface and other polarizing effects). Measurements of permeability coefficient were applied to rice roots grown in either aerated or deoxygenated solution. The results showed that P_{OPR} decreased along the root of plants grown in both conditions. However, when grown in deoxygenated media the O_2 permeability across the OPR was lower by an order of magnitude at all tested distances, compared with that of plants grown in aerated solution. This strongly correlated with the development of apoplastic barriers in the outer cell layers.

Chemical analyses showed that the amounts of suberin and lignin increased along roots of plants grown in both aerated and stagnant solutions, however, absolute loads of these monomers were markedly greater in roots grown under deoxygenated conditions. In agreement with chemical analyses, detailed histochemical studies of the OPR of rice revealed early development of exodermal Casparian bands and suberin lamellae in stagnantly grown plants. Besides suberization, early lignification of walls of densely packed sclerenchyma was found closer to root apex in these plants. Apparently, these apoplastic barriers of rice roots impede gas diffusion across the OPR. Treatment with $CuSO_4/K_4Fe(CN)_6$ resulted in formation of brown precipitates only in roots of plants grown in aerated solution. No precipitates were observed in roots of plants grown in deoxygenated medium. This indicated that well-developed apoplastic barriers in the exodermis such as Casparian bands and suberin lamellae, as well as lignified fibre cells block ion movement across the OPR. In root segments of stagnantly grown plants, no precipitates were observed even after killing with HCl, which further confirms the prominent role of the apoplastic barriers. Killing of root cells by dilute hydrochloric

acid (0.1 N) increased P_{OPR} by up to 55%. This may result from the fact that killing of cells switched off respiration in the OPR. The fact that the P_{OPR} of HCl-treated roots of plants grown in the deoxygenated solution was still significantly lower than that of untreated aerated plants further supports the view that suberization and/or lignification provides a strong barrier to oxygen flow across the OPR of rice. The strong barrier to ROL, thick roots and increased root porosity help to supply oxygen from aerial parts of the plant to root apices of plants grown in deoxygenated solution. Comparison with earlier findings of the diffusional and hydraulic water permeabilities supported the view that apoplastic barriers represented a substantial resistance to the diffusion of oxygen across the OPR but still allowed a relatively high bulk water flow. In other words, the OPR allows a rather high water flow in the presence of a high resistance to oxygen, which is an advantage for the plant. However, the above conclusion was drawn only for plants grown in aerated solution.

6.2. Transport of water in developing grape berries

The study on the hydraulic properties of the pedicel and components of the berries during development showed that the hydraulic conductance of single berries of both varieties declined during development. However, Shiraz berries had higher hydraulic conductance than Chardonnay for whole berries and all cut positions. The increase in hydraulic resistance was found in the proximal (brush) region and distal part of the berry, which was much larger in Chardonnay. For Shiraz, the increase in resistance was about half of that observed in Chardonnay. There was no evidence for changes in resistance in the pedicel or receptacle region of the berry. The reduction of hydraulic conductance could be due to combination of both: reduced aquaporin activity in the xylem parenchyma and restrictions in the xylem vessels.

7. Short summary

7.1. Transport of oxygen in rice roots: effects of anaerobic conditions

- A new technique to measure permeability coefficient of the diffusion of oxygen across the outer part of the root (OPR) has been developed. The technique was based on the perfusion of aerenchyma of root segments with moistened gas mixtures of known oxygen concentration while measuring at the same time radial flows of oxygen using cylindrical platinum electrode. Non-linear responses of rates of radial oxygen flow (J_{O2}) to increased oxygen concentration in the perfusing gas mixtures indicate that some care has to be taken when using root-sleeving platinum electrodes in the presence of high J_{O2} (high internal O_2 concentrations). At high rates of J_{O2} there might be processes tending to underestimate the actual value of J_{O2} (e.g. incomplete reduction of O_2 or other polarizing effects). These complications can be excluded, when the rates of J_{O2} are low. When high oxygen concentrations were applied around the shoot of intact plants, absolute values of radial O_2 loss were much smaller, hence, there were no problems with root-sleeving platinum electrodes.

- Deoxygenated conditions induced the early development of exodermal Casparian bands and suberin lamellae at positions closer to root apex, where it did not develop in roots grown in aerated medium. In addition to suberization, early lignification of walls of the densely packed sclerenchyma cells was found closer to root apex in plants grown in stagnant solution.

- When grown in stagnant solution, the amounts of both aromatic and aliphatic suberin in the OPR of all investigated zones were several folds greater than those of plants grown in aerated solution. In parallel to elevated suberin levels, lignin content was also higher in these roots. In roots from both conditions, there was a pronounced increase of absolute amounts of these compounds along the root from the apex to base.

- ROL from adventitious roots of intact plants grown in aerated solution was the highest at 30 mm. By contrast, roots of plants grown in stagnant solution showed the highest ROL just behind the apex and declined to almost zero at already 40

mm. It was evident that growth under hypoxic condition induced a strong barrier to radial oxygen loss. In addition, roots of stagnantly grown plants were thicker and increased the volume of air spaces.

- Oxygen permeability coefficients of the outer part of roots (P_{OPR}) of rice grown in either aerated or deoxygenated conditions were the highest at 30 mm and decreased towards the base. When grown in deoxygenated solution, the P_{OPR} of all investigated zones was several folds smaller than that of plants grown in aerated solution. These results correlated with the development of apoplastic barriers in the OPR as shown in cross sections.

- Treatment with $CuSO_4/K_4Fe(CN)_6$ resulted in formation of brown precipitates of $Cu[CuFe(CN)_6]$ or $Cu_2[Fe(CN)_6]$ only in roots of plants grown in aerated solution. In roots of stagnantly grown plants, no precipitates were observed even after killing with 0.1 N HCl. This was evidence that well-developed apoplastic barriers such as Casparian bands and suberin lamella, as well as lignified fibre cells impeded ion movement across the OPR of stagnantly grown plants.

- As a result of formation of salt precipitates in the apoplastic pores of roots grown in aerated solution, the P_{OPR} decreased by 20-5%. This is in agreement with earlier findings of diffusional and bulk water flow of Ranathunge *et al*. (2005). This suggested that the OPR of rice allows rather high water flow in the presence of relatively high resistance to O_2. However, the above conclusion was drawn only for rice grown in aerated solution. It needs to be verified in case of roots grown in under deoxygenated conditions.

- Killing of root segments by 0.1 N HCl increased the P_{OPR} by 20-55% of plants grown in both conditions. Increases may have resulted from the fact that the respiratory activity in the OPR was eliminated after HCl treatment. The oxygen permeability coefficient of HCl-treated roots grown in deoxygenated solution was still significantly lower than that of untreated roots grown in aerated solution. It was concluded that although effects due to respiration may contribute in impeding O_2 loss from rice roots, the ‘physical resistance’ rather than ‘metabolic’ was dominant.

6.2. Transport of water in developing grape berries

- The hydraulic conductance of single berries of both Shiraz and Chardonnay declined during development. However, Shiraz berries had higher hydraulic conductance than Chardonnay for whole berries and all cut positions. The increase in hydraulic resistance was found in the proximal (brush) region and distal part of the berry, which was much larger in Chardonnay. For Shiraz, the increase in resistance was about half of that observed in Chardonnay. There was no evidence for changes in the resistance in the pedicel or receptacle region of the berry. The reduction of hydraulic conductance could be due to combination of both: reduced aquaporin activity in the xylem parenchyma and restrictions in the xylem vessels.

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II

Publications

1. Measurements of oxygen permeability coefficients of rice (*Oryza sativa* L.) roots using a new perfusion technique

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Abstract

A new approach is described to analyse the barrier properties of the outer part of rice (*Oryza sativa* L.) roots towards oxygen. By using a root sleeving O_2 electrode, radial oxygen loss at different distances from the root apex was measured and related to the corresponding root structure. In addition, internal oxygen concentrations were precisely adjusted using a newly developed perfusion technique. Thus, the oxygen permeability coefficient of the outer part of root (OPR) could be calculated, since both (i) the oxygen flow across the OPR and (ii) the oxygen concentration gradient across the OPR from inside to outside were known. On the basis of the permeability coefficient, it can be decided whether or not different rates of oxygen loss across the OPR are due to changes in the OPR structure and/or to changes in the concentration gradient. The technique was applied to rice root segments, which enabled rapid perfusion of aerenchyma. In the present study, roots of rice grown under aerobic conditions were used which should have a higher O_2 permeability compared with that of plants grown in deoxygenated solution. Both radial oxygen losses and permeability coefficients decreased along the root reaching the lowest values at the basal positions. Values of oxygen permeability coefficients of the OPR were corrected for external unstirred layers. They decreased from $(2.8 \pm 0.2) \times 10^{-6}$ m s^{-1} at 30 mm to $(1.1 \pm 0.2) \times 10^{-6}$ m s^{-1} at 60 mm from the apex (n = 5; ± SE). They were similar to those measured previously for cuticles. Low diffusional oxygen permeability of the OPR suggested that the barrier to radial oxygen loss was effective. This may help to retain oxygen within the root and enhance diffusion of oxygen towards the apex in the presence of a relatively high water permeability. The results are discussed in terms of the inter-relationship between the water and oxygen permeabilities as roots develop in either aerated or deoxygenated (stagnant) media.

Key words: Aerenchyma, oxygen permeability coefficient, *Oryza sativa,* radial oxygen loss, rice root.

Introduction

Rice (*Oryza sativa* L.) is often grown in waterlogged soils, which are usually anaerobic and chemically reduced (Ponnamperuma, 1984). Under these conditions, aeration of roots depends solely on supplies of oxygen from the shoot through the aerenchyma, which provides a low resistance internal pathway for the movement of gases in plants (Armstrong, 1979). During its passage to the root tips, oxygen may be either consumed by respiration or diffuse radially to the rhizosphere. Radial oxygen loss (ROL) reduces the supply of oxygen to the roots, which would decrease the ability of roots to penetrate into anaerobic soils (Armstrong, 1979; Jackson and Drew, 1984). Forming a barrier in outer cell layers of the basal root zones diminishes losses of oxygen to the rhizosphere, enhancing longitudinal oxygen diffusion towards the root apex (Armstrong, 1979; Jackson and Armstrong, 1999; Colmer, 2003b). However, a physical barrier to ROL may also restrict water and nutrient uptake by roots (Armstrong, 1979; Koncalova, 1990).

In earlier studies, it was shown that the hydraulic conductivity of rice roots is low in comparison with other species (e.g. maize; Miyamoto *et al*., 2001; Ranathunge *et al*., 2003). This may result in problems for the roots to meet transpirational demands by the shoot (Miyamoto *et al*., 2001). Rice may suffer from water shortage during the day, even when plants are growing under waterlogged conditions (Hirasawa *et al*., 1992). The problem was related to suberization/lignification of the roots, which are required to keep the oxygen within the plant. However, it was shown that, at least for roots grown under aerated conditions, the main hydraulic resistance was located in the endodermis rather than in the outer part of roots (OPR), despite the presence of a suberized exodermis with Casparian bands and an additional layer of lignified fibre cells (Ranathunge *et al*., 2003). The ability to retain oxygen in the aerenchyma in the presence of high water permeability may be achieved by differences in the transport mechanism – diffusional versus bulk water flow (Ranathunge *et al*., 2003). This view could be supported by differences between diffusional and bulk water (*Lp*) permeabilities. The lack of data on permeability coefficients for oxygen diffusion across outer cell layers limited the understanding of the mechanisms controlling ROL and prevented quantitative comparison of the radial oxygen permeabilites in the outer cell layers (Colmer, 2003b; Ranathunge *et al*., 2004). Knowing the permeability coefficient of the OPR and, further, how to manipulate its expression may contribute to

identification of the genetic regulation of the ROL (Colmer, 2003b). Subsequent genetic manipulations may help to develop new improved cultivars and rice hybrids with higher yield than existing high-yielding varieties.

There are many data in the literature reporting different aspects of ROL from plant roots. Several techniques have been developed to evaluate ROL, with the most frequently used being the root-sleeving O_2 electrode (Armstrong, 1979; Visser *et al.*, 2000; Colmer, 2003a,b). These methods provided quantitative data of ROL. However, as the forces driving ROL could not be determined unambiguously, none of them enabled the oxygen permeability coefficients of roots to be worked out.

In the present study, a new perfusion technique was developed to measure, for the first time, oxygen permeability coefficient (Pd_{O2}) of the OPR of rice. The well-defined structure of the OPR (four cell layers) is separated from the stele by aerenchyma. Perfusion of aerenchyma of root segments with gas mixtures of known oxygen concentrations and at the same time measuring radial losses of oxygen allowed quantification of the permeability coefficients of the cell layers exterior to aerenchyma. The results indicated that absolute values of the Pd_{O2} were relatively low and decreased towards the base as roots developed. They showed that rice roots may retain oxygen in the root in the presence of rather high water permeability, which may be important for the productivity of rice plant. It should be noted that the O_2 permeability of roots grown in aerated hydroponics should be much larger than that of roots grown in stagnant (deoxygenated) conditions. However, in the present study, aerated conditions were used to (i) establish the technique and (ii) compare the O_2 permeability coefficient with the water permeability of the OPR, which has already been measured for rice grown in aerated hydroponics culture (Ranathunge *et al.*, 2003, 2004).

Materials and Methods

Plant material

Seedlings of an upland rice cultivar (*Oryza sativa* L., cv. Azucena; International Rice Research Institute, Manila, Philippines) were grown in a climatic chamber using aerated hydroponics as detailed previously (day/night rhythm: 12/12 h, 27/22 °C, light intensity: 500 µmol m^{-2} s^{-1}; Miyamoto *et al.*, 2001; Ranathunge *et al.*, 2003). The composition of the nutrient solution was (in mM): 0.09 $(NH_4)_2SO_4$, 0.05 KH_2PO_4, 0.05 KNO_3, 0.03

K_2SO_4, 0.06 $Ca(NO_3)_2$, 0.07 $MgSO_4$, 0.11 Fe-EDTA, and the micronutrients (in µM): 4.6 H_3BO_3, 1.8 $MnSO_4$, 0.3 $ZnSO_4$, and 0.3 $CuSO_4$. The overall osmotic concentration was 3 mM and the pH 5.5 to 6.0. Plants used in experiments were 30-40 d old. The overall lengths of roots used in experiments were 90-200 mm. Diameters of adventitious roots were 0.9-1.2 mm.

Measurements of ROL from roots of intact plants

Rates of ROL from adventitious roots of intact plants were measured by an amperometric mode using cylindrical platinum O_2 electrodes developed by Armstrong (1967) and Armstrong and Wright (1975). Root systems were immersed in a chamber containing a deoxygenated 0.1% agar solution with 5.0 mol m^{-3} KCl (to ensure adequate electrical conductivity) and 0.5 mol m^{-3} $CaSO_4$ (Colmer *et al.,* 1998). The presence of 0.1% of agar was sufficient to minimize convection within the solution and to provide stagnant conditions. For deoxygenation, the agar solution was boiled for 20 min and bubbled with nitrogen gas while cooling down. The shoot base was fixed to the rubber lid on the top of the chamber so that shoots were in the air and roots were in the O_2-free medium. The residual oxygen left in the medium was 0.05 µM, as measured using the O_2 electrode. The measurements were taken in climatic chambers where plants had previously been grown.

For each plant, one adventitious root was inserted through the cylindrical O_2 electrode (i.d., 2.25 mm; height, 5 mm) fitted with guides to keep the root in the centre of the electrode. Prior to measurements, plants were acclimated for 2 h in the deoxygenated medium. ROL measurements were taken along the root by positioning the centre of the electrode at distances of 10, 20, 30, 40, 50, and 60 mm from the root apex. Above 60 mm, laterals began to emerge from the primary root, which prevented further measurements. Diameters of roots at given positions were measured using a digital calliper (700-Digital, Mitutoyo, China).

To measure ROL, the polarizing voltage applied to the platinum electrode results in a reduction of oxygen to hydroxyl ions as in a Clark electrode ($O_2 + 2H_2O + 4e^- = 4\ OH^-$; Clark *et al.,* 1953). The current (in µA) required to reduce the oxygen leaving the root was converted to values of oxygen flux from the root (ROL; Armstrong and Wright, 1975). This calculation assumed that all the oxygen was completely converted

according to the stoichiometry given and that there were no deviations due to polarization effects (see below).

Responses of ROL to an increased O_2 concentration around the shoot

The roots of intact plants were immersed in a Perspex chamber in the O_2-free medium and shoots were left in the air, as described above. Then, the chamber with the plant was placed in a 12.0 l glass container flushed with humidified air (20.3% of O_2 referring to 21% O_2 of dry air) at a rate of 100 $l \cdot h^{-1}$. ROLs were measured at positions of 10, 30 or 60 mm from the root apex. When ROL at a given position reached a steady-state value, the O_2 concentration in the container was changed to 38.7 and subsequently to 58.1, and 96.8% (this equates to 40, 60 and 100% of O_2 in dry gas), and the responses to ROL were measured. After each step of different O_2 concentrations, humidified pure N_2 gas was applied to the shoot, until a minimum value of ROL was reached. This was done to remove high levels of O_2 in the plant prior to a change. Root diameters were measured using a digital calliper (700-Digital, Mitutoyo, China). The measurements were taken in the climatic chambers, where plants had previously been grown (27 °C).

Measurements of radial oxygen flow (J_{O2}) and of permeability coefficient for oxygen (Pd_{O2}) of rice root segments

In order to work out the Pd_{O2}s of the OPR, the concentration difference of oxygen in aerenchyma and in the medium has to be known. The latter was assumed to be zero to a good approximation. The former was provided by perfusing excised root segments with a moistened gas mixture of O_2 and N_2 of known oxygen concentration as shown in Fig. 1. The resulting ROL from segments was measured. Since the term ROL was always used to describe oxygen loss from intact plants, the oxygen loss from root segments will be denoted here as radial oxygen flow (J_{O2}, also in nmol m^{-2} s^{-1} as ROL).

Root segments 50 mm in length were excised at distances of 20 and 70 mm from the root apex, where aerenchyma was sufficiently developed, and inserted through the cylindrical O_2 electrode. Using polyacrylamide glue (UHU, Bühl, Germany), the cut ends of the segments were fixed to glass capillaries (inner diameters of 1.0 – 1.3 mm, depending on root diameter) placed and firmly secured onto a rack within the Perspex chamber using metal bands (Fig. 1). Subsequently, the chamber was filled with deoxygenated 0.1% agar solution (containing 5.0 mol m^{-3} KCl and 0.5 mol m^{-3} $CaSO_4$) and closed with the Perspex lid. To remove extant oxygen, the agar solution was

bubbled with pure nitrogen gas for 20 min after closing the chamber. The basal end of the root segment was connected to tanks with different mixtures of compressed O_2/N_2 of oxygen concentrations of 21, 40, 60, and 100% (tolerance ± 2%; Reissner Gase GmbH & Co KG, Lichtenfels, Germany), via a glass capillary and Teflon tubes (virtually not permeable for oxygen; i.d. 0.7 and o.d. 2.4 mm). To avoid a dehydration of root segments by dry gas, O_2/N_2 mixtures were moistened before they entered the root segment. As shown in Fig. 1, the other end of the root segment (outlet) was open to the atmosphere via another Teflon tube. Root segments were perfused with gas mixtures at overpressures of 10, 20, and 30 kPa (reference atmospheric pressure = 100 kPa), to ensure that the rate of axial flow of oxygen was higher than that of the radial loss and higher than the oxygen consumption by respiration (see Discussion).

Since the root segments immersed in deoxygenated 0.1% agar were perfused with gas of known oxygen concentrations, the corresponding $J_{O2}s$ in nmol m^{-2} s^{-1} could be measured. Permeability coefficients of the OPR were calculated from the slope of J_{O2}/C_i curves where C_i is the concentration of oxygen in the liquid phase of aerenchyma at the inner surface of OPR. Due to polarization effects at the platinum electrode, responses of J_{O2} to increasing O_2 concentrations were non-linear (see Results and Discussion). Hence, the permeability coefficients had to be calculated only from the initial slopes of the flow/concentration curves (C_i = 0-38.7% of O_2 in the humidified gas; see below).

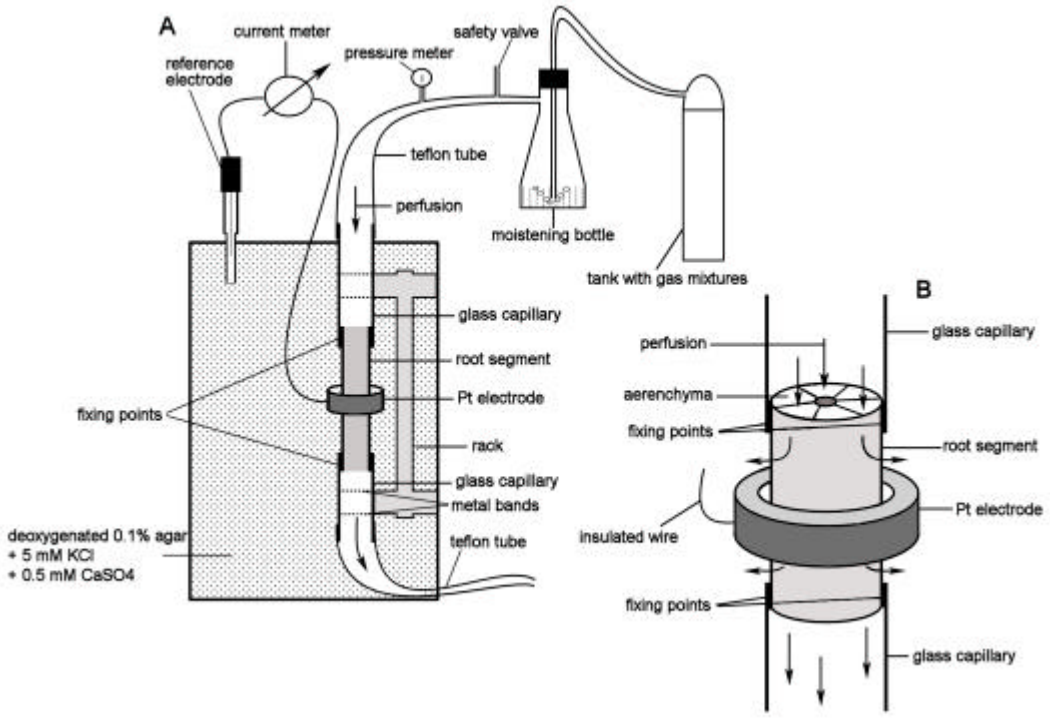


Fig. 1. (A) Schematic diagram of the experimental set-up used to measure radial oxygen flow (J_{O2}) across the outer part of rice roots (OPR). The root segment was inserted through a cylindrical platinum electrode and its ends were glued to glass capillaries. Perfusion of the aerenchyma with the moist gas mixtures at overpressures of 10, 20, or 30 kPa (reference: atmospheric pressure) was performed from the inlet side of the segment connected to tanks with different mixtures of compressed O_2/N_2 of known oxygen concentration. Radial oxygen flow (J_{O2}) was measured as an electrical current. (B) Schematic diagram to show radial oxygen flow across the outer part of the root segment during perfusion.

To test whether or not the apparent decrease of Pd_{O2} (non-linear relationship between J_{O2} and C_i) at high rates of J_{O2} was caused by polarization effects (see Discussion), tubes of silicone and Teflon (PTFE), which differed in their permeabilities for oxygen, were used for comparison. Dimensions of the polymer tubes were similar to those of roots: the length was 50 mm, internal and outer diameters were 0.5 and 0.9 mm, respectively for silicone (Laboflex, Kronlab, Dinslaken, Germany). For PTFE, dimensions were: 0.5 and 1.0 mm, respectively (Laboflon, Kronlab, Dinslaken, Germany). The tube segments were glued to glass capillaries and placed in a Perspex chamber as described above. Radial oxygen flows were measured during perfusion of gas at different oxygen concentration at an overpressure of 20 kPa at the entrance of tube segments (mean of 10 kPa see Results and Discussion).

In order to calculate Pd_{O2} of roots and polymer tubes, C_i should be given in mol m^{-3} rather than in % O_2. The internal concentration C_i was calculated from the partial pressure of oxygen according to the Henry`s law:

$$C_i = \frac{P_{O_2}}{K_H}, \qquad (1)$$

where K_H is the Henry´s law constant (74.68 kPa m^3 mol^{-1} at 25°C; Atkins and de Paula, 2007) and P_{O2} is the partial pressure of oxygen. Since the gases were humidified, P_{O2} was corrected for the saturation water vapour pressure (3167 Pa = 3.167 kPa = 31.67 mbar at 25°C; Jones, 1992). Hence, the oxygen pressure in H_2O-saturated air was 20.33 kPa and not 20.95%. The overpressure inside the system also had to be taken into account. An overpressure of 10 kPa (0.01 MPa) equates to 2.03 kPa of oxygen. Overall, the partial pressure of oxygen in air perfused across the root segments was thus 22.4 kPa. Similarly, for oxygen concentrations of 40, 60 and 100%, partial pressures were 42.6, 63.9 and 106.5 kPa, respectively. This calculation assumed nominal partial O_2

pressures given by the supplier, a mean pressure along the segments, and a rapid equilibration of the thin layer of water at the inner surface of the OPR, which is reasonable. Because of the convective mixing of the gases in the aerenchyma, there were no radial gradients in P_{O2} (and C_i) along the interior of segments. The high rates of perfusion of N_2/O_2 mixtures ensured that the J_{O2} and respiration rates were much smaller compared with the axial flow of oxygen (see Discussion), so the concentration of oxygen along the aerenchyma remained constant.

The measurements of radial oxygen flow and permeability coefficient were taken along the root at distances of 30, 40, 50, and 60 mm from the root apex. Since the radial oxygen flow could increase the oxygen concentration outside of the root, the agar solution was always bubbled with nitrogen before each change of the concentration of perfused gas mixture. Measurements were taken at 25 °C in a temperature-controlled room.

In cases, where root segments were not properly fixed to glass capillaries or were injured during handling, the experiments were stopped. Leakages were easily detected by observing air bubbles coming out at the points where capillaries were not properly fixed to segments or at places where root segments were injured during handling. Since experiments with a given segment lasted for 3-6 h, root segments had to be tested for the viability of the cells of the OPR. Root segment were randomly selected for treatment with Evan´s Blue stain (Fischer *et al.*, 1985).

Calculation of the diffusivity of oxygen across the OPR. Effects of the unstirred layer caused by the cylindrical shell of agar between root and electrode

Values of permeability coefficients obtained in the present study incorporated diffusion of O_2 across the shell of agar around the root. This unstirred layer effect might have been substantial when the overall Pd_{O2} was relatively large. The measured values of Pd_{O2} were the inverse of a resistance (or resistivity), which consisted of two resistances in series: (i) resistance of the OPR; and (ii) the resistance of the shell of agar between the root and the electrode surface. Before calculating the Pd_{O2} corrected for the USL (and diffusion coefficient of O_2 in the OPR), those two resistances had to be separated. Since the diffusion coefficient of oxygen in 0.1% agar (D_{agar}) was not known for 25 °C, this was estimated from literature data. At 22 °C, the diffusion coefficient of O_2 in 0.2-2% of agar was 2.1×10^{-9} m^2 s^{-1} (Revsbech, 1989), which relates to 2.18×10^{-9} m s^{-1} for

water at the same temperature. Using the ratio of 2.1/2.18, D_{agar} was calculated from the diffusion coefficient of O_2 in water at 25°C, which is 2.38×10^{-9} m^2 s^{-1} (Millington, 1959). This resulted in a $D_{agar} = 2.3 \times 10^{-9}$ m^2 s^{-1} for 0.1% agar at 25 °C. The oxygen permeability coefficient of the agar shell (P_{agar}) was calculated by (Ye *et al*., 2006):

$$P_{agar} = \frac{D_{agar}}{r_r \ln \frac{r_e}{r_r}} \quad , \tag{2}$$

where r_r is the radius of root, r_e is the radius of electrode. It can be easily verified that Equation 2 reduces to $P_{agar} = D_{agar}/(r_e - r_r)$, when $r_r \tilde{} r_e$. The corrected value of the permeability of the OPR (P_{OPR}) can be obtained from:

$$\frac{1}{Pd_{O2}} = \frac{r_r}{D_{OPR}} \ln \frac{r_r}{r_a} + \frac{r_r}{D_{agar}} \ln \frac{r_e}{r_r} = \frac{1}{P_{OPR}} + \frac{1}{P_{agar}} \quad . \tag{3}$$

Estimated values of P_{OPR} allowed calculation of the diffusivity of oxygen in the OPR (D_{OPR}) at different distances from the root apex according to (Ye *et al*., 2006):

$$D_{OPR} = P_{OPR} \times r_r \times \ln \frac{r_r}{r_a} \quad , \tag{4}$$

where, r_a is the radius of the root minus the thickness of the OPR [the thickness of the OPR ($r_r - r_a$) was taken as 85 µm, overall root diameter: 1 mm (Ranathunge *et al*., 2004)]. Again, for sufficiently small differences ($r_r - r_a$), equation 4 reduces to $P_{OPR} = D_{OPR}/(r_r - r_a)$.

Qualitative detection of zones of ROL using the oxidation indicator dye methylene blue

Using the redox indicator methylene blue, sites of oxygen release from roots of intact rice plants and root segments were identified (Conlin, 1986; see also Chabbi *et al.*, 2000). A solution containing 0.75% agar was prepared by heating to boiling. The mixture was then cooled to below 60 °C while stirring. At 60 °C, methylene blue was added at a concentration of 2 mg l^{-1}. The blue solution containing the oxidised dye was further cooled down to 40 °C, and then was reduced by addition of 0.75g L^{-1} sodium

dithionite ($Na_2S_2O_4$). The solution was stirred until it was completely colourless. Healthy rice root systems were carefully placed vertically in a plastic cylinder without damaging them. Then the cylinder was filled with the methylene blue-agar solution, which was cooled to 35 °C. This solution resulted in solid agar at room temperature. The shoot base was fixed to the wall of the cylinder, so that the shoots were in the air. The open surface of agar was immediately covered with plastic wrap. The cylinder was placed in the climatic chamber, where the seedlings had been grown and left for ~ 20 h. At places where oxygen was lost from the roots, methylene blue was converted from colourless (reduced) to the blue (oxidised) form, which was indicated by the formation of blue haloes around the roots. Photographs were taken after removing the roots from the agar medium.

The methylene blue technique was also used to visualize the sites of oxygen loss from perfused rice root segments. Both ends of root segments, excised at distances of 20-80 mm from the apex, were glued to glass capillaries with inner diameters of 1.0-1.3 mm. One of the glass capillaries was connected to the source of gas, and the other end remained open. Root segments were placed in a small box, covered with methylene blue agar and perfused with moistened air (20.3% of oxygen) at an overpressure of 20 kPa for 1 hour. Photographs were taken using a digital camera (Nikon D40, Nikon Corporation).

Statistical analysis

Data on ROL, J_{O2} and Pd_{O2} were analysed using one-way analysis of variance (ANOVA) to examine the effects of oxygen concentration or position along the root axis. Means are accompanied by standard errors, and were compared using the least significant difference test (LSD) at the $P \leq 0.05$ level.

Results

ROL from adventitious roots of intact plants

Measurements of ROL were taken from adventitious roots of intact plants at different distances from the root apex, with the shoot in air and the roots in the O_2-free medium. ROL was referred to 1 m^2 of root surface. Although there was a substantial variation between roots, the data given in the Fig. 2A indicate a significant maximum of ROL at ~30 mm from the apex ($F_{5,42} = 9.7$; $P \leq 0.05$). Presumably, the increase of ROL from

the distance of 10 to 30 mm was the result of the increase of porosity as roots developed aerenchyma. At later stages of development (40-60 mm), the drop of ROL was most probably due to the suberization/lignification of OPR (see Discussion; Armstrong and Armstrong, 1988; Colmer, 2003b). To minimize the effects of the variability between roots (Fig. 2A), which tended to obscure the maximum ROL at 30 mm, the data of each root were referred to 100% at 30 mm, i.e. relative rather than absolute values were given (Fig. 2B). As seen in Fig. 2B, ROL at 30 mm was significantly different from that at other distances, except at 40 mm ($F_{5,42}$ = 15.2; $P \leq 0.05$). At a distance of 60 mm, ROL was smaller by a factor of 4.3 (on average). The finding suggested that the barrier to ROL, which developed in the OPR was quite effective.

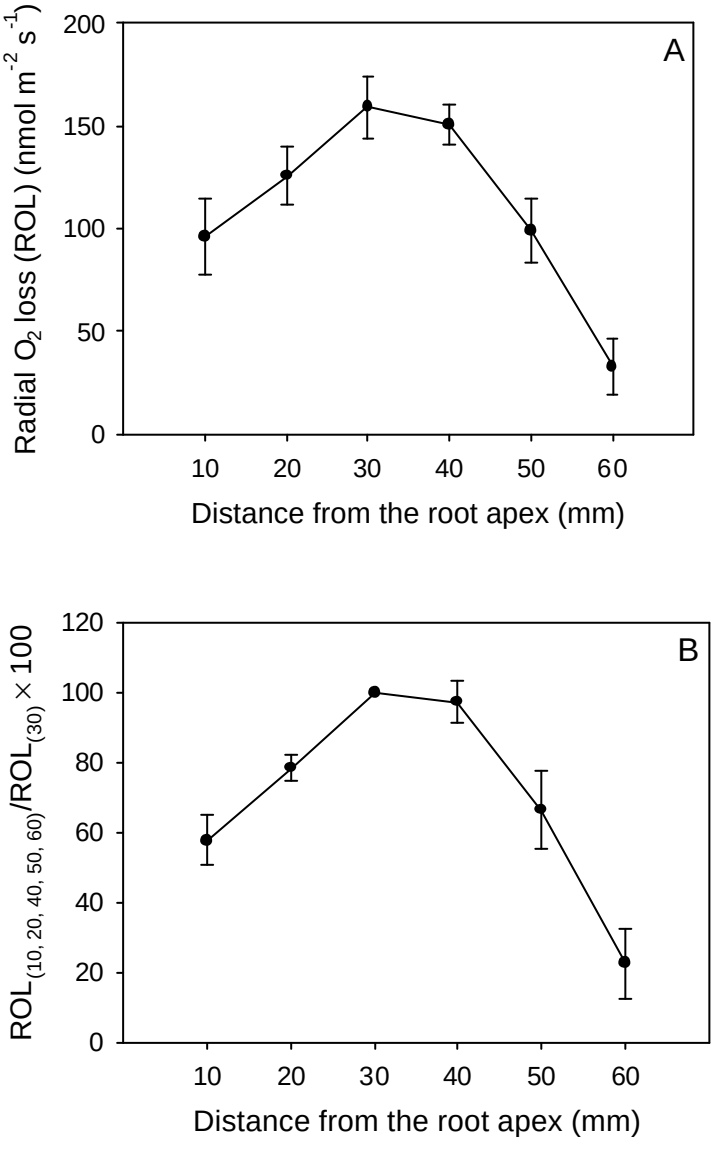
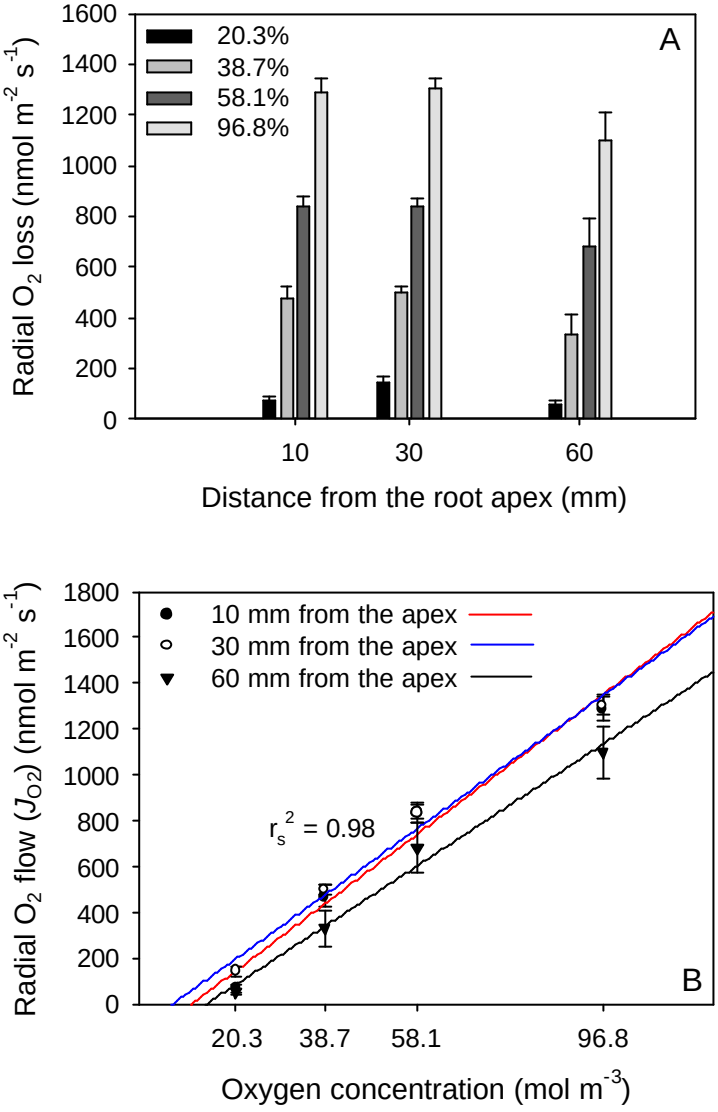


Fig. 2. (A) Rates of radial oxygen loss (ROL) along adventitious roots of intact rice seedlings grown in aerated nutrient solution for 30-40 d. Measurements were taken in a growth chamber at 27 °C for roots of 90-120 mm in length, which were in O_2-free medium, with the shoots in the air. (B) Relative values of ROL are given. To minimize variations between roots, the data for each root were referred to 100% at 30 mm. Data given are means ±SE (n = 8).

Responses of ROL to increased O_2 concentration around the shoot

Oxygen concentrations around the shoot were manipulated to test whether or not ROL increased with increasing oxygen concentration in the atmosphere as one would expect. Responses of ROL to different O_2 concentrations around the shoot (20.3-96.8% O_2/N_2 mixtures, when considering the small effect due to humidification of the gases) at different distances from the root apex are shown in Fig. 3 (10, 30, and 60 mm). As the O_2 concentration increased, rates of ROL significantly increased at all distances ($F_{3,17}$ = 142.8 for 10 mm, $F_{3,21}$ = 297.2 for 30 mm, $F_{3,14}$ = 40.1 for 60 mm; $P \leq 0.05$; Fig. 3A). However, different from the perfusion experiments with root segments (see Figs. 5, 6), rates of O_2 losses were much smaller (see Discussion). According to Figure 3B, the increases of ROL were linear, but in all cases, the regression lines did not pass through the origin. This may be explained by the fact that, at low O_2 concentrations in the cortex, most of the oxygen is used by respiration. Extrapolating the regressions to intercept x-axis provide a concentration values when the use of oxygen in the plant just compensates axial diffusional transport along aerenchyma. The compensation points would be at 11, 7 and 14% for 10, 30 and 60 mm from the apex, respectively. The linear extrapolation may be questioned, and more data are required in the range of low concentrations (see Discussion). However, for all oxygen concentrations, there was a trend that the highest ROL was observed at 30 mm from the root tip, but this was only significant for 20.3% ($F_{2,18}$ = 7.9; $P \leq 0.05$; see Fig.2 and Discussion).

Fig. 3. Effect of increasing oxygen concentration around the shoot of intact rice plants on radial oxygen loss (ROL) at distances of 10, 30 and 60 mm from the apex, when roots were in O_2-free agar solution. Measurements were taken in a growth chamber at 27 °C for roots of 90-120 mm in length. (A) For a given position, rates of ROL significantly increased as the concentration of oxygen increased ($P \leq 0.05$). Due to suberization and/or lignification of basal parts of the roots, the response in ROL at a distance of 60 mm was smaller than that of other distances for each concentration difference. (B) Increases of ROL with increasing oxygen concentration were linear. Extrapolating the regressions to intercept *x*-axis provided concentration values when the use of oxygen in the plant just compensated axial diffusional transport along aerenchyma. Red, blue, and black lines represents regressions for 10, 30, and 60 mm, respectively. Data are means ±SE (n = 5-7).



J_{O2} *and* Pd_{O2} *measured with root segments*

Perfusion of aerenchyma with different oxygen concentrations and different overpressures (axial flow rates) was performed with rice root segments placed in O_2-free medium, and radial oxygen flows (J_{O2}s) were measured. When root segments were perfused with humidified air (20.3% of O_2) at overpressures of 10, 20, or 30 kPa at the entrance of segments (Teflon tubes), there was a slight increase in J_{O2}, but this was not significant ($F_{2,21}$ = 1.1, 0.2, 0.2 and 0.3 for 30, 40, 50 and 60 mm, respectively; $P \geq 0.05$; Fig. 4). It was also not significant when data were not pooled (as in the figure) but were plotted root-by-root (data not shown). Different overpressures were used to check whether the axial perfusion of aerenchyma with oxygen was rapid enough to provide a constant internal O_2 concentration along the root interior and to compensate for losses

of oxygen by respiration (see Discussion). According to gas laws, higher absolute pressures at the inlet (110, 120, and 130 kPa) would increase the partial oxygen pressure (P_{O2}) and the actual concentration of oxygen along the tubes (segments). Assuming that the drop in pressure would be largely within the segments and would be linear for the three different pressures, average values of 105, 110, and 115 kPa were used to calculate P_{O2} and the actual O_2 concentration (see Discussion). As seen in Fig. 4, there was a drop of J_{O2} along the root from 30 mm to 60 mm (see Fig. 5). It was not possible to make measurements at distances below 30 mm (see Discussion). For the rest of the experiments, an overpressure of 20 kPa (0.02 MPa) was used at the entrance of the segments, which was sufficient to keep the constant O_2 concentration in the aerenchyma (see Discussion).

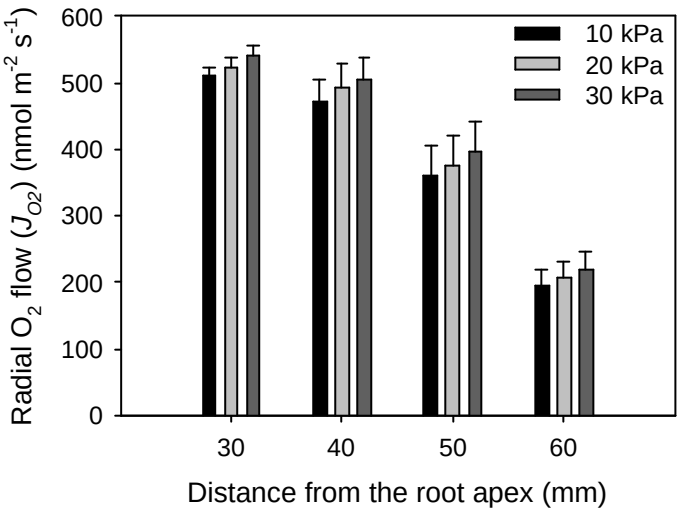


Fig. 4. Rates of radial oxygen flow (J_{O2}) along the root when perfused with 20.3% O_2 at overpressures of 10, 20, 30 kPa at the entrance of the segments (reference atmospheric pressure 100 kPa = 0.1 MPa). Assuming a linear drop in pressure along the segments for the three different pressures, average values of 105, 110, and 115 kPa were used to calculate P_{O2} and the actual O_2 concentration. For each distance from the root apex, radial oxygen flows increased with increasing overpressures, but increases were not significantly different. Measurements were taken at 25 °C in a temperature-controlled room. Data given are means ±SE (n = 8).

When individual root segments were perfused with O_2/N_2 mixtures at the oxygen concentrations of 20.3, 38.7, 58.1, and 96.8% (correction for humidification considered; for corresponding partial pressures, see above), rates of radial oxygen flow (J_{O2}) decreased along the root for each oxygen concentration, being the highest at 30 mm (Fig. 5). For each oxygen concentration, J_{O2} was not significantly different between 30 mm and 40 mm ($F_{3,92}$ = 17.6 for 20.3%, $F_{3,16}$ = 10.2 for 38.7%, $F_{3,20}$ = 9.2 for 58.1%; $F_{3,20}$ = 8.5 for 96,8%; $P \leq 0.05$). However, it was always significant between 30 mm and 50 mm, and between 40 mm and 60 mm. The marked differences between 40 mm and

50 mm from the apex and 50 mm and 60 mm were observed only, when root segments were perfused with 20.3% of oxygen. The decrease of J_{O2} in the developing root may be explained in terms of a formation of apoplastic barriers in the OPR (see Discussion). When considering a given position, there was a clear trend for J_{O2} to increase as the oxygen concentration increased. With exceptions at 50 and 60 mm, increases were always significant ($F_{3,37}$ = 173.6, 74.1, 27.5 and 14.5 for 30, 40, 50 and 60 mm, respectively; $P \leq 0.05$). At greater distances from the tip, differences in absolute values of J_{O2} tended to become smaller in response to an increasing O_2 concentration.

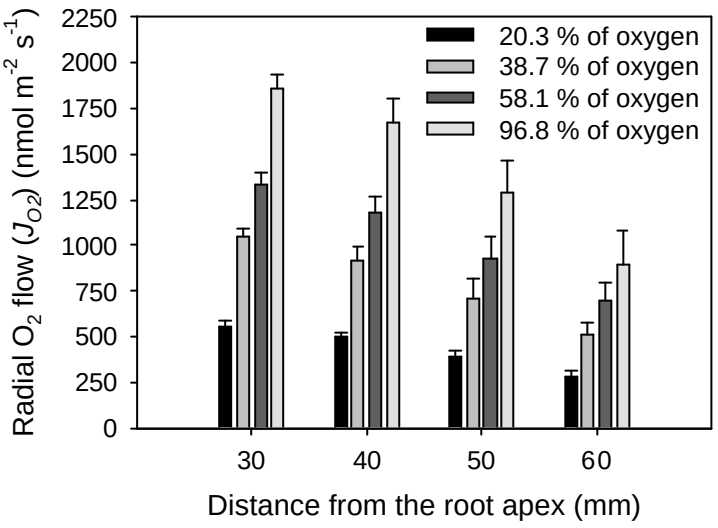


Fig. 5. Radial O_2 flow (J_{O2}) along rice root segments when perfused with moistened O_2/N_2 gas mixtures at different oxygen concentrations of 20.3, 38.7, 58.1, and 96.8% (equating to 21, 40, 60 and 100% O_2 in dry gas) at a mean overpressure of 10 kPa. For all oxygen concentrations, values of J_{O2} decreased along the root. When considering a given position, there was a clear trend for J_{O2} to increase as the oxygen concentration increased. Measurements were taken at 25 °C in a temperature-controlled room. Data given are means ±SE (n = 5-24).

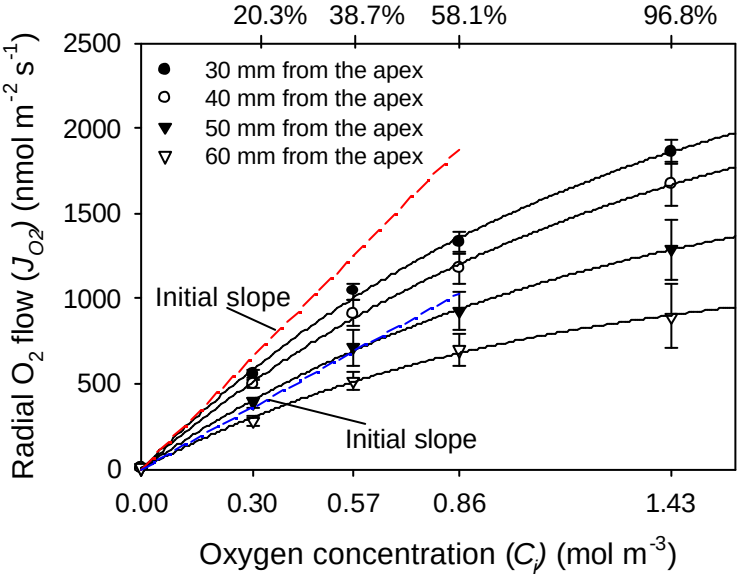
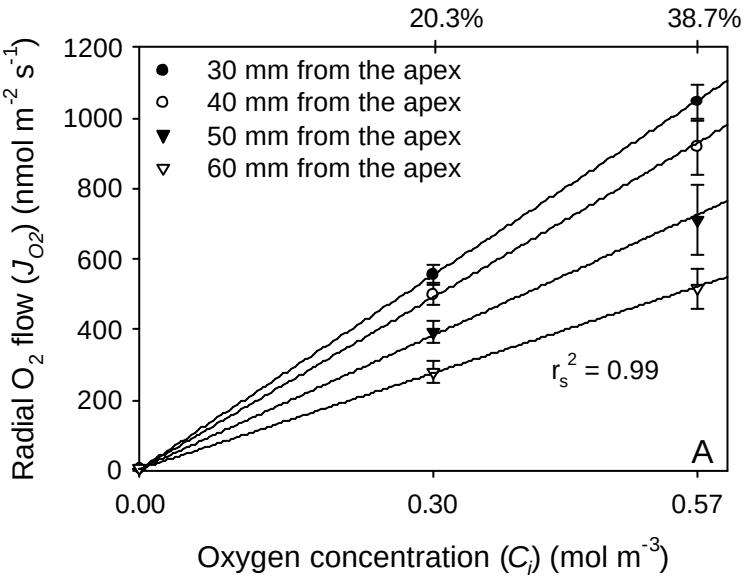


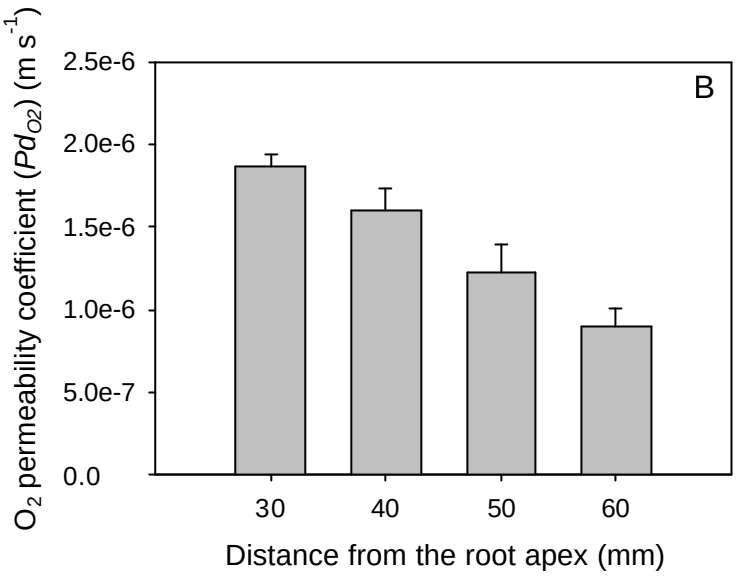
Fig. 6. Rates of radial oxygen flow (J_{O2}) along root segments plotted against the internal oxygen concentration (C_i). According to the Henry`s law, 20.3, 38.7, 58.1, and 96.8% oxygen (at a mean overpressures of 10 kPa) equated to 0.3, 0.57, 0.85, and 1.43 mol m^{-3} at the inner surface of OPR, respectively.

The radial O_2 flows (J_{O2}) increased with increasing O_2 concentrations, but increases in J_{O2} were not linear, which was due to limitations of the technique (see Discussion). Red and blue dashed lines indicate the initial slopes (0% O_2) of J_{O2}/C_i curves for 30 mm and 60 mm, respectively. Data given are means ±SD (n = 5-24 root segments).

At all four distances, increases of J_{O2} with increasing concentration were not linear (Fig. 6). This may be caused by unstirred layers outside the root, where zero concentration of oxygen at the electrode was assumed (all O_2 immediately reduced). Another reason may be an incomplete oxygen reduction, when two electrons are consumed per oxygen molecule rather than four, as described by Hahn *et al*. (1975; see Discussion). Because of the polarization effects at the Pt electrode at high J_{O2}, only the initial slopes of the curves in Fig. 6 were used to calculate the oxygen permeability coefficient (Fig. 7A). The highest values of the oxygen permeability coefficient of the OPR (Pd_{O2}) along the rice root segments were at the positions closer to the root apex (30 mm) and decreased along the root, reaching the lowest value at 60 mm (Fig. 7B). Pd_{O2} was significantly different between distances of 30 mm and 50 mm, and 40 mm and 60 mm ($F_{3,16} = 11.1$; $P \leq 0.05$). The mean value of Pd_{O2} at 60 mm was $0.9 \pm 0.1 \times 10^{-6}$ m s^{-1}, which was 2-fold smaller than that at 30 mm ($1.9 \pm 0.07 \times 10^{-6}$ m s^{-1}).

Fig. 7. (A) Because of limitations of the technique in the range of high O_2 concentrations (see Fig.6), only the initial slopes (0-38.7% O_2) of J_{O2}/C_i curves were used to calculate permeability coefficients of oxygen (Pd_{O2}). (B) The highest values of Pd_{O2} were close the root apex and decreased along the root, which is most likely due to suberization and/or lignification of roots. Data given are means ±SE (n = 5).





Radial oxygen flow across silicone and Teflon tubes

To tackle the problems arising from measurements at high J_{O2}, the radial flow of oxygen across the OPR of rice roots was mimicked using silicone and Teflon tubes of diameters similar to that of rice root segments. Tubes were perfused with the same mixture of O_2/N_2 gases (20.3, 38.7, 58.1, and 96.8% O_2), and rates of radial oxygen flow measured. The two different polymer tubes were employed, because they differed markedly in their permeability for oxygen (Zhang and Cloud, 2006). When using silicone tubes, the responses in J_{O2} to increased O_2 concentration were similar to those obtained for rice roots. In contrast, they were substantially smaller for Teflon (Fig. 8). Also, there was a non-linear response for silicone tubes (as for rice) and a linear response for Teflon (Fig. 8). The oxygen permeability coefficient (Pd_{O2}) of silicone rubber, calculated from the initial slope of the J_{O2}/C_i curve, was larger by a factor of 7.8 than that obtained for Teflon. Absolute values of permeability coefficients of silicone and Teflon tubes were $3.5 \pm 0.07 \times 10^{-6}$ m s^{-1} and $0.50 \pm 0.006 \times 10^{-6}$ m s^{-1}, respectively (n = 3; ± SE). Correcting for the resistance of shell of agar between the tube and the electrode surface, the oxygen permeability coefficient of silicone and Teflon tubes were $10 \pm 0.06 \times 10^{-6}$ m s^{-1} and $0.54 \pm 0.009 \times 10^{-6}$ m s^{-1}, respectively. As for the root segments, the data suggest limitations of the electrode technique in the presence of high ROL/J_{O2} as they occurred at O_2 concentrations >38.7% (see Discussion).

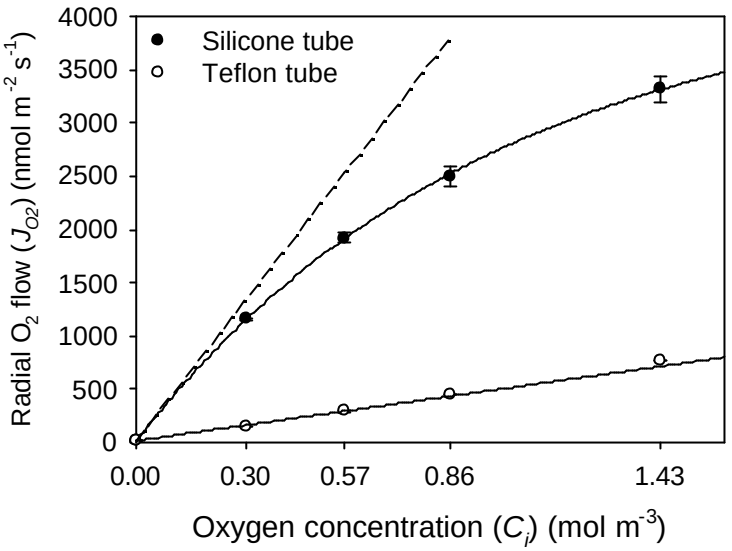
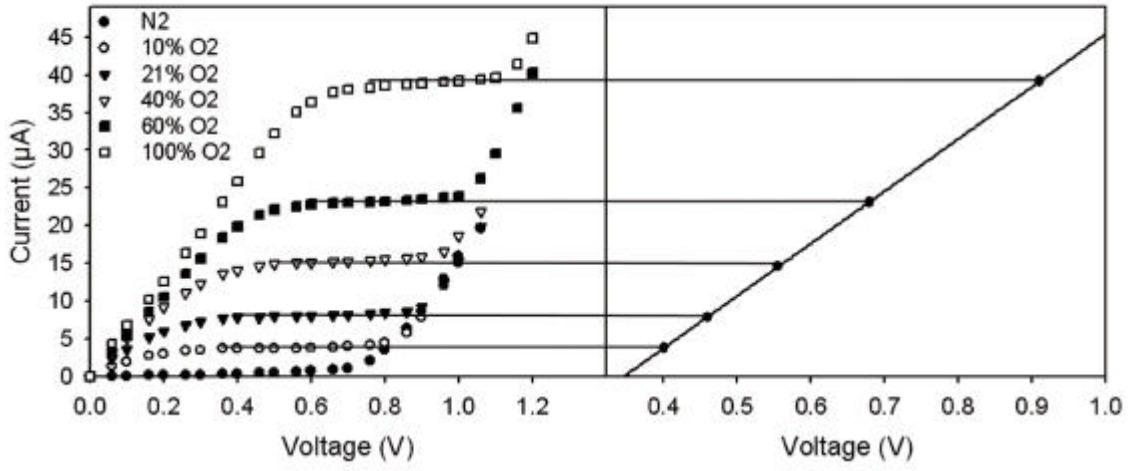


Fig. 8. Rates of radial oxygen flow from silicone (i.d., 0.5 mm; o.d, 0.9 mm) and Teflon (PTFE; i.d., 0.5 mm; o.d., 1.0 mm) tubes, when perfused with moistened gas mixtures of 20.3, 38.7, 58.1, and 96.8% concentration of O_2 at a mean overpressure of 10 kPa. As for the root segments, the relationship between J_{O2} and O_2 concentration was non-linear for silicone rubber (high permeability for O_2), but was linear over the entire range for Teflon (low O_2 permeability). The dashed-line for silicone rubber indicates the initial slope (0% O_2) of the J_{O2}/C_i curve. Data are means ±SE (n = 3).

The effects of non-complete reduction of oxygen at high rates of radial oxygen flow were tested further by increasing the voltage in steps to a polarizing voltage, which was higher than that usually used during ROL/J_{O2} measurements (around 0.5 V). Here, the voltage was increased every 20 s by 0.02 V up to 1.2 V and the resulting diffusional current was measured at each step change. Figure 9 shows the current-voltage curves for different oxygen concentrations perfused through the silicone tube at a mean overpressure of 10 kPa. (0, 9.6, 20.3, 38.7, 58.1, 96.8%; values corrected for humifidification effect). There was a linear relationship between the oxygen concentration and the applied voltage, i.e. higher oxygen concentrations required a higher voltage to reach the plateau, where the oxygen is completely reduced. It can be seen from Fig. 9 that saturation was reached at 0.46 V for 20.3% oxygen and at 0.92 V for 96.8% O_2. However, such a high plateau potential could not have been applied for 20.3% oxygen due to sharp increases of the current, which may have been caused by other effects such as the discharge of hydrogen ions at the electrode (Armstrong, 1967).

Hence, to measure J_{O2}, the usual procedure from the literature using the technique of Armstrong (1967, 1979) had to be adapted to the situation of high oxygen flow by increasing the polarizing voltage. In the literature, this limitation had not yet been made clear. This is the case because the usual ROLs (Gibberd *et al*., 1999; McDonald *et al*., 2001; Colmer, 2003a) were substantially smaller than the J_{O2}s measured in the present study. According to the present results, the measured maximum ROL was also much lower than the maximum J_{O2} (1300 nmol m^{-2} s^{-1} as compared with 1860 nmol m^{-2} s^{-1} for 96.8% O_2 at 30 mm from the apex; see Discussion).

Fig. 9. Current-voltage curves for oxygen concentrations of 0, 9.6, 20.3, 38.7, 58.1, and 96.8% perfused through the silicone tubes at the mean overpressure of 10 kPa. Polarizing voltage was increased in 0.02 V steps every 20 s up to 1.2 V, and the corresponding diffusional current was measured for each step change. Plateaus were reached when rates of oxygen diffusion to the electrode became independent of the voltage applied. It can be seen that there is a linear relationship between the oxygen concentration and the applied voltage.



Calculating the diffusivity of oxygen across the OPR

According to Equation 2, the resistivity of the shell of agar ($1/P_{agar}$) was calculated to be 1.8×10^5 s m^{-1}, which was 33 ± 1% (30 mm) and 16 ± 2% (60 mm) of the measured $1/Pd_{O2}$ ($5.4 \pm 0.2 \times 10^5$ s m^{-1} and $11.9 \pm 1.6 \times 10^5$ s m^{-1} for 30 mm and 60 mm from the apex, respectively). Hence, the agar layer did not dominate the overall resistivity, but could not be neglected. Correcting for the unstirred layers ($1/P_{agar}$) in Equation 3, P_{OPR} resulted in values of $2.8 \pm 0.2 \times 10^{-6}$ m s^{-1} (at a distance of 30 mm from the apex) and $1.1 \pm 0.2 \times 10^{-6}$ m s^{-1} (60 mm). These values related to the overall measured Pd_{O2}s of

$1.9 \pm 0.07 \times 10^{-6}$ (30 mm) and $0.9 \pm 0.1 \times 10^{-6}$ ms^{-1} (60 mm). Since the thickness of the OPR was known (85µm, Ranathunge et al., 2004), estimated values of P_{OPR} allowed calculation of the diffusivity of oxygen across the OPR according to Equation 4 (assuming a homogenous barrier). At 25 °C, the radial oxygen diffusivity, D_{OPR}, varied along the root, being $2.6 \pm 0.2 \times 10^{-10}$ m^2 s^{-1} and $1.0 \pm 0.1 \times 10^{-10}$ m^2 s^{-1} at 30 mm and 60 mm from the apex, respectively.

Determining zones of ROL using the redox dye methylene blue

Measurements of ROL with the root sleeving platinum electrode provided a quantitative measure of oxygen loss from the roots. With the methylene blue agar technique, it was possible to visualize the pattern of ROL from roots by formation of the blue haloes at the sites where roots lost oxygen. For rice roots of intact plants placed in the growth chamber for 20 h, ROL occurred from the root apex up to distances of 60-100 mm. At larger distances, only blue patches on the surface of the roots were observed. The photograph in Fig. 10A was taken after removing roots from the agar medium (methylene blue was attached to the root surface at the sites of oxygen loss).

When segments of rice roots were perfused with humidified air (20.3% O_2), blue haloes developed along the segments with time, first appearing at positions closer to root apices. After 15 min, slight blue oxidised haloes were observed up to distances of 50 mm from the apex (Fig. 10C). At this time, oxygen losses from more basal positions were not visible. After 30 min, blue haloes were well formed at positions closer to the apices, and started to develop at basal parts of the root (Fig. 10D). After 45 min, the entire root segments were surrounded by oxidised blue haloes (not shown).

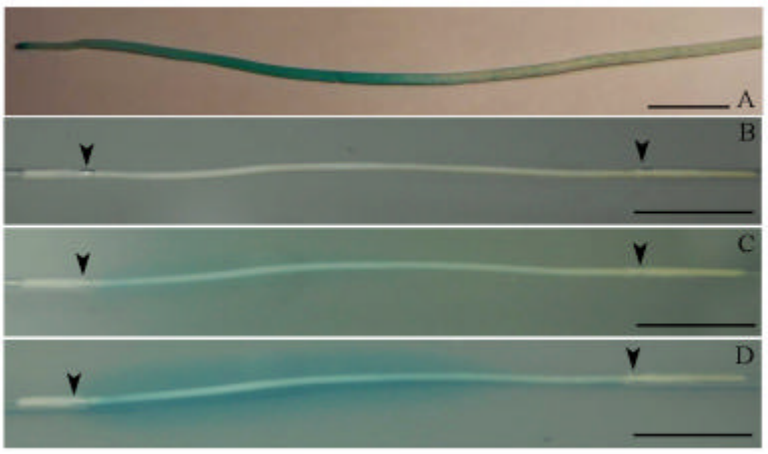


Fig. 10. (A) Development of oxidised methylene blue stains on the surface of the adventitious rice root. The rice root systems were placed for ~20 h in 0.75% agar medium, which contained the redox indicator methylene blue in reduced form. The photograph was taken after

removing roots from the agar medium. (B-D) Development of oxidised methylene blue haloes around the rice root segments. Root segments excised at the distance of 20-80 mm from the root apex were perfused with moistened air (20.3% O_2) at an overpressure of 20 kPa for 0 (B), 15 (C), and 30 (D) min. Blue haloes were formed at the sites of oxygen loss from the segments. Black arrows indicate fixing points of the root segments to glass capillaries. Bar = 10 mm.

Discussion

A new technique to measure permeability coefficients of the diffusion of oxygen across the OPR has been introduced in this study. The technique has been applied to rice roots grown under aerobic conditions, and the oxygen permeability was measured along the roots. There are not many quantitative data regarding the permeability of oxygen in plants. Most of the data refer to the permeability of cuticles isolated from leaves such as of *Citrus aurantium* L., the pericarp of *Capsicum annuum* L., and *Solanum lycopersicum* L., or from various conifer needles, or phellems isolated from trees (Lendzian, 1982; Lendzian *et al*., 1986; Groh *et al.,* 2002). Values presented for cuticles varied between 3×10^{-7} (*C. aurantium*), 1.4×10^{-6} (*C. annuum*), and 1.1×10^{-6} (*S. lycopersicum*) m s^{-1}. The range of permeabilities of cuticular membranes to oxygen for amphibious and submerged plants was 5-143 $\times 10^{-6}$ m s^{-1} (Frost-Christensen *et al*., 2003). The oxygen permeability of phellem of *Aesculus hippocastanum* was 7×10^{-6} m s^{-1}. As plant cuticles are usually regarded as rather impermeable, the present values of $(1.9 – 0.9) \times 10^{-6}$ m s^{-1} obtained for the OPR of rice roots may be regarded as rather low and the OPR an effective barrier for oxygen. This conclusion does not change when effects of unstirred layers are taken into account, which would result in values of (2.8 – 1.1) $\times 10^{-6}$ m s^{-1} for just the OPR. However, it has to be pointed out that the permeability of the OPR (Pd_{O2}) of rice roots grown under aerated conditions should be substantially larger than the Pd_{O2} of roots grown in stagnant solution. Garthwaite *et al*. (2008) assessed the oxygen permeability coefficient across the cell layers exterior to aerenchyma of stagnantly grown *Hordeum marinum*. The values were 2.9×10^{-5} at 5 mm from the apex and 1.1×10^{-6} m s^{-1} at 80 mm from the apex, which was similar to the values obtained in this paper, although there were substantial differences in the anatomy of the roots (see below).

The permeability coefficients of the outer cell layers of barley roots presented by Garthwaite *et al*. (2008) were assessed from measurements of ROL while either varying

shoot O_2 partial pressure or cooling the rooting medium to cancel out any respiratory effects. By means of these manipulations and in conjunction with calculated internal resistances (OPR, pore space, agar solution between root and sleeving electrode), the authors estimated the overall resistance of the outer cell layers (O_2 diffusion coefficient in the external cell layers). Another indirect method of calculating diffusion of oxygen across outer layers was presented by Armstrong *et al*. (2000) and applied to roots of *Phragmites australis*. It was based on the assumption that the drop of oxygen concentration across the epidermal-hypodermal cylinder was dependent on the diffusive resistance within the cylinder, the rate of oxygen consumption within it, and the radial oxygen transfer across it to the rhizosphere. Radial oxygen profiles at different distances from the root apex were obtained using Clark-type microelectrodes. The present approach differs from that of Armstrong *et al*. (2000) and Garthwaite *et al*. (2008) in that it did not require assumptions to be made about respiratory effects (the oxygen concentration in the aerenchyma was fixed by the rapid perfusion, see below), porosity, or root pore space resistances (e.g. the resistance to oxygen diffusion at the junction between shoot and root). The technique presented herein enabled, for the first time, to measure Pd_{O2} directly, since the oxygen concentration in the aerenchyma and radial flows of oxygen were known.

The experiments with rice roots segments precluded measurement of J_{O2} at distances closer than 30 mm from root apex, where the intercellular gas-filled spaces were not sufficiently developed for axial gas perfusion. In roots of the hydroponically grown rice, aerenchyma started to develop at 10-15 mm behind the apex (Ranathunge *et al*., 2003). Therefore, attempts at perfusing root segments excised at 10 mm failed, although ROL could be measured. In these experiments, the driving force for the radial oxygen flow was the difference in oxygen concentration (21, 40, 60 or 100%) between the aerenchyma and the outer O_2-free agar solution. The oxygen concentration of the gas mixtures perfused through the aerenchyma resulted in an equivalent concentration (or activity) in the water film right at the inner surface of OPR, driving the oxygen transport across the outer cell layers into the deoxygenated medium. To provide a constant oxygen concentration along the entire root segments, and to ensure that the rate of axial perfusion was much more rapid than the radial loss, perfusion had to be carried out at a certain overpressure at the entrance of segments. The overpressure of 20 kPa (average of 10 kPa = 1 m water column) was tested and found to be sufficient to provide rapid

perfusion, which could have been estimated from the fact that air bubbles appeared at the outlet side of segments. The overpressure of 30 kPa was rejected to avoid excessive pressurization of the roots. There was a slight tendency of J_{O2} to increase when the overpressure increased from 10 kPa to 30 kPa, but this was not significant (Fig. 4). Perhaps, it was caused by the higher partial pressure of oxygen or due to a slight increase in the volume of aerenchyma and a slight expansion of the OPR.

In the perfusion experiments, the forces driving the oxygen across the OPR (partial oxygen pressure in the aerenchyma) have been worked out from the oxygen concentrations in the O_2/N_2 gas mixtures taking into account that moistening the gas mixtures would reduce the partial oxygen pressure by ~3%. Also, the use of an overpressure was considered. The overpressure applied at the inlet side of the segments was assumed to drop linearly along the root. It results from the fact that the inner diameters of the tubes connecting the root segment with the moistening bottle and the air (Fig. 1) were substantially larger than those of aerenchyma, and there was no overpressure at the exit side of the segments. Hence, in all calculations of C_i, the mean value from the overpressure at the entrance and exit was used (for an overpressure of 20 kPa this was 10 kPa). As the resistance to axial gas flow was much smaller compared with radial, the assumption of a liner drop is reasonable.

As the gas flow along the aerenchyma of the root segments was viscous in nature, the volume flow in $m^3\ s^{-1}$ was calculated according to a modified Hagen-Poiseuille equation (viscous flow through a concentric annulus; see chapter 6.6 of White, 1999). The amount of oxygen supplied by the perfusion at the mean overpressure of 10 kPa was calculated to be 3.7×10^{-7} g s^{-1} at an internal oxygen concentration equivalent to 20.3% O_2. On a root volume basis, Luxmoore *et al*. (1970) calculated the overall rate of oxygen losses caused by respiration in rice root segments to be 3.9×10^{-8} g O_2 cm^{-3} of tissue s^{-1} (mean for segments excised at 20-60 mm from the apex). Since the volume of the 50 mm long segments used in this paper was ~3.9×10^{-2} cm^3, the amount of oxygen used for respiration was estimated to be 1.5×10^{-9} g O_2 s^{-1}. This is only 0.4% of the oxygen supplied by perfusion. In addition, the mean J_{O2} along the root segments for 20.3% was 1.4 g cm^{-2} s^{-1}. This equates to 2.2×10^{-9} g O_2 s^{-1} of a radial loss of O_2, or 0.58% of the oxygen supplied by perfusion. Hence, when using high rates of perfusion, a modification of the internal oxygen concentration (C_i) by either respiration or J_{O2}

could be neglected. At maximum, both effects should be ~1%. Overall, the effects of water vapour pressure (although considered), respiration, radial flows of O_2 were relatively small and did not have a significant impact on the final results. Another error could result from variations in the composition of the gas mixtures used (40, 60, 100% O_2), which were given by the supplier at an accuracy of ±2% (see above). Even more important, however, were variabilities between roots. Nevertheless, the data clearly indicate a decrease of Pd_{O2} as the roots developed (see Results), which is most probably due to a suberization/lignification of the OPR.

The present data showed that the increase in oxygen concentration in the perfusing gas did not result in a linear increase in radial oxygen flow (J_{O2}), as one may expect. Similar deviations from linearity were observed for silicone tubes of dimensions similar to that of rice roots at similar rates of radial O_2 flow. However, there was a linear response, when J_{O2}s were substantially smaller, such as with Teflon tubes. A linear relationship was also observed for intact plants, where the oxygen concentration was manipulated around the shoot, but the absolute values of ROL were relatively small, compared with those of J_{O2}. Hence, the effect of non-linearity was related to the fact that, at high rates of oxygen flow, the cylindrical platinum electrode did not give a linear response. In the usual ROL experiments, where O_2 is supplied from the shoot, the polarizing voltage of ~0.5 V was sufficient to reduce all oxygen lost from the root (Armstrong, 1967). In these experiments, absolute values of ROL were relatively small and the OPR acted as a membrane with relatively high permeation resistance such as the membrane in the Clark electrode (usually Teflon). This also applied when root segments were perfused with the oxygen concentrations of up to 40%, where the polarizing voltage of ~0.5 V was sufficient to reduce all O_2.

Non-linear effects of Clark-type electrodes were already known from other studies, but not as yet for plant roots. For example, for blood-gas analysers it was shown that the response of P_{O2} electrodes was often not linear with oxygen concentrations at high partial pressure of oxygen (P_{O2}; Crampton *et al.*, 1969; Severinghaus and Bradley, 1969; Mapleson *et al.*, 1970; Hill and Tilslay, 1973). The non-linearity was linked to an incomplete reduction of oxygen, which is reduced to hydroxyl ions in two steps (Hahn *et al.*, 1975):

$$O_2 + H_2O + 2e^- = HO_2^- + OH^-$$
$$\underline{HO_2^- + H_2O + 2e^- = 3OH^-} \quad (5)$$
$$O_2 + 2H_2O + 4e^- = 4OH^-.$$

In the first step, two electrons reduce oxygen to one hydrogen peroxide anion and one hydroxyl ion. In the second step of the reaction, two more electrons are required to reduce HO_2^- to three molecules of OH^-. As discussed by Hahn *et al.* (1975), an incomplete reduction of oxygen, where only two electrons are consumed rather than four, may lead to a considerable non-linearity, which would underestimate the J_{O2} at high oxygen concentrations. According to the overall equation for the reduction of oxygen at the platinum electrode, the Nernst equation would predict that, at a 10-fold change of P_{O2}, the electrode potential should change by 59.2/4 =14.8 mV (=$RT/4F$ at 25 °C). As the actual voltage used to overcome the increase in P_{O2} was much bigger, there should have been polarizing effects due to a concentration polarization and/or a reaction polarization (Hack, 1986). This would explain the bending of J_{O2}/C_i. It appears that the precise nature of the processes resulting in an underestimation of J_{O2} at high rates of oxygen flow is not yet known (such as with rice root segments used in the present study and silicone tubes). Overall, what remains is a *caveat*, when using platinum electrodes in an amperometric way such as the ring electrode in the presence of high J_{O2}, as done in this study. As shown, the polarizing effect can be overcome by increasing the polarizing voltage (Fig, 9). However, there may be limits to using high polarizing voltages due to reduction of hydrogen ions at the electrode, which occurs above 0.8V (Armstrong, 1967). Nevertheless, complications in terms of polarizing effects can be excluded when the rates of radial flow of oxygen are low such as during usual ROL experiments (Colmer *et al*., 1998; Gibberd *et al*., 1999; Visser *et al*., 2000; McDonald *et al*., 2002; Colmer, 2003a).

The present data of ROL may be common with many wetland plants, which contain a constitutive barrier to oxygen losses in the basal root zones (Colmer *et al*., 1998; Visser *et al*., 2000; McDonald *et al.*, 2002; Colmer, 2003a). The growth in stagnant conditions may induce a barrier in plants, where basal positions remain permeable to O_2 when grown in aerated media (Colmer *et al*., 1998; McDonald *et al.*, 2002; Colmer, 2003a). Stagnant growth may increase the barrier in plants, where the barrier is constitutively present. Thus, there may be criticism that, in the present study, roots grown in aerated solution were used, which may not reflect the natural paddy field conditions, where

Pd_{O2} of the OPR should be substantially lower. The aerated growth conditions were used to (i) develop the new technique for measuring Pd_{O2} and (ii) compare it with already existing data on water permeability across the OPR (see below; Ranathunge *et al.*, 2003, 2004). Presently, the perfusion technique is used to measure the permeability coefficient of roots of plants grown in stagnant conditions. Preliminary results showed that growth in deoxygenated solution significantly reduced Pd_{O2} along entire root axes. If this is true, the generally held view that under anoxic conditions the oxygen transport to the root tips limits root growth may be questioned, when the axial diffusional resistance is low. Knowledge about the oxygen permeability across the OPR may help to model the oxygen flow in rice roots.

The anatomical basis of the barrier to ROL was not examined in the present study. However, in previous reports it was linked to the dense arrangement of the hypodermal cylinder in *Halophila ovalis* (Connell *et al*., 1999), suberization and/or lignification of outer cell layers (Armstrong and Armstrong, 1988; Jackson and Armstrong, 1999), and a combination of densely packed cells, suberin deposits, lignification, and oxygen consumption by cells exterior to aerenchyma (Armstrong *et al*., 2000). In rice, the barrier to ROL may be related to either densely packed sclerenchyma cells with thick lignified secondary walls or suberized exodermis with Casparian bands, or both. Exodermis formed a tight juncture with the epidermis and a layer of sclerenchyma (Clark and Harris, 1981). Exodermal suberin lamellae of hydroponically grown rice were observed at ~30 mm from the tip and matured at ~50 mm (Ranathunge *et al.*, 2003, 2004). Well-developed Casparian bands were found at ~50 mm from the apex (Ranathunge *et al*., 2003). Different root structures among a range of plant species may be directly related to differences in their oxygen diffusion coefficients across cell layers exterior to aerenchyma. The diffusion coefficient of the outer part of rice roots, D_{OPR}, varied along the root being $2.6 \pm 0.2 \times 10^{-10}$ m^2 s^{-1} and $1.0 \pm 0.1 \times 10^{-10}$ m^2 s^{-1} at 30 mm and 60 mm from the apex, respectively. These values are smaller by factors of 9 and 24 than the diffusion coefficient of oxygen in water at the same temperature. According to Armstrong *et al*. (2000), the oxygen diffusivity of the outer cell layers of *P. australis* was estimated to be 5.5×10^{-11} m^2 s^{-1} at 29 mm from apex. This is smaller by about one order of magnitude than the D_{OPR} of rice roots, most probably due to the existence of a suberized multilayered exodermis (Soukup *et al*., 2006). In *Hordeum marinum*, grown in stagnant agar nutrient solution, the diffusion coefficient was reduced from 1.6×10^{-9}

$m^2\ s^{-1}$ near the root tip (5 mm), to $6.0 \times 10^{-11}\ m^2\ s^{-1}$ in the basal regions (80 mm), which was also referred to anatomical changes (suberization or lignificaton as concluded from epifluorescence; Garthwaite *et al*., 2008).

A barrier to ROL may be an adaptive feature of plants from wetland habitats, but may also have some drawbacks such as impeding water and nutrient uptake (Armstrong, 1979; Koncalova, 1990; Colmer and Bloom, 1998; Garthwaite *et al*., 2008). The diffusional water permeability of the OPR (Pd_{OPR}) was $3.0 \pm 1.6 \times 10^{-7}$ m s^{-1} for the immature root segments (20–50 mm from the root apex) and $2.0 \pm 0.7 \times 10^{-7}$ m s^{-1} for mature root segments (50–100 mm from the apex; Ranathunge *et al.,* 2004). It was smaller by an order of magnitude than the Pd_{O2}. However, it has to be taken into account that the Pd_{OPR} was smaller by a factor of 600 (20-50 mm from apex) and 1400 (50-100 mm from apex) than the hydraulic conductivity (high Lp_{OPR}/P_{fOPR} ratio; Ranathunge *et al*., 2004). The latter parameter is the one that is important during water uptake. It should be noted that the hydraulic conductivity of the OPR was larger by a factor of 30 than the overall value measured for the hydroponically grown rice roots. In other words, the water transport across the endodermis was rate limiting. It appears that, in rice, rates of water uptake can be quite high in the presence of a high capability to retain oxygen, which is favourable for the plant. It is possible to argue that rice roots have evolved an optimum balance between O_2 retention and water uptake. This may be different for roots grown under stagnant conditions, and important with regard to overall productivity of rice plant.

When considering the flow of oxygen along the whole plant from the shoot to the roots, the main resistance should be located in the OPR. It was indicated by the comparison of the slopes of the response of ROL and J_{O2} to increased oxygen concentration. In the experiments with intact rice plants, the slopes of the response of ROL to external O_2 concentrations were not significantly different at different positions, so it was possible to calculate one slope for all data. The pooled slope would represent the O_2 permeability coefficient of the whole plant (including the diffusive resistance in the shoot and in the root), which equalled $1.1 \pm 0.1 \times 10^{-6}$ m s^{-1}. This is smaller by a factor of 1.7 than the Pd_{O2} at 30 mm from the root apex and 1.2-fold larger than the Pd_{O2} at 60 mm from the root apex. The latter result of a ratio close to unity may be caused by the fact that there was some uncertainty in the measurement of both Pd_{O2}s. Overall, the comparison of the data indicates that the main resistance to oxygen flow in the plant was located in the

OPR. Considering the fact that, in the present experiments, the Pd_{O2} of roots grown in aerated medium should be higher compared with roots grown in stagnant medium, the latter conclusion should also hold for stagnantly grown plants.

In conclusion, the present study provided a new technique for measuring oxygen permeability across cell layers exterior to aerenchyma and the first quantitative data of the O_2 permeability at different positions of developing roots of rice. These data are of importance to understand the mechanisms controlling ROL and the ecophysiology of wetland species. Although the rice plants used in the present study were grown under aerated conditions, the absolute values of Pd_{O2} were relatively low, and similar to plant cuticles. Preliminary results showed that rice roots grown in stagnant conditions have much lower Pd_{O2} compared with roots of plants grown in aerated solution. Due to the resistance of the agar layer surrounding the root, the measured Pd_{O2}s were smaller by 33-16% than the actual values of P_{OPR}, which referred to just the outer cell layers. As roots matured, the resistance to radial movement of oxygen across the OPR increased from 30 mm to 60 mm from the apex by a factor of 2.5. It appears that, in rice, this is a constitutive, adaptive feature enhancing longitudinal diffusion of oxygen to the root apex. Non-linear responses of rates of radial oxygen flow (J_{O2}) to increased oxygen concentration in the perfusing gas mixtures indicated that some care has to be taken when using root-sleeving platinum electrodes (Clark-type electrodes without membranes) in the presence of high O_2 fluxes. Experiments with silicone and Teflon tubes supported that view. At high rates of J_{O2}, there may be processes tending to underestimate the actual value of radial flow of oxygen (e.g. incomplete reduction of O_2 at the electrode surface and other polarizing effects). When high oxygen concentrations were applied around the shoot of intact plant, absolute values of J_{O2} were much smaller, and there were no problems with the platinum electrodes, which also agrees with literature data of ROL.

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2. Functional and chemical comparison of apoplastic barriers to radial oxygen loss in roots of rice (*Oryza sativa* L.) grown in aerated or deoxygenated solution

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Abstract

Radial oxygen loss (ROL) and root porosity of rice (*Oryza sativa* L.) plants grown in either aerated or deoxygenated (stagnant) conditions were combined for the first time with extensive histochemical and biochemical studies of the apoplastic barriers in the roots' peripheral cell layers. Growth in stagnant solution significantly affected structural and consequently physiological features of rice roots. It increased adventitious root porosity by about 20% and decreased the ROL towards the base to zero at a distance of 40 mm from the apex. In contrast, roots of plants grown in aerated solutions revealed the highest rates of ROL at 30 mm from the apex. Differences in the ROL pattern along the root were related to histochemical studies, which showed an early development of Casparian bands and suberin lamellae in the exodermis, and lignified sclerenchyma cells in roots of plants grown in deoxygenated solution. In agreement with anatomical studies, absolute contents of suberin and lignin in the outer part of the roots (OPR) were higher in plants grown in deoxygenated solution. Regardless of growth conditions, the levels of suberin and lignin increased along the roots towards the base. It is concluded that radial oxygen loss can be effectively restricted by formation of suberized exodermis and/or lignified sclerenchyma in the OPR. However, relative contribution of suberin and lignin in a formation of a tight barrier is unclear. Knowing the permeability coefficient across OPR for roots of plants grown in both conditions will allow more precise understanding the mechanisms controlling ROL.

Key words: diffusion, outer part of root, lignin, *Oryza sativa,* radial oxygen loss, suberin, transport, waterlogging

Introduction

Rice (*Oryza sativa* L.) often is cultivated in flood-prone environments, which are usually anaerobic and chemically reduced, because of slow diffusion of oxygen in water and rapid consumption of oxygen by soil microorganisms (Ponnamperuma, 1984; Laanbroek, 1990). To overcome these threats, rice as other aquatic plants, is adapted both metabolically and structurally in numerous ways. Main adaptations include (i) the internal aeration by forming aerenchyma along the entire plant connecting the aerial parts with submerged organs, and (ii) induction of strong barriers in the root peripheral layers external to aerenchyma to impede radial oxygen loss (ROL; Armstrong, 1979; Colmer, 2003b).

Usually, the barrier to ROL in roots is related to suberization and/or lignification of the walls of root peripheral layers (Armstrong *et al*., 2000; De Simone *et al*., 2003; Soukup *et al*., 2006). In rice, the well developed outer part of the root (OPR) consists of four cell layers: rhizodermis, exodermis, sclerenchyma cells and one layer of cortical cells (Ranathunge *et al*., 2003). It is known that the suberized exodermis and/or lignified sclerenchyma cells act as a barrier to impede ROL (Kotula and Steudle, 2008). There are two consecutive developmental stages of the exodermis: (i) the formation of Casparian bands in radial and transverse walls impregnating primary cell wall pores with liphophilic and aromatic substances and (ii) the deposition of suberin lamellae to the inner surface of anticlinal and tangential cell walls (Clark and Harris, 1981; Peterson, 1989; Ranathunge *et al*., 2004). Modifications of apoplastic barriers, investigated by using histochemical staining techniques, allowed the description of developmental stages of roots in terms of structural changes in cell walls.

An insight into the chemical composition of modified cell walls can be obtained only by directly analysing the compounds occurring in such walls (Schreiber *et al*., 1999; De Simone *et al*., 2003). There is an enzymatic method for the isolation of modified root cell walls and for analysing them after chemical modification using gas chromatography and mass spectrometry (Schreiber *et al*., 1994; Schreiber *et al*., 1999). Suberin is a heterogeneous extracellular biopolymer closely attached to the inner primary cell walls (Schreiber *et al*., 1999; Bernards, 2002). It consists of aliphatic monomers (ω-hydroxy acids, diacids, primary fatty acids, primary alcohols and 2-hydroxy acids) esterified with aromatic compounds like ferulic acid and coumaric acid, and of cell wall

carbohydrates (Zeier and Schreiber, 1997; Kolattukudy, 2001). Lignin is a biopolymer bearing the three aromatic residues *p*-hydroxyphenyl, guaiacyl, and syringyl (Freudenberg, 1965; Boudet, 1998; De Simone *et al*., 2003). It has been suggested that suberin and lignin biopolymers are involved in pathogen defence, by a breakdown of the polymers by enzymes of microbes and subsequent release of toxic phenolic compounds. In addition, suberin and lignin act as mechanical barriers against pathogen invasion. They restrict entry of soil toxins at mature root zones, whereas high rates of ROL near the root apex enable oxidations in the rhizosphere including soil toxins (Armstrong, 1979; Armstrong J *et al*., 1996; Peterson, 1997; Colmer, 2003b).

In wetland plants, the barrier to ROL should affect water and nutrient uptake (Armstrong, 1979; Koncalova, 1990). When measured using a root pressure probe, the hydraulic conductivity of rice roots was 2- to 5-fold lower than that of corn (Zimmermann *et al*., 1998; Miyamoto *et al*., 2001). However, when rice was grown in aerated conditions, the main hydraulic resistance was located not in the OPR but in the endodermis (Ranathunge *et al*., 2003). A recently developed technique for measuring oxygen permeability coefficient allowed for a quantitative comparison of oxygen and water flows across the OPR (Kotula and Steudle, 2008). It was shown that diffusional water permeability was smaller by one order of magnitude than that of oxygen (Ranathunge *et al*., 2004; Kotula and Steudle, 2008). However, differences between diffusional and bulk water permeabilities indicated that rice has the ability to minimize oxygen loss from roots in the presence of high water uptake. It can be achieved by differences in the transport mechanisms – diffusional oxygen versus bulk flow of water (Ranathunge *et al*., 2004; Kotula and Steudle, 2008). Since there are no data available for rice grown in deoxygenated solution, the above conclusion may hold only for plants grown in aerated conditions. It is already known that oxygen permeability coefficient across the OPR of rice grown in stagnant solution dramatically decreased along the entire root compared with plants grown in aerated solution (Kotula *et al*., in preparation).

In the present study, radial oxygen loss from roots of plants grown in different conditions (aerated and stagnant) was related to the pattern and composition of suberin and lignin in the outer part of rice roots. Under both growth conditions, the levels of suberin and lignin in the OPR increased along the root axis. However, in plants grown in deoxygenated solution, the absolute amounts of these substances in root peripheral

layers were significantly greater developing a strong barrier to ROL. Histochemical studies correlated with biochemical analyses revealed early development of barriers to ROL in these plants. As a consequence, ROL markedly decreased along the root in plants grown in deoxygenated solution when compared with plants from aerated solution. The present results provide a basis for further studies of the oxygen permeability coefficient across the OPR, using the technique of Kotula and Steudle (2008).

Materials and methods

Plant material

Seeds of an upland rice cultivar (*Oryza sativa* L. cv. Azucena; International Rice Research Institute, Manila, Philippines) were germinated between moistened paper towels placed in Petri dishes in a climatic chamber (day/night rhythm: 12/12h, 27/22°C, light intensity: 500 µmol m^{-2} s^{-1}). Six days after germination, seedlings were transferred to a hydroponic container with aerated nutrient solution (Ranathunge *et al*., 2003; Kotula and Steudle, 2008). Seven days later, half of the seedlings were transferred to stagnant deoxygenated 0.1% agar nutrient solution. This concentration has been shown to prevent convection in the nutrient solution, thus simulating the impeded gas movements which occur in waterlogged soils (Wiengweera *et al*., 1997; Colmer *et al*., 1998). To prepare agar nutrient solution, high purity agar (Sigma-Aldrich, Steinheim, Germany) was dissolved in nutrient solution (without phosphate, Fe-EDTA and micronutrient stocks) by heating to boiling. The solution was allowed to cool to 30°C, after which phosphate, Fe-EDTA and micronutrients were added while stirring. These nutrients would have precipitated at high temperatures. Before placing the root systems in the agar solution, the medium was bubbled with nitrogen gas for several minutes for deoxygenation. Oxygen concentration in the stagnant medium measured randomly in selected days during the growth period ranged between 0.0 - 0.2 mg L^{-1}. Aerated and agar nutrient solutions were renewed weekly.

Measurements of root porosity

Using the microbalance method of Visser and Bögemann (2003), the porosity expressed as v/v (volume/volume) percent of gas space of tissue was measured on adventitious roots from plants grown in aerated and stagnant conditions. Ten millimeter long root

segments were cut with a razor blade from roots at distances of 5-15, 15-25, 25-35, 35-45, 45-55, 55-65 and 95-105 mm from the apex. After gently blotting them on tissue papers to remove adherent water, the weights of segments (w_1) were measured, and immediately transferred into Eppendorf tubes to prevent weight losses by evaporation. Then root segments were infiltrated with water under vacuum, blotted, closed in Eppendorf tubes and weighed again (w_2). The porosity was calculated from:

$$Porosity = 100 \times (w_2 - w_1) \times \frac{SW}{w_2} \quad (\%; v/v) \ , \qquad (1)$$

with SW representing the specific weight of water (=1.00 g mL^{-1}).

Measurements of radial oxygen loss

Rates of radial oxygen loss from adventitious roots of intact plants were measured using root-sleeving platinum O_2 electrodes following the method of Armstrong (1967), and Armstrong and Wright (1975). Root systems of plants from either aerated or stagnant cultures were immersed in a chamber containing deoxygenated solution of composition: 0.1% (w/v) agar, 5 mol m^{-3} KCl and 0.5 mol m^{-3} $CaSO_4$ (Colmer, 2003a; Kotula and Steudle, 2008). The shoot base was fixed to the rubber lid on the top of the chamber so that the shoot was in air (21% of O_2 v/v) and roots in the O_2-free medium. First measurements were taken 2 hours after transferring the plants to the chambers. For each plant, one adventitious root was inserted through the cylindrical platinum electrode (inner diameter: 2.25 mm, height: 5mm) fitted with guides to keep the root in the centre of the electrode. ROL was measured along the root with the centre of the electrode at positions of 5, 10, 20, 30, 40, 50 and 60 mm from the apex. Due to formation of laterals, measurements with the cylindrical electrode were not possible at positions above 60 mm. Measurements were taken at 28°C in the climatic chamber, where plants had been previously grown. Diameters of roots at given positions were measured using a digital calliper (700-Digital, Mitutoyo, China).

Histochemical detection of apoplastic barriers in the OPR

Healthy adventitious roots of rice plants grown either in aerated hydroponics or stagnant agar culture were cross-sectioned at distances of 10, 20, 30, 40, 50 and 60 mm from the apex. To detect the development of Casparian bands along the root exodermis, sections were stained for 1 h with 0.1% (w/v) berberine hemisulfate and for another hour with

0.5% (w/v) aniline blue (Brundrett *et. al*., 1988). Sections were viewed under an epifluorescence microscope using an ultraviolet filter set (excitation filter BP 365, dichroic mirror FT 395, barrier filter LP 397; Zeiss, Oberkochen, Germany). Structures containing Casparian bands were detected by bright yellow fluorescence. To detect the suberin lamellae in the exodermis, cross-sections were stained with the lipophilic fluorochrome fluorol yellow 088 (Brundrett *et. al*., 1991). The aliphatic component of suberin in cell walls was detected by yellow fluorescence under an epifluorescence microscope using ultraviolet light. To stain lignin in cell walls of the OPR, cross sections were stained for several minutes with phloroglucinol/hydrochloride at room temperature (Jensen, 1962). Under white light, stained lignin within the cell walls was observed as a bright orange/red layer.

Cell wall isolation and preparation for chemical analysis

Healthy roots selected from either aerated hydroponics or stagnant agar cultures were excised from plants. Root segments of lengths of 10 mm were prepared from 5 to 65 mm from the apex, i.e., at 5-15, 15-25, 25-35, 35-45, 45-55, and 55-65 mm. To prepare samples at given distances from the root apex, segments from 20 roots were pooled. Excised root segments were incubated for 2 to 3 weeks in enzymatic solutions containing 1% (v/v) cellulase (Celluclast, Novo Nordisk, Denmark) and 1% (v/v) pectinase (Trenolin, Erbslöh, Germany) in citric buffer (0.01 M) at pH 3.0. After this period, the walls, which were not modified by apoplastic barriers such as suberin or lignin, were digested away. Modified walls such as the endodermis or stelar xylem vessels resisted the treatment. The central cortex of rice roots is composed of a well developed aerenchyma which separates the central cylinder from the four cell layers at the root periphery in series: rhizodermis, exodermis, sclerenchyma and one layer of cortical cells (altogether named outer part of the root; OPR). For chemical analyses, OPR sleeves were separated under a binocular microscope using a pair of fine-tipped forceps, splitting root segments longitudinally and pulling the central cylinders away from the segments. Isolated wall samples were washed in borate buffer (0.01 M; pH 9.2) for 2 d replacing the solution every day, and followed by 3 washes with deionized water. Then, the samples with cell wall materials were thoroughly extracted in a mixture of chloroform and methanol (1:1; v/v) for 2 d replacing the solution several times. The resulting wall material was dried and stored over silica gel for subsequent chemical analyses.

Suberin analysis of cell wall material of the OPR

To depolymerize suberin, dried cell wall samples were transesterified using a mixture of methanol/borontrifluoride (MeOH/BF_3; Fulka, Germany) at 70 °C for 16 h as described by Zeier and Schreiber (1998). Released suberin monomers, such as fatty acids, alcohols, ω-hydroxy acids, diacids and 2-hydroxy acids, were derivatized using 20 µl of pyridine and 20 µl of BSTFA (*N*,*N*-bis-trimethylsilyltrifluoroacitamide; Machery-Nagel, Düren, Germany) at 70 °C for 40 min. Derivatization converted free carboxyl- and hydroxyl groups to their trimethylsilyl (TMS) esters and ethers, respectively. TMS-derivatives were analyzed using gas chromatography (GC) and mass spectrometry (MS; Agilent Technologies, Böblingen, Germany). Released monomers with TMS-derivatives were quantified by gas chromatography (GC) and flame ionization detection (Agilent Technologies, Böblingen, Germany) using a quadruple mass selective detector (HP 5971A, Hewlett-Packard) and referring to an internal standard (20 µg *n*-dotriacontane). Results of suberin analyses of samples from the OPR were related to root unit surface area. Surface area of the OPR was calculated from the lengths and diameters of root segments. For each treatment, three replicate analyses were used.

Lignin analyses of cell wall material of the OPR

To analyse lignin in the OPR, the peripheral root layers were isolated from enzymatically digested root segments of plants grown either in aerated hydroponics or in stagnant agar cultures. After washing in borate buffer at pH 9.2 and distilled water, cell wall preparations were extracted in a 1:1 mixture of methanol/chloroform. Dried wall samples of 1 to 10 mg were used for lignin analyses using the photometric method of Johnson et al. (1961). Wall preparations were mixed with 1 mL of acetyl bromide/acetic acid (1:3 v/v; Merck, Darmstadt, Germany) in loosely capped glass tubes and incubated in a water bath at 70 °C with occasional shaking for 30 min. Solubilized samples were cooled to about 15 °C and transferred to a mixture of 0.9 mL of 2M NaOH and 5 mL of glacial acetic acid (to hydrolyze excess acetyl bromide). To stop the reaction, 0.1 ml of 7.5 M hydroxylamine-HCl was added to the samples, followed by dilution of the solution to exactly 10 mL with more acetic acid. The absorbance of each sample was measured with a spectrophotometer (UV mini-1240, Shimadzu, Duisburg, Germany) at 280 nm. Lignin of 10 µg/mL resulted in an absorbance of about 0.24 (Johnson *et al.*, 1961). As for suberin, results of lignin

analyses of the OPR were related to unit root surface area. Three replicates were used for each treatment.

Statistical analysis

Data on root porosity, ROL, and chemical analysis were analysed using repeated measurements analysis of variance (ANOVAR) to examine the effects of treatments (between-subject factor) and position measured along roots (within-subject factor). Means are accompanied by standard errors, and were compared at $P \leq 0.05$ level using paired t-test (effect of position within one treatment) and two-sample t-test (effect of treatment within a distance).

Results

Growth of plants in aerated and stagnant solutions

In aerated nutrient solution, the greatest length achieved by shoots ranged from 640 to 690 mm. There were usually three primary tillers and 12 to 15 leaves. Growth in stagnant solution resulted in fewer tillers and slower leaf and root development (Fig. 1). The shoot usually consisted of one tiller and four to five leaves. The greatest lengths of shoots ranged from 480 to 540 mm. In aerated solution, the average length of the longest adventitious roots was between 530 and 580 mm. When grown in stagnant solution, the longest adventitious roots were only about 45% of the length of those grown in aerated solution (300-320 mm or shorter). Adventitious roots of 30 d old plants in aerated and stagnant solutions grew at rates of 31±2 mm d^{-1} and 19±1 mm d^{-1}, respectively (SE; n = 18 to 40 roots). Along the entire lengths of roots, the stagnant treatment resulted in increasing root diameters, which were significantly larger than those grown in aerated medium (two-sample t-test at $P < 0.001$). At 60 mm from the apex, the mean diameter of these roots was 1.1 ± 0.01 mm (SE; n = 120). For plants grown in deoxygenated stagnant solution, the corresponding diameter was 1.2 ± 0.02 mm (SE; n = 89 roots).

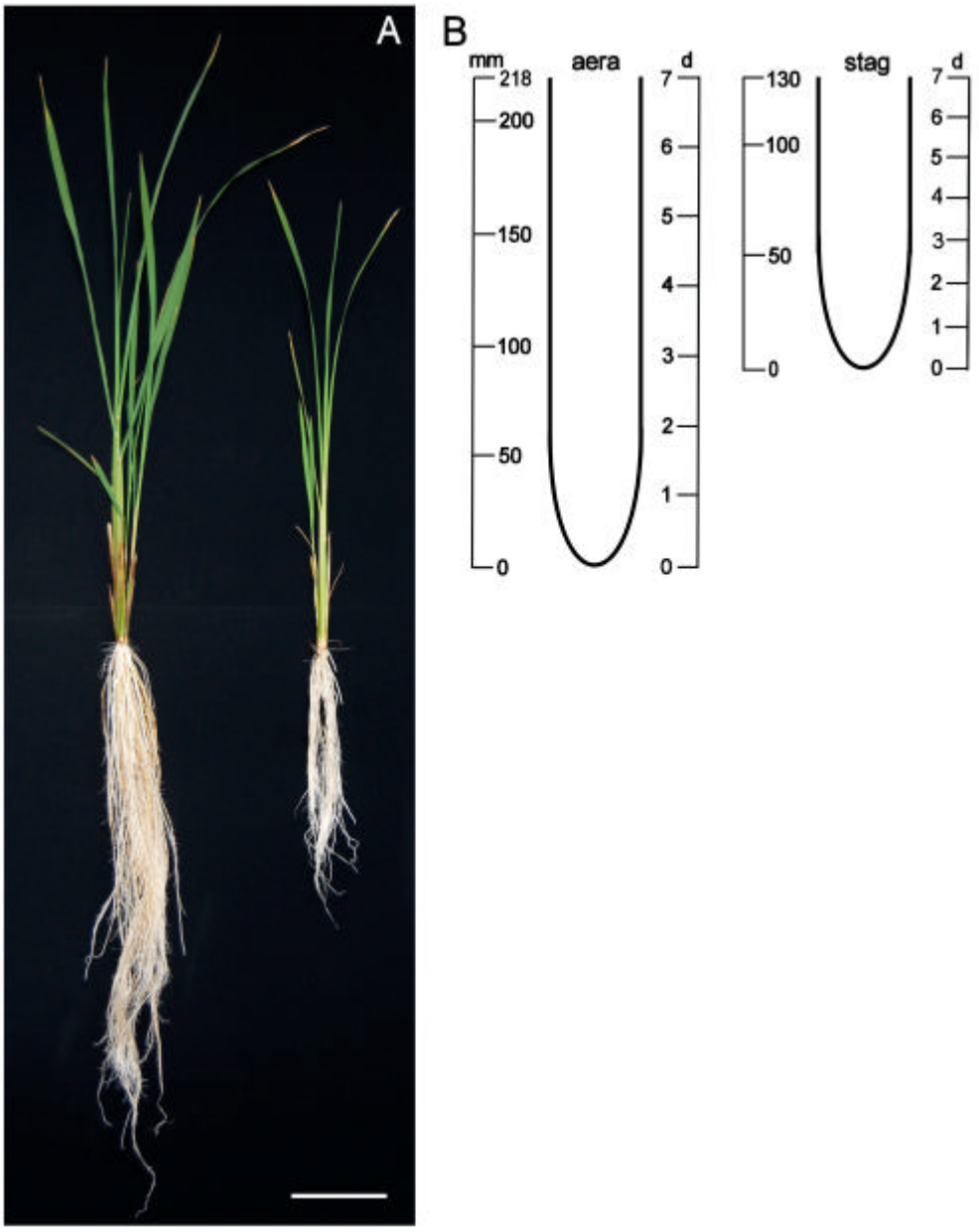


Fig. 1. (A) 30-day-old rice plants grown under either aerated or deoxygenated conditions. Compared with stagnant solution, plants form aerated culture displayed longer shoots and roots. Bar = 100 mm. **(B)** The relative growth of roots of rice plants grown in either aerated or stagnant conditions. Scales to the leftof each root indicate root length in millimetres. Scales to the right of each root indicate the tissue age in days. *aera*, aerated, *stag*, stagnant.

Porosity of adventitious roots of rice grown in aerated and stagnant deoxygenated nutrient solution

Both, aerated and stagnant conditions resulted in increases of root porosity along adventitious roots ($F_{6,48}$ = 186.0; P < 0.001). For plants grown in aerated solution, root porosity increased by a factor of 4.6 from 9.6 ± 0.7% at 10 mm to 44.0 ± 2.7% at 100 mm from the apex. Increases were, however, not significantly different between distances of 10 and 20 mm (paired t-test at $P \leq 0.05$; Fig. 2). During stagnant treatment, porosity increased 5.2-fold, from 10 ± 1% at 10 mm to 54 ± 4% at 100 mm. The marked differences for these roots were observed between each distance from the apex (paired t-test at $P \leq 0.05$). At all distances, the stagnant treatment resulted in a somewhat greater root porosity when compared with roots of plants grown in aerated solution ($F_{1,8}$ = 14.7; P = 0.005; Fig. 2). However, there were no significant differences at distances of 10 and 100 mm (two-sample t-test at $P \leq 0.05$). The highest difference of 1.6-fold was observed at 30 mm from the apex.

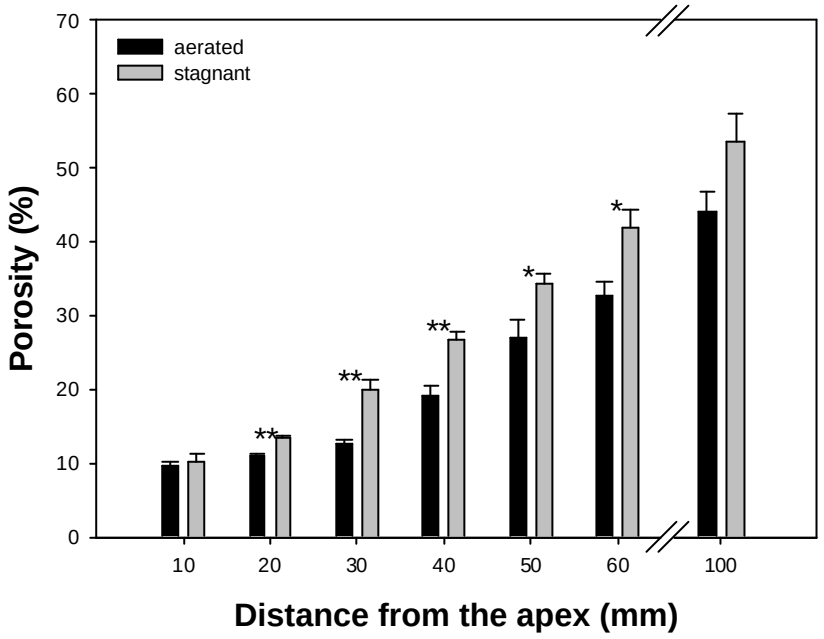


Fig. 2. Porosity as a percentage of root volume (equation 1) of adventitious roots of rice plants grown for 30-40 d in either aerated or stagnant solutions. Porosity was measured for 10 mm root segments at different distances along the root. At all distances (with exception for 10 and 100 mm), the stagnant treatment resulted in significantly greater root porosity, when compared with roots of plants grown in aerated solution. Significance levels of $P \leq 0.05$ or $P \leq 0.01$ are denoted by either * or **, respectively (two-sample t-tests). Data are means ± SE (n = 5 roots).

Radial O_2 loss (ROL) from intact adventitious roots grown in aerated or stagnant solutions

The profiles of ROL along adventitious roots of plants grown in either aerated or stagnant solutions differed considerably ($F_{6,90}$ = 83.7; P < 0.001; Fig. 3). ROL from adventitious roots of plants grown in aerated solution was the highest at 30 mm from the apex. Lower rates of ROL at positions near the root apex indicated a lower oxygen concentration within the aerenchyma due to losses of oxygen in more basal position (but not at the very base), and/or consumption of oxygen by living cortical cells. Lower rates of ROL near the basal zones indicated a formation of apoplastic barrier(s) to radial O_2 diffusion. In contrast, adventitious roots of plants grown in stagnant solution showed the highest rates of ROL just behind the apex (5 and 10 mm from the apex). When the O_2 electrode was moved towards the base, ROL significantly declined to almost zero already at 40 mm. It is evident that growth under hypoxic conditions induced formation of strong barriers to ROL.

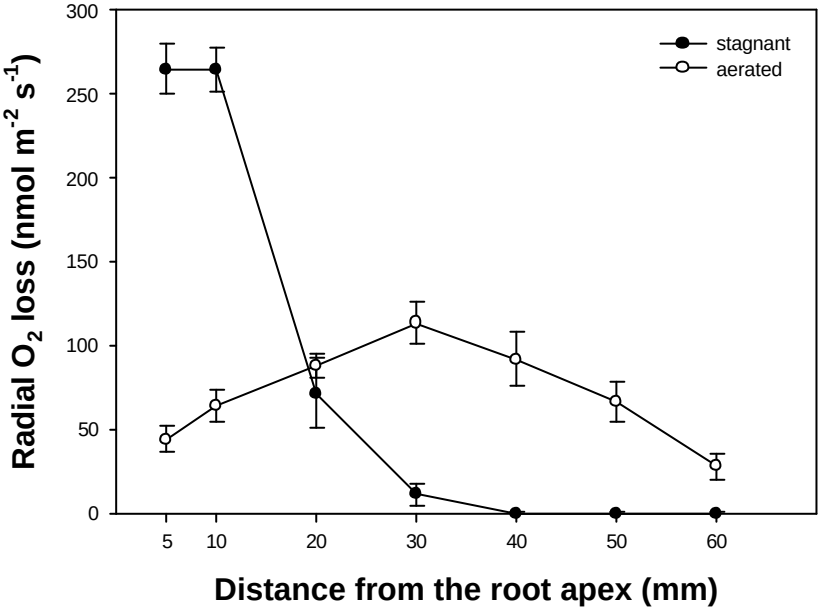


Fig. 3. Rates of radial O_2 loss (ROL) along adventitious roots of intact rice plants raised in either aerated (open symbols) or stagnant (closed symbols) solutions for 30 to 40 days. Measurements were taken in a growth chamber at 27°C with roots in O_2-free medium and shoots in the air. Lengths of the roots used in measurements were 90 to 130 mm. The figure shows that the ROL from roots grown under stagnant conditions substantially decreased along the roots towards the base, most likely due to suberization of the OPR. Data are means ± SE (n = 9 to 8 plants).

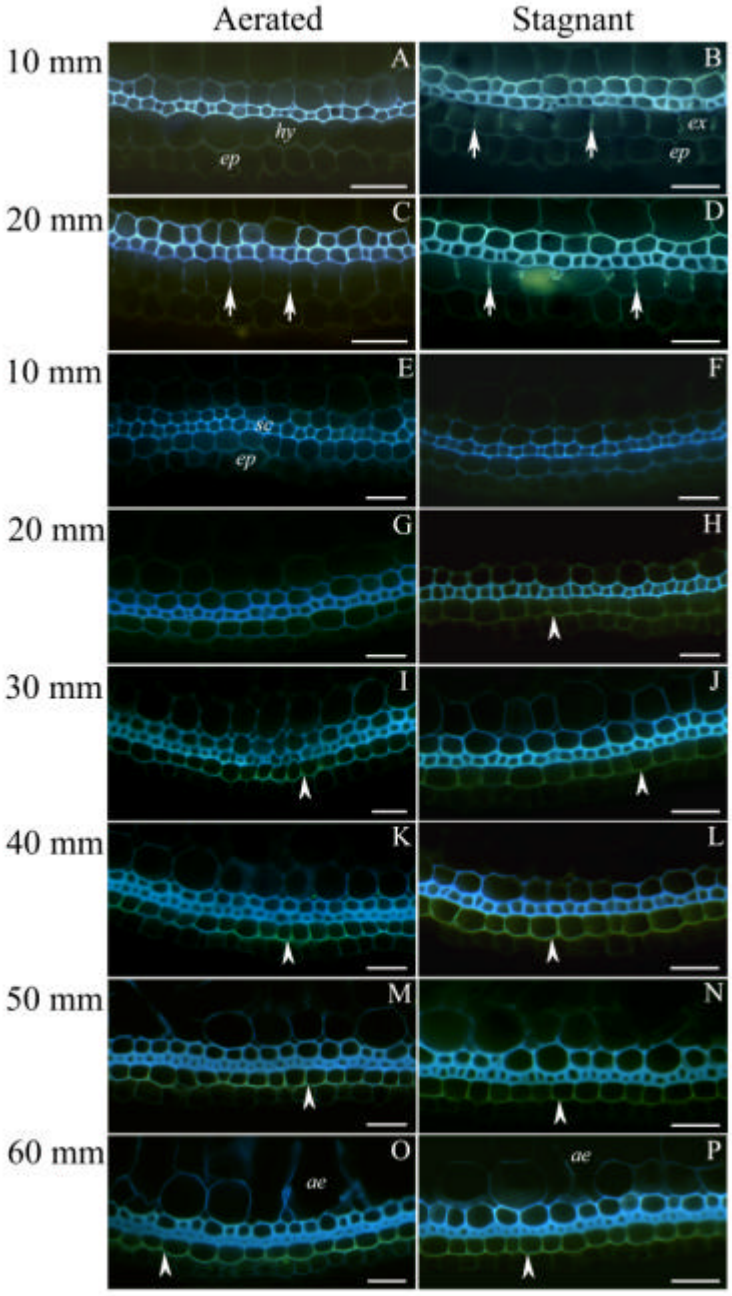
Development of Casparian bands (CBs) and of suberin lamellae in the exodermis of roots grown in either aerated or stagnant solution

Deposition of Casparian bands (CBs) in radial walls of cells of the exodermis was observed by staining cross-sections with berberine-aniline blue. At 10 mm from the apex, no CBs were observed in walls of plants grown in aerated (Fig. 4A), but they were already deposited in plants grown in stagnant solution (Fig. 4B). Faint greenish-yellow fluorescence indicated immature CBs at 20 mm of aerated plants, whereas an intense fluorescence showed mature CBs at the same distance of stagnantly grown plants (Fig. 4C, D).

Suberin lamellae in hypodermal cell walls were detected by staining cross-sections with Fluorol Yellow 088. Its presence in the exodermis was marked by a bright yellow fluorescence under the fluorescent microscope. In roots grown in both conditions, suberin was absent at 10 mm from the apex (Fig. 4E, F). It was still absent at 20 mm in roots grown in aerated (Fig. 4G), but was already discernible in roots grown in stagnant solution (Fig. 4H). In the latter, well-developed suberin lamellae were observed at the rest of the distances along roots (Fig. 4J, L, N, P). In roots grown in aerated solution, suberin lamellae started to develop at around 30 mm from the apex. However, at this distance, depositions of suberin were observed only in a few cells indicating a patchy development (Fig. 4I). At 40 mm, only about 50% of exodermal cells deposited suberin lamellae (Fig. 4K), where a majority of cells with suberin lamellae were observed at 50 mm from the apex (Fig. 4M). At this distance, few passage cells lacking suberin were detected. Fully developed suberin lamellae were present at 60 mm (Fig. 4O). The histochemical analysis indicated that development of suberin lamellae proceeded slower in roots grown in aerated than in stagnant solution.

Fig. 4. Comparison of the development of Casparian bands (A-D) and of suberin lamellae (E-P) in the exodermis of rice roots grown in either aerated (left column: A, C, E, G, I, K, M, O) or deoxygenated (right column: B, D, F, H, J, L, N, P) solution. The distances from the root apex (10 to 60 mm) are given at the left side of figures. The presence of Casparian bands was indicated by faint yellow fluorescence with berberine-aniline blue (see arrows in B, C, D). Presence of suberin lamellae was indicated by yellow-green fluorescence with Fluorol Yellow 088 (see white arrowheads). There is a blue autofluorescence in the walls of exodermis (*ex*) of aerated (E) and stagnant (F) roots at 10, and aerated (G) at 20 mm from the root apex, and a yellow-green fluorescence of walls of the exodermis (H) of a root from stagnant medium at 20 mm. In (I), a few radial walls at a distance of 30 mm from the apex exhibit a patchy green fluorescence, and in (J) there is an intense green fluorescence in all exodermal walls of roots

either from aerated or stagnant medium, respectively. By 40 mm, there is a weak, patchy green fluorescence in aerated (K) and a strong, intense fluorescence in roots from stagnant medium (L). Majority of exodermal cells with weak green fluorescing walls in aerated (M) and intense green stains in all walls of exodermis in stagnant (N), 50 mm from the apex. By 60 mm, faint yellow-green fluorescence in all exodermal cell walls of roots from aerated (O) and bright green fluorescence in all exodermal walls of roots from stagnant (P) solution. *ae* aerenchyma, *ex* exodermis, *hy* hypodermis, *sc* sclerenchyma. Bars = 50 µm.

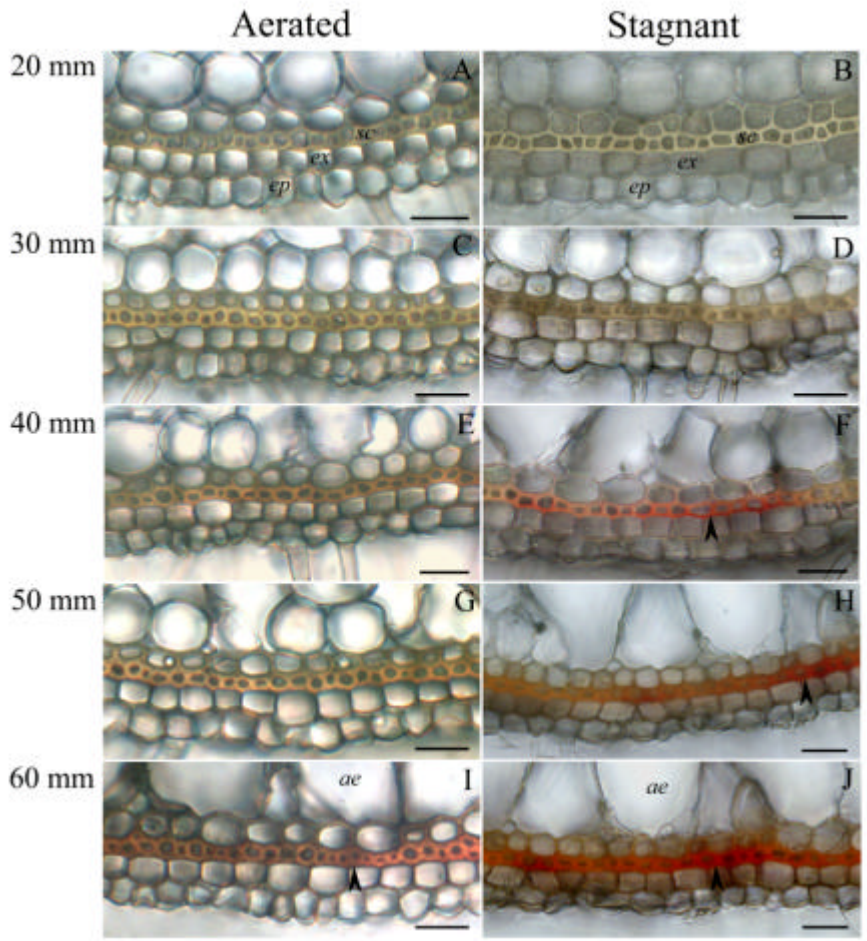


Lignification pattern of sclerenchyma cells in response to stagnant or aerated growth conditions

Lignin in cell walls was detected by an orange/red staining of cross-sections with phloroglucinol-HCl, which results from the cinnamyl aldehyde groups in the lignin. Lignin was absent in sclerenchyma cells at 20 and 30 mm from the root apex in both roots grown either in aerated hydroponics or stagnant agar medium (Fig. 5A, B, C, D). In sections taken at a distance of 40 and 50 mm, sclerenchyma cells were more compact with thick cell walls in aerated plants, though no orange/red stains were observed (Fig. 5E, G). In contrast, numerous sclerenchyma cells at 40 mm and entire layer of sclerenchyma at 50 mm stained orange/red in stagnant plants indicating an early lignification (Fig. 5F, H). In aerated plants, faint orange/red stains in walls of sclerenchyma cells at 60 mm indicated presence of lignin (Fig. 5I). In stagnant plants, intense orange stains in sclerenchyma cells showed intensive lignification under hypoxic/anoxic conditions (Fig. 5J). Lignin was also found in the walls of the adjacent cortical parenchyma cells in both aerated and stagnant plants (Fig. 5I, J). Thickening of the cell walls of parenchyma cells was already observed at around 30 mm from the apex. Taken together, the histochemical evidence indicated that the sclerenchyma cells lignified closer to the root apex and more intense during stagnant rather than aerated growth conditions.

Fig. 5. Comparison of lignin deposition in the outer part of rice roots (OPR) grown in either aerated (left column; A, C, E, G, I) or deoxygenated (right column; B, D, F, H, J) medium. Distances from the root apex are given at the right side of the figures. Lignin with cinnamyl aldehyde groups stained orange/red with phloroglucinol-HCl (indicated by black arrows). There were unstained sclerenchyma cells at 20 mm of aerated (A), stagnant (B), and 30 mm of aerated (C) and stagnant (D) roots. Unstained (E) and intense orange/red stained sclerenchyma (F) at 40 mm of aerated and stagnant roots, respectively. (G) Thick walls of sclerenchyma cells at 50 mm did not stain for lignin in aerated, but appeared with intense, complete orange stains in stagnant roots (H). Layer of sclerenchyma cells with faint orange stains of aerated (I), and with extensive bright orange stains of stagnant (J) roots at 60 mm from the apex. The absence of oxygen in the medium induced a substantial lignin incrustation, which may have affected the permeability of the OPR for oxygen.

ae aerenchyma, *ep* epidermis, *ex* exodermis, *sc* sclerenchyma. Bars = 50 µm.



Aliphatic suberin in the OPR sleeves of rice grown in aerated or stagnant solution

Transesterification with BF_3/MeOH released monomers of aliphatic suberin from OPR sleeves. The total contents per mm^2 of root surface area of aliphatic suberin in the sleeves of OPR increased significantly along the root axis in both growth conditions ($F_{5,20} = 151$; $P < 0.001$; Fig. 6). However, when grown in stagnant agar medium, the densities of aliphatics of corresponding root regions were several fold greater than those obtained from aerated hydroponics ($F_{1,4} = 125.6$; $P < 0.001$; Fig. 6). This correlated with the anatomical studies of Fig. 4. In apical zones of up to 30 mm, the increase of aliphatic suberin was more pronounced under stagnant growth compared with aerated hydroponics. The absolute values of suberin amounts in corresponding zones were 2.5- to 5.3-folds higher under stagnant growth (two-sample t-test at $P \leq 0.001$; Fig. 6). In distal zones (from 40 mm upwards), loads were approximately 1.6-fold higher in stagnant rather than in roots grown in hydroponics (two-sample t-test at $P \leq 0.01$ Fig. 6). The figure indicates that the deposition of aliphatics in the walls of OPR of stagnant

plants was complete at 40 mm from the root apex. There was a slight increase of aliphatics, which was still detectable in distal root zones grown in aerated hydroponics.

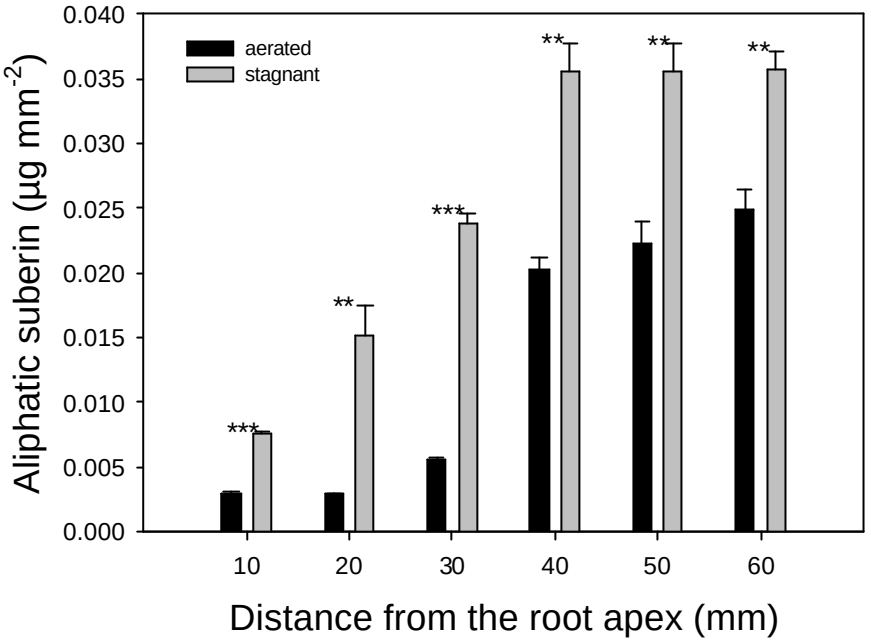


Fig. 6. Total amount of aliphatic suberin in isolated sleeves of OPR at distances of 10, 20, 30, 40, 50 and 60 mm from the apex of roots grown in either aerated or stagnant solutions. Sleeves were isolated from roots by exhaustive enzymatic digestion and manual dissection. Isolated tissues were subjected to BF_3/MeOH transesterification. Regardless of growth condition, there was a striking increase of total suberin loads in the roots from the apex to base. Compared to root zones grown in aerated medium, the deoxygenated stagnant treatment resulted in higher densities of aliphatic suberin per unit area of OPR sleeves at all root zones. Significance levels of $P \leq 0.01$ or $P \leq 0.001$ are denoted by ** or ***, respectively (two-sample t-tests). Data are means ± SE (n = 3 plants).

The aliphatic suberin of isolated sleeves of OPR was primarily composed of fatty acids (such as C16, C18, C28 and C30), ω-hydroxy acids (such as C16, C18, C26 to C30), diacids (such as C18), 2-hydroxy acids (such as C18 and C20), and primary alcohols (such as C16 and C18). Nevertheless, in both growth conditions, fatty acids and ω-hydroxy acids were the prominent components representing approximately 30% and 50% of the total, respectively (Fig. 7). Compared to aerated hydroponics, the OPR sleeves from stagnant medium had a 1.5- to 7.6-fold higher amount of fatty- and ω-hydroxy acids, depending on the position from the root apex (Fig. 7). In general, the amounts of fatty acids, ω-hydroxy acids and 2-hydroxy acids were significantly greater

in the OPR sleeves of all root zones of plants grown in stagnant medium than that of hydroponically grown plants ($F_{1,4}$ = 123 at $P < 0.001$ for fatty acids; $F_{1,4}$ = 97.2 at P = 0.001 for ω-hydroxy acids; $F_{1,4}$ = 244 at $P < 0.001$ for 2-hydroxy acids; Fig. 7). In contrast, only the apical zones had significantly higher amount of primary alcohols in OPR sleeves from the stagnant medium (two-sample t-test at $P \leq 0.05$).

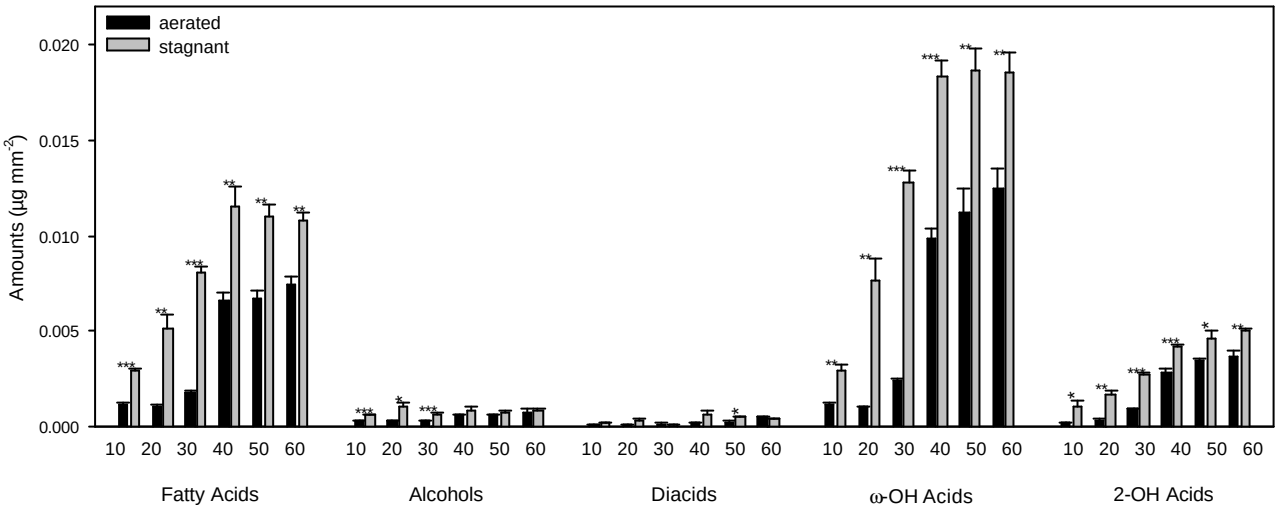


Fig. 7. Detailed composition of major aliphatic suberin monomers of OPR sleeves isolated from different root zones along the axis of roots grown either in aerated or stagnant deoxygenated medium. Isolated OPR sleeves were subjected to BF_3/MeOH transesterification to release aliphatic monomers. Fatty acids, ω-hydroxy acids and 2-hydroxy acids were the prominent components in both growth conditions. OPR sleeves from plants grown in stagnant media had significantly greater amounts of these components than sleeves from plants grown in aerated hydroponics. Significance levels of $P \leq 0.05$, $P \leq 0.01$ or $P \leq 0.001$ are denoted by *, ** or ***, respectively (two-sample t-tests). Data given are means ± SE (n = 3 plants).

Aromatic suberin in the OPR sleeves of rice grown in aerated or stagnant

In both growth conditions, there was a pronounced increase of total aromatic suberin of the OPR along the axis of the root ($F_{5,20}$ = 658; $P < 0.001$; Fig. 8). Clearly, in all root zones, OPR sleeves from stagnant medium had significantly greater amounts of aromatics than that of plants grown in aerated hydroponics ($F_{1,4}$ = 21.4; P = 0.01). Interestingly, the difference was 3- to 4-fold at the apical root zones up to 30 mm from the apex (two-sample t-test at $P \leq 0.01$) and only 1.2-fold at distal zones (≥ 40 mm). Aromatics of the OPR sleeves were primarily composed of ferulic and coumaric acids, however, the latter was prominent in the basal or mature root zones (data not shown).

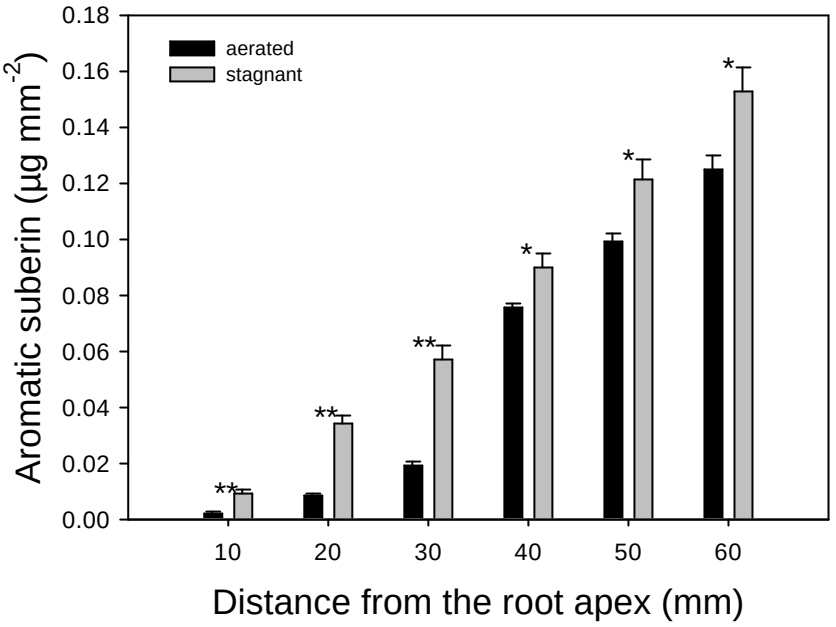


Fig. 8. Total amount of aromatic suberin monomers released from OPR sleeves of different zones along the roots from plants grown either in aerated or stagnant medium. Isolated sleeves were transesterified with BF_3/MeOH to release aromatic monomers. Regardless of growth condition, there was a steep increment of total loads of aromatics along the root axis (as for the aliphatic compounds). In all root zones, OPR sleeves from plants grown in stagnant solution had markedly greater amounts of aromatics than sleeves from plants grown in aerated medium. Significance levels of $P \leq 0.05$ or $P \leq 0.01$ are denoted by * or **, respectively (two-sample t-tests). Data are means ± SE (n = 3 plants).

Total lignin content of the OPR sleeves measured by photometric method

In both growth conditions, total contents of lignin in the OPR increased along the root as it matured ($F_{1,4}$ = 156.3; P < 0.001; Fig. 9). When comparing the quantities at different growth conditions, roots from stagnant medium had significantly higher amounts of lignin in all zones than those from aerated hydroponics ($F_{1,4}$ = 35.3; P = 0.004). The gradual accumulation of lignin in the walls of OPR (mainly in sclerenchyma cells) along the root axis was, indeed, more intense (and more rapid) in stagnant than in roots from aerated hydroponics. This suggested that this contributed to a stronger barrier to ROL. It correlated with the anatomical studies (Fig. 5), which showed early lignification of sclerenchyma cells during growth under stagnant conditions. However, it should be noted that, because of different growth rates of roots, measured amounts at corresponding zones from the apex would refer to different ages of roots.

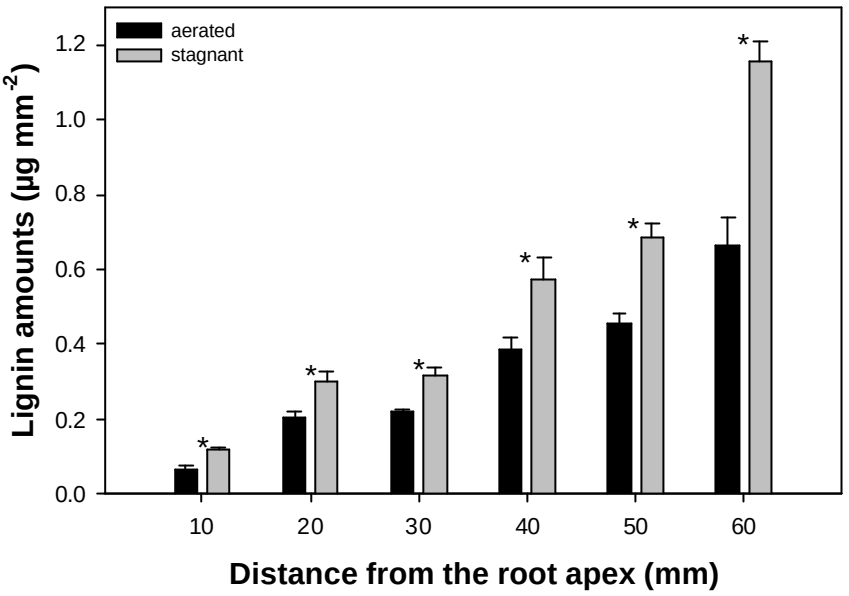


Fig. 9. Total amount of lignin released from OPR sleeves isolated from different root zones, grown either in aerated or deoxygenated stagnant media, and analysed by a spectrophotometric method. In both growth conditions, the lignin amounts increased along the roots from apex to base as roots aged. The OPR sleeves of stagnant roots had considerably greater amounts of lignin than sleeves of aerated roots. Significance levels of $P \leq 0.05$ are denoted by * (two-sample t-tests). Data are means ± SE (n = 3 plants).

Discussion

Even though, loss of oxygen from rice roots crossing the peripheral cell layers has been extensively investigated in the past using root-sleeving O_2 electrodes (e.g. Colmer *et al*., 1998; Colmer, 2003a), the precise nature of potential barriers to ROL has not yet been determined. Combining root histochemistry and biochemistry, the present study, for the first time, examined the effects of different growth conditions, i.e. aerated and deoxygenated, on changes of ROL in relation to the formation of apoplastic barriers in OPR. It has already been found that roots of rice acclimate to growth in deoxygenated conditions by increasing root porosity and the induction of a 'tight' barrier to ROL (Colmer *et al*., 1998; Colmer, 2003a). However, in the former studies, the focus was on the function (ROL) rather than on the detailed structural basis, i.e. which cell layers in the root periphery and which chemical compounds in cell walls were responsible for the strengthened barrier in rice roots. The present data showed that both elevated lignin amounts in sclerenchyma cell walls and suberin levels in the exodermis correlated with the patterns of radial oxygen loss from rice roots under stagnant conditions. It is obvious

that one or both of these substances was/were responsible for the formation of a ‘tight’ barrier to oxygen loss. However relative contribution of the two substances is not clear, and potentially one of the two might not be needed for the formation of the barrier. In addition to elevated levels of these substances in the OPR of roots grown in stagnant condition, the microstructure of the barrier i.e., how these substances deposited into the cell walls, may contribute to the tightness of the barrier. It should be noted that the flux of oxygen from aerenchyma to the rhizosphere is not only determined by the physical resistance to oxygen diffusion, but also by the consumption of oxygen by cells along the radial diffusion path (Armstrong, 1979; Colmer, 2003a).

In the past, it has been suggested that lignification and/or suberization of outer cell layers may develop barriers to impede diffusion of oxygen from roots to the rhizosphere (Armstrong and Armstrong, 1988; Jackson and Armstrong, 1999; Garthwaite *et al*., 2008). However, the knowledge of the anatomical barrier to ROL in rice roots was still scant. In this study, the anatomical distribution of barriers to ROL in the outer part of rice roots grown in either aerated or deoxygenated conditions has been extensively examined to localize the barrier. Apparently, deoxygenated conditions induced early development of exodermal Casparian bands and suberin lamellae at positions closer to root apex, where it did not develop in aerated roots. Depositions of suberin lamellae covering Casparian bands were observed in roots of plants grown in either solution. When grown in aerated solution, roots developed patchy or incomplete lamellae even in basal roots zones as also observed by Ranathunge *et al*., (2005). Due to optimum oxygen concentrations in the medium, such roots did not require tight barriers in the OPR to minimize ROL. In addition to suberization, early lignification of walls of densely packed sclerenchyma was found closer to the root apex in plants grown in stagnant solution. In the literature, it has been found that suberization and lignification of the exodermis of *Phragmites* preceded even those of the endodermis (Soukup *et al*., 2002). It is generally accepted that the presence of apoplastic barriers such as Casparian bands and suberin/lignin depositions impede water loss from roots and control solute transport through the non selective apoplastic path (Steudle and Peterson, 1998; Zimmermann et al., 1998). However, these apoplastic barriers should restrict gas diffusion as well. Confirming this idea, it has been shown that early development of suberized and lignified barriers in the outer root cell layers of *Phragmites* acted as a barrier to gas diffusion (Armstrong *et al*., 2000; Soukup *et al*., 2006). Our results clearly

indicated that there was a strong correlation between oxygen diffusion from aerenchyma to the outer medium and early suberization and lignification of the OPR in stagnantly grown rice roots.

Histochemical studies to trace suberized and lignified cell wall materials are not always sensitive enough for a precise quantitative estimation of such substances that involve in forming apoplastic barriers. Thus, quantitative chemical analysis of the isolated OPR sleeves of rice has been performed. As shown, this is a reliable and sensitive approach to identify modifications of cell walls. Suberin has been identified as a major component, which affects cell wall permeability (Zimmermann *et al*., 2000). Suberin is a biopolymer, which is known to be composed of both aliphatic and aromatic domains, but the absolute loads and monomeric composition can vary with plant species, growth conditions and/or even with the tissue analyzed (Schreiber *et al*., 1999). Our results indicated that, when grown in stagnant, oxygen deprived solution, the loads of both aromatic and aliphatic suberin in the OPR sleeves of all investigated root zones were several folds greater than those of plants grown in aerated solution. Qualitatively, the predominant components of the aliphatic constituents of the isolated OPR sleeves were ω-hydroxy acids, fatty acids and 2-hydroxy acids, which are typical components of suberin lamellae (Matzke and Reiderer, 1991; Schreiber *et al*., 1999; Kolattukudy, 2001; De Simone *et al*., 2003). In aerated and stagnant growth conditions, there was a pronounced increase of absolute monomer loads along the root axis from apex to base as was also observed for other wetland plants such as *Phragmites australis* and *Glyceria maxima* (Soukup *et al*., 2006). However, in stagnant solution amounts of suberin monomers were several folds larger than those found in aerated hydroponics. In parallel to elevated suberin levels, lignin content, which mainly deposited into walls of densely packed sclerenchyma cells, was also higher in these roots. When comparing the results of aromatic suberin domain (phenolics) using BF_3/MeOH transesterification and photometric analysis of aromatic lignin yielded a similar picture showing that stagnant treatment significantly increased the total phenolics in the OPR sleeves at all root zones compared with corresponding zones of aerated cultivation. Taking together, roots in oxygen deprived medium accelerate the deposition of suberin and lignin into cell walls of OPR close to root apex, and induce multifold greater amounts than in roots grown in aerated medium. The above plethora of responses involved in making a tight barrier at

the OPR to minimize or even to stop oxygen diffusion from roots to the anaerobic root substrate.

Plants exposed to waterlogged conditions depend on supplies of oxygen from the aerial parts of the plant. Strong barriers to ROL, thick roots and increased root porosity help to supply oxygen to root apices (Armstrong, 1979; Colmer, 2003b). This suggested that, at the whole plant level, the resistance of the OPR to oxygen rather than the axial diffusion through aerenchyma was rate-limiting (Kotula and Steudle, 2008). The present results exhibited that rice roots grown in deoxygenated conditions induced a strong barrier to ROL at the basal positions of roots, increased root diameter, and porosity. This agrees with earlier data for diverse rice genotypes and many other wetland species (Colmer *et al.*, 1998; Visser *et al.*, 2000; McDonald *et al.*, 2001; McDonald *et al.*, 2002; Colmer, 2003a). Increased root diameter and porosity not only enhance axial transport of oxygen but also enable venting of other gases such as ethylene, CO_2 and methane (Visser *et al.*, 1997, Colmer, 2003b).

When compared with aerated hydroponics, roots of plants grown in deoxygenated solution exhibited relatively higher rates of ROL just behind the apex below 20 mm. However, chemical analyses revealed higher amounts of suberin and lignin already at younger zones of stagnantly grown roots. Possible reasons would be (i) higher internal oxygen concentration in the aerenchyma of stagnantly grown plants, which results from lower resistance to longitudinal oxygen diffusion due to higher root porosity, and lower losses of oxygen in more basal positions (ii) rather high rates of oxygen consumption by respiration of cortical cells in aerated roots. These reasons may also explain the highest rates of ROL of aerated plants at a distance of 30 mm rather than 10 or 20 mm from the root apex, where lower oxygen concentrations inside the roots of younger root zones limited oxygen diffusion to the medium. Obviously, lower rates of ROL at basal root zones of aerated roots result from constitutively present barrier in the peripheral cell layers as found for many other wetland species (Colmer *et al.*, 1998; Visser *et al.*, 2000, McDonald *et al.*, 2002; Colmer, 2003a). It has also been shown that this constitutively present barrier, which is a patchy structure, has rather high permeabilities for water and solutes (Ranathunge *et al.*, 2003, 2004; Garthwaite *et al.*, 2006).

There are remarkable differences between the water and oxygen permeability of the OPR of rice roots. When comparing the diffusional permeability for water with that for oxygen, the water permeability was lower by an order of magnitude than that of oxygen

(Ranathunge *et al*., 2004; Kotula and Steudle, 2008). However, the physiologically relevant parameter was the bulk rather than the diffusional water permeability (hydraulic conductivity). The former was larger by a factor of 600 to 1400 than the latter (Ranathunge *et al*., 2004). Hence, the OPR still allows a rather high water flow in the presence of a high resistance to oxygen, which is an advantage for the plant. Despite the presence of suberin lamellae and Casparian bands in the exodermis, literature data indicate a somewhat porous apoplast in the OPR of rice roots (Ranathunge *et al*., 2004, 2005). In other words, there is a bulk (viscous) water flow across the OPR, but the oxygen flow is diffusional in nature. The former is largely around protoplasts (i.e. apoplastic), and the latter uses the entire cross section including protoplasts, where the lamellae come into play restricting the diffusion of oxygen. This suggested that in rice (as in other hygrophytes) there is an optimized balance between high rates of water uptake and low rates of oxygen loss to the environment. However, caution has to be taken since the above conclusion was drawn only for rice grown in aerated solution. Results obtained for oxygen permeability (Pd_{O2}) across the OPR of stagnant plants, using the technique recently developed in the lab (Kotula and Steudle, 2008), showed a pronounced reduction along the entire root compared with aerated plants (Kotula *et al*., in preparation). Significantly higher loads of suberin and lignin in the OPR may also affect water uptake, which still need to be investigated. This permeability (Pd_{O2}) data would also allow to quantify the oxygen flows in roots precisely in terms of mathematical models. This work is underway.

In conclusion, the present study combined measurements of radial oxygen loss with histochemistry and biochemistry. It demonstrated that elevated levels of suberin in the exodermis and lignin in the sclerenchyma of rice roots caused strong barriers to ROL, which was induced by growth in deoxygenated medium. However, the relative contribution of these substances in formation of a tight barrier remained unclear. Compared with aerated culture, rates of ROL drastically decreased in roots of plants grown in stagnant solution. This was supported by the observation that histochemical studies revealed an early development of apoplastic barriers in the OPR of plants grown in deoxygenated conditions. Under both growth conditions, the levels of suberin and lignin in the OPR increased along the root axis. However, the absolute amounts of suberin and lignin were several folds higher in plants grown in deoxygenated solution. Overall, the present study contributed to a detailed understanding of the basis of the

barrier formation to ROL. However, the flux of oxygen from aerenchyma to the rhizosphere is not only determined by the physical resistance to oxygen diffusion, but also by the consumption of oxygen by cells along the radial diffusion path (Armstrong, 1979; Colmer, 2003b). In order to understand the mechanisms controlling ROL precisely, oxygen permeability coefficients across the OPR have to be known (Kotula and Steudle, 2008).

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3. Apoplastic barriers effectively block oxygen permeability across outer cell layers of rice roots under deoxygenated conditions: roles of apoplastic pores and of respiration

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Summary

- Despite the importance of the barrier to oxygen losses of roots of hygrophytes growing in wet environments devoid of oxygen, there are few data available on permeability coefficients for O_2 across outer root cell layers (P_{OPR}) and how they may change in response to low O_2.
- A gas perfusion technique was used to measure P_{OPR} of rice (*Oryza sativa*) plants grown in either aerated or deoxygenated solution. The contributions of the apoplast and of living cells to the overall P_{OPR} were characterized either by blocking apoplastic pores with precipitates of brown $Cu_2[Fe(CN)_6]$ or by killing cells with 0.1 N HCl.
- Compared with plants from aerated hydroponics, the P_{OPR} of plants grown in deoxygenated medium was smaller by an order of magnitude. Precipitates resulting from $CuSO_4/K_4[Fe(CN)_6]$ treatment only formed in plants grown in aerated solution, where they reduced the P_{OPR} by 5 to 20%. Killing of root segments with HCl increased P_{OPR} in plants grown in both conditions by 20 to 55%.
- The results indicated that apoplastic barriers effectively restricted radial O_2 loss. The relative role of the respiratory O_2 consumption of root peripherial layers increased as P_{OPR} decreased.

Key words: apoplastic barriers, outer part of root, oxygen permeability coefficient, *Oryza sativa*, waterlogging

Introduction

When faced with oxygen depletion in flooded soil, rice (*Oryza sativa* L.), like other wetland species, develops numerous morphological and biochemical responses to reduce the impact of the stress. The aerenchyma is of particular importance. It facilitates the internal transport of gasses from well-aerated aerial shoots to underground organs exposed to anaerobic surroundings. The effectiveness of the aerenchyma can be increased by the formation of barriers in the outer cell layers of roots. These barriers inhibit radial oxygen loss (ROL) to the rhizosphere enhance longitudinal diffusion of O_2 towards the apex (Armstrong, 1979; Colmer, 2003b).

Roots of some wetland species constitutively express barriers to ROL (e.g. *Juncus effesus, Eleocharis acuta*; Visser *et al*., 2000; McDonald *et al*., 2002). In others, barriers are induced during growth in stagnant, deoxygenated media (e.g. *Oryza sativa,* wild *Hordeum* species, *Lolium multiflorum*; Colmer *et al*., 1998; McDonald *et al*., 2002; Colmer, 2003a; Garthwaite *et al*., 2003; Kotula *et al*., 2009). The barrier to ROL in roots has usually been linked to suberization and lignification of the walls of the peripheral cell layers (De Simone *et al*., 2003; Soukup *et al*., 2007; Garthwaite *et al*., 2008). Kotula *et al*. (2009) combined measurements of ROL with histochemical and biochemical analyses of the outer part of roots (OPR) of rice. They showed that, when grown in deoxygenated solution, the amounts of suberin and lignin in OPR sleeves were several folds greater than those of plants grown in aerated solution. This correlated with the pattern of ROL. It was concluded that the suberized exodermis and lignified sclerenchyma of rice roots formed a strong barrier to ROL.

Despite the importance of the barrier to ROL for roots of wetland plants, there are few data available on the permeability coefficient of O_2 across cell layers exterior to the aerenchyma. In *Phragmites australis* this permeability coefficient was assesd using O_2 concentration profiles across the hypodermis/epidermis in combination with rates of O_2 consumption in these layers (Armstrong *et al*., 2000). Using roots of *Hordeum marinum*, Garthwaite *et al*. (2008) developed another method to determine the diffusivity of O_2 across outer cell layers. Diffusivities were derived from measurements of ROL obtained while either varying shoot O_2 partial pressure or cooling the rooting medium to cancel respiratory effects. In the present study, the O_2 permeability coefficient of the OPR (P_{OPR}) of rice has been measured directly using the method

recently developed by Kotula & Steudle (2009). The technique was based on perfusing the aerenchyma of root segments with gas mixtures (O_2:N_2) of known O_2 concentration while, at the same time, measuring radial losses of oxygen using a root-sleeving platinum electrode (Armstrong, 1994). The O_2 permeability of the OPR could be calculated, as both the O_2 flow across the OPR and the O_2 concentration gradient between the aerenchyma and the surrounding medium were known.

In contrast to the previous study of Kotula & Steudle (2009), where plants were grown in an aerated solution, in the present study we examined the effects of different growth conditions on P_{OPR} by growing rice in either aerated or stagnant, deoxygenated medium. In order to estimate the contribution of the apoplast and living cells to the overall movement of O_2 across the OPR, the P_{OPR} was varied either by partially blocking apoplastic pores in the OPR with salt precipitates or by killing living tissue with 0.1 N HCl. As far as we are aware, this is the first direct quantitative comparison of O_2 permeability coefficients of peripheral layers to in roots subjected to aerated or deoxygenated treatments. Our study provides evidence of the effectiveness of apoplastic barriers in reducing O_2 and ion permeability across the root outer cell layers, when plants are grown in deoxygenated medium.

Materials and Methods

Plant material

Seeds of an upland rice cultivar (*Oryza sativa* L. cv. Azucena; International Rice Research Institute, Manila, Philippines) were germinated and cultivated in either aerated hydroponics (control) or deoxygenated 0.1% agar nutrient solution (treatment) as previously described by Kotula *et al*. (2009). After 30-40 d of growth, roots were excised and used in experiments.

Measurements of oxygen permeability coefficients (Pd_{O_2}) in rice root segments

The O_2 permeability coefficients of the outer part of rice root segments grown in either aerated or deoxygenated solution were measured as detailed previously by Kotula & Steudle (2009). Root segments of a length of 50 mm were excised from adventitious roots at distances of 20-70 mm from the apex, where the aerenchyma was sufficiently developed for axial gas perfusion, and inserted through a cylindrical platinum (Pt) electrode. Both cut ends of segments were fixed to glass capillaries and firmly secured

on the rack in a Perspex chamber. The chamber was filled with deoxygenated 0.1% (w/v) agar solution containing 5 mol m^{-3} KCl and 0.5 mol m^{-3} $CaSO_4$ and closed with a lid. The basal end of the root segment was connected to tanks with mixtures of compressed O_2 : N_2 of oxygen concentrations (v/v) of 10, 21 and 40%. The other end was open to the atmosphere. Perfusion of moistened gas mixtures through the aerenchyma of the root segments was conducted at an overpressure of 20 kPa. The corresponding rates of radial O_2 flows (J_{O2} in nmol m^{-2} s^{-1}) were measured along the segments at distances of 30, 40, 50 and 60 mm from the apex. Permeability coefficients were calculated from the slopes of J_{O2}/C_i curves where C_i (mol m^{-3}) is the concentration of O_2 in the liquid phase of the aerenchyma at the inner surface of the OPR, derived from the partial oxygen pressure (Henry's law). Measurments were taken at 25°C in a temperature-controlled room.

Calculation of the diffusivity of oxygen across the OPR (P_{OPR})

Measured values of permeability coefficients obtained form the perfusion experiment included diffusion of O_2 across the shell of 0.1% of agar (P_{agar}) between the root and the electrode surface, which acted as an unstirred layer (USL). These effects may be substantial, and were found to account for 16 to 33% of the measured Pd_{O2} by Kotula & Steudle (2009). As the diffusion coefficient of O_2 in the 0.1% of agar solution was known (2.3×10^{-9} m^2 s^{-1} at 25°C; Kotula & Steudle, 2009), and the diffusional resistances of the OPR and of the agar solution were in series, P_{OPR} was calculated using the formula:

$$\frac{1}{Pd_{O2}} = \frac{1}{P_{OPR}} + \frac{1}{P_{agar}} \,, \qquad \text{Eqn 1}$$

where P_{agar} is the O_2 permeability coefficient in the shell of agar, and P_{OPR} is the O_2 permeability coefficient of the OPR.

Blocking the apoplast of the OPR with inorganic salt precipitates

In order to investigate the contribution of the apoplast to O_2 movement across the OPR, apoplastic pores were blocked with insoluble salt precipitates using the technique of Ranathunge *et al*. (2005). Root segments (20-70 mm from the apex), previously used to measure P_{OPR}, were fixed to glass capillaries (inner diameter 1.3 mm), using polyacrylamide glue (UHU, Bühl, Germany) and connected to the perfusion apparatus.

One of the glass capillaries (inlet side) was connected to a syringe while the outlet remained open. The syringe, filled with 1 mM $K_4[Fe(CN)_6]$ was mounted on a 12-step Braun-Melsungen pump that produced defined pumping rates between 1.7×10^{-9} and 1.1×10^{-11} mm^3 s^{-1}. Solutions of $K_4[Fe(CN)_6]$ were perfused through the aerenchyma of root segments at rates of *c*. 1 10^{-10} mm^3 s^{-1}, displacing air, while the root segment was bathed in 0.5 mM $CuSO_4$ solution. Perfusion was conducted for 3 hours. The reaction between $CuSO_4$ and $K_4[Fe(CN)_6]$ resulted in rusty brown insoluble precipitates of $Cu_2[Fe(CN)_6]$ or $Cu[CuFe(CN)_6]$. Following the treatment, root segments were fixed to the gas perfusion set up, all solution n the aerenchyma was exchanged by gas perfusion and P_{OPR} re-measured.

Treatment of root segments with 0.1 N HCl to kill living cells: effects of respiration

In order to quantify the contribution of respiratory O_2 consumption to diffusion of O_2 across the OPR, 0.1 N of HCl (pH = 1) was used to kill living cells. Root segments previously used to measure P_{OPR} were attached to a syringe containing the acid (via glass capillary and Teflon tube). Hydrocholric acid was gently injected into the aerenchyma, displacing air. Then the entire root segments were immersed in 0.1 N of HCl for 20-30 min. Subsequently, root segments were carefully washed and perfused with distilled water to remove HCl from the aerenchyma and root surface. After fixing HCl-treated segments to the perfusion set up, O_2 permeability was re-measured.

Vitality test

As the O_2 permeability experiments lasted for several hours (6-7 h) and even low Cu^{2+} concentrations during precipitation experiments may be toxic to living cells (Murphy *et al*., 1999), it was essential to check the viability of cells constituting the OPR at the end of the experiments. The segments were cut into 5 mm-long pieces and soaked in 0.5% (w/w) Evan`s blue (w/w; Sigma-Aldrich, St. Louis, USA) for 30 min (Taylor & West, 1980), allowing the dye to diffuse through the aerenchyma. After thoroughly rinsing the stained segments with water several times, freehand, cross-sections and longitudinal sections were made and examined using an Axioplan light microscope (Zeiss, Oberkochen, Germany). Photographs were taken with a digital camera system (Nikon D60, Göttingen, Germany). Evan's blue stains nuclei and cytoplasm dark blue in dead cells but it does not penetrate a healthy plasmalemma of living cells. Living cells remained unstained while dead cells took on a blue coloration (Fischer *et al*., 1985).

Statistical analysis

Data on O_2 permeability coefficients were analysed using repeated measures analysis of variance (ANOVAR) to examine the effects of growth conditions or treatments (between-subject factor) and position (within-subject factor). Data are presented as means with the standard errors. Means were compared at $P \leq 0.05$ level using a paired t-test (effect of distance within growth condition or one treatment and effects of treatment within distance) and a two sample t-test (effect of growth condition within distance).

Results

Oxygen permeability coefficient of the OPR of plants grown in aerated and stagnant solutions

Measurements were made at 30, 40, 50 and 60 mm from the apex of rice plants grown in either aerated or deoxygenated solutions. Permeability coefficients of the outer part of the root (P_{OPR}) were highest at 30 mm from the apex and decreased along the root axis, reaching the lowest values at 60 mm ($F_{3,141} = 80.4$, $P < 0.001$; Fig. 1). For plants grown in aerated solution, values of P_{OPR} were always significantly different along the root (paired t-test at $P \leq 0.05$). The mean value of P_{OPR} at 60 mm was 3.8-fold smaller than that at 30 mm. During stagnant treatment, the P_{OPR} decreased by a factor of 5.5 from 30 to 60 mm; however, a significant decrease was not observed between distances of 50 and 60 mm (paired t-test at $P \leq 0.05$). Apparently, a 'tight barrier' to ROL was already present at these positions. At all distances, when compared with roots grown in aerated solution, the stagnant treatment resulted in several-fold lower O_2 permeability coefficient across the OPR ($F_{1,47} = 204.1$, $P < 0.001$). At 30 mm, the mean P_{OPR} values were 5.2 ± 0.2 and $0.6 \pm 0.1 \times 10^{-6}$ m s^{-1} for plants grown in aerated and stagnant solutions, respectively. At 60 mm, these values decreased to 1.4 ± 0.2 and $0.1 \pm 0.01 \times 10^{-6}$ m s^{-1} (SE; n = 19-30 roots). It should be noted that the P_{OPR} of plants grown in both solutions should be highest at positions closer to the root apex. However, using the perfusion technique it was not possible to measure P_{OPR} at distances below 30 mm from the apex (Colmer, 2003a; Kotula *et al.*, 2009).

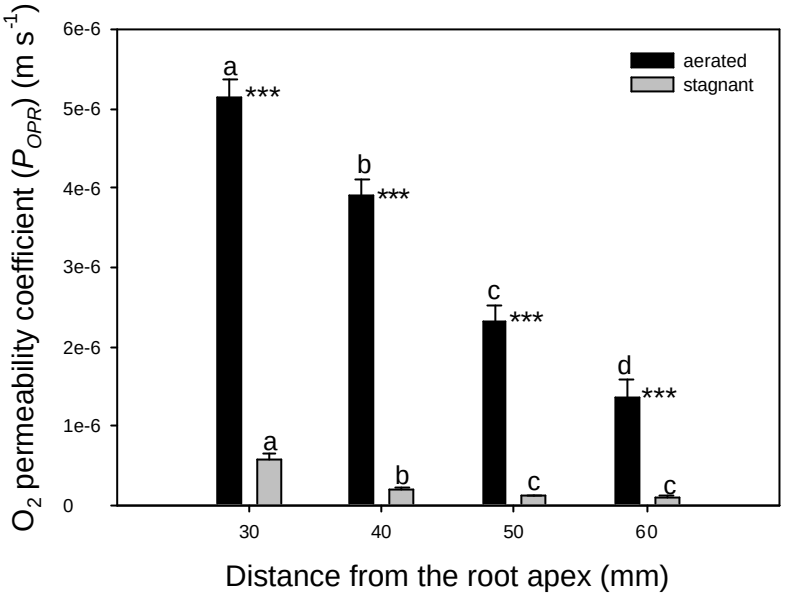

Fig. 1. Oxygen permeability coefficients of the outer part of roots (P_{OPR}) of rice grown in either aerated (black bars) or deoxygenated (grey bars) nutrient solution for 30 to 40 days. Measurements of P_{OPR} were performed at different positions along the root segments. Values of P_{OPR} were corrected for the resistance of the shell of agar between the root and the electrode surface. In both growth conditions, P_{OPR} decreased along the root. At all distances, P_{OPR} was significantly lower for plants grown in stagnant solution (significance level of $P \leq 0.001$ denoted by ***; two sample t-test). Different letters indicate significant difference between distances within a one growth condition (paired t-test). Data are means ± SE (n = 19-30 roots).

Blocking of the apoplastic path of the OPR with precipitated salt crystals

Irrespective of growth conditions, the surface of root segments untreated with $CuSO_4/K_4[Fe(CN)_6]$ appeared white along the entire length (Fig. 2a shows a root segment from an aerated plant). Because of the development of brown precipitates of $Cu_2[Fe(CN)_6]$ in cell wall pores of the OPR, the surface of roots of plants grown in aerated solution turned brown after treatment with 1 mM $K_4[Fe(CN)_6]$ and 0.5 mM $CuSO_4$ (Fig. 2b). The brown colour was more intense on the surface of immature (apical) part of the segments (30-50 mm from the apex). As apoplastic barriers develop in the basal part (50-70 mm), the brown colour gradually faded away. In contrast to roots grown in aerated solution, no precipitates were observed on the surface of root segments of plants grown in stagnant solution (Fig. 2c). Killing root segments by exposing them to 0.1 N of HCl resulted in intense brown precipitates along the entire surface of root segments in the aerated solution (Fig. 2d; compare with Fig. 2b).

Precipitates in dead root segments were uniformly distributed in the immature part but irregular and patchy in more mature parts. In roots from stagnant medium, no brown precipitates appeared even after exposing them for 30 min to 0.1 N of HCl (Fig. 2e). After 24 h treatment with $CuSO_4/K_4[Fe(CN)_6]$ only several dots of brown crystals were observed on the surface of the immature part of HCl-killed root segments (Fig. 2f).

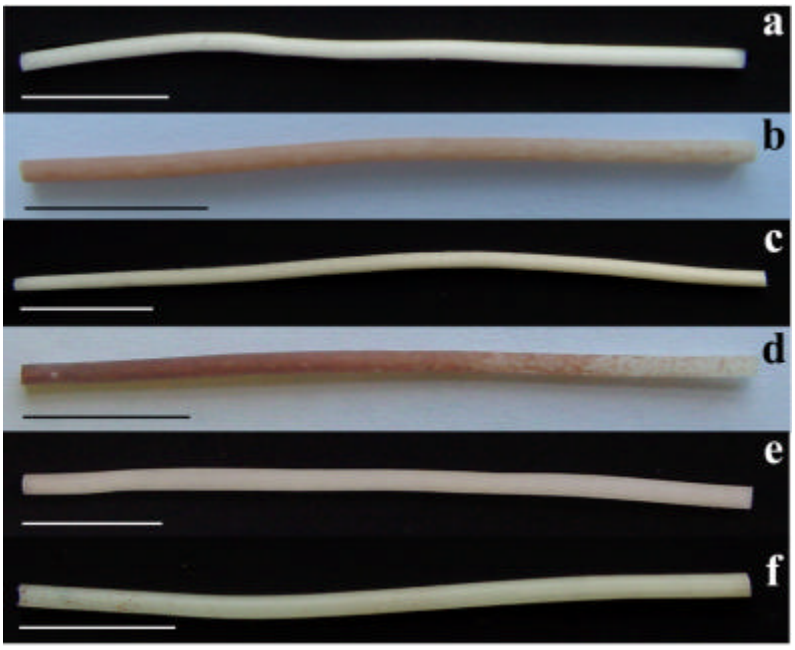

Fig. 2. Naked-eye views of the outer surface of rice root segments grown in either an aerated or a stagnant deoxygenated nutrient solution. Root segments of length 50 mm (20 to 70 mm from the apex) were placed in 0.5 mM $CuSO_4$ and aerenchyma was perfused for 3 h with 1 mM of $K_4[Fe(CN)_6]$. Brown copper ferrocyanide precipitates were formed in the cell walls of the inner cortical cell layers of the outer part of the root (OPR). (a) An untreated root segment. (b) Brown appearance of the surface of the root segment of a plant grown in aerated solution. (c) No precipitates in a root segment of a plant grown in the stagnant solution. (d) Intense brown precipitates along a root segment of a plant grown in aerated solution after exposure to 0.1 N HCl for 30 min. Precipitates were uniformly distributed in immature parts but irregular and patchy in mature parts. (e) No precipitates were formed in the root segments of plants grown in stagnant solution, even after killing of the cells with 0.1 N HCl. (f) Several dots of brown crystals (white arrowheads) were observed in the immature parts of the HCl-killed root segments grown in the stagnant solution, even after 24 h treatment with insoluble salts. Bars = 10 mm.

In cross-sections of aerated roots made after treatment with $CuSO_4/K_4[Fe(CN)_6]$, intense brown precipitates were observed in the walls of subsclerenchyma cortical cells at 30 mm from the apex (Fig. 3a). At 50 mm, less intense brown precipitates were observed in the tangential walls of the cortical layer beneath sclerenchyma (Fig. 3b). By contrast, no visible brown precipitates were detected in the cell walls at either 30 or 50

mm in stagnantly grown plants (Fig. 3c, d). This indicated that apoplastic barriers, which developed in roots grown in deoxygenated solution, blocked ion movement across the OPR.

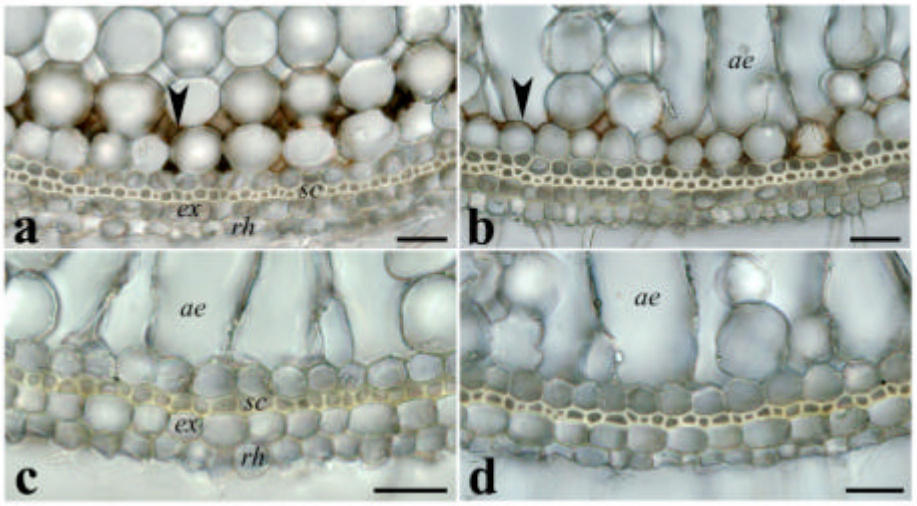

Fig. 3. Free-hand cross-section of rice root segments of plants grown in the aerated (a, b) and stagnant (c, d) solution, after they had been placed in 0.5 mM $CuSO_4$ and the aerenchyma had ben perfused with 1 mM of $K_4[Fe(CN)_6]$. Cross sections were made at 30 mm (a, c) and 50 mm (b, d) from the root apex. (a) Intense brown precipitates formed in the walls of subsclerenchyma cortical cells and (b) in the tangential walls of the cortical layer beneath the sclerenchyma of plants grown in aerated solution. (c), (d) No precipitates were formed in the root segments of plants grown in the stagnant deoxygenated solution. Bars = 50 µm.

Oxygen permeability coefficient of the OPR after blocking the apoplast with inorganic salt precipitates

The blockage of apoplastic pores of the OPR caused a decrease in the O_2 permeability coefficient (P_{OPR}) only in plants grown in the aerated solution (Fig. 4). For both control and treated root segments, P_{OPR} decreased along the root towards the base ($F_{3,15}$ = 41.2; $P < 0.001$). Compared with control, precipitates caused a decrease of P_{OPR} by 20, 16, 11 and 5 % at distances of 30, 40, 50 and 60 mm, respectively. At 30 mm, P_{OPR} decreased from 5.4 ± 0.5 to $4.3 \pm 0.3 \times 10^{-6}$ m s^{-1} and at 60 mm from 1.4 ± 0.3 to $1.3 \pm 0.3 \times 10^{-6}$ m s^{-1} (SE; n = 6). Declines were relatively small, but were significant at all distances (paired t-test at $P \leq 0.05$). By contrast, there were no treatment effects on the P_{OPR} of stagnantly grown plants.

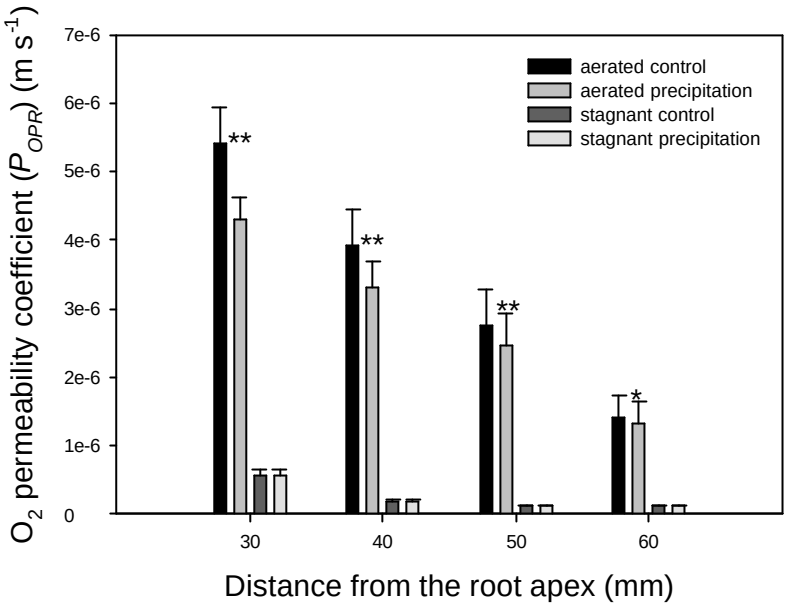

Fig. 4. Effect of treatment with insoluble salt precipitates on the oxygen permeability coefficient (P_{OPR}) of the outer part of rice roots (OPR). P_{OPR} was measured at different distances along root segments excised from plants grown for 30 to 40 d in either the aerated or the deoxygenated nutrient solution. Treatment with insoluble crystals resulted in the formation of brown precipitates only in the root segments of plants grown in aerated solution. Hence, blockage of apoplastic pores of the OPR caused a decrease in the oxygen permeability coefficient only in these plants. Significance level of $P \leq 0.05$ or $P \leq 0.01$ are denoted by * or **, respectively (paired t-test). Data are means ± SE (n = 6).

Oxygen permeability coefficient of the OPR after killing the root segments by 0.1 N HCl

In both control and HCl-treated root segments, P_{OPR} decreased along the root from apical to basal zones for plants grown in either aerated or stagnant solution ($F_{3,15} = 25.2$, $P < 0.001$ for aerated plants and $F_{3,15} = 12.5$, $P < 0.001$ for stagnantly grown plants). Compared with the control, treatment with HCl caused a significant increase in P_{OPR} at all distances along the root segments of plants grown in either aerated or stagnant conditions ($F_{1,5} = 66.9$, $P < 0.001$ for aerated plants and $F_{1,5} = 46.5$, $P = 0.001$ for stagnantly grown plants; Fig. 5). For plants grown in the aerated solution, P_{OPR} increased by a factor of *c*. 1.3. At 30 mm from the apex, the mean values were 4.8 ± 0.5 and $6.1 \pm 0.6 \times 10^{-6}$ m s^{-1} for control and HCl-killed root segments, respectively. At 60 mm P_{OPR} increased from 1.2 ± 0.5 to $1.7 \pm 0.6 \times 10^{-6}$ m s^{-1} (SE; n = 6). When plants were grown in the stagnant solution, the P_{OPR} of HCl-treated root segments was 1.6- to 2.2-fold greater than that of nontreated roots. The mean P_{OPR} values for control and

HCl-killed root segments at 30 mm were 0.6 ± 0.1 and 0.9 ± 0.2 × 10^{-6} m s^{-1}, respectively. Values at 60 mm were 0.1 ± 0.03 and 0.3 ± 0.02 × 10^{-6} m s^{-1}. The results indicate that effects of HCl treatments were significant but relatively small.

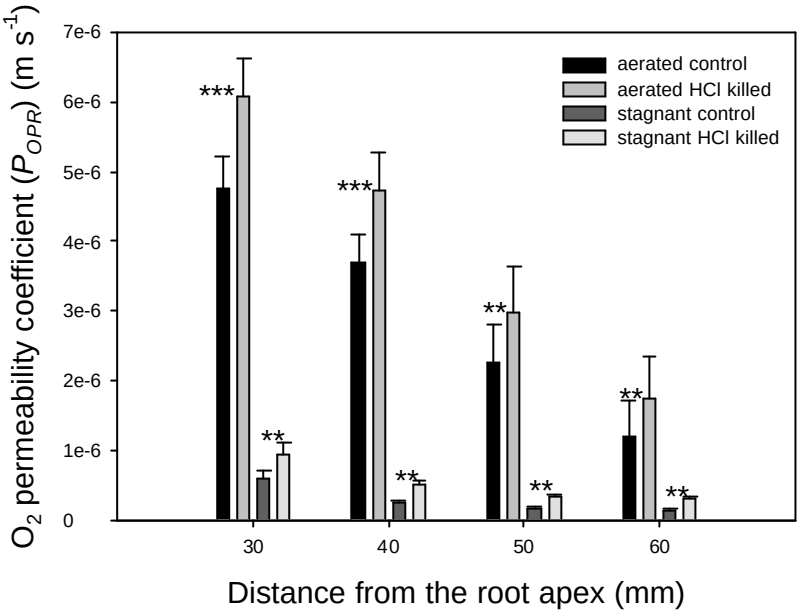

Fig. 5. Effects of 30 min of treatment with 0.1 N HCl on the oxygen permeability coefficient of the outer part of rice roots (P_{OPR}). P_{OPR} was measured at different distances along root segments excised from plants grown for 30 to 40 days in either the aerated or the deoxygenated nutrient solution. Regardless of growth conditions, there was an increase in the oxygen permeability coefficient at all distance from the apex after exposure to HCl. Significance level of $P \leq 0.01$ or $P \leq 0.001$ are denoted by ** or ***, respectively (paired t-test). Data are means ± SE (n = 6).

Tests for viability of cells in the OPR

After the experiments, the viability of cells in the OPR was examined by staining them with Evans blue. This stain cannot cross the intact plasma membrane and does not stain living cells. In killed cells lacking an intact plasma membrane, the stain could freely diffuse into the cell to stain the nucleus and cytoplasm dark blue. Unstained cells confirmed that the roots segments used for permeability experiments were alive, although a few cells in the epidermis were found to be dead (Figs. 6a, b). Treatment with $CuSO_4/K_4[Fe(CN)_6]$ did not affect the viability of cells (Figs. 6c, d). By contrast, nuclei and cytoplasm of all cells of HCl-killed root segments stained dark blue, proving that they were dead (Figs. 6e, f).

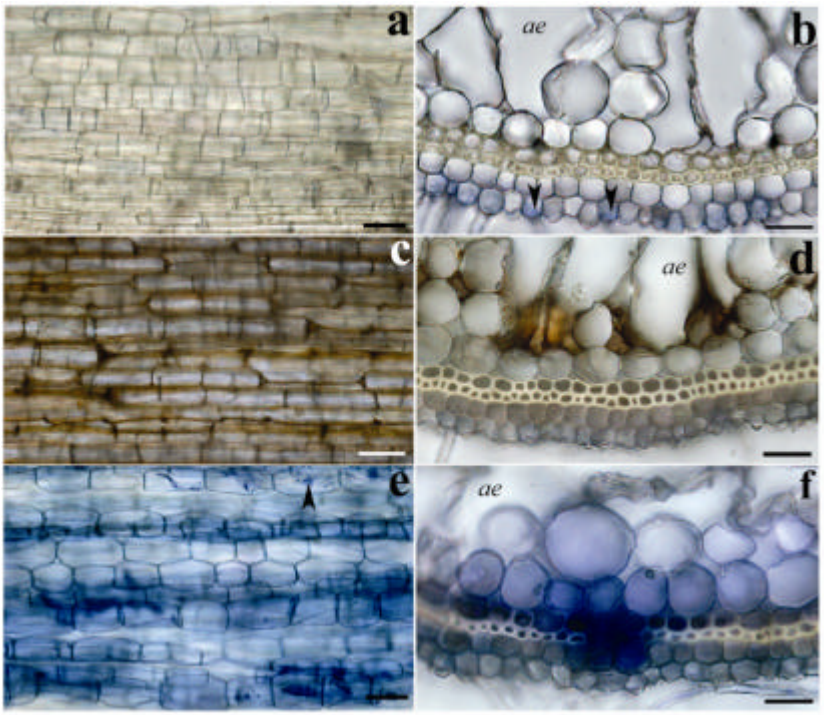

Fig. 6. Free-hand longitudinal sections (a, c, e) and cross-sections (b, d, f) of rice root segments taken *c*. 60 mm from the apex, stained with Evans bleu to check the viability of the cells at the end of the experiments. In dead cells, nuclei and cytoplasm were stained blue. (a, b) Following the gas perfusion experiment, only a few epidermal cells were found dead (arrowheads). (c, d) Treatment with insoluble crystals did not affect the viability of the cells. Only a few cells in the epidermis were found to be dead, as in controls. (e, f) Dead cells stained blue after 0.1 N HCl treatment (arrowhead). Bars = 50 μm.

Discussion

In the present study, the gas perfusion technique developed by Kotula & Steudle (2009) was used to measure oxygen permeability coefficients of the outer cell layers of roots (P_{OPR}) of rice plants grown in either aerated or stagnant, deoxygenated conditions. As far as we are aware, this is the first quantitative comparison of the permeabilities of the peripheral layers to O_2 in rice roots and how this change, when the OPR becomes modified. The results indicated that, when plants were grown in the stagnant deoxygenated solution, the P_{OPR} of all investigated zones was several folds smaller than that of plants grown in the aerated solution. When salt precipitates were formed in cell wall pores in roots of plants grown under aerated conditions, O_2 permeability decreased. By contrast, P_{OPR} increased in response to the killing of root cells with 0.1 N HCl. The

effect of the latter treatment was relatively small in roots in the aerated solution but larger in roots in the stagnant medium, suggesting a significant contribution of respiratory effects in the presence of low P_{OPR}. Overall, P_{OPR} was strongly affected by the apoplastic barriers in the roots of rce, which lowered oxygen diffusion across the peripheral cell layers.

The lower P_{OPR} in plants grown in stagnant medium as well as the reduction of P_{OPR} along the roots of plants grown in both conditions strongly correlated with the development of apoplastic barriers in the OPR. In a quantitative examination, Kotula *et al*. (2009) showed that the amounts of suberin and lignin increased along the roots of rice plants grown in both aerated and stagnant solutions, however, absolute loads of these polymers were much greater in roots grown under deoxygenated conditions. In agreement with chemical analyses, detailed histochemical studies of the OPR of rice revealed early development of exodermal Casparian bands and suberin lamellae in plants grown in stagnant conditions. In adidtion to suberization, early lignification of walls of densely packed sclerenchyma was found closer to the root apex in these plants (Kotula *et al*., 2009). Apparently, apoplastic barriers impeded gas diffusion across the OPR. Suberin, in particular, is known to offer a high resistance to O_2 diffusion (De Simone *et al*., 2003; Soukup *et al*., 2007). The present study supports these views.

Compared with the diffusion of water, O_2 diffusion across the OPR of plants grown in the aerated solution was greater by an order of magnitude. This may result from the fact that, in contrast to the O_2 molecule, water has a polar (dipole) structure, tending to reduce its diffusivity in suberized cell walls. Similar differences were found for cuticles (Lendzian, 1982; Lendzian & Kerstiens, 1991). The bulk (hydraulic) water permeability across the OPR of rice grown in an aerated soluton was much greater than diffusional water flow (Ranathunge *et al*., 2004). Modification of the apoplast of the OPR by filling intermicrofibrillar spaces (cell wall pores) with precipitates reduced the diffusional permeability of both water and O_2 by about 20%. By contrast, this treatment caused a massive 3- to 4-fold reduction of hydraulic conductivity (Lp_{OPR}; Ranathunge *et al*., 2005). This indicated that precipitates affected bulk flows of water much more than diffusive flows of water and oxygen. In contrast to bulk water flow, the diffusion of O_2 across outer cell layers ought to be appreciable over the whole inter-cell interface and was not restricted by cell membranes, which are thought to be highly permeable to O_2 (Armstrong, 1979; Nobel, 2005). The diffusion of O_2 across the OPR should be limited

by the existence of apoplastic barriers such as suberin lamellae and Casparian bands. Hence, the OPR of rice allows a rather high water flow in the presence of a relatively high resistance to O_2. This is achieved through differences in transport mechanisms: there is a bulk flow of water, but the flow of O_2 is diffusional in nature (Kotula & Steudle, 2009). This suggested that rice has evolved an optimum balance between water uptake and O_2 loss, with high rates of water uptake in the presence of low rates of O_2 loss (Ranathunge *et al*., 2004; Kotula & Steudle, 2009). However, to date, the above conclusion had been reached only for rice grown in aerated conditions. When plants were grown in a stagnant deoxygenated solution, which mimics natural paddy-field condition, roots showed several-fold greater amounts of suberin and lignin in the OPR and drastically reduced P_{OPR}. This means that pores were either completely absent in the apoplastic barriers under these conditions or diameters of pores were rather small. This situation differs from that in the cuticles, for which the precipitation technique of Ranathunge *et al*. (2005) has been used to demonstrate the existence of pores (Schreiber *et al*., 2006). In future studies, the existence or absence of pores in apoplastic barriers of roots of plants grown under deoxygenated conditions needs to be determined by comparing the diffusional and bulk permeabilities of water.

In agreement with Ranathunge *et al*. (2005), precipitation treatment of roots from aerated hydroponics produced intense brown precipitates at the apical parts of the root segments which gradually faded along the root towards the base. At least in the immature parts of roots, ions could pass through the exodermis and sclerenchyma layer. Because of the development of apoplastic barriers, ion movement across the OPR was reduced in more mature parts, but was still present. At distances of 50-60 mm, salt precipitates revealed a patchy structure, which may correlate with the maturation of the exodermis (Fig.2d). In cross-sections of salt-treated roots, brown precipitates were localized to the side where $K_4[Fe(CN)_6]$ was applied, suggesting that Cu^{2+} rather than $[Fe(CN)_6]^{4-}$ passed through the barrier of the OPR in plants grown in aerated conditions. Obviously, the ferrocyanide anion with its four negative charges moves across the barrier much more slowly than the positively charged copper kations, probably because of repulsion by the negative fixed charges of the cell wall matrix. In contrast to plants grown in aerated conditions, no precipitates were observed in the roots of plants grown in the deoxygenated medium. This is evidence that well-developed apoplastic barriers in the exodermis and lignified fibre cells impeded ion movement across the OPR. The

concentration of Cu^{2+} and $[Fe(CN)_6]^{4-}$ ions in the apoplast remained too low to cause precipitation. In addition, suberin lamellae may block ion transport through membranes. It was already suspected that the suberization/lignification of rice roots may form a barrier that reduces the uptake of ions, such as Fe^{2+} (Armstrong & Armstrong, 2005). Colmer & Bloom (1998) showed that NH_4^+ and NO_3^- uptake in basal regions of roots of *Oryza sativa* was *c*. 30% of that in *Zea mays*, even when plants were grown in aerated solution. The present results suggest that the barrier to ROL, which is induced during growth under deoxygenated conditions, reduced the transport of ions. Under stagnant conditions, the number and size of pores in the apoplast may be also reduced.

When cell wall pores were blocked with precipitates, the greatest reduction in P_{OPR} was observed closer to the root apex, where the exodermis was not yet fully developed. The effect was reduced at the more basal parts of the root. The dense precipitates formed in the OPR hindered the radial O_2 diffusion through the transcellular pathway, which is also done by suberin lamellae in well-developed roots (Ranathunge *et al*., 2005). However, it should be noted that the precipitates may not have completely blocked the pores in the cell walls. Thus, the reduction in O_2 permeability was relatively small. Overall, the effect of salt precipitates was twofold: firstly an additional barrier was created in series to the apoplastic barriers, and secondly pores within the barriers may have been blocked. No reduction in O_2 permeability in plants grown in the deoxygenated solution resulted from the lack of salt precipitates in cell wall pores.

At all tested distances and growth conditions, the acid treatment caused a relatively small increase in P_{OPR}. The increase may have resulted from cancelling of respiratory O_2 consumption by living cells in the OPR. It was possible to estimate the respiration rates of the OPR from the differences in radial O2 flows (in nmol m^{-2} s^{-1}) between control and HCl-treated roots. Assuming the thickness of the OPR to be 85 μm and the root diameter to be 1 mm, the respiration rate of a 40-mm-long root segment at atmospheric O_2 concentration would be 3.9×10^{-11} and 2.6×10^{-11} g O_2 mm^{-3} s^{-1} for plants grown in aerated and deoxygenated solutions, respectively (for a reference see Kotula & Steudle, 2009). Although there was a higher respiration rate in plants grown in the aerated solution, the relative increase in P_{OPR} after the cells had been killed was smaller than that found in plants grown in the stagnant medium. This suggested that, in plants grown in the aerated solution, the effect of respiration on P_{OPR} should be relatively small in relation to high rates of J_{O2}. This may differ from the situation in

plants grown in stagnant conditions, where J_{O2} is relatively low. The data indicated that respiratory O_2 consumption contributed much more in conjunction with a reduced O_2 permeability. It may be argued that, in addition to the respiratory activity, the acid treatment may affect the integrity of the apoplastic barrier by causing disintegration of the suberin or lignin polymers, allowing higher O_2 diffusion. However, as both suberin and lignin are known to be chemically resistant (Johansen, 1940; Ranathunge *et al*., 2008), 0.1 N HCl probably only had a small effect on them. This conclusion agrees with the findings that, firstly, the P_{OPR} of HCl-killed roots grown in the deoxygenated solution was still significantly smaller than that of untreated roots grown in the aerated solution and, secondly, in root segments from plants grown in stagnant conditions, no precipitates were observed after killing with HCl. Overall, the 'physical resistance' played a dominant role in impeding O_2 loss from rice roots, although respiration may also have an effect, when rates of radial O_2 loss were low. The results support the findings of previous studies by Armstrong *et al*. (2000) and Garthwaite *et al.* (2008) on roots of *Phragmites australis* and *Hordeum marinum*, respectively. In H. marinum, the physical barrier appeared to account for 84% of the reduction and respiratory activity for 16% (Garthwaite *et al*., 2008).

In conclusion, the quantitative comparison of O_2 permeability coefficients across the OPR (P_{OPR}) of rice grown in either an aerated or a deoxygenated solution indicated that the formation of apoplastic barriers strongly decreased P_{OPR}. Treatment with $CuSO_4/K_4[Fe(CN)_6]$ resulted in the formation of brown precipitates only in the roots of plants grown in the aerated solution, indicating that the apoplast of the OPR has a somewhat porous structure. By contrast, no precipitates were observed in roots of plants grown in the deoxygenated medium. It was concluded that well-developed apoplastic barriers in the exodermis such as Casparian bands and suberin lamellae, as well as lignified fibre cells blocked ion movement across the OPR. Even after killing of the root segments of plants grown in stagnant conditions, no precipitates were observed.This confirmed that apoplastic barriers had a substential effect on ion movement. The formation of precipitates in the OPR of aerated plants reduced P_{OPR} by 5 to 20%, depending on the development of the apoplastic barriers along the root. Comparison with earlier findings concerning diffusional and hydraulic water permeabilities supports the view that apoplastic barriers provided substantial resistance to the diffusion of O_2 across the OPR but allowed high bulk water flow. It appears that in rice, the OPR

allows a rather high water flow in the presence of a high resistance to O_2, which is an advantage for the plant. Killing of root cells by dilute hydrochloric acid increased P_{OPR} by up to 55%. This suggested a significant, although not dominating role of respiratory O_2 consumption in living cells of the OPR.The fact that P_{OPR} of HCl-treated roots of plants grown in the deoxygenated solution was still significantly lower than that of untreated aerated plants further supports the view that suberization and/or lignification provides a strong barrier to O_2 flow across the OPR of rice.

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4. Direct measurement of hydraulic properties in developing berries of *Vitis vinifera* L. cv Shiraz and Chardonnay

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Abstract

Berries of *Vitis vinifera* L. cv Shiraz can undergo weight loss during later stages of ripening. Existing published views on how weight loss occurs are based on changes in capacity of the vascular system to import water during development (McCarthy and Coombe, 1999). One important element of these views is the proposed cessation of water flow through the xylem after veraison. We have now measured the water flow into berries of Shiraz and Chardonnay as they develop using the pressure probe and the high pressure flow meter (HPFM). The pressure probe connected to the pedicel of individual berries provided measurements of single berry hydraulic conductance. By systematic excision of tissue segments of the berry and pedicel we determined where in the pathway hydraulic conductance changed during development. The HPFM was used on whole bunches showing that berries (including pedicels) represent parallel high hydraulic resistances and that the hydraulic resistance of the whole bunches was rather small. The hydraulic conductance per berry could be determined from excision experiments. There was close agreement between the pressure probe and HPFM measurements. Both showed a ten-fold reduction in hydraulic conductance of whole berries from veraison to full ripeness. Shiraz had hydraulic conductances that were 2- to 5-fold higher than those for Chardonnay. Shiraz maintained a higher hydraulic conductance past 90 days after flowering than Chardonnay. The decrease in hydraulic conductance occurred in both the distal and proximal parts of the berry for both varieties. The pressure probe also provided measurements of the xylem pressure that non-transpiring berries could develop. These pressures were –0.2 to –0.1 MPa until veraison and increased to zero when the juice osmotic potential reached about –3 MPa in Chardonnay and –4 MPa in Shiraz. The results suggest values of the reflection coefficient of the osmotic barrier around the xylem vessels of about 0.1-0.2 at veraison decreasing to 0 at harvest. It is suggested that in addition to changes in xylem anatomy, aquaporins in berry membranes may play a role in regulating hydraulic conductance. Water movement from the berry back to the parent vine via the xylem (backflow) may be an important component of berry weight loss in Shiraz, particularly if the phloem ceases functioning at high osmotic potentials near maximum weight. Backflow could account for a weight loss of 43 mg per day in Shiraz berries for a relatively small gradient of 0.1 MPa.

Abbreviations

L_o hydraulic conductance; **L_p** hydraulic conductivity; **R_o** hydraulic resistance; **DAF** days after flowering, **HPFM** high pressure flow meter

Keywords: Vitis vinifera, *berry ripening, xylem function, xylem pressure, hydraulic conductance, hydraulic conductivity, pressure probe, HPFM, aquaporins*

Introduction

Weight loss of *Vitis vinifera* L. berries during advanced stages of ripening may have impacts on sugar metabolism and flavour development (Coombe and McCarthy, 2000), mineral nutrient concentrations of berries (Rogiers et al. 2000), and the practical estimation of final yields by viticulturists (McCarthy, 1999). The most studied variety in this context has been Shiraz, which can loose over 20% of the maximum berry weight depending on maximum weight achieved (McCarthy, 1997; Rogiers et al. 2004a,b). Weight loss in Shiraz berries has been observed in a range of climates (Smart et al. 1974, Davies and Robinson 1996, McCarthy 1997, Rogiers et al. 2000) and under different irrigation regimes (McCarthy 1997). McCarthy observed that the onset of weight loss in field grown vines occurred at about 90 days after flowering (DAF) irrespective of seasonal differences or irrigation regime. For potted Shiraz vines, deficit irrigation, nitrogen nutrition and rootstock seemed to have an effect on the timing of the onset of weight loss with maximum weight being recorded between 74 and 85 DAF (Rogiers et al. 2004a). The extent of weight loss appeared to be related to the size of berries at their maximum weight that varied according to treatments. Larger berries at maximum volume (2 cm^3) showed larger percentage loss than smaller berries (1 cm^3) (Rogiers et al. 2004a).

To better define the cause of berry weight loss in Shiraz, McCarthy and Coombe (1999) calculated the components of berry weight during weight loss to show that it was loss of water that accounted for most of the loss of weight. The rate of accumulation of solutes per berry slowed after about 90 DAF and reached a plateau at 105 to 115 DAF. The changes in weight during different periods of berry development were explained in terms of variable contributions from xylem and phloem to berry water inflow. They proposed that xylem flow accounted for most of the water inflow up to veraison, during and immediately after which the xylem became non-functional. Water flow into the berry after veraison was proposed to occur predominantly via the phloem. Just before maximum weight was achieved it was proposed that the phloem became progressively non-functional resulting in water loss via transpiration exceeding water inflow. This model provides a good working hypothesis of berry weight loss where both xylem and phloem play key roles.

Historically the change in xylem functionality during ripening has been investigated in three ways. Firstly the uptake of water soluble dyes has been used to examine the xylem transport in both excised (Findlay et al. 1987, Düring et al. 1987, Creasy et al. 1993) and attached berries (Rogiers et al. 2001). These have all demonstrated that an apparent blockage occurred in the peripheral and axial xylem vessels of post-veraison berries. The general pattern from studies on Muscat Gordo Blanco (Findlay et al. 1987), Pinot Noir and Merlot (Creasy et al. 1993), Riesling (Düring et al. 1987) and Shiraz (Rogiers et al. 2001) is that during veraison, dye accumulation virtually ceased in the peripheral xylem and became much reduced or ceased beyond the brush region of the axial xylem. Breaks in tracheids mainly in the peripheral xylem have been linked to cessation of dye transfer (Findlay et al. 1987, Creasy et al. 1993). These studies imply that there is a marked change in xylem function that militates against the use of certain dyes used as a tracers. Studies based on dye molecules that are charged and of large molecular weight do not exclude the possibility that water may still enter or leave the berry via the xylem and connections to the berry apoplast (Rogiers et al. 2001).

A second method to assess xylem function has been to measure the changes in K and Ca content of berries as they mature. The rationale is that Ca is xylem mobile and phloem immobile, while K is both xylem and phloem mobile. Lang and Thorpe (1989) recalculated data on de Chaunac grapes from Hrazdina et al. (1984) to show that Ca accumulation ceased at veraison. Creasy et al. (1993) showed for Pinot Noir that K uptake increased rapidly after veraison but Ca did not. Contrasting to these results, Rogiers et al. (2000) showed that Ca content continued to increase in post-veraison berries of Shiraz, although the K content increased at a greater rate so that the K/Ca ratio increased in post veraison berries. This suggests that Shiraz berries may have a greater degree of xylem function after veraison compared to other varieties.

The third method of assessing the role of xylem and phloem during development has been to measure berry volume, weight or diameter, after treatments that impede the function of the phloem. These treatments, which have included girdling or heating of the pedicel, are compared to untreated attached berries, and berries that have been completely detached from the bunch but left in place to allow normal transpiration. With such experimentation Lang and Thorpe (1989) observed flow through the xylem from the berry to the parent plant (coined ‘backflow’). For Italia grapes that had ceased

volume expansion at full ripeness, 36% of the net water loss could be accounted for by xylem backflow.

In a highly original experiment Greenspan et al. (1994) examined the diurnal swelling and shrinking patterns of treated berries of potted Cabernet Sauvignon and Zinfandel. They concluded that the role of the xylem in berry volume changes was much reduced after veraison while the contribution by phloem increased substantially. A subsequent study on field vines of Cabernet Sauvignon under different irrigation regimes confirmed the potted vine study, but also showed that some backflow through the xylem occurred (Greenspan et al. 1996). However, most water lost by the berry could still be accounted for by transpiration. From measurements of bunch and stem water potentials, Greenspan et al. (1996) observed that the water potential gradients were predominately directed into the berry. More recently, Rogiers et al. (2001) have used girdling experiments on Shiraz berries. Girdling at 67 DAF reduced the volume of the berries as expected, but detached berries reduced volume to a much greater extent. This indicated that water inflow still occurred through the xylem despite the apparent blockage reported by tracer dyes. If the xylem in the brush region remains connected to the berry apoplast in Shiraz berries after veraison, it would also be possible for backflow to occur if the pressure gradient is in the appropriate direction. Thus xylem backflow could account for some berry weight loss in Shiraz, which accompanies the cessation of phloem function proposed by McCarthy and Coombe (1999).

The pressure gradients that generate flow via the xylem/apoplast of the berry will depend on the degree to which the stem water potential of the parent plant is transmitted to the pedicel xylem and apoplast of the berries, and on the counter tension that the berry can develop within the apoplast. Water potentials of the xylem of the bunch stem have been observed to be similar or slightly more negative than the main stem xylem (Greenspan et al. 1996), indicating that stem xylem tension from the vine can be transmitted through to at least the xylem in the bunch rachis. Although berries develop strongly negative osmotic potentials (up to -5 MPa) these may not counter the apoplast tensions developed by the parent vine. It has been indicated that membrane selectivity of pericarp cells is lost during ripening, i.e. the reflection coefficient is reduced (Lang and Thorpe 1989; Lang and Düring 1991). The reflection coefficient of a membrane is by definition the extent to which an osmotic gradient is translated into a pressure gradient across a membrane. It is likely that the barrier between the berry lumen and

xylem lumen will have a low reflection coefficient even when membranes of pericarp cells have intact selective membranes (high reflection coefficient). Any reduction in reflection coefficient of berry membranes could render the berry's negative osmotic potential ineffective in preventing water loss to the parent vine.

From the discussion above, a key factor for understanding the role of the xylem in berry weight loss in ripening fruit is the way in which the hydraulic conductance and selectivity of the xylem-berry interface changes during development. This interface is likely to be analogous to a composite osmotic barrier consisting of pathways with different hydraulic conductances and reflection coefficients analogous to the composite transport model of membranes or roots (Steudle and Peterson 1998, Steudle and Henzler 1995). According to McCarthy and Coombe (1999) we would expect to find a sudden reduction in hydraulic conductance during veraison. Rogiers et al. (2004b) also hypothesize that a reduction in net vascular flow into the berry, concomitant with continued transpiration, leads to berry weight loss. Despite the importance of this reduction there appear to be no quantitative measurements of overall xylem hydraulic conductance, nor any elucidation of where changes in hydraulic conductance might occur within either the berry or the pedicel-berry system.

Here we report for the first time quantitative measurements of hydraulic conductance of pedicel-attached berries and parts thereof by using the root pressure probe (Steudle et al. 1993) and high pressure flow meter (HPFM, Tyree et al. 1995). Comparisons are made between Shiraz and Chardonnay berries. Chardonnay was chosen because it is not known to undergo substantial weight loss during later stages of ripening. We expected to observe that the hydraulic conductance of Shiraz was higher than that of Chardonnay and that the main region within the berry where hydraulic conductance changed during development would be in the brush region. Using the pressure probe we were also able to measure the xylem tensions that could be developed by non-transpiring detached berries. We expected to find a reduction in xylem tensions if the membranes in the osmotic barrier separating the xylem from the berry interior were to lose semipermeability during ripening or if the berries would tend to become hydraulically isolated due to a high hydraulic resistance in the xylem.

Material and Methods

Fruit material

Hydraulic measurements were conducted on pre-veraison and post-veraison berries collected from *Vitis vinifera* cvs Shiraz (BVCR12) and Chardonnay (I10V1) growing on own roots in the Coombe Vineyard, Waite Campus of the University of Adelaide during the 2002-2003 and 2003-2004 seasons. In 2002-2003 Shiraz berries were selected from twelve vines that formed part of a randomized block rootstock trial. The Chardonnay berries were from twelve adjacent vines. Whole shoots were cut from the vine in the morning, the cut stem placed into water and transported to the laboratory (500 m). Berries were cut from the bunch with the pedicel underwater immediately before experiments. For 2003-2004 six replicate Shiraz and Chardonnay vines were selected from across the block and used for all measurements. Whole shoots collected with two bunches attached were transported to the laboratory with the cut stem in water. Samples were collected at least three times a day, because when the shoots were more than 3 hours in water the conductance appeared to fall sharply, presumably because of xylem occlusion caused by bacterial growth. Once in the laboratory the bunches were detached immediately prior to experiments at the proximal end of the peduncle by cutting with a sharp razor blade under water. The bunch closest to the proximal part of the shoot was used for HPFM measurements.

The dates of flowering (time for 50% capfall across the entire vineyard) were recorded for both seasons so that changes in berry physiology could be related to days after flowering (McCarthy, 1999).

Pressure probe measurements

The root pressure probe (Figure 1a) was used to measure the water relations of whole berries and berries cut back at different positions. The root pressure probe is normally used to measure equilibrium root pressure and root hydraulic conductance (e.g. Steudle et al. 1993). Analogous to this we have connected the probe to the pedicel of single berries and assumed that the xylem provides a hydraulic connection between the probe and berry (analogous to the use of the instrument with roots). Dye injection experiments confirmed that the xylem was connected hydraulically to the pressure probe. To seal the pedicel with the instrument a piece of tubing 15-20 mm long (Tefzel-Schlauch tubes of

ID 1.0 and 1.6 mm (OD 1.6 and 3.2 mm) depending on pedicel diameter) was flared at one end in order to provide a snug fit to the pedicel. The pedicel was gently pushed into the flared end and sealed with a cyanoacrylate glue (Super Glue or Loctite 401 or 406 adhesive with tubing treated with Loctite 770 primer). This provided a pressure tight seal which was tested as part of the measuring procedure (see below). The berry and pedicel connected to the tube were sealed into the end of the pressure probe taking care to eliminate any air from the system. The water used to fill the tubing and probe was Millipore (0.2 μM) purified and de-aerated by vacuum treatment. The berry was covered with wet tissue paper to prevent transpiration. As the seal was tightened around the tubing the pressure was elevated in the system (Figure 1b). Measurements were made in the laboratory at temperatures between 20-22 ^{o}C.

Steady state pressure

The pressure of the internal hydraulic system was allowed to relax to a steady state level after sealing (Figure 1b). In pre-veraison berries the pressure often became quite negative and in some instances caused cavitation in the probe. When this occurred it was not possible to carry out further measurements unless a constant water flow into the berry was allowed at higher pressures.

Elasticity

Subsequent to a steady state pressure being achieved, elasticity of the 'system' (measured as $\beta_s = DP/DV$) was determined by changing volume (using the micrometer screw and oil-water interface position in the measuring capillary) and recording the change in pressure (Figure 1c). 'System' in this case refers to a pedicel-attached berry plus probe and seals. The elasticity of the probe (β_p) with a blocked piece of tubing inserted in place of the pedicel was also measured. Figure 1d shows the change in volume with change in pressure for connected berries and blocked tubing. If β_s was equal to or higher than the value for blocked tubing (β_p), then it is possible that hydraulic continuity through the pedicel had not been obtained. Other two criteria were used to test for hydraulic continuity: (1) glue was used to block the cut pedicel which should then show a large reduction of hydraulic conductance; (2) at the end of an experiment the cut surface of the pedicel was allowed to dry. If this caused a negative pressure to develop in the xylem, which returned to zero when a drop of water was placed on the cut surface it was assumed that a hydraulic connection was present.

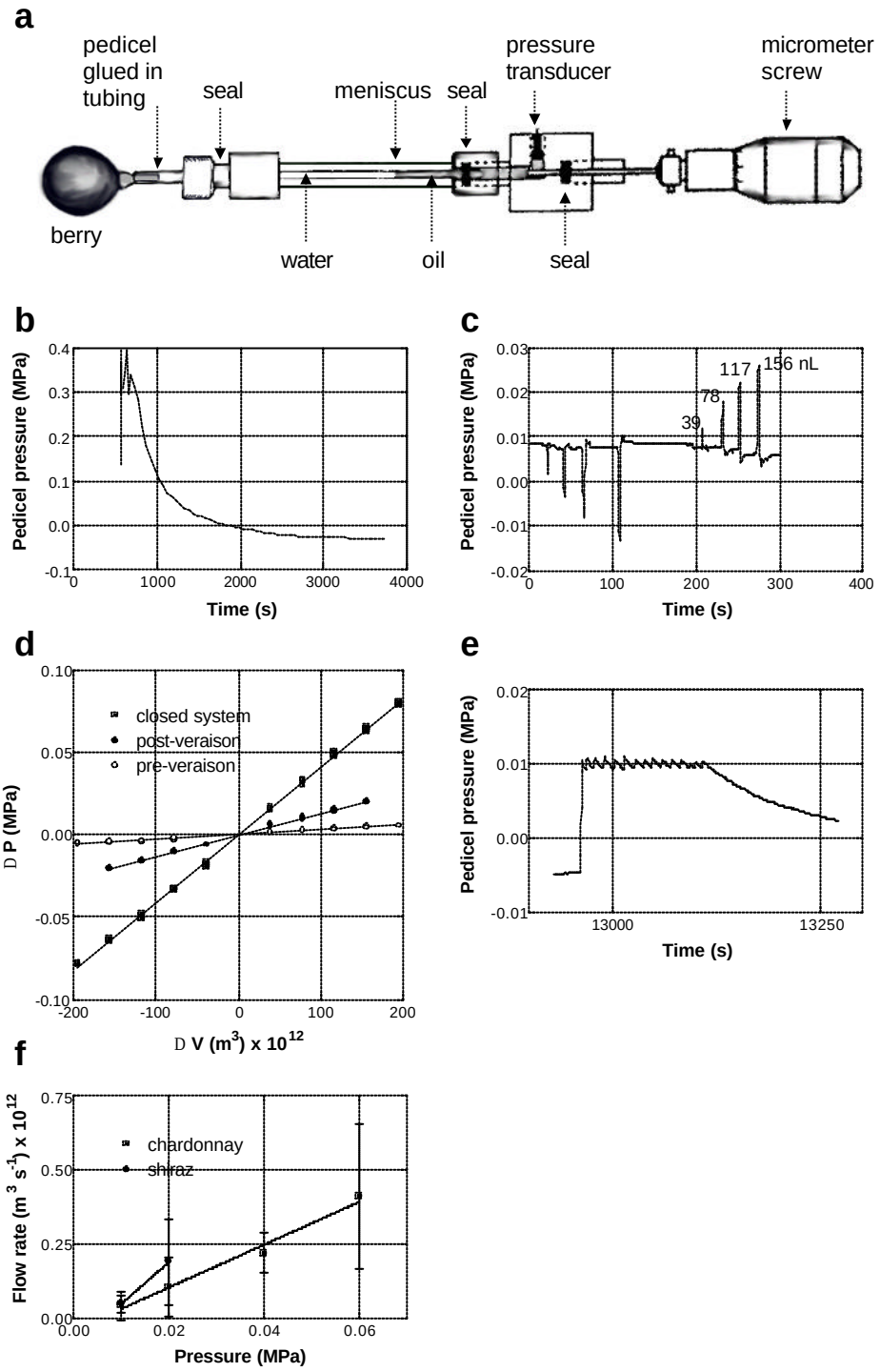

Figure 1. The pressure probe technique for measurement of berry hydraulic conductance and xylem tensions in the pedicel. (**a**) Diagram of the "berry" pressure probe illustrating the method of attaching the berry pedicel to a piece of plastic tubing that is sealed into the probe. The oil/water meniscus can be used to measure volume flow into or out of the berry. The pressure transducer is connected to a computer and the micrometer screw is used to inject or withdraw water from the pedicel-attached berry system. (**b**) Initial equilibration stage after sealing the berry to the probe. The pressure slowly relaxes to a steady level that was always negative in berries pre-veraison and became positive during veraison and after veraison.

(c) Pulses of pressure recorded when different volumes were injected or withdrawn from the berry. These are used to calculate elasticity of the system. (**d**) Pressure change as a function of volume change used to calculate elasticity. A closed (blocked) system was measured in order to check the hydraulic connection with the berry. (**e**) A pressure clamp experiment. The pressure is held at a constant level in this case by continued injection of volume into the berry. (**f**) The rate of volume injected is recorded and plotted as a function of the pressure. The slope of the relationship in (f) enables calculation of the hydraulic conductance of the berry. Berries were cut back at different positions during an experiment (see Figure 7a) in order to determine respective positions of major hydraulic resistances.

Hydraulic conductance

To determine the hydraulic conductance, a pressure clamp procedure was used (Figure 1e). Initial experiments demonstrated that pressure relaxation curves combined with measurements of β, as usually used with roots (Steudle 1994), overestimated the hydraulic conductance through cut pedicels. Pressure clamp (volume relaxation) experiments entailed elevating the pressure by a certain recorded amount (between 0.01 and 0.05 MPa) and maintaining this increment in pressure while recording the rate of volume flow into the berry, when this was steady or nearly so (as during the HPFM technique). This could be done either using the movement of the oil-water interface or by following the movement required on the micrometer screw (both gave the same volume flow). Figure 1f shows steady flow rate as a function of pressure into whole berries from which hydraulic conductance was calculated. To determine limiting hydraulic conductances in parts of the berry and pedicel, distal portions were excised progressively. These positions coincided with: the base of the seeds, base of the berry; and base of the receptacle (torus) (Figure 7a). Hydraulic conductance (L_o) was divided by the berry surface area to give a value for 'hydraulic conductivity' (L_p) for comparison with other measurements on fruit in the published literature (Fishman and Génard, 1998). The assumption behind using the fruit surface area is that the actual composite membrane surface area is proportional to berry size.

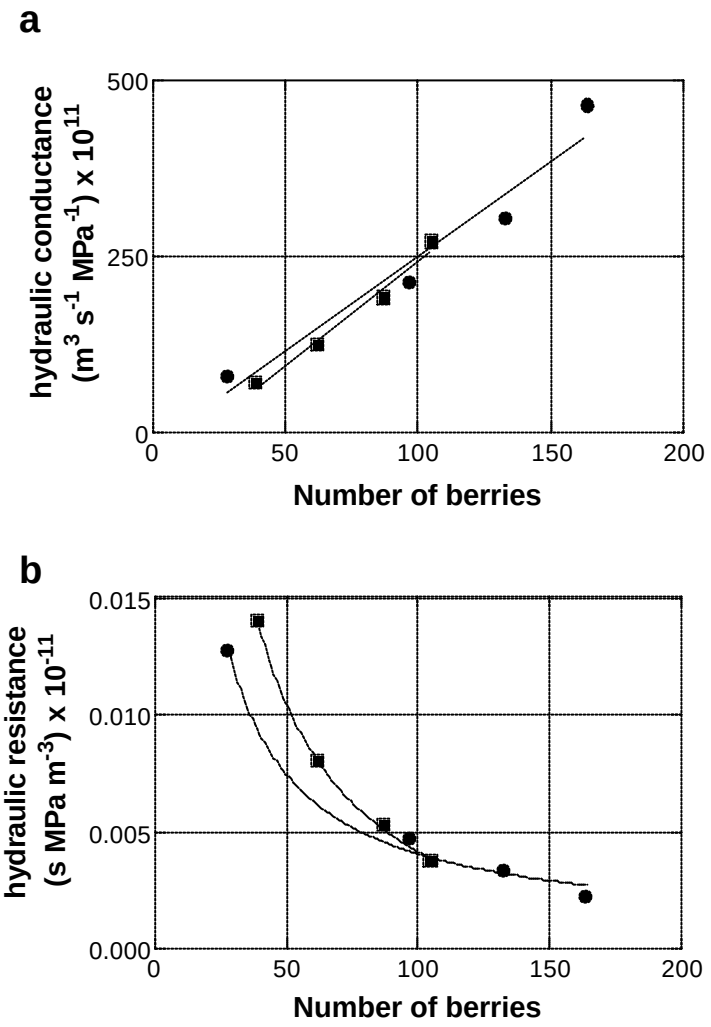

Figure 2. High pressure flow meter experiments performed on a whole bunch while excising successive segments of the bunch (distal to proximal) gave a close to linear relationship between hydraulic conductance and the number of berries (**a**) where two separate experiments are shown. (**b**) Resistance as a function of the number of berries could be fitted to an equation of the form $R_o = A + B/(\text{berry number})$. *A* was small indicating that the rachis hydraulic conductance was much larger than that of the berries.

Hydraulic conductance of whole bunches using the High Pressure Flow Meter (HPFM)

An hydraulic conductance flow meter (HPFM-XP Dynamax, Houston Texas, USA) was used to measure hydraulic conductance of whole bunches and cut-back bunches. Use of the HPFM has been described for measuring root and shoot systems hydraulic conductance by Tyree and colleagues (Tyree et al. 1995; Bogeat-Triboulot et al. 2002). The HPFM was used as per manufacturer's instructions in either the transient or steady state mode of operation. The temperatures at which the measurements were made were close to that used for the calibration of the instrument (between 20-22 ^{o}C). After

connecting the bunch via the cut peduncle and ensuring that no air had entered while sealing, transient measurements were begun. The pressure was slowly elevated at a constant rate while flow was recorded with the instrument. Flow was measured as the drop in pressure across a manifold connected with tubing of different and known hydraulic conductances. It was sometimes necessary to undertake a few trial measurements in order to use the appropriate flow range on the instrument and to test for the presence of air in the system after sealing. The hydraulic conductance was determined from the slope of the linear part of the volume flow versus pressure relationship (i.e. for steady flows, when all relevant capacities for water were infiltrated within the pedicel or berry). The pressure range for transient measurements was normally between 0.2 and 0.45 MPa. Measurements using the quasi steady state flow meter mode of the instrument where a constant pressure for a period of time was applied and flow rate measured (analogous to the pressure-clamp measurements with the pressure probe), gave hydraulic conductances not significantly different from those measured in transient mode on the same bunches (P=0.27, two ANOVA with repeated measures on six replicated bunches, cut to the four segments, see below). When using either the HPFM or pressure clamp it has to be ensured that infiltration of tissue by relatively large amounts of water does not change the hydraulic conductance being measured). No evidence of this was seen, and this is consistent with the tiny volumes injected relative to the volume of berries.

After at least four replicate measurements of hydraulic conductance, the measured bunch had the distal most portion (about 25% of berries) severed with a sharp razor and the cut rachis (main bunch stem) was sealed with Super Glue. Another set of transient measurements was taken before the next most distal 25% (approx.) of berries was removed and the rachis again sealed. This process was continued until at least three segments of the bunch were removed. This provided hydraulic conductances for: (1) the whole bunch, (2) bunch minus first distal segment, (3) bunch minus first and second distal segments, (4) bunch minus first, second and third distal segments; called proximal segment. Berries were counted and weighed for each segment. Surface areas could be calculated from the known relationship between surface area and berry weight (see below).

Plots of hydraulic conductance versus the number of berries yielded linear to slightly concave-up curves (Figure 2a). Although in most cases a linear equation was

statistically a better fit, we used an exponential equation to fit the relationship between hydraulic conductance and number of berries. The hydraulic conductance per berry was estimated from these fits assuming that the bunch stem and branches had a much higher hydraulic conductance than the berries themselves. This was checked by examining the hydraulic resistance (1/hydraulic conductance) as a function of berry number (Figure 2b). That relationship could be best fitted by an equation of the form: *Resistance = A + B/number of berries,* where *A* was close to zero, to a god approximation. A value of *A* close to zero indicates a high conductance of bunch stem and brunches. Hence, the whole bunch acted as a simple system of similar parallel hydraulic resistors, wher component conductances were additive.

Berry weight

During the 2003-2004 season mean berry weight was recorded from 50-100 berries as 5-berry samples from proximal mid and distal parts of bunches on the six replicate vines. Berries were collected by the same method during 2002-2003 for Brix, pH and osmolality measurements.

Brix, pH and osmolality

Approximately 50 mL of juice was collected from berries selected for weight measurements. Brix was measured using a digital refractometer (temperature compensated, ATAGO Model PR101). pH was measured using a Cyberscan pH 310 Series (Eutech Instruments). Osmolality was measured on juice using a Wescor (Model 5500) water vapour pressure osmometer and converted to osmotic potential ($\Psi_\pi = -RTC$, where R=8.3143 J/mol.K and T = absolute temperature in K).

Stem water potential

One leaf from each replicate vine was sealed in foil-covered zip-lock bags for at least one hour before water potential was recorded using a pump-up portable pressure chamber (PMS Instrument Co.). Measurements were taken between 12 noon and 2 pm local time.

Berry deformability

Berry deformability was measured across the mid section of the berry at the widest point using a set of calipers that placed a constant force generated by a spring equivalent to 2.06 N. The deformation was measured as the millimeter reduction in diameter in response to the force. Five berries from each of four bunches of the six replicate vines were randomly selected to measure deformation.

Berry surface area

Berry surface area was calculated from measurements of length and diameter using the formula for the surface area of a prolate spheroid. For surface areas of berries on whole bunches used for the HPFM, a calibration was first obtained between berry surface area and berry weight on separately harvested berries. The relationship was best fitted by a quadratic equation of the form; surface area (mm^2) = A + B weight (g) + C weight $(g)^2$, where for Shiraz A=544.6 mm^2, B=1305 mm^2/g, C = -90.68 mm^2/g; and Chardonnay A= 486.7 mm^2 B= 1455 mm^2/g^{-1}, C= -132.8 mm^2/g. Surface areas for HPFM experimental berries were then determined from the weights of the berries on the segments of the bunches.

Results

Pedicel equilibrium pressure related to post-veraison berry development

Pedicel equilibrium pressures were initially negative (sub-atmospheric) at veraison, which for the 2002-2003 growing season occurred at approximately 50 days after flowering (DAF) in both Shiraz and Chardonnay (Figure 3a). Pre-veraison berries could be measured, but often the negative pressures of less than -0.1 MPa generated would cause cavitation in the pressure probe. Through veraison and afterwards, the pedicel equilibrium pressures gradually increased and approached an asymptote at about zero, or at slightly positive pressures of no more than about 0.02 MPa. For both Chardonnay and Shiraz the pedicel equilibrium pressures reached zero MPa at about 80-90 DAF (see fitted curves in Figure 3a). The timing of this corresponded to juice osmotic potentials of about –3 and –4 MPa for Chardonnay and Shiraz, respectively (Figure 3b). TSS and pH increased to reach full ripeness values at about 110 DAF (about 22 ^{o}Brix and pH 4 respectively) (Figure 3c).

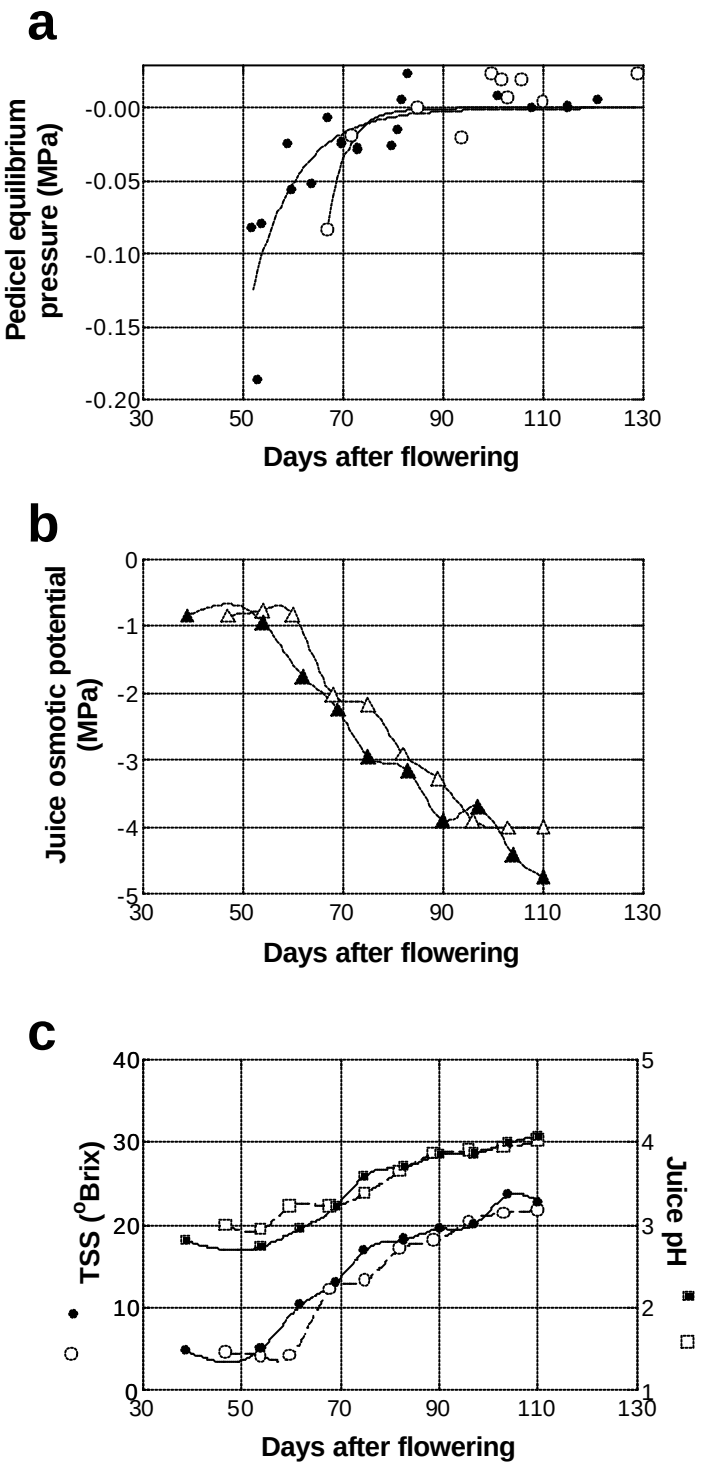

Figure 3. Pedicel equilibrium pressure (**a**) and ripening responses (juice osmotic potential, (**b**); TSS and pH (**c**)) of berries of Shiraz (closed symbols) and Chardonnay (open symbols) as a function of days after flowering collected during the 2002-2003 season. Comparison of pedicel equilibrium pressure with juice osmotic pressure at 50 DAF resulted in a estimate of the reflection coefficient of the osmotic barrier within the berry of 0.15.

In the 2003-2004 growing season, more data were collected on berry hydraulic parameters over the time between veraison and about 100 days DAF. The pedicel equilibrium pressures behaved essentially as was observed in 2002-03, with a gradual increase from subeatmospheric values of between –0.025 MPa and –0.05 MPa to slightly positive values of up to 0.025 MPa (Figure 4a). Similar to behaviour recorded in 2002-03, observations in 2003-04 showed that Chardonnay reached pressures near zero near 80 DAF while Shiraz reached zero pressure at 90 DAF. This again corresponded to osmotic potentials of the juice of –3 MPa in Chardonnay and –4 MPa in Shiraz (Figure 4b). Osmotic potential of the juice declined more rapidly over time than for 2002-03, but reached similar peak values. Subsequent to these peak values of about –4.5 MPa in Chardonnay and –4.8 MPa in Shiraz, the osmotic potential increased again to values above –4 MPa (Figure 4b). For both Chardonnay and Shiraz there was a significant loss of berry weight after day 79 in Chardonnay and after day 75 in Shiraz (Figure 4c). This corresponded to a slight reduction in stem water potential measured at midday on the vines bearing the fruit (Figure 4d). However, subsequent irrigations that increased water potential to above –1 MPa for Shiraz (Figure 4d) did not correspond to a gain in weight of Shiraz berries (Figure 4c). Berry deformability increased as expected during ripening reaching a maximum for Shiraz at 96 DAF (Figure 4e). A full series of measurements was not possible for Chardonnay due to the selected vines being harvested. Despite veraison occurring later in 2003-04 than in 2002-03, the berries reached maturity quicker with full ripeness (about 22 ^{o}Brix) being reached at day 89 in Chardonnay and after day 96 in Shiraz (Figure 4f).

Hydraulic conductance of single berries measured with the pressure probe

Figure 5 shows the hydraulic conductances of Shiraz (a, c, e, g, left side) and Chardonnay (b, d, f, h, right side) berries during ripening in the 2002-03 (open symbols) and 2003-04 (closed symbols) and for the berries cut back at different positions (see Figure 7a). Different operators of the pressure probes collected data in each season yet there was good agreement in the magnitude of hydraulic conductances measured.

There was a trend of declining hydraulic conductance during ripening for both seasons and this was significantly so for whole berries of Shiraz (Figure 5a). Shiraz appeared to have higher hydraulic conductances than Chardonnay for whole berries and all cut positions (compare Figure a with b, c with d, e with f, g with h).

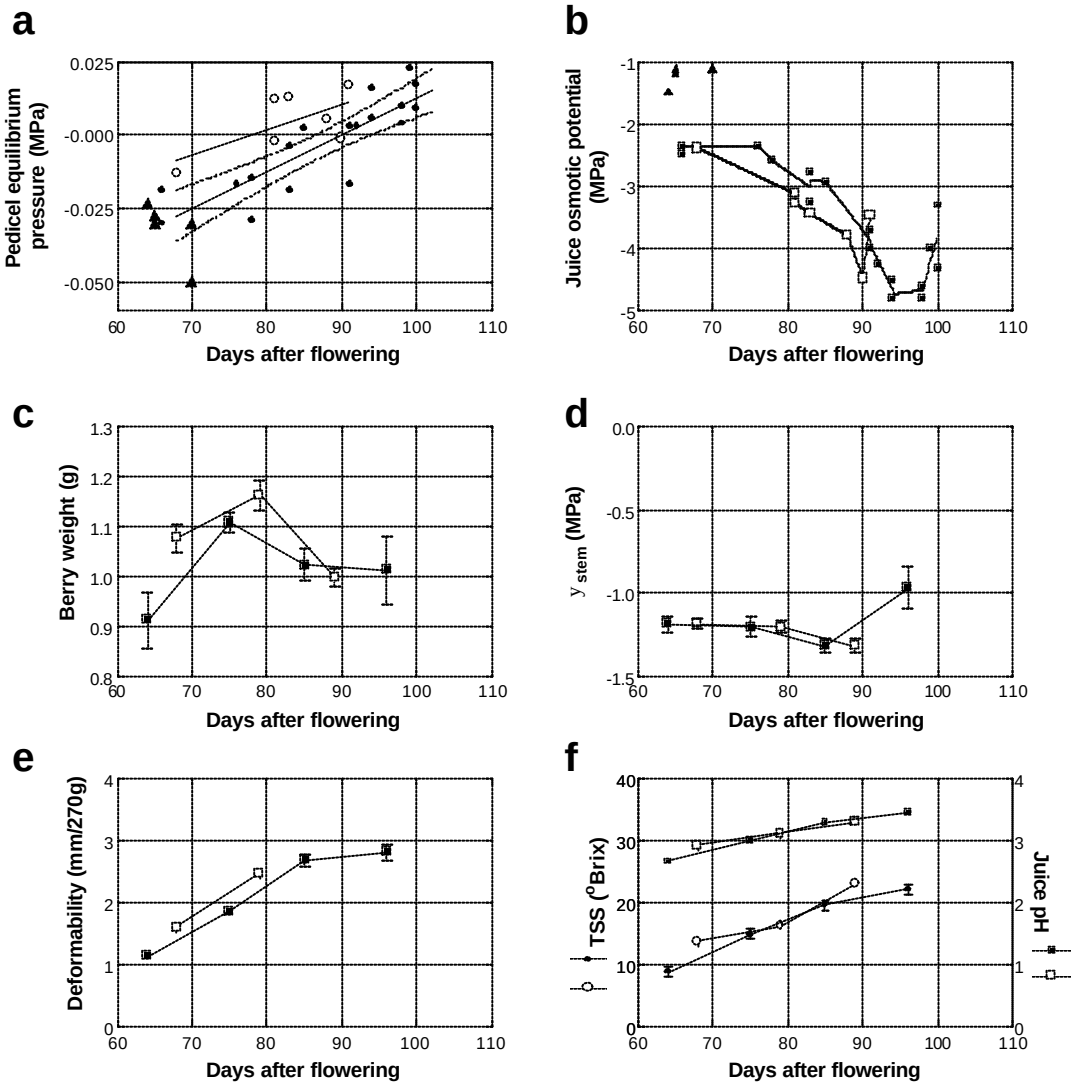

Figure 4. Pedicel equilibrium pressure (**a**) and berry physiological parameters of Shiraz (closed symbols) and Chardonnay (open symbols) as a function of days after flowering collected during the 2003-2004 season. Shown are: juice osmotic potential for pre-veraison berries (solid triangle), and post-veraison berries (solid square) (**b**); berry weight **(c)**; stem water potential (**d**); deformability (**e**); and juice TSS and pH (**f**). With respect to pedicel equilibrium pressure, the same berries that were used in Figure 5 are shown here and were taken from the same shoots used for the HPFM experiments (Figure 6). All vales in figures c, d, e, and f are means (±SEM) from six replicate vines.

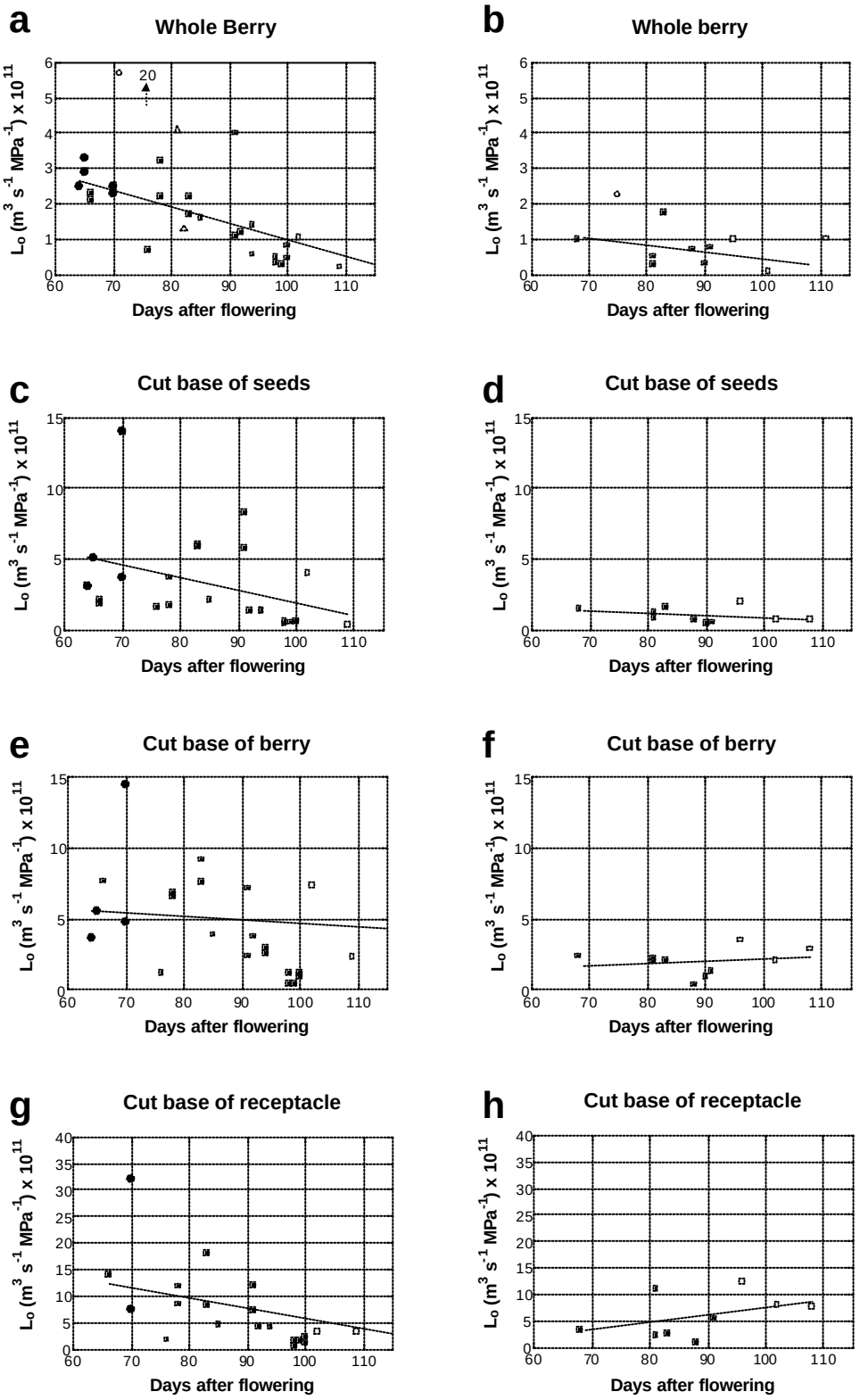

Figure 5. Berry hydraulic conductances *versus* days after flowering for Shiraz (**a, c, e, g, left side**) and Chardonnay (**b, d, f, h, right side**) measured with the pressure probe in two seasons (2002-2003 open symbols; 2003-2004 closed symbols). Berry tissue was excised at successive locations as indicated in Figure 7a. Regressions were fitted to the combined 2002-2003 and 2003-2004 data.

Hydraulic conductance of berries determined using the HPFM

Using the method of cutting back whole bunches while measuring the change in hydraulic conductance (see methods) it was possible to measure hydraulic conductance per berry during ripening in the 2003-04 season (Figure 6). These measurements correspond to the closed symbols in Figure 5a and 5b using the pressure probe on single whole berries. Since averages are taken over many more berries with the HPFM measurement there was inherently less scatter in the measurements of berry hydraulic conductance. There was remarkable agreement between the HPFM and pressure probe measurements of berry hydraulic conductance (compare Figure 6 with Figures 5a, 5b). Regression against DAF (fitted lines) showed a significant decline in berry hydraulic conductance for both varieties confirming the trends observed with the pressure probe measurements. Furthermore, Shiraz had significantly higher berry hydraulic conductance than Chardonnay, also confirming the validity of pressure probe measurements.

Hydraulic conductances have been converted to hydraulic conductivities (on a berry surface area basis) for the HPFM measurements (Table 1). A two-way repeated measures analysis of variance on the corresponding measurement dates showed that the berry hydraulic conductivity was significantly larger for Shiraz than the Chardonnay at the first and last sampling date corresponding to 68 DAF (Chardonnay) and 64 DAF (Shiraz), and 89 DAF (Chardonnay) and 85 DAF (Shiraz).

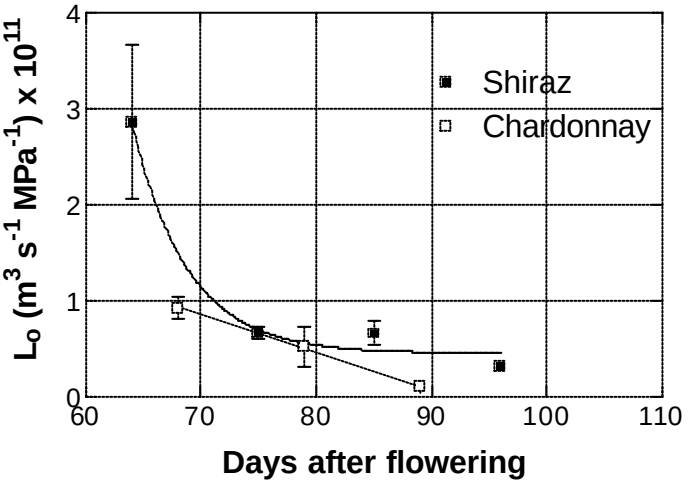

Figure 6. Berry hydraulic conductances *versus* days after flowering from HPFM experiments conducted in 2003-2004. Values are the mean of six replicate vines (one bunch per vine). Linear regression equations are significantly different between varieties (P<0.05) and are: Shiraz (closed symbols); slope =

-0.0755×10^{-11} m^3/s.MPa.day, intercept = 7.3×10^{-11} m^3.s.MPa. For Chardonnay (open symbols); slope = -0.040×10^{-11} m.s.MPa.day, intercept = 3.6×10^{-11} m^3/s^1,MPa. For Shiraz an exponential equation was statistically a better fit to the data (solid line). For the exponential equation: span = 1.4×10^{-5} m^3/s.MPa, plateau = 0.44×10^{-11} m^3/s·MPa, rate constant = 0.21/day.

Discussion

Studies reported in this present paper on two grapevine varieties have provided the first published data on hydraulic properties of the pedicel and components of berries during development. Since the phloem would become blocked when the pedicel is excised, we assumed that the measurements reflect water transport through the xylem and composite osmotic barrier within the grape berry that are in series to the xylem. This has been confirmed by dye injection experiments (J. Tilbrook pers. comm.) and is also the underlying assumption in previous pedicel feeding experiments (Findlay et al. 1987) and in root pressure probe measurements (Steudle et al. 1993). When used in the pressure-clamp mode, the pressure probe and HPFM techniques gave values of berry hydraulic conductance that were in good agreement. The HPFM may be more generally suitable for measuring hydraulic conductances for other fruits during development.

The magnitudes of the ‘hydraulic conductivity’ (conductance divided by berry surface area, Table 1) are similar to the estimates made from modeling the dynamics of peach fruit expansion ($2\text{-}3 \times 10^{-4}$ g/cm.h, Fishman and Génard, 1998). The actual composite membrane hydraulic conductivity in the berry will be determined by the relationship between berry surface area and the effective surface area of a composite membrane (apoplast and symplast) within the berry. However, it is not clear what constitutes the composite membrane within a berry since there is no clear endodermis with a casparian band analogous to roots in berries, and the reflection coefficient of the osmotic barrier is close to zero in ripening fruit.

Date	Shiraz			Chardonnay			
	DAF	Berry Lp ($m^3/s.m^2.MPa$) $\times 10^9$	SEM ($m^3/s.m^2.MPa$) $\times 10^9$	DAF	Berry Lp ($m^3/s.m^2.MPa$) $\times 10^9$	SEM ($m^3/s.m^2.MPa$) $\times 10^9$	*P* value (Bonferroni post-tests)
21.01.04	64	22.5	6.18	68	5.71	0.67	P<0.05
01.02.04	75	4.16	0.35	79	3.26	1.49	P>0.05
11.02.04	85	3.78	0.79	89	0.52	0.14	P<0.001
21.02.04	96	1.88	0.19				

Source of Variation	% of total variation	P value
Interaction	6.44	0.0275
variety	24.19	P<0.0001
date	45.58	P<0.0001

Table 1. Hydraulic conductivities of berries determined with the HPFM during the 2003-04 season on Shiraz and Chardonnay. Each measurement value is the mean of six replicates on bunches from different vines. A two way repeated analysis of variance was conducted on the data for the first three sample dates. The sources of variation and their significance are shown at the bottom of the table. A post-test (Bonferroni) was conducted to determine when differences occurred between varieties.

Our direct measurements show a substantial decrease in hydraulic conductance during and after veraison, as was previously implied by indirect measurements. Our data do not indicate that the hydraulic conductance goes to near zero right after veraison. Rather, there is a continuous decline during ripening. This was particularly the case for Chardonnay. For Shiraz, higher hydraulic conductances were measured during all stages of development, which might correlate with this variety's propensity to exhibit berry weight loss. We must, however, acknowledge that during our measurements on Chardonnay, there was also berry weight loss. Interestingly the onset of berry weight loss seemed to occur at similar hydraulic conductances for the two varieties (about 5×10^{-12} m/s.MPa from HPFM measurements).

Using the regression equations of hydraulic conductance *versus* DAF for different positions in the berry (Figure 5), we can calculate the hydraulic resistances ($1/L_o$) at two time points (70 and 100 DAF). These are plotted against cut positions (Figure 7a) for Shiraz and Chardonnay in Figure 7b and 7c respectively. Assuming that the resistances

are in series, we can calculate by subtractions the intermediate series hydraulic resistances through each position in the berry. For example the hydraulic resistance between the base of the receptacle and the base of the berry (between position 3 and 2 respectively) is given by the hydraulic resistance measured for cut position 2 minus that measured for cut position 3. These component hydraulic resistances are plotted in Figure 7d and 7e for Shiraz and Chardonnay respectively.

Comparison of Figure 7d and 7e shows that the two varieties show similar behaviour in the way in which berry hydraulic resistances increase during development, but with large differences in the magnitude of the changes. In Chardonnay there is a much larger increase in hydraulic resistance through the proximal (brush) region and distal part of the berry. For Shiraz, the increase in resistance is about half of that observed in Chardonnay. We found no evidence for changes in resistance in the pedicel or receptacle region of the berry.

The large differences in the changes in hydraulic resistance, and hence hydraulic conductance, should have large effects on the hydraulic behaviour of the berries during ripening. For Shiraz, the berry does not become as hydraulically isolated in the brush region as was anticipated from dye uptake experiments (Rogiers et al.2001). This may account for the continued Ca uptake after veraison (Rogiers et al. 2000). For Chardonnay the hydraulic isolation is more complete due to larger increases in hydraulic resistances in the proximal and distal parts of the berry. The behaviour of Chardonnay is in accord with experiments on Muscat Gordo Blanco showing dye traveling only to the brush region of the berry vasculature (Findlay et al. 1987).

It is interesting to speculate on the nature of the reduction in hydraulic conductance through berry development. Generally anatomical changes to the xylem vessels have been implicated in the interpretation of the dye movement studies. However one cannot exclude the possibility that xylem parenchyma cells that surround the berry xylem vessels become less water permeable during development. Aquaporins are largely responsible for high water permeabilities of plant cell membranes (Tyerman et al. 2002) and have been implicated in berry development (Delrot et al. 2001). Also composite transport in roots can have a large contribution from aquaporins (Tyerman et al. 2002), thus it would be surprising that this did not occur within berries given the similar hydraulic conductivities that we observe. Picaud et al. (2003) observed an increase in expression after veraison and at harvest, of one group of aqupaorins associated with the

plasma membrane (PIP1). However, this group of aquaporins generally show low water permeability (Tyerman et al. 2002) that was also observed for the grapevine PIP1 by Picaud et al (2004), and they may be more involved in neutral solute transport (Picaud et al. 2004). It remains to be seen what changes occur in the PIP2 group that generally show very high water permeabilities. It would be worthwhile to test the water permeability of berry plasma membranes during development.

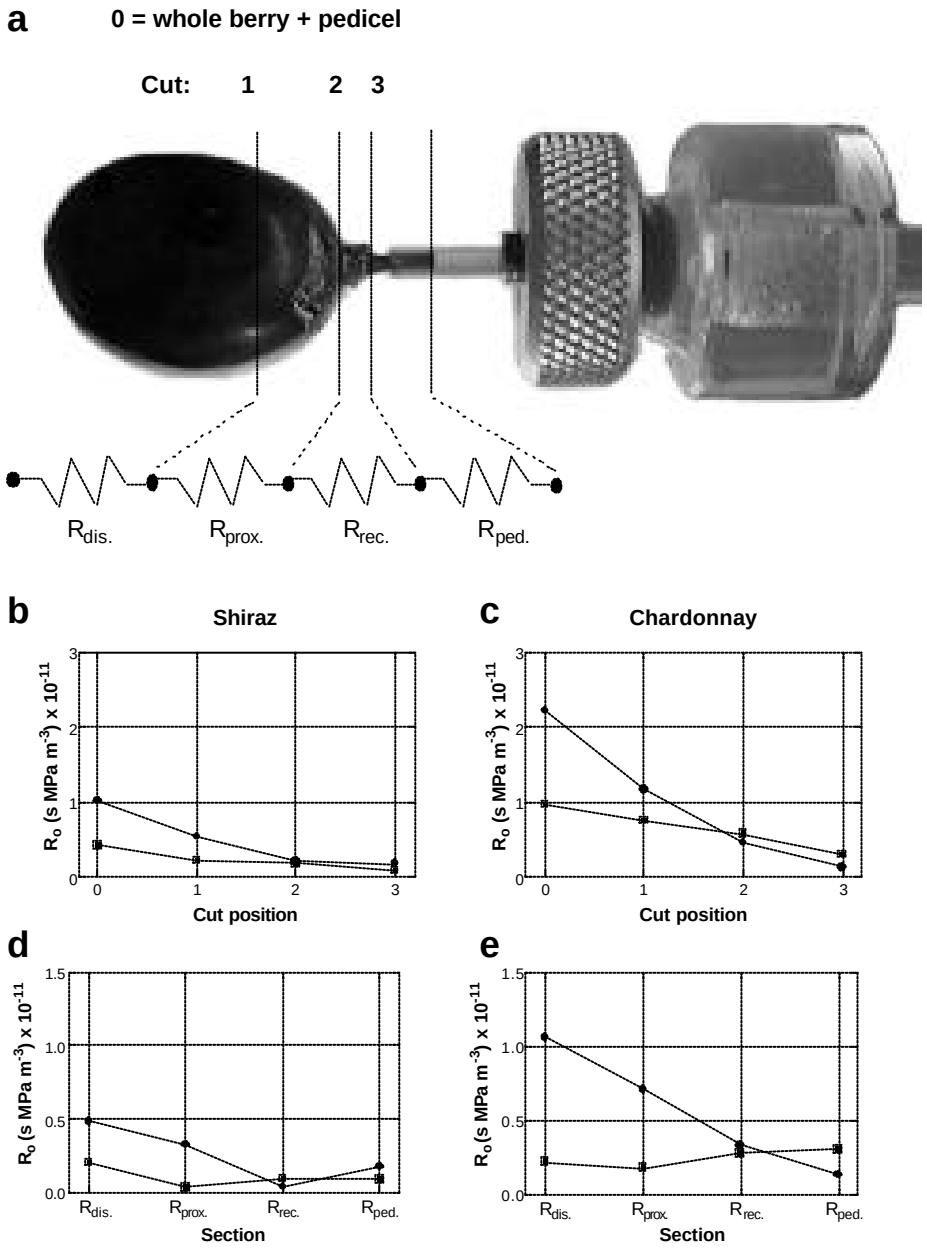

Figure 7. Calculating the positions in berries where hydraulic resistance (R_o) changed between 70 DAF (solid squares) and 100 DAF (solid circles). (**a**) Diagram of the cut positions along the berry, where condition 0 corresponds to the intact whole berry. (**b, c**) Hydraulic resistance as a function of cut position for Shiraz and Chardonnay respectively. (**d, e**) Hydraulic resistance for each section (see **a**) for Shiraz and

Chardonnay respectively. This is calculated as R_o at cut position 2 minus R_o at cut position 3, R_o at cut position 1 minus R_o at cut position 2, and R_o in condition 0 minus R_o at cut position 1.

The degree of hydraulic connection between the berry and the parent vine may influence the propensity for berry weight loss at late stages of ripening in two ways. First, if transpiration continues from the berries and is able to generate sufficient xylem tensions by cohesion tension from capillarity forces in berry cell walls, then higher berry hydraulic conductance (lower resistance) might limit berry weight loss by allowing greater water flow into the berry. On the other hand if the berry cannot generate sufficient tension in the xylem to counter the tension in the parent vine, as suggested by our measurements, then hydraulic isolation (low hydraulic conductance) would predispose towards prevention of weight loss.

If it is the effectiveness of hydraulic isolation from the parent vine at a particular stage of development that reduces the likelihood of berry weight loss, we can develop an hypothesis to explain why continued hydraulic connection via the xylem and berry composite membranes can lead to weight loss. Xylem backflow should be larger for an equivalent gradient directing water flow into the vine when the hydraulic conductance between the berry and the xylem of the pedicel is larger. Our measurements only provide hydraulic conductances for flow into the berry so it must be confirmed that reverse flow (corresponding to backflow) yields similar hydraulic conductances. Lang and Thorpe (1989) have shown for *cv* Italia that backflow can account for 36% of the total water loss from the berry. Given that Greenspan et al. (1996) considered backflow to be a minor component in Cabernet Sauvignon, these contrasting results suggest that there are probably large varietal differences as indicated in the present study.

Xylem backflow can only occur if there is an appropriate gradient to drive water flow through the composite membrane system in the berry to the adjacent stem xylem from the berry mesocarp cells. What is clear from the pressure probe measurements of equilibrium pressures of pedicel xylem in non-transpiring berries, is that the very low osmotic potentials in the berry (down to –5 MPa) are not translated to negative pressures in the xylem, at least not for post-veraison berries. Rather there is a gradual increase in xylem pressures toward zero, which follows the pattern of declining hydraulic conductances. The berry pericarp cells have recently been reported to have much reduced turgor (almost zero) after veraison (Thomas et al. 2004), and unpublished results of ours using the cell pressure probe also indicate very low or zero turgor

pressures in post-veraison pericarp cells. This might suggest that the water potential of these cells could be as negative as –5 MPa (the measured osmotic potentials). However, this is not transmitted through to the xylem conduits of the pedicel or adjacent vine stem. An apparent breakdown in compartmentation has been proposed for grape berries by Lang and Düring (1991). It would appear that the berry could only develop xylem tension through berry transpiration, and this is likely to decline during berry ripening (Rogiers et al. 2004b). Tensions developed by the parent vine would then prevail, and berries would shrink.

Our working hypothesis of how berry shrinkage might occur is illustrated in Figure 8. Due to solute accumulating in the apoplast or loss of semipermeability in the cell membranes of the xylem parenchyma cells, the berry is not able to develop xylem tensions despite the large osmotic potentials in the pericarp cells or berry apoplast (Figure 8b). Corresponding to this loss of *effective* osmotic gradients within the berry there would be a developmentally programmed decline in hydraulic conductance within the brush region and in the general distal region of the berry, as we have recorded. This reduction in hydraulic conductance could be due to a combination of reduced aquaporin activity in the xylem parenchyma or restrictions in the xylem vessels (Figure 8b,c). This hydraulic isolation would be required for two reasons: (1) to reduce xylem backflow; (2) to prevent an excessive water uptake from the vine that could result in the berry reaching dangerously high pressure (but note loss of selectivity in pericarp cells). Even with incomplete hydraulic isolation, provided phloem translocation continued, there would be ample flow into the berry to prevent weight loss. If on the other hand the hydraulic conductance does not decline sufficiently, then backflow will be larger, particularly during the day. If the phloem ceased to function, as proposed by McCarthy and Coombe (1999), this would exacerbate the situation by cutting off any supply of water to the berry (Figure 8c). Thus through a combination of reduced phloem function and relatively high hydraulic conductances within the xylem/composite membranes of the berry, Shiraz berries are more likely to display weight loss. The recent observation that berry size matters, that is, that larger berries are more likely to display weight loss (Rogiers et al 2004a), may not be due to higher transpiration from larger berries as was proposed, but rather a higher hydraulic conductance of the internal composite membrane and xylem for larger berries which would increase the rate of backflow from the berry.

We can calculate the efflux of water due to backflow for a given pressure gradient using the hydraulic conductances that were measured for berries just before berry weight loss was observed (5×10^{-12} m^3/s.MPa), and which were sustained over time in Shiraz berries (Figure 7). For a relatively small average gradient of 0.1 MPa, Shiraz berries could loose 43 mg per day, which after one week would amount to about 30% of the weight of an average sized berry. Taking transpiration into account, some 15 mg per day estimated by Rogiers et al. (2004b), there is the potential for substantial weight loss through a combination of cessation of phloem loading, transpiration and backflow.

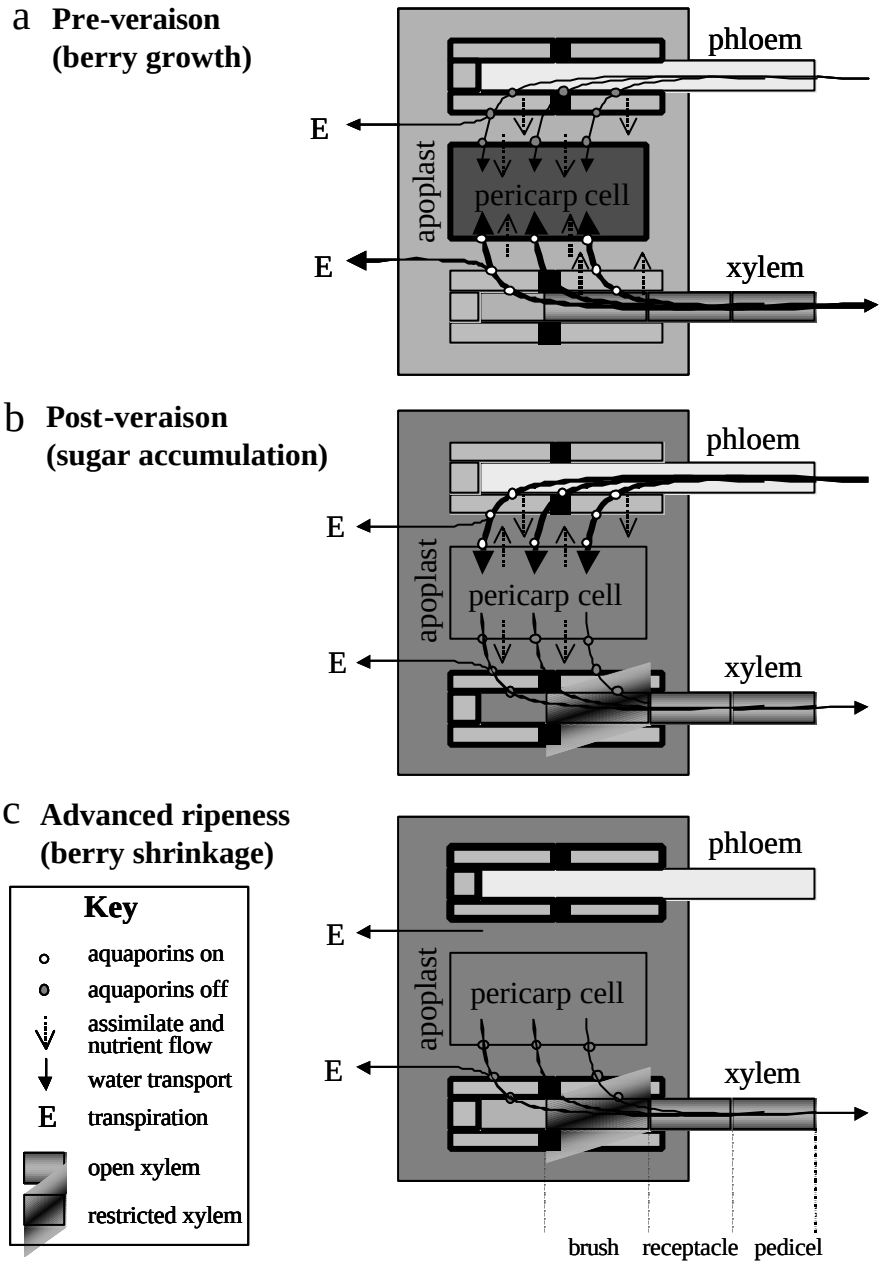

Figure 8. Cartoon summary of the hydraulics in grape berries incorporating the McCarthy and Coombe (1999) hypothesis and observations made in this report. It is hypothesized that aquaporins may be involved in changing flow during development in addition to xylem restrictions. (**a**) Pre-veraison berry has osmotically competent cells (reflection coefficient = 1) and the apoplast has low solute concentrations. Transpiration and cell expansion drive water inflow predominantly via the xylem. Aquaporins are activated in xylem parenchyma cells. (**b**) Berry after veraison where pericarp cell membranes lose osmotic selectivity (reflection coefficient goes to zero) and/or berry apoplast solute concentrations increase. Apoplast water potentials rise and cannot counter the tensions that could develop in the parent plant. Xylem/composite membrane hydraulic conductance has now become substantially reduced through a combination of xylem restriction and possibly closure of aquaporins in xylem parenchyma cells reducing backflow. Phloem translocation continues more rapidly and aquaporins are activated in the phloem. (**c**) Berry in the shrinkage phase where phloem translocation has ceased and where the xylem/composite membrane hydraulic conductance has not reduced sufficiently (e.g. Shiraz) to achieve hydraulic isolation from the parent vine, so that through a combination of transpiration and backflow the berry loses weight.

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5. Summary

5.1. Radial oxygen flow in rice (*Oryza sativa* L.) roots: effects of anaerobic conditions and the role of apoplastic barriers

When cultivated in waterlogged soils, rice, as other hygrophytes, is exposed to anaerobic and reduced environment. To reduce the impact of these stresses, rice develops numerous responses. It forms extensive aerenchyma, which facilitates the internal transport of gases from well-aerated shoots to underground submerged organs. The effectiveness of the aerenchyma can be increased by the formation of barriers in the outer cell layers of roots. These barriers inhibiting radial oxygen loss (ROL) to the rhizosphere enhance longitudinal diffusion of O_2 towards the apex (Armstrong, 1979; Colmer, 2003). Despite the importance of the barrier to ROL for roots of rice, its the precise nature has not yet been determined. A major deficiency in knowledge was lack of the oxygen permeability coefficient across outer cell layers of roots, which limited understanding of the mechanisms controlling ROL.

To determine the nature of the barrier to oxygen diffusion from aerenchyma to rhizosphere, measurements of radial oxygen loss from rice roots grown in either aerated or deoxygenated conditions were combined, for the first time, with root histochemisty and biochemistry. It was shown that the pattern of ROL from rice roots of stagnantly grown plants correlated with development of the apoplastic barriers in the root peripheral layers. Deoxygenated conditions induced early development of exodermal Casparian bands and suberin lamellae at positions closer to root apex, where it did not develop in aerated roots. In addition to suberization, early lignification of walls of densely packed sclerenchyma cells was found closer to root apex in stagnantly grown plants. Supporting these findings, biochemical analyses revealed that, when grown in stagnant solution, the amounts of both aromatic and aliphatic suberin in the OPR sleeves of all investigated zones were several folds greater than those of plants grown in aerated solution. In parallel, lignin content was also higher in stagnantly grown roots. In both conditions, there was a pronounced increase of absolute loads of these compounds along the root axis from apex to base. Similarly, the highest rates of radial oxygen loss from plants grown in deoxygenated conditions were observed just behind the apex and decreased dramatically towards the base. By contrast, ROL from adventitious roots of plants grown in aerated solution was the highest at 30 mm from the apex. It was

concluded that elevated levels of suberin and lignin in the OPR caused strong barrier to ROL, which was induced by growth in stagnant solution.

A new perfusion technique has been developed to measure, for the first time, the oxygen permeability coefficient of the outer part of root (OPR) of rice. The well-defined structure of the OPR (four cell layers) is separated from the stele by aerenchyma. Perfusion of aerenchyma of root segments with mixtures of O_2/N_2 of known oxygen concentration and at the same time measuring radial losses of oxygen allowed quantification of the permeability coefficient of the cell layers exterior to aerenchyma. The data showed that the increase in oxygen concentration (above 40%) in the perfusing gas did not result in linear increase in radial oxygen flow (J_{O2}). Similar deviations were observed for silicone tubes, which have similar permeability to rice roots grown in aerated solution. A linear response was observed for Teflon tubes, when J_{O2}s were substantially smaller, and for intact plants, where the O_2 concentration was manipulated around the shoot, but the absolute values of ROL were relatively small. It was concluded that at high rates of oxygen flow there might be process tending to underestimate the actual value of J_{O2} (e.g. by an incomplete reduction of O_2 at the electrode surface and other polarizing effects). Thus, some care has to be taken, when using platinum O_2 electrodes of the Clark type in the presence of high J_{O2} fluxes. Complications in terms of polarizing effects can be excluded when rates of J_{O2} are low. The perfusion technique could have been successfully used when roots segments were perfused with oxygen concentrations of up to 40%.

The new method for measuring the O_2 permeability of the outer part of roots (P_{OPR}) was applied to rice grown in either aerated or deoxygenated conditions. This was the first quantitative comparison of the radial permeability of peripheral layers to O_2 in roots. The results showed that P_{OPR} decreased along the root of plants grown in both conditions. However, when grown in deoxygenated medium, the O_2 permeability across the OPR was lower by an order of magnitude at all tested distances compared with plants grown in aerated solution. At 30 mm the mean P_{OPR}s were 52 and 6×10^{-7} m s^{-1} for plants grown in aerated and stagnant solutions, respectively. At 60 mm, permeabilty declined to 14 and 1.0×10^{-7} m s^{-1}, respectively. The lower P_{OPR} in roots grown in stagnant solution as well as the reduction of P_{OPR} along the roots of plants from both conditions strongly correlated with the development of apoplastic barriers in the OPR.

The radial flux of oxygen from aerenchyma to the rhizosphere is not only determined by physical resistance to O_2 diffusion, but also by the consumption of oxygen by cells along the radial diffusion path. Thus, in order to estimate the contribution of apoplast and living cell for the overall movement of O_2 across the OPR, the P_{OPR} was affected either by blocking the apoplastic pores in the OPR with salt precipitates or by killing the living cells with 0.1 N HCl.

Treatment with $CuSO_4/K_4Fe(CN)_6$ resulted in formation of brown precipitates only in roots of plants grown in aerated solution. Precipitates were intense at the apical part of the root segments and gradually faded away along the root towards the base as apoplastic barriers developed. This suggested that at least in immature parts of the segments, ions (mainly Cu^{2+}) could pass through the exodermis and sclerenchyma layer. This, in turn, indicated an existence of pores in the apoplastic barriers. In contrast to plants grown in aerated solution, no precipitates were observed in root segments of stagnantly grown plants, even after killing with 0.1 N HCl. This is strong evidence that well-developed apoplastic barriers such as Casparian bands and suberin lamella, as well as lignified fibre cells impeded ion movement across the OPR in these plants.

As a result of the formation of salt precipitates in the apoplastic pores of roots grown in aerated solution, the P_{OPR} decreased by 20-5%. This is in agreement with earlier findings of diffusional and bulk water flow of Ranathunge *et al*. (2005). The blockage of the apoplast with precipitates reduced the diffusional water permeability by about 20% and caused a massive 3- to 4- fold reduction of hydraulic conductivity. This suggested that the OPR of rice allow rather high water flow in the presence of relatively high resistance to O_2. It is achieved by differences in transport mechanisms: there is a bulk flow of water, but the flow of oxygen is difusional in nature. However, the above conclusion was drawn only for rice grown in aerated solution.

Killing of root segments by 0.1 N HCl increased the P_{OPR} by 20-55% of plants grown in both conditions. At least in part, these increases may result from vanishing the respiratory activity in the OPR. It was possible to estimate the respiration rates of the OPR from the differences in radial O_2 flows (in nmol m^{-2} s^{-1}) between control and HCl-treated roots. The calculation suggested that contribution of respiratory activity should be small in cases where P_{OPR} is high, and may contribute more in the presence of strong apoplastic barriers at low P_{OPR}. The oxygen permeability coefficient of HCl-treated roots grown in deoxygenated solution was still significantly lower than that of untreated

roots grown in aerated solution. Overall, the physical resistance plays a dominant role in impeding O_2 loss from rice roots, although effects due to respiration may contribute, namely, in the presence of low rates of radial oxygen loss.

5.2. The hydraulic properties in developing berries of *Vitis vinifera* L. *cv.* Shiraz and Chardonnay

Berries of *Vitis vinifera cv*. Shiraz can undergo weight loss during later stages of ripening. This may have impact on sugar metabolism and flavour development, and consequently on the quality of the vintage. It was hypothesized that the reduction in net vascular flow of water into the berry, concomitant with transpirational loss, leads to berry weight loss. There may be also some back flow of water from the berry to the parent plant along the xylem. The present study provided first data on the hydraulic properties of the pedicel and components of the berries during development. Comparisons were made between Shiraz and Chardonnay, which is not known to undergo substantial weight loss. It was shown that the hydraulic conductance of single berries of both varieties declined during development. However, Shiraz berries had higher hydraulic conductance than Chardonnay for whole berries and all cut positions. The increase in hydraulic resistance was found in the proximal (brush) region and distal part of the berry, which was much larger in Chardonnay. For Shiraz, the increase in resistance was about half of that observed in Chardonnay. There was no evidence for changes in resistance in the pedicel or receptacle region of the berry.

The reduction of hydraulic conductance could be due to combination of both: reduced aquaporin activity in the xylem parenchyma and restrictions in the xylem vessels. If the hydraulic conductance does not decline sufficiently, then back flow will exceed the inflow of water through the phloem, particularly during the day. The results suggested that in Shiraz due high hydraulic conductance of the whole berries, berries components and pedicel, water movement from the berry back to parent vine *via* the xylem may be an important component of berry weight loss during last stages of ripening.

6. Zusammenfassung

6.1. Radialer Sauerstoffffluss in Reiswurzeln *(Oryza sativa L.)*: der Einfluss anaerober Bedingungen und apoplastischer Barrieren

Wenn sie in mit Wasser gesättigten Böden wachsen, sind Reis und andere Hygrophyten anaeroben und chemisch reduzierenden Bedingungen ausgesetzt. Um die Auswirkungen der daraus resultierenden Stresseffekte zu reduzieren, hat der Reis eine Vielzahl von Strategien entwickelt. So kommt es vor allem zur Ausbildung eines Aerenchyms, um den internen Gastransport vom ausreichend mit Sauerstoff versorgten Spross zu den unterirdischen Organen zu gewährleisten. Die Effizienz des Aerenchyms wird durch die Ausbildung von Sauerstoffbarrieren in den äußeren Zellschichten der Wurzel (OPR) gesteigert. Diese Barrieren verhindern radiale Sauerstoffverluste (ROL) in die Rhizosphäre und steigern die longitudinale Diffusion von Sauerstoff in Richtung des Wurzelapex (Armstrong, 1979; Colmer, 2003). Trotz der Bedeutung des OPR für den Sauerstofftransport von Reiswurzeln sind die Eigenschaften dieser Barrieren bis jetzt noch nicht genau untersucht worden. Eine große Wissenslücke stellt dabei der Permeabilitätskoeffizient für Sauerstoff über die äußeren Zellschichten der Wurzeln dar.

Um die Diffusion von Sauerstoff durch die Barrieren aus dem Aerenchym in die Rhizosphäre zu quantifizieren, wurden Messungen zum radialen Sauerstoffverlust (ROL) von Reiswurzeln durchgeführt. Diese Messungen wurden an Wurzeln durchgeführt, die entweder unter sauerstoffreichen oder sauerstoffarmen Bedingungen angezogen worden waren. Zum ersten Mal wurden solche Messungen mit histochemischen und biochemischen Analysen kombiniert. Es wurde gezeigt, dass beim Wachstum von Reiswurzeln in stagnierender Lösung (sauerstoffarm) der ROL mit der Entwickung apoplastischer Barrieren in den äußeren Wurzelschichten (OPR) korreliert. Sauerstoffarme Bedingungen verursachten eine frühe Entwicklung von Casparyschen Streifen in der Exodermis und von Suberinlamellen nahe der Wurzelspitze. Unter sauerstoffreichen Wachstumsbedingungen wurden diese Effekte nicht beobachtet. Zusätzlich zu den Suberinisierungseffekten wurde eine frühe Lignifizierung der Zellwände der dicht gepackten skelerenchymatischen Zellen nahe der Wurzelspitze gefunden (sauerstoffarme Wachstumsbedingungen). Biochemische Analysen zeigten, dass die Mengen von aromatischem und aliphatischem Suberin in den OPRs in allen untersuchten Wurzelzonen unter sauerstoffarmen Wachstumsbedingungen um ein

Mehrfaches höher war als bei Anzucht in sauerstoffreichem Milieu. Zusätzlich war der Gehalt an Lignin bei Anzucht unter sauerstoffarmen Bedingungen höher. Die absoluten Mengen von Suberin und Lignin erhöhten sich für beide Anzuchtbedingungen entlang der Wurzel. Bei Wurzeln, die unter anaeroben Bedingungen angezogen worden waren, wurden die höchsten Raten des radialen Sauerstoffverlustes knapp hinter der Wurzelspitze gemessen. Danach nahmen sie in Richtung zur Wurzelbasis drastisch ab. Der ROL von Adventivwurzeln war am höchsten 30 mm entfernt von der Wurzelspitze. Daraus wurde geschlossen, dass eine erhöhte Einlagerung von Suberin und Lignin im OPR zu ausgeprägten Barrieren für den ROL führt, welche durch die Aufzucht in sauerstoffarmen Wachstumsmedien verstärkt induziert wurden.

Eine neue Perfusiontechnik erlaubte es erstmals, den Permeabilitätskoeffizienten von Sauerstoff in den äußeren Schichten der Reiswurzel (OPR) zu bestimmen. Die Struktur des OPR (vier Zellschichten) ist von der Stele durch das Aerenchym getrennt. Durch die Perfusion des Aerenchyms von Wurzelsegmenten mit O_2/N_2-Gasgemischen mit bekannter Sauerstoffkonzentration und die simultane Messung des radialen Sauerstoffverlustes wurde der Permeabilitätskoeffizient der äußeren Zellschichten quantitativ bestimmt. Die Daten zeigten, dass eine Erhöhung der Sauerstoffkonzentration auf über 40 % im Perfusionsgas (O_2/N_2-Gemisch) nicht zu einem linearen Anstieg des radialen Sauerstoffflusses (J_{O2}) führte. Ähnliche Beobachtungen wurden an Silikonschläuchen gemacht, welche eine ähnliche Permeabilität wie die Reiswurzeln hatten. Ein linearer Zusammenhang wurde bei der Verwendung von Teflonschläuchen beobachtet, d.h. für relative kleine J_{O2}s und für intakte Pflanzen bei denen die Sauerstoffkonzentration am Spross manipuliert wurde. Es wurde gefolgert, dass bei großen Sauerstoffflüssen Elektrodenprozesse den eigentlichen Wert von J_{O2} unterschätzen (z.B. durch die unvollständige Reduktion von O_2 an der Oberfläche der Platinelektroden und andere polarisierende Effekte). Deshalb sollte man bei der Verwendung von Platinelektroden zur O_2-Messung (Clark-Elektroden) darauf achten, dass zu grosse J_{O2}-Flüsse vermieden werden. Bei niedrigen J_{O2}-Flüssen konnten Probleme aufgrund von Polarisierungseffekten ausgeschlossen werden. Die Perfusiontechnik kann erfolgreich eingesetzt werden, wenn die gewählte Sauerstoffkonzentration im Aerenchym unter 40 % bleibt.

Die neue Methode zur Messung der O_2-Permeabiltiat des äußeren Wurzelteils (P_{OPR}) wurde angewandt für Reis, welcher unter sauerstoffreichen bzw. -armen Bedigunen

aufgezogen worden war. Dies war der erste quantitative Ansatz, um Änderungen der radialen O_2- Permeabilität für die peripheren Schichten von Wurzeln zu bestimmen. Die Ergebnisse zeigten, dass bei beiden Wachtumsbedingungen P_{OPR} entlang der sich entwickelnden Wurzel abnimmt. Unter sauerstoffarmen Wachstumsbedigungen war die O_2-Permeabilität des OPR an allen untersuchten Positionen entlang der Wurzel um eine Größenordnung niedriger als bei Pflanzen, die unter sauerstoffreichen Bedingungen angezogen worden waren. In einer Entfernung von 30 mm von der Wurzelspitze lagen die mittleren P_{OPR}s bei 52×10^{-7} m s^{-1} bei Wurzeln aus sauerstoffreichem und bei 6×10^{-7} m s^{-1} bei Wurzeln aus sauerstoffarmem Milieu. In einer Entfernung von 60 mm von der Wurzelspitze nahmen diese Werte auf 14 bzw. $1{,}0 \times 10^{-7}$ m s^{-1} ab. Die niedrigeren P_{OPR}s von Wurzeln in sauerstoffarmer Lösung wie auch die Reduzierung der P_{OPR} entlang der Wurzeln von Pflanzen beider Wachstumsbedingungen korrelierten deutlich mit dem Entwicklungszustand der apoplastischen Barrieren im OPR (siehe oben).

Der radiale Fluss des Sauerstoffs aus dem Aerenchym in die Rhizosphäre wird nicht nur durch den physikalischen O_2-Diffusionswiderstand beeinflusst sondern auch durch den Sauerstoffverbrauch der Zellen, die sich entlang des radialen Diffusionsweges befinden. Um den Beitrag des Apoplasten und der lebenden Zellen am Gesamtsauerstofftransport zu bestimmen, wurde P_{OPR} entweder manipuliert durch die Ausfällung unlöslicher Salze in den apoplastischen Poren (die die Poren verstopfen sollten) oder durch Abtöten lebender Zellen mit 0.1 N HCl.

Bei Wurzeln von Pflanzen, die in sauerstoffreicher Lösung angezogen worden waren, führte die Behandlung mit $CuSO_4/K_4[Fe(CN)_6]$ zu braunen Präzipitaten im OPR, wobei in der Nähe der Wurzelspitzen die intensivsten Ausfällungen beobachtet wurden. Zur Wurzelbasis hin kam es zu einer sukzessiven Verminderung der Ausfällung, die mit der sukzessiven Entwicklung von apoplastischen Barrieren einherging. Zumindest in den relativ wenig entwickelten Wurzelbereichen scheinen Ionen (vor allem Cu^{2+}) die Exodermis und die sklerenchymatischen Zellschichten passieren zu können. Dies wiederum deutet auf die Existenz von Poren in den apoplastischen Barrieren hin. Im Gegensatz zu Pflanzen, die in sauerstoffreicher Lösung angezogen worden waren, wurden unter sauerstoffarmen Wachstumsbedingungen keine Präzipitate beobachtet, sogar nach dem Abtöten der Zellen mit 0.1 N HCl. Das ist ein klarer Hinweis darauf, dass stark entwickelte apoplastische Barrieren (Casparysche Streifen, Suberinlamellen und lignifizierte Zellen) einen Transport von Ionen durch den OPR unterbinden.

Durch die Bildung von Präzipitaten in den apoplastischen Poren von Wurzeln aus sauerstoffreichem Milieu nahm P_{OPR} um 20 - 5% ab. Dies war vergleichbar mit früheren Ergebnissen von Ranathunge *et al*. (2005) zum Wasserfluss aufgrund von Diffusion (P_{dOPR}) oder Massenfluss (P_{fOPR}). Die Inhibierung des Apoplast durch die Bildung von Ausfällungen erniedrigte danach die diffusive Wasserpermeabilität um etwa 20%. Sie verursachte dagegen eine 3-4fache Abnahme der hydraulischen Leitfähigkeit. Diese Unterschiede zeigen, dass der OPR von Reis eine vergleichsweise große hydraulische Leitfähigkeit erlaubt bei relativ großen diffusiven O_2-Widerständen. Dies lässt sich anhand der unterschiedlichen Transportmechanismen von Wasser und Sauerstoff erklären: der Wasserfluss ist ein Massenfluss, der Sauerstofffluss hingegen ein Diffusionsfluss. Da zur Zeit die entsprechenden Daten zum Wasserfluss für Pflanzen fehlen, die unter anaeroben Bedingungen aufgezogen wurden, lässt sich diese Schlussfolgerung zur Zeit nur bei Reispflanzen ziehen, die aerob angezogen worden waren.

Unter beiden Wachstumsbedingungen steigerte sich die P_{OPR} um 20–55% nach der Abtötung von Zellen durch 0.1 N HCl. Dieser Anstieg lässt sich teilweise mit einem Rückgang der respiratorischen Aktivität der OPR erklären. Der Beitrag der Respiration zum Gesamtsauerstofffluss wurde anhand von Unterschieden im radialen Sauerstoffverlust abgeschätzt (in nmol m^{-2} s^{-1}). Die Abschätzung zeigte, dass der Beitrag der respiratorischen Aktivität relativ klein ist, ween P_{OPR} relativ groß ist. In Gegenwart starker apoplastischer Barrieren sollte die Respiration aber eine wichtige Rolle spielen bei niedrigem P_{OPR}. Der O_2-Permeabilitätskoeffizient von HCl-behandelten sauerstoffarmen Wurzeln immer noch signifikant niedriger war als der von unbehandelten sauerstoffreichen Wurzeln. Zusammenfassend lässt sich sagen, dass der physikalische Widerstand eine dominierende Rolle bei der Verhinderung des Sauerstoffverlustes in Reiswurzeln spielt. Respiratorische Effekte sollten aber bei relativ kleinen radialen Sauerstoffverlusten nicht vernachlässigt werden.

6.2. Die hydraulischen Eigenschaften von sich entwickelnden Beeren von *Vitis vinifera* L. *cv.* Shiraz and Chardonnay

Beeren von *Vitis vinifera* cv. Shiraz können im späten Zustand der Reife unter Gewichtsverlust leiden. Dies hat Auswirkungen auf den Zuckergehalt, die Entwicklung des Geschmacks und konsequenterweise auch auf die Qualität der Weines. Es wurde

angenommmen, dass die Reduktion des vaskulären Nettowasserflusses und auch Transpirationsverluste zu Gewichtsabnahmen der Beeren führen. Ein anderer Grund könnte ein Rückfluss von Xylemwasser aus den Beeren in die Mutterpflanze sein. Die aktuelle Studie behandelte erstmals die hydraulischen Eigenschaften der Beeren während ihrer Entwicklung, vor allem die des Stieles der Beere. Vergleiche wurden angestellt zwischen Shiraz und Chardonnay, welcher nicht unter substantiellem Gewichtsverlust der Beeren leidet. Es zeigte sich, dass Beeren von Shiraz eine höhere hydraulische Leitfähigkeit hatten als die Beeren von Chardonnay. Dies wurde für verschiedene Messpositionen entlang der Beeren beobachtet. Ein Anstieg des hydraulischen Widerstandes wurde für die proximale Region und den distalen Bereich der Beeren gefunden, wobei dieser bei Chardonnay viel größer war. Für Shiraz war der Anstieg des Widerstandes ungefähr halb so groß wie für Chardonnay. Es gab keinen Hinweis auf Veränderungen des Widerstandes im Stiel der Beeren (Pedicel) oder der rezeptalen Region der Beeren.

Die Verminderung der hydraulischen Leitfähigkeit könnte von einer verminderten Aquaporinaktivität im Xylemparenchym herrühren und/oder von einer Verengung der Xylemelemente. Falls die hydraulische Leitfähigkeit sich nicht substantiell ändert, dann würde der Wasserausstrom den Wassereinstrom durch das Phloem vor allem während des Tages übertreffen. Die Ergebnisse deuten darauf hin, dass für Shiraz, aufgrund der höhern Leitfähigkeit der Beere, der Wasserausstrom von der Beere in die Mutterpflanze durch das Xylem eine wichtige Rolle für den Gewichtsverlust der Beere während der Reife spielt.

Erklärung

Hiermit erkläre ich, dass ich die Arbeit selbständig verfasst und keine anderen als die von mir angegeben Quellen und Hilfsmittel benutzt habe.

Ferner erkläre ich, dass ich anderweitig mit oder ohne Erfolg nicht versucht habe, diese Dissertation einzureichen. Ich habe keine gleichartige Doktorprüfung an einer anderen Hochschule endgültig nicht bestanden.

Bayreuth, den 3. Juli 2009

(Lukasz Kotula)