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Research

Ontogenetic changes in root traits and root-associated fungal community composition in a heteroblastic epiphytic bromeliad

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Heteroblastic variations among leaf traits is a well-known process, especially in bromeliad species that show abrupt changes, but little effort was directed to test whether comparable ontogenetic variation occurs among root traits and their fungal partners. Usually considered for their mechanical role, roots of bromeliads may also play a role in resource use that we expect to differ between ontogenetic stages, namely nutrient pulse-supplied atmospherics (young stage) and continuously-supplied tanks (later stage). On a selection of adventitious roots of the heteroblastic epiphyte *Lutheria splendens*, we explored 1) ontogenetic variations in morphological and anatomical root traits, namely the number of adventitious roots and tips, total root length, average root diameter, root tissue density, specific root length and root tissue areas, and 2) ontogenetic changes in the composition of the root-associated fungal communities using ITS1 region-based high-throughput sequencing. Plant size, taken as an indicator of gradual ontogenetic variations, and plant stage had significant effects on root traits, unless for root tissue density. The number of adventitious roots and tips, the total root length, average root diameter and all root tissue areas (i.e. velamen, outer cortex, inner cortex, vascular cylinder) increased gradually with ontogeny while the specific root length decreased abruptly. The root-associated fungal communities varied with plant stage. The tank stage showed higher proportion of mycorrhizal fungi and a lower abundance of dark septate endophyte as compared to atmospheric stage. Overall, we demonstrated significant ontogenetic variations of root traits and fungal partners in bromeliad species that suggest important changes in the strategy for resource use as plants mature. We invite future root trait studies to better account for ontogenetic variations.

Keywords: Bromeliaceae, fungal functional guilds, heteroblasty, ITS metabarcoding, ontogeny, root trait gradients



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Introduction

Across plant species and growth forms, our understanding of plant strategies for resource use has developed with a strong bias towards leaf traits that fall along a ‘leaf economics spectrum’ that reflect changing strategies for light resources acquisition (Wright et al. 2004). Whether an equivalent unidimensional spectrum of root traits exists for belowground resource acquisition is debated (Roumet et al. 2016, Weemstra et al. 2016, Bergmann et al. 2020). Root traits, notably morphological, anatomical and physiological traits, and root-associated fungal partners reflect plant strategies for soil resource use, and vary across plant species and environmental conditions (Freschet et al. 2017, Valverde-Barrantes et al. 2017, Ma et al. 2018). On a variety of woody and non-woody species observed at plant maturity, belowground trait-based approaches showed two main axes of variations in root traits and their fungal partners (Bergmann et al. 2020). One axis relates to the conservation of resources ranging from ‘fast’ short life span roots with a high nitrogen content and metabolic rate to ‘slow’ long-lived roots with high tissue investment such as root tissue density. The other axis corresponds to variations in collaboration strategies ranging from the ‘do-it-yourself’ strategy characterised by high specific root length enabling soil exploration by the fine roots to the ‘outsourcing’ strategy characterised by a high investment of carbon in mycorrhizal partners that explore soil resources. The high mycorrhization rate usually requires large root cortex tissues and thus absorptive roots of larger diameter.

Similar axes of variations among growth forms may also be found within a single plant species across environmental gradients (Hajek et al. 2013, Isaac et al. 2017, Wang et al. 2021, Westerband et al. 2021) and/or with ontogeny (Houter and Pons 2012, Mason et al. 2013, Mediavilla et al. 2014). Here again, variations of plant trait with ontogeny were also more investigated in terms of leaf/aboveground traits, whereas ontogenetic variations of root traits remains particularly limited (Alvarez-Flores et al. 2014, Garbowski et al. 2021). Ontogenetic changes are the result of a developmentally programmed growth trajectory and are referred to as ‘apparent plasticity’ (Weiner 2004). In general, this apparent plasticity is relatively subtle and gradual (i.e. homoblastic species), but some species show abrupt changes (i.e. heteroblastic species). For example, European ivy *Hedera helix* (Poethig 2010), phyllodineous *Acacia* (Leroy and Heuret 2008) and tank bromeliads (Zotz et al. 2011) are heteroblastic species with well-known abrupt changes in leaf traits. By contrast, research considering the influence of ontogeny on root trait variations of heteroblastic species are to our knowledge missing.

In the Bromeliaceae family, many species of the subfamily Tillandsioideae are characterized by a sudden change in morphology during ontogeny with two (Schmidt and Zotz 2001, Zotz 2004) or even three (Meisner and Zotz 2011) ontogenetic stages. Small juveniles have morphological characteristics typical of atmospheric tillandsioids. They form rosettes with small, linear leaves, densely covered with trichomes.

Large bromeliad form tanks or phytotelmata by overlapping broad leaf bases that can store substantial amounts of rainwater and organic matter. Gradual changes in leaf morphology, anatomy and physiology were related to plant size (Schmidt and Zotz 2001, Zotz 2004, Meisner et al. 2013). Juvenile atmospheric and tank individuals rely on different strategies of nutrient and water acquisition (Males 2016). Epiphytic atmospheric bromeliads represent an extreme life form capable of absorbing water and nutrients directly from atmospheric sources (e.g. rainfall, wind-borne dust, nitrogenous gases) thanks to leaf-absorbing trichomes (Leroy et al. 2016, Supporting information). As a consequence, atmospheric bromeliads grow in nutrient pulse-supplied environments. By contrast, continuously supplied tank bromeliads store water and nutrients that are absorbed by the leaf-absorbing trichomes, and thus buffer irregular precipitation inputs and reduce water and nutrient stresses (Leroy et al. 2016). Numerous studies have thus focused on the structure and role of leaf-absorbing trichomes (Nyman et al. 1987, North et al. 2013), leaving the root system overlooked.

In the seedlings of Bromeliaceae, the primary root is quickly replaced by roots originating from the stem, i.e. adventitious roots (Krauss 1949). Usually, the roots of epiphytic bromeliads are reduced to their mechanical role as holdfasts (Martin 1994, Winkler and Zotz 2009). However, some recent investigations showed that the roots of some bromeliad species also play a role in water and nutrient uptake (Vanhouette et al. 2016, Carvalho et al. 2017, Leroy et al. 2017, 2019b). Furthermore, anatomical inspection of bromeliad roots pointed a multiseriate epidermis similar to the velamen of terrestrial and epiphytic species (Pita and Menezes 2002, Proença and das Graças Sajo 2008, Silva and Scatena 2011, but see Kowalski et al. 2019). The velamen is a specific tissue common among monocotyledons (Zotz et al. 2017). In the root system of bromeliad species, the velamen is suggested to contribute to the water and nutrient supply, and to the mechanical protection of the root system (Zotz and Winkler 2013, Zorger et al. 2019). In several bromeliad species, the more distal part of the velamen (epivelamen) exhibit root hairs, suggesting its role in water and nutrients absorption (Segecin and Scatena 2004, Silva and Scatena 2011).

Furthermore, bromeliad roots are associated to mycorrhizal and dark septate endophytes (DSE) fungi (Allen et al. 1993, Rabatin et al. 1993, Rowe and Pringle 2005, Grippa et al. 2007, Lugo et al. 2009). Recently, Leroy et al. (2021) found bromeliad roots to have diverse fungal assemblages with 1) mycorrhizal fungi such as AMF, ectomycorrhizal fungi (EcMF), orchid mycorrhizal fungi (OMF) and ericoid mycorrhizal fungi (ErMF), and 2) non-mycorrhizal fungi such as fungal endophytes (FE) including DSE. Fungal endophytes in bromeliad roots benefit plant nutrition and growth (Leroy et al. 2019a). Complementary to the role of leaves, bromeliad roots may play an important role in nutrient acquisition, especially in oligotrophic areas (Zotz and Hietz 2001, Laube and Zotz 2003) thanks to fungal associations.

But little is known about the relations between root morphological and anatomical traits of bromeliads and their fungal partners along ontogenetic variations.

Our study aimed to explore the ontogenetic variations of 1) morphological and anatomical traits of adventitious roots and 2) their associated fungal community composition in the heteroblastic epiphytic bromeliad *Lutheria splendens*. Variations in root traits and fungal partners are taken as indicators of changes in soil resource use as plants mature. Based on previous findings on the vegetative part of heteroblastic bromeliads, we expected that the morphological and anatomical structure of the adventitious roots change with ontogenetic drift. Additionally, because water and nutrient uptake strategies differ between nutrient pulse-supplied atmospheric and continuously-supplied tanks, we expected ontogenetic variations in root resource use strategies, notably related to the collaboration with fungal partners.

Material and methods

Sampling site and bromeliad species

This study was conducted in a lowland rainforest located near the Petit-Saut Dam, Sinnamary (5°04'22.4"N, 52°58'35.4"W; elevation < 80 m a.s.l.). The climate is tropical moist with 3000 mm of annual precipitation, little seasonal variation in air temperature (monthly averages range from 20.5 to 33.5°C), and a relative humidity oscillating between 70 and 100%. There is a major reduction in rainfall between July and December (the 'dry season') and another shorter and more irregular dry period in March. The remaining months constitute the 'rainy season'.

Lutheria splendens (Bromeliaceae: Tillandsioideae) is an epiphytic and heteroblastic tank-forming bromeliad commonly found in the understory of the area studied (Fig. 1).

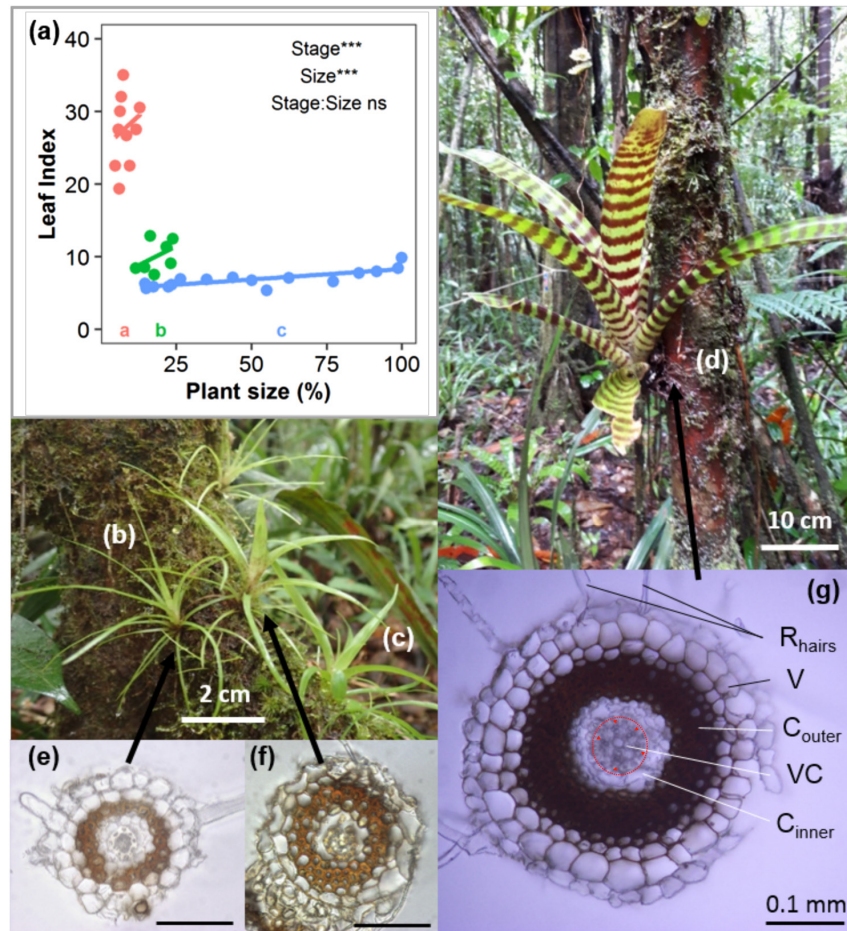


Figure 1. (a) Changes in leaf index as a function of plant size. Each point represents a value for adventitious roots according to their plant stages: atmospheric in red, transitional in green and tank in blue. Analyses of covariance (ANCOVAs) were used to examine the effect of plant size (size), plant stages (stage) and their interaction (size:stage) on the different morphological and anatomical root traits. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant. Small letters indicate significant differences among plant stages (Tukey's post hoc test, $p < 0.05$). Heteroblastic change from (b) atmospheric, (c) transitional to (d) tank stages in *Lutheria splendens* in French Guiana. Light micrographs of hand-cut transverse sections near the apex of the root of (e) atmospheric, (f) transitional and (g) tank stages. R_{hairs} = root hairs, V = velamen, C_{outer} = outer cortex, VC = vascular cylinder (red dashed-line circle), C_{inner} = inner cortex. Red triangles indicated protoxylem poles.

To reliably distinguish heteroblastic from homoblastic species, the leaf index has usually been used (Zotz et al. 2011, Meisner and Zotz 2012). The leaf index was calculated as the ratio between leaf length and width and the plant size was expressed as a percentage of maximum leaf length (Fig. 1a). Variations in the leaf index point a shift between three stages (Fig. 1a). 1) Small juveniles show morphological characteristics of atmospheric, possess non-impounding rosettes of small, linear leaves, densely covered by the shields of foliar trichomes. 2) Large tanks feature broad leaves, which overlap basally forming water-filled chambers (Fig. 1d). Finally, 3) an ephemeral transitional stage presents a mixture of small linear leaves and broad leaves (Fig. 1b–c, Supporting information). As for all bromeliad species, *L. splendens* have adventitious roots.

Root morphology

To compare the root morphology of the three plant stages of *L. splendens*, we collected 10 atmospheric, 7 transitional and 16 tank individuals of different sizes at man height (ca 1.6 m from the soil). First, we washed the adventitious root system with running tap water to free them from organic matter. We counted the number of adventive roots per bromeliad and two to three representative adventive roots per bromeliad were further cleaned, using a binocular, by removing all mosses and small remaining organic debris attached to the roots. Each root was scanned using a high-resolution flatbed scanner (600 DPI resolution, 256-level gray-scale, TIFF format) and WINRHIZO basic software. From these images, we obtained the following morphological root traits: total root length (TRL, cm), average diameter (ARD, mm), surface area (cm²), root volume (cm³) and numbers of tips. Then, all adventive roots were oven-dried for 48 h at 60°C, and weighted with precision scale (0.1 mg). We calculated the specific root length (SRL, cm mg⁻¹) as the ratio between the total length of the root and its dry weight and the root tissue density (RTD, g cm⁻³) was calculated by dividing root dry weight by its fresh volume.

Root anatomy

For each root system, we selected one adventitious root for anatomical analyses. The selected root was fixed in FAA (5% formalin, 5% acetic acid, 50% ethanol) for 1–2 weeks, and transferred in 70% ethanol for long term storage. Transversal sections were made by freehand with razor blades at 1 cm from the apex of the main root (Fig. 1e–g). Then, the root sections were observed with an inverted microscope and images were acquired with a digital camera. From these images, we measured the following anatomical traits: the velamen area, the exoderme area, the internal cortex area, the root stele area and the root section area. Analyses were done with Adobe Photoshop CS5 following a three steps procedure. First, each tissue was isolated on a single image layer. Then, the area of each layer was automatically calculated using a Java script on ImageJ2 software. Finally, the sclerenchyma wall thickness

(μm) was measured for thirty randomly selected cells in the internal cortex of each transversal section.

Molecular fungal identification

To characterize the fungal community composition, the roots of atmospheric and tank stages were collected from 9 replicate individuals (*L. splendens*) at man height. First, the length of the longest leaf was measured for each individual to determine the plant size. Then, three to four different parts of the root system were sampled and stored in a humid paper towel in plastic bags and transported to the lab in a cool box. The roots were gently washed and brushed under tap water to remove any organic matter and dried with a paper towel. Then, all roots were cut in small pieces and directly immersed in a CTAB 2X Buffer (100 mM Tris–HCl (pH 8), 1.4 M NaCl, 20 mM Na₂ EDTA, 2% CTAB), and kept at 4°C until DNA extraction.

The total DNA was extracted from 20 mg per individual using the DNeasy Plant Mini Kit following the manufacturer's instructions. The Internal Transcribed Spacer 1 (ITS1) of the ribosomal DNA was targeted using the ITS5/5.8SF (5'-GGAAGTAAAAGTCGTAACAAGG-3'/5'-CAAGAGATCCGTTGTTGAAAGTK-3') primer set (White et al. 1990, Epp et al. 2012) with reverse primer as modified by Taberlet et al. (2018) to account for Glomeromycota as well as Ascomycota and Basidiomycota sequences. Amplifications were carried out in 20 μl reactions containing 10 μl of Ampliqaq Gold 360 Mastermix 2 \times (Applied Biosystems, Foster City, USA), 1 μl of 5 μM for each primer, 5 μl of 5% PVP, 1 μl milliQ water and 2 μl of template DNA. The PCR was performed on a DNA Engine Peltier thermal cycler (BIO-RAD, PTC-200, Foster City, CA, USA) with a program consisting of an initial denaturation at 95°C for 10 min, 40 cycles of repeated reactions (denaturation at 95°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 1 min) and a final elongation of 7 min at 72°C. PCR products were loaded onto a 1% standard agarose gel for electrophoresis stained by 5% (v/v) GelRed (Biotium, Hayward, USA). Unsuccessfully amplified samples were subjected to multiple amplifications at various DNA concentrations. Positive PCR products were purified by centrifugation on a Sephadex G-50 Superfine mini column in Multiscreen-HTS 96-well plates (MSHVN4550; Millipore), respecting the manufacturer's specifications. After quantification with the QUBIT 2.0 (Thermo Fisher Scientific, Waltham, USA), the PCR products were mixed in equimolar amounts to prepare sequencing libraries. The libraries were paired-end sequenced using the Illumina MiSeq technology (2 \times 300 bp) by Fasteris (Planles-Ouates, Switzerland).

MiSeq sequence libraries were analysed with a custom pipeline named EGRETТА1.3 (Leroy et al. 2021) using public tools and personal python scripts. Contigs of paired end reads were assembled along with sequence curation (e.g. offset, short construct, chimera removal) and dereplication. Sequences were clustered into 97% similarity operational taxonomic units (OTUs) with an Uparse wrapper (Edgar 2013).

ITS1 positions were annotated with HMMs approaches (Bengtsson-Palme et al. 2013). Taxonomic assignments were made using BLASTn (Camacho et al. 2009) against a personal database which contains fungal and outgroups sequences (mostly Chlorobionta) from the International Nucleotide Sequence Database Collaboration (INSDC). Sequences were cured and annotated following, inter alia, recommendations of UNITE literature (Kõljalg et al. 2013). We removed unassigned OTUs and singletons from the main dataset before statistical analyses. Then, each taxonomically defined fungal OTU was assigned with its fungal functional guild using the FunGuild database (Nguyen et al. 2016) and completed with individual research in the literature. In this study, our selected fungal functional groups (hereafter, FFGs) of interest were symbiotrophs with the corresponding fungal ecologies: arbuscular mycorrhizal fungi (AMF), ectomycorrhizal fungi (EcMF), ericoïd mycorrhizal fungi (ErMF), orchidoïd mycorrhizal fungi (OMF), fungal endophytes (FE) and dark septate endophytes (DSE).

Data analysis

All statistical analyses were conducted using R, ver. 3.4.4 (<www.r-project.org>), and were evaluated at a 95% confidence level. The results are presented as means \pm SE throughout the text.

Multivariate relationships among root morphological traits were analyzed with a principal component analysis (PCA) from the FactoMineR package (Lê et al. 2008). All plant trait variables were scaled to unit variance prior to analysis. Only the first two principal components were retained, as they had eigenvalues greater than one and consistently explained more than 80% of the variance in the data (Supporting information). In addition, the plant size was added to the PCA as a proxy of plant ontogeny. To check any differences in the three ontogenetic stages, permutational multivariate analyses of variance (PERMANOVAs) were performed with the *adonis* function in *vegan* (Oksanen et al. 2019) using Bray–Curtis distances and 999 permutations. If the PERMANOVA results were significant, a post hoc multilevel pairwise analysis was performed using the *pairwise.adonis* wrapper function in the *Pairwise-adonis* package with Bonferroni-corrected p values (Martinez Arbizu 2019). The *dimdesc* function was used to calculate the correlation coefficient between variables and the principal component axes, and also to assess the significance of the correlation. Then, Pearson correlation analyses (PerformanceAnalytics package) were used to test for relationships between root morphological traits.

Analyses of covariance (ANCOVAs) were used to examine the effect of plant stage (i.e. atmospheric, transitional and tank stages) as fixed factor, plant size (used as a proxy of ontogeny) as covariate and their interaction on the different morphological and anatomical root traits. The variables were log- or rank-transformed, if necessary, to normalize distributions and reduce heterogeneity of variance. A significant effect of plant stage indicated that the intercept of the regression lines changed with the plant stage. A change in the slope

of the regression lines was indicated by a significant interaction between plant stage and plant size. Post hoc Tukey's honestly significant difference (HSD) multiple comparisons were used to test for differences among the three plant stages.

The sampling effort was estimated with accumulation curves to show the relationship between the OTUs abundance and the number of fungal OTUs obtained, using the *specaccum* function in the *vegan* package (Oksanen et al. 2019). The community overlap was determined using a Venn diagram from the *VennDiagram* package. Taxonomic and functional fungal assemblage patterns were visualized by the non-metric multidimensional scaling (NMDS) analysis as implemented in the *metaMDS* command of the R package *Vegan* using a Bray–Curtis dissimilarity index on a Hellinger-transformed data. To check whether the fungal community compositions were significantly affected by the plant stage and plant size, permutational multivariate analysis of variance (PERMANOVA) were performed with the 'adonis' function in *vegan* package using Bray–Curtis distances and 999 permutations (Oksanen et al. 2019). To explore the correlations between FFGs with taxonomic fungal community composition, FFGs characteristics factors (i.e. AMF, EcMF, ErMF, OMF, FE and DSE) were fitted as vectors onto the NMDS plot using the function *envfit* from the *vegan* package. The plant size was also fitted as a vector on the NMDS plot to link FFGs with root morpho-anatomical traits. Then, goodness-of-fit statistics (R^2) for environmental variables fitted to the NMDS ordinations of fungal communities were calculated using the *envfit* function of *vegan* with p-values based on 999 permutations.

Results

Adventitious root morphology

The two first axes of the PCA accounted for more than 80% of total trait variation. The first PCA axis (65.3% of total trait variation) represented a covariation between SRL and average root diameter (Fig. 2). These two variables were negatively correlated ($r = -0.74$, $p < 0.0001$, Table 1). SRL was also negatively correlated with the number of roots ($r = -0.63$, $p < 0.0001$), the total root length ($r = -0.54$, $p < 0.0001$) and the number of tips ($r = 0.50$, $p < 0.0001$) and positively correlated with RTD ($r = 0.27$, $p < 0.01$). The second axis (17.9% of total trait variation) represented a gradient of RTD. RTD was negatively correlated to ARD ($r = -0.40$, $p < 0.0001$). The three plant stages were significantly differentiated along the first PCA axis with small-size atmospheric and large-size tanks on two sides, and the transitional stage in the centre. The atmospheric stage showed a significantly lower number of roots, total root length, number of tips and average root diameter and a higher RTD and SRL compared to the tank stage. The transitional stage has intermediate root trait values. The number of roots, total root length, number of tips and average root diameter increased significantly with plant stage and size, whereas SRL decreased (Fig. 3,

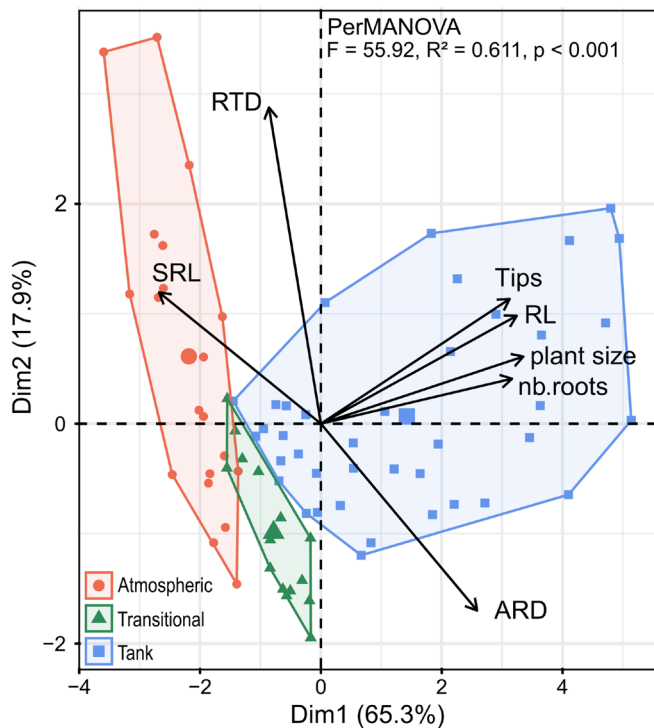


Figure 2. Principal component analysis (PCAs) of six root traits along with plant size in *Lutheria splendens* bromeliad. Measured traits were average root diameter (ARD), number of adventitious roots (nb. roots), root tissue density (RTD), specific root length (SRL), number of tips (Tips) and total root length (TRL). Individual projection (points) were grouped by plant stage with atmospheric, transitional and tank stages in red, green and blue, respectively.

Supporting information). RTD did not vary with plant size but significantly varied with plant stage. The number of adventitious roots, total root length, number of tips, RTD and SRL showed significant interaction between plant stage and plant size, which means that the slope of the regression line between root traits and plant size varied with plant stage (Supporting information). The effect of plant size on root traits was significant for each of the three plant stages respectively, unless for RTD (Supporting information).

Adventitious root anatomy

The roots of *L. splendens* consisted of the velamen, a heterogeneous cortex (outer and inner cortex) and a vascular cylinder

(Fig. 1e–g). The outermost layer of the velamen was the epivelamen, composed of few cells differentiated into root hairs. The remaining layers of the velamen were formed by 1–4 layers of thin-walled cells. The outer cortex had 1–4 layers of cells with thickened, lignified walls and reduced lumen forming a sclerenchymatous ring. The inner cortex was parenchymatous and formed by 1–3 layers of rounded, thin-walled cells radially arranged around the endodermis. The pericycle was one-layered and the vascular cylinder was polyarch, with 3–8 protoxylem poles (Fig. 1g, Supporting information). The total root section area, the area of the different root tissues (velamen, outer cortex, inner cortex and vascular cylinder) and the sclerenchyma wall thickness increased significantly according to plant stage and plant size (Fig. 4, Supporting information). The interaction between plant stage and plant size was not significant indicating no significant differences in the slope of the regression lines among plant stages (Supporting information). For each of the root tissues, the ratio between the area of the root tissue and the area of the total root section did not vary with plant stage and size (Supporting information). This lack of relation indicates that the velamen, outer and inner cortex and vascular cylinder increased proportionally with ontogeny and plant size.

Adventitious root-associated fungal community composition

The metagenomic analysis provided 11 796 cured non-singleton reads of strict symbiotrophic fungi which were clustered to a 97% similarity level in 800 OTUs. These OTUs corresponded to 143 taxa, 11 classes, 34 orders, 54 families and 75 genera. The rarefaction curves were asymptotic for the two plant stages suggesting that the sequencing depth was sufficient to capture the diversity of root-associated fungi with 399 and 401 OTUs corresponding to 112 and 93 taxa for the atmospheric and tank stages, respectively (Fig. 5a). Fungal diversity (Shannon and Simpson diversity indexes) showed similar patterns of richness for these two plant stages (Supporting information). The Venn diagram showed that 50 taxa (35%) were specific to the atmospheric stage, 31 taxa (22%) were only found in the tank stage and 62 taxa (43%) were shared by both plant stages.

At the phylum level, the fungal community was dominated by Ascomycota representing 67% and 57% of all OTUs in the atmospheric and tank stages, respectively. Basidiomycota accounted for 19% and 22% of all OTUs and

Table 1. Pearson correlation coefficients between the six morphological root traits corresponding to the number of adventitious roots (Nb. roots), total root length (TRL), average root diameter (ARD), number of tips (Tips), root tissue density (RTD) and specific root length (SRL). The significance level is as follows: * $p < 0.05$, *** $p < 0.001$.

	Nb. roots	TRL	ARD	Tips	RTD	SRL
Nb. roots	1					
TRL	0.81***	1				
ARD	0.56***	0.53***	1			
Tips	0.78***	0.97***	0.44***	1		
RTD	-0.15	-0.074	-0.40***	-0.062	1	
SRL	-0.63***	-0.54***	-0.74***	-0.50***	0.27*	1

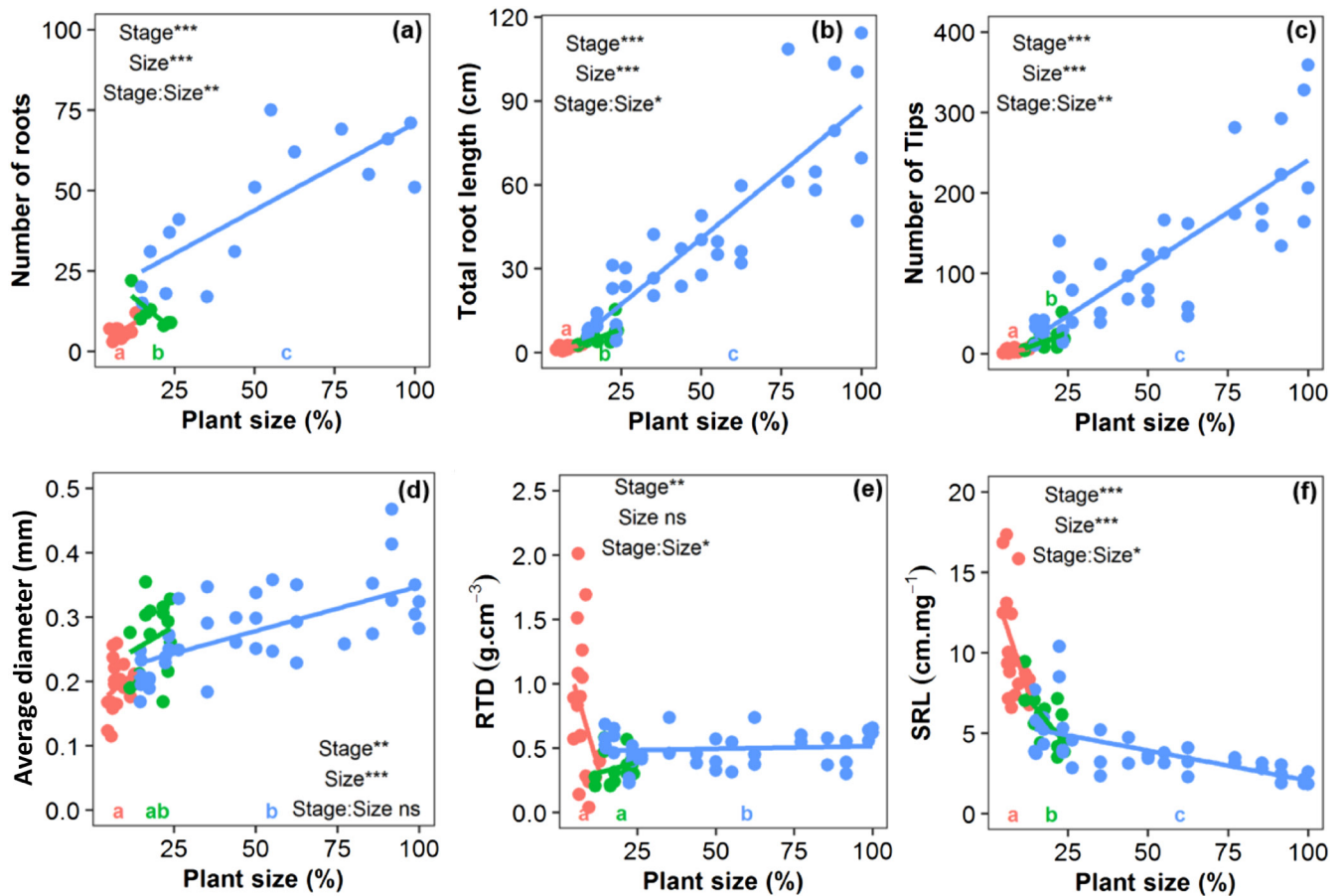


Figure 3. Relationships between (a) number of roots, (b) total root length (cm), (c) number of tips, (d) average diameter (mm), (e) root tissue density (g cm^{-3}) and (f) specific root length (cm mg^{-1}) and plant size (%) in adventitious roots of *Lutheria splendens*. Each point represents a value for adventitious roots according to their plant stage: atmospheric in red, transitional in green and tank in blue. Analyses of covariance (ANCOVAs) were used to examine the effect of plant stage (stage), plant sizes (size) and their interaction (stage:size) on the different morphological and anatomical root traits. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant. Small letters indicate significant differences among plant stages (Tukey's post hoc test, $p < 0.05$).

Glomeromycota for 14% and 22%, respectively. Among the Ascomycota, Hypocreales (39% and 44%) and Eurotiales (12% and 9%) were the two most abundant fungal orders out of 21 found in the atmospheric and tank stages, respectively. With 10 orders, Basidiomycota were represented mainly by Agaricales (49% and 58%) and Trechisporales (19% and 17%) in atmospheric and tank stages, respectively, and to a lesser extent by Cantharellales (11%) in the tank stage. In both plant stages, Glomeromycota were exclusively represented by Glomerales (98%). Non-mycorrhizal fungi accounted for more than a half of the total OTUs with 66% FE of which 12% were DSE in the atmospheric stage and 53% FE of which 6% were DSE in the tank stage (Fig. 5c). Within FE of the atmospheric and tank stages, *Fusarium* (21% and 24%, respectively) and *Trichoderma* (16% and 17%, respectively) were the two more represented genera (Supporting information). More specifically, in DSE, *Cladosporium* accounted for 51% of the OTUS in the atmospheric stage and 34% in the tank stage. In the atmospheric and tank stages, mycorrhizal fungi were mainly represented

by AMF (14% and 21%, respectively) and EcMF (16% and 21%, respectively). In particular, *Amanita* accounted for 30% and 28% in the atmospheric and tank stages and *Cortinarius* for 23% in the tank stage.

The non-metric multidimensional scaling (NMDS) plots of OTUs showed distinct separation of the atmospheric and tank stages (Fig. 5b). Community composition consistently differed between atmospheric and tank stages (PerMANOVA, $F = 1.82$, $R^2 = 0.102$, $p = 0.002$). Among the fitted vectors on the NMDS plot, EcMF and DSE were significantly correlated with the ordination, indicating that they were significant factors structuring the fungal community composition in the roots of *L. splendens* (Table 2). We found significant differences in the FFGs between the two plant stages (PerMANOVA, $F = 4.56$, $R^2 = 0.22$, $p = 0.002$) and along plant size with mycorrhizal fungi associated with larger plants while FE was correlated with small plant size. These differences were mainly related to the higher (double) proportion of DSE in the small-size atmospheric stage compared to the tank one (Anova, $F = 12.67$, $p = 0.002$, Fig. 5c). AMF

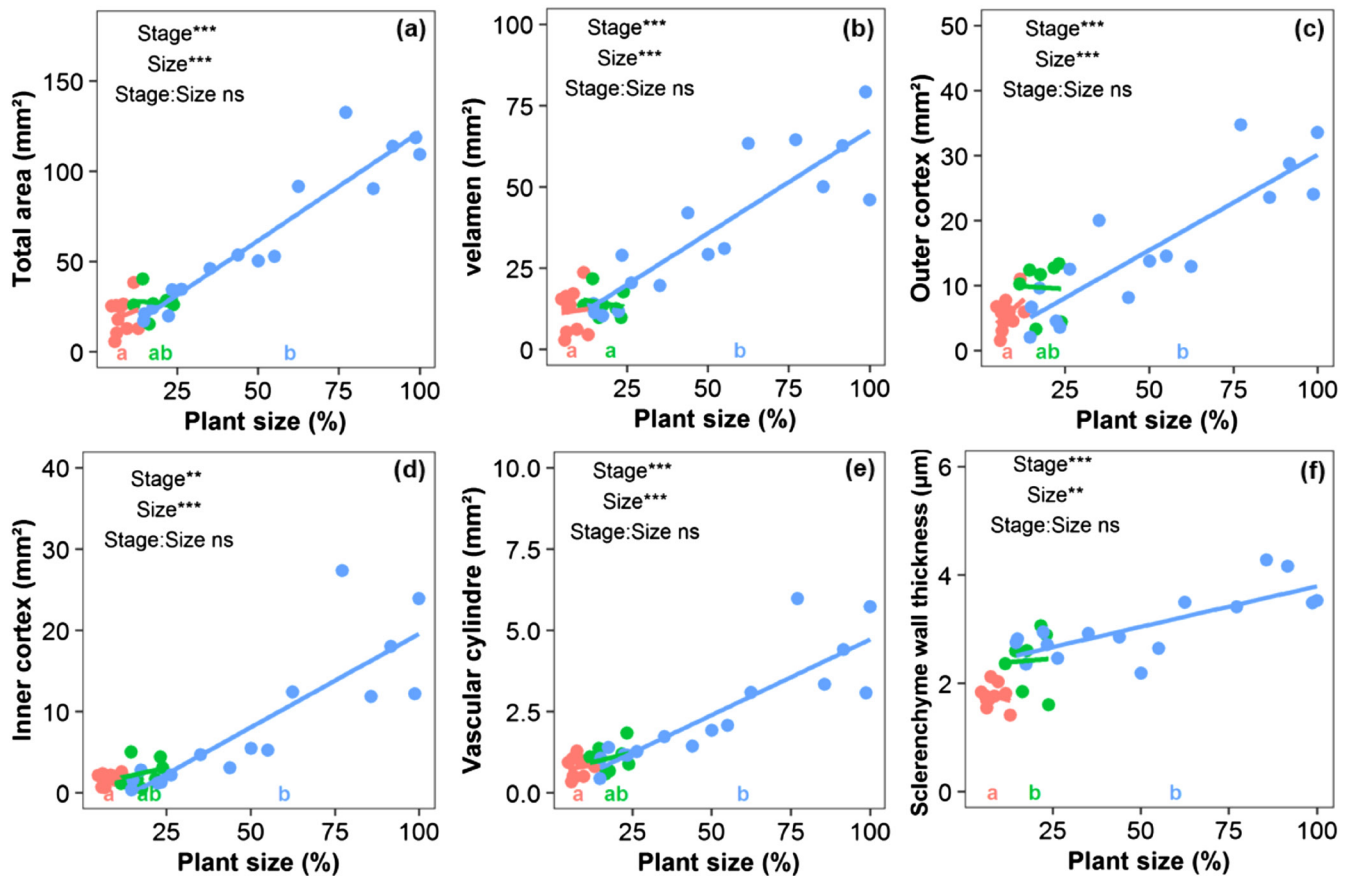


Figure 4. Relationships between (a) total section area (mm^2), (b) velamen area (mm^2), (c) outer cortex area (mm^2), (d) inner cortex area (mm^2), (e) vascular cylinder area (mm^2) and (f) sclerenchyma wall thickness (μm) and plant size (%) in adventitious roots of *Lutheria splendens*. Each point represents a value for adventitious roots according to their plant stage: atmospheric in red, transitional in green and tank in blue. Analyses of covariance (ANCOVAs) were used to examine the effect of plant stage (stage), plant sizes (size) and their interaction (stage:size) on the different morphological and anatomical root traits. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant. Small letters indicate significant differences among plant stages (Tukey's post hoc test, $p < 0.05$).

was marginally significantly higher in the large-size tank stage (Anova, $F = 3.48$, $p = 0.072$).

Discussion

Plant size, taken as an indicator of ontogenetic gradual variations, and plant stage had significant effects on root traits, unless for RTD that did not vary significantly with plant size. Specifically, the number of roots and tips, total root length, average root diameter, the areas of the different root tissues (velamen, outer cortex, inner cortex and vascular cylinder), and sclerenchyma wall thickness increased gradually whereas SRL decreased abruptly as plants mature. Along ontogeny, size-related variations in morphological and anatomical root traits of *L. splendens* occurred simultaneously to leaf step changes from atmospheric to tank bromeliads. In addition, we found contrasting root-associated fungal communities along plant size and between atmospheric and tank stages. This study underscores the importance of explicitly considering ontogeny when assessing root trait variations in bromeliad species.

Ontogenetic changes in root morpho-anatomy

We found that large tank bromeliads were characterised by a high number of adventitious roots and tips, long and thick roots, a large velamen and cortex area, and thick sclerenchyma wall. On the contrary, small-sized atmospheric bromeliads had thin roots, high SRL, a small velamen and cortex area and thin sclerenchyma wall. SRL reflects the absorptive capacity of roots per unit biomass invested (Roumet et al. 2016) and, as its aboveground equivalent (specific leaf area, SLA), SRL is expected to decrease through plant ontogeny (Tobner et al. 2013, Garbowski et al. 2021). Our results suggest that the atmospheric stage has morphological root traits that are usually associated with a reduced life span and an increased capacity for resource exploration per unit of root biomass invested. On the contrary, the tank stage had low SRL and high average root diameter that usually reflect a more conservative strategy for resource use, and in our case can relate to their larger velamen area, as observed in orchids and aroids (Wagner et al. 2021).

As reported for other species of Bromeliaceae, the presence of root hairs originating from the velamen may be associated

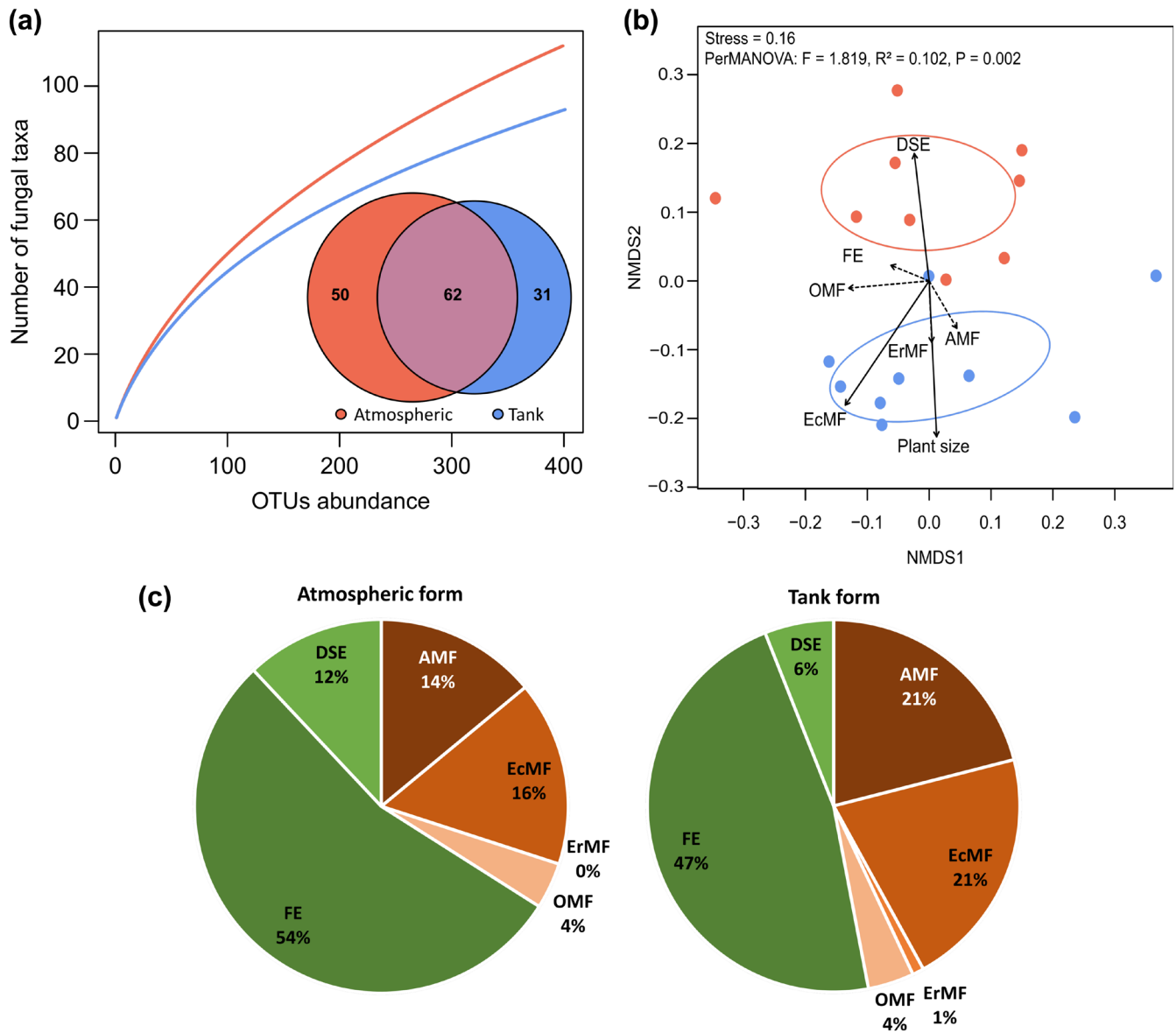


Figure 5. (a) Species accumulation curves and Venn diagram of root-associated fungal taxa according to *Lutheria splendens* stages (i.e. atmospheric in red and tank in blue). (b) Nonmetric multidimensional scaling (NMDS) ordinations of *L. splendens* root fungal communities associated with atmospheric (red dots) or tank (blue dots) stages based upon their OTU composition derived from Hellinger-transformed data and Bray–Curtis distances matrix. Vectors represent the strength/direction of the weight of fungal functional guilds (FFGs) on fungal communities along with plant size. Significant factors are shown by black solid lines, while the non-significant factors are indicated using dotted lines (Table 2). Ellipses indicate one SD around group centroids. FFGs: AMF, arbuscular mycorrhizal fungi; DSE, dark septate endophytes; EcMF, ectomycorrhizal fungi; FE, fungal endophytes; ErMF, ericoid mycorrhiza fungi; OMF, orchid mycorrhiza fungi. (c) Proportions of the functional guilds observed in atmospheric and tank stages based on assignment with FunGuild database (Nguyen et al. 2016).

with the epiphytic life (Pita and Menezes 2002, Segecin and Scatena 2004, Proença and das Graças Sajo 2008, Silva and Scatena 2011). Presumably, the epivelamen play a role in the plant strategy for water and nutrients absorption and may contribute to the plant mechanical fixation (Segecin and Scatena 2004, Silva and Scatena 2011, Zorger et al. 2019). But, in our study we could not evaluate any variation in the density of root hairs along ontogeny because observation of root hairs was not reliable from a transversal section to another

one within and between plant stages (Supporting information). In spite of that, we showed a progressive increase in the size of the root meristem during ontogeny with increased radial area of each tissue along ontogeny. Although larger velamen in the tank stage is subject to greater construction costs (Enquist et al. 1999), it generally confers higher ability to absorb water and nutrients, and higher resistance to water loss and mechanical damage (Zotz and Hietz 2001, Pita and Menezes 2002, Segecin and Scatena 2004, Proença and das

Table 2. Goodness-of-fit statistics (R^2) for parameters fitted to the nonmetric multidimensional scaling (NMDS) ordination of fungal community composition. The significance estimates were based on 999 permutations. Significant factors (Bonferroni corrected $p < 0.05$) are indicated in bold.

Vector	R^2	p-value
EcMF	0.7266	0.001
DSE	0.4956	0.003
ErMF	0.1142	0.423
OMF	0.2422	0.114
AMF	0.0962	0.615
FE	0.0618	0.618

Graças Sajo 2008, Silva et al. 2020). Thus, the velamen is presumably an important regulator to enhance the adaptability of *L. splendens* to the epiphytic environment.

The outer cortex composed of sclerenchymatous cells with thickened secondary walls may also reflect an adaptation to the epiphytic habit as it provides rigidity to anchor the plants on tree stems (Segecin and Scatena 2004, Kowalski et al. 2019). A larger ring of sclerenchyma tissue in the tank stage is likely to efficiently anchor mature individuals characterised by a higher leaf biomass as compared to the atmospheric bromeliads. In addition, the sclerenchyma ring could also minimise transpiration by reducing water loss from the inner to the outer region of the cortex (Zotz and Winkler 2013), a crucial role for roots of epiphytes that are continuously exposed to air and thus particularly prone to desiccation. But, the sclerification of the outer cortex may also impose constraints to the plant in terms of nutrient transportation from the epidermis to the vascular cylinder via the cortex (Males 2016, Kowalski et al. 2019). Tradeoffs between root functions are common as plant roots are multifunctional (Freschet et al. 2021). They acquire water and nutrient resources, ensure the mechanical anchorage of the plant, and may also act as storage organs. Each of these root functions are associated with specific combinations of root trait values (morphology, physiology, biochemistry), with some synergies and tradeoffs between traits values (Freschet et al. 2021). In the case of *L. splendens*, the more intense sclerification of the outer cortex in the adult tank stage suggests that the mechanical constraints are dominant at this stage for plant anchorage. We expect that counterpart of the investment in sclerified tissues is the lower ability of roots to absorb and transport nutrients. Given that tank bromeliads have reservoirs that buffer stresses in nutrient and water absorbed by leaf trichomes, the lower ability of roots to acquire resources in the tank stage may not be critical for the plant to survive. The root absorption is presumably a secondary and alternative path to acquire nutrients.

Ontogenetic changes in community composition of root-associated fungi

We found the same fungal functional groups in the atmospheric and tank stages, but in different proportions. In the atmospheric stage, DSE, AMF and EcMF had the same proportions whereas in tank stage the proportion of AMF and EcMF increased at the extent of DSE that was two times less

abundant. Fungal endophytes are widespread fungal partners in the roots of many plants (Schulz and Boyle 2006, Ruotsalainen et al. 2021). Likewise mycorrhizal fungi, some DSE improves nutrient acquisition, promotes plant growth (Hiruma et al. 2016, Almario et al. 2017) and increases resistance against pathogens and herbivores (Clay and Schardl 2002). In addition, DSE are particularly widespread under adverse abiotic conditions including salinity and drought, and under temperature, nutrient and heavy metal stresses (Eid et al. 2019, Rodriguez et al. 2009). The higher proportions of DSE in the roots of the atmospheric stage may reflect that the latter explore more stressful habitats (pulse-supplied water and nutrients) as compared to tank stage that have a permanent external water storage which buffer water stress. *Cladosporium*, the most abundant Ascomycetous genera found in the roots of the atmospheric stage, has been described as a phyllosphere endophytic fungus of mangrove species (Yao et al. 2019). However, it is also known as a root endophyte promoting plant growth through synthesis of gibberellin hormones and diverse volatile organic compounds (Hamayun et al. 2009).

Effects of host ontogenetic stage on root-associated fungal diversity and/or community composition have been reported in various plant species of different growth forms (Borovec and Vohník 2018, Vukicevich et al. 2019, Qin et al. 2021, Šmilauer et al. 2021). In grasses and forbs, Šmilauer et al. (2021) showed a difference in AMF community composition between seedling and adult stages and an increase of AMF diversity in the adult stage as compared to the seedlings. On the contrary, while AMF fungal communities did not vary with ontogeny in grapevine, AM fungal structures did with more arbuscules in young roots and more vesicles and/or spores in older roots (Vukicevich et al. 2019). In the root of the orchid *Pleione bulbocodioides*, Qin et al. (2021) found a relative constant OMF assemblage and increased fungal endophytes in the root with plant ontogeny. In a marine context, Borovec and Vohník (2018) showed an ontogenetic variation in fungal communities associated to the seagrass *Posidonia oceanica* with young seedlings lacking DSE colonization and adult plants possessing abundant DSE while AMF were absent in both ontogenetic stages. These studies along with our results report an ontogenetic effect on the taxonomy and/or functional group composition of root-associated fungal communities with contrasting and species-dependent trajectories.

Shifts in root-associated fungal composition with ontogeny might be related to ontogenetic shifts in root traits. For instance, in the seagrass *P. oceanica* the absence of fungal colonization in seedlings was linked to the presence of dense root hairs whereas adult plants with abundant DSE lacked root hairs (Borovec and Vohník 2018). In our study, the increased average root diameter and velamen areas with ontogeny may favour mycorrhizal colonisation whereas the sclerified cortex might impair fungal symbiosis. Across plant species and growth forms, thick root species show higher mycorrhizal fungi as compared to thin root species (Comas et al. 2014, Kong et al. 2014, Ma et al. 2018). This difference suggests that plants with thick roots rely more on mycorrhizal fungi

for resource acquisition. AMF and EcMF are well-known root mutualistic symbionts that improve host performance by enhancing nutrient and water uptake (Wipf et al. 2019). Thus, we suggest that mycorrhizal fungi in the tank stage may facilitate the nutrient influx from the substrate.

Ontogenetic variations of root traits follow a two-dimensional spectrum

The two PCA axes of root trait variations along plant ontogeny of the bromeliad *L. splendens* presented similarities to the two main dimensions of the ‘root economics space’ evidenced by Bergmann et al. (2020) on a set of mature plant species spanning a wide range of growth forms. The first axis (65.3%) was related to variations in SRL and average root diameter, the latter being related to changes in root-associated fungal communities. By analogy with the collaboration axis identified by Bergmann et al. (2020), we suggest that this first axis of variation indicate a shift from thin roots of atmospheric bromeliads that rely more on the roots themselves to absorb nutrients to coarser absorptive roots of large-sized tank bromeliads that rely more on fungal collaborations to absorb nutrients. The second axis (17.9%) represents a gradient of RTD that reminds the conservation axis of Bergmann et al. (2020), without clearly distinguishing the three plant stages though.

The negative relationships between SRL and average root diameter along the ontogenetic gradient of *L. splendens* mimics the widely observed equivalent relation in the context of interspecific variations of mature plants (Kong et al. 2014, Roumet et al. 2016, Bergmann et al. 2020, Spitzer et al. 2021, Wagner et al. 2021). By contrast, the relationship between SRL and RTD across species is less clear (Kong et al. 2014, Valverde-Barrantes and Blackwood 2016, Weemstra et al. 2016). Across species, SRL can either correlate negatively (Ma et al. 2018, Bergmann et al. 2020, Garbowski et al. 2021), positively (Holdaway et al. 2011, Kong et al. 2014) or not correlate (Craine et al. 2001, Valverde-Barrantes and Blackwood 2016) with RTD. Along the ontogenetic gradient of *L. splendens*, we found a positive correlation between SRL and RTD. This positive correlation was also found in the context of interspecific variations of vascular epiphytes (Wagner et al. 2021) and suggest that variations in SRL in epiphytes are more related to changes in root diameter than root density. The negative correlation between RTD and average root diameter observed here was in accordance with other studies on trees (Weemstra et al. 2016, Ma et al. 2018) and on vascular epiphytes (Wagner et al. 2021). It indicates that larger roots of tank forms are less dense, whereas thin roots of atmospheric plant stages are denser. Presumably, the higher density of thin roots of atmospheric or small tank stages that do not present external storage of resources reflect a strategy to cope with irregular amounts of water and nutrients. The root traits combinations of epiphytic bromeliads along ontogeny suggest that they have evolved alternative morphological and ecophysiological adaptations in response to varying stresses in resource and drought as plants mature.

Conclusion

Our study shows ontogenetic variations in root morphological and anatomical traits, and in the composition of root-associated fungal communities in the epiphyte bromeliad *L. splendens*. The pulse-supplied atmospheric bromeliads showed root trait syndromes that may reflect a ‘do-it-yourself’ strategy (high SRL, low reliance on fungal mycorrhizae symbiont), whereas the continuously-supplied tanks forms showed coarser diameter roots associated to a larger diversity of mycorrhizal fungal symbionts, that suggest an ‘outsourcing’ strategy. Overall, our results highlight important ontogenetic changes in roots traits and root-associated fungal communities of epiphytes, and points that poorly studied ontogenetic variations of root traits display similarities to the well-investigated spectra of roots traits related to interspecific variations. Finally, beyond the bromeliad case study, we invite future root trait studies to better account for ontogenetic variations.

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Author contributions

Celine Leroy: Conceptualization (lead); Data curation (equal); Formal analysis (lead); Funding acquisition (lead); Investigation (equal); Methodology (equal); Writing – original draft (lead); Writing – review and editing (lead). **Arthur Q. Maes:** Data curation (equal); Formal analysis (supporting); Writing – original draft (supporting); Writing – review and editing (supporting). **Eliane Louisanna:** Investigation (equal); Methodology (equal); Writing – original draft (supporting); Writing – review and editing (supporting). **Nathalie Séjalon-Delmas:** Investigation (equal); Methodology (equal); Writing – original draft (supporting); Writing – review and editing (supporting). **Amandine Erktan:** Formal analysis (supporting); Writing – original draft (equal); Writing – review and editing (equal). **Heidy Schimann:** Formal analysis (equal); Methodology (equal); Writing – original draft (supporting); Writing – review and editing (supporting).

Data availability statement

Data are available from the Zenodo Digital Repository: <<https://doi.org/10.5281/zenodo.6325316>> (Leroy et al. 2022), as well as in the NCBI Sequence Read Archive under the BioProject PRJNA694348 (<www.ncbi.nlm.nih.gov/sra/PRJNA694348>).

Supporting information

The supporting information associated with this article is available from the online version.

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