Anatomy of the gas canal system of *Nelumbo nucifera* (Gaertn.)

Abstract

*Nelumbo nucifera* grows by extending a creeping rhizome through anaerobic sediments. Nodes form at intervals along the rhizome, each producing a single leaf, and gas canals channel air from the leaves throughout the petioles and rhizomes. The gas flow pathway was mapped by casting the canals in growing shoots with silicone and by blowing air through complexes of rhizomes and petioles. Air from a leaf flows to a rhizome through one of two petiolar canal pairs, joining with the lowermost of three canal pairs in the rhizome through a chamber in the node. The lowermost canal pair links these nodal chambers along the length of a rhizome, allowing gas from a node to flow both forwards, towards a growing shoot, and backwards, towards preceding leaves. These linked chambers also connect with the middle pair of canals on their proximal side, enabling flow to proceed backwards along the rhizome to an adjacent node. A chamber in the next node then diverts the flow into the upper canal pair. This pair leads to a third node and chamber from which the air vents to the atmosphere through the second petiolar canal pair. Thus flow proceeds away from the growing tip in a stepped fashion. Forward flow also ventilates a shoot’s growing tip, with air from the lowermost canal pair entering a chamber in the developing node which, as described above, connects with the middle canal. This allows the air to reverse direction and enter the vent flow pathway.
Introduction

Low oxygen levels found in flooded sediments are intrinsically problematic for macrophytes. Lack of oxygen can damage roots, but an anoxic environment also lowers the redox potential of the sediment, leading to the production of toxic levels of reduced ions such as Fe$^{2+}$ and Mn$^{2+}$ (Grosse et al. 1996b; Santruckova et al. 2001). This situation is worsened by anaerobes that produce methane and other metabolites that can accumulate to harmful levels (Armstrong et al. 1996). These problems are solved if buried roots or rhizomes receive an abundant supply of oxygen (Colmer 2003; Grosse et al. 1996b), either directly from photosynthesis, or from the atmosphere. Consequently, most secondarily aquatic plants possess continuous airspaces within their stems, leaves and rhizomes that allow for the movement of oxygen and other gases.

Oxygen diffusion through air is approximately 250,000 times faster than through water, but it is not as effective as convection in rapidly moving large volumes of gas over long distances. Convection of gas occurs from a region of high pressure to a region of low pressure. Therefore, if aquatic plants are to take advantage of convection to supply oxygen to their submerged and buried organs, they must possess an interconnected system of lacunae with separate entry and exit points as well as a means of generating a pressure gradient across them.

Air within the emergent organs of many aquatic plants pressurises passively due to a process termed Knudsen diffusion. This diffusion regime occurs in the presence of a temperature and humidity difference across a micro-porous partition, with diffusion of gas occurring towards the warmer, more humid side (Armstrong et al. 1996). Micro-porous partitions occur in the leaves of many plants. The small diameter of the pores (<0.1 μm) permits diffusion into the humid interior of the leaf, but prevents convective flow back to the atmosphere (Dacey 1987). Under these conditions gas continually diffuses into the leaf against a pressure gradient but cannot flow back through the partition, resulting in a pressure difference capable of driving convective flow.

Convective gas transport in aquatic plants was first reported in 1841, when Raffeneau-Delile made observations of gas bubbles emerging through pools of water trapped on the concave surface of sacred lotus leaves (*Nelumbo nucifera* Gaertn.) (Grosse et al. 1996a). Since then, many other emergent and floating-leaved wetland plant species have been found to be capable of passively generating pressure within their leaves. For early investigators into this phenomenon, it was primarily the
mechanism of pressurisation, and not the physiological or anatomical significance of flow, that was of interest. Thus it was not until over 100 years later that studies on *Nuphar luteum* (the yellow water lily) revealed the first pathway of pressurised gas flow, from young leaves capable of generating pressure (influx leaves), to old, leaky leaves attached to the same rhizome (efflux leaves) (Dacey 1980; Dacey 1981). This pattern has since been found in other *Nuphar* and *Nymphoides* species (Grosse and Bauch 1991; Grosse *et al.* 1991). In contrast, *Nelumbo nucifera* leaves are capable of acting as both influx and efflux points. This ability was first proposed by Dacey (1987) before being definitively identified by Mevi-Schutz and Grosse (1988) through ethane tracer gas experiments.

Although *N. nucifera* is among the best known and most spectacular of the aquatic plants known to pressurise, the anatomy of its gas transport system has so far received little attention. Interest has been focused principally on the large emergent leaves and their ability to pressurise, and little consideration has been given to the complex gas canals present in the extensive creeping rhizome. Only recently have the first major gaps in our understanding of *N. nucifera*’s gas canal anatomy been explored (Vogel 2004).

*N. nucifera* is an aquatic perennial that re-shoots in spring from tuberous rhizomes buried in flooded soils (Mevi-Schutz and Grosse 1988). The plant grows by extending a long creeping rhizome below the sediment. Nodes form at intervals along the rhizomes and produce an axillary rhizome shoot, adventitious roots, and either an emergent or floating leaf (Esau and Kosakai 1950)(fig. 1A). Each leaf is roughly oval, with the petiole inserting in the centre of the leaf lamina. The insertion point of the petiole is visible in the centre of the abaxial leaf surface as a whitish, central plate (Mevi-Schutz and Grosse 1988). The leaf, petiole and rhizome all display a bilateral symmetry relative to the long axis of rhizome growth, as do the gas canals within (fig. 1B). The petiole possesses two major and two minor pairs of gas canals (A and B constitute the major pairs, c and d the minor pairs; fig. 1C). Gas canal A conducts gas upward, connecting to the atmosphere through giant stomata located above the central plate (Vogel 2004). Canal B connects with approximately one-third of the leaf ray canals that duct pressurized gas from the leaf lamina downward. The remaining two-thirds of the leaf ray canals connect with canals A, c and d under the petiolar insertion point (Mevi-Schutz and Grosse 1988; Vogel 2004). Canal A, which connects to the atmosphere, is considered to be the efflux point, while canal B ducts the pressurized
Fig. 1. *Nelumbo nucifera* anatomy. A. Arrangement of internodal rhizomes, axillary shoot and petiole at a node. B. Bilateral symmetry of lotus rhizome, axillary shoot and petiole at a node. Petiole (C.) and rhizome (D.) in cross-section showing arrangement of gas canals.

Gas from the leaves down to the rhizome (Mevi-Schutz and Grosse 1988; Vogel 2004). Emergent leaf petioles have a tendency to bend just below the petiolar insertion point, thus orienting the leaf lamina at an incline, with the major A pair uppermost.

The rhizome contains three lateral major pairs of gas canals (I, II, III) and two single major canals, located ventrally (IV) and centrally (V) (fig. 1D). Occasionally a smaller canal pair (i) is found dorsally just above pair I – usually in thicker rhizomes. Vogel (2004) recently examined the connections between the gas canals of the petiole and rhizome. However, due to a lack of material, he was only able to examine connections between two adjacent petioles and a terminal bud. His major findings were that the petiolar canals A and B remained completely separate, with no connection between the upstreaming and downstreaming gas flows in a single node. Therefore, through-flow from the atmosphere to the rhizome, and back again, could not occur in the petiole of an individual leaf. He also found that canal B connected to
the rhizome canals II and III, while the venting A canal connected to I only. His examination of connections between the two adjacent nodes also failed to reveal communication between the pressurizing canals of one leaf and the vent canals in the adjacent leaf. The growing tip was considered to be without cavities and its contribution to the flow pathway was therefore ignored. Thus the location of the crossover point between pressurized and venting canals, as well as gas canal connections within a growing shoot, remained unresolved.

To understand the mechanism of pressurized ventilation in *N. nucifera* more completely it is necessary to identify the gas flow pathway from influx points in the leaves, through the rhizomes and back out to the atmosphere. Effective ventilation of the rhizome can only be achieved if connections between influx and efflux points are repeated at predictable intervals along the length of the rhizome, rather than occurring as the result of random merging between otherwise un-branched gas canals. To map this gas flow pathway, the lacunal connections between the petiole and rhizome, as well as between adjacent nodes, were examined using gas flow tests on lengths of rhizome possessing multiple leaves and nodes. The air canals within growing shoots were also similarly examined, as well as being cast using silicone to determine their suitability as a juncture between pressurising and venting gas streams.

**Methods**

*Plant material*

*Nelumbo nucifera* tubers with growing shoots were collected from an open-air pond at the Adelaide Botanic Gardens in mid-October 2004. They were then planted in 10 plastic pots, 38 cm in diameter by 34 cm deep, containing sandy loam, cow manure and Osmocote Plus (Scott’s-Sierra, Horticultural Products Company, USA). Pots were submerged in round fibreglass ponds 50 cm deep and 146 cm in diameter. The plants were grown under natural light conditions in a glasshouse until April 2005, at which time they became dormant.

*Casting growing shoots*

Growing shoots were collected from the glasshouse ponds by cutting the rhizomes above the water surface. The cut ends were dried with a paper towel to remove any latex oozing from the wound. In the laboratory, the shoots were prepared for casting by wrapping a 2 cm wide strip of aluminium foil around the cut end of the rhizome, creating a collar. The shoots were then held vertically with the cut end
uppermost, and low-viscosity silicone casting material (SilGel 604, Wacker Gmbh, Germany) was gently introduced from a disposable syringe into canal III until it emerged from canal II. The remaining canals and then the collar were filled with silicone, and the cast was left to set for at least 8 h. The solidified casts were cleaned of plant tissue using a procedure modified from Vogel (2004) whereby the casts were boiled in KOH (20 % w/w) for approximately 1 h, rinsed in water, then immersed in concentrated nitric acid for 20 min and rinsed again, before finally being boiled in KOH for 1 h. Fine forceps were then used to clean the casts of all remaining tissue. Images of the casts were taken using a Nikon Coolpix 4300 digital camera mounted on a Leica WILD M28 light microscope.

**Air flow pathways**

The air connections between the rhizomes, petiole and growing tips of shoots were investigated by flowing pressurized air into selected canals and observing the efflux canals. Three separate lengths of primary rhizome, possessing multiple nodes, leaves, growing tips and auxiliary shoots, were collected from the glasshouse ponds in March 2005. In total the tested sections comprised seven nodes, six axillar shoots, five healthy emergent leaves, two dying emergent leaves and two floating leaves. These sections had grown out of their pots and into the pond, allowing them to be pulled above the surface and then cut. This prevented water from entering the gas canals. They were taken to the laboratory and placed in a water-filled trough with their cut ends held above water. The length between adjacent nodes (length of internodal rhizome), the type of leaf at each node (floating or emergent) and the condition of each leaf were recorded. Each node was prepared by cutting off the axillar shoot, two adjacent internodes and the petiole with a razor blade, leaving three lengths of rhizome and a petiole, each between 10 to 20 cm long, united at the node. The newly cut ends were patted dry with a paper towel to prevent latex from the wounds entering the gas canals. To test the connections between an internode and an adjacent petiole, two of the three cut ends of the internodes were sealed with a putty (Blu-tak, Bostik, UK). Air was supplied through a 200 μl disposable pipette tip attached by a length of tubing to a piston air pump (Reciprotor, Copenhagen, Denmark) regulated by a needle valve and monitored using a 0-500 ml min⁻¹ flow meter. The pipette tip was firmly inserted into the cut end of each of the major gas canals in turn and pressurised air was blown through. While this was done, the cut end of the petiole was kept slightly submerged and the location of emerging bubbles,
indicating a connection, was recorded. This process was repeated to test the
c connetions between all internodes and attached petioles as well as between
internodal gas canals.

Growing rhizome shoots were tested for connections between canals I, II and
III by inserting 20-cm lengths of 0.8 mm internal diameter flexible PVC tubing into
all three canals and then sealing around them with Blu-tak. The free ends of the tubes
were then submerged in water, and air from the pump was then blown into each of the
tubes in turn. The occurrence of bubbling from the submerged tubes was then
recorded.

Results

Casts

The growing shoot is essentially the repeating unit of N. nucifera’s growth
form, as it possesses a developing node, petiole, distal internode and axillar shoot, as
well as all the air canals associated with these structures when fully grown. Five
growing shoots were collected from glasshouse-grown plants in various stages of
development, from tightly sealed buds surrounded by cataphylls to shoots possessing
emerging furled leaves. Cross-sections taken through other growing tips revealed a
network of caverns and canals in the growing shoot, always filled with a network of
white, spongy material, thought to prevent flooding of the air system if the plant is
broken under water (Blaylock and Seymour 2000). Despite the fine pores of the
spongy material, it was possible to cast such narrow gas spaces by allowing the
silicone fluid to infiltrate slowly into the vertically-held shoot.

Gas canals from the proximal internode entered the growing shoot, where they
connected with spongy nodal caverns (Vogel 2004). Like the gas canals, these
caverns were bilaterally symmetrical, with two sets of caverns – each the mirror
image of the other, and not interconnecting. Only canal IV, which was divided by the
plane of symmetry, was very weakly attached to the adjacent chamber pairs by a thin
webbing of silicone. This broke away in all of the casts during the cleaning process,
leaving two pairs of caverns connected to their respective canals (I to III; fig. 2A).
The exterior of the cavern cast appeared soft and porous, and was studded with
projections marking shallow holes at insertion points of adventitious roots. The
interior of the nodal caverns was markedly different, with casts revealing chambers
directly beneath the spongy outer layer connected with the gas canals from the
Fig. 2. Silicone casts of nodal chambers and gas canals of growing shoots. Scale bars are 2 mm.  A. Internal view of growing shoot with proximal internode canals, nodal chambers, petiolar canals, distal internode canals and developing node (far right). B. Inside view of chambers X and Y.  Developing axillar shoot canals marked with an asterisk.  C.  External view of young node with vertical cavity encircling all chambers indicated by arrow.  D.  External view of chambers X and Y of old node, with reduced vertical cavity indicated by arrow.  E.  Internal view of chamber Z with bifurcation of distal canal III marked by arrow.
rhizome. These chambers divided the nodal caverns into three distinct regions. The uppermost cavern connecting with canals I and i from the rhizome was vertically divided into a proximal (X) and distal (Y) chamber, with both I and i connecting to chamber X (fig. 2A, B). The nodal caverns connecting with canals II and III from the proximal internode fused to form the third chamber, Z (fig. 2A). All chambers (X, Y, Z) appeared to be connected by a vertical cavity that encircles the distal end of the nodal caverns and ran between chambers X and Y vertically across chamber Z (fig. 2C). However, in older, more developed growing tips this cavity appeared to be greatly reduced, thereby separating all three chambers (fig. 2D). The casts of all of these chambers could be split easily along a line of weakness into two pairs, one containing chambers X and Y, the other chamber Z.

The air canals of the petiole, distal internode and axillar shoot developed from the distal side of the nodal caverns. Air canals A, c and d in the petiole clearly arose from chamber Y, as did canal I of the distal internode (fig. 2B). The remaining petiolar air canal, canal B, and canal III of the distal internode, both developed from chamber Z (fig. 2A). Distal canal III connected to chamber Z with a bifurcated junction which ensured a connection across nodal chamber Z between distal canal III and proximal canals II and III (fig. 2E). Chamber X gave rise to distal canal II. Air canals for the axillar shoot arose from the top two chambers, X and Y (fig. 2B). Canal I of the axillar shoot connected with chamber X, and canals II and III arose from chamber Y.

**Airflow pathways**

As the rhizome is bilaterally symmetrical, only one of the canals in a pair is considered here, although both were tested. Air flow tests began at the oldest (proximal) end of the primary rhizome, working toward the (distal) growing tip. The tests were conducted on excised nodes with attached lengths of internode and petiole, as described in the methods. It was found that pressurised air administered to canal I of the proximal internode did not connect with any petiolar canals, but exited from canal II of the distal internode. Air supplied to canals II and III exited only from canal III of the distal internode and from canal B of the petiole. Air blown back through a node through canal I of the distal internode vented through canal A of the petiole, while distal canal II connected with proximal canal I, and distal canal III connected with proximal canals II, III and petiolar canal B. The single canal IV was
continuous between proximal and distal internodes, but did not connect with any other canals. Canal V was always blind-ending, terminating just before a node. These patterns were invariant along the length of all the rhizomes tested.

The connections between the axillar rhizome and the proximal and distal internodes were more difficult to determine, due to twisting of the axillar rhizome and the absence, in many cases, of one or both members of canal pair I, a useful marker in orienting the major canal pairs. However, the pattern that emerged showed that air blown from canal I of the distal internode vented from canals I and II in the axillar rhizome, while air from canal I of the proximal and canal II of the distal internode exited from canal III of the axillar rhizome. The leaves growing from the axillar rhizome were all floating leaves. Canal A from the petiole connected with both canals I and II of the axillar shoot, while canal B connected to canal III.

Some of the rhizomes possessed leaves that were beginning to senesce, indicated by yellowing. In these leaves, the B canals were blocked at the node, while the A canals remained open. The blockage results from centripetal cell growth of the surrounding the canal wall, filling in the cavity (Vogel 2004). All petiolar canals were completely blocked in old, withered leaves.

Air flow tests of growing tips revealed a turnaround point in the node. Air blown into canal III exited canal II and, to a lesser extent, canal I. No connection occurred between canal IV and any other canal pairs.

Discussion

This study has revealed that *N. nucifera* has a complex system of gas flow. Unlike the simple leaf-to-leaf or culm-to-culm flow patterns present in other aquatic macrophytes (Dacey 1981; Dacey and Klug 1982b; Grosse and Mevi-Schutz 1987; Tornbjerg et al. 1994), the sacred lotus possesses a repeating arrangement of gas canals that precisely channel air flow throughout the rhizome.

The connections observed in the silicone casts of air canals in the growing shoots completely agree with the flows observed using pressurised air across intact nodes and internodes. The flow pattern is summarised in fig. 3 and the repeating unit is illustrated in fig. 4. Pressurised gas from a leaf flows down through canal B into nodal chamber Z in the rhizome. Nodal chamber Z connects with canal III on both its proximal and distal side, allowing the pressurised gas to flow forwards or backwards along the length of the rhizome. However, for ventilation to occur, the pressurised gas must flow out of the rhizome and back to the atmosphere. Theoretically, this can
happen in two ways. The most common pattern of flow occurs in a distal to proximal direction along the length of the rhizome. Chamber Z also connects with proximal canal II, allowing pressurised air to flow from chamber Z in one node back along the internodal rhizome through canal II to the preceding node. In this node, canal II joins chamber X, which is connected with proximal canal I. The air then continues to flow back along the rhizome through tube I until it reaches yet another node. Within this node canal I enters chamber Y, from which the petiolar canals A, c and d arise, allowing the pressurised gas to vent up the petiole and out through the porous petiolar plate. In this manner pressurised gas from one leaf must flow backwards through two nodes before it returns to the atmosphere (fig. 3).

Fig. 3. Lateral cross-section of gas canal anatomy within a rhizome possessing a growing tip and three nodes (axillary shoots not shown). Dark gray canals indicate pressurised canals connecting with pressurising petiolar B canals. Light gray canals indicate low pressure canals connecting with venting A canals. Arrows indicate direction of flow in petiolar canals.
The second way flow can occur is forward along the rhizome toward the growing shoot. Pressurised air from canal III enters chamber Z within the developing node. When it flows into chamber Z, the air comes to a dead end in the still-developing distal canal III, but as canal II arises from the proximal side of chamber Z, air can then reverse and flow back down the rhizome through canal II, repeating the pattern described above. It also appears that in young nodes, air can move from chamber Z to chamber X through the vertical cavity, and thus flow back through proximal canal I. In both these examples, the connections between axillary shoots would not disrupt the pattern, as the pressurising canals II and III from the axillary rhizome join the vent chamber Y, while vent canal I joins chamber X.

The flow of air through canals and chambers within the rhizome is regular, but circuitous, and this suggests that some evolutionary advantage might be gained from such a complex arrangement. If the selective pressure were for ventilation of the node alone, then the simple evolutionary step of joining the A and B petiolar canals in the node would suffice. Similarly, if internodal ventilation were also important, fusion of chambers X and Z would allow the efflux and influx canals of adjacent leaves to alternate, thereby ventilating both the node and internode. However, in *N. nucifera* the pressurised gas from all leaves flows into the same canal pair (III) and can then only reach a vent canal in a petiole after flowing backwards through two nodes.

The flow pattern within the rhizomes of *N. nucifera* has three potential benefits. Firstly, canal III is continuous for the length of the rhizome and all leaves contribute to pressurising the gas within. As a result, all leaves can access pressurised.
air to ventilate their nodes and internodes, regardless of their individual pressurising status. This has the advantage of avoiding pockets of stale air under leaves that may not be pressurising. It has also been observed that withering leaves block their B canals first, thereby preventing depressurisation of canal III while allowing the aging leaves to continue acting as vent points through their A, c and d canals. Secondly, the nodal caverns ensure that all rhizome canals connect only with a vent point and do not reconnect with each other, ensuring that all canals are ventilated along their entire length. If canals in the rhizome were to reconnect in the node, then flow would primarily occur in the canal with the least resistance, partly bypassing the other two canals. Finally, this system of canals allows ventilation to occur right to the growing tip. Air from canal III flows into chamber Z at the tip of the growing shoot and then back out through canal II and down the internode, ensuring that oxygen is supplied to the rapidly growing tissues, where it is most needed.

The significance of return flow back through two nodes is unclear. Ventilation of nodes and internodes supplies oxygen to the plants tissues, but the structure of the nodal chambers implies that gas exchange with the rhizosphere through the roots is also important. The spongy walls of chambers X and Z are transected by adventitious roots which then grow out through the wall of the node into the surrounding sediment. As each root is surrounded by a thin cavern as it grows out through the node wall, giving the surface of the nodal caverns the appearance of villi when cast with silicone (fig. 2A). Chamber Z, the largest, possesses approximately two-thirds of the roots, and receives pressurised gas directly from the leaf. Thus the majority of the roots would receive the highest oxygen levels, which could then diffuse into the surrounding sediment through the roots. However, chamber X, which possess a third of the roots, would receive gas only after it had flowed through chamber Z in a preceding node, and would therefore receive only less oxygenated gas. If flow could reverse, however, and flow through petiolar canal A into chamber Y (which does not possess roots) and then through distal canal I into chamber X, then this group of roots would also be oxygenated.

With the connections between all the gas canals now established, it is interesting to speculate on the function of the giant stomata located over the vent canal, and their possible role in determining flow direction. The stomata are capable of closing rapidly, thereby preventing gas from venting through canal A (Vogel, 2004 and personal observation). The stimulus for this is not known. The gas that vents through the petiolar stomata originates not only from the rhizome, but also from the
adjacent leaf lamina. In this way pressurised gas from two-thirds of the leaf vents directly to the atmosphere without contributing to ventilation of the rhizome (Vogel 2004). Stopping this efflux, and blocking canal A, would have several important consequences. Assuming that a pressure gradient existed between the gas within the leaf and canal III, then gas would flow down the petiole through canal A, and forward along the rhizome through canals I, II and into III. This would have the benefit of oxygenating the roots associated with chamber X, as discussed previously, as well as allowing a single leaf to contribute atmospheric oxygen to two sections along the rhizome. However, if the efflux stomata of a non-pressurising or ‘leaky’ leaf were to close, then gas would flow up through all of the petiolar canals and vent through the entire surface of the leaf lamina. On warm, sunny afternoons, efflux through the leaf surface has been observed on both emergent and floating leaves of *N. nucifera*.

Although the gas canal anatomy of *N. nucifera* has been described here, the internal pattern of flow is likely to prove highly dynamic. Influx and efflux points may vary due to environmental conditions as well as physiological responses such as the opening and closing of petiolar stomata.

Acknowledgements
We gratefully acknowledge the Adelaide Botanical Gardens for supplying plant material and assistance.