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EMBOLIZED CONDUITS OF RICE (*ORYZA SATIVA*, POACEAE) REFILL DESPITE NEGATIVE XYLEM PRESSURE[1]

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Embolism reversal in rice plants was studied by testing the plant's ability to refill embolized conduits while xylem pressures were substantially negative. Intact, potted plants were water-stressed to a xylem pressure of −1.88 ± 0.1 MPa and a 66.3 ± 3.8% loss of xylem conductivity (PLC) by cavitation. Stressed plants were carefully rewatered, allowing xylem pressure to rise, but not above the theoretical threshold of c. −0.15 MPa for embolism collapse. Despite xylem pressures being more negative than this threshold, the PLC fell significantly (28.5 ± 5.6%), indicating the refilling of vessels. Above c. −1.0 MPa, almost all plants regained their maximum hydraulic conductivity. Dye uptake experiments showed the same pattern of embolism refilling despite negative pressure. Refilling was prevented in plants that were light-starved for 5 d, suggesting the unknown mechanism is dependent on metabolic energy. Results are among the first showing that herbaceous plants can reverse embolism without bulk xylem pressures rising near or above atmospheric.

Key words: embolism; novel refilling; *Oryza*; rice; xylem cavitation; xylem pressure.

According to the cohesion theory, evaporation of water from leaves creates a pulling force that is transmitted through the entire soil–plant continuum (Tyree, 1997; Steudle, 2001). A consequence of the cohesion–tension transport mechanism is that water in the xylem is under negative hydrostatic pressure (P_x) when the soil water potential is negative or when the plant is transpiring. This makes xylem sap inherently vulnerable to cavitation (Tyree and Sperry, 1989). Cavitation occurs when the water column breaks and a void is created. The void is initially filled with water vapor, but air can diffuse into it, forming an embolism.

We now know that drought-induced xylem cavitation is by no means a rare event. It has been shown to occur in roots (Sperry and Hacke, 2002), stems (Pockman and Sperry, 2000), and leaves (Salleo et al., 2001; Stiller et al., 2003). The direct result of cavitation in plants is a reduced hydraulic conductivity and steeper pressure (P_x) gradients along the xylem. More negative pressures may cause additional cavitation, causing even steeper P_x gradients, unless water loss is reduced by stomatal closure (Tyree and Sperry, 1988; Jones and Sutherland, 1991). In a recent study, Stiller et al. (2003) have shown that rice leaves (*Oryza sativa*) are especially vulnerable to xylem cavitation. The authors found the percentage loss of conductivity (PLC) from cavitation to be >60% even in well-watered plants and that rice was able to refill embolized conduits on a daily basis in association with nightly root pressure.

Positive root pressures have been linked to vessel refilling in a variety of plants, such as corn (*Zea mays*, Miller, 1985; Tyree et al., 1986), grapevines (*Vitis labrusca* and *V. riparia*, Sperry et al., 1987), trees (*Acer saccharum*, Sperry et al., 1988; *Acer pseudoplatanus* and *Betula pendula*, Hacke and Sauter, 1996), and a vinelike bamboo (*Rhipidocladum racemiflorum*, Cochard et al., 1994), as well as numerous species of tropical vines (Ewers et al., 1997). However, several recent studies have reported refilling without positive root pressures, even when xylem pressures are still substantially negative (Salleo et al., 1996; Holbrook and Zwieniecki, 1999; Tyree et al., 1999; Holbrook et al., 2000; Hacke and Sperry, 2003). This ''novel refilling'' phenomenon has been most convincingly demonstrated in just a few woody species: bay laurel (*Laurus nobilis*, Salleo et al., 1996, 2004; Tyree et al., 1999; Hacke and Sperry, 2003) and two tree species of the Brazilian savanna (*Schefflera macrocarpa*, *Caryocar brasiliense*, Bucci et al., 2003). It is not known how common this type of refilling is and whether it is restricted to certain groups or functional types of plants. Furthermore, the mechanism of this refilling is currently unknown.

There is general agreement that the embolized conduits are somehow pressurized independently of the transpiration stream and that osmosis is the ultimate source of the pressure, but beyond this, all is speculation (Holbrook and Zwieniecki, 1999; Tyree et al., 1999; Holbrook et al., 2000). Some experiments suggest an important role of the phloem and other living cells, presumably for secretion of the osmoticum, if not also the delivery of water, to the embolized conduit (Salleo et al., 1996, 2004; Holbrook et al., 2000; Zwieniecki et al., 2000). Starch hydrolysis in vascular parenchyma cells has also been closely linked to novel refilling in bay laurel (Salleo et al., 2004) and the two Brazilian species (Bucci et al., 2003).

In the present study, we investigated whether novel refilling occurs in rice. Although in our earlier work we demonstrated daily refilling of embolized vessels in rice leaves in association with root pressure, whether refilling could occur in the absence of root pressure was not conclusively tested (Stiller et al., 2003). Refilling despite negative pressure could be important for rice plants that are grown under upland (aerobically) or rain-fed lowland conditions because these plants are subjected to unpredictable periods of soil drought (Chaudhary and Rao,

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1982). Under these conditions, rice plants lose their ability to generate root pressure after a few days without irrigation (Stiller et al., 2003), and grain yield is greatly reduced. Novel refilling could provide an important means for maximizing photosynthetic performance and thus lessen drought-associated yield loss.

MATERIALS AND METHODS

Plant material and growth conditions—Rice plants (*Oryza sativa* L. cv. IR64, Poaceae) were grown from seed (supplied by the International Rice Research Institute [IRRI], Los Banos, Philippines) in a greenhouse at the University of Utah in 15-L pots under natural light. Soil was fritted clay (Balcones Mineral Corporation, Flatonia, Texas, USA). During growth the pots were kept in water-filled trays (5 cm water level) and plants were frequently watered. Plants were grown for 60–90 d at 22°C and approximately 60% relative humidity until early flowering, at which stage experiments began.

Dehydration–rehydration treatment—To induce water stress and cavitation, plants were allowed to transpire without watering until the leaves began to roll. Some plants were drought-stressed in the greenhouse for up to 7 d under relatively low evaporative demand. Other plants were stressed more rapidly in the laboratory by placing them under constant light (1500 μmol photons $\cdot$ $m^{-2} \cdot s^{-1}$) and relatively high evaporative demand (air temperature near 25°C and relative humidity approximately 40–50%) with air well stirred by fans for 24 continuous hours. The two groups of plants behaved no differently in the experiment, and the more rapid stress induction was employed as a matter of convenience.

The stressed plants were moved to a dark room to suppress transpiration. This was done to promote water potential equilibration between plant and soil at all times so that xylem pressure (P_x) could be carefully monitored and controlled during the rehydration phase. The xylem pressure and PLC of leaves were measured periodically in tillers of similar size as plants were carefully watered to avoid excessive rehydration. Each pair of PLC/P_x measurements required the removal of one entire tiller (rhizome tip plus leaves—see P_x and PLC measurements), so the number of measurements that could be made per plant was limited to between two and four. One PLC/P_x measurement was made prior to rewatering to give the PLC at maximum stress. The remaining PLC/P_x measurements were made during rehydration. We monitored P_x by itself more frequently than PLC because this required only removing one leaf tip and allowed us to make sure the xylem pressure was always below the minimum xylem pressure (P_r) that would induce passive refilling through bubble collapse. P_r was calculated from the capillary equation as $P_r = P_{wv} - (2T/r)$ (Eq. 1), where r equals the radius of the bubble, P_{wv} equals the vapor pressure of water, and T equals the surface tension of water (Yang and Tyree, 1992). The minimum r in minor veins was c. 3 μm, meaning P_r was approximately −0.05 MPa for an air bubble and −0.15 MPa for a vapor embolism. A total of nine plants were taken through a dehydration–rehydration cycle to determine whether the PLC decreased during rehydration.

Light-starved plants—To investigate the influence of metabolism on novel refilling, we repeated the dehydration–rehydration experiment with light-starved plants. The experiment followed the same protocol described, except the plants were kept in the dark room for 5 d after the dehydration phase before being rewatered and monitored for P_x and PLC. Five plants were taken through the dehydration–rehydration cycle under light-starved conditions.

P_x and PLC measurements—The P_x and PLC were monitored throughout the rehydration phase. We took special care to insure that the P_x measured with the pressure chamber reflected the xylem pressure at the site of the PLC measurement. This was achieved by keeping the plants in the dark during the rehydration phase and so suppressing transpiration. In addition, for every measurement of P_x and PLC we harvested an entire tiller (rhizome tips plus all attached leaves) and equilibrated them further inside a humid box for 20 min. P_x was measured on the first and third youngest mature leaves with a Scholander type pressure chamber (Soil Moisture Equipment Corporation, Santa Barbara, California, USA). These two measurements never differed by more than 0.1 MPa and were averaged to obtain the estimate of the xylem pressure in the middle leaf (the second youngest mature leaf), which was used for the PLC measurement.

To measure the PLC on the middle leaf, we cut it from the tiller underwater to avoid introducing additional embolism. The leaf was trimmed so that 1–2 cm of the leaf sheath was attached to the blade and the apical 4–5 cm of the blade was removed. Such leaf segments usually lacked continuous aerenchyma lacunae that would interfere with the PLC measurement. The hydraulic conductivity (K_{native}) of the leaf blade was calculated as the flow rate of water divided by the pressure gradient creating the flow. To make these measurements, we attached the leaf at the ligule with a rubber gasket to a tubing system that was connected to a water-filled container that could be lowered approximately 60 cm to create a negative pressure head. The tip of the leaf blade was submerged in a small water-filled cup that was placed on an electronic balance (BA210S, Sartorius, Goettingen, Germany). We used four different pressure heads to measure the flow rates of water off the balance and calculated K from the linear regression between flow rate and applied pressure. After lowering the container, we allowed 2–3 min for the plastic tubing to equilibrate with the changed water pressure and for flow rates to reach steady state before measurements commenced. After completing flow measurements and after calculation of K_{native}, the leaves were flushed at 100 kPa for 30 min with deionized and filtered (0.2 μm) water to reverse any embolism. During flushing, the cut apical end remained submerged under water. If large air bubbles emerged from the cut surface during flushing, the leaf was discarded because this indicated a continuous aerenchyma lacuna. After flushing, we waited for 15 min to allow the tubing system to decompress before the maximum hydraulic conductivity (K_{max}) was measured using the same protocol as for K_{native}. The percentage that K_{native} was below K_{max} gave the segment's PLC due to reversible xylem embolism.

Dye-uptake experiments—The extent of dye uptake and staining in excised leaves was used as an independent method to assess embolism reversal during the rehydration phase. Nine plants were stressed and rehydrated as described for the PLC measurements, and the P_x measured on the first and third youngest mature leaves of tillers equilibrated as usual. Instead of using the middle leaf for PLC measurements, it was excised under water and the cut base put into 15 mL polystyrene centrifuge tubes that were filled with approximately 2 mL of safranin solution (0.02% w/w). The tops of the tubes were sealed with Parafilm (American Can Company, Neenah, Wisconsin, USA) to minimize evaporation. The sealed tubes with the leaves were weighted and placed under sodium vapor lights (1500 μmol photons $\cdot$ $m^{-2} \cdot s^{-1}$) until they had taken up the equivalent amount of dye (per leaf area) that completely stained all vascular bundles in nonembolized leaves. Staining was assessed by counting the number of dye-filled leaf veins at ¾ the distance to the leaf tip using a stereomicroscope at 40× magnification. We calculated the percentage of unstained veins (PUV) as a measure for the amount of xylem embolism. The amount of dye taken up (per leaf area) was kept as constant as possible for all leaves to insure that any lack of staining was not simply an artifact of slower dye uptake due to stomatal closure. Thus, stressed leaves with low uptake rates were left in the dye for much longer time periods than unstressed leaves.

The PUV was also measured in leaves of five never-stressed control plants harvested early in the morning when leaf P_x was maximum.

RESULTS AND DISCUSSION

Stressed rice plants were highly embolized with a PLC as high as 80 (Fig. 1, open circles). Dye experiments also showed reduced staining of veins in stressed plants (Figs. 2B, 3, open circles). The PLC and PUV of stressed leaves agreed remarkably well (66.3 ± 3.8% vs. 65.0 ± 6.4%, respectively).

During rehydration there was a significant drop in PLC despite the fact that P_x never rose above the P_r threshold for

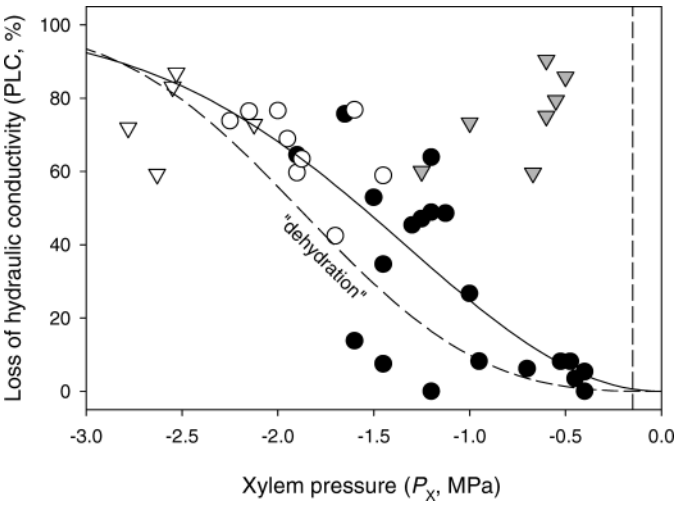

Fig. 1. Percentage loss of hydraulic conductivity (PLC) in leaf xylem vs. xylem pressure (P_x) in potted rice plants. Each plant was measured once at maximum dehydration (open circles) and one or more times during subsequent rehydration (solid circles). The solid ''rehydration'' curve is a Weibull function fit to pooled data from nine separate plants showing the reduction in PLC with rehydration. The dashed ''dehydration'' curve is a Weibull function fit showing PLC induction in progressively dehydrated plants of the same variety (Stiller et al., 2003). Five plants that were light-starved for 5 d (triangles) did not reduce PLC during rehydration (compare open triangles at dehydration vs. gray triangles during hydration). The vertical dashed line indicates the P_r threshold above which bubbles would dissolve (Eq. 1).

refilling (Fig. 1, solid circles). The same result was seen in the dye staining experiments: PUV decreased during rehydration without P_x ever reaching the P_r threshold (Fig. 3, solid circles). The rehydrated PUV approached the minimum seen in never-stressed control plants measured at maximum P_x (Fig. 3, solid diamonds). A Weibull function fit (Neufeld et al., 1992) to both the PLC and PUV data was not significantly different (F test, $P = 0.56$). Both methods independently confirm an unknown refilling mechanism at work that did not require the xylem pressure in the transpiration stream to rise to near atmospheric pressure.

The decline in PLC during rehydration (Fig. 1, solid curve) closely paralleled the rise in PLC during dehydration recovery as determined previously for the same rice variety (Fig. 1, dashed ''dehydration'' line: Stiller et al., 2003). The maximum rehydration time was 8 h, but significant PLC recovery was observed after rehydration times of as little as 1.5 h. This result suggests that in rice there is very little hysteresis in the P_x vs. PLC curve, whether it was obtained during a dehydration or rehydration phase. Xylem cavitation and embolism reversal appears to be a very dynamic process in rice that closely tracks the instantaneous xylem pressure. Similarly, rapid refilling was observed in the first experiments on the novel refilling phenomenon on *Laurus nobilis* (Salleo et al., 1996) for embolism induced by air injection, but this species refilled much more slowly when naturally embolized by soil drought (Hacke and Sperry, 2003).

Light-starved plants did not exhibit novel refilling—the PLC during rehydration was no different than during stress (Fig. 1, gray triangles). This result makes it unlikely that the refilling was simply an artifact of the hydraulic method for detecting embolism. It furthermore suggests that the refilling requires an active energy metabolism, consistent with results from other work (Salleo et al., 1996, 2004; Holbrook et al., 2000; Zwieniecki et al., 2000; Bucci et al., 2003; Hacke and Sperry, 2003). The minimum P_x leaf of light-starved plants tended to be lower than light plants (Fig. 1, compare open triangles and circles), which may also mean that refilling was inhibited by excessive water stress. However, the uniformity of the hydrated PLC values in the light-starved plants (Fig. 1, gray triangles) despite overlap in stressed P_x values between the two treatments suggests that the P_x during stress was not the dominant factor preventing refilling.

The K_{max} (hydraulic conductivity after embolism reversal by flushing) in both light and light-starved groups was not significantly different (2.18 ± 1.53 E^{-3} mg $\cdot$ s^{-1} $\cdot$ kPa^{-1} and 2.83 ± 1.17 E^{-3} $\cdot$ mg $\cdot$ s^{-1} $\cdot$ kPa^{-1} mean $\pm$ SD for plants under light and light-starved plants, respectively). This indicates that tylosis formation or other significant vessel occlusions did not influence the PLC results. Vessels occlusions would result in a decrease in the PLC but no actual refilling.

Although the mechanism of novel refilling is unknown, it is unlikely to violate the P_r threshold within the refilling vessel. Somehow the xylem pressure in refilling vessels is able to rise above P_r while neighboring water-filled vessels are at a much more negative pressure (Holbrook and Zwieniecki, 1999; Tyree et al., 1999; Holbrook et al., 2000; Hacke and Sperry, 2003). If this is the case, one might expect to see heterogenous balancing pressures when using a Scholander pressure chamber to measure P_x in refilling stems: the refilling vessels at higher xylem pressure should exude their sap at very low applied chamber pressure, while the full vessels at negative pressure will not exude until the ''true'' balance pressure is applied. We detected no heterogenous sap exudation during the pressure chamber measurements of rehydrated material. However, as Fig. 1 indicates, by the time we measured the xylem pressure after rewatering the refilling was already complete. This time span was a minimum of 1.5 h. A finer time course of pressure measurement would be needed to test for ''premature'' sap exudation from refilling conduits.

Novel refilling may require metabolic energy for generating the driving force to move water into the embolized vessel. The driving force is likely to be osmosis created by energy-demanding solute gradients. The osmosis could be directly into the conduit itself in response to solute accumulation within the conduit. Although the bulk xylem sap does show an increase in osmotic concentration in refilling stems (Hacke and Sperry, 2003), no experiment has yet shown sufficient osmoticum in the refilling conduits for the necessary pressurization (Tyree et al., 1999). Alternatively, osmosis into vascular parenchyma cells could be stimulated by solute mobilization, resulting in turgor-driven flow directed into the conduit by the appropriate asymmetry in membrane permeability. In this case, the osmotic potential in the vessels themselves is potentially uncoupled from the pressurization and so does not need to be above any threshold (Vesala et al., 2003). The recent evidence for a role of starch hydrolysis in refilling (Bucci et al., 2003; Salleo et al., 2004) suggests that mobilization of sugars is linked to the osmotic gradients either directly if the sugars themselves are the osmoticum or indirectly if their catabolism provides the energy. The former seems more likely given the cyclical nature of starch disappearance and reappearance implied by this work.

Another problem for the refilling mechanism is how the pressure in the refilling conduits can be so much greater than in neighboring water-filled ones. One possibility is that air-

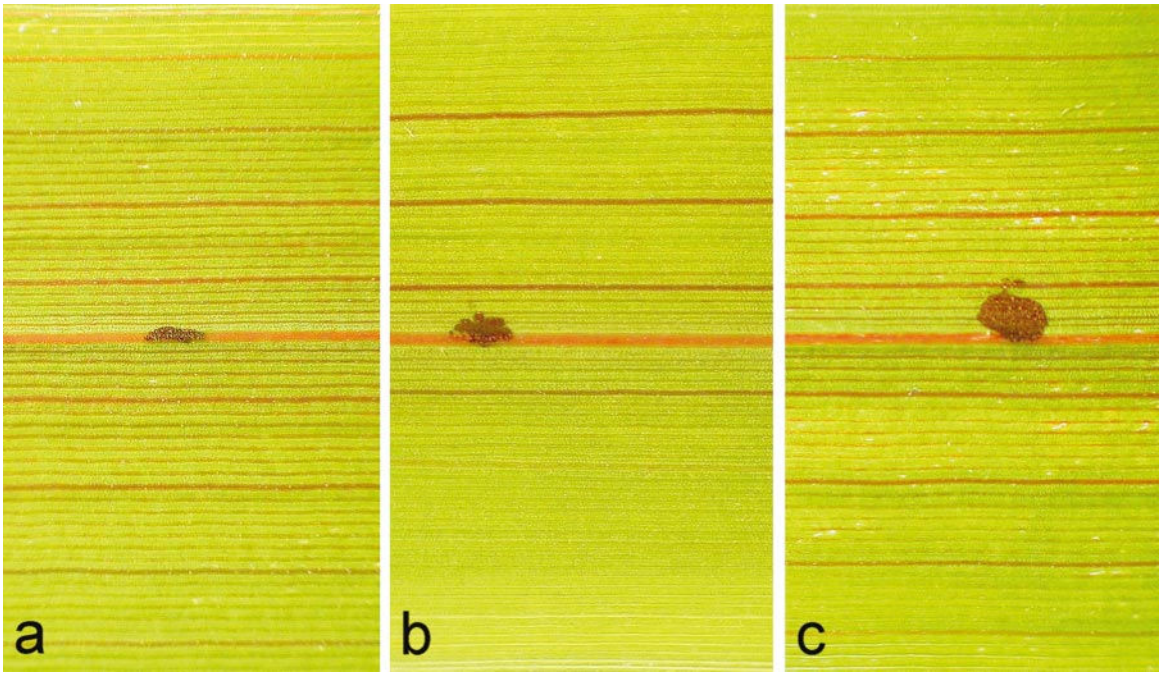

Fig. 2. Typical images of the abaxial surface of rice leaves that were allowed to take up safranin during transpiration. In well-watered control plants all veins were stained (a), whereas in stressed plants a large number of veins remained unstained (b). In rewatered plants that were allowed to recover for some time most veins were stained (c). The black mark at ¾ the distance to the leaf tip served as a visual aid for the calculation of the percentage of unstained veins (PUV).

filled pit chambers isolate the pressure in the refilling vessel (Holbrook and Zwieniecki, 1999; Holbrook et al., 2000; Zwieniecki and Holbrook, 2000; Konrad and Roth-Nebelsick, 2003). This would require that all pit chambers remain air filled until refilling of the conduit lumen is complete. The dissolution of all remaining air bubbles within the pit chambers must happen instantaneously as to avoid expansion of ''leftover'' bubbles when hydraulic contact to neighboring conduits is reestablished. This mechanism would be most effective for species with slitlike pit apertures and lacking pit chamber vestures (Zwieniecki and Holbrook, 2000), such as rice (U. G. Hacke, University of Utah, personal communication) Alternatively, the osmoticum could act at the pit membrane, pulling water directly from the transpiration stream across the pit membranes and into the pressurizing embolized conduit (Hacke and Sperry, 2003). This mechanism would restrict the refilling to species with very tight (or dense) pit membranes that could act as semipermeable membranes to a relatively large organic osmoticum, such as a disaccharide or larger sugar.

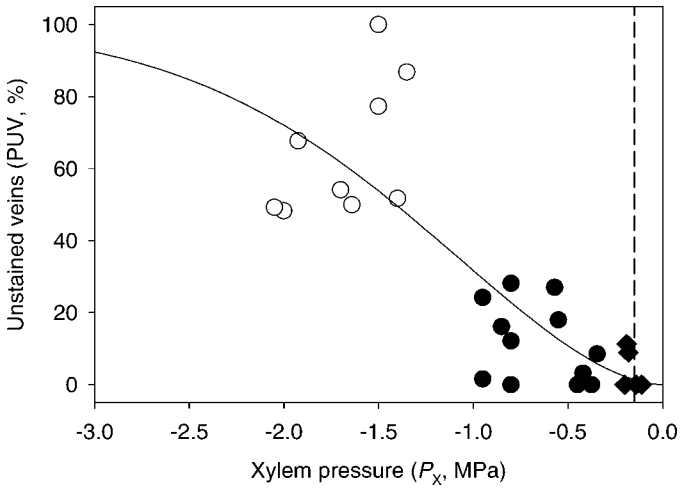

Fig. 3. Percentage of unstained veins (PUV) in leaf xylem vs. xylem pressure (P_x) in potted rice plants. Leaf veins from five well-watered control plants measured early in the morning at maximum P_x were almost completely stained (solid diamonds, PUV: 4.0 ± 2.5%, mean and SE). Nine plants were stressed and rehydrated. At maximum stress, PUV was 65.0 ± 6.4% (open circles, mean and SE). During recovery for 60–220 min in the dark, PUV decreased significantly to 11.6 ± 3.2% (solid circles, mean and SE). The vertical dashed line indicates the xylem pressure threshold (P_t) above which bubbles would dissolve (Eq. 1).

Conclusions—Rice leaves were able to refill embolized conduits despite prevailing negative pressure in the xylem. Our experiments show for the first time that novel refilling occurs in an herbaceous crop plant. ''Rehydration curves'' showing the refilling of embolized vessels as xylem pressure becomes less negative almost exactly traced ''dehydration curves'' that show the development of embolism as pressures become more negative. Light starvation prevented refilling, consistent with a requirement for metabolic energy. Little is known of the mechanism, and it remains a question of major significance in plant water relations. Rice xylem is very vulnerable to cavitation even under well-watered conditions and novel refilling may be crucial for restoring hydraulic conductivity. Although at least some rice cultivars also can refill by root pressure (Stiller et al., 2003), the additional presence of a novel refilling mechanism could be important when soil drought prevents root pressure or for cultivars that lack strong root pressure.

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