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Palm snorkelling: leaf bases as aeration structures in the mangrove palm (*Nypa fruticans*)

GUILLAUME CHOMICKI FLS[1]*, LUC P. R. BIDEL[2], WILLIAM J. BAKER FLS[3] and CHRISTIAN JAY-ALLEMAND[4]

[1]*Systematic Botany and Mycology, Department of Biology, University of Munich (LMU), Munich 80638, Germany*
[2]*INRA Montpellier, UMR AGAP, Montpellier, France*
[3]*Royal Botanic Gardens, Kew, Richmond, Surrey, TW9 3AB, UK*
[4]*University of Montpellier 2, UMR DIADE, Place Eugène Bataillon, F-34 095, Montpellier, France*

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Mangrove species have evolved specialized structures, such as pneumatophores, to supply oxygen to the roots, but, in *Nypa fruticans*, the only mangrove palm, no such structure has been reported. This study aimed to determine the adaptations of *N. fruticans* to the mangal habitat with special reference to the air-supplying structure. Following senescence, the rachis is abscised at the zone of junction with the leaf base. Simultaneously, lenticels develop so that, when abscission is completed, a network of mature lenticels covers the leaf base. Expansigenous aerenchyma with increasing porosity towards the stem junction occurs in the leaf base. The first two root branching orders present a subero-lignified rhizodermis and exodermis, and the cortex consists of schizo-lysigenous aerenchyma with wide lacuna, limiting radial oxygen loss and facilitating longitudinal oxygen transport to living tissues. Lifespan estimation suggests that leaf bases can live for up to 4 years following abscission, ensuring the persistence of aeration structures. This study provides structural evidence indicating that *N. fruticans* has evolved a unique type of air-supplying structure in the mangal habitat. © 2013 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2013, **00**, 000–000.

ADDITIONAL KEYWORDS: aerenchyma – Arecaceae – expansigeny – hypertrophic lenticel – leaf lifespan – Palmae – rachis abscission – root oxygen supply – schizo-lysigeny – tannins.

INTRODUCTION

The success of mangrove species in waterlogged, and therefore anoxic and highly reduced, environments is classically attributed to the remarkable morphological adaptations of their root systems (Scholander, Van Dam & Scholander, 1955; Tomlinson, 1995). These typically aerial root adaptations include pneumatophores [e.g. *Avicennia marina* (Forsk.) Vierh.], adventitious aerial roots that can emerge ectopically from high branches, such as in the red mangle (*Rhizophora mangle* L.), or form a cluster at the base of the trunk, known as stilt roots, such as in *Rhizophora stylosa* Griff, knee roots [e.g. *Bruguiera gymnorrhiza* (L.) Lamk.], buttress roots, such as those of *Heritiera littoralis* Aiton, or sinuous buttresses, so-called plank roots, of *Xylocarpus granatum* Ridl. (Saenger, 1982; Tomlinson, 1995). All of these structures possess high densities of lenticels, which enable gas transfer whilst preventing water entry (Tomlinson, 1995). In mangrove species, it has long been assumed that such adaptive root systems enable oxygen transport to drowned tissues, hence ensuring aerobic metabolism in an anaerobic environment (Scholander *et al*., 1955; Saenger, 1982; Tomlinson, 1995).

Beyond the morphological adaptations of mangrove root systems, the key anatomical trait that enables tolerance to inundation and soil anoxia is the formation of aerenchyma with interconnected air

*Corresponding author. E-mail: g.chomicki@biologie.uni-muenchen.de; guillaume.chomicki@gmail.com

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channel systems throughout the plant. Consistently, an increase in tissue porosity and the formation of air channels are pivotal to increase the rate of oxygen diffusion in plant tissues (Arber, 1920; Armstrong, 1979). Aerenchyma types fall into three main categories according to the mode of development: lysigeny, schizogeny and expansigeny (Seago *et al*., 2005). In lysigenous aerenchyma, the air lacunae arise from cell lysis and collapse, whereas, in schizogenous aerenchyma, cell separation of adjacent cell layers occurs, resulting in the joining of intercellular spaces into lacunae. Expansigeny is somewhat different from these two processes in that it involves the enlargement of intercellular spaces by cell division and expansion, but not by cell death, collapse or separation (Seago *et al*., 2005).

Aerenchyma structure and development have been described in detail for several mangrove species, including *Rhizophora mangle* (Gill and Tomlinson, 1977) and *Avicennia marina* (Purnobasuki and Suzuki, 2005). Moreover, in mangrove species, the pattern of oxygenation of the root system is dependent upon tides, with oxygen concentration peaking at low tides (Scholander *et al*., 1955; Allaway *et al*., 2001).

Nypa fruticans Wurmb. is the only extant mangrove palm, and is distributed from India and Sri Lanka in the west through South-East Asia to Australia and the Solomon Islands (Dransfield *et al*., 2008). *Nypa* Steck is a monotypic genus that arose as an early diverging lineage near the base of the palm phylogeny (Asmussen *et al*., 2006; Baker *et al*., 2009), dated at 93.5 Mya (Baker & Couvreur, 2013).

Nypa has an extensive fossil record dating back to the upper Cretaceous (Maastrichtian) (Tralau, 1964; Morley, 2000; Gee, 2001; Harley, 2006; Dransfield *et al*., 2008; Gomez-Navarro *et al*., 2009), with occurrences reported from far outside its modern range, e.g. in the Americas, Europe and Africa. Recent palaeobotanical data indicate that extinct *Nypa* spp. were also found in waterlogged environments and that changes towards a drier climate in the late Eocene–early Miocene coincided with a dramatic decline in the distribution of *Nypa* (Rull, 1998; Harley, 2006; Gomez-Navarro *et al*., 2009). Ancient records of *Nypa* coupled with swamp palaeoenvironments are used as a marker of the mangrove biota by palaeontologists (Gee, 2007). This suggests that the mangrove habit, and therefore the adaptation to the mangal habitat, is synapomorphic for the genus and not a recent adaptation in *N. fruticans*.

Nypa fruticans possesses an unusual habit for a mangrove plant (Fig. 1). In contrast with eudicot mangrove trees and shrubs, which have an orthotropic shoot system, *N. fruticans* has a plagiotropic stem that branches in a dichotomous manner, which, in turn, allows vegetative colonization (Tomlinson, 1971, 1995; Fig. 1). One remarkable adaptation of *Nypa* to the mangal habitat is the viviparous seedlings which germinate following seed maturation, before being shed from the fruiting head that terminates the inflorescence (Tomlinson, 1971, Fig. 1C). As a result of the plagiotropic habit of *Nypa* and tides, its stem is often immersed, the leaves being the only emergent part of the plant (Fig. 1). *Nypa* does not produce breathing roots like many other mangrove species, including some palm species living as mangal associates in wet habitats, which develop thin pneumatophores (Tomlinson, 1995). Thus, the air-supplying structures should be the leaves, as they are the only emergent part of the plant. Tomlinson (1995: 105) hypothesized that *Nypa* leaves play the role of a 'giant pneumatophore', based on their spongy texture and that of the rhizome ground tissue, and further suggested that this role may continue in the leaf base, noting that they break at an apparently similar position.

In this study, we explore the role of the *Nypa* leaf in the mangrove habit. We show that leaves of *N. fruticans* follow a distinctive developmental path, whereby the distal part of older leaves abscises, leaving behind persistent leaf bases that have attributes of pneumatophore-like aeration structures. This indicates that *N. fruticans* has evolved an air-supplying structure that is unique among mangrove species.

MATERIAL AND METHODS

Plant material

Plant material was collected from a mature specimen of *N. fruticans* growing at the Montgomery Botanical Center, Miami, FL, USA. The specimen was planted in 1982 (accession number 82473B). Additional root and leaf material was collected at the Royal Botanic Gardens, Kew, UK (accession number 2007-1564).

Anatomy, staining and microscopy

All anatomical sections were hand-cut apart from the lenticel sections in Figure 7, which are microtome sections. Staining for lignin was performed with a 5% w/v phloroglucinol solution (dissolved in ethanol) for 30 s, followed by concentrated hydrochloric acid (HCl) for 30 s. To detect suberin and cutin, we used a solution of 5% w/v Sudan IV in ethanol. Light microscopy (LM) was performed using a Leica DM LB100 microscope equipped with a Nikon Coolpix 4500 camera. For stereomicroscopy (for Fig. 7C only), we used a Leica KL300, and images were acquired with the same Nikon Coolpix 4500 camera. Epifluorescence microscopy was carried out on a Nikon epifluorescence microscope eclipse E600 fitted to a Nikon

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Figure 1. *Nypa fruticans* habit, specimen growing at the Montgomery Botanical Center, FL, USA, planted in 1982. A, Habit and general morphology of *N. fruticans*. B, Young inflorescence showing the pleonanthic habit (axillary inflorescences) of *N. fruticans*. C, Inflorescence. D, Young dichotomizing stem showing the two sister shoots and the subtending leaf with the two enclosing grooves. Scale bars: B, 15 cm; C, D, 10 cm.

camera Ds-Fi 1. A Nikon DAPI (4′,6-diamidino-2-phenylindole) filter (excitation spectrum, 380–420 nm) was used to track lignin in tissues. Under this filter, bright blue–white fluorescence is attributed to lignin autofluorescence and blue autofluorescence depicts all other cell walls (Liu *et al*., 1994). A Nikon B2-A filter (excitation spectrum, 450–480 nm) was used on diphenylboric acid 2-aminoethyl ester (DPBA)-stained slides to seek flavonoid distribution in tissues; yellow–gold or yellow–green fluorescence is associated with flavonoids (typically quercetin and kaempferol, respectively) (Sheahan & Rechnitz, 1992).

Image analysis and porosity measurements

Image analysis, cell and lacunae measurements and aerenchyma area measurements were performed on imageJ (http://rsb.info.nih.gov/ij). Porosity was estimated from transverse sections of the rachis and leaf base. The porosity of the leaf base and rachis was determined by the quantification of aerenchyma lacunae over the total area (Purnobasuki & Suzuki, 2004). For each of the three positions in the leaf axis, the total area was determined for three cross-sections per position and the area of each lacuna was determined for each cross-section with the ‘polygon selec-

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Table 1. Parameters measured for the estimation of the leaf base lifespan in *Nypa fruticans*

Shoot number (n = 5)	Number of living leaves (L_e)	Number of leaf base (B_{abs})
1	5	7
2	4	7
3	6	6
4	6	8
5	5	13
Average	5	7.5

tion' and 'measure' tools of imageJ. The percentage of porosity was obtained as follows: porosity (%) = 100 × (area of air spaces/total cross-sectional area) (Armstrong, 1979; Maricle & Lee, 2002; Visser & Bögemann, 2003).

Leaf base lifespan estimation

With the hypothesis that the leaf base of *Nypa* fulfils an oxygenation role, it was pivotal to evaluate the leaf base lifespan. The leaf base lifespan is the sum of the whole leaf lifespan and post-abscission lifespan. Tomlinson (1990) provided equations for the calculation of the leaf lifespan. The continuous growth of palms establishes that the lifespan of a single leaf A can be obtained from the product of the plastochrone interval P (i.e. the inverse of the number of leaves produced per year) and the total leaf number n, $A = Pn$ (Tomlinson, 1990). The total leaf number is expressed as the sum of the exposed L_e and unexposed L_u (still enclosed in the crown) leaves, and can generally be estimated by means of observation as the number of exposed and unexposed leaves is similar, hence $n = L_u + L_e \sim 2L_e$ (Tomlinson, 1990), although this assumption is not always met (Dalrymple & Fisher, 1994). To obtain the lifespan of the leaf bases, the equation should be modified so that the number of leaf bases n_{lb} corresponds to the number of exposed leaves L_e plus the number of unexposed leaves L_u, equivalent to L_e, plus the number of living leaf bases post-abscission B_{abs}, i.e. $n_{lb} = 2L_e + B_{abs}$. Therefore, the leaf base lifespan is obtained from the equation $A = Pn_{lb}$, that is $A = P(2L_e + B_{abs})$. L_e and B_{abs} were directly measured independently in five different shoots on one specimen (Table 1). The plastochrone interval P, however, requires longer times to be estimated accurately and was therefore taken from the literature. Plastochrone intervals for *Nypa* measured in wild populations varied from 3.04 ± 0.8 (Fong, 1986) to 2.0 (Rozainah & Aslezaeim, 2010) leaves per year. Therefore, the leaf base lifespan A was calculated for values of P = 1/2, P = 1/2.5 and P = 1/3. The lifespan of the leaf bases post-abscission (A_{LBabs}) was obtained using the equation $A_{LBabs} = P(B_{abs})$. The different plastochrone intervals probably account for the error range because of the difference in the unexposed versus exposed leaves. Moreover, the lifespan of the post-abscission leaf base is independent of this parameter. A summary of the parameters measured and used for leaf lifespan evaluation is provided in Table 1.

RESULTS

Leaf abscission in *N. fruticans*

Leaves of *N. fruticans* can be > 7 m long (Tomlinson, 1971, 1995). They consist of a wide (25–40 cm) leaf sheath or leaf base and a long rachis (6–8 m) in which reduplicate leaflets are inserted. The leaf sheath is open and has an adaxial groove, forming a well-defined leaf base that is separated from the rachis by a short petiolar transition zone. Leaves that subtend a dichotomy enclose two shoots and, as a result, possess two grooves (Fig. 1D). Abscission occurs at the transition zone between the leaf base and the rachis (Fig. 2A).

The abscission process starts with a distal to proximal senescence of the rachis and of leaflets, which stops just before the leaf base. The observation of mature *Nypa* specimens suggests that the first step in abscission is mechanical and consists of the folding of the rachis at the transition zone with the leaf base, just above the adaxial groove (Fig. 2A). A plot of diameter against leaf axis length reveals a large variation in diameter in the leaf base (35.7 cm m^{-1}), but a small variation (1.4 cm m^{-1}) in the rachis (Fig. 2C). To gain further insights into the mechanism of abscission, leaves undergoing abscission were sectioned longitudinally. The intact leaf base aerenchyma is separated by a clear abscission zone that is followed by a zone of degradation (Fig. 2D). Importantly, cells in the zone of abscission and in the adjacent degraded zone have thick-walled, non-lignified cell walls (Fig. 2E–G). In contrast, cells in the adjacent aerenchyma of the leaf base and the rachis are characterized by thin cell walls (Fig. 2D, E).

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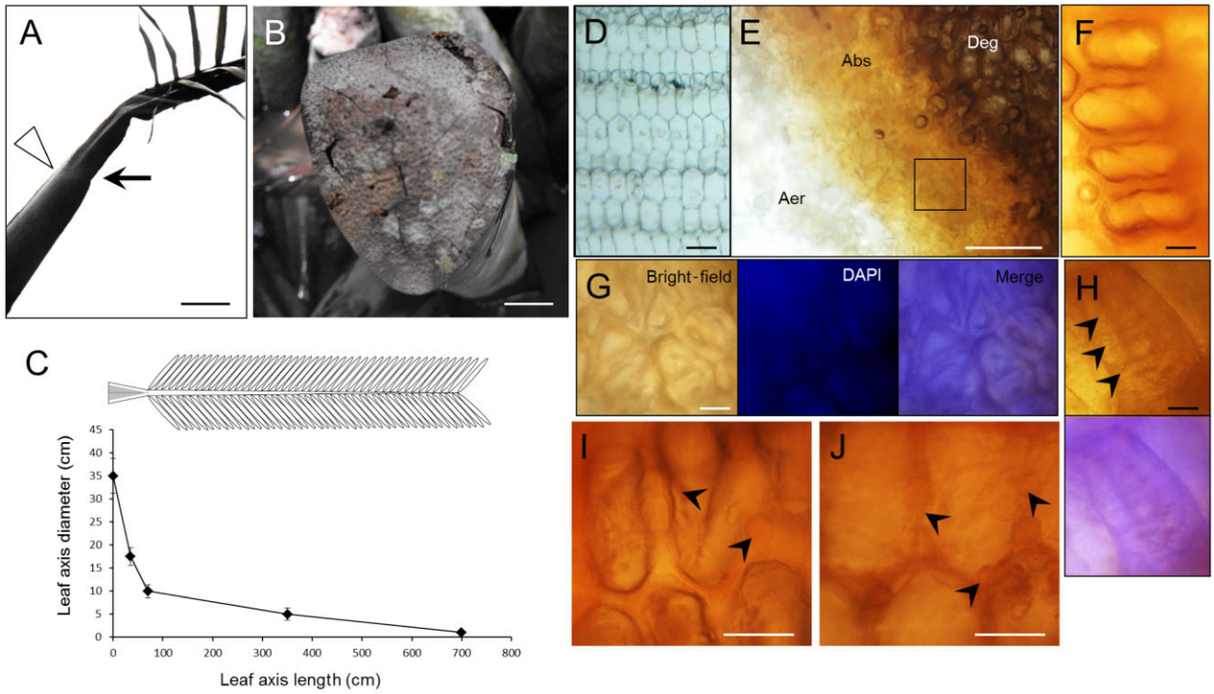

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Figure 2. Abscission pattern in *Nypa fruticans*. A, First stage of abscission, involving the distal to proximal necrosis of the leaf to the abscission zone. White arrowhead points to the beginning of the groove. Black arrow shows abscission zone. B, Abscission scar in surface view. C, Leaf axis width plotted as a function of the leaf distance from the basal-most point (as shown by leaf diagram). D, Longitudinal section of the aerenchyma in the leaf base. E, Longitudinal section in the abscission zone. F, Details of the cells that are above the abscission zone (in the Deg zone in E). G, Young non-lignified fibres in the abscission zone (frame in E). H, Recently divided cells in the abscission zone. Top panel represent a bright-field image, bottom panel represents a merged with DAPI (4′,6-diamidino-2-phenylindole) image. Arrowheads point to the cell walls parallel to the division plane. I, J, Cell wall hydrolysis during abscission in the Deg zone shown in E. Arrowheads show the degraded cell wall and cellulose agglomeration. Scale bars: A, 10 cm; B, 1.2 cm; D, 60 μm; E, 200 μm; F, 40 μm; G, 30 μm; H, I, 20 μm; J, 15 μm. Abbreviations: Abs, abscission zone; Aer, aerenchyma; Deg, degraded cells.

Furthermore, cells in the abscission zone are small and often present in rows of three to four cells, indicating recent division (Fig. 2H). Cells distally adjacent to the abscission zone (Deg in Fig. 2E) exhibit substantial cell wall hydrolysis signs (Fig. 2F, G), with string-like detachment of cellulose microfibrils (Fig. 2I) and glomerular agglomeration of degraded cell walls (Fig. 2J), suggesting high activity of cell wall hydrolases, such as cellulase. Once the rachis is completely removed from the leaf base, the exposed surface of the zone ultimately becomes cutinized (Fig. 2B). After completion of the abscission process, the leaf base remains as a living appendage.

Leaf base lifespan in *Nypa*

Following rachis abscission, leaf bases do not senesce, but persist for a long time. The leaf base lifespan in *Nypa* was evaluated using an equation modified from Tomlinson (1990) who used palm canopy parameters (see Material and Methods). Total leaf base lifespan (before and after abscission) was estimated to be over 8 years on average (Fig. 3A), with the rachis living from 3.0 to 5.5 years according to the plastochrone interval (Fig. 3B). The leaf base lifespan after abscission was found to be 4.1, 3.3 and 2.7 years, on average, for the respective plastochrone intervals used (Fig. 3C). Fong (1986) calculated that the leaf lifespan in *Nypa* is 5.5 years in wild populations in Malaysia, providing a cross-validation of our results.

Lenticels result from transient meristematic activity below the stomata in leaf bases of *Nypa*

Stomata are present on the abaxial epidermis of the leaf base (which represents most of the surface, the adaxial epidermis being restricted to the groove) and

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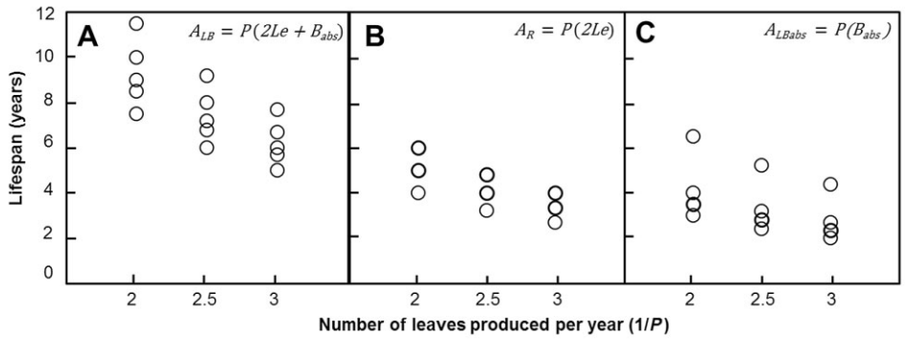

Figure 3. Evaluation of leaf base lifespan. A, Total leaf base lifespan (A_{LB}) (before and after abscission). B, Rachis lifespan (A_R). C, Post-abscission leaf base lifespan (A_{LBabs}). Each data point represents one shoot. For each plastochrone interval modelled, the same dataset with the number of exposed leaves (L_e) and the number of post-abscission leaf bases recorded from five independent shoots of a 27-year-old *Nypa* specimen [82473B, Montgomery Botanical Center (MBC)] was used.

rachis at a density of *c*. 20 cm^{-2}. Leaf bases (most notably after abscission) possess a network of lenticels, the density of which is equivalent to the stomatal density. The examination of the leaf base epidermis at different ages reveals that lenticels develop in the place of existing stomata (Fig. 4A–D). On the leaf base, stomata stay functional for only a short time after leaf initiation (Fig. 4A). Cutin rapidly accumulates around the stomata and guard cells, the latter probably accumulating other phenolic substances, resulting in the maintenance of stomata in the open state (Fig. 4B). Subsequently, stomata become longitudinally split by the appearing lenticel (Fig. 4C). Finally, the lenticel develops and all stomatal traces disappear (Fig. 4D). Although this process occurs all along the leaf axis (leaf base and rachis), mature lenticels (Fig. 4D) do not occur on the distal part of the rachis as it is abscised before they can develop.

To understand the mechanisms underlying lenticel formation, anatomical sections were performed at different stages of lenticel development (i.e. for differently aged leaf bases). Cork anatomy consists of an epidermis with four underlying cell layers of tannin-rich cells, termed tannin idioblasts (TI), followed by about four layers of more or less round cells that have a suberized and lignified thick cell wall with a small, tannin-rich lumen, and are referred to as tannin brachysclereids (TB) (Fig. 5A).

Longitudinal and transverse sections in young stomata show rows of aligned cells suggestive of a meristematic zone (Fig. 5B–D) and, in turn, indicate that lenticel development starts at an early stage. The meristematic activity emanates from the parenchymatous cells under each stoma, and the pattern of the disposition of rows of three of four cells in Figure 5B–D indicates that each parenchymatous cell gives rise to three or four daughter cells, and hence has a limited meristematic potential. Following division, the cells differentiate centripetally into TIs and cells that resemble dicot phellem cells, termed phellem-like cells (Fig. 5E, F).

The alternation of phellem-like tissue and TIs uncovers a transient mode of functioning (Fig. 5F). That is, once a definite set of parenchyma cells has divided and produced TIs and phellem-like tissue, another set of parenchymatous cells inner to the first set divides and produces a new TI and phellem-like tissue. This results in an alternation of TIs and phellem-like cells in mature lenticels (Fig. 5F). A lenticel showing such organization, i.e. with more than one cycle of parenchyma differentiation, is termed secondary.

Expansigenous aerenchyma provides an air pathway from lenticels towards the roots

The ground tissue of the leaf axis in *Nypa* consists of aerenchyma (Fig. 6). The air canals or lacunae of the aerenchyma are established along a gradient of diameter; the more proximal, the wider is the lacuna (Fig. 6A–F), with a marked increase in lacuna diameter towards the leaf base (Fig. 6G). Increased lacuna density and increased lacuna diameter result in a dramatic porosity increase towards the leaf base (Fig. 6I).

To determine the origin of the difference in lacuna diameter along the leaf axis, lacunae were observed at different positions along the leaf base/rachis axis using serial transverse sections. Distinct types of lacuna are identified according to the number of cells that border each lacuna and the degree of elongation of these cells (Fig. 6K–O). Transitional stages with cells in division were found in young leaf bases

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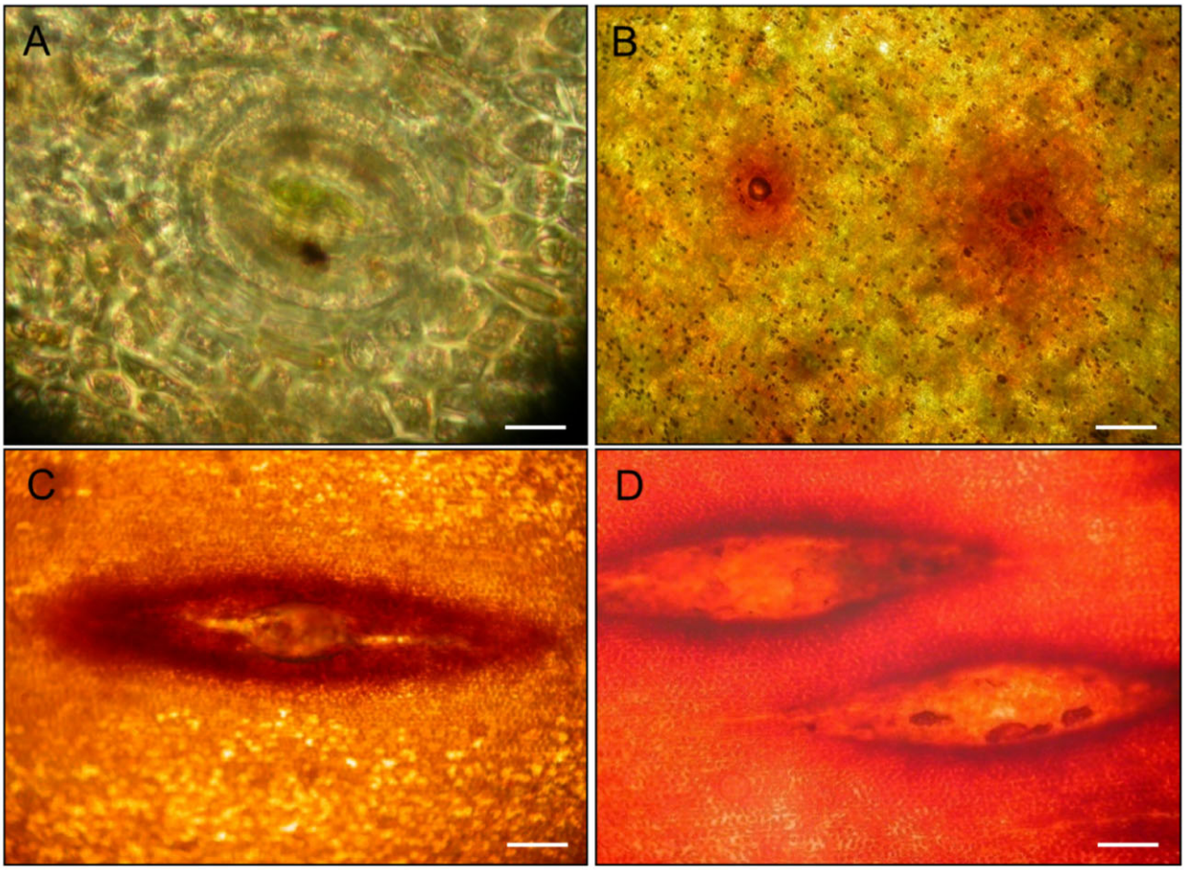

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Figure 4. Emergence of lenticels from stomata on *Nypa* leaf bases. A, Stomata on a leaf base of a young leaf. B, Guard cells accumulate cutin and are 'frozen' open on a slightly older leaf base. C, In a young leaf base, a lenticel forms in the place of a stoma. The location of the stoma is clearly visible. D, Young lenticel on an adult leaf base. Scale bars: A, 25 μm; B, 300 μm; C, 75 μm; D, 1.5 mm.

(Fig. 6P, Q), suggesting that these stages form a developmental series. The number of cells bordering a lacuna increases gradually towards the leaf base (Fig. 6J). However, cell length increases only in the leaf base (Fig. 6H), rather than gradually along the leaf axis.

A longitudinal section revealed that air lacunae form closed air pockets (Fig. 6R). These air pockets are separated by a single-celled layer in the leaf base (Fig. 6O), and this increases to over three cell layers in the rachis. The lacunae are interconnected by a pattern of cell wall pitting on the walls of certain cells (Fig. 6R, S).

Root architecture and anatomy in *Nypa*

Nypa roots are present at high density in the first 10 cm of substrate. In *N. fruticans*, the root system presents an architecture characterized by three branching orders in the architectural system of Hallé *et al*. (1978) (Fig. 7A). In contrast with previous studies, all three axis orders were examined, and it appears that the different orders vary substantially in their anatomy and potential functionality (Fig. 7).

The first order (AX1) is about 0.5 cm in diameter and presents a thick rhizodermis with slightly lignified outer walls, and the subsequent four or five cell layers constitute the exodermis which is suberized and interspersed with tanniferous cells (Fig. 7C, G). The lignified rhizodermis is followed by four or five suberized exodermal cell layers that contain interspersed tanniferous cells (arrow in Fig. 7G). The second axis (AX2) is 2–3 mm in diameter and has a layer of highly tanniferous and probably suberized cells that follows the rhizodermis, and three

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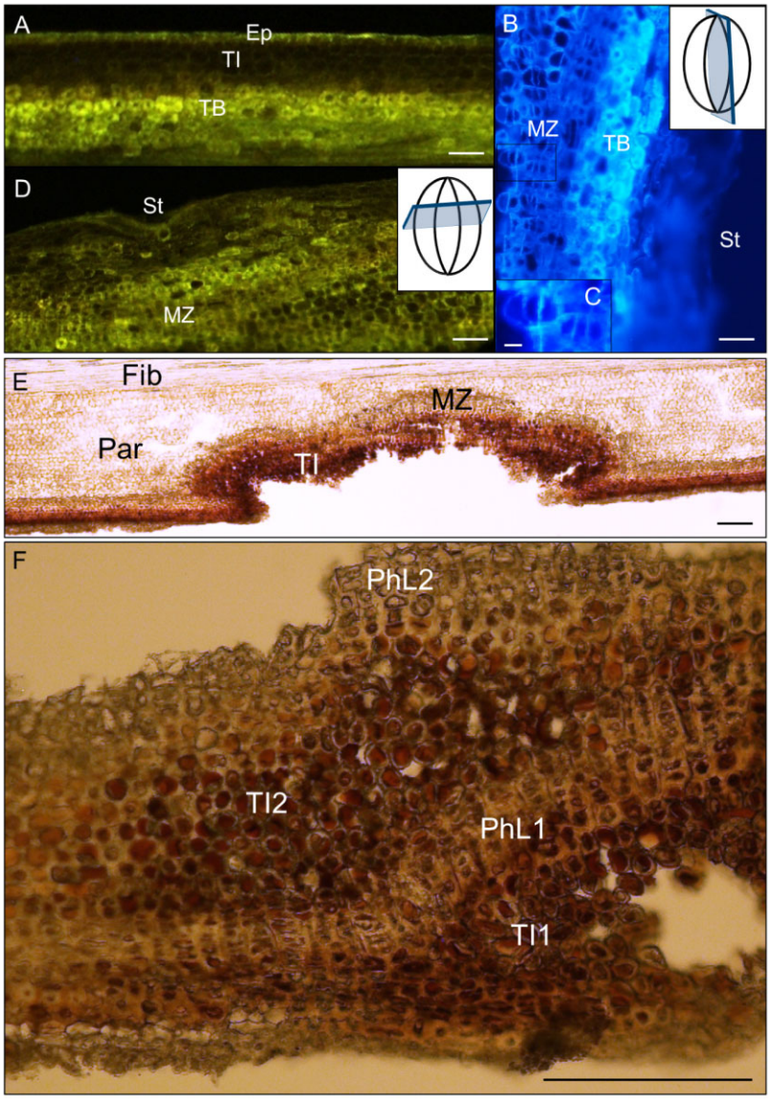

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Figure 5. Transient meristematic activation underneath stomata underpins lenticel development on *Nypa fruticans* leaf base. A, Anatomy of the 'cork' (longitudinal section). B, Longitudinal section of a stoma on a young leaf base. C, Close-up of the frame shown in B pointing out two clusters of recently divided cells. D, Transverse stomatal section through a stoma. E, Transverse section of a young lenticel. F, Transverse section of a mature lenticel. Microscopy type and staining: A, D, epifluorescence with a B2A filter and diphenylboric acid 2-aminoethyl ester (DPBA) staining; B, C, epifluorescence with a 4′,6-diamidino-2-phenylindole (DAPI) filter; E, F, light microscopy with Sudan IV staining. Scale bars: A, 40 μm; B, 80 μm; C, 30 μm; D, 30 μm; E, 350 μm; F, 200 μm. Abbreviations: Ep, epidermis; Fib, fiber; MZ, meristematic zone; Par, parenchyma; PhL1 and PhL2, phellem-like cells 1 and 2, respectively; St, stoma; TB, tannin brachysclereid; TI, tannin idioblast; TI1 and 2, primary and secondary tannin idioblast, respectively.

non-lignified, parenchymatous cell layers which are followed by lignified cells less dense than the other (Fig. 7H). The identification of the dark brown cells as tanniferous is in accordance with Seubert (1996), who reported highly tanniferous cells in the first three cell layers of AX1 roots in *N. fruticans*. Total delayed reiteration (reproduction of the total root architectural system) occurs, at least in cases of traumatism (Fig. 7A, black arrows; white arrow shows a partial reiteration).

Aerenchyma type and development are equivalent in AX1 and AX2, with the exception that the ratio

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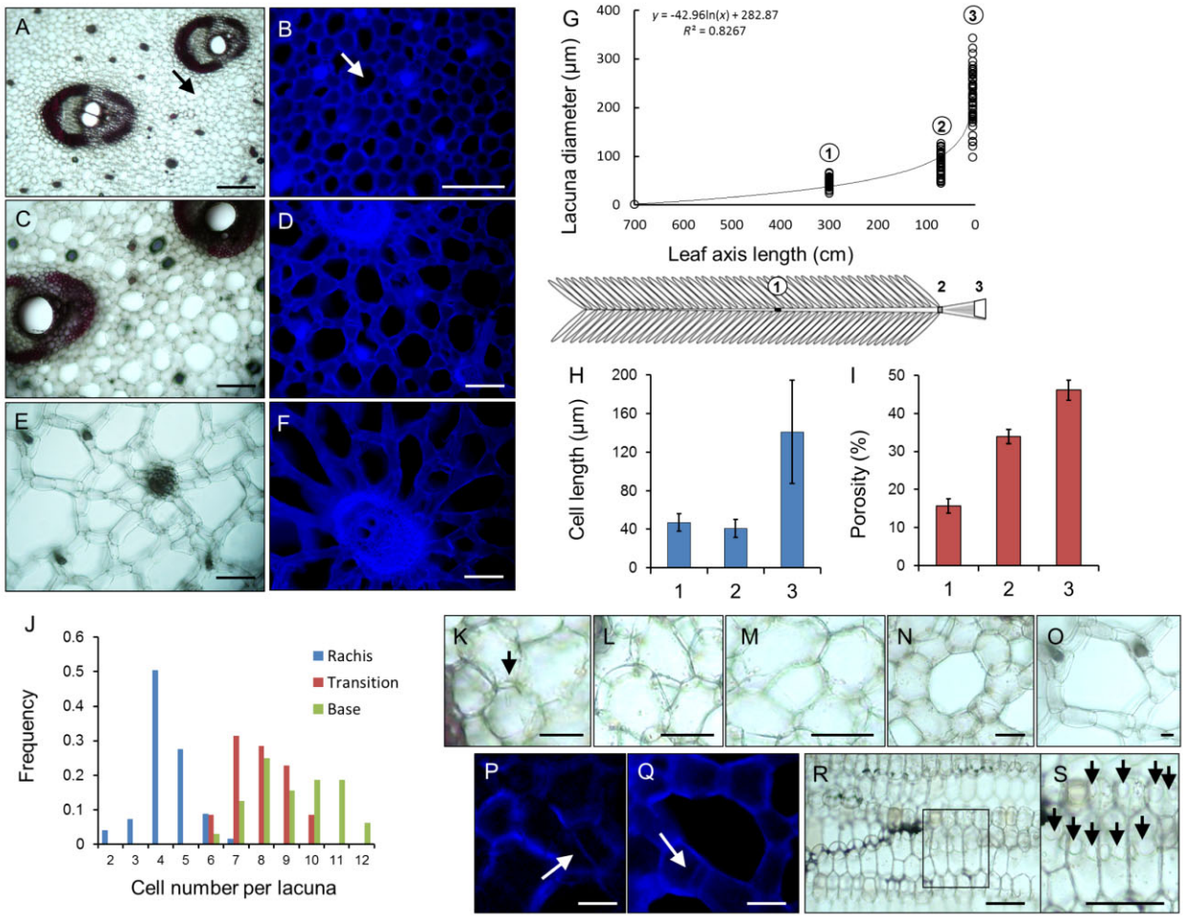

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Figure 6. Acropetal gradient of expansigenous aerenchyma in *Nypa* leaf base. A, B, Rachis transverse section; arrow points to a lacuna. C, D, Transition zone transections. E, F, Leaf base transection (position 3 in the leaf scheme). G, Lacuna diameter in the zones of the leaf axis shown in the scheme. H, Cell length of the aerenchyma in the zones shown in the scheme, n = 77, 88 and 128 for positions 1, 2 and 3, respectively. All cell measurements were made from transverse sections of the leaf axis. I, Porosity (volume of air spaces) in the three positions studied, n = 3. J, Distribution of cell number per lacuna in transverse sections in the rachis, transition zone and base of the leaf axis. K–O, Developmental anatomy of the aerenchyma. Arrowhead in K points to the nascent lacuna. P, Q, Cell division occurring in aerenchyma cells. R, S, Longitudinal section in the leaf base showing cell wall pitting on the side of the lacunae. Microscopy type and staining: A, C, E, K–O, R, S, light microscopy (LM) with phloroglucinol-HCl staining; B, D, F, P, Q, epifluorescence with a 4′,6-diamidino-2-phenylindole (DAPI) filter. Scale bars: A, B, 200 µm; C, D, 130 µm; E, F, 150 µm; K–O, 40 µm; P, Q, 50 µm; R, S, 50 µm.

occupied by aerenchyma diminishes in AX2 as ~15 cell layers of parenchyma follow the exodermis, by contrast with AX1 (Fig. 7C, D), thus reducing the porosity in AX2. Aerenchyma development shows features of schizogenous development (i.e. cell separation) (Fig. 7I, J) and lysigenous development (i.e. cell lysis and collapse) (Fig. 7D, K). Lysigeny directly follows schizogenous cell separation (Fig. 7I, J), resulting in an apparently entirely lysigenous aerenchyma. A peculiar cell type in the diffuse inner cortex is not affected by either schizogeny or lysigeny, and is characterized by larger cells that are connected to many more cells than those of the diffuse inner cortex which forms aligned cell rows (Fig. 7L, M). These 'scaffolding cells' apparently help to maintain the root structure in AX1 and AX2 by keeping lacunae together (Fig. 7L). Furthermore, these cells are concentrated towards both the surface tissues and the stele to prevent aerenchyma formation in these zones (Fig. 7M).

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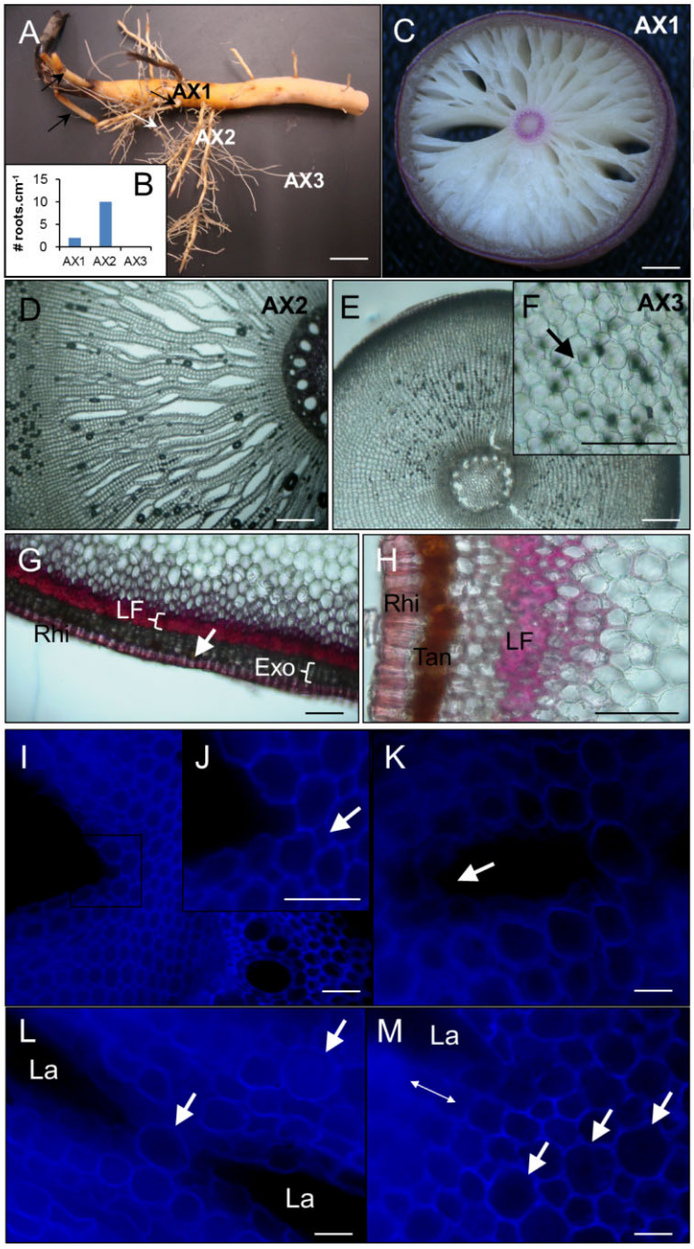

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Figure 7. *Nypa* root architecture and anatomy. A, General architecture of *Nypa fruticans* roots. Black arrows indicate reiteration of AX1. White arrow indicates fourth root order, showing reiteration of AX1. B, Branching capacity of each root order as evaluated by the number of root branches per centimetre. C, Anatomy of the first root order (AX1). D, Anatomy of the second root order (AX2). E, Anatomy of the third root order (AX3). F, Detail of the anatomy of AX3. Arrow points to an intercellular space (radial expansigenous aerenchyma). G, Anatomy of the surface layers in AX1. Arrow points to a tannin idioblast. H, Anatomy of the surface layers in AX2. I, Detail of the aerenchyma in AX1, showing a schizogenous leading end (frame). Arrow shows an intercellular space. J, Close-up of the frame illustrated in I showing a schizogenizing end. Arrow indicates an intercellular space. K, Lysigeny in AX1. Arrow points to cells in the process of lysis. L, Scaffolding cells in AX1 lysigenous aerenchyma (arrows). M, Concentration of scaffolding cells (arrows) at the outer end of the aerenchyma. Double arrow indicates a cell file in the aerenchyma. Microscopy type and staining: C, D, E, light microscopy (LM) with phloroglucinol-HCl staining; G, H, co-stained with phloroglucinol-HCl and Sudan IV; I–M, epifluorescence with a 4′,6-diamidino-2-phenylindole (DAPI) filter. Scale bars: A, 1 cm; C, 200 mm; D, 200 μm; E, F, 120 μm; G, H, 70 μm; I–M, 50 μm. Abbreviations: Exo, exodermis; LA, lacuna; LF, lignified fibres; Rhi, rhizodermis; Tan, tanniniferous layer.

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Figure 8. Tannin accumulation in the leaf base of *Nypa fruticans*. The leaf bases were collected from the same shoot, A being the youngest (distal-most) and C the oldest (basal-most). The scheme at the top left illustrates the position of the leaf bases A, B and C.

In contrast, aerenchyma in the third and last root order (AX3) is non-lysigenous and non-schizogenous and is reduced to small intercellular spaces (Fig. 7E, F).

DISCUSSION

The abscission type in *Nypa*

Tomlinson (1990) emphasized that leaf abscission, despite being a developmentally and ecologically important phenomenon, is a neglected area of palm biology. A major dichotomy was, however, recognized early with self-cleaning versus non-self-cleaning palm trunks, as related to the sheath structure (La Floresta, 1904; Schoute, 1915; Tomlinson, 1990). Clearly, the abscission pattern exhibited by *N. fruticans* does not fall into these categories because abscission concerns only the rachis and not the whole leaf.

The presence of non-lignified fibres and of larger thick-walled non-lignified cells in the abscission zone (Fig. 2E–G) is a crucial determinant of the abscission mechanism. This cellulose-rich ground tissue becomes, at the time of abscission, easy to degrade by the activation of cell wall-degrading enzymes, such as cellulase and polygalacturonase, which have long been known to play a key role in abscission in other species, including the palm *Elaeis guineensis* Jacq. (Horton & Osborne, 1967; Lewis & Varner, 1970; Reid *et al*., 1974; Riov, 1974; Addicott, 1982; Bonghi *et al*., 1992; Henderson *et al*., 2001). Cells with degraded cell walls, as well as mucilage found in the abscission region (data not shown), clearly indicate that rachis abscission in *Nypa* is an active process. The development of a distinctive abscission zone, as evidenced by anatomy and recently divided cells, is consistent with this hypothesis.

Cell wall degradation in the abscission zone, resulting in weakening, associated with the physiognomy of the *Nypa* leaf, would then induce the folding of the leaf by external factors such as wind, which, in turn, would exert a mechanical action facilitating the abscission, notably by helping fibres embedded in the ground tissue break.

The evolution of such a unique abscission mechanism dependent on both the mechanics of the leaf physiognomy and the expression of cell wall hydrolases in the transition zone suggests a functional selection pressure for the remaining leaf base.

Tannin accumulation and leaf lifespan

A striking feature of leaf bases in *N. fruticans* is the change in colour of the internal parenchymatous tissues as they age (Fig. 8). These compounds are

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soluble and dark red, and are most likely tannins. The occurrence and protective role of tannins has been widely reported in roots, leaves and bark of mangrove species (Kimura & Wada, 1989; Tomlinson, 1995; Hernes *et al*., 2001; Zhang *et al*., 2010). As antioxidants, tannins have been shown to play protective functions against bacterial and fungal pathogens (Smith, Imlay & Mackie, 2003; Smith & Mackie, 2004; Ishida *et al*., 2006) and in protecting roots from reduced and acidic soil conditions by association with ferric ions (Kimura & Wada, 1989). The generalized accumulation of tannins in leaf base ground tissue is consistent with a protective role in the leaf base.

A recent study evaluating the corrosion inhibition potential of tannin solutions of *N. fruticans* indicated that the leaf base and inflorescence stem are the most proficient against corrosion, and therefore the most tannin-rich (Satar *et al*., 2012). Tannin induction and subsequent gradual accumulation is a marker of leaf base aging (Fig. 8). The heterotrophic and semi-immersed condition of leaf bases of *Nypa* after abscission makes them sensitive to pathogens. Therefore, this process of tannin accumulation is probably a protective mechanism against such pathogens. Thus, tannin accumulation probably plays a role in the long lifespan of *Nypa* leaf bases.

Aerenchyma development in *Nypa*

Following the taxonomy of Seago *et al*. (2005), three types of aerenchyma can be identified in *N. fruticans*: honeycomb expansigenous aerenchyma in the leaf base and the proximal part of the rachis; schizo-lysigenous aerenchyma in the first- and second-order roots; and radial expansigenous aerenchyma in the third-order root. Drabble (1904) described lysigenous aerenchyma in the roots of *Areca* L. and *Howea* Becc. Generally, it has been argued that aerenchyma development, which occurs in the roots of many palm species, is of lysigenous origin (Tomlinson, 1990; Tomlinson, Horn & Fisher, 2011), although Tomlinson (1990) speculated on the existence of schizogeny in palm roots. Although other studies have claimed to demonstrate schizogeny in palm roots, none has provided microscopic evidence (e.g. Mahabale & Udwadia, 1959; Seubert, 1996). Therefore, this is the first unequivocal evidence for this type of aerenchyma in the family. Schizogeny appeared to be particularly promoted by intercellular spaces of the parenchymatous cells (i.e. radial expansigeny), which must be present at an early stage in root development, facilitating cell separation; this relation was also noticed by Seubert (1996).

The developmental gradient of honeycomb expansigenous aerenchyma present in leaf bases of *Nypa* provides an efficient way of increasing porosity towards the stem junction. This type of aerenchyma is also present in the stem of palm species such as *Archontophoenix alexandrae* (F.Muell.) H.Wendl. & Drude and *Roystonea regia* (Kunth) O.F.Cook, where it provides a mechanism for stem enlargement in the absence of secondary growth (Tomlinson *et al*., 2011). The disproportional physiognomy of the leaf base in comparison with the rachis, which is thought to play a role in the abscission process, is a consequence of the expansigenous aerenchyma gradient.

Air pathway from the leaf base to the roots

In addition to the role of the leaf base as an aeration structure in *N. fruticans*, this study provides a preliminary account for the air pathway. Oxygen entry into the leaf base of *Nypa* is thought to be greatly facilitated by hypertrophic lenticels, which have long been known to enhance air entry into tissues in mangrove and non-mangrove woody plants (Scholander *et al*., 1955; Armstrong, 1979). Following air entry from lenticels, the interconnected lacuna provided by the expansigenous aerenchyma in the leaf base allows a conduction of the oxygen down to the junction of the stem. Although the stem was not studied here for practical reasons, it is known that the stem of *Nypa* is also aerenchymatous (Tomlinson, 1995; Tomlinson *et al*., 2011). Following entry into the roots, oxygen is conducted in the schizo-lysigenous aerenchyma in the first two root orders (AX1 and AX2). The impermeable ligno-suberized rhizodermis and exodermal layers of AX1 and AX2 probably reduce radial oxygen loss, thus enhancing longitudinal oxygen transport to the third and last root order (AX3).

Quantification of the branching level of each root order revealed that the second branching order (AX2) is five-fold more branched than the first branching order (AX1), corresponding to a 1 : 2 : 10 ratio of the three branching orders (Fig. 7B) This 1 : 10 ratio of large over fine roots in *Nypa* is significant because it has been shown that fine roots consume much less oxygen than major roots (Bidel *et al*., 2000). Thus, the architecture of the root system in *Nypa*, with the 1 : 2 : 10 abundance of the three root branching orders, may reduce the oxygen demand of the flooded root system. This large number of fine roots certainly also produces a large surface to volume ratio, enhancing nutrient uptake. The absence of hydrophobic surface layers on the fine roots (AX3) certainly greatly enhances radial oxygen loss. This probably helps to create microaerobic oxidizing conditions that minimize phytotoxin entry, consistent with previous studies (Armstrong, Armstrong & Beckett, 1992; Youssef & Saenger, 1996).

CONCLUSION

This study provides structural evidence for a new aeration structure of a mangrove species. *Nypa* leaves

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undergo a unique abscission process by which only the rachis is shed, leaving the leaf base in place. A lenticel network develops on the leaf base whilst the rachis senesces. These hypertrophied lenticels connect to the aerenchyma network that occurs from the leaf base to the roots. Leaf bases remain alive for up to 4 years after abscission, perhaps aided by protection from tannins, ensuring that the aeration system remains functional for a long time. Further studies are needed to investigate the physiology of root oxygenation in *N. fruticans*.

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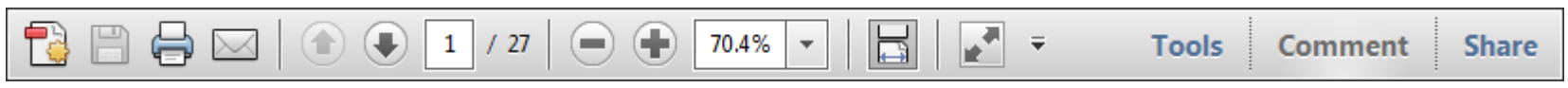

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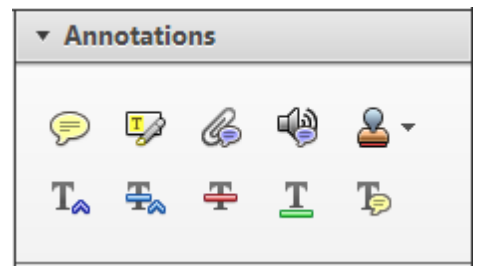

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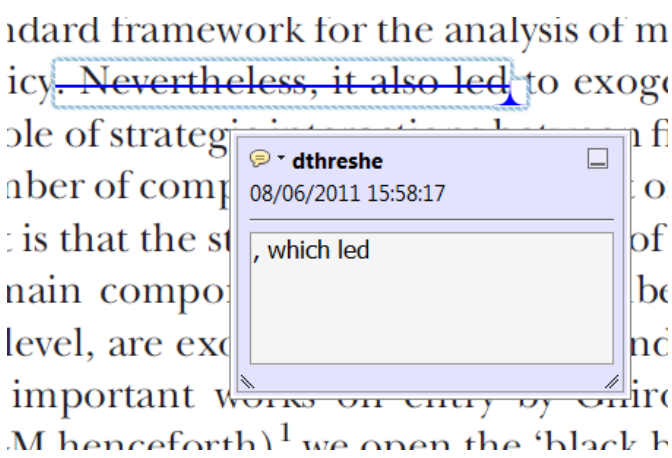

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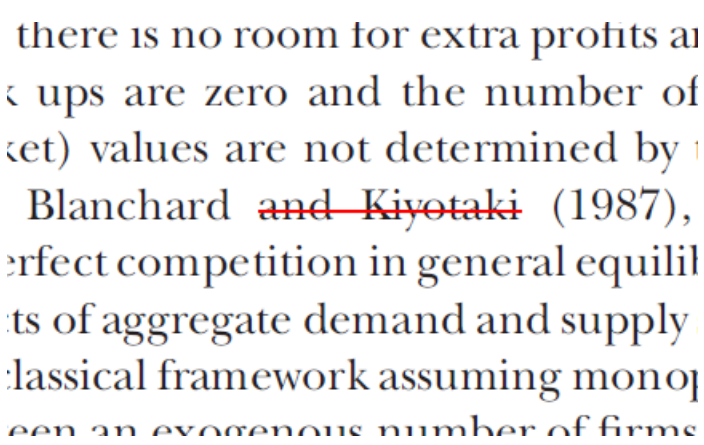

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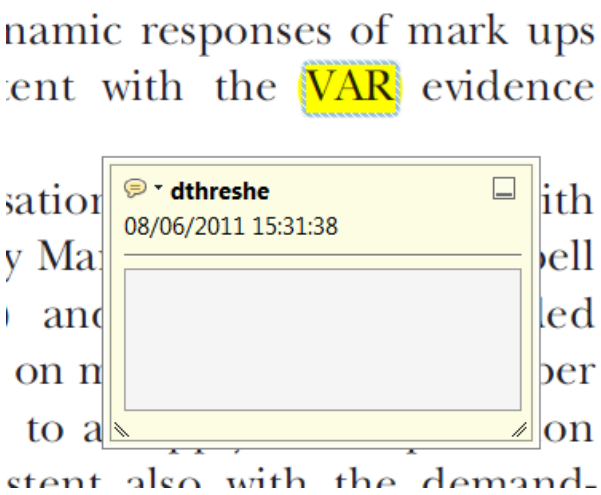
- Click on the Add note to text icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.

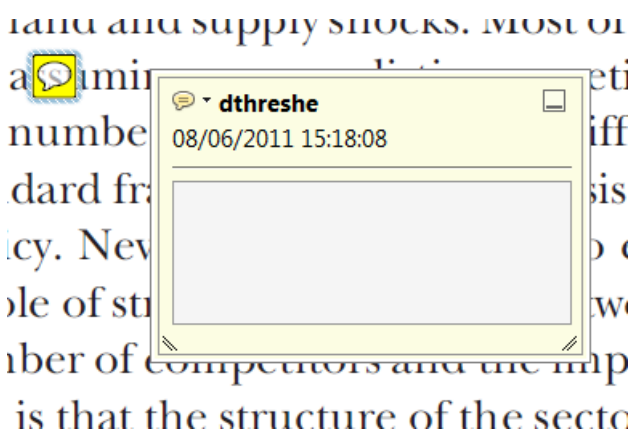


4. Add sticky note Tool – for making notes at specific points in the text.

Marks a point in the proof where a comment needs to be highlighted.

How to use it

- Click on the Add sticky note icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.



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Comment citer ce document :
Chomicki, G. (Auteur de correspondance), Bidel, L., Baker, W. J., Jay-Allemand, C. (2014). Palm snorkelling: leaf bases as aeration structures in the mangrove palm (Nypa fruticans). Botanical Journal of the Linnean Society, 174 (2), 257-270. DOI : 10.1111/boj.12133

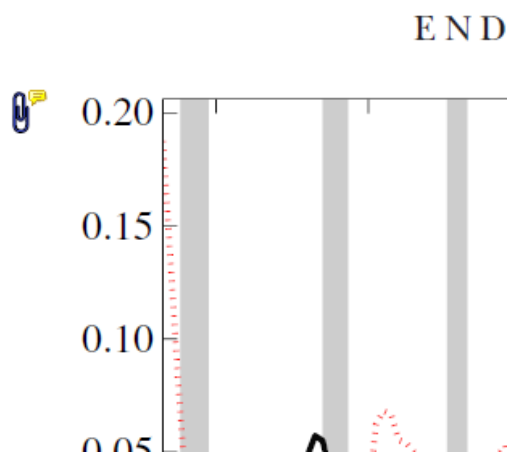
USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

5. Attach File Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate pace in the text.

How to use it

- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.

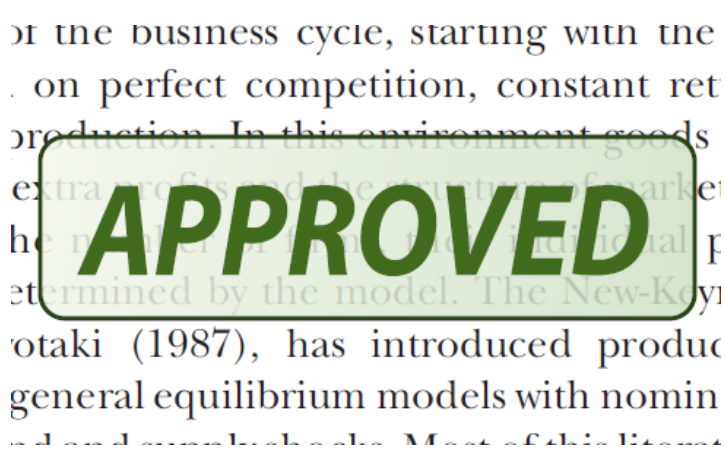


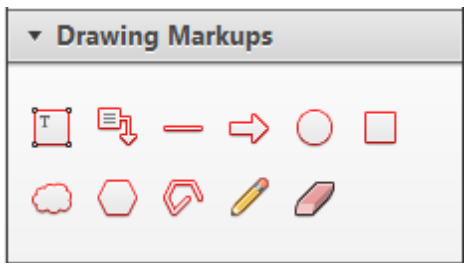
6. Add stamp Tool – for approving a proof if no corrections are required.

Inserts a selected stamp onto an appropriate place in the proof.

How to use it

- Click on the Add stamp icon in the Annotations section.
- Select the stamp you want to use. (The Approved stamp is usually available directly in the menu that appears).
- Click on the proof where you'd like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).



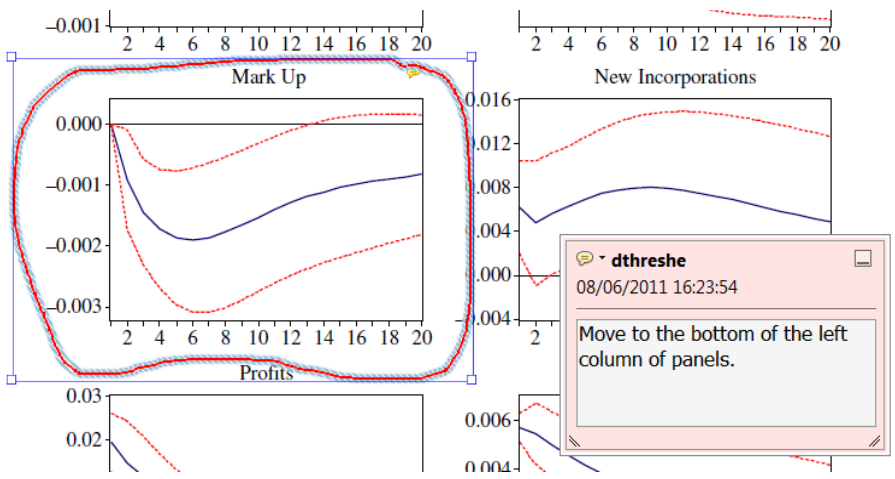


7. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

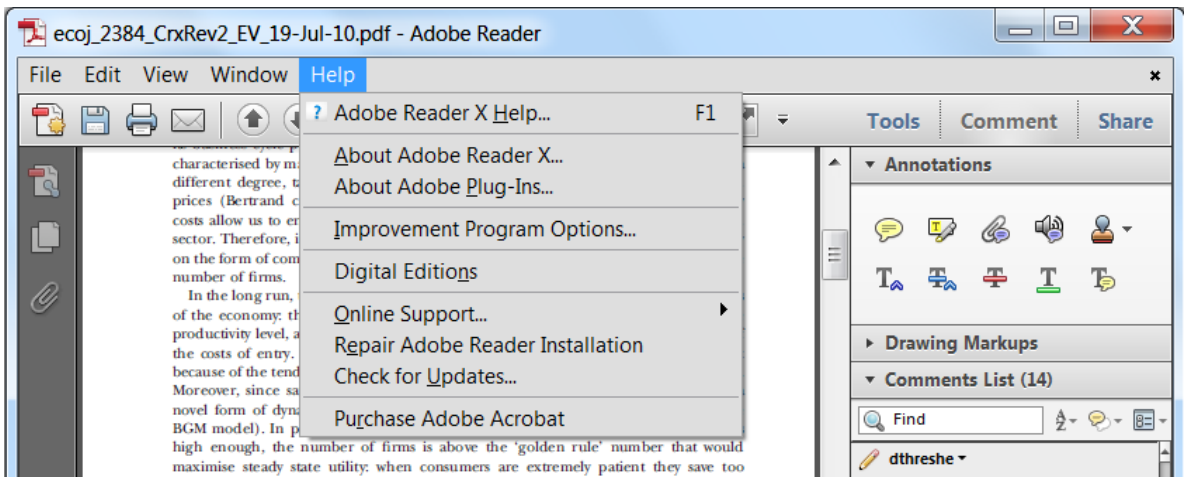
Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks..

How to use it

- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.



For further information on how to annotate proofs, click on the Help menu to reveal a list of further options:



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