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Phytobiomes Journal
A Transdisciplinary Journal of Sustainable Plant Productivity

RESEARCH

Drought Tolerance Traits in Neotropical Trees Correlate with the Composition of Phyllosphere Fungal Communities

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Accepted for publication 27 May 2022.

ABSTRACT

Plant-associated microorganisms have been shown to aid plants in coping with drought. However, the underlying mechanisms are poorly understood and there is uncertainty regarding which microbial taxa and functions are mostly involved. We explored these issues in Neotropical rainforests and identified foliar microorganisms that may play a role in drought tolerance of trees. Our objectives were to (i) test the relationship between drought tolerance traits in Neotropical trees and the diversity and composition of their foliar fungal and bacterial communities and (ii) identify leaf microbial taxa positively or negatively associated with drought tolerance traits. Our results showed that the composition of leaf fungal communities but not bacterial communities was related to drought tolerance. We identified 27 fungal amplicon sequence variants whose relative abundance covaried with drought tolerance traits. Most variants were assigned to fungal clades often described as plant pathogens and increased in abundance with drought susceptibility. This greater relative abundance of leaf pathogens in the most drought-susceptible trees might increase their vulnerability to climate change. Moreover, we identified the *Strelitziana* and *Ochroconis* fungal genera as potential candidates for future culture-dependent studies aimed at understanding and improving drought tolerance in Neotropical forests.

Keywords: abiotic stressors, bacterial communities, drought tolerance, fungal communities, microbiota, phyllosphere

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M. C. Cambon and D. Cartry are co-first authors. H. Schimann and C. Vacher are co-last authors.

Author contributions: M.C.C. performed bioinformatic and statistical analyses, interpreted the results, and wrote the manuscript. D.C. processed all samples and performed molecular biology work and preliminary analyses. E.C. provided methods and tools for molecular biology and performed sequencing and demultiplexing. C.Z., S.C., C.S., and J.-Y.G. performed leaf ecophysiological measurements (Ptlp, Pmd, and gmin); S.L. performed leaf embolism resistance measurements; and S.D. performed stem embolism resistance measurements. B.B., C.S., C.Z., E.L., J.C., J.-Y.G., M.B., P.H., P.L., S.C., S.L., T.F., and Y.R. contributed to field or lab work. T.F. made the drawings and participated in the figure realization. D.B. and M.R. had the original idea for the project and gathered the consortium. H.S. designed and coordinated the leaf sampling campaign. C.V. coordinated the analyses and wrote the manuscript. All authors discussed the results and revised the manuscript.

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Funding: This study was supported by funding from the Conseil Régional Aquitaine (Athene project number 2016-1R20301-00007218) for lab equipment for C. Vacher and S. Delzon, the Agence Nationale de la Recherche (ANR) (NGB project ANR-17-CE32-0011) for M. C. Cambon's post-doctoral grant, LABEX CEBA (DROUGHT project ANR-10-LABX-25-01), LABEX ARBRE (ANR-11-LABX-0002-01), LABEX COTE (Micromic project ANR-10-LABX-45) for molecular biology reagents for C. Vacher, and the Université de Bordeaux for T. Fort's Ph.D. grant.

e-Xtra: Supplementary material is available online.

The author(s) declare no conflict of interest.

Plants and trees live in association with diversified communities of microorganisms. These communities include some microbial species that promote plant growth (Compant et al. 2010; Hardoim et al. 2015), contribute to plant resistance to microbial pathogens (Hacquard and Schadt 2015; Hacquard et al. 2017; McLaren and Callahan 2020; Vannier et al. 2019) and insect pests (Pineda et al. 2017; Vacher et al. 2021), and contribute to tolerance to abiotic stressors such as heat, drought, or salinity (Lata et al. 2018; Rho et al. 2018a; Rodriguez et al. 2008). A current challenge is to discover and isolate the microbial strains or consortia that alleviate biotic and abiotic stresses, and to integrate them into pest and disease biocontrol (Berg et al. 2017; Compant et al. 2005; Poudel et al. 2016), plant biostimulation (du Jardin 2015; Rouphael and Colla 2018), and soil biofertilization strategies (Bhardwaj et al. 2014; Mahanty et al. 2017). In particular, manipulating the microbiota to alleviate drought stress is a priority to maintain food and fiber production under current and future climate conditions. Several bioprospection campaigns aimed at discovering microbial strains that foster crop growth under drought stress have been conducted successfully (Eke et al. 2019; A. L. Khan et al. 2016; Timmusk et al. 2014; Zhang et al. 2020) and the key microbial players and the mechanisms are now better understood, at least for the belowground compartment. Belowground microorganisms that strengthen drought tolerance include arbuscular mycorrhizal fungi (Boutasknit et al. 2020; Zhang et al. 2014), rhizospheric bacteria (Chaín et al. 2020; Tiepo et al. 2018), and, in trees, ectomycorrhizal fungi. The latter enhance water and nutrient uptake under drought conditions by extending the absorbing surface area and stimulating water transport mediated by root aquaporins (Brunner et al. 2015; Lehto and Zwiazek 2011). By contrast, less is known about the relationship between aboveground microorganisms and tree drought tolerance. The aim of the present study is to start filling this gap.

Fungi and bacteria colonizing the aerial part of plants, sometimes termed the phyllosphere microbiota (Vacher et al. 2016; Vorholt 2012), might contribute to tree water balance through at least three groups of mechanisms involving (i) the tree water transport system, (ii) stomatal behavior, and (iii) leaf residual water losses. First, fungi and bacteria colonizing the xylem sap can interfere with tree hydraulic functioning. For instance, vascular wilt pathogens induce the formation of tyloses inside xylem conduits that block the water transport (Oliva et al. 2014; Yadeta and Thomma 2013). Second, leaf fungi and bacteria can influence stomatal functioning. There is evidence that they can modulate stomatal conductance and stomatal density, with sometimes opposite effects depending on the microbial strain and the plant species (Arnold and Engelbrecht 2007; Rho et al. 2018a). For instance, in oak, leaf infection by the biotroph phytopathogenic fungus *Erysiphe alphitoides* decreases stomatal conductance by 15 to 30% (Hajji et al. 2009), whereas infection by the endophytic entomopathogenic fungus *Beauveria bassiana* tends to increase stomatal conductance under drought stress (Ferus et al. 2019). Moreover, the hyphae of foliar fungi might contribute to foliar water uptake by favoring the formation of liquid water films on the stomatal walls that connect the apoplast and the leaf surface, thus enabling the penetration of water through stomata (Burkhardt et al. 2012; Schreel and Steppe 2020). This may be particularly true for tropical rainforest tree species that have relatively long-lived evergreen leaves, potentially hosting a diversity of epiphytes. Third, leaf microorganisms can alter cuticular permeability by producing biosurfactants. For instance, *Pseudomonas syringae* can reduce cuticular permeability to up to 50% by syringafactin production, which could alter the leaf residual water loss (Aragón et al. 2017; Burch et al. 2012).

Due to the diversity of microbial species and potential mechanisms, the overall effect of the aboveground microbiota on tree growth under drought conditions is difficult to assess based on current knowledge. A couple of studies isolated leaf endophytes from tree species and experimentally demonstrated their positive influence on drought tolerance (A. L. Khan et al. 2016; Rho et al. 2018a). For instance, leaf endophytes isolated from poplar and willow trees growing in stressful environments increased drought tolerance in poplar (Z. Khan et al. 2016), and also increased drought tolerance when inoculated in rice by influencing stomatal density, stomatal conductance, and leaf acid abscisic concentration (Rho et al. 2018b). To go further along this promising research avenue, we need to better understand the relationships between leaf microbiota and plant traits related to drought tolerance (Rosado et al. 2018).

In the present study, we investigated whether the phyllosphere microbiota could play a role in tree drought tolerance. Our objectives were to (i) test the relationship between several ecophysiological traits indicative of drought tolerance in trees and the diversity and composition of their foliar fungal and bacterial communities and (ii) identify leaf microbial taxa positively or negatively associated with drought tolerance. We hypothesized the leaf microbiota would be mainly associated with drought tolerance traits related to the stomatal behavior and the leaf residual water losses rather than drought tolerance traits related to the water transport system. We also hypothesized that, among drought tolerance traits related to the tree water transport system, the leaf xylem functioning would be more associated with the leaf microbiota than the stem xylem functioning. To test these hypotheses, we characterized foliar fungal and bacterial communities (epiphytic and endophytic, without distinction) of 22 tree species using single-gene community profiling approaches, then analyzed the relationship between community structure and 11 physiological traits contributing to tree drought tolerance. The traits were available from previous studies (Levionnois et al. 2021; Ziegler et al. 2019) and were related to either the tree water transport system, the stomatal behavior, or the leaf residual water losses. The data were collected on rainforest canopy tree species in French Guiana. This part of the Amazonian Forest undergoes seasonal drought, the intensity of which can induce severe mortality, especially when associated with strong El Nino Southern oscillation events (Phillips et al. 2009).

MATERIALS AND METHODS

Study site and sampling design. Tree leaves were collected by tree climbers from January to July 2017 in the Paracou Research Station, which is located in the tropical rainforest of French Guiana (5°18′N, 52°53′W) (https://paracou.cirad.fr/). Leaves were sampled from 88 trees belonging to 22 angiosperm species, encompassing 6 orders, 9 families, and 18 genera (Supplementary Tables S1 and S2). Four trees per species were sampled (Fig. 1). We selected trees in the undisturbed forest plots of the research station (P6, P11, P15) (Gourlet-Fleury et al. 2004), which were located less than 2 km from each other and had very similar soil and rain conditions. We selected healthy dominant or codominant trees that form the canopy, except for *Gustavia hexapetala*, which is a subcanopy species. Climbers collected a canopy branch of 2 to 3 m in length on each tree. Sun-exposed branches were sampled as much as possible, within the safety limits of the climbers (approximately 25 to 30 m above ground level). On each branch, four mature, nonsenescing and healthy leaf samples, each measuring approximately 15 by 10 cm, were collected. Depending on the tree species, a sample corresponded to an entire leaf, a piece of leaf, or several leaflets. Leaves were collected with gloves and sterilized scissors and placed immediately into individual sterile paper envelopes in a box filled with silica gel. This storage method gives

similar results to snap freezing in terms of leaf microbiota composition and diversity (Qiu et al. 2020), and allowed us to overcome the difficulty of bringing dry ice or liquid nitrogen into the field in a tropical environment. At the end of the sampling campaign, silica gel-dried leaf samples were shipped to the sequencing facility (PGTB, Cestas, France) and stored at −80°C until processing.

Ecophysiological measurements. Several ecophysiological traits indicative of drought tolerance were measured on the same trees and have already been published and discussed elsewhere (Levionnois et al. 2021; Ziegler et al. 2019). In the present study, we used 11 traits indicative of drought tolerance, measured on 50 tree individuals encompassing 18 species (Fig. 1; Table 1; Supplementary Table S3). These traits were related to either (i) the water transport system, (ii) stomatal behavior, or (iii) leaf residual water losses. During a drought event, failure of the water transport system (i) can arise because of increasing water tension due to high evaporative demand or water shortage. Gas bubbles form with increasing tension, which creates embolism, consequently blocking water transport in xylem conduits, and drives the loss of xylem conductivity. Drought-induced xylem embolism resistance is quantified by measuring the xylem pressure (−MPa) at 12, 50, and 88% loss of branch hydraulic conductivity (P12, P50, and P88, respectively). The more negative the P50, the more resistant the xylem is to embolism, which was shown to be associated with whole-plant drought resistance (Blackman et al. 2019a; Urli et al. 2013). Embolism resistance was measured with the flow-centrifuge method and the data were published and discussed by (Ziegler et al. 2019). Moreover, during drought, stomatal closure (ii) prevents excessive water losses with increasing evaporative demand or water shortage, with an early stomatal closure improving drought resistance (Martin-St-Paul et al. 2017). The leaf turgor loss point (Ptlp) represents the leaf water potential at which leaf cells lose turgor and was shown to be associated with stomatal closure (Bartlett et al. 2016; Brodribb and Holbrook 2003). The more negative the Ptlp, the more resistant the plant is to drought. Ptlp was measured with an osmometer (Bartlett et al. 2012) and the data were published and discussed by (Ziegler et al. 2019). Finally, during water stress and after stomatal closure, there are still leaf residual water losses (iii) through the cuticle and not perfectly closed stomata. These leaf residual water losses can be quantified with the minimum leaf conductance (gmin in milimoles per square meter per second), which is indicative of the rate at which plant water reservoirs are depleted (Duursma et al. 2019). Thus, low gmin value is indicative of drought tolerance. The gmin was measured by following the weight loss of detached leaves and the data have been published by Levionnois et al. (2021). In addition, we computed several hydraulic safety margins (HSMs) as proxies of stem hydraulic safety, which can be defined as the strength of the hydraulic system compared with the intended hydric stress (Oliveira et al. 2021), expressed as the dry-season leaf water potential at midday (Pmd). Pmd reflects the actual hydric stress trees experience during the dry season. It was measured with a pressure chamber and the data were published and discussed by Ziegler et al. (2019). In the present study, we computed three xylem HSMs ($HSM^{Pmd\text{-}P12}$, $HSM_{Pmd\text{-}P50}$, and $HSM_{Pmd\text{-}P88}$) (Meinzer et al. 2009) and stomatal safety margins ($SSM^{Pmd\text{-}P12}$, $SSM_{Ptlp\text{-}P50}$, and $SSM_{Ptlp\text{-}P88}$) (Martin-St-Paul et al. 2017) with 12, 50, and 88% loss of branch hydraulic conductivity, respectively. For simplicity, HSMs and SSMs will be referred to as HSM_12, HSM_50, and HSM_88 and SSM_12, SSM_50, and SSM_88 respectively. Large margins corresponded to higher drought resistance, and both HSM and SSM have been demonstrated to be strong determinants of drought resistance and drought-induced mortality, including in tropical ecosystems (Anderegg et al. 2016; Blackman et al. 2019a; Martin-St-Paul et al. 2017).

In addition to the 11 traits presented in Table 1, measurements of leaf xylem embolism resistance were available for a subset of trees ($n = 35$) encompassing 17 species. Like stem xylem, leaf xylem is prone to hydraulic failure, and leaf embolism resistance was shown to be an important component of the whole-plant drought resistance (Blackman et al. 2019b; Creek et al. 2020; Levionnois et al. 2021). Although these data are partial, we included them in the study because we expect a stronger relationship between leaf microorganisms and leaf xylem functioning than between leaf microorganisms and stem xylem functioning. Leaf embolism resistance can be measured with the same metrics as stem embolism resistance; that is, by measuring the xylem pressure (−MPa) at 12, 50, and 88% loss of leaf xylem hydraulic conductivity (P12leaf, P50leaf, and

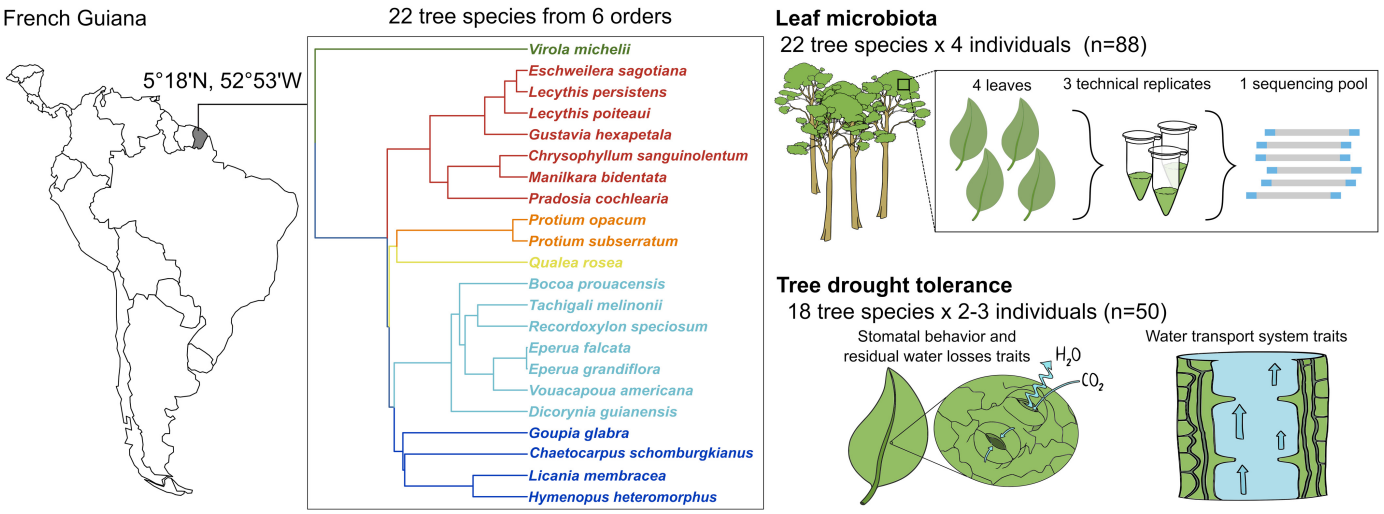

Fig. 1. Summary of the sampling design. In total, 88 trees growing in the Paracou Research Station in French Guiana were selected for the study. The trees belonged to 22 Neotropical species encompassing 6 orders represented in different colors in the phylogeny. From top to bottom: Magnoliales (green), Ericales (red), Sapindales (orange), Myrtales (yellow), Fabales (light blue), and Malpighiales (dark blue). For each tree, four leaves were collected and processed to characterize leaf microbiota composition. Ecophysiological traits indicative of tree drought tolerance were measured on a subset of 50 trees, encompassing 18 species.

P88leaf, respectively). It was measured with the optical visualization method and the data have been published and discussed by Levionnois et al. (2021).

Leaf processing and microbiota sequencing. Leaf sample processing was performed in February 2018 in the sterile environment of a class II microbiology safety cabinet (Thermo Scientific MSC-Advantage). Leaf samples were first cut into small pieces of 2 cm^2 using sterilized tools. Leaf pieces of the same individual tree were placed in the same sterile Petri dish and mixed. For each tree individual, a subset of randomly chosen leaf pieces, weighing 0.9 g in total (Practum; Sartorius), was then transferred into a polyoxymethylene vial (Labman Automation), each containing a 32-mm inox bead. Before use, opened pots, cap undersides, and beads were placed for 20 min under the UV lamp of the biological safety cabinet. Grinding was performed in a Skandex SK 550 1.1 grinder (Fast & Fluid; Idex Corporation) for 5 min at 850 rpm.

To prevent contamination and cross-contamination of the samples, leaf powder was transferred from grinding pots to individual extraction microtubes in the sterile environment of the microbiology safety cabinet. For each tree individual, three technical replicates of 20 to 30 mg of powder were weighed (Practum; Sartorius) and placed into 1.4-ml microtubes with screw caps (Micronic). Cap undersides and opened microtubes were exposed for 20 min under the UV lamp of the biological safety cabinet before use. We obtained 264 samples, corresponding to 22 tree species × 4 tree individuals × 3 technical replicates (Fig. 1). Leaf samples were randomized in three 96-well extraction plates. Each plate contained one replicate of each tree and eight empty tubes that were used subsequently as extraction negative controls. Plates were stored at −20°C until extraction in March 2018.

DNA extraction was performed in a laboratory of the PGTB sequencing facility (Cestas, France) dedicated to environmental DNA analysis. This laboratory had filtered air under positive pressure and was cleaned by UV radiation between each use. DNA was extracted under a hood equipped with both carbon filters and HEPA filters to provide protection against external particulate contamination (Captair; Erlab). The DNeasy 96 Plant Kit (Qiagen) with some modifications of the manufacturer's protocol was used for DNA extraction: samples were incubated for 10 min at 65°C after adding the lysis solution, all centrifugations were made at 5,650 rpm, only 450 µl of the first washing buffer (AW1) buffer was added in each tube because we obtained 300 µl of supernatant per tube, and elution was performed in two steps, using 50 µl of the elution buffer (AE) each time. The absence of DNA in extraction negative controls was

TABLE 1
Mean values of drought tolerance traits per species[a]

Species	Code	P12	P50	P88	Ptlp	gmin	HSM_12	HSM_50	HSM_88	SSM_50	SSM_88	SSM_12	Nmicro	Ntraits
Bocoa prouacensis	Bp	−2.16	−3.89	−5.62	−1.85	3.51	−0.26	1.48	3.21	2.04	3.78	0.31	2	2
Chaetocarpus schomburgkianus	Csch	−0.83	−2.01	−3.18	−1.56	8.15	−0.44	0.74	1.92	0.39	1.57	−0.78	3	2
Chrysophyllum sanguinolentum	Csan	−3.68	−4.05	−4.43	−1.73	7.37	2.10	2.47	2.84	2.32	2.69	1.95	5	4
Dicorynia guianensis	Dg	−1.11	−2.17	−3.22	−1.30	6.50	−0.80	0.29	1.37	0.86	1.92	−0.19	4	3
Eperua falcata	Ef	−2.66	−3.68	−4.70	−1.66	3.01	1.26	2.28	3.30	2.03	3.05	1.00	4	3
Eperua grandiflora	Eg	−5.58	−6.15	−6.73	−1.86	3.34	4.17	4.74	5.32	4.29	4.86	3.71	3	3
Eschweilera sagotiana	Es	−2.42	−2.70	−2.98	−1.58	2.22	1.17	1.44	1.72	1.11	1.39	0.83	3	3
Goupia glabra	Gg	−2.72	−4.72	−6.71	−2.13	5.31	1.93	3.93	5.92	2.59	4.59	0.59	3	3
Gustavia hexapetala	Gh	−6.78	−7.63	−8.49	−2.03	2.85	6.39	6.76	7.13	5.64	6.49	4.79	4	3
Lecythis persistens	Lper	−2.46	−3.39	−4.31	−1.77	2.79	1.14	2.06	2.99	1.62	2.54	0.69	4	4
*Lecythis poiteauii**	Lpoi	NA	NA	NA	−2.28	4.13	NA	NA	NA	NA	NA	NA	4	0
*Licania heteromorpha**	Lh	NA	NA	NA	−1.39	14.01	NA	NA	NA	NA	NA	NA	2	0
Licania membracea	Lm	−1.44	−2.81	−4.19	−1.71	7.98	0.19	1.56	2.94	1.10	2.48	−0.27	4	3
Manilkara bidentata	Mb	−3.68	−7.96	−12.25	−2.18	5.00	1.24	5.52	9.81	5.78	10.07	1.50	2	1
Pradosia Cochlearia	Pc	−4.52	−6.06	−7.60	−1.76	4.90	1.26	3.31	5.36	4.29	5.83	2.76	3	3
Protium opacum	Po	−1.49	−2.38	−3.26	−1.97	4.30	−1.02	−0.13	0.76	0.30	1.19	−0.59	4	2
Protium subserratum	Psub	−1.44	−2.93	−4.41	−2.08	5.33	−0.53	0.96	2.44	0.71	2.20	−0.78	4	1
Qualea rosea	Qr	−0.99	−1.86	−2.72	NA	6.41	−0.41	0.46	1.32	NA	NA	NA	4	2
*Recordoxylon speciosum**	Rs	NA	NA	NA	−1.61	4.67	NA	NA	NA	NA	NA	NA	4	0
Tachigali melinonii	Tm	−2.52	−4.24	−5.96	−1.72	4.53	0.85	2.58	4.30	2.53	4.25	0.80	4	4
Virola michelii	Vm	−4.24	−5.38	−6.52	−1.62	6.45	3.34	4.48	5.62	3.77	4.91	2.62	4	4
*Vouacapoua americana**	Va	NA	NA	NA	−1.88	2.88	NA	NA	NA	NA	NA	NA	3	0

[a] Eleven traits indicative of tree drought tolerance were selected in the present study. P12, P50, and P88 = xylem pressure at 12, 50, and 88% loss of branch hydraulic conductivity. Ptlp = leaf turgor loss point and gmin = minimum leaf conductance. HSM_12, HSM_50, and HSM_88 = hydraulic safety margins at 12, 50, and 88% loss of branch hydraulic conductivity, respectively. HSM_12, HSM_50, and HSM_88 = xylem hydraulic safety margins at 12, 50, and 88% loss of branch hydraulic conductivity, respectively. SSM_12, SSM_50, and SSM_88 = stomatal safety margins at 12, 50, and 88% loss of branch hydraulic conductivity, respectively. Drought tolerance increased with decreasing values of all traits, except for HSMs and SSMs. Nmicro = the number of individuals of each species for which we obtained both fungal and bacterial community data (77 individuals in total) and Ntraits = the number of individuals of each species for which both community data and ecophysiological traits data were available (50 individuals in total). Ecophysiological data at the individual level are available in Supplementary Table S3. Asterisks (*) indicate tree species that were not included in statistical analyses of the relationships between leaf microbiota and drought tolerance traits due to missing values.

checked by electrophoresis and the DNA extracts were then quantified using the NanoDrop 2000/2000c Spectrophotometer (Thermo Scientific).

Two barcode regions were then amplified using tagged primers (Supplementary Table S4), by assigning the same tag combination to the three technical replicates of each tree individual. The V5-V6 region of the bacterial 16S ribosomal RNA (rRNA) gene was amplified using the primer pair 799F-1115R (Chelius and Triplett 2001; Redford et al. 2010), which excludes chloroplast DNA. To avoid a two-stage PCR protocol that would increase amplification biases, and to reduce sequencing biases, each primer contained the Illumina adaptor sequence, a tag, and a heterogeneity spacer, as described by Laforest-Lapointe et al. (2017) (799F: 5′-CAAGCAGAAGACGGCATACGAGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTxxxxxxxxxxxxHS-AACMGGATTAGATACCCKG-3′ and 1115R: 5′-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTxxxxxxxxxxxxHS-AGGGTTGCGCTCGTTG-3′, where HS represents a 0- to 7-bp heterogeneity spacer and "x" a 12-nucleotide tag). The ITS1 region of the fungal internal transcribed spacer (ITS) was amplified using the ITS1F-ITS2 primer pair (Gardes and Bruns 1993; White et al. 1990), with each primer containing the Illumina adaptor sequence and a tag (ITS1F: 5′-CAAGCAGAAGACGGCATACGAGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTxxxxxxxxxxxxCTTGGTCATTTAGAGGAAGTAA-3′ and ITS2: 5′-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTxxxxxxxxxxxxGCTGCGTTCTTCATCGATGC-3′, where "x" represents a 12-nucleotide tag).

The PCR mixture (20-µl final volume) consisted of 4 µl of 5× mix HOT FIREPol (Solis BioDyne), 1 µl of each primer (2 µM), 1 µl of DNA, and 13 µl of pure H_2O (Invitrogen Nuclease-Free Water, Fisher Scientific). A 1:5 dilution was applied to DNA extracts before amplification of the fungal ITS region. PCR cycling reactions were conducted in a VERITI 96 Well (Applied Biosystems) thermocycler with the following conditions: initial denaturation at 95°C for 15 min; followed by 35 cycles at 95°C for 15 s, 60 or 55.8°C (for bacterial 16S and fungal ITS, respectively) for 45 s, and 72°C for 60 s; with a final extension at 72°C for 5 min. Size and quality of PCR products were checked by electrophoresis using a 20-min migration at 100 V on agarose gels with GelRed coloration (Nucleic Acid Gel Stain, 10,000×; Biotium, Glowing Products for Science).

In addition to leaf samples, each PCR plate contained eight controls: three extraction negative controls, three PCR-negative controls, and two PCR-positive controls. The three extraction negative controls were each represented by an extraction well without a DNA template, extracted, and amplified like all other samples. They were chosen randomly among the eight extraction negative controls present on the extraction plates. The PCR negative controls were each represented by a PCR well with 1 µl of pure water (Invitrogen Nuclease-Free Water; Fisher Scientific), amplified like all other samples. The PCR positive controls were each represented by 1 µl of DNA at 10 ng μl^{-1} of a pure marine strain unlikely to be found in leaves (*Sulfitobacter pontiacus* or *Vibrio splendidus* for bacteria, and *Candida oceani* or *Yamadazyma barbieri* for fungi), amplified like all other samples. PCR plates were only validated if the six negative controls were negative on electrophoresis gel and the two positive controls were positive.

PCR products representing the same tree individual (i.e., technical replicates) were pooled (Fig. 1). Then, PCR products were purified, quantified (Quant-it PicoGreen dsDNA assay kit; Thermo Fisher Scientific), and equimolarly pooled (Hamilton Microlab STAR robot). Average fragment size was checked using a Tapestation (Agilent Technologies). Libraries were sequenced on a MiSeq (Illumina) with reagent kit v2 (500 cycles). Bacterial 16S and fungal ITS libraries each contained 96 samples (88 leaf samples and 8 control samples) and were sequenced on separate runs. Both runs each contained a total of 192 samples, because they included samples collected on the same trees and at the same time for another study. Sequence demultiplexing (with exact index search) was performed at the PGTB sequencing facility (Genome Transcriptome Facility of Bordeaux, Pierroton, France) using DoubleTagDemultiplexer.

Bioinformatic analyses. Sequences were processed using the dada2 package (v1.12.1) (Callahan et al. 2016) in R (v4.0.3) (R Core Team 2020) following the dada2 tutorial for 16S rRNA gene data v1.12 and the data2 tutorial for ITS data v1.8. Heterogeneity spacer and primer sequences were removed using Cutadapt v3.0 (Martin 2011). Poor-quality sequences (defined as sequences having more than one expected error based on quality scores, at least one ambiguous nucleotide, or shorter than 100 bp) were removed with the *filterAndTrim* function. Amplicon sequence variants (ASVs) were inferred for each sample using the *dada* function, forward and reverse reads were paired using the *mergePairs* function, and chimeric sequences were removed using the *removeBimeraDenovo* function and default parameters. Taxonomic assignments were performed using the RDP Naive Bayesian Classifier algorithm (Wang et al. 2007) implemented in the *assignTaxonomy* function of dada2, with an 80% confidence threshold. The classifier was trained with the SILVA database v138 (Quast et al. 2012) and the UNITE database (v8.2) (Abarenkov et al. 2021) for bacteria and fungi, respectively.

Positive and negative controls were then used to identify contaminants. The PCR and extraction negative controls were first used to identify contaminant ASVs using the "frequency" method of the decontam package and default parameters (v1.8.0) (Davis et al. 2018), which assumes that contaminant ASVs have a higher frequency in samples with low DNA concentration before equimolar pool, whereas the frequency of noncontaminant ASVs is independent from DNA concentration. Contaminant ASVs were removed from the whole dataset. Then, positive and negative controls were used to identify additional contaminant sequences within each sample by following the method described by Galan et al. (2016). The cross-contamination threshold was defined as the maximal number of sequences of each ASV found in negative or positive control samples. The false-assignment threshold was defined as the highest sequence count of a positive control strain in a noncontrol sample, divided by the total number of sequences of the strain in the whole run and multiplied by the total number of sequences of each ASV. ASVs were removed from all samples where they harbored fewer sequences than either the cross-contamination or false-assignment threshold. All samples containing less than 100 reads at the end of the data curation were discarded. All 16S and ITS ASVs unassigned to the phylum level or matching chloroplastic sequences were removed. At the end of the filtering procedure, the dataset contained bacterial and fungal data for 77 tree individuals. A detailed description of read losses through the bioinformatic pipeline can be found in Supplementary Methods S1.

Statistical analysis. Variations in leaf microbiota composition across tree individuals were analyzed using a permutational analysis of variance with 999 permutations and sequential addition of terms, and was performed with the *adonis2* function of the vegan package v2.5-7 (Oksanen et al. 2019). Compositional dissimilarities among samples were estimated with the Bray-Curtis index (Beals 1984). The model had sample sequencing depth as the first explanatory variable, followed by tree species, sampling date, tree location, and the interaction between tree species and sampling date. Sequencing depth was defined as the total number of sequences per sample,

after the filtering steps. Sampling date was defined as the number of days elapsed since the first day of sampling. Tree location consisted of the eigenvectors of a principal coordinates of neighbor matrice (PCNM) based on the Euclidean distance of tree geographical coordinates, calculated with the *pcnm* function of the vegan package v2.5-7 (Oksanen et al. 2019). The model included the four factors as simple effects and the interaction between tree species and sampling date but not the interaction between tree species and tree location, because this interaction represented too many parameters to estimate.

The relative contribution of the leaf microbiota to variations in drought tolerance across tree individuals was then assessed by performing a sequence of redundancy analyses (RDA) with the *rda* function of the vegan package v2.5-7 (Oksanen et al. 2019). The RDA included 50 tree individuals encompassing 18 species, for which at least four drought tolerance traits were available and bacterial and fungal community data were obtained (Table 1). The RDA response matrix was the same in all analyses and represented the values of the 11 traits related to drought tolerance (Table 1; Supplementary Table S3) for the 50 selected trees. Seventeen missing values were approximated with the *estim_ncpPCA* and *imputePCA* functions from the missMDA package v1.17 (Josse and Husson 2016) and trait values were scaled to unit variance. The RDA explanatory component differed among analyses. The first set of RDAs had either leaf fungal community α-diversity, leaf fungal community β-diversity, leaf bacterial community α-diversity, leaf bacterial community β-diversity, tree species phylogeny, or tree location as an explanatory component. Each explanatory component was represented by several explanatory variables (defined below). For each component, the variables contributing significantly to variations in drought tolerance were selected with a forward stepwise selection model based on 499 permutations, performed with the *ordiR2step* function of the vegan package. The selected variables were included as explanatory variables in the final RDA, which was then used for variance partitioning with the *varpart* function of the vegan package. In the first set of RDAs, community α-diversity was represented by three explanatory variables: community richness, measured with the Chao1 index; community diversity, measured with the Shannon index; and community evenness, measured with the Pielou's index. The Chao1 and Shannon indexes were calculated using the *diversity* and *estimateR* functions of the vegan package, respectively, while the Pielou's index was defined as $S/[\log(N)]$, where S is the Shannon index and N the number of ASVs of a given sample, as suggested in the vegan package documentation (Oksanen et al. 2019). Community β-diversity, tree species phylogeny, and tree location were represented by the PCNM eigenvectors of the Bray-Curtis distance (Beals 1984) between samples, the cophenetic distance (Sneath and Sokal 1973) between tree species, and the Euclidean distance of tree geographical coordinates, respectively. PCNM eigenvectors were calculated with the *pcnm* function of the vegan package v2.5-7 (Oksanen et al. 2019). Tree species phylogeny was extracted from the megatree by (Zanne et al. 2014) using the brranching package v0.6.0 (Chamberlain 2020). The tree species *Chaetocarpus schomburgkianus* was not present in the megatree; therefore, we replaced it with a closely related species, *Jatropha curcas*, to extract phylogenetic information. The cophenetic distance between tree species was calculated with the *cophenetic.phylo* function of the ape package v5.4.1 (Paradis and Schliep 2019). Moreover, to further test for a phylogenetic signal on the drought tolerance traits, we calculated Pagel's λ and Blomberg's K for each trait with the phylosignal package (Keck et al. 2016). We also checked for the robustness of the RDA results for variations in sampling depth because these latter might influence our measures of community α- and β-diversity (Weiss et al. 2017). We performed the same RDA after rarefying the data with the first quartile of the read number distribution as a rarefaction threshold and recalculating diversity indices.

Then, we investigated which ASVs covaried with drought tolerance, using the TITAN2 package v2.4 (Baker et al. 2019), which was designed to investigate changes in community composition along an ecological gradient. In our case, the ecological gradient was represented by tree drought tolerance trait values. The TITAN2 analysis was first performed on the same dataset as RDA, representing the drought tolerance traits and fungal communities of 50 tree individuals belonging to 18 species. Microorganisms associated with drought tolerance traits were searched within the set of fungal ASVs having more than 100 reads in total and present in at least three samples, as required by TITAN2. Then, the same analysis was applied to the subset of 35 tree individuals for which the leaf resistance to embolism was available (P12leaf, P50leaf, and P88leaf). Variations in drought tolerance trait values due to phylogenetic signals were removed before TITAN2 analysis by applying a phylogenetic correction to each trait, performed with the *phylo.correction* function of the phyloint package v0.1 (Eklöf and Stouffer 2016). The TITAN2 analysis allowed us to quantify, for each ASV and each drought tolerance trait, the change in ASV relative abundance related to the phylogenetically corrected value of each trait. The intensity and the direction of the relationship between each trait and the relative abundance of each ASV were represented by a z-score. The z-score sign was inverted for gmin, Ptlp, P12, P50, P88, P12leaf, P50leaf, and P88leaf, so that a positive value always represented a positive association between an ASV and drought tolerance. ASVs whose relative abundance covaried with the phylogenetically corrected value of at least one trait, with the same direction, in 95% of 500 bootstraps of the dataset were considered as significantly associated with drought tolerance and were selected for subsequent analysis. Their putative ecology was determined based on their taxonomy at the family or genus level when available. The fungal guild was determined using the FunGuild database (Nguyen et al. 2016) and additional information was retrieved from the literature.

Finally, to confirm the influence of the TITAN2-selected ASVs on drought tolerance, we replaced the PCNM eigenvectors representing community β-diversity with the relative abundance of the selected ASVs in the final RDA model and again performed a partition of variance.

RESULTS

The dataset was composed of 7,950 bacterial variants and 8,748 fungal variants (ASVs) distributed among 86 and 79 leaf samples, respectively, collected from 88 tree individuals from 22 species growing in the Paracou Research Station in French Guiana. The bacterial community was dominated by ASVs assigned to the orders *Rhizobiales, Deinococcales*, and *Chloroflexales*, while the fungal community was dominated by ASVs that could not be assigned at the order level, followed by variants assigned to orders Capnodiales and Xylariales (Fig. 2). Both the bacterial and the fungal community differed significantly among the 22 tree species selected for the study (Supplementary Table S5). Tree species accounted for more than 35% of the variation in community composition among tree individuals (Supplementary Table S5). Tree localization did not have any impact on the leaf microbiota composition (Supplementary Table S5).

RDA based on 50 tree individuals and 18 species revealed that the composition of leaf fungal communities but not bacterial communities contributed to variations in drought tolerance among tree individuals. In the first set of RDA, leaf fungal community α-diversity, leaf fungal community β-diversity, leaf bacterial community

α-diversity, leaf bacterial community β-diversity, tree species phylogeny, and tree location were represented by 3, 26, 3, 28, 20, and 6 variables, respectively, of which 11 variables were found to be significantly related to variations in drought tolerance among tree individuals (Table 2). Among these 11 variables, 9 represented tree species phylogeny, 1 represented leaf fungal community β-diversity, and 1 represented leaf bacterial community β-diversity (Table 2). The final RDA including the 11 significant variables revealed that the phylogenetic distance between tree individuals explained 41% of the variance in their drought tolerance traits, although we did not find any phylogenetic signal on individual traits, except for gmin (Supplementary Table S6). The composition of the foliar fungal and bacterial community explained 3 and 0% of the variance, respectively. Moreover, 5% of the variance was explained by both tree phylogeny and fungal community composition, which raised the total percentage of variance explained to 49% (Table 2). We obtained similar results after rarefying the data (Supplementary Table S7).

Based on RDA results, TITAN2 analysis was only performed for the leaf fungal community. The analysis identified 27 fungal ASVs (Table 3) whose relative abundance changed significantly with the value of at least one phylogenetically corrected drought tolerance trait. Among these ASVs, 21 decreased in abundance with drought tolerance and 6 increased in abundance. No ASV decreased in abundance with a trait but increased with another, and conversely (Fig. 3A). The drought tolerance trait associated with the highest number of fungal ASVs was HSM_88. HSM_88 was associated with nine ASVs, followed by HSM_50, P50, and SSM_50, which were associated with seven, six, and five ASVs, respectively. All other traits were associated with fewer than five ASVs (Fig. 3A). In total, leaf resistance to embolism traits (P12leaf, P50leaf, and P88leaf) were associated with three ASVs, while xylem resistance to embolism traits (P12, P50, and P88) were associated with nine ASVs. Two of the ASVs associated with leaf resistance to embolism traits were also associated with one and two traits in the complete analysis (Fig. 3A). The z-score obtained for those leaf traits can be found in Supplementary Table S8.

The 27 fungal ASVs significantly associated with drought tolerance belonged to 10 putative fungal orders (out of 65 identified in the whole dataset). The Xylariales order has the highest number of TITAN2-selected ASVs, with one ASV positively and six ASVs negatively associated with drought tolerance traits (Fig. 3B). Putative taxonomic assignments at the family or species level could be obtained for 15 TITAN2-selected ASVs. Thirteen of these ASVs were described as plant pathogens in the FunGuild database and literature (Table 3).

Finally, RDA confirmed the contribution of TITAN2-selected ASVs to variations in drought tolerance among tree individuals. Variance partitioning showed that the relative abundance of the 27 TITAN2-selected ASVs explained 7% of the variance in drought tolerance traits (Table 4), which is higher than the amount of variance explained by the composition of the whole fungal community (3%) (Table 2).

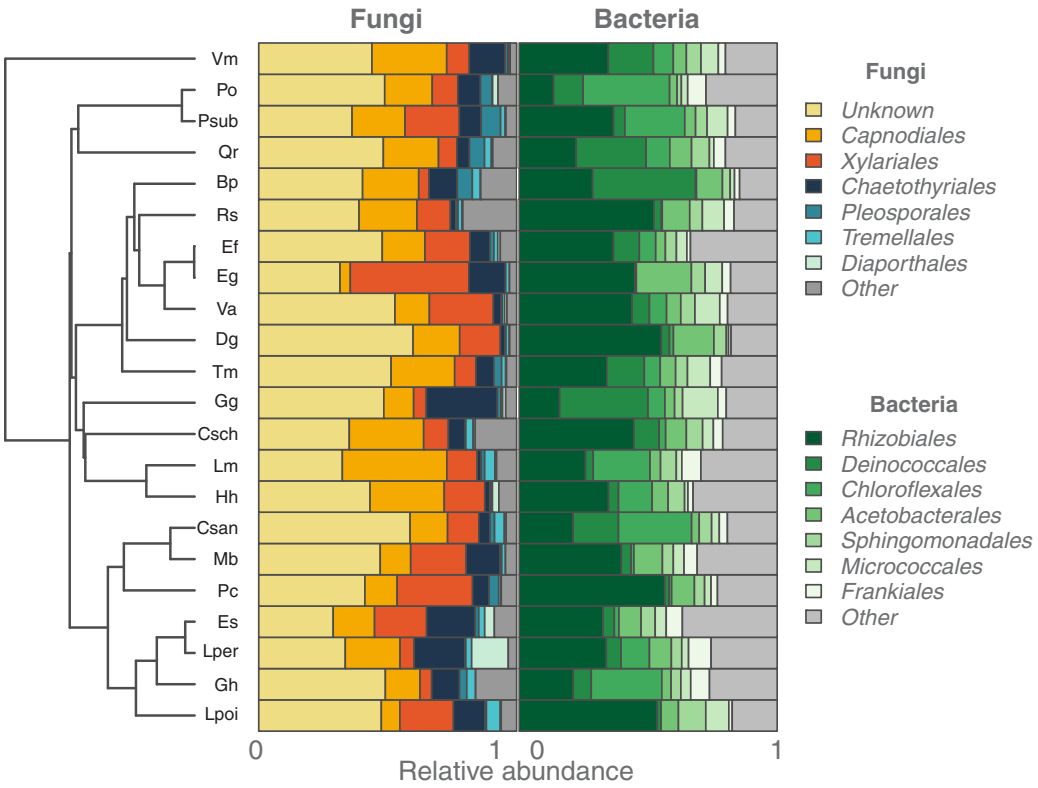

Fig. 2. Leaf microbiota composition of Amazonian tree species growing in the Paracou Research Station (French Guiana). Tree species phylogeny (left side) was extracted from the megaphylogeny of Zanne et al. (2014). Leaf microbiota composition (right side) is the average community composition of two to four individuals per tree species. Relative abundances of the most abundant fungal and bacterial orders are represented in different colors, while less abundant orders have been pooled in the "Other" category. Sequences that could not be assigned at the order level have been pooled in the "Unknown" category. Csch = *Chaetocarpus schomburgkianus*, Ef = *Eperua falcata*, Eg = *Eperua grandiflora*, Es = *Eschweilera sagotiana*, Qr = *Qualea rosea*, Gh = *Gustavia hexapetala*, Lm = *Licania membracea*, Csan = *Chrysophyllum sanguinolentum*, Vm = *Virola michelii*, Gg = *Goupia glabra*, Lper = *Lecythis persistens*, Pc = *Pradosia cochlearia*, Po = *Protium opacum*, Rs = *Recordoxylon speciosum*, Lpoi = *Lecythis poiteauii*, Dg = *Dicorynia guianensis*, Mb = *Manilkara bidentata*, Bp = *Bocoa prouacensis*, Tm = *Tachigali melinonii*, Va = *Vouacapoua americana*, Psub = *Protium subserratum*, and Hh = *Hymenopus heteromorphus*.

DISCUSSION

In this study, we explored the diversity of leaf microbiomes in a Neotropical forest to identify microbial taxa that may influence tree drought tolerance. Our specific objectives were to (i) test the relationship between drought tolerance traits and the diversity and composition of foliar fungal and bacterial communities and (ii) identify leaf microbial taxa positively or negatively associated with tree drought tolerance. We also tested two hypotheses. First, we tested the hypothesis that drought tolerance traits related to stomatal behavior and leaf residual water loss were more strongly associated with leaf microbes than drought tolerance traits related to the tree water transport system. Second, we tested the hypothesis that, among drought tolerance traits related to the water transport system, the relationship with the leaf microbiota was stronger for leaf xylem functioning than for stem xylem functioning.

Our results confirmed the high diversity of phyllosphere bacterial and fungal communities in tropical forests. We detected more than 15,000 ASVs in less than 100 leaf samples. The leaf bacterial community was dominated by ASVs assigned to the orders *Rhizobiales*, *Deinococcales*, and *Chloroflexales*, which were previously described in other phyllosphere studies (Donald et al. 2020; Flores-Núñez et al. 2020; Noble et al. 2020; Sun et al. 2021; Toju et al. 2019), while the fungal community was dominated by ASVs that could not be assigned at the order level, followed by orders Capnodiales and Xylariales, which were also previously described in another study conducted in French Guiana (Donald et al. 2020).

Our results also confirmed the high variability of drought tolerance traits across tree species, in line with previous studies of Ptlp and xylem embolism resistance (P12, P50, and P88) (Maréchaux et al. 2015; Oliveira et al. 2019; Ziegler et al. 2019). We found that tree phylogeny was the main driver of variation in drought tolerance traits among tree individuals. It explained 41% of the variance in the 11 drought tolerance traits measured, while the other factors (tree location, microbiota diversity, and composition) accounted for only 8% of the variance. Because the RDA we performed was not

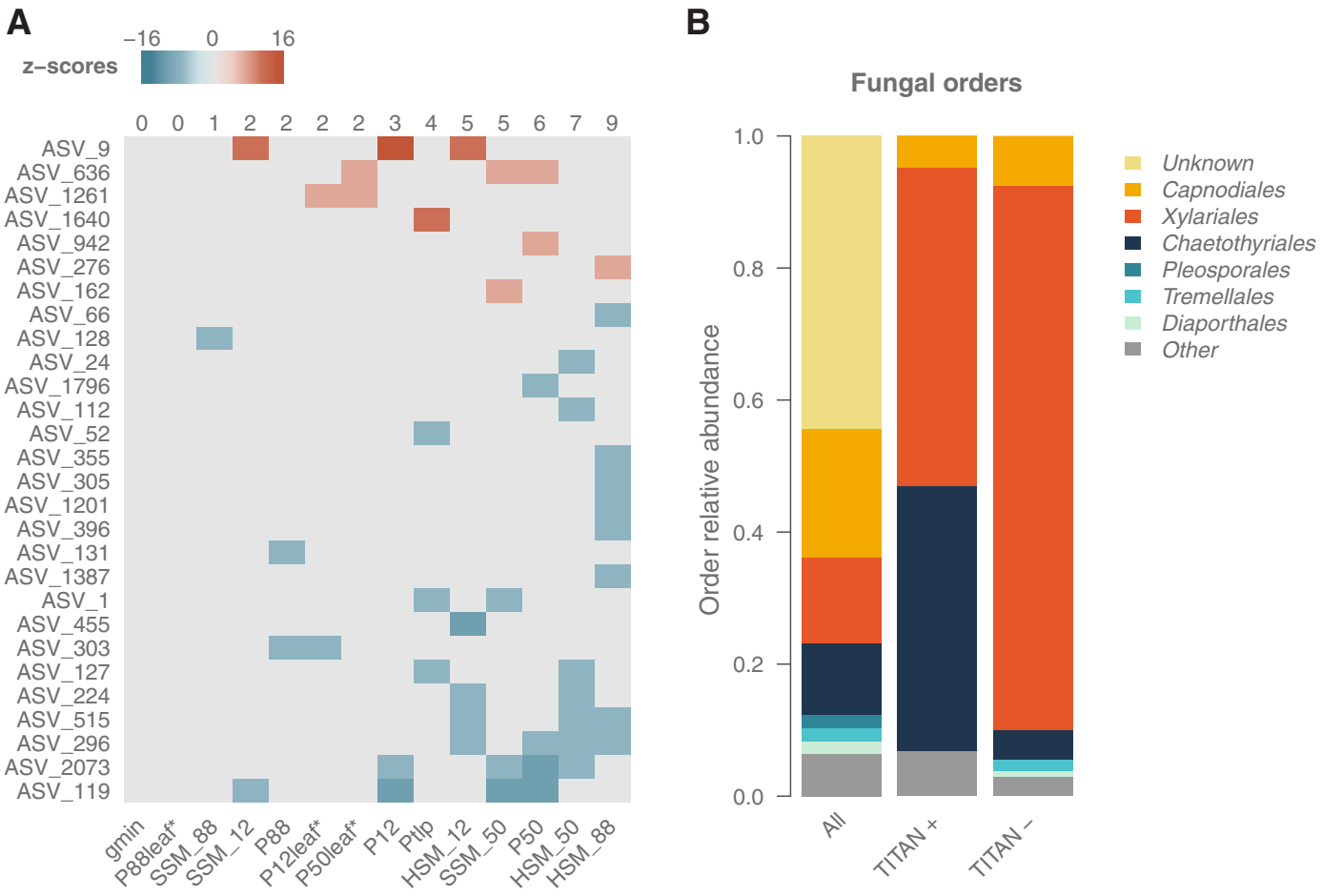

Fig. 3. Fungal amplicon sequence variants (ASVs) associated with drought tolerance in 50 tree individuals belonging to 18 Amazonian tropical tree species, according to TITAN2 analysis. **A,** Association scores (i.e., z-scores) between the relative abundance of fungal ASVs and the value of drought tolerance traits. Only ASVs significantly associated with at least one trait are shown. The z-score sign was inverted for gmin, Ptlp, P12, P50, and P88 so that a positive value always represents a positive association between an ASV and drought tolerance. As a result, ASVs represented in red are positively associated with drought tolerance, while ASVs represented in blue are negatively associated with drought tolerance. ASVs have been ordered according to their mean z-score, and drought tolerance traits have been ordered according to their number of associated ASVs (indicated as numbers above each bar). Stars indicate leaf resistance to embolism traits, which could only be analyzed on a subset of 35 samples. **B,** Taxonomic composition, at the order level, of the whole foliar fungal community and the community subsets significantly associated with drought tolerance. "All" corresponds to the taxonomic distribution of all fungal ASVs in the dataset (n = 6,615 ASVs). "TITAN +" (n = 6 ASVs) and "TITAN –" (n = 21) indicate the taxonomic composition of the community subsets positively and negatively associated with drought tolerance, respectively, according to TITAN2 analysis. Relative abundances of the most abundant fungal orders are represented in different colors, while less abundant orders have been pooled in the "Other" category. ASVs that could not be assigned at the order level have been pooled in the "Unknown" category.

designed to test for a phylogenetic signal per se, we further tested for a phylogenetic signal in each drought tolerance trait by using Pagel's λ and Blomberg's K indices (Keck et al. 2016); however, we did not find any except for gmin. These results are consistent with those of Fontes et al. (2020), who did not detect any phylogenetic signal in xylem resistance to embolism (P50) and other drought tolerance traits in Amazonian tree species. Thus, the major effect of tree phylogeny we found in the RDA reflects the fact that individuals of the same species are both phylogenetically close and share similar ecophysiological characteristics. This does not imply that individuals belonging to phylogenetically close species share similar ecophysiological characteristics, which would be a true phylogenetic signal.

Our analyses suggested that the second most important driver of drought tolerance was the composition (β-diversity) of the leaf fungal community, which explained 3% of the variance in the drought tolerance traits. By contrast, the diversity (α-diversity) of the leaf fungal community was not related to tree drought tolerance. The contribution of fungal community β- but not α-diversity to variations in drought tolerance across trees suggests that tree drought tolerance depends on the abundance of specific fungal taxa, rather than the diversity of the whole fungal community. This result is in agreement with previous studies showing that specific fungal endophytes can modulate stomatal conductance or alter the formation of water films on the leaf surface (Arnold and Engelbrecht 2007; Burgess and Dawson 2004; Ferus et al. 2019; Hajji et al. 2009; Schreel and Steppe 2020). The overall contribution of foliar fungi to 3% of the variance in drought tolerance among tree individuals belonging to different species can be considered low. However, it is difficult to compare this value with others because most studies have been looking at the impact of plant functional traits on the microbiota composition (Kembel et al. 2014) and not the other way around. Moreover, we expect variations in drought tolerance traits across species to be mainly determined by genetics (Moran et al. 2017), with a minor impact of other factors, which is coherent with the 3% of variance we found explained by the fungal community composition. Surprisingly, we found that the bacterial community composition did not explain any variation in drought tolerance traits, in contrast to the fungal community composition. This absence of relationship between phyllosphere bacterial communities and tree physiology contrasts with the results obtained by Kembel et al. (2014). In their study, the composition of the leaf bacterial community was found to be linked to several plant functional traits in 57 Neotropical tree species. However, the functional traits included leaf elemental chemistry, leaf morphology, wood density, sapling growth rate, and sapling mortality rate but no drought tolerance traits. In addition, Kembel et al. (2014) assessed how the tree functional traits shaped the leaf microbiota and not the other way around, which could explain the difference observed with our findings. Moreover, in our study, sampling date did have a significant effect on bacterial but not on fungal community composition, suggesting that leaf bacterial communities have a greater temporal turnover than fungal communities, and that associations between trees and leaf bacteria are more labile than tree–fungus associations. This may explain why we did not find any relationship between the taxonomic composition of the leaf bacterial community and drought tolerance traits. A functional or phenotypic approach based on metagenomic data could give a different picture (Barberán et al. 2017; Fierer et al. 2014; Madin et al. 2020).

TABLE 2
Relative contribution of tree species phylogeny, tree geographic location, and leaf microbiota diversity and composition to variations in drought tolerance across 50 tree individuals belonging to 18 Amazonian tropical tree species[a]

Source of variation	Total number of variables (number of significant variables)	Explained variance (%) (adjusted R^2)
Tree phylogeny	20* (9*)	0.41
Tree location	6* (0)	–
Fungal α-diversity	3 (0)	–
Fungal β-diversity	26* (1*)	0.03
Tree phylogeny + fungal β-diversity	–	0.05
Bacterial α-diversity	3 (0)	–
Bacterial β-diversity	28* (1*)	0.00
Tree phylogeny + Bacterial β-diversity	–	0.00

[a] Variation in drought tolerance trait values across tree individuals was analyzed using redundancy analyses. The phylogenetic and geographic distance between trees, and the compositional dissimilarity (β-diversity) in their foliar fungal and bacterial communities, were summarized using principal coordinates of neighbor matrices eigenvectors (*), while the α-diversity of foliar fungal and bacterial communities were each represented by three indices (Chao1, Shannon, and Pielou). A stepwise selection procedure was used to select the variables significantly related to drought tolerance. The number of selected variables is indicated in parentheses. These significant variables were used for variance partitioning. Their corresponding source of variation is indicated in bold.

In addition, our analyses allowed us to identify 27 fungal ASVs whose relative abundance was significantly associated with one or more drought tolerance trait values. Together, these fungal ASVs contributed to 5% of the variance in drought tolerance traits. The contribution of these 27 ASVs is of the same order of magnitude as the contribution of the whole fungal community composition, suggesting that we have identified, among the 8,748 fungal ASVs forming the community, the few that are related to drought tolerance. The statistical framework that we have developed for this study, which combines RDA with TITAN2 (Baker et al. 2019) analysis, thus allowed us to identify a small subset of microbial taxa whose abundance covary with plant quantitative functional traits, among thousands of microbial taxa. Therefore, this statistical framework could be of interest to all studies searching for key players in host–microbiome interactions. It allowed us to test our hypothesis about the relationships between the leaf microbiota and the three groups of traits indicative of drought tolerance (i.e., related to the tree water transport system, stomatal behavior, or leaf residual water losses).

In contrast to our first hypothesis, traits related to stomatal behavior and leaf residual water loss (namely Ptlp, SSM_12, SSM_50, SSM_88, and gmin) were less associated with the leaf microbial community than traits related to water transport (P12, P50, P88, and HSMs). The drought tolerance traits associated with the highest number of fungal ASVs were the xylem HSMs HSM_88 followed by HSM_50, and the xylem embolism resistance P50. SSM_50 ranked only fourth in terms of the number of associations with fungal ASVs. In contrast with our second hypothesis, leaf resistance to embolism traits (P12leaf, P50leaf, and P88leaf, associated with three ASVs) were not more associated with leaf fungi than xylem resistance to embolism (P12, P50, and P88, associated with nine ASVs). These findings might be accounted for by two alternative hypotheses. First, the fungi detected in the leaves could be present or even originate from the branch xylem and have a direct effect on water-transport-related traits (Oliva et al. 2014). Alternatively, because the water-transport-related traits are known to be integrative traits that reflect the whole-plant drought resistance

TABLE 3
Taxonomic assignment and putative ecology of TITAN-selected fungal amplicon sequence variants (ASVs)[a]

ASV ID	Prev[b]	TITAN[c]	Phylum	Class	Order	Family[d]	Genus	Putative ecology	References
ASV_1	74	−	Ascomycota	Sordariomycetes	Xylariales	Sporocadaceae	*Pestalotiopsis*	**Plant pathogen,** widely distributed on trees throughout tropical and temperate regions	(Solarte et al. 2017; Tedersoo et al. 2014)
ASV_9	14	+	Ascomycota	Sordariomycetes	Xylariales	…	…	…	…
ASV_24	48	−	Ascomycota	Sordariomycetes	Xylariales	Sporocadaceae	*Pseudopestalotiopsis*	Plant pathogen, widely distributed on trees throughout tropical and temperate regions	(Maharachchikumbura et al. 2014)
ASV_52	20	−	Ascomycota	Sordariomycetes	Xylariales	Sporocadaceae	*Pestalotiopsis*	**Plant pathogen,** widely distributed on trees throughout tropical and temperate regions	(Solarte et al. 2017; Tedersoo et al. 2014)
ASV_66	20	−	Ascomycota	Eurotiomycetes	Chaetothyriales	…	…	…	…
ASV 112	22	−	Ascomycota	Dothideomycetes	Capnodiales	…	…	…	…
ASV_119	25	−	Ascomycota	Sordariomycetes	Xylariales	Sporocadaceae	*Pestalotiopsis*	**Plant pathogen,** widely distributed on trees throughout tropical and temperate regions	(Solarte et al. 2017; Tedersoo et al. 2014)
ASV_127	13	−	Basidiomycota	Tremellomycetes	Tremellales	…	…	…	…
ASV_128	26	−	Ascomycota	Dothideomycetes	Botryosphaeriales	Phyllostictaceae	…	Plant pathogen, widely distributed on trees throughout tropical and temperate regions	(Wikee et al. 2011)
ASV_131	7	−	Ascomycota	Dothideomycetes	Capnodiales	Mycosphaerellaceae	*Pseudocercospora*	**Plant pathogen,** widely distributed on trees throughout tropical and temperate regions	(Crous et al. 2013; Tedersoo et al. 2014)
ASV_162	9	+	Ascomycota	Eurotiomycetes	Chaetothyriales	…	…	…	…
ASV_224	19	−	Ascomycota	Dothideomycetes	Capnodiales	…	…	…	…
ASV_276	18	+	Ascomycota	Eurotiomycetes	Chaetothyriales	Chaetothyriales *fis*	*Strelitziana*	**Plant pathogen,** leaf epiphyte frequent in tropical plants	(Crous et al. 2015; Tedersoo et al. 2014)
ASV_296	25	−	Basidiomycota	Agaricomycetes	Polyporales	…	…	…	…
ASV_303	16	−	Ascomycota	Dothideomycetes	Capnodiales	Tetratosphaeriaceae	*Devriesia*	**Plant pathogen**	(Crous et al. 2012; Tedersoo et al. 2014)
ASV_305	16	−	Ascomycota	Sordariomycetes	Diaporthales	Diaporthaceae	*Diaporthe*	**Endophyte, plant pathogen,** wide range of plant hosts	(Gomes et al. 2013; Tedersoo et al. 2014)
ASV_355	10	−	Ascomycota	Eurotiomycetes	Chaetothyriales	…	…	…	…
ASV_396	25	−	Ascomycota	Sordariomycetes	Xylariales	Xylariaceae	*Xylaria*	…	…
ASV_455	9	−	Ascomycota	Dothideomycetes	Capnodiales	Teratosphaeriaceae	*Devriesia*	**Plant pathogen**	(Crous et al. 2012; Tedersoo et al. 2014)
ASV_515	18	−	Ascomycota	Dothideomycetes	Capnodiales	…	…	…	…
ASV_636	12	+	Ascomycota	Dothideomycetes	Venturiales	Sympoventuriaceae	*Ochroconis*	**Undefined saprotroph, plant pathogen,** endophyte, cosmopolitan distribution	(Tazik et al. 2020; Tedersoo et al. 2014)
ASV_942	10	+	Ascomycota	Dothideomycetes	Capnodiales	…	…	…	…
ASV_1201	8	−	Basidiomycota	Agaricomycetes	Russulales	Peniophoraceae	*Peniophora*	**Plant pathogen, wood endophyte,** mainly described in temperate trees	(Boddy and Rayner 1983; Rishbeth 1963; Tedersoo et al. 2014)
ASV_1261[e]	11	+	Ascomycota	Sordariomycetes	Hypocreales	Hypocreales *fis*	…	…	…
ASV_1387	8	−	Ascomycota	Sordariomycetes	Xylariales	…	…	…	…
ASV_1640	11	+	Ascomycota	Dothideomycetes	Capnodiales	…	…	…	…
ASV_1796	6	−	Basidiomycota	Agaricomycetes	…	…	…	…	…
ASV_2073	11	−	Basidiomycota	Tremellomycetes	Tremellales	Cuniculitremaceae	…	Plant pathogens	(Kirschner et al. 2001)

[a] The putative ecology of ASVs was derived from their taxonomy at the family or genus level when available. The putative fungal guild (in bold) was determined using the FunGuild (Nguyen et al. 2016) database when available, and additional information (in regular font) was retrieved from the literature.
[b] Prevalence (Prev) indicates the number of trees where the ASV was found (total = 88 tree individuals).
[c] Symbols + and − indicate the direction of the relationship between TITAN-selected ASV and drought tolerance.
[d] Abbreviation: *fis* = fam Incertae sedis.
[e] ASV which was only selected in the TITAN2 analysis of leaf resistance to embolism, based on 35 tree individuals.

(Blackman et al. 2019a; Urli et al. 2013), they might correlate with some other plant features that could be directly influenced by leaf microbes.

Over the thousands of fungal ASVs colonizing leaves of Neotropical trees that we studied, our analyses identified 21 fungal variants that were negatively associated with drought tolerance traits. Those for which we could infer a putative ecology belonged to taxonomic groups usually described as fungal pathogens. The fungal ASV that was the most negatively associated with drought tolerance traits covaried with SSMs (SSM_12 and SSM_50) and xylem resistance to embolism (P12 and P50). This ASV (ASV_199) was assigned to the *Pestalotiopsis* genus. Two other ASVs (ASV_1 and ASV_52) that were also assigned to the *Pestalotiopsis* genus covaried with Ptlp, a trait reflecting stomatal behavior, together with SSM_50 for ASV_1. These results suggest that the presence of *Pestalotiopsis* spp. alters both leaf-related and water-transport-related traits. The *Pestalotiopsis* genus contains plant pathogens with widespread distribution in tropical areas that are responsible for a wide range of diseases on aerial plant parts (Espinoza et al. 2008; Maharachchikumbura et al. 2014). Species from the *Pestalotiopsis* genus have been reported to primarily cause leaf lesions and, subsequently, disseminate in stems (Chen et al. 2012), which could account for their effect on both leaf-related and water-transport-related traits. Similarly, ASV_2073 was negatively associated with both SSM (SSM_50) and drought tolerance traits related to the water transport system (P12, P50, and HSM_50). This ASV was assigned to the Cuniculitremaceae family, which contains putative bark pathogens (Kirschner et al. 2001). Overall, these results suggest that some foliar fungi tend to decrease tree drought tolerance because they also colonize and cause disease in woody parts of trees, altering the water transport system.

The fact that we found a higher abundance of putative plant pathogens in trees with a lower drought tolerance could actually result from two mechanisms. On the one hand, the alteration of tree physiology by drought favors pathogen establishment (Desprez-Loustau et al. 2006). Woody organs, in particular, have an increased susceptibility to pathogen attacks under drought stress (Jactel et al. 2012). In our study, lower safety margins represent a higher probability of drought stress for trees and could imply an increased susceptibility to pathogens. On the other hand, fungal pathogens can reduce hydraulic performance and, thus, lower tree drought tolerance (Oliva et al. 2014). For instance, they can reduce stomatal conductance (Hajji et al. 2009) or induce the formation of tyloses blocking water transport and reducing xylem conductance (Yadeta and Thomma 2013). Thus, the negative relationships we found between some fungal ASVs and xylem resistance to embolism or SSMs may be the result of a direct impact of fungi on tree hydraulic functions. In both cases, the fact that taxa described as putative plant pathogens were more abundant in the most drought-susceptible trees suggests that drought-susceptible trees could suffer from additional threats in case of drought events.

Moreover, our analyses identified six fungal variants positively associated with drought tolerance in Neotropical trees. Among them, two were taxonomically assigned to the genus level. The first one (ASV_276) covaried with the tree HSM_88 and was assigned to the *Strelitziana* genus, within which several species have been described as leaf endophytes (Chen et al. 2020; Crous et al. 2015). The second (ASV_636) covaried with both the SSM and xylem resistance to embolism (SSM_50 and P50) and was assigned to the *Ochroconis* genus. There is no straightforward hypothesis to explain this result because species in the *Ochroconis* genus have various lifestyles; several species have been described as opportunistic pathogens with a wide host range (Tazik et al. 2020) while others have been found as root endophytes in nonsymptomatic plants (Zeng et al. 2021).

To go further, culture-dependent studies are needed. Isolation, culture, and inoculation of leaf fungal species belonging to the *Strelitziana* and *Ochroconis* genera are the only ways to confirm their positive effect on drought tolerance in Neotropical trees and to analyze the underlying mechanisms. Therefore, our results provide guidance for future culture-dependent studies that will investigate the influence of the tree microbiota on tree drought tolerance. The leaf fungal community of the most drought-tolerant trees of the Paracou Research Station (French Guiana) could be isolated using high-throughput isolation methods (Collado et al. 2007) that include plant based media (Sarhan et al. 2019) and screened for the two genera of interest. The fungal isolates could then be inoculated into tree saplings, as described in previous work (Arnold and Engelbrecht 2007; Arnold et al. 2003), and their effect on drought tolerance traits could be measured and compared in the lab or in common gardens. Fungal isolates enhancing drought tolerance could then be tested on other plant species, including crops (Rho et al. 2018b).

Although our results may guide future studies, they need to be considered carefully. Indeed, this study revealed statistical correlations between the relative abundances of several fungal variants and drought tolerance traits in Neotropical trees. These statistical correlations seemed consistent and robust because no fungal ASV decreased in abundance with a trait but increased with another, and conversely. We preferentially interpreted them as a potential impact of leaf fungi on tree drought tolerance for two reasons. First, such an effect is plausible according to the literature (Arnold and Engelbrecht 2007; Ferus et al. 2019; Hajji et al. 2009; Oliva et al. 2014). Second, our variance partitioning approach tested the amount of variance in drought tolerance traits explained by the variance in leaf microbial communities. However we cannot exclude the possibility that the actual causality, if any, is the other way around. As mentioned above, experimental approaches are required to determine the causal relationship between fungal taxa and tree drought tolerance, and to discover underlying mechanisms. A way to get an insight into the mechanisms would be to use metagenomic approaches such as in Lajoie et al. (2020) to associate specific genes and functions of the leaf fungal community with drought tolerance traits. However, even doing this, culture-dependent analysis with single or multiple strains will ultimately be necessary to validate the effect of leaf microbiota on drought tolerance (Compant et al. 2020).

TABLE 4
Relative contribution of tree species phylogeny and foliar fungi to variations in drought tolerance across 50 tree individuals belonging to 18 Amazonian tropical tree species[a]

Source of variation	Number of variables	Explained variance (%)[b]
Tree phylogeny	9*	0.55
Fungal β-diversity	27	0.07
Tree phylogeny + fungal β-diversity	–	0.00

[a] Variation in drought tolerance trait values across tree individuals was analyzed using redundancy analyses. Traits did not include P12leaf, P50leaf, and P88leaf, which were only available for 35 individuals. The phylogenetic distance between trees was summarized using principal coordinates of neighbor matrices eigenvectors (*). Foliar fungi were represented by 27 variables, corresponding to the relative abundance of the 27 fungal amplicon sequence variants (ASVs) associated with drought tolerance according to TITAN2 analysis. This excludes ASV_1261, which was only associated with P12leaf and P50leaf. All variables were used for variance partitioning.

[b] Adjusted $R = S/[\log(N)]$, where S is the Shannon index and N the number of ASVs of a given sample.

Culture-dependent approaches are also required to validate the taxonomy and functions of the fungal taxa we detected using metabarcoding approaches. Indeed, taxonomic identification based on a small region of the ITS does not always allow an identification to the species level, especially in tropical environments where reference databases are not exhaustive. Then, the large number of fungal pathogens we found among the taxonomically assigned ASVs might be due to their overrepresentation in taxonomic reference databases and in databases linking taxonomy to function. Moreover, the fact that some taxa were described as pathogens in the literature does not mean that they were actually pathogens in the asymptomatic leaves we collected for the study because fungi can alternate between commensal and pathogenic stages (Kuo et al. 2014). Despite these methodological limitations, our results represent a breakthrough in the study of the functional role of the phyllosphere microbiota because they narrow the scope of possibilities for future experimental studies aimed at identifying microorganisms that influence drought tolerance. Based on our results, studies should first focus on fungi, rather than bacteria, and focus first on the fungal genera identified by our analyses such as *Strelitziana* and *Ochroconis.*

Conclusion. In this study, we combined ecophysiological measurements and microbial community analyses to better understand the drivers of drought tolerance in Neotropical forests. We found a significant relationship between the composition of the leaf mycobiome and variations in drought tolerance across Neotropical trees and identified, among the thousands of fungal taxa colonizing leaves, approximately a dozen fungal taxa that may contribute to these variations. Among this small set of fungal taxa associated with variations in drought tolerance, most taxa were described as putative plant pathogens in the literature and were more abundant in the most drought-susceptible trees. This suggests that the hypothesis of drought-susceptible trees being associated with fungal pathogens representing an additional threat in case of drought events might deserve some attention in future forest management research. We also identified two fungal genera that increased in abundance with drought tolerance. These genera may be good candidates for future culture-dependent studies aimed at identifying microorganisms that influence drought tolerance. The isolation and experimental study of strains belonging to these two genera might allow us to improve our knowledge of the mechanisms of drought tolerance in Neotropical trees, and strains that effectively improve drought tolerance might be considered for plant biostimulation under drought stress.

Data availability. The raw sequence data were deposited in the European Nucleotide Archive under the study accession number PRJEB42566 (https://www.ebi.ac.uk/ena/browser/view/PRJEB42566). The individual accession numbers of each sample are found in Supplementary Table S1. The filtered ASV tables, sample metadata, and codes to produce figures and tables are available at https://gitlab.com/marccamb/drought-microbiota-paracou-draft.

ACKNOWLEDGMENTS

We thank the editor and the reviewers for their relevant comments that greatly improved the manuscript; all members of the tree climbing team (Valentine Alt, Samuel Counil, Anthony Percevaux, and Elodie Courtois) for their contribution to the sampling campaign; all members of the PGTB sequencing facility for providing molecular biology labs, reagents, and protocols; Marie Simonin for helpful advice on the statistical analyses; and Usman Hussain for checking the English language of the manuscript.

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