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Living far from the ground: Strategies of forest epiphytes

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Living far from the ground: Strategies of forest epiphytes

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ORIGINAL RESEARCH
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Getting a Grip on the Adhesion Mechanism of Epiphytic Orchids – Evidence From Histology and Cryo-Scanning Electron Microscopy

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Plants and animals evolve different attachment structures and strategies for reversible or permanent adhesion to different substrate types. For vascular epiphytes, having the ability to permanently attach to their host plants is essential for establishment and survival. Unlike mistletoe roots, roots of vascular epiphytes do not penetrate the host tissues but instead achieve attachment by growing in close contact to the surface of the substrate. However, the fundamental understanding of the attachment functions of epiphytic roots remains scarce, where majority of studies focused on the general root morphology, their functional properties and the descriptions of associated microbial endophytes. To date, research on attachment strategies in plants is almost entirely limited to climbers. Therefore, this study aims to fill the knowledge gap and elucidate the attachment functions of roots of epiphytic orchids. With the use of histology and high-resolution cryo-scanning electron microscopy (cryo-SEM) technique with freeze fracturing, the intimate root-bark substrate interface of epiphytic orchid *Epidendrum nocturnum* Jacq was investigated. Results showed a flattened underside of the root upon contact with the substrate surface, and the velamen layer appeared to behave like a soft foam, closely following the contours of the substrate. Root hairs emerged from the outermost velamen layer and entered into the crevices in the substrate, whenever possible. A layer of amorphous substance (glue-like substance) was observed on the surface of the root hairs. Combining the observations from this study and knowledge from previous studies, we hypothesised that epiphytic orchid roots produced a layer of glue-like substance to adhere the root to the substrate. Then root hairs are produced and enter into the voids and crevices of the substrate. This further generates a mechanical interlocking mechanism between root and substrate, thus reinforcing the attachment of the root (and hence the whole plant) to its substrate.

Keywords: adhesion, attachment, epiphytes, root hairs, glue-like substance, attachment strategy

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INTRODUCTION

Attachment structures and strategies that evolved in animals and plants allow reversible or permanent adhesion under diverse environmental conditions and/or to different substrate types (Gorb, 2008). Attachment is achieved by a diversity of biological structures: hooks and spikes (Darwin, 1875; Rowe and Isnard, 2009), smooth pads (Gorb, 2001, 2007; Drechsler and Federle, 2006; Barnes, 2007), thin hairs (Autumn et al., 2002; Geim et al., 2003;

Gao et al., 2005), suction cups (Ditsche and Summers, 2019), byssus plaques (Silverman and Roberto, 2007), or attachment pads on adventitious roots (Groot et al., 2003; Seidelmann et al., 2012). As summarized in the review of Gorb (2008), these structures fulfill different biological functions including: (1) firm anchorage for stability (e.g., Walker, 1981; Flammang, 2006); (2) reversible adhesion for effective locomotion (e.g., Irschick et al., 1996; Federle and Endlein, 2004; Tian et al., 2006); (3) firm grip on prey (e.g., Bauer and Kredler, 1988; Betz and Kölsch, 2004); (4) temporary attachment for copulation (e.g., Aiken and Khan, 1992; Voigt et al., 2017); or (5) attachment to a surface in the context of, e.g., pollination or dispersal (Kiviniemi, 1996; Krenn et al., 2005). Both the first and last function aptly apply to the lifeform of vascular epiphytes since they are permanently attached to their host plants (Zotz, 2016).

Epiphytes are, by definition, non-parasitic plants structurally dependent on trees. This lifeform takes advantage of previously unexploited spatial resources (Lüttge, 2007) such as crotches and branches in a tree canopy. Apart from having to cope with the low and intermittent supply of water and – to a lesser degree – nutrients associated with epiphytism (Zotz et al., 2021b), a major challenge for epiphytes is to stay attached to their hosts. Compared to terrestrial plants that develop deep root systems for stable anchorage (Coutts, 1983; Ennos, 2000), many epiphytes grow on bare bark with little to no substrate. Unlike mistletoe roots, roots of vascular epiphytes do not penetrate host tissue, but typically achieve attachment by growing in close contact to the surface of the substrate. Secured attachment is vital for the survival of epiphytes, because the chances of survival on the ground are low (Matelson et al., 1993) in case of dislodgement by wind or heavy rain (Rodríguez-Robles et al., 1990; Tremblay, 2008). Surprisingly, the fundamental understanding of how these plants achieve attachment to their host *via* their roots is still lacking. Most of the available studies on epiphytic roots focus on general root morphology (e.g., Benzing et al., 1982; Brighigna et al., 1990; Oliveira and Sajo, 1999; Moreira and Isaias, 2008; Thangavelu and Ayyasamy, 2017), functional properties such as the water and nutrients absorption capacity of the orchid velamen (e.g., Pridgeon, 1987; Zotz and Winkler, 2013; Joca et al., 2017), and descriptions of associated microbial endophytes (e.g., Bernard, 1911; Pereira et al., 2003; Tsavkelova et al., 2007; Sathiyadash et al., 2012).

To date, research on attachment strategies in plants is almost entirely limited to climbers (Isnard and Silk, 2009). Climbing is achieved *via* different devices or mechanisms, such as twining (e.g., *Ipomoea purpurea*; Scher et al., 2001), by hooks (e.g., *Galium aparine*; Bauer et al., 2011), by tendrils (e.g., *Parthenocissus tricuspidata*; Steinbrecher et al., 2010), or with adventitious pads originating from roots (e.g., *Hedera helix*; Melzer et al., 2010). Several attachment systems of climbers from various families are well-characterized: for example, Moraceae (*Ficus pumila*; Groot et al., 2003), Araliaceae (*H. helix*; Zhang et al., 2008; Melzer et al., 2010, 2011), Vitaceae (*Parthenocissus quinquefolia*, *P. tricuspidata*; Scherge and Gorb, 2001; Bowling and Vaughn, 2008; Steinbrecher et al., 2010, respectively), Bignoniaceae (*Amphilophium crucigerum*; Seidelmann et al., 2012), and Passifloraceae (*Passiflora discophora*; Bohn et al., 2015). Most recently, the attachment mechanism of seagrass using a combination of mechanical interlocking of subdividing root hairs and glue-like substances was described (*Posidonia oceanica*; Zenone et al., 2020a,b).

Considering that epiphytes attach *via* their roots to the host plant, their attachment mechanism may be similar to that of root climbers and seagrasses that adhere to either bare bark or rocks. Hence, their adhesion strategies might shed some light on the attachment mechanism of epiphytes. For example, Melzer et al. (2011) state that the root climber English Ivy *H. helix* attaches to nearly any surface *via* natural-forming glue and shape-changing root hairs. On the underside of its stem, ivy produces numerous adventitious roots with root hairs that secret a glue-like substance, allowing the plant to adhere onto surfaces, such as tree bark, rocks or walls. For a permanently secured hold to the substrate, the root hairs apparently take on a spirally cracked shape and interlock themselves within the crevices of the substrate (Melzer et al., 2011). Spirally cracked root hairs were also reported in the climber *Syngonium podophyllum* (Yang and Deng, 2017), again suggesting a link between these structures and anchorage. Spirally cracked root hairs have also been found in seagrasses *P. oceanica* (Tomasello et al., 2018). In *P. tricuspidata*, the attachment pads appear to adapt their surface to the substrate geometry and exude an adhesive fluid that fills any space underneath the pad for adhesion to the substrate (Scherge and Gorb, 2001; Steinbrecher et al., 2011). Finally, Zenone et al. (2020b) found that the root hair tips of seagrasses branch and form a pad-like structure, increasing the contact area with the corrugated rock surface. Additionally, a glue-like substance fills gaps between the pad base and the substrate, which further enhances contact area and increases adhesion. However, further investigations are necessary to validate the functions of the various root hair forms (i.e., branches and spirals): do they also increase nutrients absorption by increasing root hair surface-volume ratio, and do they only enhance root contact to the substrate?

Orchidaceae is one of the largest families of flowering plants (Dressler, 1993), with 75% of all the species in the family being epiphytic, constituting the largest percentage (68%) of all epiphytes (including hemiepiphytes) (Zotz et al., 2021c). Epiphytic orchids produce roots that either remain aerial (i.e., free-hanging and not attached) or attach to a substrate. Several studies noted that attached roots are no longer spherical in shape: roots become hemispherical, with one side flattened to follow the contours of the substrate, with root hairs being restricted to the side in contact with the substrate (e.g., Dycus and Knudson, 1957; Stern and Judd, 1999; Stern, 2014; Ponert et al., 2016; Thangavelu and Ayyasamy, 2017; Balachandar et al., 2019a; Deseo et al., 2020). Root hairs of epiphytic orchids have been reported in older parts of the root as well (Dycus and Knudson, 1957; Almeida et al., 2016), further indicating that the root hairs may have other functions beside nutrient and water uptake. Orchid root hairs also exist in various forms, apart from the usual cylindrical shape: there are branched, spiraled and club-shaped thickened versions of root hairs (Leitgeb, 1865; Balachandar et al., 2019a). Spirally cracked root hairs form mainly *via* the rupturing of the outer cell wall layer, which subsequently causes the inner wall layer to

FIGURE 1 | (A) *Epidendrum nocturnum* in its natural habitat in Panama. The green fleshy roots are growing on the surface of the substrate. **(B)** An example of a root segment that was collected and preserved in 50% ethanol.

stretch and break (Bernal et al., 2015). However, the phenomenon may also be an artifact following the forceful detachment of roots from their substrate. Some authors hypothesized that these spiral root hairs aid in capillary water absorption or in improved adherence to the substrate (Bernal et al., 2015; Almeida et al., 2016). The similarities with climbing plants and seagrasses (i.e., root hairs on root surface contact substrate; presence of spiral-shaped root hairs) are good starting points to study the attachment mechanism of epiphytic orchids.

Given the lack of reports demonstrating exactly how orchid roots are attached to their host plant, the present study aimed at filling that knowledge gap and elucidate the attachment functions and mechanism of roots of epiphytic orchid. With the use of histology and a high-resolution cryo-scanning electron microscopy (cryo-SEM) technique combined with freeze fracturing, we directly studied the intimate interface between the root and its substrate. From these images and knowledge from previous publications, we propose a schematic diagram, to summarise our hypothesis of how epiphytic orchid roots (and hence the whole plant) adhere to their substrates.

MATERIALS AND METHODS

Plant Materials

Fieldwork was conducted in April 2019 in the Barro Colorado Natural Monument on Lake Gatun, Panama. To collect root samples for microscopy analyses, three adult epiphytic orchids, *Epidendrum nocturnum* Jacq attached to different *Annona glabra* tree branches were identified (**Figure 1A**). *A. glabra* are restricted to the marshes around the coast and islands, and the trees have a short stature, rarely growing to a height of more than 7 m (Croat, 1978) and have a relatively open crown structure. The trees were accessed with a boat for samples collection, therefore the roots were mainly collected from orchids which were maximum 2 m above the boat. For each plant, one root (with 5 cm measured from the root cap discarded) was collected together with the bark substrate still attached. The remaining roots were about 5 cm long and were further divided into three parts, ca. 1.5 cm each, totaling nine pieces of root segments (**Figure 1B**). To examine if free hanging orchid roots had any root hair development, roots that were not attached to any substrate were also collected from another three independent *E. nocturnum* plants. The roots were prepared into nine root segments as stated above. These root segments were preserved in individual Eppendorf tubes with 50% ethanol for export to Germany, where further microscopy analyses were carried out.

Initial Examination of Roots – Freehand Thin Sections

To examine if there were any of root hairs on the orchid roots, both attached and free hanging root segments were examined. Three root segments from each group were randomly selected to perform the freehand cuts. For the root segments with attached substrate, the substrates were carefully removed to better visualize any root hairs that were growing onto the substrate. Freehand cuts were prepared with a disposable razor blade. The cuts were bleached for 30 min in a solution of 2% NaClO to clear cell contents. Next, the cuts were rinsed three times in distilled water and then stained for 1 min with safranin (0.1 g in 100 ml deionized water) to stain lignified/suberized cell walls red. These cuts were further soaked for 2–3 h in 70% ethanol to remove excess stain. Fifteen cuts from each group (free-hanging and attached roots) were randomly selected and were viewed at 2–8× zoom ratio with a stereo microscope Olympus Type SZX16 (objective lens total magnification 2.1–690×, Olympus, Japan). Pictures were taken with a connected digital camera (Olympus UC30, 3.2 megapixel, Olympus, Japan).

Histology – TECHNOVIT®

To examine the root hair-substrate interface and to understand how the root is attached to the substrate, histological examinations of the root hair-substrate cross-sections were carried out. To prepare the samples for histological examinations, another three root segments of *E. nocturnum* (with attached substrate) were selected and further sliced into 0.2 cm root sections. This was to ensure that the root sections were as small as possible for effective dehydration and infiltration of the resin into the root and bark cells. During the process of cutting the root segments into smaller sections, some bark substrates were separated from the root. Among those roots that were still attached to a piece of bark substrate, 10 pieces

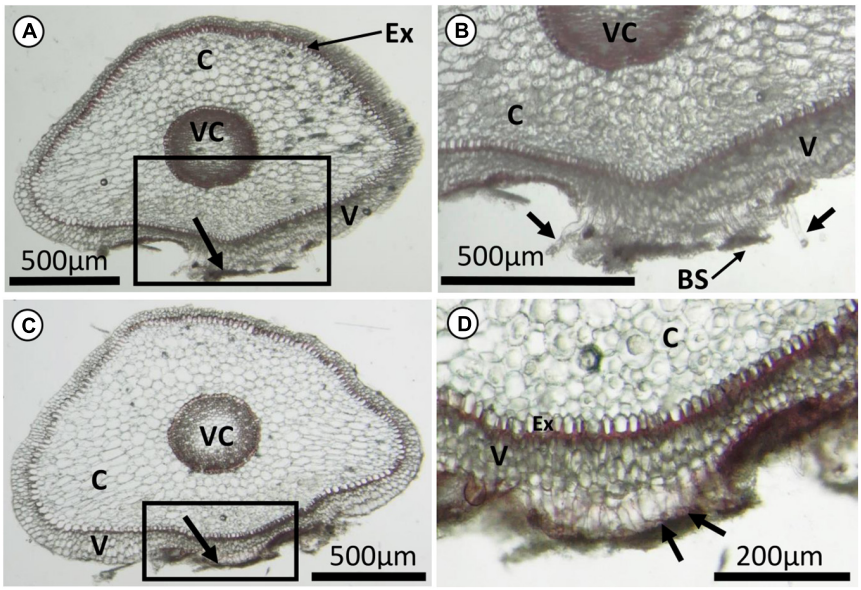

FIGURE 2 | Freehand cuts of roots of *Epidendrum nocturnum* with most of the substrate removed. **(A,C)** Non-cylindrical root sections, that were flattened at the underside of the root in contact to the bark substrate (indicated by black arrows). **(B,D)** Enlarged images corresponding to the boxes in **(A,C)**, respectively. There were extensions of tubular root hairs from the velamen layer. Some of the root hairs tips were probably broken off when the substrate was removed before making the cuts (black arrows in **B**), and some were still adhering to the substrate (black arrows in **D**). Ex, exodermis with U-thickened walls; C, cortical parenchyma; V, velamen; VC, vascular cylinder; BS, bark substrate.

were randomly selected for infiltration and embedding in TECHNOVIT® 7100 (Kulzer GmbH, Wehrheim, Germany). TECHNOVIT® is a polymerization system based on 2-hydroxyethyl methacrylate that remains transparent after polymerization. The root segments were already preserved in 50% ethanol, therefore, further dehydration proceeded in an ascending ethanol concentration series as the following: 70, 90, 96, 96, and 99% EtOH, changed every other hour, last step overnight. Then, the samples were pre-infiltrated with a solution made from equal parts of 99% EtOH and TECHNOVIT® 7100, at 4°C overnight. If samples were floating after the change in ethanol/pre-infiltration solution, they were placed in a vacuum desiccator for at least 10 min (or until samples fall to the bottom of the Eppendorf tube), to remove all moisture within the samples. Lastly, samples were infiltrated with a solution made of TECHNOVIT® 7100 and Hardener 1, at 4°C overnight. This step was repeated to ensure that the bark substrate was fully infiltrated. For polymerization, the infiltrated samples were arranged carefully into the Teflon Histoform embedding cavities (Size: S, Kulzer GmbH, Wehrheim, Germany), and filled with 0.7 ml TECHNOVIT® polymerization solution each. Polymerization was completed in 2 h at room temperature. To release the polymerized blocks from the mold, a Histobloc carrier part was glued to the polymerized blocks with TECHNOVIT 3040 (2-component resin based on methyl-methacrylate) (Kulzer GmbH, Wehrheim, Germany).

The polymerized blocks (total 10 blocks) were clamped in a microtome (Reichert-Jung SUPERCUT 2050, Wetzlar, Germany). Thin sections (5 µm) of the blocks were cut by HISTOBLADES (Kulzer GmbH, Wehrheim, Germany). Firstly, the blocks were sliced at 8 µm to remove any uneven surfaces. Then, thin sections of 5 µm were made. During this process, it was common that thin sections were curling onto themselves and were unusable. Nonetheless, at least 10 undamaged sections of each block were collected in a water bath at 20–25°C. Thin sections were air dried for at least 2 h on microscope slides (VWR®), subsequently stained in 0.05% toluidine blue aq. (toluidine chloride 1272, Merck KGaA, Darmstadt, Germany) for 1 min, washed three times in distilled water for 30 s each, and air dried again overnight. Toluidine blue is a polychromatic dye used to stain carboxylated polysaccharides with pinkish purple; lignin and tannins with greenish blue or bright blue; and nucleic acids with purplish or greenish blue (O'Brien et al., 1964). Lastly, these thin sections were mounted in ENTELLAN® (107961, Merck KGaA, Darmstadt, Germany). Four microtome cuts from each of the 10 undamaged sections made were randomly selected and viewed at 4–25 × magnification (with a 10 × eyepiece magnification) using a light microscope POLYVAR Type 300602 (Reichert-Jung, Wetzlar, Germany). Pictures were taken with a connected camera (Olympus UC30, 3.2 megapixel, Olympus, Japan).

Cryo-Scanning Electron Microscopy

The remaining three root segments of *E. nocturnum* (with attached substrate) were prepared for cryo-SEM examination. The samples were shock-frozen (−140°C) to study the root-substrate interface in a SEM Hitachi S-4800 (Hitachi High-Technologies Corp., Tokyo, Japan) equipped with a Gatan ALTO 2500 cryo-preparation system (Gatan Inc., Abingdon, United Kingdom). For details of sample preparation and

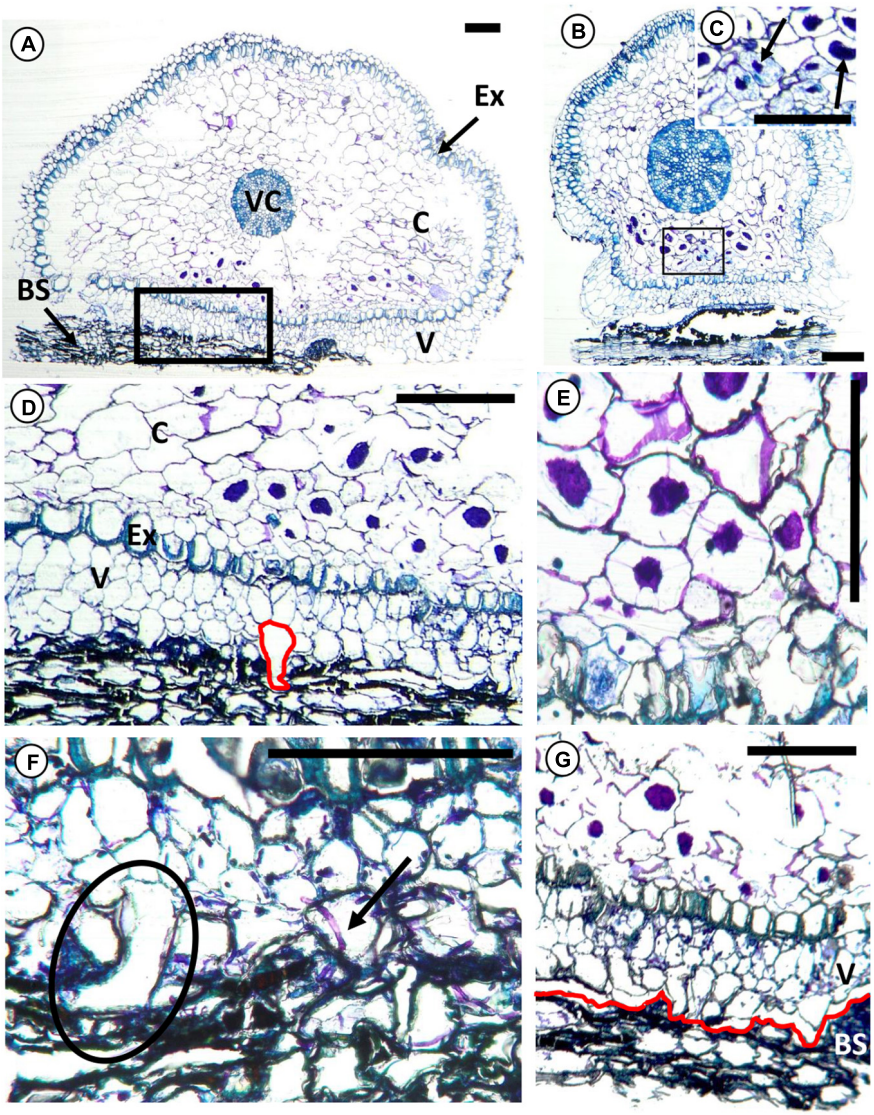

FIGURE 3 | Transverse histological sections of root segment of *Epidendrum nocturnum* in contact with the bark substrate of the tree *Annona glabra*, with toluidine blue staining. Only the outermost portion of the phellem, is shown. **(A,B)** Non-cylindrical root segment with root hairs adhering to the bark substrate. **(C)** Cortex fungus (in association to the mycorrhizae) forming pelotons (arrows). They are mainly found in cells next to the substrate. **(D)** Root hairs enter crevices in the bark substrate. The root hair, highlighted in red, has a vertical length of 120 μm. **(E)** Adjacent cells with pelotons communicated by hyphae. **(F)** Root hair growing onto bark substrate (circle) and a piece of mycorrhizal hyphae extending from the bark substrate into the epidermal cells (arrow). **(G)** The outermost velamen layer replicated the contours of the substrate layer (highlighted in red). The root got separated from the bark substrate during the slicing procedure with the microtome. Ex, exodermis with U-thickened walls; C, cortical parenchyma; V, velamen; VC, vascular cylinder; BS, bark substrate. Scale bars, 200 μm.

mounting for cryo-SEM, see Gorb and Gorb (2009). Cryo-SEM is useful, where preservation of the natural morphology of cells and tissues is desired. Additionally, it allows visualization of fluids, which might be crucial in the case of root-substrate attachment. Mounted root segments with bits of bark substrate attached were sputter-coated in frozen conditions with gold-palladium (thickness 10 nm) and examined at 3 kV acceleration voltage and temperature of −120°C in the microscope.

To ensure that the results of the cryo-SEM analyses were not artifacts due to the preparative procedures and long storage in ethanol, three 1 cm fresh root samples of another epiphytic orchid, *Dendrobium koordersii* J.J.Sm, mounted onto *Robinia pseudoacacia* bark, were collected in January 2021 in the greenhouse in the Botanical Garden Kiel, Germany. All plant names follow The Plant List (2013).

RESULTS

The majority of the freehand cuts of the *E. nocturnum* roots (with attached substrate) displayed a semi-circular shape, with the underside of the root being flattened out to the substrate surface

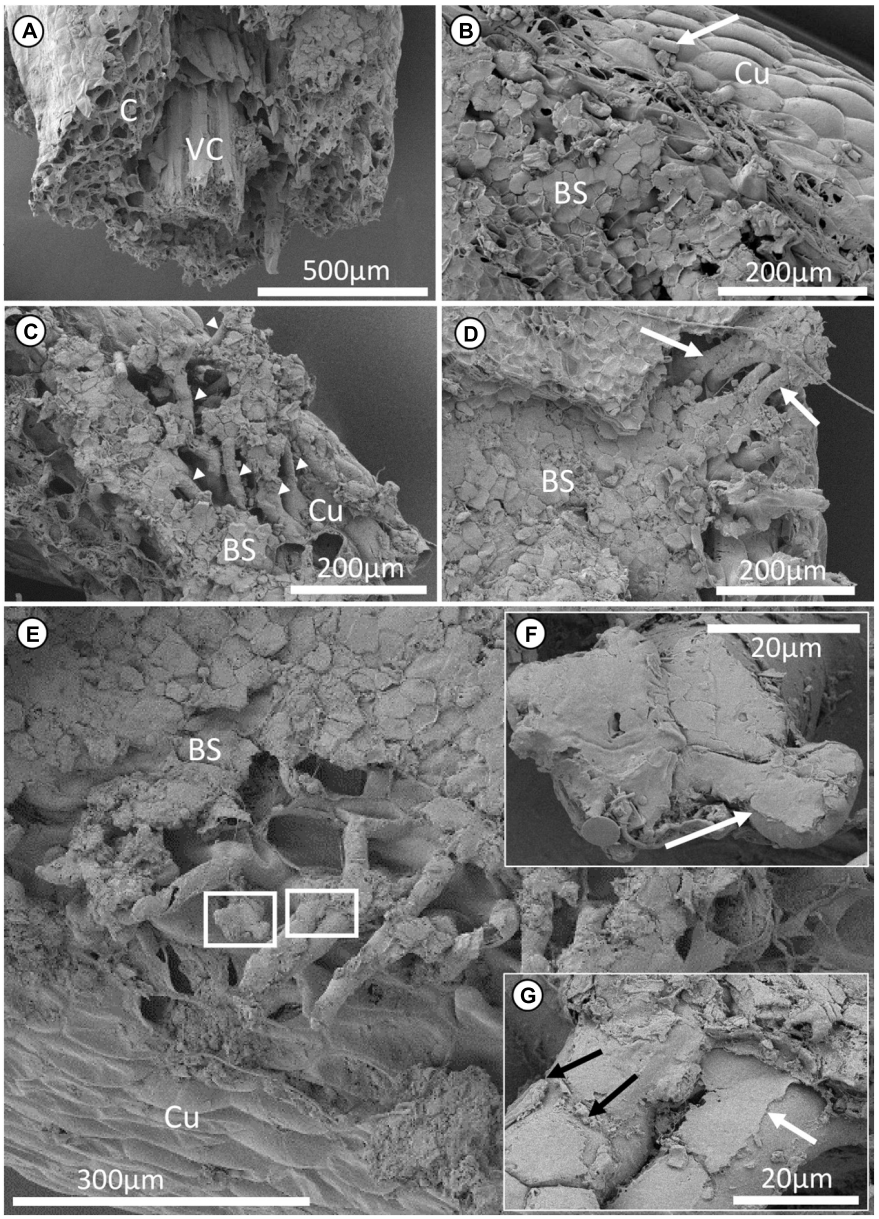

FIGURE 4 | Cryo-scanning electron microscopy micrographs of a segment of *Epidendrum nocturnum* root attached to bark substrate of *Annona glabra*. **(A)** Transverse section of the root segment. **(B–E)** View of how root hairs are attached to the bark substrate. **(B)** Bark substrates are attached to the cuticle of the root. Some diatoms (together with other micro-organisms) can also be observed on the root surface. White arrow points at a diatom. **(C,D)** Root hairs growing between the crevices of the layers of bark substrate (white arrowheads and arrows). Pockets of hollow spaces are present between the root hairs. **(F,G)** Enlarged images corresponding to the boxes in **(E)**. **(F)** The end of a single root hair that is flattened due to the extremely thin cell wall of the root hair, probably appressed to the substrate and molded according to the microstructure of the substrate. **(G)** A layer of amorphous substance (glue) is adhering to the root hair (white arrow, also labeled in **F**). Pieces of bark substrates were attached or imprinted (black arrows) to the root hairs. C, cortical parenchyma; VC, vascular cylinder; BS, bark substrate; Cu, cuticle.

(**Figures 2A,C**). This was in comparison to the free-hanging roots that remained spherical in shape, without any flattened sides (**Supplementary Figure 1**). From the roots with attached substrate, root hairs were clearly present as extensions of the velamen layer, from the part of the root that was attached to the substrate (**Figures 2B,D**). However, on the free-hanging roots, no root hairs were observed in any of the cuts from the sample (**Supplementary Figure 1**). Both attached and free-hanging roots did not have a uniform number of velamen layers in the cross-sections (**Figures 2A,C** and **Supplementary Figure 1**).

To have a better illustration of how the root hairs interacts with the substrate, histological thin sections were examined. In some cases, the root and bark cells were not well infiltrated with Technovit, hence, those thin sections were not successful

and there were incomplete cell structures (not shown). For those thin sections with successful infiltration, the root cells also showed a flattened side on the part that was in contact with the substrate (**Figures 3A,B**). Root hairs were emerging from the outermost velamen layer facing the substrate (**Figures 3A,D,F,G**), growing into the crevices of the substrate (**Figures 3D,F**). On the underside of the root, the outermost velamen layer appeared to behave like a soft foam, closely following the contours of the adhered substrate (**Figures 2D**, **3G**).

For the cryo-SEM observations, root segments were examined (**Figure 4**) and a layer of attached substrate was gently removed to reveal the root hairs underneath (**Figures 4B,C**). Comparing the preserved and fresh samples, the preserved samples had more micro-organisms observed on the root and substrate surfaces. For examples, many diatoms were seen on the *E. nocturnum* roots (**Figure 4B**) that came from Panama but not on the *D. koordersii* samples from the greenhouse in Kiel. Nonetheless, the samples were similar in terms of having root hairs distributed along the length of the root segments and root hairs interacting with the substrate. Root hairs were growing between the layers of the substrate and in the crevices wherever possible (**Figures 4C–E** and **Supplementary Figures 2C–E**). Root hair size and length varied: hairs stop growing and flattened out at the tip once contacting the substrate (**Figure 4F** and **Supplementary Figure 2E**). A layer of amorphous substance ("glue-like substance") was observed to be coated on the surface of the root hair (**Figures 4F,G** and **Supplementary Figures 2F,G**) and it appeared to be a very thin layer. On top of this glue-like layer, there were pieces of bark substrate that were either adhered directly on the glue or imprinted on the surface (**Figure 4G**). Root hairs of *D. koordersii* were also observed to bend and twist to hook onto smaller particles (**Supplementary Figures 2D,E**).

The cell wall of the root hairs of *E. nocturnum* and *D. koordersii* was very thin ca. 1 μm (**Figure 5A** and **Supplementary Figure 2B**). This extremely thin cell wall allowed the root hairs to be flattened against the surface of the substrate and molded to the microstructures of the substrate (**Figure 4F** and **Supplementary Figure 2E**). A cracked root hair was observed in *E. nocturnum* (**Figure 4E**), and a more obvious spirally cracked root hair was observed in *D. koordersii* when the root hair was ruptured (**Supplementary Figure 3**). The ruptured area of the root hairs consisted of several broken layers of cell wall material (**Figures 5B,C** and **Supplementary Figure 3**).

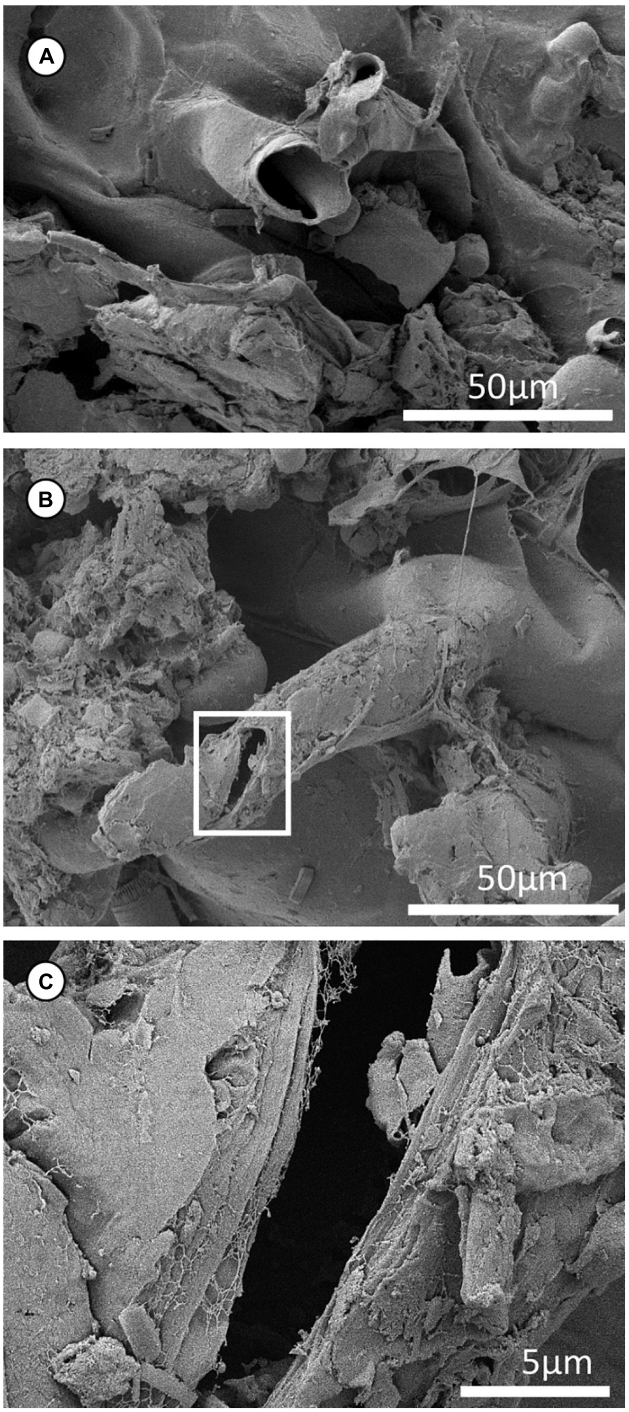

FIGURE 5 | Cryo-scanning electron microscopy micrographs of a segment of *Epidendrum nocturnum* root hairs. **(A)** A piece of broken root hair; the cell wall of the root hair is very thin (i.e., <0.5 μm). **(B)** A single root hair that had a flattened tip and was broken with a crack. **(C)** Enlarged box in **(B)**. Cracked root hair, with broken layers.

DISCUSSION

In a typical ground-rooted terrestrial plant, root hairs are ephemeral and their growth is restricted to the area directly behind the zone of root elongation (Dickison, 2000; Grierson and Schiefelbein, 2002). The growth of root hairs is typically interpreted as an increase in surface area for nutrient uptake, microbe interactions and anchorage in the soil (Jungk, 2001; Grierson and Schiefelbein, 2002). The number and length of root hairs is largely regulated by the supply of mineral nutrients, in particular phosphate and nitrate (Foehse and Jungk, 1983; Bates and Lynch, 1996). In epiphytic orchids, however, root hairs are not restricted to the distal region of the root apex (Bibikova and Gilroy, 2002). Roots in epiphytic orchids are found along the length of the root, even in the older parts of the roots, which

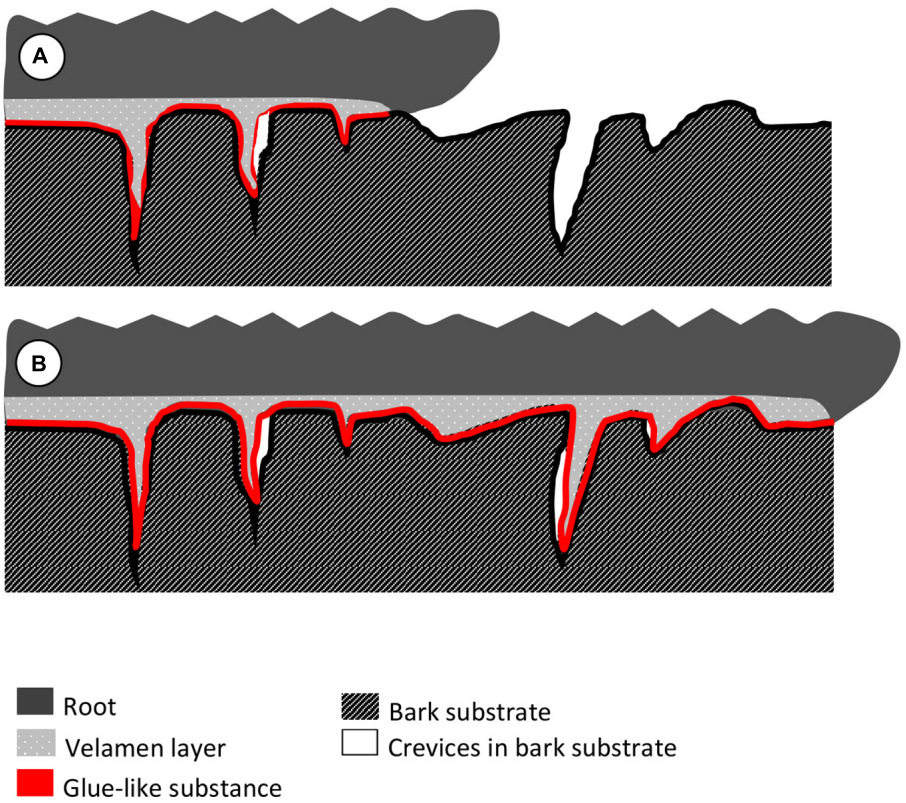

FIGURE 6 | Hypothesis on how epiphytic orchids attach to their substrate *via* their roots as deduced from the observations. **(A)** The underside of the root, which is in contact to the substrate, produces root hairs from the outer velamen layer. The outermost velamen layer resembles a foam and molds closely to the microstructures of the substrate. A layer of glue-like substance is in between the root and the substrate, which could help the root adhere to the substrate. **(B)** With continued root growth along the length of the substrate, root hairs persist in the cavities, generating additional long-lasting mechanical interlocking on top of the adherence *via* glue.

yields a large contact area for adhesion to the substrate. Studies found that contact to substrate is necessary for the development of root hairs since the presence of root hairs are only recorded on root surface facing the substrate (Groom, 1893; Chomicki et al., 2014; Thangavelu and Ayyasamy, 2017; Balachandar et al., 2019b). Free-hanging aerial roots remained spherical/cylindrical in shape without any root hair development (**Supplementary Figure 1**), while those attached to substrate were flattened on the side that was in contact to the substrate (**Figures 2**, **3**). A flattened surface suggests a maximization of contact area for water and nutrient uptake (Thangavelu and Ayyasamy, 2017). Root hairs emerged from the outermost layer of the immature velamen in this flattened area. This is in agreement with other studies, where root hairs were also observed in the regions with direct contact to the substrate (Almeida et al., 2016; Thangavelu and Ayyasamy, 2017). It suggests that root hairs promote the attachment of orchids to its substrate by increasing the contact area of the root to the substrate.

We observed that there was a very tight contact between root and substrate, where the outermost velamen layer acts like a soft foam, molding closely onto the contours of the substrate for effective adhesion (**Figures 2D**, **3G**). Then, there are root hairs, which have very thin cell walls (i.e., ca. 0.5 μm, measured from **Figure 5A**). Literature data for comparison are very limited. For example, cell wall thickness of young fresh root hairs of *Lyginia barbata* is similar with 0.5 μm (Shane et al., 2011), but *P. oceanica* have root hair cell wall thickness of 1.07 ± 0.15 μm (Zenone et al., 2020a). Given the differences in the type of environments and habitats that these plants grow in, the root hairs also serve different purposes. In the case of epiphytic orchid root hairs with the function of adhesion, having a thin cell wall would allow the root hairs to be readily flattened against the substrate or to protrude into voids, crevices and spaces between the layers of substrate (**Figure 4D** and **Supplementary Figures 2D,E**). This most likely promotes attachment as a mechanical interlocking mechanism between root and substrate. In the SEM images, an amorphous substance was observed to coat the surface of the root hairs (**Figure 4G** and **Supplementary Figure 2F**). This glue-like substance probably helps to hold the root in place. The presence of glue had also been reported in epiphytic ferns (Moran unpublished observation in Zotz et al., 2021a), in climbing figs, root climbers and in seagrasses (Groot et al., 2003; Melzer et al., 2011; Steinbrecher et al., 2011; Huang et al., 2016; Zenone et al., 2020b). Melzer et al. (2009) stated that English Ivy's root hairs exude a glue-like substance as roundish excretes. Further analysis is needed to confirm if this is also true for orchid roots. Nonetheless, the repeated observation of glues on root hairs of epiphytic and climbing plants suggests that there

may be a "universal" mechanism for attachment of non-parasitic plants to a host.

To remain securely attached to their host plants, epiphytic orchids have to withstand gravity and mechanical disturbances, e.g., wind. Principally, on very rough bark with deep fissures, when the whole root can grow into gaps, effective orchid root attachment to the substrate might be possible without root hairs. However, especially on smooth bark, which lacks larger fissures, the growth of many fine root hairs into microscale crevices on the surface provides reinforcement of attachment by mechanical interlocking, which may be critical for a strong and secure attachment. Root hairs were observed along the length of the root, in between the layers of substrates (**Figures 3D,F**, **4C**), and they were present in different sizes and lengths (**Figure 4E** and **Supplementary Figure 2C**). Therefore, this further implies that root hairs were growing into voids and crevices, whenever present, to increase the overall contact of the root to the substrate.

Some studies placed emphasis on the spirally cracked root hairs, stating that the shape change in the root hairs played a role in providing stronger attachment of the plant to their substrate and also facilitating water uptake by capillarity (Melzer et al., 2010; Almeida et al., 2016; Yang and Deng, 2017). However, none of these additional functions has yet been shown experimentally. There is no consensus on how these spirally cracked roots are formed. One possibility is the rupturing of the outer cell wall of the root hair (caused by external force), followed by stretching and breakage of the inner walls (Bernal et al., 2015). Alternatively, it could simply result from the helical arrangement of the cellulose microfibrils in the cell wall, inducing the spiral form when the cells stretch due to growth, by some form of pressure (Frei and Preston, 1961; Lloyd and Chan, 2002; Smyth, 2016), or when root hairs dry out and shrink (Melzer et al., 2010). Yang and Deng (2017) argued that the spiral shaped root hairs in climber plant *S. podophyllum* have two functions. Firstly, ruptured cell walls of the root hairs release a glue-like substance that eventually helps in the adherence of root and substrate. Secondly, the spirally cracked root hairs act as microsprings for shock absorption when the root is loaded by external forces. For example, when the root is pulled, root hairs extend and crack helically to allow for more flexible elongation of the cell, which helps in dissipating energy to prevent complete detachment of the plant from its substrate. From this study, a cracked root hair was observed in *E. nocturnum* (**Figure 5B**) and a slightly spiral shaped root hair was observed in *D. koordersii* (**Supplementary Figure 3**). Although none of the observed cracks followed the length of the root hair or were clearly orthogonal to it but the cracks were always angled to the length of the root hair, and this may eventually cause the root hair to have a spiral shape. The spirally cracked root hair seemed to result from the rupturing of several cell layers on different points along the root hair (**Supplementary Figure 3A**), which may eventually cause the root hair to break completely and twist spirally, as suggested by Bernal et al. (2015). However, the presence of spirally cracked root hairs was only scarcely observed in this study. While the arguments regarding the origin and function of spiral root hairs may also be plausible in the case of epiphytic orchids, a larger sample size and further physiological and ontogenetic studies with orchids are necessary to put these ideas to a rigorous test.

CONCLUSION

This study focused at the root hair-substrate interface, with the combination of freehand cuts, histological sectioning and cryo-SEM techniques, to elucidate the attachment mechanism of epiphytic orchids to the bark substrate of their host plants. This study provides an in-depth, thorough understanding of how epiphytic orchids attach to their hosts *via* their roots. A glue-like substance is observed on the surface of the root hairs, and that root hairs enter the crevices of the substrate suggesting a mechanical interlocking mechanism between the root and the substrate for a more secure anchorage. Based on the knowledge from previous studies and the new observations from this study, we suggest a schematic diagram that summarizes our current understanding and our hypothesis on how epiphytic orchids attach to their substrate *via* their roots. This diagram illustrates only the attachment of orchid roots on relatively smooth bark substrates (without deep fissures) with microscale crevices on the surface (**Figure 6**):

a) The underside of the root, which is in contact to the substrate, produces root hairs from the outer velamen layer. The outermost velamen layer acts like a foam and molds closely to the microstructures of the substrate. In addition, there is a layer of glue-like substance between root and substrate, which could be an additional help for the root to adhere to the substrate.
b) As the root grows along the substrate, root hairs continue to grow into voids and crevices on the substrate, and generate an additional mechanical interlocking mechanism between root and substrate, further reinforcing the attachment of the root (and the whole plant itself), to the host. Meanwhile, old root hairs maintain attachment along the entire length of the root.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

GZ, HE, and JT conceived and designed the research. SG contributed analytical tools. JT conducted the experiments and analysis, analyzed the data, and wrote the manuscript. GZ, SG, and HE revised the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ffgc.2021.764357/full#supplementary-material

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Quantification and Variation of Microclimatic Variables Within Tree Canopies - Considerations for Epiphyte Research

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Forest canopies are incredibly complex self-maintaining biological structures. Conditions above and within the canopy can differ vastly, often resulting in a vertical gradient of microclimate conditions. Canopy epiphytic plants have to deal with climatic variability on much more variable scales compared to many other plant groups. The difficulty of sensor installation and their high cost can explain why it has been ignored in many studies on canopy epiphytes. Direct measurements of microenvironmental conditions are the only accurate way to assess specific intra-canopy environmental conditions, as there is also still a lack of methodologically and financially viable alternatives to allow the collection of this type of data. This study aims to make recommendations for the direct use of microclimate measurements in epiphyte research and to summarize key discussion points concerning the number and placement of sensors required for different types of epiphyte studies. In addition, we presented high-resolution field data from the United Kingdom, where we employed over 56 microclimate sensors, to demonstrate the spatial and temporal variability of radiation, temperature, and relative humidity (RH) in a tree canopy. Our data demonstrated that sensor height in the tree and leaf-set were the most important factors determining microclimate variability in the canopy. For the first time, we have made recommendations regarding the placement and number of sensors required in studies that specifically require the use of microclimate sensors in epiphyte studies in forest canopies.

Keywords: light, temperature, relative humidity, forestry, sensors, climate, microclimate, canopy

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INTRODUCTION

Forest canopies are the assemblage of all branches, foliage, the interconnecting space between them and their flora and fauna (Parker, 1995; Moffett, 2000; Nadkarni et al., 2004). The stratification of forest canopies and their inherent variability and complexity often results in a vertical stratification of microclimate conditions (e.g., radiation, temperature, and relative air humidity) and the associated differences in the distribution of canopy organisms (Foggo et al., 2001; Ozanne et al., 2003). It has been shown that canopies are incredible biodiversity hotspots

(Nakamura et al., 2017). Forest canopies support about 40% of all living species, of which 10% are thought to be canopy specialists (Ozanne et al., 2003). For example, 10% of all known vascular plants have been estimated to be epiphytes (Zotz et al., 2021).

High variation in canopy conditions and its structural complexity have been shown to drive high species richness in forest canopies (Ozanne et al., 2003). The distribution of epiphytes within the canopy is believed to be to some extent driven by microclimate differences. For example, the lower canopy in many tropical forests is often dominated by large-leaved species that allows plants to capture radiation more efficiently. Similarly, the outer canopy is often dominated by more drought tolerant and atmospheric species. For some species (e.g., many filmy ferns), their high levels of specialization, can make them very susceptible to change in the environment, whereas other species are well adapted to withstand large changes (e.g., many bromeliads). Microclimatic conditions in vertically layered vegetation are directly regulated by radiation (Leuchner et al., 2012; Zellweger, 2019; Brüllhardt et al., 2020) intensifying or attenuating temperature and water availability including relative humidity (Baldocchi et al., 2000). Concerning spatial variation, radiation within a tree crown can be modified by structural crown features (Ventre-Lespiaucq et al., 2016) such as branch distribution patterns and orientation, as well as the presence, shape, or absence of leaves.

In forest ecosystems, the changes in the energy balance as a consequence of canopy architecture (e.g., canopy height, density, etc.), plant transpiration, and climate conditions (Song et al., 2017; Pau et al., 2018; de Andrade et al., 2021) results in within canopy temperatures and relative humidity levels to be markedly different from conditions outside the canopy (Jones, 1992; Pau et al., 2018). This buffering effect of the upper canopy on lower branches has prominent importance both over short and long-time scales. Over a shorter period, the buffering can drive temperatures to increase less during the day and decrease less during the night, while relative humidity often shows the reverse pattern (Aussenac, 2000; Von Arx et al., 2012; Prévosto et al., 2020). Over longer periods the buffering of the canopy produces lower annual and seasonal maximum temperatures, with higher recorded minimum temperatures and relative air humidity (Renaud et al., 2011; Gaudio et al., 2017; Prévosto et al., 2020). As incident solar radiation is selectively absorbed by leaves (Endler, 1993; Hartikainen et al., 2020), light properties (e.g., quantity and the spectrum) are modified as light reaches the lower layers of the tree crown. When light is intercepted by the canopy, the proportion of diffuse radiation increases, and, on the other hand, the proportion of direct radiation received decreases (Ventre-Lespiaucq et al., 2016). In several forest types, less than 2% of the photosynthetically active radiation (PAR) incident above the canopy may actually reach the forest floor (Chazdon and Pearcy, 1991).

It is important to highlight that radiation patterns change spatially and temporally, according to time of day, season, latitude, cardinal orientation, the position of the tree within the stand, and the architectural features of the canopy. In addition, gaps in the tree crown can increase the crown's heterogeneity as they allow direct sunlight to penetrate it, causing sunflecks that can move along the canopy depending on the sun's position (Chazdon and Pearcy, 1991). Within sunflecks, the radiation intensity can be 10 to 100 times higher than in the shade (Wagner and McGraw, 2013) and may account for 10-90% of the daily amount of radiation in forest canopies (Chazdon et al., 1988; Pfitsch and Pearcy, 1989) and up to 50% of the daily amount of radiation in the understory (Chazdon and Pearcy, 1991). Thus, canopy organisms deal with climatic variability on much sharper scales than it is generally measured by local meteorological stations that only measure above canopy conditions (Geiger et al., 1995; Potter et al., 2013; Kovács et al., 2020).

Frequently in canopy studies, below-canopy radiation is estimated by quantifying canopy traits, in terms of cover and canopy openness (Jennings et al., 1999; Lieffers et al., 1999; Zellweger et al., 2019). Although canopy openness, compared to cover, is a more accurate representation for below canopy radiation conditions (Alexander et al., 2013), both approaches can be used to estimate functional variables commonly used such as Leaf Area Index (LAI) (Binkley et al., 2013; Schleppi and Paquette, 2017; Zellweger, 2019). These approaches, however, can be unreliable, as they can be time-consuming and ineffective for mapping radiation regimes at high resolution across the canopy (Zellweger, 2019). This last point specifically might not represent a major issue for studies seeking to answer questions related to broad within-canopy conditions (e.g., open vs. closed canopy). However, for studies that require a more detailed account of the conditions within the canopy (e.g., physiological studies on epiphytes), more precise methods are needed (Gendron et al., 1998). Similarly, canopy temperature and relative humidity have been measured using approaches that rarely cover all canopy sections (e.g., along the vertical profile). Currently, the experimental designs used in studies to measure microenvironmental conditions have been diverse and range from sensors placed vertically along towers (e.g., Feigenwinter et al., 2010) to sensors placed vertically on the trunks of trees (e.g., Rambo and North, 2009), to sensors placed both above the canopy and in the understory (e.g., Phillips et al., 1999; Gotsch et al., 2015; Tymen et al., 2017; Parker et al., 2019). Alternative approaches to sensors include the use of RATP (radiation absorption, transpiration and photosynthesis) models, which simulate the spatial distribution of radiation and leaf-gas exchange within canopies as a function of canopy structure, canopy microclimate and physical and physiological leaf properties (Sinoquet et al., 2001). These models can provide high resolution of the microclimate data from within the tree canopy. However, the difficulty of parameterizing them has made the use of these difficult and costly.

Few field studies on epiphytes have used sensors to obtain direct measurements of the intra-canopy microclimatic parameters. For example, Sillett and Van Pelt (2007) used radiation and temperature sensors to study vascular epiphyte on *Sequoia sempervirens* Endl. in California. In particular, they were interested in assessing how epiphytes and their substrates can modify intra-canopy microclimates. Another example is the study by Rascher et al. (2012), who used temperature

and radiation sensors to investigate differences in physiological responses of epiphytes. Our study, therefore, prompted by the understanding that direct measurements of microenvironmental conditions are the only uncontroversial way to assess the specific intra-canopy environment and that it is essential to build not only financially, but also methodologically viable alternatives to encourage this type of measurement in canopy research. In order to improve our understanding of the variability of microclimate conditions within forest canopies, we deployed an array of radiation, temperature, and relative humidity sensors in a forest canopy in the United Kingdom. Our research questions were: (1) How do microclimate conditions change within a tree canopy?; (2) Does the microclimate variability differ in the same tree before and after leaf-set?; (3) How many microclimate sensors are needed to capture variability of microclimate conditions in one forest tree? Answering these questions will allow us to provide recommendations for vascular epiphyte studies that aim to assess direct microclimate measurements and we will bring together key discussion points concerning the number and placement of sensors required for different types of epiphyte studies.

MATERIALS AND METHODS

Study Site and Tree Selection

This study was carried out at Scutcher Acres (53.5617306, −2.8679865), a 13-hectare Local Nature Reserve in West Lancashire, United Kingdom. Elevation of the site was 25 m a.s.l. The site has a history of rural land use and is currently under forest restoration. In 1997 the land was bought by its current owner to enhance its value for wildlife. Many of the trees were planted over the last few years and the forest is currently a mosaic of well-established oak *(Quercus* sp.*)* and European beech *(Fagus sylvatica* L.*)* trees and smaller stands of oak, *Pinus* spp., and mixed native and non-native trees. The understory is thin and composed of some shrubs and young trees, with a well-established herbaceous layer mostly formed by bluebells (*Hyacinthoides hispanica* (Mill.) Rothm.) and brambles (*Rubus fruticosus* Pollich). The canopy trees in our study area were between 20 and 25 m tall, forming a predominantly continuous canopy. For this study, due to the number of sensors needed for a detailed canopy study, and their prices (very expensive), we selected only one European beech tree (*F. sylvatica*). The tree was selected based on a) its dominant size in the area, and b) being a representative species of the canopy in the study site, and a species commonly found in United Kingdom woodlands. In addition, being a deciduous species, *F. sylvatica* allowed us to sample microclimatic conditions over a period from leaf-out to full leaf development, an important element accounting for spatial and temporal variation of canopy conditions (Jones, 2013; Wen et al., 2020). The array of sensors was installed in the selected tree on April 26th 2020, and kept in the canopy for 45 days. The leaves on the tree were fully developed by May 11th and data collection extended until June 10th. Thus, in this study, we considered 27th April to May 10th as the leaf-out phase and May 11th to June 10th as the foliaged phase.

Installing the Sensors

In order to measure canopy microclimate conditions for the first time at a very high spatial resolution, we equipped the selected tree with 40 temperature/relative humidity sensors (EA EL-USB-2 Temperature & Relative Humidity USB Data Logger – Lascar Electronics Ltd., United Kingdom, typically $< \pm 3\%$ of accuracy), 15 pyranometers (SKS 1110/I Pyranometer sensors - Skye Instruments Ltd., United Kingdom typically $< \pm 3\%$ of accuracy, wavelength 400-1100 nm), and one PAR (photosynthetically active radiation) sensor (SKE 510/I – Skye Instruments Ltd., United Kingdom typical calibration error $< 3\%$). The PAR sensor was calibrated against a reference lamp of known output and pyranometers were calibrated under open-sky conditions, against reference pyranometers. Pyranometers and PAR sensors were connected to two data loggers (SDL 5400 – 8 channel DataHog2 Skye Instruments Ltd., United Kingdom) to store all readings. Radiation measurements were logged for 10 s, every 6 h, while temperature and relative humidity sensors logged at 30-min intervals. The intervals were selected because smaller intervals would have filled up the memory too quickly when employed for longer under field conditions (see discussion for further detail).

The canopy was accessed using rope climbing techniques that allowed direct access to the upper canopy without the use of 'high-tech' and financially more costly methods (Picart et al., 2014). The installation of the sensors took two full days. We placed the sensors in positions that were believed to represent the variability in microclimate conditions within the tree. In some cases, sensor positioning was limited by accessibility issues. For example, the maximum branch diameter that could safely be accessed was 35 cm. We measured horizontally the distance between each sensor to the center of the tree and vertically to the floor (see **Supplementary Table 1**) using a tape measure to test the microenvironmental spatial variation within the canopy (**Figure 1**).

Each individual pyranometer was installed on a 15 × 15 cm wooden platform. The platforms were custom-built and included a spirit level and a plastic bracket that held the sensors in place using silicon screws (**Figure 2**). Each platform was securely attached to each branch using cable ties and wood screws (**Figure 2**). It was ensured that each sensor platform was horizontally leveled during installation. Temperature/RH sensors were attached directly to branches with cable ties. Sixteen temperature/RH sensors were directly attached to the pyranometer platforms. The sensor platform's attachment was visually assessed from the ground every week from its installation until the end of the recording interval. By the end of the collection phase, one of the platforms was slightly unlevelled. After assessing the readings of the sensor and discussing the data with the manufacturer of the sensors, the data was included in all further analyses.

Data Management and Analysis

Because our temperature sensors were not shielded from direct sunlight, we calculated the relationship between temperature and radiation. We analyzed only the temperature sensors that were paired with radiation sensors during day time and during times

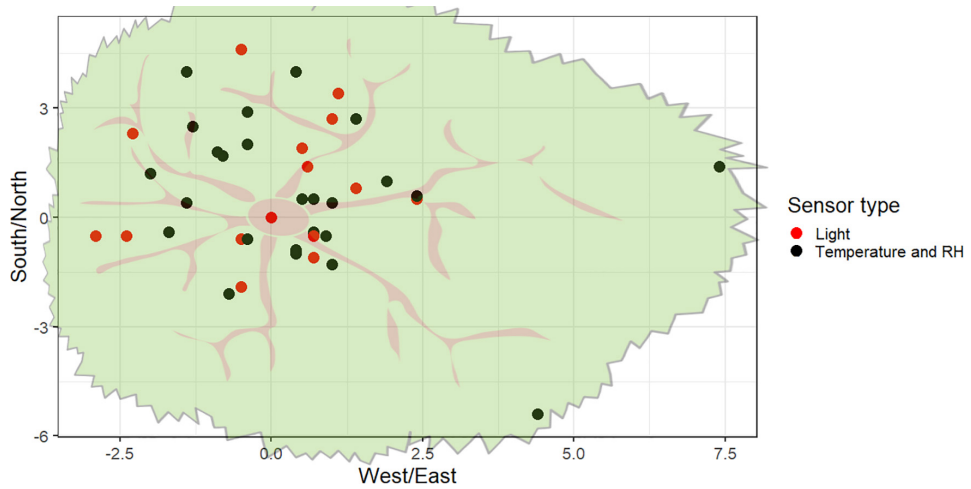

FIGURE 1 | Horizontal distribution of radiation (red dots) and temperature/RH sensors (black dots) within the tree canopy. All radiation sensors had paired temperature sensors coupled to the platforms.

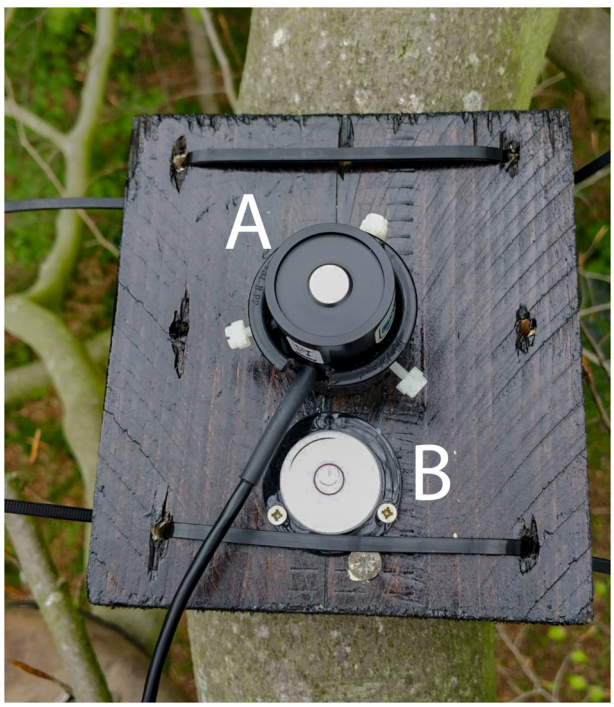

FIGURE 2 | Example of costume-build radiation sensor platform used, showing the pyranometer **(A)** and a spirit level **(B)**.

when both the light and temperature sensors synchronized their measurements. We tested the correlation separately for the above and within canopy sensors. A moderate correlation was found between radiation and temperature when assessing the above sensors (r = 0.46; p = 5.5-06). However, only a weak correlation was found when assessing the within canopy sensors (r = 0.21, p = 6.4-14). Due to the moderate heat-effect of radiation on the above canopy temperature sensor, we only used within canopy temperature and RH measurements for the remaining analysis (see below for a more detailed discussion).

To identify the most important explanatory variables that affected microclimate conditions within the tree, a random/mixed-effects meta-regression model was used. We used the 'glmulti' package in R Core Team, 2020 for this analysis following Anderson (2007). Three meta-models were run, one for radiation, one for temperature, and one for RH. Tree characteristics that were included in the meta-model included leaf-set, sensor height, sensor distance to the tree center, orientation and day- and night-time. Akaike information criterion (AICc) was used to compare models and select the best-fit models. We used multimodal inference to help us to make inferences about our predictors using the "metafor" package. This method takes into consideration the relative weights of each model combination.

To compare in more detail how much each microclimate variable (e.g., radiation, temperature, and RH) was affected by tree height and by the leaf-set, we divided our tree canopy into three equal parts (e.g., 1st third = Lower canopy; 2nd third = Middle canopy; 3rd third = Upper canopy), similar to the zonation model proposed by Johansson (1974) (e.g., Johansson's zones III, IV, and V). However, unlike the large and emergent trees sampled by Johansson, our tree is located inside a forest stand, surrounded by other trees of similar dimensions. Under such conditions, the radiation that penetrates the crown comes mainly from the top and not from its sides. Moreover, as our mixed effect model suggested that height was the most consistent and important explanatory variable for radiation, temperature, and RH, only vertical height was used to classify the different canopy zones. The sensors positioned within the range of each zone (e.g., 11 m to 14.5 m = Lower canopy; 14.6 m to 18.4 m = Middle canopy; 18.5 m to 22 m = Upper canopy) were used to calculate the mean values of each response variable, for each day, from April 27th to June 10th. The

canopy zone classifications were then used to plot density plots of radiation, temperature, and RH, for the leaf-out and foliaged phases.

We also calculated the variance of each response variable captured by each one of the sensors for the leaf-out and the foliaged phases to visualize whether different portions of the canopy experience more variable conditions. We calculated the percent of variance that each sensor captured and plotted the sensors according to their position in the tree canopy (e.g., sensor's vertical height and horizontal distance to the tree center).

Finally, a Monte Carlo analysis was undertaken to test the change of standard error when reducing or increasing the number of sensors. Firstly we averaged the data by day and secondly by sensor. The analysis was run using 10,000 permutations without replacements. We analyzed the increase of the error when reducing sensors from 15 to five in the model for radiation and 39 to 15 in the models for temperature and RH.

RESULTS

The most important explanatory variable affecting the within-canopy microclimate for all variables was sensor height (**Table 1** and **Figure 3**). In addition, day time and leaf-set were explanatory variables that showed also high importance in the models. For temperature, the cardinal orientation of the sensor on the tree was important, whereas this was not the case for RH or radiation. In general, increased sensor height resulted in higher observed radiation and temperature and lower RH values.

When assessing the temporal changes of microclimate conditions, radiation, temperature and RH showed the same patterns of variability over time, with synchronized shifts and higher values for the above canopy readings, followed by smaller difference between the upper, middle and lower canopy (**Figures 4**, **5**). Although the above temperature sensor was not included in the inference model analysis, we included the data here for illustrative purposes. RH, on the other hand, was higher in the lower, compared to the middle and upper canopy. Temperature was higher and RH lower during leaf-out when compared to the foliaged phase (**Table 1** and **Figure 5**).

In order to test how the standard error changed with the number of sensors used, a Monte Carlo analysis was performed and showed that a significantly smaller number of sensors could have been employed in our study tree (**Figure 6**). For example, by removing four radiation sensors within the canopy would have kept the error below 25% of the total error (**Figure 6A**). For temperature and RH on the other hand, a total of 13 sensors could have been removed, to still capture 75% of the total error (**Figures 6B,C**). No difference was found when comparing the leaf-set and folliaged phases (data not shown).

In addition, an analysis of the percentage of the variance showed that four out of five radiation sensors that captured less than 5% of the radiation variance for leaf-out phase were located below 15 m in the tree (**Figure 7A**). It was also found that during the leaf-out phase 23 of the temperature sensors (**Figure 7C**) and 22 of the RH sensors (**Figure 7E**) captured less than 2.5% of the total temperature and RH variance respectively. For the foliaged phase, nine of the radiation sensors captured less than 5% of the radiation variance (**Figure 7B**), while 19 temperature sensors (**Figure 7D**) and 16 RH sensors (**Figure 7F**) accounted for less than 2.5% of the total variance of temperature and RH, respectively.

DISCUSSION

We found that sensor height was a better predictor of microclimate variables than the distance of sensors to the tree center for all three variables measured. This could be explained by the location of the tree within the forest stand, as the surrounding trees would have increased the buffering potential and thus decreased the horizontal microclimate variation. For example, incident radiation levels are likely to be higher in the upper compared to the lower canopy, but can differ depending

TABLE 1 | Summary of multi-model inference results, testing the effect of tree height, horizontal distance to the tree center, cardinal orientation, day- and night-time, and leaf-set on radiation, temperature, and RH.

Response Variable	Best model parameters	Estimate	Std error	*z*-value	*P*-value	ci.lb	ci.ub	Importance
Radiation	Height	0.2055	0.0329	6.2377	0	0.1409	0.27	1
	Day time:Middle	6.48	2.6306	2.4633	0.0138	1.3242	11.6359	1
	Day time:Upper	23.2885	3.6708	6.3442	0	16.0938	30.4832	1
Temperature	Leaf-set:Without	−2.1566	0.0303	−71.1008	0	−2.216	−2.0971	1
	Height	0.0703	0.0033	21.4035	0	0.0639	0.0767	1
	Day time:Night	−3.2888	0.0341	−96.3269	0	−3.3558	−3.2219	1
	Orientation:Northeast	0.189	0.0946	1.9978	0.0457	0.0036	0.3744	1
	Orientation:Northwest	0.1566	0.0985	1.5896	0.1119	−0.0365	0.3496	1
	Orientation:Southeast	0.3403	0.0945	3.6013	0.0003	0.1551	0.5255	1
	Orientation:Southwest	0.3475	0.0978	3.5546	0.0004	0.1559	0.5391	1
RH	Leaf-set:Without	2.2015	0.1156	19.049	0	1.975	2.428	1
	Height	−0.3121	0.0128	−24.3666	0	−0.3372	−0.287	1
	Day time:Night	12.1098	0.1301	93.0873	0	11.8548	12.3647	1
	Distance	0.1284	0.0428	2.9978	0.0027	0.0445	0.2124	0.981

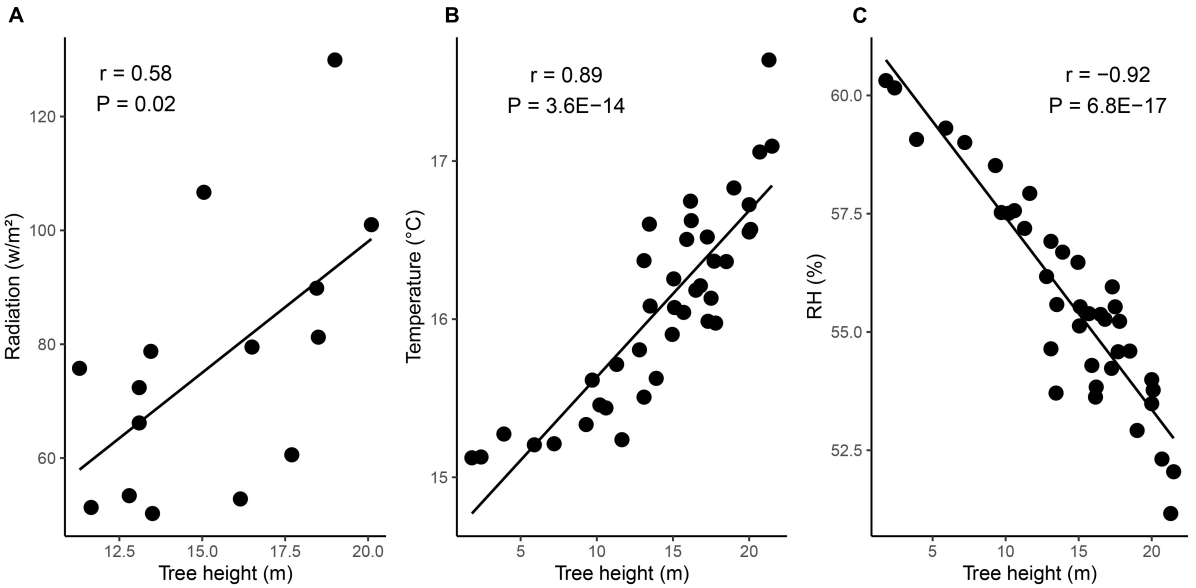

FIGURE 3 | Regression plots showing how radiation **(A)**, temperature **(B)** and RH **(C)** changes with height in the tree.

on the surrounding forest stand (e.g., trees growing close to gaps or even emergent trees can experience greater amounts of radiation incident laterally in the lower portions of their crowns). The upper canopy is often more heterogeneous and has more sudden changes in conditions during the day, compared to the middle and lower canopy or canopy positions that are more sheltered by other surrounding trees. This stabilized microclimate configuration in the middle of the canopy could facilitate vascular epiphyte species establishment (Hietz and Briones, 1998). It is important to highlight that the relative stability of the microclimate in the middle of the canopy is also accompanied by different characteristics of the substrate when compared to other canopy positions. For example, the middle canopy is one of the oldest parts of the canopy, providing longer colonization potential (Benzing, 1990), and often has the highest surface area, which provides more space for colonization (Bonnet and De Queiroz, 2006). In addition, these areas of the canopy are also characterized by a higher abundance of moss cover and organic material that can supply and retain water and nutrients better, thus facilitating vascular epiphyte establishment (Nadkarni, 1984; Freiberg and Freiberg, 2000). Direct measurements of microclimate are therefore valuable in explaining stratification along structurally different portions of the canopy, as shown for bryophytes by Man et al. (2022). To our knowledge, no study has rigorously tested whether it is canopy age, surface area and/or microclimate stability that primarily drives epiphyte species richness and abundance in the middle of the canopy.

Measuring radiation across a forest canopy is much more difficult compared to measuring temperature and RH, due to the radiation's high temporal and spatial variability. Sunflecks can particularly increase the heterogeneity of radiation in the canopy (Chazdon and Pearcy, 1991). For example, we recorded radiation changes between 0.22 and 427.33w/m^2 for the same sensor on different days. These extreme fluctuations in the radiation environment occurred regardless of the vertical or horizontal position of the sensors. Thus, it becomes important to position several radiation sensors across the canopy, but also have a high recording frequency. In the case of our study, although we used a large number of sensors in a single tree, the frequency of logging intervals per radiation sensor was quite low. Ideally, to account for temporal variability, radiation measurements should be taken at a higher frequency compared to our study. However, the frequency of records will need to be balanced against the storage and battery capacity of the sensors used. Especially studies in remote locations and where access to the data loggers is difficult, it might become important to carefully consider data storage. Network systems are now available that allow almost instantaneous remote download of data, these networks are often very expensive and require the use of a mobile phone network. This makes the use of these systems more challenging in remote locations and more inclusive to well funded canopy studies.

In terms of temperature and RH, leaf-set markedly changed the temperature and RH in our surveyed tree. The buffering effect of the crown to atmospheric conditions (De Frenne et al., 2019) was particularly noticeable by a decrease in the incident radiation after leaf-set, which was followed by a decrease in overall canopy temperature, and an increase in RH during the foliaged phase. However, leaf-set was an important factor in our inference model only for temperature and RH (**Table 1**). In terms of canopy epiphytes, high species richness has often been observed in biomes that are often characterized by high temperatures and higher water availability, but fewer seasonal changes (Zotz and Winter, 1994; Cardelús, 2007; Zotz, 2016). Most of these biomes are dominated by broadleaved evergreen tree species (Zotz, 2005; Einzmann et al., 2015), indicating that

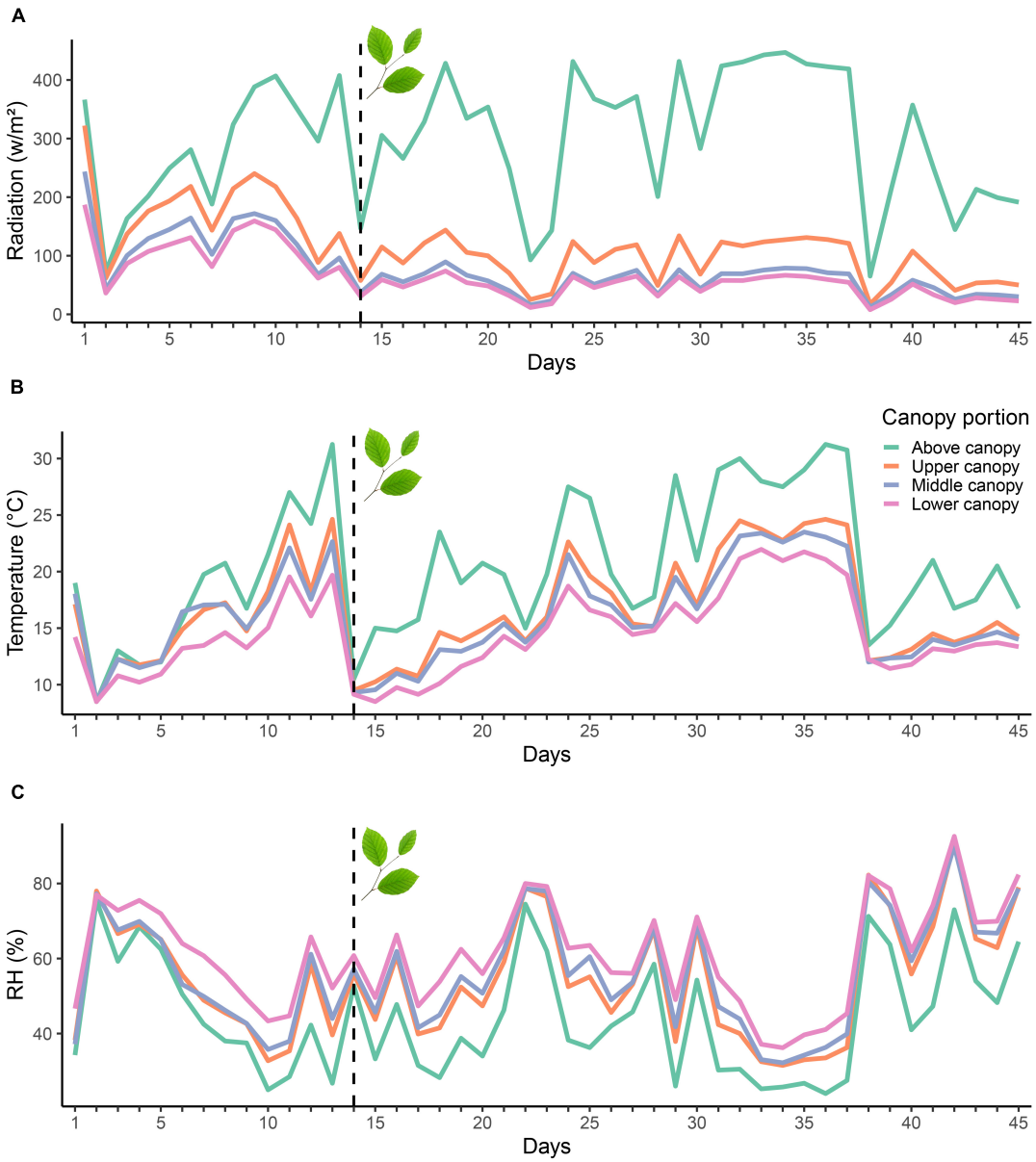

FIGURE 4 | Mean radiation **(A)**, temperature **(B)** and RH **(C)** variability measured above and within the tree at different canopy positions (lower, middle, and upper) during the study period (May-June). The dashed line represents the transition from the leaf-out to the foliaged phase.

the deciduous nature of most northern hemisphere trees makes the forest canopy a more challenging environment to grow in (but see Zotz, 2005). For example, Einzmann et al. (2015) has shown that differences in luminance between evergreen and deciduous broad leaved trees is 3-4 times higher in deciduous species, but that broadleaved evergreens trees support up to nine times more vascular epiphytes compared to deciduous ones.

The height of the sensors explained more microclimate variability then any of the other variables included in the analysis. We found a strong positive correlation of temperature (R^2 = 0.89) and strong negative correlation of RH with sensor height (R^2 = −0.92). Radiation was weak, positively correlated to sensor height (R^2 = 0.58). This gradient of microclimate conditions has been reported previously (Freiberg, 1996; Wagner and McGraw, 2013; Fauset et al., 2017; Meeussen et al., 2021) and has been shown to be more distinct in taller tree species (Richards et al., 2020). Our data showed that the presence of the canopy (e.g., when comparing leaf out and foliaged phase) acted as a buffer for microclimate conditions, especially in the lower canopy. However, it needs to be noted that our temperature sensors were not shielded from direct radiation and placed under well ventilated shields, which makes a comparison with above canopy temperature conditions more difficult. Ideally, studies that employ temperature sensors should be using radiation

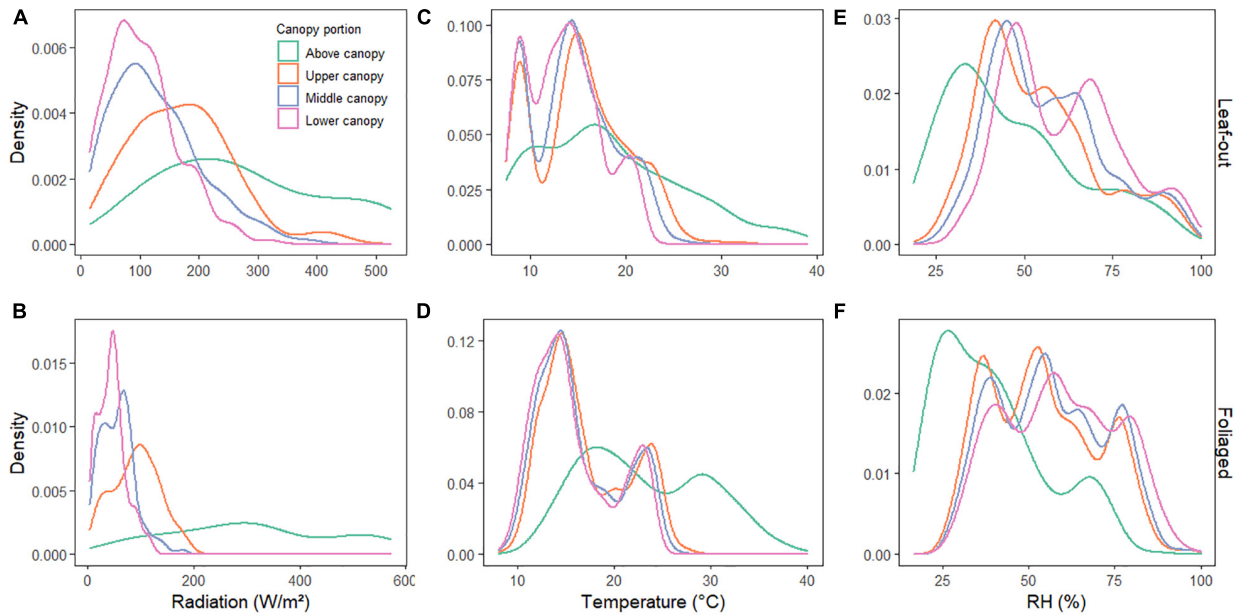

FIGURE 5 | Density plots of daytime records of radiation **(A,B)**, temperature **(C,D)**, and RH **(E,F)** for each canopy zone and for the leaf-out **(A,C,E)** and foliaged phases **(B,D,F)**.

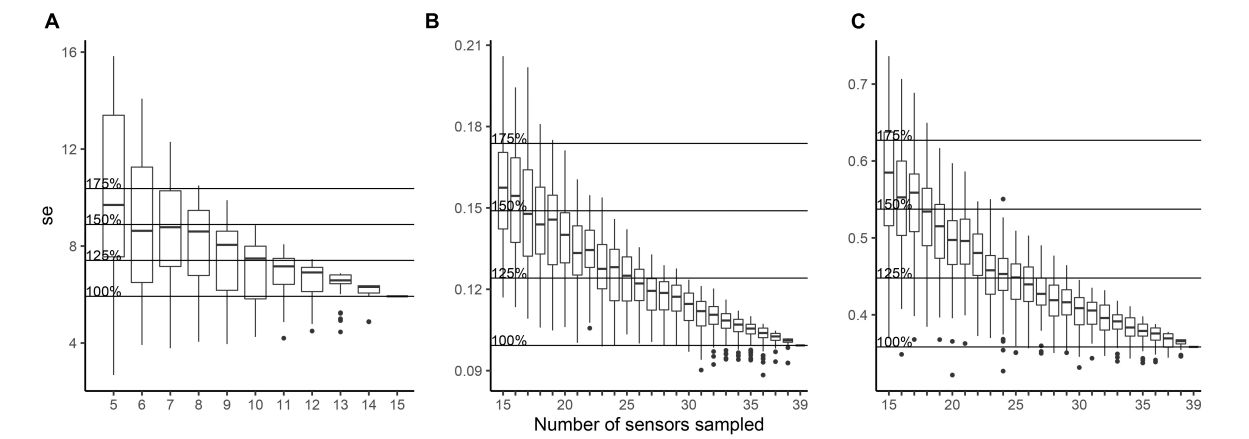

FIGURE 6 | Monte Carlo analysis of the standard error for radiation **(A)**, temperature **(B)**, and RH **(C)**, according to the number of sampled sensors. The horizontal lines show the total standard error of the data (e.g., 100%) and the consequential increase of the error (e.g., 1.25, 1.5, and 1.75 * the standard error) as the number of sensors is decreased.

shields to ensure that temperature measurements are not affected by changes in radiative force. For the epiphytes, tree height has been one of the key predictive variables of epiphyte richness in a local context (Flores-Palacios and García-Franco, 2006; Krömer et al., 2007). Taller trees often harbor a higher richness, biomass and abundance of epiphytes, compared to smaller trees (Flores-Palacios and García-Franco, 2006; Elias et al., 2021; Mitchell et al., 2021). The stratification of epiphytes along the vertical profile of trees has thus been the focus of several scientific studies in the past (see Johansson, 1974 and Zotz, 2007). Distinct patterns of occupation can often be observed, where many drought resistant species occupy drier and more exposed sections of the canopy (e.g., the upper and outer branches) and less tolerant species the lower branches and trunk of the tree. For example, many filmy fern species (Hymenophyllaceae) are most frequently found in canopy positions, where RH and water availability are high, as well as temperature and light are low (Proctor, 2003). Even though these vertical distribution patterns of epiphytes are generally well documented, there is still a scarcity of studies that have quantified epiphyte distributions in terms of microclimate gradients across canopies.

A review of the literature highlighted the scarcity of studies investigating intra-canopy microclimatic conditions, especially

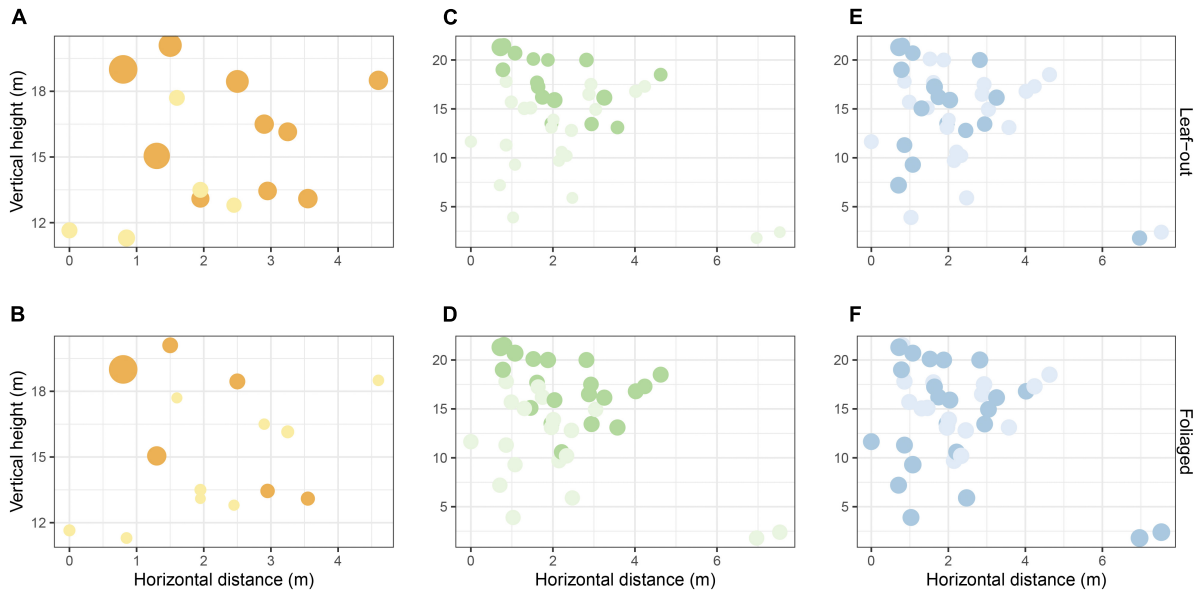

FIGURE 7 | Percentage of total variance captured by each sensor when plotted against tree height and horizontal distance to the tree center; Light yellow dots represent radiation sensors that captured less than 5% of total radiation variance and orange dots represent radiation sensors that captured more than 5% of total radiation variance **(A,B)**; Light green dots represent temperature sensors that captured less than 2.5% of total temperature variance and dark green dots represent temperature sensors that captured more than 2.5% of total temperature variance **(C,D)**; Light blue dots represent RH sensors that captured less than 2.5% of total RH variance and dark blue dots represent RH sensors that captured more than 2.5% of total RH variance **(E,F)**. All dots were size-mapped for percentage variance values.

in relation to epiphytes. Only four studies discussed the use of climate sensors in forest canopy epiphyte research and only two (Sillett and Van Pelt, 2007; Rascher et al., 2012) used sensors to measure the canopy microclimate in relation to vascular epiphytes. In addition, we found 30 studies that systematically quantified forest canopy microclimate conditions of radiation, temperature, and/or relative humidity. However, all of them used only a few sensors. These studies are summarized in **Supplementary Table 2**, in addition to Sillett and Van Pelt (2007) and Rascher et al. (2012) studies. Sixty nine percent of the reviewed studies only used one type of sensor (e.g., only temperature sensors) and/or only sampled part of the canopy. In addition, 56% of the studies only used one single sensor to quantify microclimate canopy conditions, and only 9% of the studies have employed several sensors along the full vertical profile. Thus, our single tree experiment is an important contribution to the field highlighting some key issues regarding the number of sensors that were employed in a single tree.

Currently, most studies that have employed microclimate sensors in forest canopies have often used (a) a very small number of sensors per tree (minimum = 1 and maximum = 12 sensors/tree), (b) limited their measurements to only certain parts of the vertical canopy (e.g., lower canopy only), (c) neglected the horizontal canopy profile and/or d) only sampled one microclimate variable (e.g., radiation, RH or temperature) (**Supplementary Table 2**). For example, only nineteen of the studies (63%), we found in our literature search, used sensors in the forest understory, positioning them at most two meters above the ground (e.g., Pecot et al., 2005; Lindner, 2011; Putzenlechner et al., 2019). Studies by Feldhake (2002),Wieser et al. (2002) and Awada et al. (2013) used fixed sensor positions along the height of the canopy. However, their studies did not account for vertical differences, as we did in the present study, and their very targeted sensor installation limits their ability to capture microclimate variability within the whole canopy. On the other hand, Fauset et al. (2017) employed one PAR sensor in the open sky and 18 sensors, connected at one-meter intervals along the tree trunk, to characterize the vertical radiation environments of forests along a disturbance gradient. In general, the use of many sensors in canopy studies, particularly in epiphyte studies, is rare.

It is not always required to install a large number of sensors, especially in studies that are more broadly interested in quantifying canopy conditions for epiphytes. However, for physiological studies or studies that aim to investigate habitat preferences at fine scale, a more comprehensive employment of sensors would be more recommended. In our single tree, we installed 56 microclimate sensors and demonstrated that 36 would have still been required in order to account for 75% of the variation. This demonstrates that a large number of sensors are necessary to quantify microclimate conditions in a single tree well. However, sensor cost most likely has been an important obstacle when considering the low number of sensors used in past studies. For example, the 15 pyranometers and 39 temperature and RH sensors, used in this study, have a unit price of £215 and £40, respectively. The total costs of all sensors used was £4,785.

Based on our Monte Carlo analysis, we could have reduced the overall number of sensors by 15 and still captured 75% of their measurement variation, therefore saving £1,420 in costs.

The main caveat of our study is that our analysis is based on a single tree. However, it still highlights the importance of the number and placements of sensors that are required to quantify microclimate conditions in a tree canopy well. Key aspects that need to be considered include the height of the tree, the density of the canopy (e.g., during different times of the year) and the position of the study tree within the forest. In cases where a tree is located in an open pasture, the canopy is very large or less sheltered by the surrounding trees, the horizontal gradient would also need to be considered during sensory installation. Based on our results and when reviewing the literature, making recommendations on the absolute number of sensors needed is difficult. However, our data suggests that a much larger number of sensors is required to capture microclimate variability within each tree than previously recognized. Radiation in particular was more variable across the canopy compared to temperature and RH. We are suggesting under limited resources, researchers should focus on the sensor installation along the vertical profile. In addition, we found that microclimate variability was much higher in the upper canopy (top 10 m) compared to the rest of the canopy, thus suggesting more sensors should be placed in this section. Methodologically, we are also recommending that radiation shields are used when measuring microclimate variables such as temperature. When measuring radiation, it is important that sensors are installed on stable platforms before they are attached directly to the tree. In the case of our study, we made customized platforms that included a spirit level and we only attached platforms to branches greater than 35 cm diameter to avoid excessive movement and also ensure safe access. Finally, the logging frequency chosen should be as high as possible, to capture temporal variability at a high resolution. However, this needs to be carefully weighed against the data storage and battery requirements of the equipment used, as a high logging frequency can lower the longevity of the sensors under fieldwork conditions, especially in remote or difficult to access locations.

CONCLUSION

In conclusion, we found that tree height is the most important factor determining microclimate conditions in our sampled tree. We further observed differences in microclimate conditions between phenological phases during leaf set and demonstrated that a large number of sensors is required to accurately determine microclimate conditions within a canopy. Despite the limitation imposed by the fact that we only sampled one tree, our study makes a genuine contribution to the development of field approaches for the direct measurement of three important microclimatic variables (radiation, temperature, and RH). We believe that our recommendations can highlight important issues when researching canopy dwellers, by informing researchers on how to overcome methodological challenges. This will contribute to future research on forest strata and its biodiversity.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

FNR, MM, and SB conceived and designed the research. MM and SB conducted the experiments. MM conducted the analysis, analyzed the data, and wrote the manuscript. RA conducted Monte Carlo analysis. FNR, MD, and SB revised the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ffgc.2022.828725/full#supplementary-material

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Microorganisms of the Phyllosphere: Origin, Transport, and Ecological Functions

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Microbes are ubiquitous residents of the atmosphere, including the air that we breathe. They are also widely present in terrestrial, marine, and aquatic environments. Typical microbes include viruses, fungi, archaea, bacteria, algae, and bryophytes. Many are of edaphic origin and play significant ecological roles in the soil. Propagules are exceedingly lightweight and small, generally measured in microns (millionths of a meter). Propagules achieve airborne status in the wind, where they may travel from a few millimeters to thousands of kilometers. Most have been recorded at least as high as the stratosphere. While airborne, microbes may pass through multiple generations. Microbes in the atmosphere are often accompanied by vast clouds of dust. They perform a variety of essential functions such as raindrop and snowflake condensation nuclei, without which there would be little or no precipitation. It is important to realize that all solid things that are carried up into the atmosphere must eventually fall back down to the Earth. When precipitated or deposited back onto the Earth, they may land on and occupy any surface, including trees and other plants where they become epiphytic residents. They have been documented on broad-leaved and needle-leaved trees from deserts to tropical rainforests. If they land on bare soil, they often participate in biological soil crusts that are important for soil stabilization and for water and nutrient cycling.

Keywords: viruses, fungi, archaea, bacteria, algae, bryophytes, aerobiology

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INTRODUCTION

Microbes are nearly ubiquitous in the atmosphere, including in the air that we and other animals breathe. It has been estimated that billions of microbes are descending from the atmosphere at all times of every day (Weisberger, 2018). Typical microbes include viruses, fungi (free-living, lichenized, and mycorrhizal), archaea, bacteria (cyanobacteria, chemoheterotrophic, and diazotrophic), algae including diatoms, and bryophytes (mosses, liverworts, and stoneworts) (Koskella, 2020; Warren and St Clair, 2021). Some microbes reproduce sexually, but most rely primarily on asexual means of reproduction (Warren et al., 2019). Common forms of asexual reproduction among microorganisms include replication, fragmentation, binary fission, cloning, budding, mitotic cell division, asexual sporogenesis, etc. Many of the asexual propagules, and even some of the mature microorganisms are very small, measured in microns (millionths of a meter). Given their small size and weight, microbes and/or their propagules are easily lifted into the atmosphere (Després et al., 2012; Fröhlich-Nowoisky et al., 2016) at least as high as the stratosphere (DasSarma et al., 2020). They are dispersed aerially over extensive distances (Mayol et al., 2017;

Reche et al., 2018), including intercontinentally and interhemispherically (Prospero et al., 2005). Microbes are often accompanied by vast clouds of dust from the Earth's arid areas (Griffin, 2020; Hu et al., 2020).

As microbes and their propagules return to the Earth's surface, they may be deposited onto bare soil where they can be incorporated into biological soil crusts (Belnap and Lange, 2001). Where bare soil is absent, as in tropical rainforests, the microorganisms occupy the duff or litter layer (Tripathi et al., 2016). Deposited microbes are very abundant, ranging up to 10^7 living cells of bacteria alone per square centimeter of surface area (Lindow and Brandl, 2003). Airborne microorganisms may alternatively fall onto lava beds (Lavoie et al., 2017), mine tailings (Gypser et al., 2016), or sand dunes (Smith et al., 2004). They may land on bodies of freshwater (Benson et al., 2019) or saltwater (Ul-Hasan et al., 2019). Some may land on snow (Yakimovich et al., 2020), glaciers (Anesio et al., 2017), rocks (Coleine et al., 2021), stone monuments (Li et al., 2016), gravestones (Villanueva et al., 2019), building roofs and facades (Barberán et al., 2015), or animals (Kaup et al., 2021). Others may be inhaled by humans or other animals (Barberán et al., 2015).

Given the theme of this special issue, many microbes and/or their propagules are known to fall from the atmosphere and land on trees where they become epiphytic residents of the phyllosphere, i.e., the aboveground parts of plants exposed to the atmosphere (Koskella, 2020). It can be logically concluded that all plants have epiphytic microbes. Microorganisms have been documented on coniferous trees and shrubs (Neitlich and McCune, 1997; Sevgi et al., 2019) and broad-leaved trees and shrubs (Wallace et al., 2018; Herrmann et al., 2021), fruit trees (Michavila et al., 2017; Janakiev et al., 2019), and nut trees (Pardatscher and Schweigkofler, 2009; Valverde et al., 2017).

In addition to trees, all other plants have a phyllosphere occupied by microorganisms (Partida-Martínez and Heil, 2011), including grasses and grains (Aydogan et al., 2020; Bowsher et al., 2021), ferns (Jackson et al., 2006), vegetables, fruits, and ornamental flowers (Lopez-Velasco et al., 2011; Mamphogoro et al., 2020), as well as cacti and other desert plants (Fonseca-Garcia et al., 2016; Flores-Nuñez et al., 2020). This includes trees and other plants in all climates from tropical rain forests (Kim et al., 2012), to hyperarid deserts (Al-Ashhab et al., 2021), to frigid areas such as Antarctica (Cid et al., 2017). Epiphytic microbes are even known to occur on emergent seagrass (Agawin et al., 2016). While microbes are precipitated onto exposed tree and other plant surfaces, their arrival my vary spatially and seasonally (Lighthart, 1997; Grady et al., 2019).

FUNCTIONAL ROLES OF EPIPHYTIC MICROBES

Epiphytic microorganisms are dispersed passively by wind (Cusimano et al., 2016) and are often accompanied by great clouds of dust (Gannet Hallar et al., 2011). However, as dust particles coalesce and become heavier, and as windspeeds subside, the airborne microorganisms and accompanying dust particles are precipitated back to Earth (Itani and Smith, 2016).

Epiphytic microbes may have either positive or negative impacts on their hosts (Rastogi et al., 2013). Bacteria, fungi, and viruses are often antagonistic pathogens, although some may act as mutualists of the host, promoting plant growth and tolerance of environmental stressors (Stone et al., 2018). As an example, the bacterium *Pseudomonas syringae*, a well-known plant pathogen, is also a biocontrol against plant viruses and other plant bacteria (Passera et al., 2019). Epiphytic microorganisms also fix or consolidate plant nutrients, particularly nitrogen (Fürnkranz et al., 2008), thus promoting growth of the host plant. Phyllosphere microorganisms can promote plant growth in other ways as well (Wagi and Ahmed, 2017; Yurimoto et al., 2021). Phyllosphere bacteria may also alter susceptibility to insect herbivory (Wielkopolan and Obrępalska-Stęplowska, 2016). Others have been shown to induce tolerance to drought stress (Kumar Devarajan et al., 2021).

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Diversity and Vertical Distribution of Epiphytic Angiosperms, in Natural and Disturbed Forest on the Northern Coast of Jalisco, Mexico

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Background and Aims: Epiphytes are an important component of tropical forests, also they are sensitive to disturbance and deforestation caused by humans, since they depend on their host trees and the micro environmental conditions that these provide. The aim of this study was to analyze the differences in species richness, composition, and vertical distribution of epiphytic angiosperms between areas with natural and disturbed forest at the Northern Coast of Jalisco state, Mexico.

Methods: The presence/absence of epiphytic angiosperms was evaluated in each vertical zone of a selected tree, as well as those present in the understory, both in natural and disturbed sites in three types of vegetation (gallery forest, oak forest, tropical semideciduous forest) with a total of 30 plots of 20 m × 20 m in six sites. Alpha diversity was calculated for each site, as well as species turnover (beta diversity) between habitats. An analysis of variance was performed to determine if there was a significant difference in species richness between sites and, also to compare the height and diameter at breast height (DBH) of the host trees. Multivariate analyzes were used to group the sites according to their floristic composition. Furthermore, a linear regression was performed to detect any relationship between the number of species and the phorophyte structure.

Results: We recorded 45 species, 29 genera and nine families of epiphytic angiosperms. The most diverse families were Bromeliaceae and Orchidaceae and the richest genus was *Tillandsia*. Although the disturbed sites had more species, a significant difference in richness was not found, except for the disturbed gallery forest. Epiphytic angiosperms presented a high beta diversity, since the sites shared only between 2 and 18% of the recorded species. The inner portion of the canopy (Z3 and Z4) hosted most of the species in all sites and the understory had a high representation of epiphytes except for the disturbed oak forest, where these were absent. A relationship between the DBH and the number of species was found only at the disturbed sites, however, it was highly influenced by the high number of taxa registered in disturbed gallery forest. Therefore, the size of the trees could not be considered a factor in determining the diversity of epiphyte species.

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Conclusion: The diversity of epiphytic angiosperm species from the North Coast of Jalisco has not been severely affected by the human disturbance. Most of the species have morphological and physiological adaptations that allow their establishment and survival in adverse climatic conditions. Our results suggest that epiphytic angiosperms cannot be considered as a good indicator for natural or disturbed environments in this region but should be considered in environmental conservation, as they present a high beta diversity.

Keywords: beta diversity, bromeliads, disturbance, floristic composition, orchids

INTRODUCTION

Currently, there is a great pressure on biodiversity due to anthropic impact, even though factors such as climate change and wildlife trafficking have also negative effects on biological communities, land-use change is considered the principal cause of biodiversity loss and ecosystem degradation (Sala et al., 2000; Wilson et al., 2016). Tropical forests are often the hardest hit, which is particularly serious, because they are containing more than half of the world's flora, while covering less than 7% of the Earth's surface (Sodhi et al., 2008; Newbold et al., 2015). Some species are sensitive to environmental changes and can be used as bioindicators, since aspects such as their distribution, abundance, dispersal or reproductive success might indicate the environmental conditions of interest (Holt and Miller, 2011). The level of disturbance and succession in which the forest habitat is found, will determine the plant community's permanence, as well as the composition and richness (Cuevas-Reyes and Vega-Gutiérrez, 2012).

Epiphytes are principally tropical plants that grow on other plants without being parasitic. This group includes both non-vascular (lichens and bryophytes) and vascular plants, the majority belong to ferns and monocots (e.g., orchids, bromeliads, and aroids) (Benzing, 1990; Zotz, 2016). There exist around 31 311 vascular epiphyte species in the world (ca. 10% of the global flora), of which 28 227 are angiosperms (Zotz et al., 2021). Mexico has a record of 1 813 vascular epiphytes and 84.4% (1 531) are angiosperms (Espejo-Serna et al., 2021). In Jalisco occur around 301 vascular epiphyte species (273 angiosperms) and the state is positioned on sixth place after Chiapas, Oaxaca, Veracruz, Guerrero, and Puebla. These plants are mostly distributed in humid montane forests, which encompass between 30 and 66.6% of their whole richness (Hietz, 2010; Espejo-Serna, 2014); however, they are also an important component in other tropical forests, where they play a crucial role in the community dynamics and constitute habitat and food for many vertebrates and invertebrates (Cruz-Angón et al., 2009; Gotsch et al., 2016; Sabagh et al., 2017). In addition to their floristic and ecological contribution, many epiphyte groups have economic importance, since several species are used as ornamental plants, others have medicinal value and some, especially the bromeliads, are an important element on religious ceremonies (Beutelspacher and Farrera, 2007; Flores-Palacios and Valencia-Díaz, 2007; Haeckel, 2008; Mondragón, 2015; Krömer et al., 2018; Jiménez-López et al., 2019).

Epiphytes are sensible to anthropic disturbance and deforestation, as they depend on their host trees and microenvironmental conditions that these provide (Köster et al., 2009; Larrea and Werner, 2010; Werner et al., 2011; Krömer et al., 2014). Water availability is the most important environmental factor, since it can cause local geographical shifts or species disappearance in certain areas (Benzing, 1998; Zotz and Hietz, 2001), because these plants do not have direct access to water and nutrients from the ground (Gentry and Dodson, 1987; Zotz, 2016). This sensibility has caused a richness decrease in disturbed and secondary forests compared to natural forests in different regions in Mexico (Wolf, 2005; Krömer et al., 2014; Pérez-Peña and Krömer, 2017), as well as in other neotropical countries (Barthlott et al., 2001; Köster et al., 2009, 2013; Werner and Gradstein, 2009). Another factor that influences the distribution, richness, and survival of epiphytes are the characteristics of the phorophytes they are associated with. Their size, diameter, bark texture, and chemical characteristics, as well as water and nutrient availability can positively or negatively affect epiphyte establishment and growth (Mehltreter et al., 2005; Martínez-Meléndez et al., 2008; Jiménez-López et al., 2017). Due to the high vulnerability that epiphytes present to changes in environmental conditions, these plants have been considered as excellent indicators of environmental quality (Turner et al., 1994; Krömer et al., 2014). Epiphytes that depend on mature host trees and have high humidity requirements, will be the most affected by disturbance and microclimatic changes, while epiphytes that are tolerant to high light exposure and drought stress will benefit (e.g., some xeromorphic species of the genus *Tillandsia*) (Reyes-García et al., 2007; Menini-Neto et al., 2009; Ochoa-López, 2009; Krömer et al., 2021). Therefore, the number of species, the floristic composition, and/or the abundance of epiphytes, can be good indicators of the ecosystem condition, succession status or habitat conservation.

Tropical dry forests can be structurally less complex than humid tropical forests; however, they have a high species turnover (beta diversity) between communities, which is expressed in a high level of endemism (Ceballos et al., 2010). There are few studies on epiphytes from tropical dry forests in Mexico (Aguirre et al., 2010; Cuevas-Reyes and Vega-Gutiérrez, 2012; Trejo-Cruz et al., 2021), which mostly report that disturbance affects species richness according to different land-use types. In a dry forest of Ecuador, less richness in disturbed areas has also been found, but density (number of species per tree) did not

vary significantly between the different studied land-use types (Werner and Gradstein, 2009).

The North Coast region of Jalisco has a great plant species diversity and a high level of endemism and includes a part of the most conserved and extensive tropical deciduous and semideciduous forests of Mexico (Arriaga et al., 2000; Vázquez-García et al., 2000). However, these are threatened by land-use changes, like the removal of vegetation for agricultural use, the heavy grazing or periodic fires that prevent the growth of new trees or stop the process of succession, as well as the logging of timber species and the excessive growth of the tourism sector (Ceballos et al., 2010; Morales-Hernández et al., 2016).

According to the available information about human impact on epiphyte diversity, our hypothesis states that, if epiphytic angiosperm richness and its floristic composition are negatively affected by land-use change, then their diversity will decrease in disturbed sites, and the composition will be different between habitats. Therefore, the objective of this study is to observe the differences of the epiphytic angiosperm richness and its floristic composition between areas with natural and disturbed forest at the North Coast of Jalisco state, Mexico.

MATERIALS AND METHODS

Study Area

The study was conducted in the municipality of Cabo Corrientes, located in the western part of Jalisco state, Mexico, between 20° 10′ 55″ and 20° 31′ 00″ latitude N and 105° 10′ 00″ and 105° 41′ 25″ longitude W (**Figure 1**). Cabo Corrientes belongs to the North Coast region together with the municipalities of Puerto Vallarta and Tomatlán [Comisión Estatal del Agua Jalisco [CEA], 2021] and forms part of the biogeographic provinces Pacific Coast and Sierra Madre del Sur (Morrone et al., 2017). Approximately 53% of the municipality has mountainous terrain, and its elevation ranges from sea level to 1 920 m a.s.l. [Instituto de Información Estadística y Geográfica [IIEG], 2018]. The predominant vegetation type is tropical deciduous forest (TDF), located on the southern coast, followed by tropical semideciduous forest (TSF), which extends its distribution along the north coast (Órgano informativo oficial del municipio de Cabo Corrientes, Jalisco, 2012). On higher elevations there are oak forest (OF) and pine-oak forest (POF) that can sometimes also be found at 300 m a.s.l., associated with tropical species. Besides, there is presence of gallery forest (GF) along rivers, while cloud forest (CF) is restricted to steep slopes in the areas with presence of POF (Rzedowski and McVaugh, 1966). The predominant climate is warm sub-humid with a dry season in winter and a rainy season in summer, and semi-warm semi-humid on the East side (higher elevations). The mean annual temperature is 24.6°C with a maximum of 36°C and minimum of 13°C and the mean annual precipitation is 1 624 mm [Instituto de Información Estadística y Geográfica [IIEG], 2018].

Sampling Methods

Field work was conducted in three types of forest vegetation (GF, OF, TSF), according to the classification of Rzedowski (1978), because the epiphytic angiosperms in this region are better represented in these communities. For each vegetation type two kinds of habitat were chosen, following the criteria proposed by Gómez-Díaz et al. (2017) based on Newbold et al. (2015): (a) natural forest are dominated by mature trees and show no or little signs of logging and other human impacts, with little presence of shrubs and a low canopy opening; and (b) disturbed forest show an evident degree of alteration (selective logging, grazing), a lower presence of mature trees, and a higher percentage of shrubs and grasses. The sampling of epiphytes was based on the method proposed by Krömer and Gradstein (2016), which consists in selecting mature trees with a diameter at breast height (DBH) greater than 10 cm and with a high load of epiphytes. Around the selected tree, a non-permanent 20 m × 20 m plot was established, where also the small trees, shrubs and shoots that constitute the understory were sampled (**Figure 2**). Five plots were surveyed in each vegetation type (GF, OF, TSF) and habitat (N: natural and D: disturbed) with a total of 30 plots in six study sites (**Figure 2**) corresponding to an area of 12,000 m^2.

We considered true epiphytes or holoepiphytes (that spend their entire life cycle in the canopy without having contact to the ground), primary hemiepiphytes (that begin their cycle in the canopy and then establish root contact with the ground), and secondary hemiepiphytes (that start their life cycle rooted to the ground and then may lose contact while climbing a phorophyte) (Kress, 1986; Benzing, 2012). However, the use of the last term has been questioned by some authors, since there is no evidence that these plants really lose their connection to the ground to pass to an epiphytic state, and thus the use of "nomadic vine" is suggested for all climbing plants that germinate in the ground (Zotz, 2013; Bautista-Bello et al., 2021).

For each selected tree, we recorded the presence/absence of the epiphytic angiosperms located within five vertical zones (Z1: basal part of trunk, 0–2 m high, Z2: upper part of the trunk to the first ramification, Z3: basal part of the large branches, up to the second ramifications, about a third of total branch length, Z4: second third of branch length, Z5: outer third of branch length) according to Johansson (1974). We also noted the presence/absence of all epiphytes in the understory within each 20 m × 20 m subplot. To document the habitat of the epiphytes, the following host tree data were recorded: taxonomic identification (when fertile material was available), height (estimation by observation), and DBH (Krömer and Gradstein, 2016). For a more complete sampling of the epiphytes, the single rope technique was used in several humid montane forest studies (Flores-Palacios and García-Franco, 2001; Krömer et al., 2007a). However, it was not necessary to use this method in our study, due to the simple structure of the phorophyte species (not very dense canopy with trees over 20 m high or trees of low height), which allowed an adequate observation from the ground using binoculars and digital camera.

The species that could not be identified at the sampling site, were collected and herborized; in some cases when epiphytes did not present reproductive structures, live plants were kept in cultivation until flowering, in order to guarantee its correct identification. The collected samples were prepared according to Aguirre-León (1986) and deposited in the

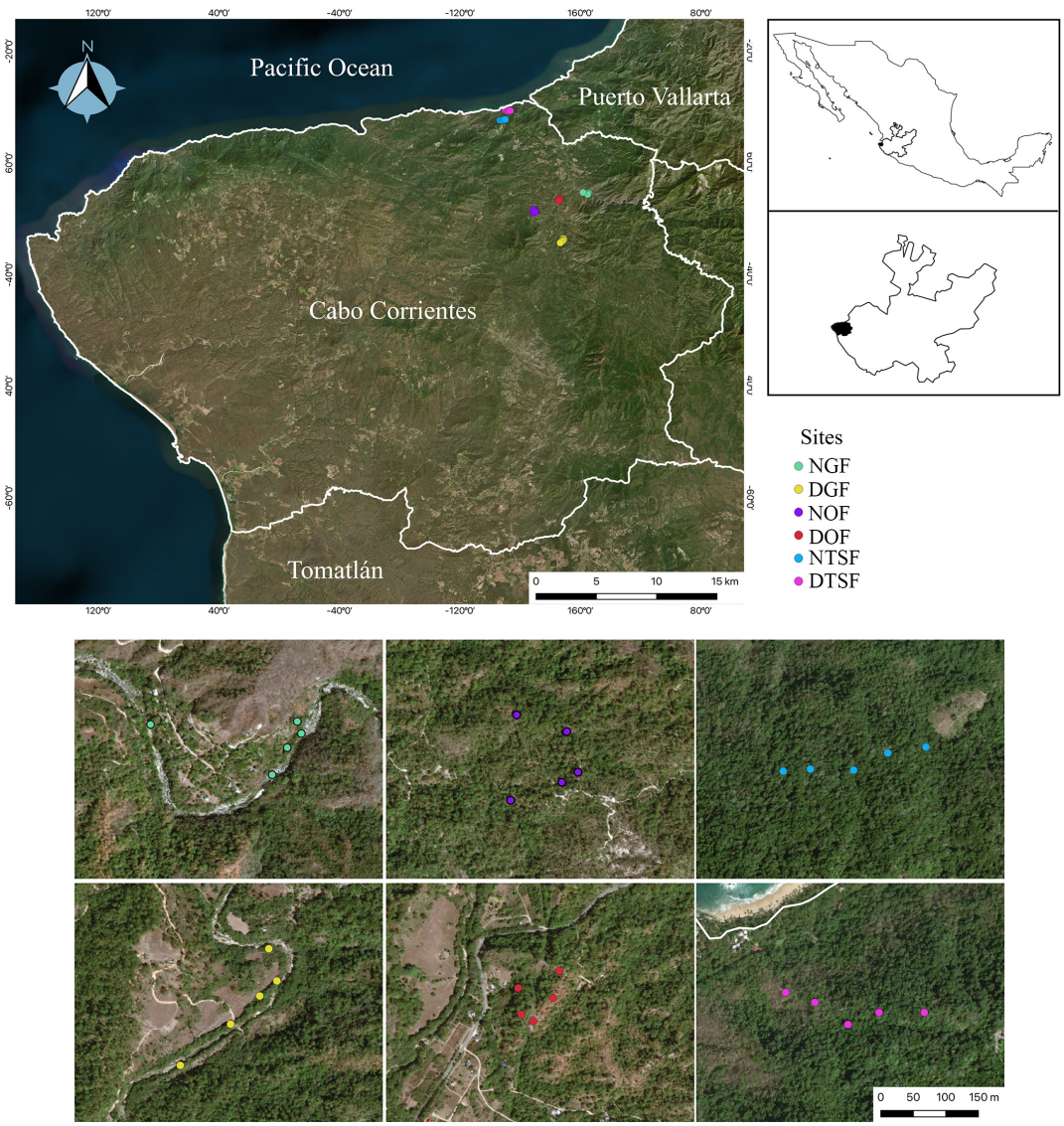


FIGURE 1 | Study area and location of the six sampling sites (NGF = natural gallery forest, DGF = disturbed gallery forest, NOF = natural oak forest, DOF = disturbed oak forest, NTSF = natural tropical sub-deciduous forest, DTSF = disturbed tropical sub-deciduous forest) in Cabo Corrientes, Jalisco, Mexico. (Google Earth Pro, 2020).

Herbario Metropolitano (UAMIZ) of the Universidad Autónoma Metropolitana-Iztapalapa in Mexico City, Mexico. For the circumscription of the epiphyte families, we followed the classification of APG IV (The Angiosperm Phylogeny Group, 2016) and at the species level that of The Plant List (2013) and Tropicos (2020). In the case of some families such as Bromeliaceae, Cactaceae, and Orchidaceae, the studies of some taxonomic specialists were considered (Schuiteman and Chase, 2015; Cruz et al., 2016; Espejo-Serna and López-Ferrari, 2018).

Data Analysis

To verify the sampling representativeness, species accumulation curves for the different sampled vegetation types and habitats were made, applying the estimator of potential species richness based on Chao2 presence-absence data using the Estimate*S* statistical package (Colwell, 2019). Species richness was determined for each vegetation type, habitat, and plot (alpha diversity), as well as the species turnover between vegetation and habitats (beta diversity), using the Sørensen dissimilarity index (Hao et al., 2019). An analysis of variance (ANOVA) was performed to determine if there was a significant difference in species richness between the six sites (NGF, DGF, NOF, DOF, NTSF, DTSF), and to compare the height and the DBH of the hosts. The normality was analyzed with the Skewness and Kurtosis (Omnibus) test and the homogeneity with the *F*-test. In case of a significant difference, the Tukey-Kramer test was used to compare each site. The significance level used is 0.05, therefore, the null hypothesis (H0) is not rejected if the probability value is

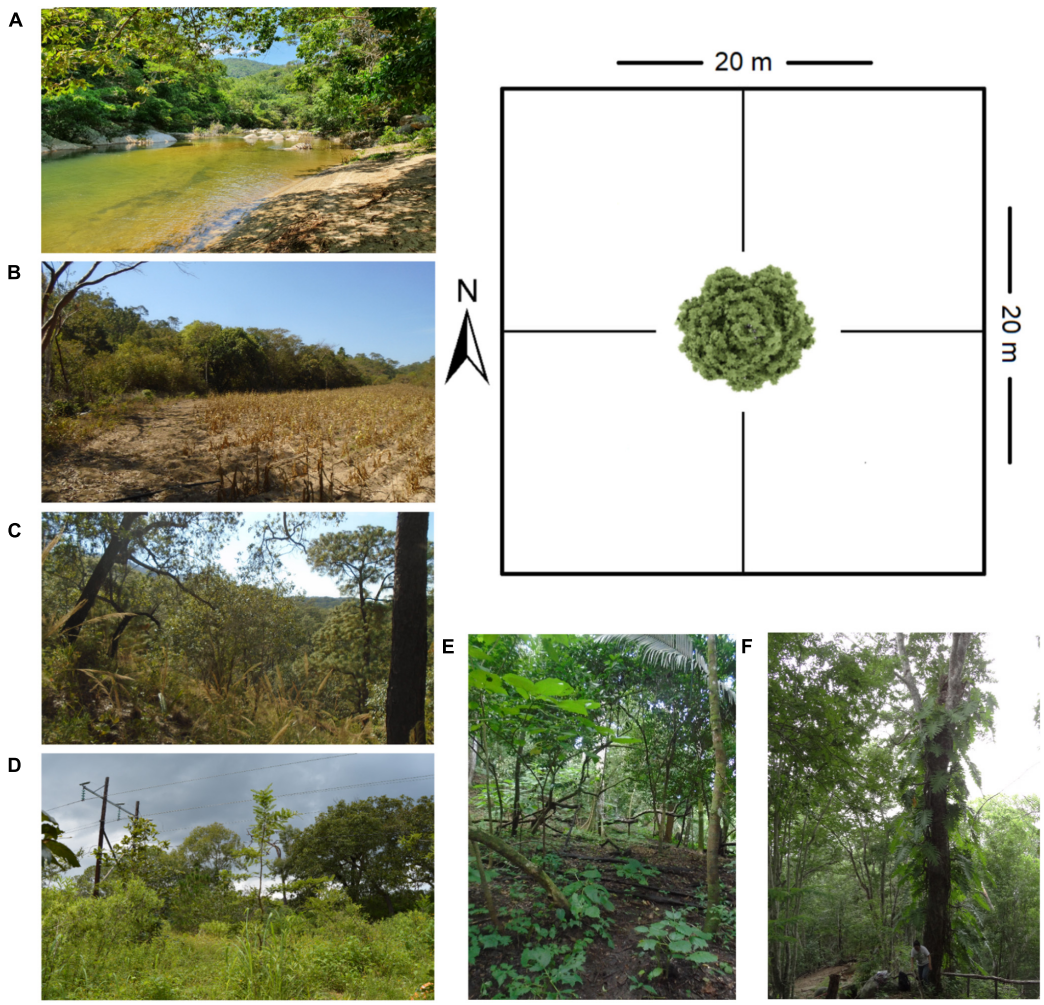


FIGURE 2 | Diagram of the plot and view of the sampling sites. **(A)** NGF = natural gallery forest, **(B)** DGF = disturbed gallery forest, **(C)** NOF = natural oak forest, **(D)** DOF = disturbed oak forest, **(E)** NTSF = natural tropical sub-deciduous forest, **(F)** DTSF = disturbed tropical sub-deciduous forest.

equal to or greater than 0.05 (there is no significant difference), and it is rejected if it is less than 0.05 (at least one is significantly different). To determine if there was a relationship between the number of species and the phorophyte structure (height and DBH) a simple linear regression was performed for both natural and disturbed sites. A cluster analysis was performed using the hierarchical, agglomerative method in order to group the sampled sites based on the floristic composition. Regarding the vertical distribution and understory structure, the non-metric multidimensional scaling (NMDS) analysis was applied between each vertical phorophyte zone (Z1-5), and the understory of every sampling site based on Sørensen dissimilarity index. All analyzes were carried out in the statistical program NCSS (2018).

RESULTS

We found 45 species, 29 genera and nine families of epiphytic angiosperms (**Supplementary Appendix 1**). The most diverse families were Bromeliaceae and Orchidaceae with 17 and 15 species, respectively. The richest genus was *Tillandsia* with 13 species. The recorded species include 37 true epiphytes, four primary hemiepiphytes, and four secondary hemiepiphytes. None of the species found are registered in the Official Mexican Standard (NOM-059-SEMARNAT-2010); however, 15 species are endemic to Mexico (8 of them exclusive to the Pacific slope), two endemic to western Mexico and two endemic to Jalisco (**Supplementary Appendix 1**). According to the Chao2 estimator, we observed mostly more than 90% of the estimated species. The lowest numbers of observed species were recorded in NGF with 15 (83%) and in DTSF with 13 (68%) (**Figure 3**).

Besides being the most diverse families, Bromeliaceae and Orchidaceae were represented in all sampling sites. Bromeliaceae stands out for high species numbers in NOF (7; 64%) and DTSF (6; 46%), while Orchidaceae dominates in NGF (6; 40%), DGF (10; 43%), and DOF (7; 58%). Araceae represented the highest richness in NTSF (4; 44%) (**Figure 4**). The remaining families contributed less than 15% in each site. *Guarianthe aurantiaca*

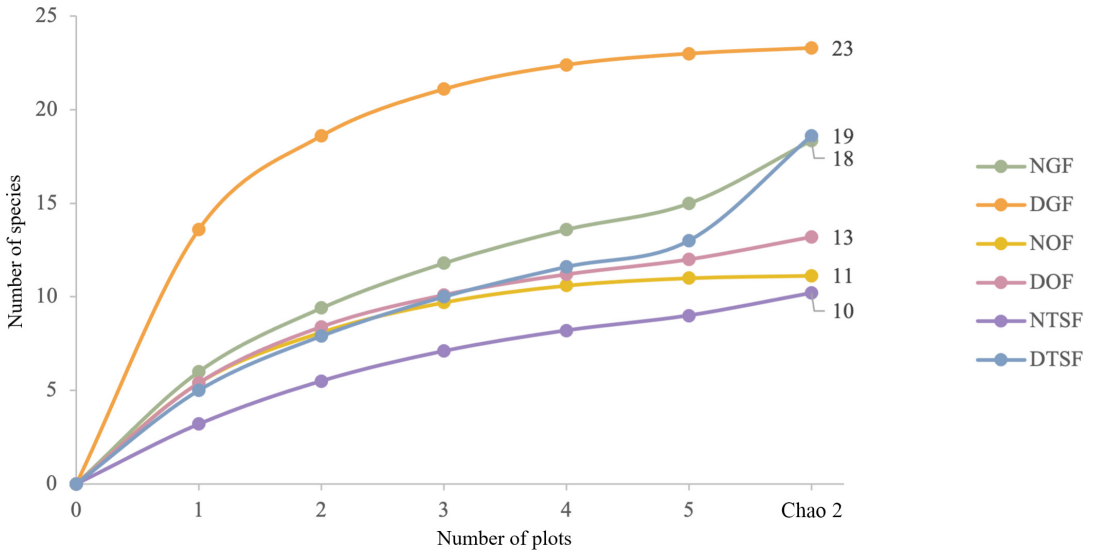


FIGURE 3 | Accumulation curves of epiphytic angiosperm species for the six sampling sites (NGF = natural gallery forest, DGF = disturbed gallery forest, NOF = natural oak forest, DOF = disturbed oak forest, NTSF = natural tropical sub-deciduous forest, DTSF = disturbed tropical sub-deciduous forest).

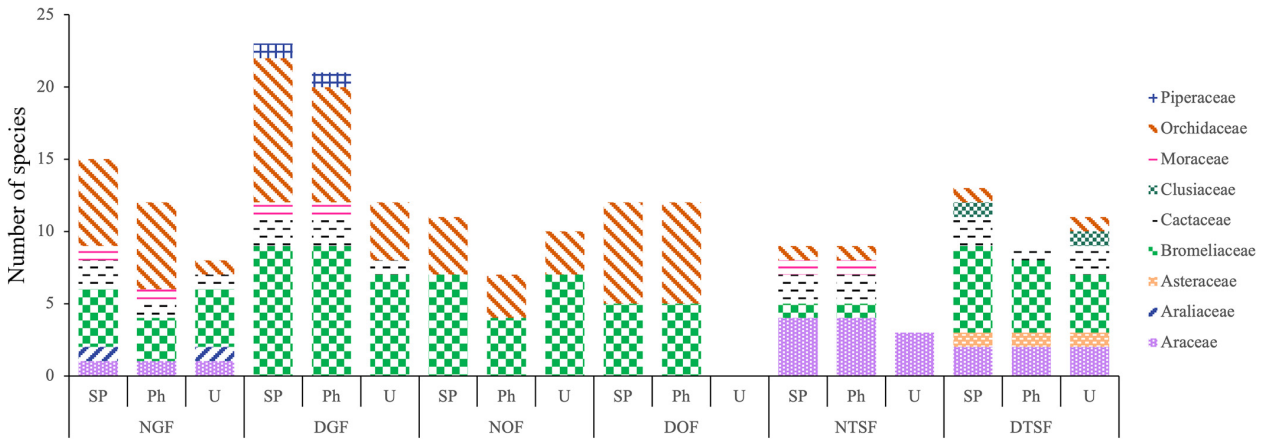


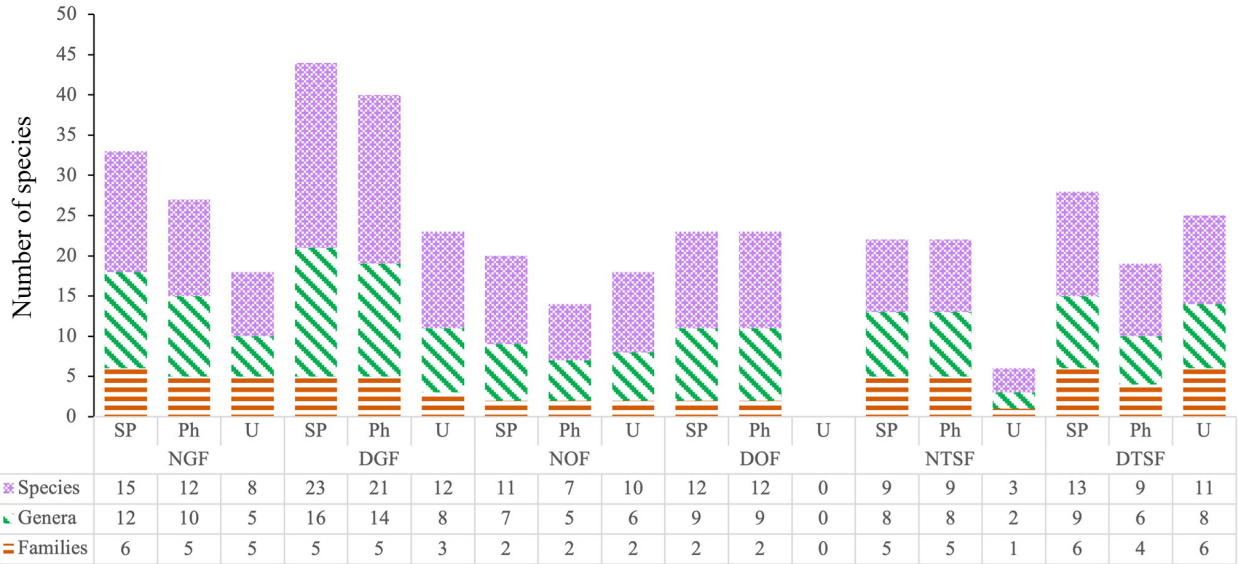
FIGURE 4 | Number of species per family in the entire sampled plot (SP), the phorophytes (Ph) and the understory (U) in each sampling site (NGF = natural gallery forest, DGF = disturbed gallery forest, NOF = natural oak forest, DOF = disturbed oak forest, NTSF = natural tropical sub-deciduous forest, DTSF = disturbed tropical sub-deciduous forest).

(Bateman) Dressler and W. E. Higgins and *Tillandsia schiedeana* Steud. were present in five of the six sampling sites, while 49% of the epiphyte species were found in only one site, being DGF the site with the highest number (7) of exclusive species.

Effects of the Sampling Sites and Vertical Zones on Species Richness

The DGF harbored most species (23) and the NTSF the lowest number (9) of epiphytes (**Figure 5**). Although the ANOVA indicated that the richness was significantly different between the six sites ($F = 24.6683$; $p < 0.001$), the Tukey-Kramer test showed that the only different site in relation to the number of species was the DGF ($p < 0.001$; **Figure 6**).

The sampled phorophytes were hosting a total of 39 species, 25 genera, and seven families of epiphytic angiosperms (**Figure 5** and **Supplementary Appendix 2**) and showed an average height of 18 ± 5 m and a DBH of 77.4 ± 41.7 cm. The highest tree was located in NTSF measuring 32 m and the smallest was in NOF with 10 m. The widest tree corresponded to the DGF with a DBH of 181.4 cm and the thinnest belonged to the NOF with 29.6 cm (**Supplementary Appendix 2**). According to the ANOVA, there are significant differences between the height of the trees from the sampling sites ($F = 14.0225$; $p < 0.001$), as well as in the DBH ($F = 8.3824$; $p < 0.001$) (**Figure 7**). The simple linear regression model showed that there is no relation between phorophyte height and epiphytic angiosperm richness for both natural ($a = 4.7011$, $b = -0.0569$, $r^2 = 0.1332$, $p > 0.05$) and



	SP	Ph	U	SP	Ph	U	SP	Ph	U	SP	Ph	U	SP	Ph	U	SP	Ph	U
	NGF			DGF			NOF			DOF			NTSF			DTSF		
Species	15	12	8	23	21	12	11	7	10	12	12	0	9	9	3	13	9	11
Genera	12	10	5	16	14	8	7	5	6	9	9	0	8	8	2	9	6	8
Families	6	5	5	5	5	3	2	2	2	2	2	0	5	5	1	6	4	6

FIGURE 5 | Families, genera and species in the entire sampled plot (SP), phorophytes (Ph) and understory (U) per sampling site (NGF = natural gallery forest, DGF = disturbed gallery forest, NOF = natural oak forest, DOF = disturbed oak forest, NTSF = natural tropical sub-deciduous forest, DTSF = disturbed tropical sub-deciduous forest).

disturbed sites ($a = 12.7123$, $b = -0.3677$, $r^2 = 0.2095$, $p > 0.05$) (**Figure 8**). As for DBH, no correlation was found with the number of species in the natural sites ($a = 4.1123$, $b = -0.0079$, $r^2 = 0.0263$, $p > 0.05$), but it was found in the disturbed sites ($a = 1.6553$, $b = 0.0657$, $r^2 = 0.4426$, $p < 0.05$) (**Figure 8**).

The vertical distribution of epiphytes was principally concentrated in the zone Z4 with 28 species, while Z1 had the lowest richness with 12. The zones Z3 and Z4 hosted the greatest richness in almost all sampled sites, while Z1 and Z2 had the lowest (**Figure 9**). *Aechmea bracteata*, *Hylocereus purpusii* (Weing.) Britton and Rose, *Philodendron scandens* Kunth, and *Tillandsia jaliscomonticola* Matuda were present in all five zones and 26% of the species were exclusive to one zone (**Supplementary Appendix 1**). On the other hand, the understory had 32 species, 19 genera, and eight families of epiphytic angiosperms. There was a higher richness in the understory than in the phorophytes in the NOF and in the DTSF, but we did not observe any epiphyte species in the DOF understory (**Figure 5** and **Supplementary Appendix 2**). Bromeliads were present in the understory at all sites, except for NTSF where only species of Araceae were found (**Figure 4**).

Effects of the Sampling Sites and Vertical Zones on Species Composition

The sampling sites shared between 2 and 18% of the recorded species in which the DGF and the NOF were least different with a dissimilarity coefficient of 0.68, while the sites with greatest dissimilarity were NTSF with both NOF and DGF, with values of 0.91 and 0.89, respectively (**Supplementary Appendix 3**). On the other hand, the cluster analysis divided the sites into two main groups: (1) NOF, DOF, and DGF; (2) NTSF, DTSF, and NGF (**Figure 10**). According to the NMDS analysis, there is a clear difference in the epiphytic species represented in the zones and in the understory in almost all sites (**Figure 11**). The highest dissimilarity index was between the zones Z3 and Z4 in NOF, as they share 86% of the species, and the zone Z2 in DOF was the

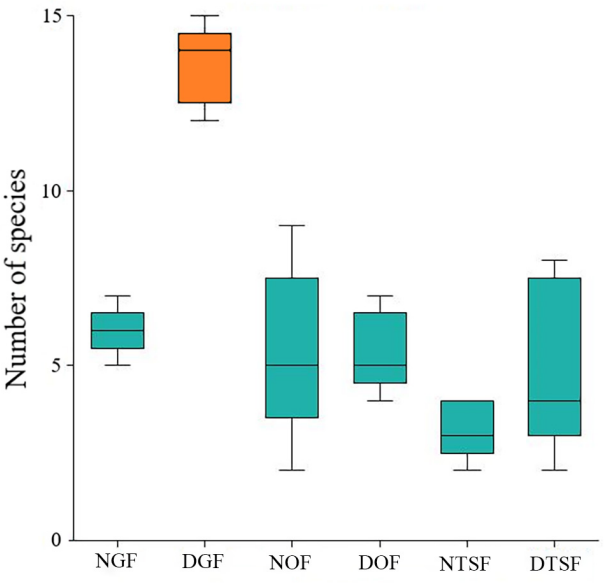


FIGURE 6 | Variation in number of epiphytic angiosperm species per sampling site (NGF = natural gallery forest, DGF = disturbed gallery forest, NOF = natural oak forest, DOF = disturbed oak forest, NTSF = natural tropical sub-deciduous forest, DTSF = disturbed tropical sub-deciduous forest). Green: no significant difference; Orange: with a significant difference.

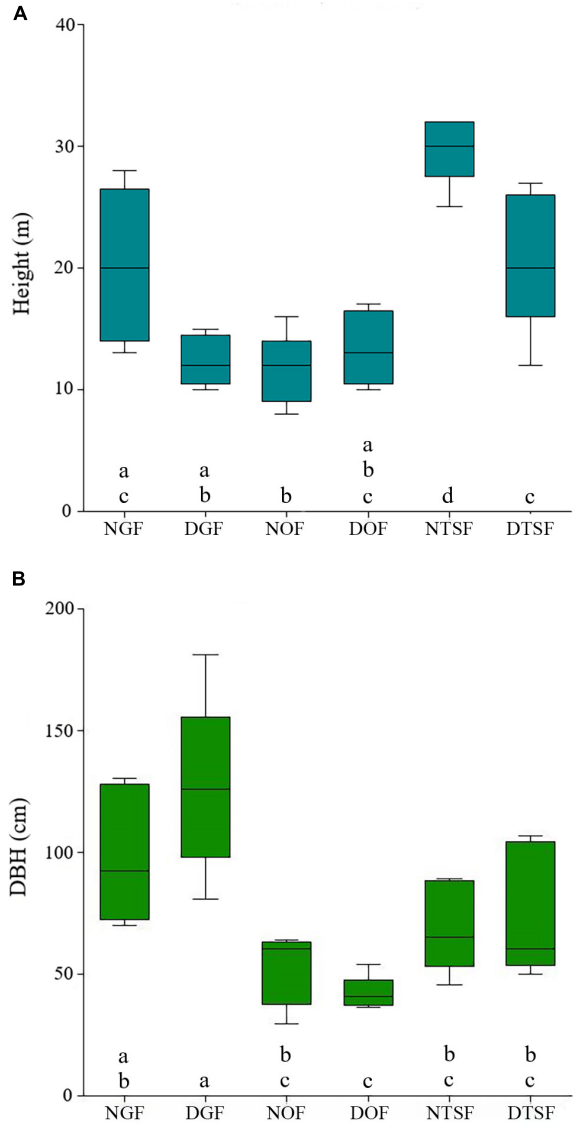


FIGURE 7 | Variation in the height **(A)** and diameter breast height (DBH) **(B)** of the phorophytes in each sampling site (NGF = natural gallery forest, DGF = disturbed gallery forest, NOF = natural oak forest, DOF = disturbed oak forest, NTSF = natural tropical sub-deciduous forest, DTSF = disturbed tropical sub-deciduous forest). Sampling sites that do not have the same letter are significantly different.

most different from all, as it presented only one species, that was also found in zone Z2 in NTSF. The understory from all sites were more similar to the canopy zones than to the trunk area.

DISCUSSION

When comparing the number of species obtained in the present study, with the total of angiosperm epiphytes registered for Jalisco (Espejo-Serna et al., 2021), we recorded 16.5% of these in less than one km^2 (12,000 m^2). There is also a considerable richness compared to that reported in two previous floristic inventories made for the region: Ramírez-Delgadillo and Cupul-Magaña (1999) recorded 29 taxa of epiphytic angiosperms for the municipalities of Bahía de Banderas, Cabo Corrientes, and Puerto Vallarta. On the other hand, Vázquez-García et al. (2000) registered 28 species for the municipality of Cabo Corrientes in their contribution for the North Coast of Jalisco. We obtained more than 80% of the estimated epiphyte species richness for the study area, which indicates that the sampling effort was sufficient.

The high richness of Bromeliaceae and Orchidaceae registered in the area, was an expected result, since both are the richest families among the angiosperm epiphytes at the national and state level in Mexico (Ceja-Romero et al., 2010; Krömer et al., 2020; Miguel-Vázquez et al., 2020; Espejo-Serna et al., 2021). In some local or regional studies carried out in Mexico this pattern has also been found, both in the case of humid forests (Flores-Palacios and García-Franco, 2008; Martínez-Meléndez et al., 2008; Krömer et al., 2013) and dry forests (Wolf, 2005; Trejo-Cruz et al., 2021), with the Orchidaceae family predominating. Other studies have registered a greater number of Bromeliaceae species, as in the case of a dry forest in Chamela, Jalisco (Cuevas-Reyes and Vega-Gutiérrez, 2012), or that of El Cielo Biosphere Reserve, in Tamaulipas (de la Rosa-Manzano et al., 2017). However, in many studies carried out in other Neotropical countries, it has been found that Araceae, including the nomadic vines, occupies the second place especially in lowland rainforests, while in humid montane forests the ferns have a similar richness to that of orchids (Kreft et al., 2004). The dominance of the Araceae in tropical semideciduous forest and its absence in oak forest, is due to the fact that the representatives of this family generally do not inhabit dry and/or temperate forests (Wolf, 2005; Cuevas-Reyes and Vega-Gutiérrez, 2012), and also because their species number tends to decrease with increasing elevations, since the plants are not well adapted, both structurally and physiologically, to arid or cold conditions (Krömer et al., 2005; Acebey and Krömer, 2008).

The Piperaceae family is generally diverse in humid montane forest and tropical evergreen forest (Barthlott et al., 2001; Krömer et al., 2005, 2007b), because the species of the genus *Peperomia* have a marked preference for warm or temperate areas, but with high humidity conditions (Vergara-Rodríguez et al., 2017), which may also explain the presence of one of its species in the disturbed gallery forest of the study area. The other registered families (Araliaceae, Asteraceae, Cactaceae, Clusiaceae, and Moraceae), most of them present with only one species, had a low diversity, situation that has been reported in other studies (Olmsted and Gómez-Juárez, 1996; Gomez-Escamilla et al., 2019; Krömer et al., 2020; Miguel-Vázquez et al., 2020). It is also important to consider that the presence of the epiphytic habit among its representatives, is lower than in the case of the four main families mentioned above (Araceae, Bromeliaceae, Orchidaceae, Piperaceae), which concentrate 87% of the epiphytic angiosperms in the world (Zotz et al., 2021).

Tillandsia was the richest genus; many of its species present special adaptations (e.g., atmospheric forms, absorbent foliar trichomes, CAM photosynthesis), which allows them to resist and survive in conditions of dry air and high temperatures

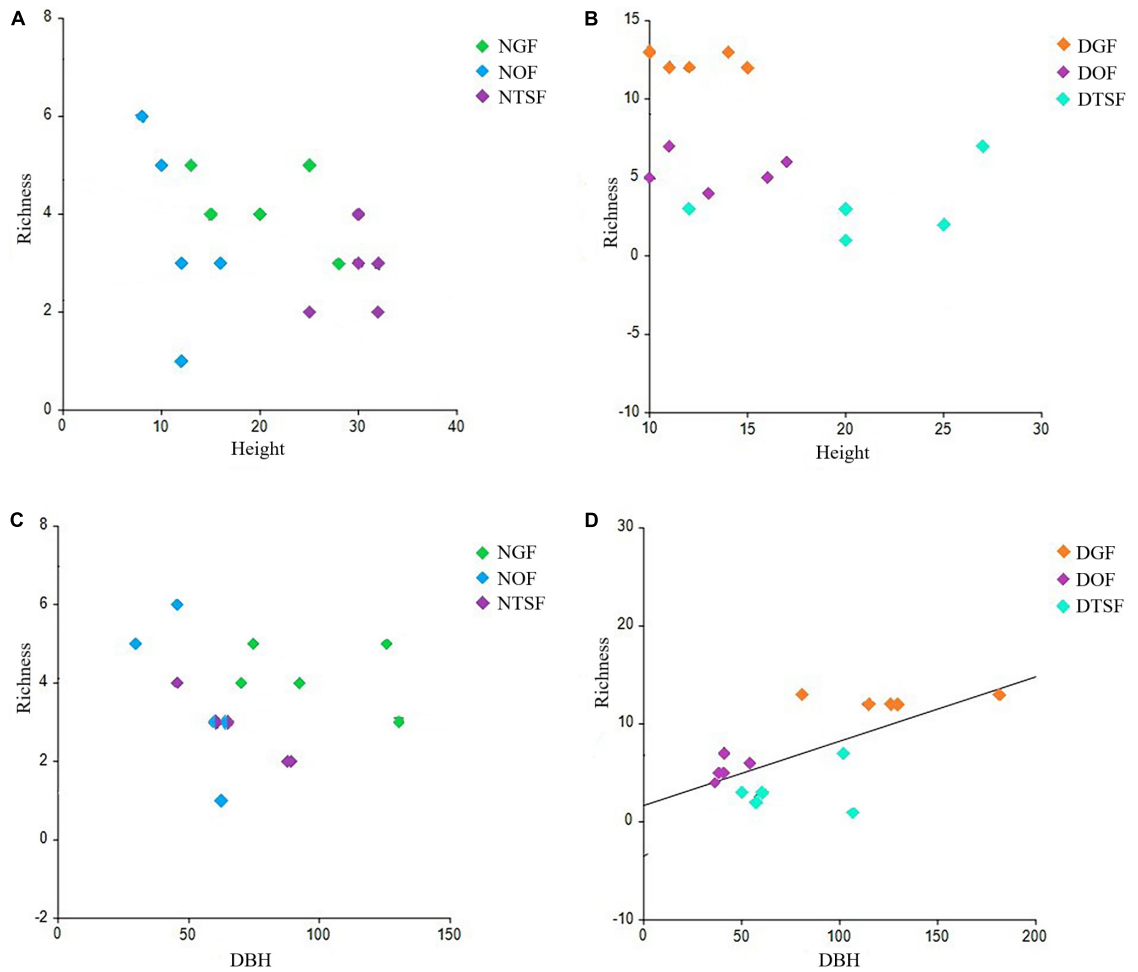


FIGURE 8 | Simple linear regression between phorophyte height and species richness of epiphytic angiosperms in natural sites **(A)** and disturbed sites **(B)**. Simple linear regression between phorophyte DBH and species richness of epiphytic angiosperms in natural sites **(C)** and disturbed sites **(D)**. NGF = natural gallery forest, DGF = disturbed gallery forest, NOF = natural oak forest, DOF = disturbed oak forest, NTSF = natural tropical sub-deciduous forest, DTSF = disturbed tropical sub-deciduous forest.

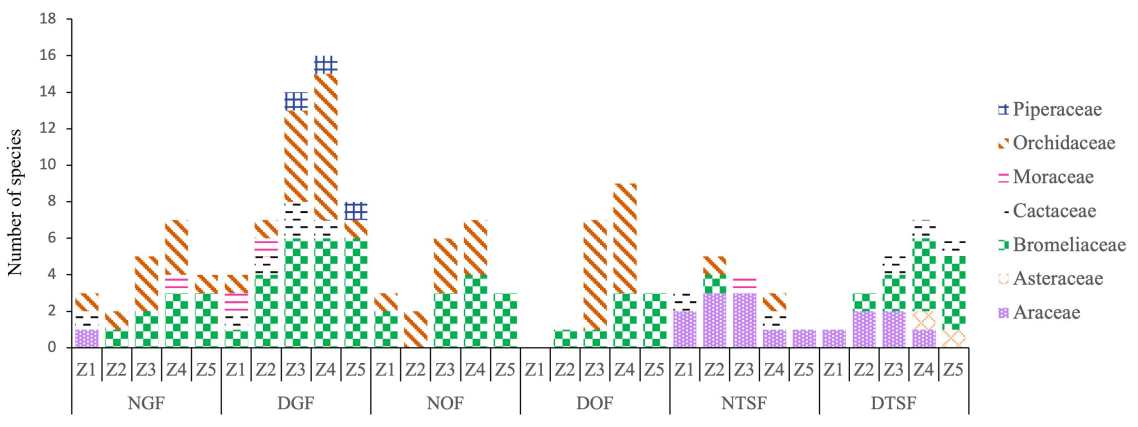


FIGURE 9 | Number of species per family in the vertical zones (ZI, Z2, Z3, Z4, Z5) in each sampling site (NGF = natural gallery forest, DGF = disturbed gallery forest, NOF = natural oak forest, DOF = disturbed oak forest, NTSF = natural tropical sub-deciduous forest, DTSF = disturbed tropical sub-deciduous forest).

(Zotz and Andrade, 2002; Benzing, 2012; Krömer et al., 2014), as well as surpassing other species, both in richness and abundance (Wolf, 2005; Cascante-Marín et al., 2006; Hietz et al., 2006; Cuevas-Reyes and Vega-Gutiérrez, 2012). In addition, faster growth rates have been found in seedlings of some *Tillandsia* species that grow in more exposed sites than those in shady places (Winkler et al., 2005; Flores-Palacios and García-Franco, 2006; Einzmann and Zotz, 2017). This may explain the low presence of species and individuals of the genus in the tropical semideciduous forest, which is the site with the most closed canopy.

Effects of the Sampling Sites and Vertical Zones on Species Richness

In the three types of forest vegetation sampled in the study area, surprisingly the disturbed sites presented a greater number of epiphyte species. This is in contrast to published data (Barthlott et al., 2001; Wolf, 2005; Krömer et al., 2007b, 2014; Köster et al., 2009, 2013; Pérez-Peña and Krömer, 2017), where generally the highest specific richness of epiphytes, both in Mexico and in the Neotropics, has been found in natural forests, while few authors found no differences (Hietz-Seifert et al., 1996; Larrea and Werner, 2010), or even reported the opposite situation. Aguirre et al. (2010) recorded a greater richness of epiphytes in disturbed sites in a forest dominated by the palm *Sabal mexicana*, on the Gulf coast in Veracruz, mainly due to the accumulation of hemiepiphytic species of low abundance (e.g., *Ficus* spp.) and the presence of accidental epiphytes, although these are not normally considered in studies of this type. Furthermore, Guzmán-Jacob et al. (2020) observed that, along the altitudinal gradient of the Cofre de Perote in Veracruz, richness of epiphytes in a degraded tropical semideciduous forest at the 500 m elevation, was higher than in adjacent old-growth forest.

It should be noted that, in general terms, there was no significant variation among the six sampling sites, except for the DGF. This could indicate that epiphyte richness did not respond significantly to disturbance in dry forests, due to the physiological and morphological pre-adaptations of the mostly drought tolerant species (Werner et al., 2011; Guzmán-Jacob et al., 2020). Some species such as *Philodendron warszewiczii* Koch and C. D. Bouché, *Aechmea bracteata*, and *Tillandsia jaliscomonticola*, had preference for phorophytes from disturbed and open areas, however, they were not exclusive to disturbed sites. Therefore, the changes of dominance in these species, could be used as indicators to determine the effects of anthropic disturbance (Krömer et al., 2014).

Although all the sampling sites presented exclusive species, only nine taxa were restricted to the natural ones; however, most are common and wide-ranging species such as *Tillandsia ionantha* and *Syngonium neglectum*. Therefore, the species that can be considered as indicators of environmental quality in this part of the country could not necessarily be used in other regions. The preference for certain type of microenvironments, regardless of whether the site was natural or disturbed, was more notable in the case of some species: for example, *Trichocentrum oestlundianum* (L. O. Williams) M. W. Chase and N. H. Williams was collected at both habitats in the gallery forest but showed a preference for more closed and humid sites. Some species were also observed to benefit from disturbance, such as several of the genus *Tillandsia*, which were more abundant in open spaces, or

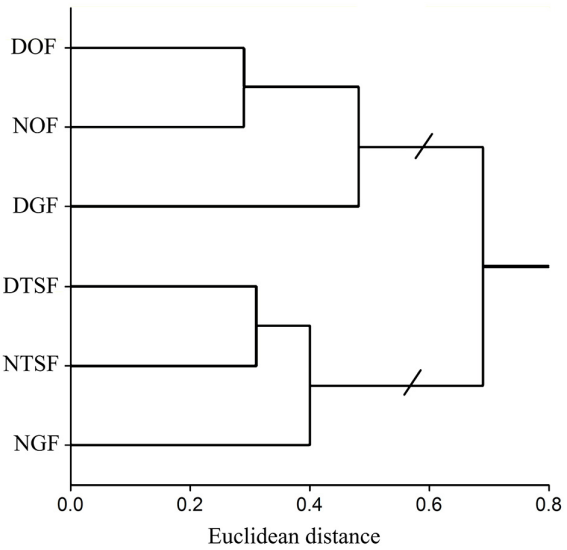


FIGURE 10 | Cluster analysis of epiphytic angiosperms floristic composition between the six sampling sites (NGF = natural gallery forest, DGF = disturbed gallery forest, NOF = natural oak forest, DOF = disturbed oak forest, NTSF = natural tropical sub-deciduous forest, DTSF = disturbed tropical sub-deciduous forest).

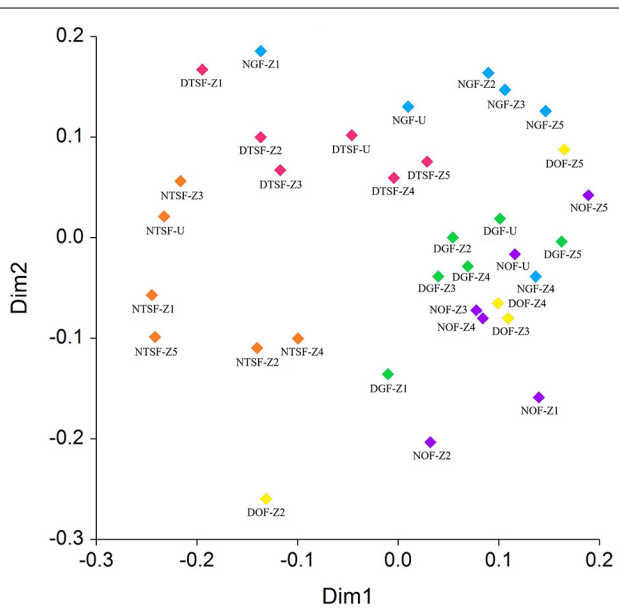


FIGURE 11 | Ordination site scores in two dimensions generated by non-metric multidimensional scaling (NMDS) using the Sørensen dissimilarity index of epiphytic angiosperms floristic composition per vertical zone (Z1, Z2, Z3, Z4, Z5) and understory (U) in each sampling site (NGF = natural gallery forest, DGF = disturbed gallery forest, NOF = natural oak forest, DOF = disturbed oak forest, NTSF = natural tropical sub-deciduous forest, DTSF = disturbed tropical sub-deciduous forest).

the hemiepiphyte *Philodendron warscewiczii* that had a wider vertical distribution and also a much higher frequency in the disturbed site (DTSF) (see **Supplementary Appendix 1**). The changes in dominance of these species could be used to determine effects of anthropic disturbance (Krömer et al., 2014). The greater richness and frequency of the species recorded in the disturbed sites in the study area, could be explained with the hypothesis of intermediate disturbance (Connell, 1978; Kun et al., 2009), which suggests that the presence of intermediate disturbance levels allow the coexistence of many species, more than would exist in the absence of these alterations or in the case of more drastic environmental changes. If the habitat is not highly fragmented, populations can filter through clusters of habitable sites, favoring the spread of a population.

The size (height and DBH) of the phorophytes was highly variable in the six sampling sites, even among the same forest types. The structure of trees in NGF was very similar to those of DTSF, however, the greater richness recorded in the former, may be mainly due to its proximity to a stream. The phorophytes of the disturbed gallery forest were structurally more complex than those of the other sites, offering a greater diversity of ecological niches due to the variety of sizes and positions of their branches, thus presenting pitchforks and nodes, that facilitate colonization by epiphytes (Arévalo and Betancur, 2006), as well as variable spaces, from shady and humid places near the trunk base to lighted and dry areas on the outer part of the crown (Böhnert et al., 2016), which caused the greatest richness of this site. In the case of the oak forest of the study area, all the host trees were of the genus *Quercus*. The rough bark of their trunks and branches offers a porous substrate with higher humidity due to its high-water retention capacity; this facilitates the anchoring of seeds (Callaway et al., 2002; Malizia, 2003) and makes them ideal phorophytes for the epiphytes in the dry environments of this type of vegetation. The phorophytes of NTSF had the same number of species as those of DTSF (**Figure 5**), although the average of all trees per site was lower (2.8) compared to the site with disturbed vegetation (3.2), which is probably explained by the presence in this last site of a single phorophyte with many epiphytes, probably due to its proximity to a stream of water (see **Supplementary Appendix 2**). The scarce presence of epiphytes in the phorophytes of tropical semideciduous forest is a consequence of their structure, since they have long and straight trunks and their branches in the canopy have little surface area for the establishment of epiphytic plants. In addition, the closed canopy of these plant communities, especially in the natural sampling site, did not allow the passage of sunlight, affecting the development of heliophilous species.

There is evidence of a positive relationship between the richness and/or abundance of epiphytic species and the DBH or height of the phorophytes (Hietz and Hietz-Seifert, 1995; Burns and Dawson, 2005; Flores-Palacios and García-Franco, 2006; Ochoa-López, 2009; Gómez-Díaz, 2010; Cuevas-Reyes and Vega-Gutiérrez, 2012; Dislich and Mantovani, 2016; Jiménez-López et al., 2017). However, in the study area, the disturbed sites were the only ones in which this positive relationship was observed, although probably the high number of taxa registered in DGF influenced this result, since both in DOF and DTSF, few species were recorded on trees with greater DBH. In other documented cases, such as that of a temperate forest in the Sierra Norte de Oaxaca (Bautista et al., 2014), and that of a tropical dry forest in the state of Morelos (Vergara-Torres et al., 2010), this relationship between the DHB of the phorophytes and the richness of epiphytes, was also not found. Additionally, it is important to consider that a mature tree does not necessarily have to be large, but rather its age, that is, the time it has been available for the colonization of epiphytes, could be the factor that most influences the species richness. Köster et al. (2011) observed that relatively small, but mature trees, in secondary vegetation derived from a cloud forest, harbored a high number of epiphytic species. Therefore, the structure and the specific identity of the phorophyte, as well as the type of vegetation in which it is found, are the determining factors, rather than the size of the trees.

The greatest epiphyte richness was found inside the canopy (Z4 y Z3), while the lowest was on the trunk (Z1 y Z2) of the trees. The inner part of the canopy has a suitable microenvironment for a successful establishment of many epiphytes, there is abundant organic matter on thick, vertical branches (ter Steege and Cornelissen, 1989; Freiberg, 1996), as well as favorable climatic conditions, such as lower solar incidence and higher humidity, which favors the survival of epiphytes in tropical dry forests. The zone Z5 of the phorophytes in humid montane forest is considered as harsh environment, because it is more exposed to strong insolation, high temperatures, and the action of winds as well as a low air humidity (Krömer et al., 2007a). However, the observed abundance of bromeliads, most of them xeromorphic or atmospheric *Tillandsias* in the outer part (Z5) of the canopy may be related to the availability of rainwater and fog and the accumulation of dew, conditions that prevail in this zone of the phorophytes in dry environments (Graham and Andrade, 2004; Reyes-García et al., 2007).

The restriction of orchids to the inner zone of the canopy may be due to the conditions required for their seed's germination and anchorage, since they lack endosperm and need an adequate mycorrhizal association (Mondragón et al., 2007). Even so, some species of this family, such as *Prosthechea chacaoensis*, presented a wide vertical distribution, due to adaptations to tolerate water stress, such as the presence of pseudobulbs and succulent leaves, which generally store water, as well as CAM metabolism (Zotz, 2004; Krömer et al., 2007b, 2014; Kerbauy et al., 2012).

According to Jácome et al. (2004), the members of the Araceae family in the rainforests of the Pacific coast of Colombia, grow preferentially in the first 10 m from the base of the phorophytes, due to the presence of leaves with a thin cuticle and a broader foliar area, which represents a disadvantage compared to the xeric adaptations present in other epiphytes (Benzing, 1990; Mayo et al., 1997). In the study area, the Araceae showed this same pattern, since the hemiepiphytic species of the genera *Philodendron* and *Syngonium* were observed mainly in the zones Z1 and Z2 of the phorophytes, while the vertical distribution of the holoepiphyte *Anthurium halmoorei* extended to higher levels (Z3 and Z4), which is mainly due to the rosette arrangement of its leaves that favors the accumulation of organic matter and rainwater, similar to tank-bromeliads. Probably the most important biotic factors for the establishment

of epiphytes in the different zones of the phorophytes, were the microenvironmental characteristics (branch position and surface area, bark type, canopy shape, etc.) that each of them offered and the type of vegetation in which it grew. For example, *Tillandsia jaliscomonticola* was present in all five zones in the disturbed gallery forest, while in the disturbed oak forest it was found only in zones Z4 and Z5. It has been observed that denser crowns (with more branches) reduce the wind speed and limit the dispersal of anemochore seeds (Victoriano-Romero et al., 2017); this could explain the different vertical distribution of this species in the two sites.

The epiphyte richness of the understory in all sampling sites represented 71% of the total, and six species grew only in this stratum. The environmental conditions of the understory are, in many cases, conducive to the successful establishment of many epiphytes, since there is less solar incidence, grater humidity, and less influence of the wind (Krömer et al., 2007a). The absence of epiphytes in the disturbed oak forest understory, could be due to the alteration of this site by grazing, which has prevented the development of small trees that function as hosts for the epiphytes in this stratum.

Effects of the Sampling Sites and Vertical Zones on Species Composition

The two groups obtained in the cluster analysis represent, in the case of the first one, the sites with species related to the oak forest, and in the second, those that are mainly made up to tropical semideciduous forest species. The taxa that define the first group belong mostly to the Bromeliaceae and Orchidaceae, while the second includes Araceae and other hemiepiphytes such as Araliaceae, Asteraceae, and Moraceae, these four families being exclusive of this second group. This can explain the high beta diversity in the study area. In addition, dry forests can have a higher beta diversity than humid forests (Ceballos et al., 2010); the topographic and climatic conditions present on the Pacific slope create barriers that confine species in a restricted distribution and, therefore, the species composition will change more rapidly from one locality to another (Espinosa and Ocegueda, 2008); Cuevas-Reyes and Vega-Gutiérrez (2012) also report a high dissimilarity in a dry tropical forest with different degrees of disturbance, sharing between 37 and 42.8% of their species. On the other hand, Flores-Palacios and García-Franco (2008) found a high turnover of species among different habitats of a humid montane forest, due to their isolation and the distance at which they were from a permanent source of water. In another study carried out in a montane forest in the Andes of Venezuela, it was also found that the composition of epiphytes was differing more and more with the degree of disturbance, which causes the plants to be exposed to increased insolation (Barthlott et al., 2001).

As in the cluster analysis, a difference can also be observed between each site in the NMDS analysis; however, some zones were very different compared with the ones from the same site, probably due to the presence of species with low abundance like *Billbergia pallidiflora* Liebm. or because they share few to no species with the other zones. The high similarity between the understory and the inner canopy zones can be explained because the understory also presents environmental conditions similar to those of the inner canopy (Krömer et al., 2007a).

Threats and Conservation

Despite the great capacity that epiphytes present to resist environmental alterations caused by humans in the dry forests of the study area, threats of land use change due to agriculture or tourism, could generate a long-term negative effect. The introduction of cattle or goats implies the simplification of the vegetation structure and provokes the replacement of native species by introduced ones. On the other hand, the removal of vegetation cover for the development of large tourist complexes could generate greater habitat fragmentation, impeding connectivity between epiphyte populations (Fischer and Lindenmayer, 2007). An alternative that has been proposed and carried out for the conservation of epiphytes in other parts of Mexico is the establishment of Environmental Management Units (UMAs), in order to reproduce and propagate some rare or endemic species, either through *in vitro* laboratories or in a more rustic way (Damon et al., 2005). However, the sustainable use of populations must be based on a management plan for the maintenance of their diversity (Mondragón and Ticktin, 2011; Toledo-Aceves et al., 2014; Francisco-Ventura et al., 2018). In the case of this region, population studies specially of the species with restricted distribution (see **Supplementary Appendix 1**) would be required before presenting concrete proposals on their management.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

AF-A, AE-S, and AL-F conducted the field trips and samplings. AF-A analyzed the data and wrote the manuscript. TK contributed with the study design and data analysis. AE-S, AL-F, and TK revised the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ffgc.2022.828851/full#supplementary-material

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The Role of Secondary Metabolites and Bark Chemistry in Shaping Diversity and Abundance of Epiphytic Lichens

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Diversity of secondary lichen metabolites was studied in epiphytic lichens on six phorophytes—spruce, pine, birch, alder, aspen and poplar in the Middle Urals of Russia. Atranorin, usnic, fumarprotocetraric acid, zeorin, and gyrophoric acid were found in 31, 24, 23, 18, and 14 species, respectively, of 237 taxa collected. Seventy-seven species (i.e., 32% of total species documented) contained no secondary metabolites. Spectra of secondary metabolites of fruticose and foliose lichens varied on different phorophytes, while in crustose species the strong dependence on the tree species was not detected. This is different to the pH dependence of saxicolous lichens where crustose lichens were more susceptible to the rock chemistry. The results of Canonical Correspondence Analysis reveal the affinity of species containing depsides, depsidones or usnic acid to acidic substrata and those lacking secondary metabolites or containing terpenes and antraquinones to the pH-neutral bark. We suppose that phenolic compounds and flavonoids, as chemical constituents of bark, may interact with lichen symbioses and elements in phellem, and similarly to the lichen acids shape the affinity of species to the substrata.

Keywords: substrate ecology, phorophyte, flavonoids, terpenes, Middle Urals, CCA analysis

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INTRODUCTION

Lichens contain from 800 (Elix and Stocker-Wörgötter, 2008) to 1,050 (Stocker-Wörgötter, 2008; Goga et al., 2018) secondary metabolites, or lichen acids, that belong to various groups, including aliphatic acids, antraquinones, phenolic compounds, quinones, pulvinic acid derivatives, steroids, terpenes, and xanthones (Elix, 2014). Some secondary lichen metabolites act as photoprotectors (Legouin et al., 2017; Phinney et al., 2019; Beckett et al., 2021), or cause an ability of species to withstand high temperatures or a prolonged drought (Asplund et al., 2017; Lutsak et al., 2017), others act as allelopathic agents (Giordano et al., 1997), or may alter a permeability of the cell membrane of phycobionts (Lawrey, 1986).

Secondary lichen metabolites play an important role in interaction of lichens with their substrates Hauck and Jürgens, 2008; Hauck et al., 2010). The property as weaker or stronger acids enables lichen substances shape preferences of species to the substrate pH (Bačkor et al., 1997; Hauck and Jürgens, 2008). This action of usnic acid is based on its activity as a protonophore

that aids penetration of hydrogen ions to plasmalemma and acidification of protoplasts (Hauck et al., 2009b), the feature that made this metabolite a widely known antimicrobial, antitumor and anti-inflammatory agent (Antonenko et al., 2019). Lichen acids with a lower constant of dissociation such as fumarprotocetraric, perlatolic, or thamnolic may impart lichens a higher acidity tolerance (Hauck et al., 2009a). These chemical properties of secondary lichen metabolites result in differential selection of substrates by lichens containing different lichen acids. Crustose species that specialize in siliceous rock substrates produce more lichen substances than those on calcareous substrates (Spier and Aptroot, 2005) and the abundance of species containing particular chemical constituents depends on the rocky substrate (Paukov et al., 2019). Variation of secondary chemistry is found between species of the same genus growing on different rocks (Favero-Longo et al., 2015).

Rock and bark offer lichens a different set of conditions. Lichens on most rock types interact with substantially higher concentrations of metals (Purvis and Halls, 1996; Favero-Longo et al., 2004). Unpolluted bark has a lower amount of elements (Purvis et al., 2005) but contains variable organic compounds produced by a tree species and is more susceptible to changes caused by airborne pollutants (van Dobben et al., 2001). Both bark and rocks in natural conditions vary from more acidic to neutral or basic, however, bark has lower minimal values and higher variation rates compared to rocks (Skye, 1968; Paukov et al., 2019).

The working hypothesis was that there is an interaction of secondary metabolites in lichens and bark that results in different species composition and abundance of epiphytes on a variety of phorophytes. The aims of the paper were: (1) To evaluate if epiphytic lichens producing different secondary metabolites have distinct affinities to substrates with different pH and (2) to evaluate if organic chemical constituents of phorophyte bark affect the species diversity of epiphytic lichens. We estimated lichen diversity and the variability of secondary metabolites on six different tree species ranging from acid to neutral bark, Siberian spruce (*Picea obovata* Ledeb.), Scotch pine (*Pinus sylvestris* L.), common birch (*Betula pendula* Roth), speckled alder [*Alnus incana* (L.) Moench], European aspen (*Populus tremula* L.), and balsam poplar (*Populus balsamifera* L.) in protected regions of the southern part of Sverdlovsk region (Middle Ural, Russia) and the abundance of lichens in relation to their secondary chemistry on these phorophytes.

MATERIALS AND METHODS

Study Area

Epiphytic lichens were collected in Pripyshminskiye Bory, Olenyi Ruchyi (Paukov, 2003; Paukov and Teptina, 2013) and Reka Chusovaya (57°16′N, 59°17′E) national parks, and in the vicinity of Dvurechensk settlement (**Figure 1**). These territories are situated far from sources of atmospheric pollution that prevented possible contamination of the bark by airborne pollutants. The territory of Pripyshminskiye Bory national park belongs to West Siberian plain, hemiboreal small-leaved forests zone. Olenyi Ruchyi and Reka Chusovaya national parks and Dvurechensk belong to the mountain taiga zone. The territory is covered by coniferous forests dominated by Scotch pine and Siberian spruce and secondary forest vegetation with birch and aspen. The climate of the territory is moderately continental, with a cold winter and a warm summer. Average annual temperature varies from –0.7 to + 1.3°C (Ogureeva et al., 2018).

FIGURE 1 | Study area and sampling localities. Modified from maps at d-maps.com. (**1**) Pripyshminskiye Bory national park, (**2**) Olenyi Ruchyi national park, (**3**) Reka Chusovaya national park, (**4**) Dvurechensk settlement.

Field Survey

The biodiversity of lichens in the protected territories of Middle Urals was studied by a route method (Vassiliyeva, 1959). The routes were defined using vegetation maps so as to visit various forest stands dominated by different phorophytes. Within every stand thorough collecting of epiphytic lichens was undertaken within 10 × 10 m plots. A list of species was compiled for every phorophyte, which was used for comparison the diversity of secondary metabolites of lichens. The most species-rich phorophytes were then selected for recording epiphytic lichen groupings. Epiphytes of pine, birch and poplar were recorded near Dvurechensk settlement (56°36′N, 61°02′E) (**Figure 1**). The main type of vegetation near Dvurechensk is pine forests with domination of *Vaccinium myrtillus* L. and *Pteridium pinetorum* C.N. Page and R.R. Mill. Birch stands are distributed along rivers or as a secondary vegetation after logging. Poplar is an alien species that was widely used in townships of the region some 50–100 years ago. Now it is losing its popularity but remains a very common tree in older parts of settlements. For the characterization of epiphytes on this phorophyte we used poplars in a village of Klyuchi (56°37′N, 61°03′E), 3 km NE from Dvurechensk. Lichens of aspen, spruce, and the rest of birch trees was described in Reka Chusovaya nature park where the dominated vegetation types are spruce forests intermixed with aspen and birch stands as a secondary vegetation. Vertical trees of every phorophyte species with the breast height diameter not less than 40 cm were selected. Epiphyte lichen groupings on alder were not described as this phorophyte has mostly slanted trunks. Thirty trees of poplar, 50—of spruce, 90—of both aspen and birch, and 100—of pine were studied. Every tree was

photographed with a superposed 10 × 10 cm quadrate mesh subdivided into 100 1 × 1 cm squares twice, at the base, and at the height of 1.3 m. We followed the approach of preferential sampling (Pennino et al., 2019), describing the most species-rich lichen assemblages and avoiding trees devoid of lichens at either height above the ground. The total number of quadrats used in this study is 720. Bark samples from every tree species were collected together with lichens from the photographed quadrats on the height of 1.3 m to the depth of 2–3 mm. Ten samples for each phorophyte were arbitrarily selected for the determination of pH, and five samples for the determination of phenolic compounds, terpenes, and flavonoids. Care was taken to select the bark populated by lichens as it differs from bare bark in an elevated levels of phenolic compounds (Latkowska et al., 2015).

For determination of species most crustose lichens (genera Lecanora, Lepraria, and others) and foliose-fruticose lichens known for their variable chemistry (Bryoria, Usnea) were studied chromatographically. Specimens of taxa known for their constant chemistry were periodically checked to approve the absence of chemosyndromes in the studied region. Selected thalli of species photographed during the registration of the cover of lichen groupings were taken for the control determination of the composition of secondary metabolites in a laboratory.

Analytical Procedures

Secondary lichen products were analyzed using WinTab software (Lafferty et al., 2021) by applying standard thin-layer chromatography techniques (Culberson and Kristinsson, 1970; Orange et al., 2001) in solvent systems A (toluene:1,4-dioxane:acetic acid, 180:45:5), B (hexane:diethyl ether:formic acid, 140:72:18) and C (toluene:acetic acid, 170:30). For rapid data sorting and operation an electronic table was created where collected species were arranged in rows and phorophytes together with secondary lichen metabolites were arranged in columns. The presence of a species on a particular phorophyte was marked as "1." Similarly, the presence of a particular metabolite in a lichen species was marked by "1."

The determination of pH of water extraction was performed following the protocol of Kricke (2002) with some modifications. For this, 2 g of powdered bark samples were poured into 20 mL of distilled water and left for 24 h with periodical shaking. The pH was measured after precipitation of particles with a pH meter (Anion 4100, Novosibirsk, Russia) in ten repetitions for every phorophyte.

Bark chemistry was analyzed using thin layer chromatography (Wagner et al., 1984; Ermoshin et al., 2021) in a solvent system toluene:ethyl acetate:formic acid, 30:18:2. One hundred milligrams of bark of six tree species in 3 repeats each were extracted in 1 mL acetone and 14 mkL of every extract was applied on a chromatographic plate. Quercetin, gallic, salycilic, and ferulic acid solutions (4 mkg in every spot) were used as a reference. Plates were developed in 2% solutions of aluminum chloride, iron chloride, and phosphotungstic acid in ethanol (Sorescu et al., 2018; Shaikh and Patil, 2020). For a quantitative analysis of chemical constituents 120 mg of bark were poured into 1.5 mL of ethanol and extracted at 55°C in an ultrasound chamber (Grad 80–35, Proton LLC, Moscow, Russia) for 35 min. The extract was centrifuged and the extraction from the same bark sample was repeated three times. Supernatants taken from the same bark sample were united and topped to the volume of 6 mL so as to 1 mL corresponded the extract from 20 mg of bark. A total content of phenolic compounds was determined photometrically (Infinite M200pro photometer, Tecan, Grödig, Austria) using Folin-Ciocalteu reagent with addition of a sodium carbonate. The concentration of phenolic compounds in solutions was expressed in relation to gallic acid. Flavonoids were determined photometrically after addition of an aluminum chloride solution and the concentration was expressed in relation to rutin (Larayetan et al., 2019).

Statistical Analyses

Matrices of (a) metabolite occurrence with respect to lichen species, (b) pH and organic compounds in bark of phorophytes, (c) cover of species with different metabolites on every 10 × 10 cm plot were assembled as Microsoft Excel electronic tables. To avoid the influence of species-specific patterns in the distribution of lichens, depending on the parameters of microhabitats, the cover of species containing the same lichen metabolite was summed in every description within 10 × 10 cm plot. If a species contained several lichen metabolites, its cover was counted correspondingly several times for every lichen acid in a thallus. The abundance of lichens containing different secondary metabolites in relation to the content of organic compounds and the pH of bark was analyzed as raw data in CANOCO 5.0 (Šmilauer and Lepš, 2002). The analyses were separately performed for crustose and foliose-fruticose species for comparison. The response data had a gradient of 7.8 and 6.3 SD units long, respectively, so unimodal constrained ordination (CCA) was used. Organic compounds contained in a one phorophyte species only were not included into the CCA analysis. Differences in percentage of species containing various metabolites were analyzed using χ^2 criterion and differences of bark pH were estimated using a Mann-Whitney *U*-test in Statistica 13.0 (Statsoft, Tulsa, United States). Cluster analysis was performed by a single-linkage method in the PAST 3.18 package (Hammer et al., 2001). The data on secondary chemistry and abundance of epiphytic lichens were compared with similar data on saxicolous species (Paukov et al., 2019).

RESULTS

Species and Secondary Metabolite Diversity

During the general inventory 237 lichen species were found inhabiting tree bark in the protected territories of Middle Urals. The species recorded contained 76 lichen metabolites. Of them, six groups of metabolites represented a half of the total diversity: Nine of them belonged to β-orcinol depsidones, eight—to β-orcinol depsides, and eight to pulvinic acid derivatives. The groups of aliphatic acids and orcinol depsidones were both represented by seven metabolites.

Thirty-five secondary lichen metabolites were found in only one species, 21 were present in 5 or more, and six—in 10 or

more epiphytic species. The most common among them was atranorin, which was found in 31 species (**Table 1**). Usnic, fumarprotocetraric acid, zeorin, and gyrophoric acid were found in 24, 23, 18, and 14 species, respectively. Seventy-seven species (i.e., 32% of total species documented) contained no secondary metabolites. Divaricatic, fumarprotocetraric, lecanoric, squamatic and usnic acids are contained in a larger amount of species compared to saxicolous lichens with the same metabolites, and in the saxicolous group antraquinones, gyrophoric, lobaric, norstictic, psoromic, and stictic acids were contained in a relatively larger amount of species compared to epiphytes.

Foliose lichens were the most chemically diverse and contained 32 metabolites in 52 species (ratio 0.62), while fruticose lichens had 21 metabolites in 39 taxa (ratio 0.54), and 144 crustose species contained 55 lichen acids (ratio 0.38). Three, 35, and 40 percent of crustose, foliose and fruticose species, respectively, contained no secondary metabolites. Epiphytes are more diverse in lichen metabolites compared to saxicolous species. The latter had metabolite to species ratios of 0.30 for foliose, 0.44 for fruticose, and 0.16 for crustose taxa.

Secondary Metabolite Diversity on Different Phorophytes

The most species-rich substrate was aspen (88 species) followed by birch, alder, spruce and pine (**Table 2**). The diversity of secondary metabolites did not correlate with the species diversity on a particular tree species but, unlike in the group of saxicolous lichens, the metabolite to species ratio on different phorophytes in epiphytes gradually decreased from spruce to aspen. Similarly, the average number of metabolites in a lichen species decreased in the same direction, the phenomenon that was previously noted also in the group of saxicolous lichens.

The proportion of crustose species without lichen acids on different phorophytes varies but the variability is statistically insignificant ($\chi^2 = 6.8$, $p = 0.2$) (**Figure 2A**). Lecanoric and vulpinic acids were more common in crustose lichens on conifers and birch ($p < 0.05$). The only metabolite that increased its percentage in species on aspen was anthraquinone (parietin). No particular trend was seen for other major metabolites, however, zeorin was slightly more abundant in lichens on alder and aspen. This is different to the chemistry of saxicolous crustose lichens where the proportion of species without lichen acids was five times higher on limestone compared to that on quartzite.

Within foliose and fruticose lichens there were 26 percent of species on aspen that contained no secondary metabolites and as low as 1 or 2 percent on coniferous trees (**Figure 2B**). Species with anthraquinone were the most diverse on aspen ($\chi^2 = 21.9$, $p = 0.0005$) and species with usnic acid were more abundant on conifers and birch ($\chi^2 = 12.05$, $p = 0.034$). Other species that were significantly confined to spruce, pine and birch contained thamnolic, barbatic ($\chi^2 = 18.2$, $p = 0.002$ and 11.2, $p = 0.05$, respectively), fumarprotocetraric and divaricatic acid, but the dependence of the two latter on the phorophyte species was insignificant. The substrate-dependent variation of secondary metabolites was more pronounced in epiphytic foliose and fruticose compared to saxicolous species as the only statistically supported difference in the latter group was found for species lacking secondary metabolites. Generally, the secondary chemistry of epiphytic foliose and fruticose species is more dependent on the phorophyte as opposed to saxicolous lichens where this dependence is more pronounced in crustose species.

The taxonomic structure of epiphytic lichen groupings is dependent on the species of phorophyte. In crustose lichens the most pronounced differences are found in Caliciaceae family, which is more diverse on conifers, and Ramalinaceae, which is more diverse on deciduous trees (**Figure 3A**). In both families species growing on pine and spruce contain secondary metabolites and the most taxa found on alder and aspen are lacking "lichen acids." The taxonomic structure of epiphytic foliose-fruticose species is more simple compared to that of crustose species but the differences between epiphytes of five phorophytes are also visible (**Figure 3B**). Fruticose Ramalinaceae unlike crustose species belonging this family contain secondary metabolites and found on conifers only. Physciaceae are diverse on aspen and represented by species containing atranorin, zeorin or no lichen substances. As opposed to Physciaceae, Parmeliaceae family, which is rich in secondary metabolites, is more diverse on conifers and birch.

TABLE 1 | The quantity of epiphytic and saxicolous lichen species containing the most frequently recorded secondary metabolites.

Metabolites	Epiphytic lichens (237 species)		Saxicolous lichens (543 species)	
	Number of species	Percent of total	Number of species	Percent of total
Atranorin	31	13.1	73	13.4
Usnic acid	24	10.1	42	7.7
Fumarprotocetraric acid	23	9.7	21	3.9
Zeorin	18	7.6	37	6.8
Gyrophoric acid	14	5.9	41	7.6
Norstictic acid	10	4.2	36	6.6
Lecanoric acid	9	3.8	14	2.6
Anthraquinones	6	2.5	35	6.4
Squamatic acid	6	2.5	10	1.8
Divaricatic acid	6	2.5	5	0.9
Stictic acid	5	2.1	32	5.9
Xanthones	4	1.7	10	1.8
Lobaric acid	1	0.4	11	2.0
Psoromic acid	1	0.4	10	1.8

Data on saxicolous lichens are according to Paukov et al. (2019).

Chemical Properties of Bark

Bark of phorophytes varied in chemical composition and pH of water extracts. Thirty-six metabolites belonging to flavonoids, phenolic compounds, quinones, and terpenes were detected chromatographically in acetone extracts. Fifteen of them were found in bark of two or more tree species, and 21 metabolites contained in one species of phorophyte only. Poplar and aspen contained the highest diversity of flavonoids (6 and 7,

TABLE 2 | Species and secondary metabolite diversity on different phorophytes.

Measured feature	Spruce	Pine	Birch	Alder	Aspen
Total number of species	57	55	85	69	88
Number of secondary metabolites	44	41	45	36	41
Metabolite to species ratio	0.77	0.75	0.53	0.52	0.47
Average number of metabolites in one species	1.68 ± 0.18^{A}	1.62 ± 0.15^{A}	1.58 ± 0.12^{A}	1.31 ± 0.16^{A}	1.14 ± 0.13^{B}

Values in the last row marked by the same letter are statistically insignificant at p = 0.05.

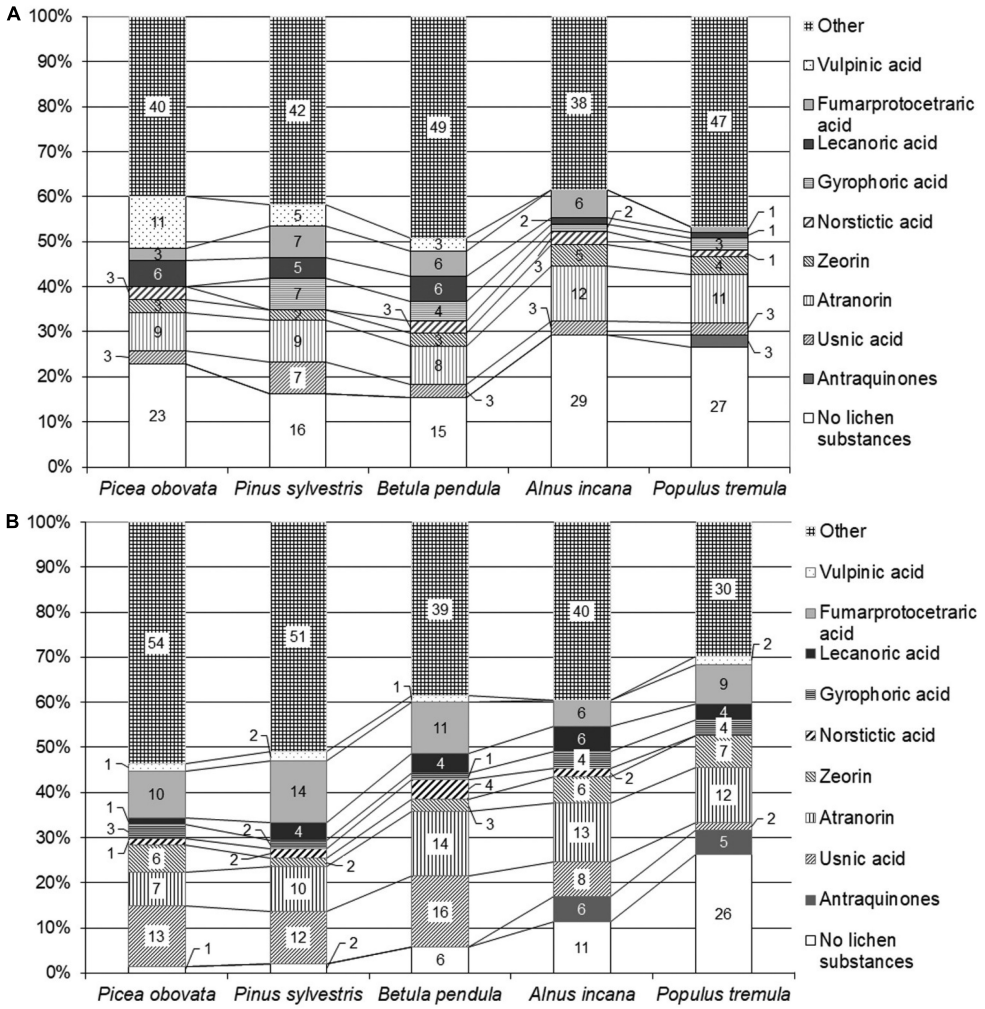

FIGURE 2 | Percentage of lichen species containing major secondary metabolites on different phorophytes. **(A)** Crustose species, **(B)** foliose and fruticose species.

respectively) (**Table 3**). Poplar also contained four phenolic compounds followed by pine with three metabolites. Terpenes are the most diverse in birch with eight compounds followed by alder with six metabolites. The chemical composition of bark of studied phorophytes has a low similarity even regarding species belonging to the same family. Pine and spruce have the most similar chemistry (**Figure 4**).

Flavonoids had their highest content in poplar and aspen (44.11–61.86 mg/g) and were the least abundant in alder and birch (1.87–3.51 mg/g) (**Table 4**). Phenols had the highest concentration in bark of alder (61.06 mg/g); were in a range 44.16–49.24 mg/g in spruce, aspen, and poplar, and had the lowest content in pine and birch (25.21 and 30.62 mg/g, respectively). Bark pH gradually rises from 3.68 in spruce to 6.52 in aspen.

Secondary Metabolites and Chemistry of Bark

Canonical correspondence analysis (CCA) on relationships between metabolites and the chemical composition of bark showed similar results for crustose and foliose-fruticose species (**Figure 5**). In both analyses, pH of bark, phenols, flavonoids and

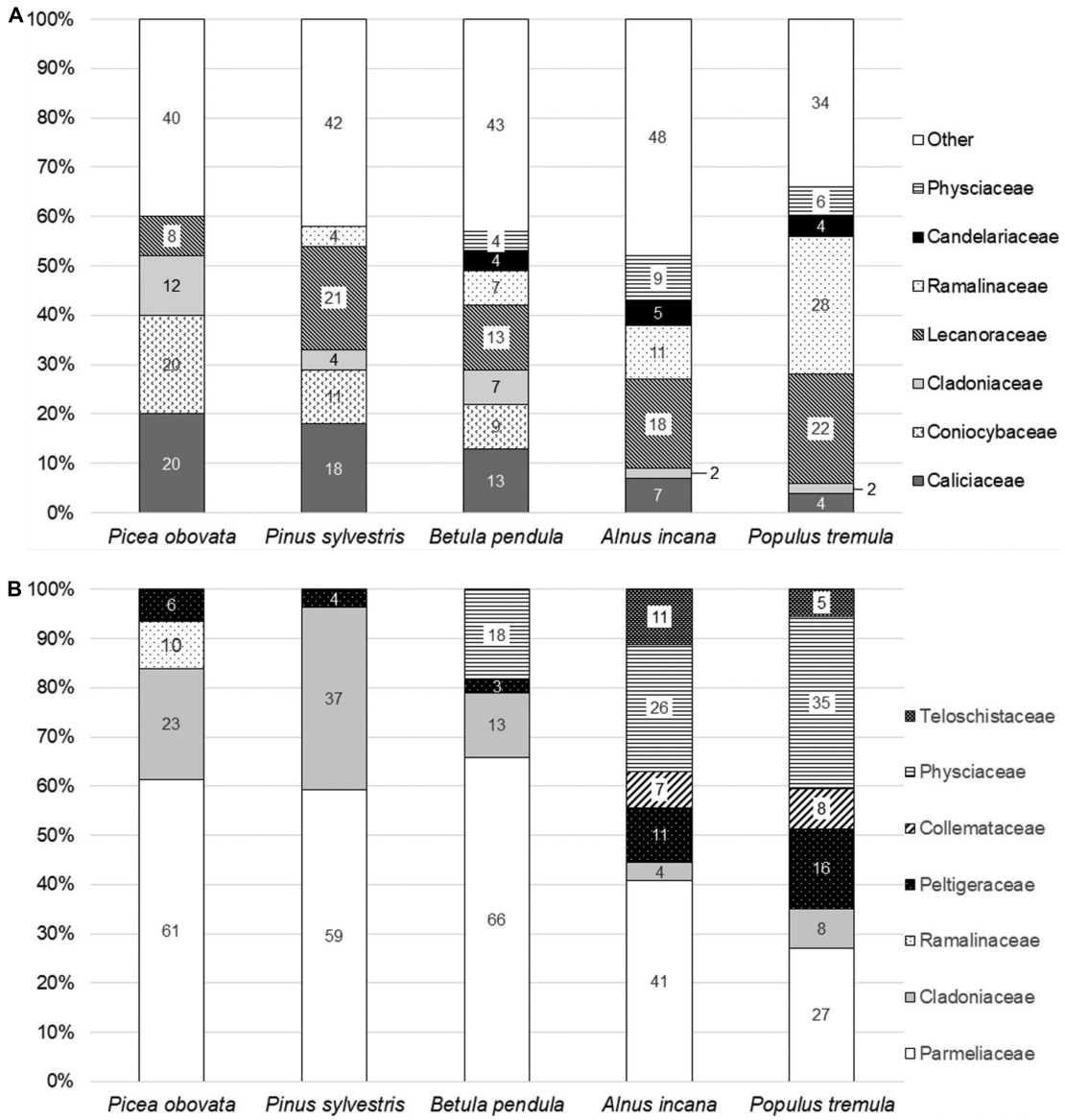

FIGURE 3 | Taxonomic structure of lichen assemblages on five phorophytes. **(A)** Crustose species, **(B)** foliose and fruticose species.

TABLE 3 | Diversity of studied groups of metabolites in bark of phorophytes.

Group of metabolites	Spruce	Pine	Birch	Alder	Poplar	Aspen
Flavonoids	3	3	2	1	6	7
Phenols	2	3	2	1	4	1
Quinones	0	1	0	0	0	1
Terpenes	3	5	8	6	3	5

two terpenes had high loading on the first axis (63.9% of variance for foliose-fruticose and 34.8% for crustose species) and the second axis (14.7 and 21.7%, for foliose-fruticose and crustose, respectively) reflects a loading of a combination of terpenes as well as flavonoid quercetin with salycilic acid for foliose-fruticose lichens, and phenols with flavonoids for crustose species. The forward-selection procedure implemented in Canoco showed a low impact of the position of lichen groupings (tree base or 1.3 m) compared to other recorded factors.

In the CCA, in crustose lichens the species lacking secondary metabolites or with anthraquinones had the highest cover on substrates with higher pH, and content flavonoids and phenols (**Figure 5A**). A moderate affinity to the substrates with the same characteristics was displayed by species containing stictic,

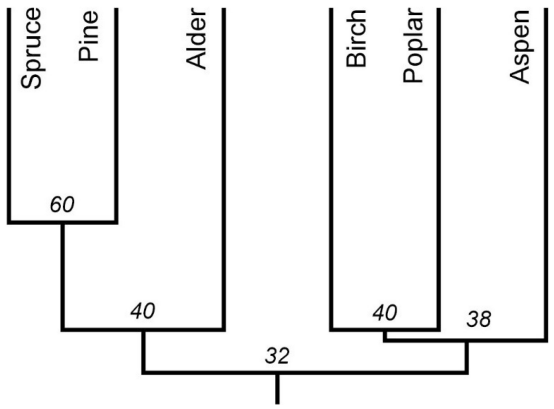

FIGURE 4 | Similarity dendrogram of chemical composition of bark of five phorophytes based on the Sørensen's coefficient of similarity.

roccellic acids and gangaleoidin, which are more common on aspen compared to poplar. Atranorin, barbatic, divaricatic, fumarprotocetraric, jackinic, and to a lesser extent gyrophoric, lecanoric, usnic acids, and zeorin were associated mostly with conifers and birch with a relatively low pH and a higher content of terpenes.

In foliose-fruticose species, like in the group of crustose lichens, species without secondary metabolites, containing antraquinone parietin, and to a lesser extent β-orcinol depsidone salacinic acid were the most abundant on *Populus balsamifera* and *P. tremula*, on substrates with a higher pH and containing higher concentrations of phenolic compounds and flavonoids (**Figure 5B**). Species containing divaricatic, fumarprotocetraric, norstictic, physodalic, and usnic acid had an affinity to substrates with a low pH and a low concentration of flavonoids and phenolic compounds but containing higher concentrations of terpenes. Fruticose species containing barbatic and squamatic acids were limited to birch and spruce. An aboveground height of lichen groupings performed a little or no effect on the secondary chemistry both for crustose and foliose lichens and was not shown on the graphs.

Generally, both crustose and foliose-fruticose species containing a variety of metabolites were more abundant on substrates with a lower pH, a lower concentration of flavonoids and phenolic compounds and a higher content of terpenes. In both morphological groups only species without secondary metabolites and containing antraquinones are generally confined to a "rich" bark. The former fact is also true for saxicolous species where taxa without "lichen acids" are more abundant on basic substrates and their abundance correlated better with the amount of calcium and strontium, not the pH itself (Paukov et al., 2019).

DISCUSSION

The paper continues our research into the dependence of secondary chemistry of saxicolous lichens on the chemistry of their substrates. Both saxicolous lichens and epiphytes are similar in the relationship of their secondary chemistry to the properties of the substrate. Species growing on rock and bark with a low pH have a more diverse chemistry compared to species on neutral or weakly alkalic substrates that is evident both in metabolite to species ratio and an average number of metabolites in one species. Unlike saxicolous lichens, the dependence of the composition of secondary metabolites on the substrate pH in epiphytes is better visible in foliose-fruticose rather than crustose lichens. A relative amount of species containing gyrophoric, lecanoric, vulpinic acid and antraquinones were dependent on the pH of bark in crustose lichens. In foliose-fruticose species the same was true for species containing no metabolites or usnic acid, and antraquinones. Some metabolites were found in species growing mainly on a particular tree species.

Rocky substrate and bark are different in their chemical properties. The pH values of different kinds of rocks are close to neutral and have a narrower range (6.16 ± 0.06 to 8.18 ± 0.08) compared to that of bark which is more acidic in most phorophytes (3.68 ± 0.10 to 6.52 ± 0.15). Further, the substantial difference between two types of substrates is in the content of metals and transition elements, which is generally much higher in neutral of slightly basic ultramafic rocks compared to unpolluted bark (Bates and Brown, 1981; Rajakaruna et al., 2012; Parzych et al., 2017). Third, bark as opposed to rocky substrates, contains a multitude of secondary metabolites of a non-lichen origin, but are similar to secondary lichen substances, namely terpenes, flavonoids and phenolic compounds.

No rocks with such a low pH were found in the region. The marked shift in pH of bark and rocky substrates causes a rise in a percentage of some metabolites in lichens on a conifer bark. The higher proportion of species containing usnic, thamnolic, and fumarprotocetraric acids (the latter, however, insignificantly) in foliose-fruticose species, vulpinic and lecanoric acids in crustose species on acid bark proves the ability of these components to impart the acidity tolerance to lichens (Hauck and Jürgens, 2008; Hauck et al., 2009a,b). However, provided that pH levels in rock and bark generally do not intersect, the similar tendencies of changes in metabolite spectra in both substrate groups implies that other factors, not only pH, affect the distribution of species containing different metabolites. Additionally, bark with similar pH (aspen and poplar vs. quartzite and granite) has a much higher proportion of species without secondary metabolites.

Lichen secondary metabolites with a high affinity to metals may seemingly play a controversial role. On the one hand, they may chelate elements thus protecting lichens from an excess of toxic metals and preventing them from absorption to the apoplast (Purvis et al., 1987, 1990; Hauck and Huneck, 2007). On the other hand, lichen acids prevent lichens from deficiency of elements (Hauck et al., 2009c). Metabolites that may prevent lichens from excessive metals are those, which are more abundant in saxicolous lichen compared to epiphytes. In our study norstictic, psoromic and stictic acids are components of a higher share of saxicolous lichens. Norstistic and psoromic acids have a high affinity to copper and iron (Purvis et al., 1987) and stictic acid may have similar effect in respect to other metals. Bark pH affects the accumulation rate of metals in lichens (Asplund et al., 2015), and a higher proportion of barbatic, thamnolic and vulpinic

TABLE 4 | pH of samples and a content of phenolic compounds and flavonoids (mg/g) in bark of studied phorophytes.

Measured feature	Spruce	Pine	Birch	Alder	Poplar	Aspen
pH	3.68 ± 0.10^{A}	3.85 ± 0.04^{A}	4.50 ± 0.17^{B}	5.10 ± 0.10^{C}	6.48 ± 0.08^{D}	6.52 ± 0.15^{D}
Flavonoids	7.22 ± 0.58^{A}	10.26 ± 1.48^{B}	3.51 ± 0.64^{C}	1.87 ± 0.54^{C}	44.11 ± 4.90^{D}	61.86 ± 0.20^{E}
Phenols	44.16 ± 3.03^{A}	25.21 ± 1.91^{B}	30.62 ± 1.07^{B}	61.06 ± 2.89^{C}	49.24 ± 0.90^{A}	48.53 ± 1.22^{A}

Values in rows marked by the same letter are statistically insignificant at p = 0.05.

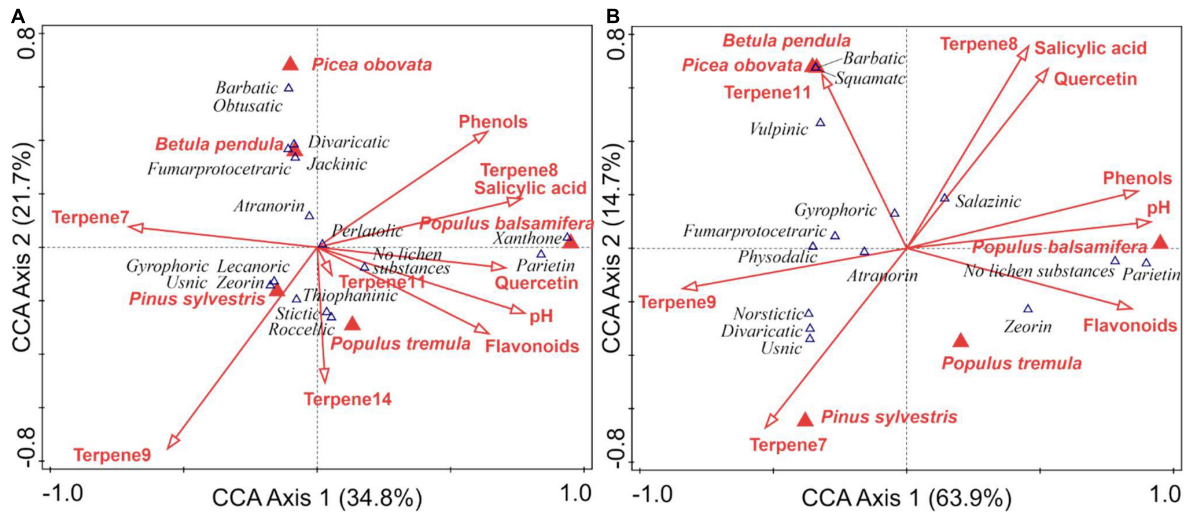

FIGURE 5 | Canonical correspondence analysis (CCA) ordination plot showing the cover of lichens containing different secondary metabolites in relation to phorophytes and their chemical properties. **(A)** Crustose species, **(B)** foliose and fruticose species. Bark metabolites found in several phorophytes are shown.

acid-containing species on acid bark in our research may also prove the contribution of these metabolites into accumulation of elements. This property of secondary metabolites may have a crucial role for lichens growing on bark compared to most types of rocks as phellem of phorophytes contains lower concentrations of minerals and an ability of "lichen acids" to chelate them at low pH helps lichens attract elements.

The third difference of bark compared to rocks is a presence of organic compounds of a plant origin. As it is shown in the CCA analysis both epiphytic crustose and foliose-fruticose species lacking secondary metabolites and containing antraquinones are more abundant on bark with a higher pH and containing higher concentrations of flavonoids and phenolic compounds. Species containing most of major metabolites are common on a conifer and birch bark with a lower pH and containing more terpenes. Many phenolic compounds and flavonoids are chemically similar to many lichen metabolites in having hydroxyl groups which bear a high affinity to metals, iron and copper (Přemysl et al., 2011; Říha et al., 2014), likewise the group of secondary lichen metabolites. The complex formation is the most active at higher pH (Mira et al., 2002), at the levels, which were found in alder, poplar, and aspen. Higher pH conditions reduce the availability of Fe, Cu (Lucas and Davis, 1961) and a complex formation may increase a bioavailability of these elements. Phenolic compounds have a similar effect as chelators of transition metals and have an ability to quenching of free radical reactions (Foti, 2007; Kulbat, 2016). Thus, the presence of phenols and flavonoids in bark can be a substitution for lichen metabolites such as melanins, lecanoric, and usnic acid (Solhaug and Gauslaa, 1996; Luo et al., 2009; Prokopiev et al., 2018) in prevention thalli from a damage from reactive oxygen species under high UV-B levels. A close contact of crustose epiphytes with their substrates and a variability in an amount of phenols and flavonoids in bark may be a reason for a lower dependence of a secondary chemistry of crustose species on bark pH. Terpenes are used by plants in many ways, including physiological regulation and defense responses (Tholl, 2015). Eighteen species of epiphytic lichens contained terpene zeorin, and fourteen compounds were additionally found in bark. Both in crustose and foliose-fruticose groups species containing zeorin were the least abundant on birch as the most terpene-rich substrate. In lichens zeorin acts as an antiherbivorous substance (Nimis and Skert, 2006; Asplund and Gauslaa, 2010) and other terpenes contained in bark may have a similar protective action on thalli.

Our results show that the secondary chemistry of saxicolous and epiphytic lichens reflects differences of substrate pH in a similar way but the variability of metabolites and distribution of species is refined by a different set of additional parameters, such as metal availability and a presence of organic compounds in bark. This is not the only factor determining distribution of lichens on phorophytes as far as other functional traits like photobiont type, ascomata type and asexual reproduction of mycobionts affect the affinity of lichens to tree species (Łubek et al., 2021). Studies using genera with a variable chemistry and their distribution on different species of trees as

well as direct application of bark metabolites on lichens will give additional confirmation on the effect of lichen chemistry as one of the important traits in the selection of substrate by lichen species.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

AP: conceptualization and methodology. AP, AT, AE, EK, and LS: investigation. AP and AT: writing. All authors contributed to the article and approved the submitted version.

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Tree Size Drives Diversity and Community Structure of Microbial Communities on the Bark of Beech (*Fagus sylvatica*)

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Tree bark constitutes an ideal habitat for microbial communities, because it is a stable substrate, rich in micro-niches. Bacteria, fungi, and terrestrial microalgae together form microbial communities, which in turn support more bark-associated organisms, such as mosses, lichens, and invertebrates, thus contributing to forest biodiversity. We have a limited understanding of the diversity and biotic interactions of the bark-associated microbiome, as investigations have mainly focused on agriculturally relevant systems and on single taxonomic groups. Here we implemented a multi-kingdom metabarcoding approach to analyze diversity and community structure of the green algal, bacterial, and fungal components of the bark-associated microbial communities of beech, the most common broadleaved tree of Central European forests. We identified the most abundant taxa, hub taxa, and co-occurring taxa. We found that tree size (as a proxy for age) is an important driver of community assembly, suggesting that environmental filtering leads to less diverse fungal and algal communities over time. Conversely, forest management intensity had negligible effects on microbial communities on bark. Our study suggests the presence of undescribed, yet ecologically meaningful taxa, especially in the fungi, and highlights the importance of bark surfaces as a reservoir of microbial diversity. Our results constitute a first, essential step toward an integrated framework for understanding microbial community assembly processes on bark surfaces, an understudied habitat and neglected component of terrestrial biodiversity. Finally, we propose a cost-effective sampling strategy to study bark-associated microbial communities across large spatial or environmental scales.

Keywords: algae, bacteria, biofilm, community ecology, dermosphere, forest management, fungi, metabarcoding

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INTRODUCTION

The aboveground surfaces of plants are ideal substrates for microbial colonization. The bark surface [or dermosphere; Lambais et al. (2014)], in particular, is one of such important aboveground substrates in forests. The bark provides a range of microhabitats that promote colonization of microbial communities with varied ecologies (Whitmore, 1963). On the one hand, microsites such

as holes, cracks, and lenticels retain humidity and nutrients, thus constituting stable microhabitats suitable for slow-growing, stress-sensitive microbes. On the other hand, the exposed surfaces of the bark may harbor more stress-resistant microbial communities that can cope with environmental challenges (Vorholt, 2012; Aguirre-von-Wobeser et al., 2021), such as low nutrient availability, increased exposure to light, fluctuating moisture conditions and desiccation (Lindow and Brandl, 2003; Vorholt, 2012; Leff et al., 2015), and presence of compounds that are resistant to microbial degradation (e.g., suberin), or that directly inhibit microbial growth (Baldrian, 2017).

Compared to other aboveground components, such as leaves, branches or fruits, that undergo seasonal and diurnal changes (Vitulo et al., 2019), bark represents a stable, long-lived substrate that supports microbial colonization (Leff et al., 2015). Further, the bark surface is often screened from excessive precipitation- and/or UV radiation by the tree canopy and changes slowly during development over several years (Whitmore, 1963). A number of studies have investigated the bark-associated microbial diversity, especially for fungi and bacteria, in various systems, e.g., grapevine plants (Martins et al., 2013; Arrigoni et al., 2018), bark beetle-infested spruce (Strid et al., 2014), *Ginkgo* (Leff et al., 2015) and avocado trees (Aguirre-von-Wobeser et al., 2021). These studies report that the tree bark supports microbial communities that are often distinct from spatially-close substrates like leaves and roots (Martins et al., 2013; Leff et al., 2015; Arrigoni et al., 2018), indicating niche differentiation and a clearly structured habitat (Aguirre-von-Wobeser et al., 2021). Furthermore, the dermosphere constitutes a reservoir for microbial diversity (Arrigoni et al., 2018; Hagge et al., 2019; Kobayashi and Aoyagi, 2019), potentially harboring undiscovered specialist taxa (Aschenbrenner et al., 2017), and taxa that facilitate the colonization of other epiphytes, including lichens (Aschenbrenner et al., 2017). The microbial communities on tree bark, and the biofilm which they form, can indeed be considered the basis of a food web that supports photosynthetic epiphytes (e.g., mosses and lichens), as well as a diverse microfauna (Andre, 1985). With an estimated more than 3 trillion trees in the world (Crowther et al., 2015), bark communities could thus be particularly important reservoirs of biological diversity. However, bark is a poorly explored habitat with respect to microbial diversity and community structure, compared to other substrates such as the phyllosphere and rhizosphere.

Biological and environmental factors driving diversity and community assembly in bark-associated epiphytes have been linked to forestry management, e.g., management intensity (Boch et al., 2021), forest homogeneity (Lamit et al., 2015), deadwood abundance (Boch et al., 2021), and tree age. For the latter, higher epiphyte diversities have been linked to the availability of large, old-growth trees (Aude and Poulsen, 2000; Nascimbene et al., 2013; Boch et al., 2021), probably because of higher niche partitioning in older trees (Łubek et al., 2020). At smaller spatial scales, abiotic drivers of bark-associated diversity and community structure include ultraviolet radiation, water shortages and correlated desiccation, and poor nutrient availability (Lindow and Brandl, 2003; Vorholt, 2012; Leff et al., 2015), while biotic drivers include host traits, such as maturity of the substrate and host genotype (Arrigoni et al., 2018, 2020). Community composition is therefore, to some degree, host specific. A few studies showed that the trends observed for macroepiphytes (e.g., bryophytes, lichens) or components of the phyllosphere also appear in bark-associated microbes (e.g., Vorholt, 2012; Arrigoni et al., 2020). However, our understanding of the factors shaping the different components of the highly diverse bark-associated microbial communities is still limited. Most of the studies focus on non-natural, commercially driven ecosystems like orchards or vineyards (Martins et al., 2013; Arrigoni et al., 2018) and are often conducted over small spatial scales with small sample sizes (e.g., Leff et al., 2015). Lastly, the focus often lies on only a single group of microorganisms, with bacteria and fungi far outweighing terrestrial algae (Aschenbrenner et al., 2017; Petrolli et al., 2021). Integrative sampling of major microbial contributors over regional or potentially even global scales can help identifying not only the diversity of microorganisms but also potential cooperative and competitive interactions among them. Revealing the diversity and structure of these rather unique microbial communities is essential to predict their responses in a changing environment. Furthermore, considering the importance of fungi, bacteria and algae to ecosystem nutrient and energy budgets in terrestrial habitats, gaining information on the bark-associated microbial communities and their dynamics is essential and directly relevant for ecosystem service assessment.

In this study we present one of the first integrated investigations of the bark microbiome in temperate forests. Here we use the term microbiome following the definition by Berg et al. (2020). Specifically, we study the three main components of the bark microbiome, i.e., green algae, bacteria and fungi. We sampled bark surfaces in forests under different management regimes, ranging from highly-managed stands to relatively undisturbed sites in the core zone of a national park. We used metabarcoding to analyze microbial diversity, community structure and species interactions from the tree to the landscape level. Specifically, we asked the following questions: (i) What is the microbial diversity found on the bark of the most common broadleaved tree in central Europe (*Fagus sylvatica*)?, (ii) Which species co-occur and who are the main players in the identified ecological modules?, (iii) Which factors, i.e., management intensity and tree-size classes (as a proxy for tree age), affect the bark-associated microbiome, both at tree and landscape level?

The comparison of diversities among trees of different size classes within a spatially-explicit framework allowed us to test for the effects of sampling design on the estimation of microbial diversity. This information is essential for further, larger scale sampling campaigns.

MATERIALS AND METHODS

Study Site and Sampling

Sampling sites are situated within the central European region of Hainich-Dün (Thuringia, Germany), one of the three regions of the Biodiversity Exploratories (Fischer et al., 2010). The Hainich-Dün region is characterized by soils stemming from calcareous

bedrock, an annual rainfall between 500–800 mm, and a mean temperature of 6.5–8°C at an elevation of 285–550 m above sea level (Fischer et al., 2010).

Sample collection took place in autumn between the 13th and 15th of October 2020. We chose a subset of 16 out of the established 50 experimental plots (Fischer et al., 2010), sampling a subplot of 20 m × 20 m within the 100 m × 100 m experimental plots. These plots were chosen to represent two regimes of land-use intensity, namely a high and a low intensity forest management (eight plots each), according to the Forest Management Index (ForMI, high > 1, low < 1). This is an index combining measures of harvested stem volume, occurrence of non-natural species and deadwood stemming from harvest (Kahl and Bauhus, 2014). The plots had an average stand density of 485 trees/ha (min = 152 trees/ha, max = 1,830 trees/ha). We defined three size classes: large [i.e., > 30 cm diameter at breast height (DBH)], medium (15–30 cm DBH) and small (5–15 cm DBH). We sampled two trees per size class, resulting in a total of 96 samples. When one tree-size class was not available (three plots), we sampled more trees of the other size classes depending on which was highly abundant in the direct vicinity as judged in the field. Within each plot we recorded the spatial position of the trees relative to each other by measuring distance (m) and azimuth (degrees) from the nearest sampled tree.

We collected microbial bark surface communities using individually wrapped sterile nylon-flocked medical swabs with a 30 mm breakpoint, typically used for medical specimen collection (FLOQSwabs™, Copan, Brescia, Italy). The breakpoint mechanism minimizes the possibility of contamination when transferring the swab into the Eppendorf tube. Prior to collection the bark was moisturized with deionized water to mobilize the surface biofilms. Then the tree was swabbed at approximately 150 cm height in a 3 cm-wide band around the trunk, rolling the swab and moving it up and down while applying gentle pressure. While swabbing, we took care to include smooth surfaces as well as cracks and crevices in the bark, to ensure a good representation of micro-habitats. If present, large patches (>10 cm) of bryophytic epiphytes were excluded. Conspicuous, larger lichen thalli were not present in the swabbed areas, however, small lichen thalli/propagules were included during the swabbing. The swab head was broken off into Eppendorf tubes pre-filled with 750 μl Nucleic Acid Preservation (NAP) buffer (Camacho-Sanchez et al., 2013). Tubes were immediately placed in styrofoam boxes with ice and the samples were subsequently stored at 4°C until DNA extraction.

DNA Extraction

Prior to DNA extraction we added 750 μl ice-cold phosphate-buffered saline (PBS) into the Eppendorf tube and centrifuged the sample for 15 min at 6,000 × *g* as recommended by Menke et al. (2017). The supernatant was then discarded without disturbing the swab head or pellet. DNA was extracted using the Quick-DNA Fecal/Soil Microbe Microprep kit (Zymo Research Europe GmbH, Freiburg, Germany). Initial tissue lysis was achieved through mechanical disruption by bead beating, using the beads included in the extraction kit. We modified the kit protocol by directly adding the beads and bead-beating buffer into the tube containing the pellet and swab and shaking for a total of 6 min (SpeedMill PLUS, Analytik Jena, Jena, Germany). In the later steps we followed the manufacturer's protocol, using DNAse-free water as elution buffer. We included six extraction blanks as contamination controls, that were sequenced as well. Extraction blanks consisted of one unused swab, unpacked and transferred to the NAP buffer in the field and subsequently treated in the same way as regular samples. DNA extracts were frozen at −20°C until PCR.

PCR Amplification and High-Throughput Sequencing

Algal, bacterial and fungal fractions of the extracted microbial DNA were amplified, using universal primers for the ITS2 region for fungi and algae, and the 16S hyper-variable region V3–V4 for bacteria (**Table 1**).

All samples were amplified in duplicate with forward and reverse primers individually tagged with octamers allowing for a double index multiplexing approach. Each duplicate contained eight PCR negative controls (i.e., master mix without sample), that were sequenced as well, meaning that a total of 110 × 2 samples were obtained after PCR. Additionally we included 16 "Multiplex Controls" (i.e., empty wells) to allow detection of potential primer jump during sequencing (Schnell et al., 2015). We set up 15 μl PCR reactions containing 5 ng of DNA, 7.5 μl of MyTaq™ HS Mix, 2x (Bioline GmbH, Luckenwalde, Germany), 0.6 μl 10 μM of each primer, and 4.3 μl DNAse free water. Cycling conditions differed in cycle number and annealing temperature among organismal groups. Conditions were as follows: an initial denaturation at 95°C for 1 min, followed by 30 (algae, bacteria) or 35 (fungi) cycles of denaturation at 95°C for 15 s, annealing a 54°C (algae), 59°C (bacteria) or 56°C (fungi) for 15 s and elongation at 72°C for 10 s, with a final extension at 72°C for 1 min. The number of PCR cycles was determined prior to sampling, using initial test PCRs with material obtained in the same manner. The algal and bacterial amplicons reached a homogenous PCR amplification across all samples after 30 cycles, while the fungal amplification required 35 cycles. Samples were

TABLE 1 | Primer names and sequences used in this study.

Direction	Name	Sequence	Sources
Algae			
Forward	ITS-Cha3	CAACTCTCRRCAACGGATA	Cheng et al., 2016
Reverse	ITS u4	RGTTTCTTTTCCTCCGCTTA	Cheng et al., 2016
Bacteria			
Forward	341F (modified)	CCTACGGGWGGCWGCAG	Muyzer et al., 1993; Vieira et al., 2020
Reverse	785R	GACTACHVGGGTATCTAATCC	Herlemann et al., 2011
Fungi			
Forward	FITS7 (modified)	GTGARTCATCGAATCTTTG	Ihrmark et al., 2012
Reverse	ITS 4 (modified)	TCCTCCGCTTATTGATATGC	White et al., 1990

randomly distributed over two 96-well plates, with both replicates following the same placement scheme.

The amplicons were individually cleaned using magnetic beads (MagSI-NGS^{Prep} Plus, magtivio B.V., Geelen, Netherlands) and DNA concentration was quantified with fluorescence measurement using the Qubit dsDNA HS assay (Thermo Fisher Scientific, MA, United States) as specified by the manufacturer. The replicates were equimolarly pooled within the respective organismal groups, creating a total of three pools for sequencing. The pooled amplicons were send for library preparation and sequencing to Fasteris SA (Plan-les-Ouates, Switzerland). Libraries were prepared for each pool according to the Fasteris MetaFast protocol[1], in order to avoid PCR for library preparation and thus minimizing additional PCR bias and chimera creation. The samples were sequenced on an Illumina MiSeq (Illumina Inc., San Diego, CA, United States) with 2 × 300 bp paired-end reads.

Bioinformatics

Adapter-trimmed reads trimmed with Trimmomatic (Bolger et al., 2014) were supplied by the sequencing provider. We demultiplexed the reads using Cutadapt v3.3 (Martin, 2011) following the demultiplexing combinatorial dual-indexes section of the manual. The error rate was set to 0.15, allowing no insertions or deletions, and discarding reads shorter than 50 bp. Commands were run a second time with the octamer tags in the reverse order to account for amplicons in mixed orientation resulting from PCR-free library preparation. The resulting files were merged to obtain one R1 and one R2 file per replicate. Reads were checked for remaining primer sequences, which were removed using Cutadapt, if present.

The demultiplexed reads were further processed with the DADA2 pipeline (Callahan et al., 2016). Filtering and trimming operations used DADA2 default parameters, except for setting a truncation length [truncLen = c(250,260)] for bacteria, but not for algae and fungi since the length of the ITS2 region can vary between taxa (Schoch et al., 2014). Furthermore, the maximum error rates were relaxed to maxEE = c(5,5) for bacteria and maxEE = c(6,6) for algae and fungi. After de-noising and sample inference, pairs were merged within each replicate, chimeras were removed and one amplicon sequence variant (ASV) table was constructed per replicate. To account for the mixed orientation of the libraries we checked the tables for reverse complement sequences, reversed them and added their counts to the respective complement sequence using DADA2s *rc()* function. Finally, the replicates were merged by summing up the read counts.

For taxonomic assignment the sequences were matched against publicly available databases, namely UNITE general release 8.2 (Abarenkov et al., 2020) for fungal reads and SILVA 138.1 SSU Ref NR 99 (Quast et al., 2012) for bacteria. Since no similar database is currently available for green algae we used the program SEED2 v2.1.2 (Větrovský et al., 2018) to conduct a BLASTn search against GenBank (Clark et al., 2016, last accessed 30.03.2022).

[1]https://www.fasteris.com/en-us/NGS/DNA-sequencing/Metabarcoding/Metagenomics-16S-18S-ITS-or-custom-PCR-amplicons

We then checked the reads for potential contamination with the *decontam* package (Davis et al., 2018), using the combined prevalence and frequency approach. For all organismal groups d*econtam* only showed low numbers (algae = 0, bacteria = 8, fungi = 4) of potential contaminant ASVs, which were discarded. The *decontam*-filtered ASV tables were curated using the LULU algorithm (Frøslev et al., 2017) to merge highly similar ASVs and obtain more reliable diversity metrics. Taxonomic information for all ASVs can be found in **Supplementary Table 1**.

Diversity and Community Structure Analyses

All analyses were conducted in R (R Core Team, 2021, version 4.0.4) through RStudio (RStudio Team, 2021). ASV tables, taxonomic information and accompanying metadata were combined using the *phyloseq* R package (McMurdie and Holmes, 2013) to ease analyses. Figures were created with *ggplot2* (Wickham, 2016) and *gridExtra* (Auguie, 2017). Samples were not rarefied, as recommended by McMurdie and Holmes (2014) and instead treated as compositional count data (Gloor et al., 2017). Scripts of all analyses are available on GitHub at https://github.com/LukDrey/beech_micro_communities.

Intra-Group Diversities

We calculated the Shannon Index (Shannon, 1948) as a measure of alpha diversity, using the function *estimate_richness()* on the full untransformed ASV table as obtained from DADA2 and LULU. Differences in Shannon diversity between tree sizes and management category were tested with an Analysis of Variance (ANOVA) with the function *aov()* and verified *via* a Tukey Honest Significant Differences test (Tukey HSD). Furthermore, we tested whether the Shannon diversity for trees within a plot was spatially autocorrelated. For this purpose, we computed Moran's I (method from Gittleman and Kot, 1990) as a measure of spatial autocorrelation with the function *Moran.I* from the *ape* R package (Paradis and Schliep, 2019).

To create the community barplots we aggregated taxa at the order rank and subset the datasets to the 25 relatively most abundant taxa with *get_top_taxa()* (Teunisse, 2017). The resulting subsets were transformed to reflect their compositional nature by *transform()* and plotted using *plot_composition()*, both from the *microbiome* R package (Lahti and Shetty, 2017).

Inter-Group Differences

Before comparing differences in community composition of differently sized trees and management regimes, each full dataset was transformed based on centred log-ratios (CLR) with *transform()*. After the transformation we conducted a principal component analysis (PCA) on the clr-transformed datasets using the *phyloseq* function *ordinate()* ("RDA" method) which for clr-transformed data is the same as PCA. In the ordination plots we show the first two Principal Components (PC), with the axes scaled to the proportion of variance the PC explains, as recommended by Nguyen and Holmes (2019).

To test if groups showed similar within-group variance, we used the *betadisper()* function and verified the results with

the accompanying permutation test *permutest()* from the *vegan* package (Oksanen et al., 2020). To test for differences in community composition between tree sizes and management intensity we performed Permutational Analysis of Variance (PERMANOVA) with a distance matrix based on Aitchison's distance (method = "euclidean" with the *phyloseq* function *distance()* for clr-transformed data). The PERMANOVA was computed using the *vegan* function *adonis2()* examining marginal effects of tree size and management intensity together.

Species Interactions

The interaction networks were generated with the *SPIEC-EASI* method (Kurtz et al., 2015), a robust method for the sparse and compositional nature of microbiome datasets implemented in the R package *SpiecEasi*. Before network inference the ASV tables were subset to contain only ASVs contributing at least one percent of the total reads to ease both visualization and computational load. The main SPIEC-EASI algorithm was set to use the meinshausen-bühlmann's neighborhood selection (Meinshausen and Bühlmann, 2006) and Bounded StARS model selection (Müller et al., 2016) on 50 subsamples (rep.num = 50), with lambda.min.ratio = 0.1, nlambda = 100, pulsar.select = TRUE and seed = 10010. We calculated one network per organismal group, as well as one containing all three groups together.

The obtained models were refit, turned into *igraph* (Csardi and Nepusz, 2006) objects and loaded in *Gephi* v0.9.2 (Bastian et al., 2009) for visualization. Modularity and betweenness centrality (for visualization purposes) were computed with *Gephi*s internal algorithms (Brandes, 2001; Blondel et al., 2008). The graph layouts were constructed using the Fruchterman-Reingold algorithm (Fruchterman and Reingold, 1991). For each network, hub taxa were calculated based on vertex betweenness centrality using the *igraph* function *betweeness()* with default parameters, except setting directed = FALSE. The top five hub taxa, based on vertex betweenness centrality, were extracted.

Differential Abundance Analysis

Differential abundance analysis was conducted using *ALDEx2* (Fernandes et al., 2013, 2014; Gloor et al., 2016). We compared abundances of two groups, i.e., high/low management intensity, large/small, large/medium and medium/small trees, for each organismal group. *ALDEx2* generates Monte Carlo samples (N = 128), drawn from the Dirichlet distribution for each individual sample, and tests differences between specified groups through Wilcoxon rank-sum tests. *ALDEx2* is a robust choice for compositional datasets because the data is clr-transformed internally. Taxa were declared differentially abundant if they showed a Benjamini-Hochberg corrected p-value < 0.05.

RESULTS

Intra-Group Diversities

In total we obtained on average 59,324 reads per sample for algae (min = 22,913, max = 99,255), 58,259 reads for bacteria (min = 24,830, max = 124,263) and 45,403 reads for fungi (min = 18,510, max = 163,736). The extraction blanks had on average 888 reads for algae (min = 283, max = 1,679), 12,836 for bacteria (min = 462, max = 19,723), and 34,211 for fungi (min = 6171, max = 109,074), while the PCR negative controls contained on average 1,152 reads for algae (min = 272, max = 3,745), 625 reads for bacteria (min = 196, max = 2,076), and 3,241 for fungi (min = 196, max = 15,752). The negative controls were discarded from the analysis after controlling for possible contaminant sequences with *decontam* (Davis et al., 2018). From these reads, we retrieved 216 algal, 1,742 bacterial and 992 fungal ASVs.

Overall algae and fungi displayed similar Shannon alpha diversity, while bacteria showed a slightly higher diversity (**Figure 1**). Neither algae, fungi nor bacteria exhibited statistically significant differences in alpha diversity when comparing low and high management intensity plots (**Figures 1A–C**). Considering differences between tree sizes, smaller trees displayed higher alpha diversity for algae and fungi (**Figures 1D,F**). Overall, bacterial diversities were more uniform, but displayed higher median Shannon diversity values for larger trees (**Figure 1E**).

This trend was corroborated by the results of an ANOVA comparing the three tree-size classes. For algae, we found a significant overall effect of tree size ($F = 4.163$, $p < 0.05$), that was driven by a significant difference between large and small trees (Tukey HSD p-value < 0.05). No significant differences were found between large/medium and medium/small trees. Tree size had a significant effect overall ($F = 17.33$, $p < 0.001$) in fungi, with significant differences between large and medium ($p < 0.01$), as well as large and small trees ($p < 0.001$). For bacteria we found no significant overall effects.

Spatial autocorrelation tests showed that only in four of 48 cases (three organismal groups × 16 plots) the null-hypothesis of no spatial correlation could be rejected (**Table 2**). This indicates that the effect of spatial autocorrelation within plots is negligible. Trees belonging to the same plot showed very similar Shannon alpha diversity values. One exception is plot HEW8 where alpha diversity was significantly spatially autocorrelated for both algae and bacteria.

Bark microbial communities were similar among trees, with only minor differences in rare orders for all three organismal groups. For algae, the predominant orders were Trebouxiales and Chlorellales, with Trebouxiales contributing more than 50% of the reads in many plots (**Figure 2A**). Rare orders displayed a relatively high inter-plot variability, with Prasiolales being almost absent for the plot HEW5. Compared to algae, bacteria were more homogeneous, with the same four orders—Rhizobiales, Sphingomonadales, Acetobacterales and Cytophagales—displaying comparably high relative abundances in all plots (**Figure 2B**). We found a higher rare-order diversity in bacteria and fungi compared to algae. Capnodiales was by far the most abundant fungal order, and dominant in all plots (**Figure 2C**). Compared to bacteria and algae, a higher proportion of fungal reads could not be assigned at the order rank. These unassigned reads contributed more than 25% of the total reads in some samples. The three relatively most abundant ASVs in the algal dataset belong to the genera *Symbiochloris* (12%), *Apatoccocus* (12%) and *Desmococcus* (8%), and in the

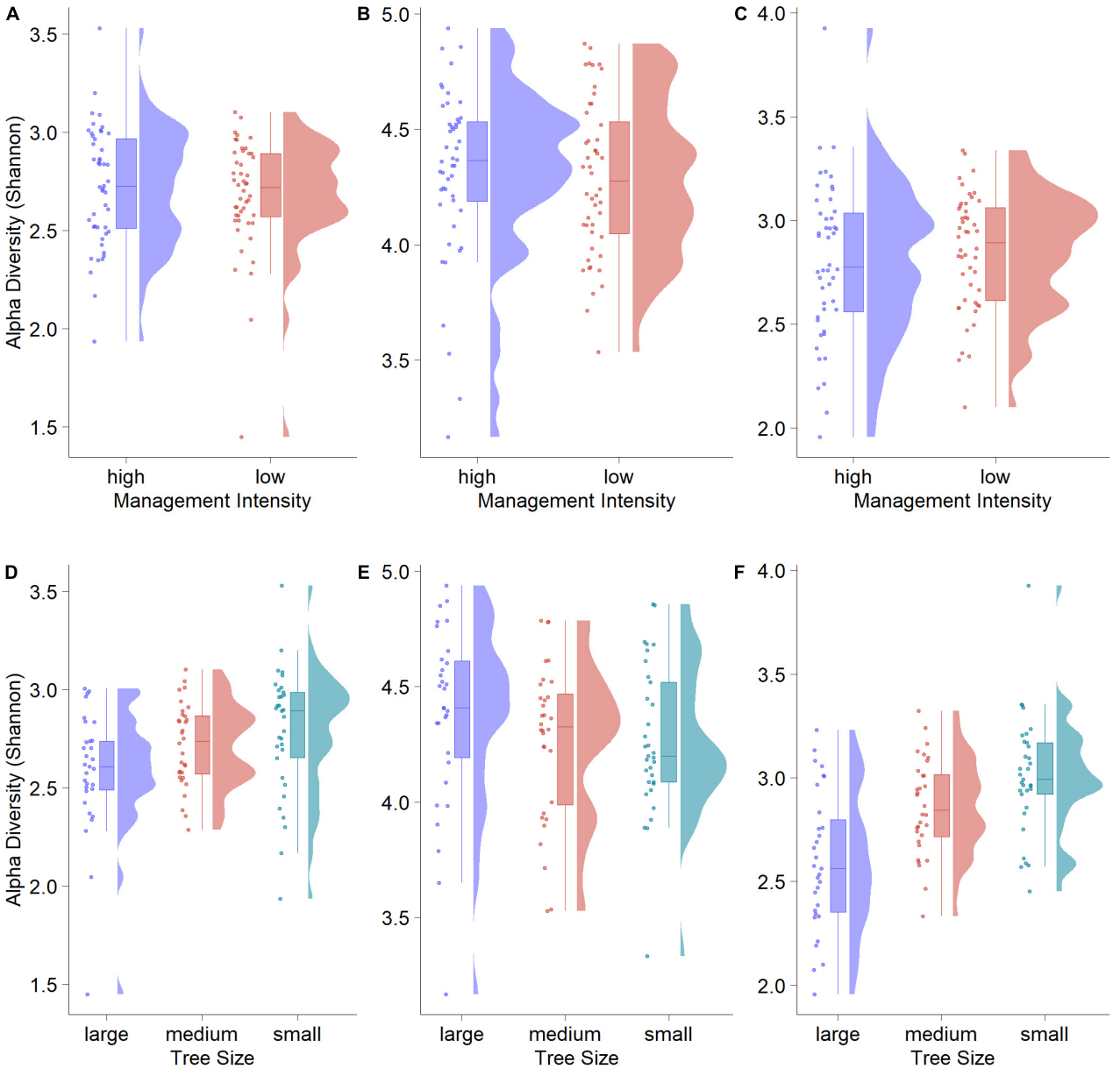

FIGURE 1 | Rain-Cloud plots of alpha diversity (Shannon) against management regime and tree size for algae **(A,D)**, bacteria **(B,E)** and fungi **(C,F)**. Differences are visible from the boxplots, while the original data structure is visible from raw data scatters (randomly jittered) and raw data distribution.

bacterial dataset to *Acidiphilium* (10%), 1174-901-12 (4%) and *Methylocella* (4%). Only one of the three most abundant fungal ASVs could be assigned to a genus—namely *Scoliciosporum* (4%)—while one was assigned to Capnodiales (26%) and one only to Ascomycota (7%).

Intra-Group Interactions

We inferred ASV interaction networks for all three microbial groups (**Figure 3**). Each ASV entering the networks contributed at least 1% of the total reads, resulting in 129 ASVs for the algae, 624 for bacteria and 289 for fungi. The algal network (**Figure 3A**) had a diameter of 10 (i.e., the longest shortest path between two nodes was through ten edges), an average path length of four and a modularity score of 0.575. Modularity scores > 0.4 indicate strong modularity (Newman, 2006). The diameter for the fungal network (**Figure 3C**) was 7, with an average path length of ∼3.2 and a modularity slightly lower than the algae at 0.434. The bacterial network (**Figure 3B**) was denser and more interconnected with a diameter of 5, an average path length of ∼2.7 and a modularity of 0.335.

The algal network could be subdivided into nine different modules, five of which consisting of more than 10 ASVs (see **Table 3**). There were also 8 ASVs that did not interact with any other taxon in the network. The algal module with the highest

TABLE 2 | Overview of Moran's I (MI) values for all three organismal groups in the studied plots.

	Algae				Bacteria				Fungi			
Plot ID	**MI (observed)**	**MI (expected)**	**sd**	***P* value**	**MI (observed)**	**MI (expected)**	**sd**	***P* value**	**MI (observed)**	**MI (expected)**	**sd**	***P* value**
HEW11	−0.19	−0.2	0.17	0.95 (n.s.)	−0.09	−0.2	0.12	0.32 (n.s.)	0	−0.2	0.24	0.41 (n.s.)
HEW20	−0.41	−0.2	0.16	0.17 (n.s.)	−0.15	−0.2	0.14	0.75 (n.s.)	−0.43	−0.2	0.16	0.13 (n.s.)
HEW26	−0.26	−0.2	0.24	0.8 (n.s.)	−0.55	−0.2	0.21	0.09 (n.s.)	−0.24	−0.2	0.23	0.86 (n.s.)
HEW27	−0.46	−0.2	0.2	0.19 (n.s.)	−0.08	−0.2	0.19	0.53 (n.s.)	−0.27	−0.2	0.24	0.77 (n.s.)
HEW28	0.32	−0.2	0.3	0.09 (n.s.)	−0.31	−0.2	0.22	0.60 (n.s.)	−0.39	−0.2	0.31	0.53 (n.s.)
HEW31	−0.09	−0.2	0.11	0.31 (n.s.)	−0.35	−0.2	0.16	0.33 (n.s.)	0.02	−0.2	0.14	0.12 (n.s.)
HEW32	0.04	−0.2	0.13	0.06 (n.s.)	−0.19	−0.2	0.09	0.92 (n.s.)	0.05	−0.2	0.15	0.09 (n.s.)
HEW33	−0.22	−0.2	0.25	0.93 (n.s.)	−0.21	−0.2	0.08	0.95 (n.s.)	0.04	−0.2	0.29	0.41 (n.s.)
HEW34	−0.3	−0.2	0.16	0.52 (n.s.)	−0.3	−0.2	0.19	0.61 (n.s.)	−0.35	−0.2	0.2	0.47 (n.s.)
HEW35	−0.35	−0.2	0.14	0.29 (n.s.)	−0.14	−0.2	0.15	0.68 (n.s.)	0.09	−0.2	0.13	0.03 (*)
HEW36	−0.31	−0.2	0.12	0.39 (n.s.)	−0.19	−0.2	0.11	0.90 (n.s.)	−0.12	−0.2	0.09	0.37 (n.s.)
HEW37	0.01	−0.2	0.11	0.07 (n.s.)	−0.2	−0.2	0.09	0.95 (n.s.)	−0.27	−0.2	0.11	0.53 (n.s.)
HEW43	−0.31	−0.2	0.15	0.48 (n.s.)	−0.34	−0.2	0.09	0.13 (n.s.)	−0.34	−0.2	0.08	0.08 (n.s.)
HEW49	−0.15	−0.2	0.1	0.58 (n.s.)	−0.38	−0.2	0.11	0.11 (n.s.)	−0.13	−0.2	0.08	0.44 (n.s.)
HEW5	−0.06	−0.2	0.09	0.09 (n.s.)	−0.29	−0.2	0.1	0.40 (n.s.)	−0.01	−0.2	0.09	0.04 (*)
HEW8	0.31	−0.2	0.22	0.02 (*)	0.39	−0.2	0.26	0.02 (*)	0.25	−0.2	0.28	0.11 (n.s.)

An observed higher significant p-value (< 0.05) vs. expected indicates positive autocorrelation, whereas lower MI values indicate negative autocorrelation.

number of nodes was module 2 (gold color, **Figure 3A**) with the most abundant ASV belonging to the genus *Desmococcus* (relative abundance = ∼47% in the module, **Table 3**).

The bacterial network consisted of seven modules, all of which included more than 10 ASVs and no taxa with no connections. In this case, module 6 (purple color in **Figure 3B**) was the module with the highest count of taxa, with an ASV assigned to the genus *Abditibacterium* being the predominant strain (12% of reads in the module, **Table 3**).

For fungi, the network clustered into nine different modules, all containing more than ten ASVs and no unconnected taxa. Also in this case, module two (purple color, **Figure 3C**) was the module with the highest number of taxa for the fungal network, with an ASV belonging to the order Capnodiales—not assignable more specifically (**Table 3**)—with the highest relative abundance in the module (14%).

Network structure was examined by identifying nodes with the highest number of shortest paths going through them (betweenness centrality), indicating taxa that are important for the connectivity of the network. We defined so called hub taxa as the five taxa with the highest betweenness centrality (**Table 4**). In the algal network these hub taxa belonged to two orders, Chlorellales and Trebouxiales, and three different genera. Three ASVs were assigned to the genus *Apatoccocus*, and one to *Trebouxia* and *Symbiochloris,* respectively. Bacterial hub taxa showed a higher diversity than the algae with hub taxa belonging to five different orders. The genera include *Tundrisphaera*, *Actinomycetospora* and *Oligoflexus*. One of the ASVs was assigned to the group 1174-901-12, a group of uncultured bacterial strains within the order Rhizobiales and one to the family *Chitinophagaceae*. Many of the fungal hub taxa were not assigned at the genus rank, with the exception of two ASVs belonging to the genera *Tremella* and *Aureobasidium*. Two more ASVs were assigned to the order Capnodiales while one was only assigned at the phylum rank.

Inter-Group Interactions

The combined network of algae, bacteria and fungi (**Figure 4**) displayed a diameter of 4, an average path length of ∼2.6 and a modularity of 0.259, making this network more densely connected than the bacterial network. Out of the eight modules, modules two (29%) and three (26%) accounted for more than half of the total reads.

The top orders for algae, bacteria and fungi in the relatively most abundant module (module 2, pink color in **Figure 4B**) were Trebouxiales, Sphingomonadales and Capnodiales, respectively. Algae and fungi accounted for up to 36 and 26% of the module reads, respectively, while Sphingomonadales only contributed 5% of the reads. Trebouxiales contributed 87% of the algal reads, Sphingomonadales 30% of bacterial reads, and Capnodiales 77% of the fungal reads. In total, algae contributed 42%, bacteria 19%, and fungi 34% of the reads assigned to module 2.

In the second most abundant module (module 3, blue color in **Figure 4B**). Chlorellales were the most important algal order, Rhizobiales the most important bacterial order, while the most important fungal order was again Capnodiales. Chlorellales contributed 18%, Rhizobiales 16% and Capnodiales only 5% of the module reads. Chlorellales accounted for 43% of the algal reads, Rhizobiales 48% of the bacterial reads, and Capnodiales 34% of the fungal reads. Overall, module three consisted of 42% algae, 34% bacteria and 10% fungi.

The hub taxa of the combined network (**Table 4**) contained members of two microbial groups, specifically three bacteria and two fungi. Four ASVs could be assigned down to genus rank, while one fungal ASV—a hub taxon present also in the fungal network—could only be assigned to order rank (Capnodiales).

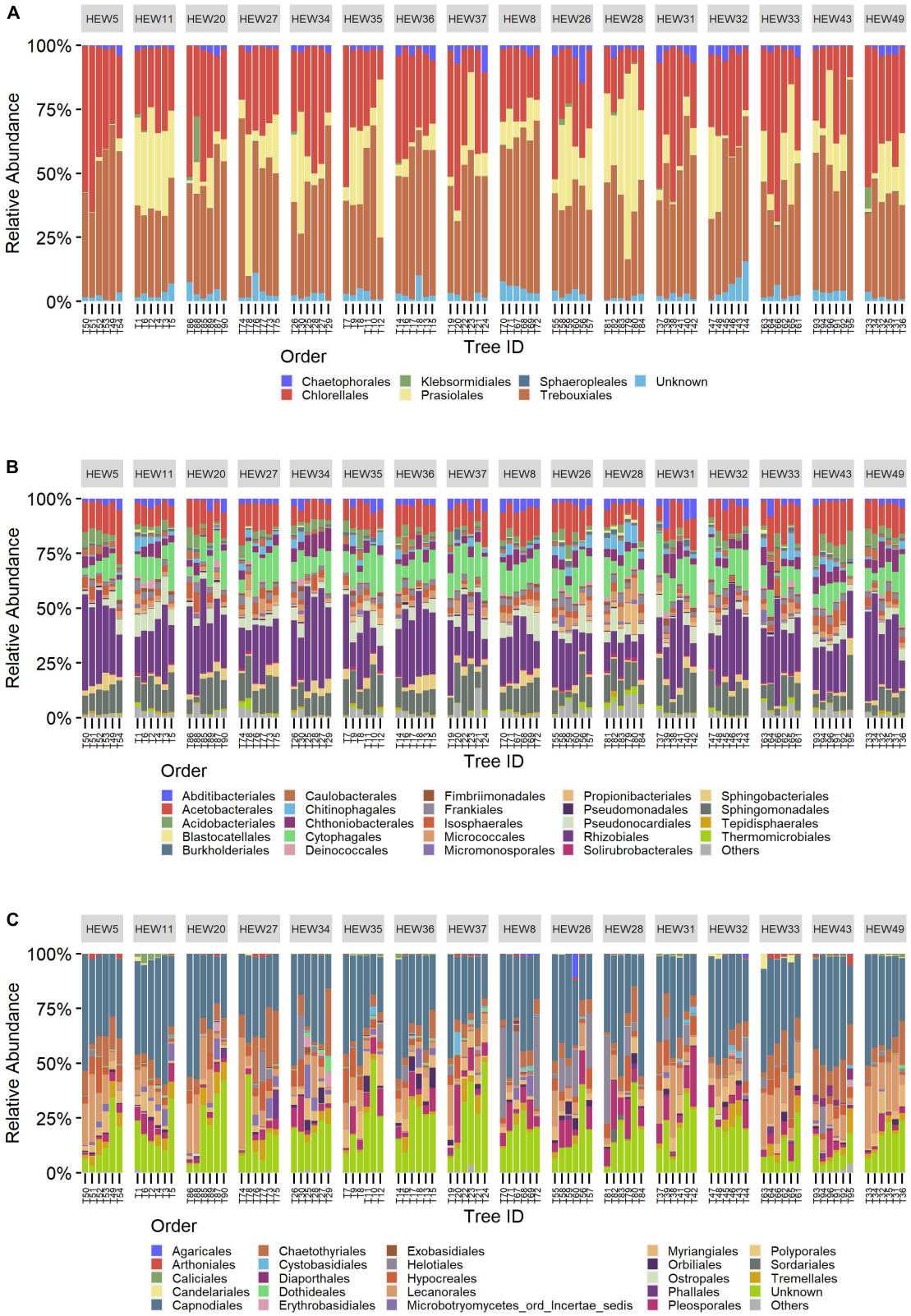

FIGURE 2 | Community bar charts showing the relative abundance of the 25 most abundant orders split by sampling plots for algae **(A)**, bacteria **(B)** and fungi **(C)**. From left to right: the first eight plots are under a low-, and the next eight under a high-management regime. Bars within plots represent individual trees, from large to small tree-size class (2 trees each), from left to right.

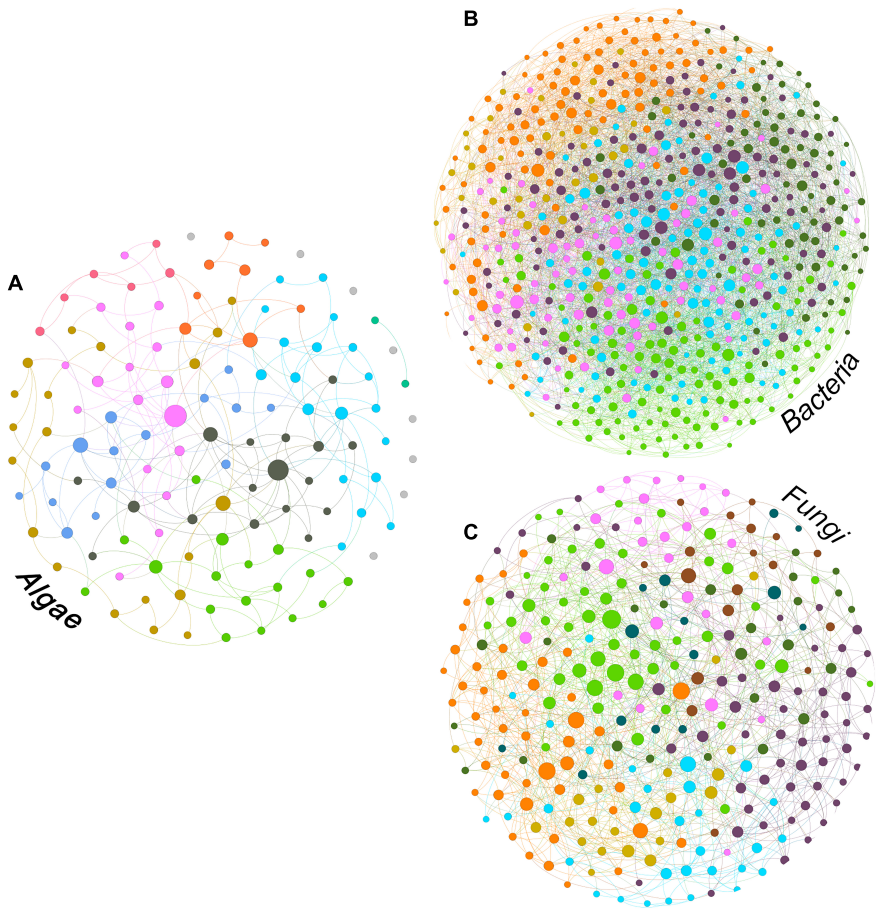

FIGURE 3 | Intra-group ASV interaction network for algal **(A)**, bacterial **(B)** and fungal **(C)** fractions of the bark microbiome. The size of the nodes is proportional to the value of betweenness centrality (based on Brandes (2001)) and colors correspond to modules (Blondel et al., 2008).

The other fungus—also a fungal hub taxon—belonged to the genus *Aureobasidium*, while the three bacterial ASVs belonged to the genus *Edaphobaculum,* the uncultured group LD29 within the order Chthoniobacterales, and the genus *Kineococcus*.

Drivers of Changes in Community Composition

To investigate differences in community composition we plotted the results of the PCA (**Figure 5**). The differences between the high and low management intensity are not readily visible by looking at the clusters of the two groups. Yet, there are significant differences between the groups as revealed by the PERMANOVA results (algae: $p < 0.05$, bacteria: $p < 0.01$, fungi: $p < 0.001$). The dispersion permutation test was significant (algae: $p < 0.05$, bacteria: $p < 0.05$, fungi: $p < 0.05$) indicating a heterogeneous dispersion within groups. If on one hand this might suggest that the results are not reliable, Anderson and Walsh (2013) showed that PERMANOVA is not sensitive against heterogeneity, compared to other analyses. Management intensity explained only ∼2% of the variance in the dataset, suggesting a subtle yet significant effect.

The tree sizes clustered in a much clearer structure, with less overlap of the 95% confidence interval ellipses. The PERMANOVA analysis confirmed this observation, with highly significant results for all microbial groups ($p < 0.001$ for all), while the permutation test indicated homogenous dispersion within the bacteria and fungi ($p > 0.05$), but not the algae ($p < 0.05$).

Further examination of the community structure showed that the differences in community composition were also visible through significantly differentially abundant taxa (**Supplementary Table 2**). The difference between management intensities was little, with only four differentially abundant fungal ASVs. The tree-size classes, however, showed a different signal and confirmed the stronger differences indicated by the PERMANOVA results. Between large and medium trees we found nine algae, four bacteria and 10 fungal taxa that showed significant abundance differences. A larger difference in abundances could be seen between large and small trees, with 19 algae, 43 bacteria and 34 fungi that were differentially abundant. Almost no difference could be observed between communities of medium and small trees with only three algal and one bacterial ASVs with significant changes in abundance.

TABLE 3 | Taxonomic assignment of the most abundant ASVs and their relative abundance for modules with more than 10 ASVs.

ASV ID	#	Kingdom	Phylum	Class	Order	Family	Genus	Rel. abund.
Algae								
ASV 4	2	Viridiplantae	Chlorophyta	Trebouxiophyceae	Prasiolales	Stichococcaceae	Desmococcus	0.47
ASV 1	4	Viridiplantae	Chlorophyta	Trebouxiophyceae	Trebouxiales	Trebouxiaceae	Symbiochloris	0.8
ASV 7	5	Viridiplantae	Chlorophyta	Trebouxiophyceae	Trebouxiales	Trebouxiaceae	Trebouxia	0.4
ASV 2	7	Viridiplantae	Chlorophyta	Trebouxiophyceae	Chlorellales	Chlorellaceae	Apatococcus	0.38
ASV 33	9	Viridiplantae	Chlorophyta	Trebouxiophyceae	Prasiolales	Stichococcaceae	Diplosphaera	0.44
Bacteria								
ASV 2	0	Bacteria	Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	Acidiphilium	0.47
ASV 8	1	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	Methylocella	0.13
ASV 210	2	Bacteria	Abditibacteriota	Abditibacteria	Abditibacteriales	Abditibacteriaceae	Abditibacterium	0.08
ASV 54	3	Bacteria	Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	Acidiphilium	0.11
ASV 38	4	Bacteria	Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	Acidiphilium	0.11
ASV 6	5	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	1174-901-12	0.15
ASV 46	6	Bacteria	Abditibacteriota	Abditibacteria	Abditibacteriales	Abditibacteriaceae	Abditibacterium	0.13
Fungi								
ASV 11	0	Fungi	Ascomycota	Lecanoromycetes	Lecanorales	Lecanoraceae	Scoliciosporum	0.19
ASV 23	1	Fungi	Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	Capronia	0.58
ASV 51	2	Fungi	Ascomycota	Dothideomycetes	Capnodiales			0.14
ASV 32	3	Fungi	Ascomycota	Dothideomycetes	Capnodiales	Mycosphaerellaceae		0.25
ASV 43	4	Fungi	Ascomycota	Dothideomycetes	Capnodiales			0.39
ASV 4	5	Fungi	Ascomycota					0.36
ASV 6	6	Fungi	Ascomycota	Leotiomycetes	Helotiales			0.3
ASV 1	7	Fungi	Ascomycota	Dothideomycetes	Capnodiales			0.88
ASV 61	8	Fungi	Ascomycota					0.18

TABLE 4 | Taxonomic information for the hub taxa of the respective network, identified based on their betweenness centrality.

ASV ID	Kingdom	Phylum	Class	Order	Family	Genus
Algae						
ASV 10	Viridiplantae	Chlorophyta	Trebouxiophyceae	Chlorellales	Chlorellaceae	Apatococcus
ASV 11	Viridiplantae	Chlorophyta	Trebouxiophyceae	Chlorellales	Chlorellaceae	Apatococcus
ASV 13	Viridiplantae	Chlorophyta	Trebouxiophyceae	Trebouxiales	Trebouxiaceae	Symbiochloris
ASV 22	Viridiplantae	Chlorophyta	Trebouxiophyceae	Trebouxiales	Trebouxiaceae	Trebouxia
ASV 8	Viridiplantae	Chlorophyta	Trebouxiophyceae	Chlorellales	Chlorellaceae	Apatococcus
Bacteria						
ASV 162	Bacteria	Planctomycetota	Planctomycetes	Isosphaerales	Isosphaeraceae	Tundrisphaera
ASV 19	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	1174-901-12
ASV 372	Bacteria	Bacteroidota	Bacteroidia	Chitinophagales	Chitinophagaceae	
ASV 79	Bacteria	Actinobacteriota	Actinobacteria	Pseudonocardiales	Pseudonocardiaceae	Actinomycetospora
ASV 855	Bacteria	Bdellovibrionota	Oligoflexia	Oligoflexales	Oligoflexaceae	Oligoflexus
Fungi						
ASV 16	Fungi	Ascomycota	Dothideomycetes	Capnodiales		
ASV 18	Fungi	Basidiomycota	Tremellomycetes	Tremellales	Tremellaceae	Tremella
ASV 4	Fungi	Ascomycota				
ASV 56	Fungi	Ascomycota	Dothideomycetes	Dothideales	Aureobasidiaceae	Aureobasidium
ASV 65	Fungi	Ascomycota	Dothideomycetes	Capnodiales		
Combined						
ASV 56	Fungi	Ascomycota	Dothideomycetes	Dothideales	Aureobasidiaceae	Aureobasidium
ASV 65	Fungi	Ascomycota	Dothideomycetes	Capnodiales		
ASV 116	Bacteria	Verrucomicrobiota	Verrucomicrobiae	Chthoniobacterales	Chthoniobacteraceae	LD29
ASV 395	Bacteria	Bacteroidota	Bacteroidia	Chitinophagales	Chitinophagaceae	Edaphobaculum
ASV 187	Bacteria	Actinobacteriota	Actinobacteria	Kineosporiales	Kineosporiaceae	Kineococcus

DISCUSSION

In this study we used a multi-kingdom metabarcoding approach to investigate the microbiome (algae, bacteria, fungi) on the bark of beech from sites with different forest management intensity in the Hainich-Dün region, Thuringia, Germany. We provide a first characterization of aboveground bark-associated microbial communities in beech forests, as well as an

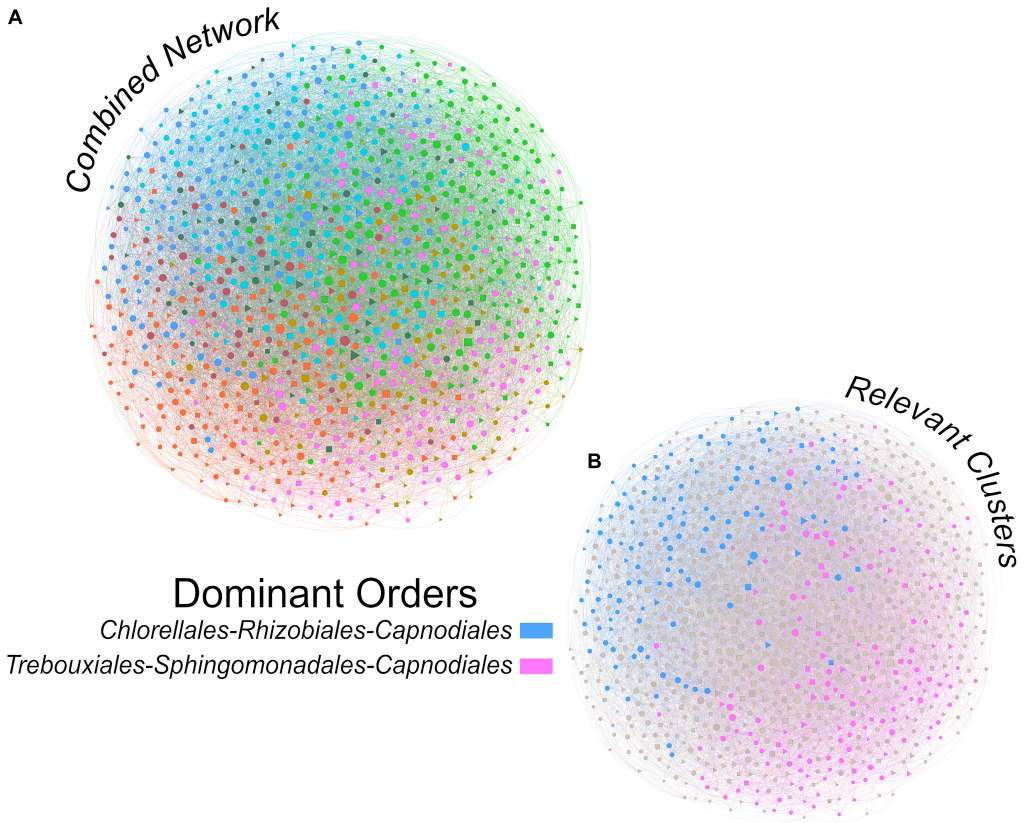

FIGURE 4 | Inter-group ASV interaction network integrating algal, bacterial and fungal taxa for a description of the interactions within the complete bark microbiome **(A)**. The two colored modules **(B)** are the two most abundant modules of the whole dataset, accounting for 29% (pink) and 26% (blue) of the total reads. The size of the nodes is proportional to the value of betweenness centrality (based on Brandes (2001)) and colors correspond to modules (Blondel et al., 2008).

account of microbial inter-kingdom interactions. Additionally, by testing how community diversity and structure of the different organismal groups change in relation to land-use intensity and tree size, we identify potential drivers of community assembly and provide sampling recommendations for studying the bark microbiome at broader spatial scales.

Diversity of the Bark-Associated Beech Microbiome

Our results show that the bark of beech harbors highly diverse algal, bacterial and fungal communities.

The modules and hub-taxa of the algal interaction networks are mainly represented by species of the families Trebouxiophyceae and Chlorellaceae, in particular by genera commonly found in subaerial environments and already detected in forest settings (Štifterová and Neustupa, 2015). Algae of the genus *Apatococcus*, a "flagship" taxon of above-ground ecosystems (Rindi, 2007), are abundant in the modules and interconnected members of the algal microbiome. Additionally, *Apatoccocus* is a known photobiont of *Scoliciosporum* (Sanders and Masumoto, 2021), a very common fungal genus in our dataset. Another abundant alga is *Desmococcus* sp., which typically forms visible powdery, greenish layers on the bark of trees in association with *Apatococcus* and other subaerial green algae (Rindi, 2007). Both are part of what is possibly the most tolerant subaerial algal community, being able to thrive even in urban areas (Barkman, 1958). Furthermore, our results confirm *Symbiochloris* as an important component of the dermo- and phyllosphere (Škaloud et al., 2016; Zhu et al., 2018), as well as other Trebouxiales, an order consisting of free-living as well as lichen-forming green algae (Sanders and Masumoto, 2021). One of the algal hub taxa comes from the genus *Trebouxia,* the most common lichen forming alga (Sanders and Masumoto, 2021).

Compared to the algae and fungi, the important taxa of the bacterial network modules are far more diverse. Similarly to Aschenbrenner et al. (2017), the bacterial community is dominated by the class Alphaproteobacteria, in particular Rhizobiales and Acetobacteriales. Among the Rhizobiales we detected *Methylocella* sp., a facultative methanotroph adapted to various nutrients (acetate, pyruvate, succinate, malate, and ethanol) (Dedysh and Dunfield, 2011), and 1174-901-12, previously described as an early colonizer of aerial environments (Romani et al., 2019) and a known member of the phyllosphere (Ares et al., 2021). In the case of Acetobacterales, *Acidiphilium* is among the taxa contributing the most to the modules. The genus consists of aerobic bacteria with photosynthetic pigments, with a pH range that matches well to the pH of beech bark

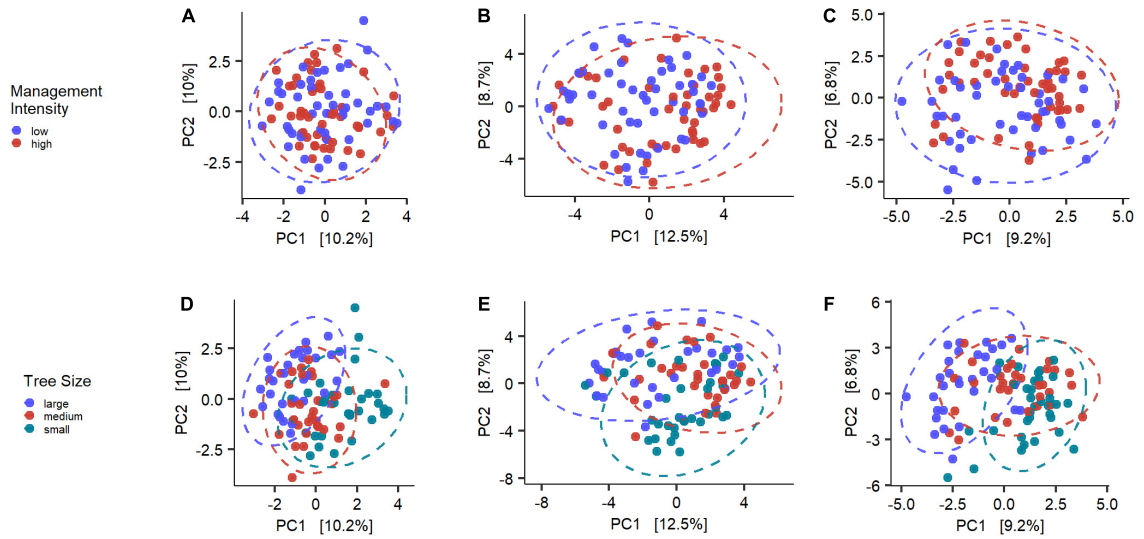

FIGURE 5 | Overview of the Principle Component Analysis for algae **(A,D)**, bacteria **(B,E)** and fungi **(C,F)**. Colors indicate the tested groups and axes are scaled to the proportion of variance explained by the displayed Principal Component (Nguyen and Holmes, 2019).

(~4.4 according to Asplund et al. (2015)) and that do not overlap in metabolic demands with *Methylocella* (Hiraishi and Imhoff, 2015). Another well represented class in the modules are the Abditibacteria, especially the genus *Abditibacterium* whose representatives are well adapted to low-nutrient conditions and have been reported on tree bark before (Tahon et al., 2018; Kobayashi and Aoyagi, 2019). Among the bacterial hub taxa we find genera from classes already reported for tree-associated microbiomes, such as *Oligoflexus* in bryophytes (Ma et al., 2017), *Actinomycetospora* and 1174-901-12 on tree bark and lichens (Yamamura et al., 2011; Ares et al., 2021), and *Tundrisphaera*, previously only isolated from lichen-dominated tundra soils (Kulichevskaya et al., 2017). One ASV belongs to *Chitinophagaceae*, a family associated with the degradation of fungal cell-walls (Carrión et al., 2019). As found for the algae, some of the most abundant genera are important components of the bacterial networks. Contrary to Aschenbrenner et al. (2017), we did not find major contributions from the genera *Burkholderia* and *Pseudomonas*, possibly indicating that these genera are specific to the bark of sycamore maple (*Acer pseudoplatanus*).

The investigation of the important fungal diversity was somewhat hindered by low taxonomic resolution with many ASVs that could not be assigned past phylum rank. This may result from a lack of resolution in public fungal databases combined with the presence of several unknown taxa in our dataset. Important members of the fungal networks in the beech bark community are the so-called black yeasts, e.g., *Capronia* and *Aureobasidium,* which are known to occur on tree bark and leaves (Untereiner and Malloch, 1999; Andrews et al., 2002), but also decaying wood (Cooke, 1959) and on other fungi or lichens as secondary saprobionts (Untereiner and Malloch, 1999). Other important network components belong to the genus *Tremella,* known mycoparasites (Zugmaier et al., 1994). Among the lichen-forming fungi, one of the most common fungi in our dataset belongs to the genus *Scoliciosporum*, a genus of crustose lichens that was already reported on beech bark (e.g., Dymytrova, 2011). The biggest contributors at the order rank are members of the Capnodiales (Dothideomycetes), whose species have been shown to associate with the lichen microbiome (Smith et al., 2020) and are abundant in the beech phyllosphere as well (Unterseher et al., 2016). Yet, more research into these orders is needed as they are taxonomically and ecologically highly diverse and include a large diversity of life forms, from lichenized, to mycoparasytic, epi-, ecto-, endophytic, as well as saprobiontic species. In contrast to Unterseher et al. (2016) we could not find a larger contribution of Helotiales, suggesting a specialization of this fungal order to the phyllosphere. The most abundant ASVs of two fungal modules, as well as one of the most abundant ASV overall, could not be assigned further than Ascomycota, highlighting that important components of the fungal bark microbiome remain undescribed. Sampling in different seasons may reveal an even higher—described and undescribed—fungal diversity on tree trunks (Beck et al., 2014). In conclusion, more research is needed in order to confirm the role of the bark habitat as a reservoir of novel fungal diversity. This could be done by combining genetics and culture-based approaches.

Biotic Interactions and Inter-Kingdom Synergies in the Bark Microbiome

The higher modularity scores of the fungal and especially algal networks may indicate higher specialization or niche differentiation in these groups (Augustyn et al., 2016). In contrast, bacteria are less clearly divided into ecological modules, which potentially indicates closer interactions between all taxa as there seems to be no split into specialized groups. Further analyses based on a broader dataset are needed to exclude that the observed patterns are an artifact of the overall higher diversity found in bacteria.

The results from the combined, inter-kingdom co-occurrence analysis indicate that algal and fungal specialists might be connected through a common set of bacteria. It is tempting to speculate that the interactions between Rhizobiales and Chlorellales (mostly represented by members of the genus *Apatococcus*) observed in the main ecological cluster in our dataset are of symbiotic nature, as Rhizobiales are well-known beneficial partners in plant-microbe interactions and common associates of lichens (Erlacher et al., 2015; Grube et al., 2015). Positive interactions among Sphingomonadales, Trebouxiales, and Capnodiales—all known occupants of bark substrates—characterize the second most important cluster. The bacterial genus *Sphingomonas* is very common in above-ground forests habitats (Vorholt, 2012), exhibiting facultative photosynthesis.

Finally, we identified the most highly connected taxa (hubs), i.e., taxa that are crucial for the stability of the ecological network (Banerjee et al., 2018). For bacteria, the hub taxa belong to the genera LD29 (Verrucomicrobiota), *Edaphobaculum* (Bacteroidetes) and *Kineococcus* (Actinobacteria). Little is known about their ecology, with LD29 particularly abundant in lichen thalli (Aschenbrenner et al., 2017), *Edaphobaculum* previously found in soils where it contributes to the creation of biofilms (Keuschnig et al., 2021), and *Kineococcus* isolated from soil samples as well as the rhizosphere (Normand and Benson, 2015). As for the fungi, both of the inter-kingdom hub taxa are also found as hub taxa in the fungal network. One of them belongs to the genus *Aureobasidium*, common on leaves of apple trees (Andrews et al., 2002) but also linked to the decay of bark (Cooke, 1959). The other could not be assigned below the order rank and is a member of the Capnodiales.

Bark Microbiome Responds to Tree Size, but Not to Intensity of Forest Management

The intensity of the forest management regime has virtually no effect on microbial community diversity and structure in our study area. This might be a result of a forest management plan that avoids clear cuts and carefully selects trees to harvest, which leads to a uniform forest structure in the study area (Schall et al., 2020). Based on a broader sampling including this and other two large forest areas in Germany, Boch et al. (2021) showed that an increase in forest management intensity is linked to reduced lichen species richness. A larger sampling effort covering a broader gradient of land-use intensity is therefore required to test whether the response of the bark-associated microbiome differs from that of the macroepiphytes.

We found significant differences in diversity and composition of the bark microbiome according to different tree-size classes. The lower microbial diversity found on larger (older) trees for algae and fungi is probably the result of environmental filtering on highly heterogeneous pioneer communities over time. This is particularly evident when comparing large and small trees, thus suggesting slow succession of these microbiomes toward final community composition. Our results from the spatial autocorrelation analysis underpin random assembly of the microbial bark community at the local (plot) level, with a high heterogeneity between trees. Lastly, a finer-scale comparison of micro-habitats, i.e., different exposures, bark crevices/cracks/holes, would be of great interest for disentangling micro-scale interactions that our approach cannot reliably identify.

Conclusions and Sampling Recommendations

In this pioneering study we provide novel insights into the diversity, spatial context, and biotic interactions that characterize the beech bark microbiome in Central European forests. We showed that there are predictable community shifts depending on tree age. These represent the first steps toward proposing a framework of community assembly on forest tree bark, a ubiquitous, ecologically relevant, yet overlooked component of terrestrial habitats.

Taken together, our results show that a single tree does not adequately characterize the bark-associated microbial community at plot level. To capture most of the microbial diversity, considering the spatial randomness shown by the spatial autocorrelation analysis, we recommend sampling using a spatially random approach with a balanced representation of the main tree-size classes present in the plot. Samples taken from multiple trees can then be combined into a composite sample. The use of composite samples ensures relatively low costs for obtaining adequate sequencing depths while maximizing the spatial range of the study and the number of plots, allowing for easy upscaling to large areas and/or environmental gradients. This data can then be used to assess the effects of factors, e.g., related to forest structure, such as stand density and canopy openness.

DATA AVAILABILITY STATEMENT

The raw sequences are deposited in the NCBI SRA repository, accession numbers SRR18461106, SRR18461107, and SRR18461108. All scripts and additional data necessary to replicate the analysis are available at https://github.com/LukDrey/beech_micro_communities. The data on the Forest Management Index are available at https://www.bexis.uni-jena.de/underAccessionnumber16466.

AUTHOR CONTRIBUTIONS

FD and IS secured the funding. FD and LD devised the sampling and analytical methods, conducted the fieldwork and sampling, and wrote the manuscript with contributions from IS. LD performed the laboratory work and analysis. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ffgc.2022.858382/full#supplementary-material

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The Amazon Epiphyte Network: A First Glimpse Into Continental-Scale Patterns of Amazonian Vascular Epiphyte Assemblages

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Epiphytes are still an understudied plant group in Amazonia. The aim of this study was to identify distributional patterns and conservation priorities for vascular epiphyte assemblages (VEA) across Amazonia. We compiled the largest Amazonian epiphyte plot database to date, through a multinational collaborative effort of 22 researchers and 32 field sites located across four Amazonian countries – the Amazonian Epiphyte Network (AEN). We addressed the following continental-scale questions by utilizing the AEN database comprising 96,448 epiphyte individuals, belonging to 518 vascular taxa, and growing on 10,907 tree individuals (phorophytes). Our objectives here are, first, to present a qualitative evaluation of the geographic distribution of the study sites and highlight regional lacunae as priorities for future quantitative inventories. Second, to present the floristic patterns for Amazonia-wide VEA and third, to combine multivariate analyses and rank abundance curves, controlled by major Amazonian habitat types, to determine how VEA vary geographically and ecologically based on major Amazonian habitat types. Three of the most striking patterns found are that: (1) VEA are spatially structured as floristic similarity decays with geographic distance; (2) a core group of 22 oligarchic taxa account for more than a half of all individuals; and (3) extensive floristic sampling gaps still exist, mainly across the highly threatened southern Amazonian

deforestation belt. This work represents a first step toward unveiling distributional pattern of Amazonian VEA, which is important to guide future questions on ecology and species distribution ranges of VEA once the collaborative database grows allowing a clearer view of patterns.

Keywords: Amazon environments, epiphytes habitat, distribution, Neotropics, oligarchic species, rain forest, species richness

INTRODUCTION

Amazonia, here defined as the tropical moist forest of the Amazon basin and the Guiana Shield, occupies approximately 40% of South America, and is home to the largest expanse of the world's remaining contiguous tropical rain forest harboring an estimated 50,000 seed plant species, of which 14–16,000 are estimated to be trees (ter Steege et al., 2013, 2020). The Amazon basin supports approximately 40% of the world's remaining rainforest, accounts for some 10% of global terrestrial primary productivity, provides 16–18% of the world's freshwater and harbors 10% of the world's plant species, of which over half are considered endemic to the region (Latrubesse et al., 2017; Science Panel for the Amazon, 2021). Such astounding numbers are attributed to the regions' continental size, high levels of primary productivity, spatial variability in edaphic and climatic conditions, and long-term geological stability (Malhado et al., 2013).

Due to a combination of logistical and financial impediments, large expanses of the Amazonian flora and fauna remain woefully understudied (Hopkins, 2007). This is even more evident for some specialized groups with difficult access, such as vascular epiphyte assemblages: plants which depend on the structural support of trees during some stages, if not all, of their life cycle. They contribute substantially to global species richness, accounting for approximately 10% of all vascular plant species (Zotz et al., 2021), and in some tropical forests up to 50% of the local plant species richness (Kelly et al., 2004). This pattern is not different in Amazonia, and the Andean-Amazon interfaces one of the most important speciation centers for this particular group (Kreft et al., 2004). Likewise, lowland Amazonia harbors a relatively rich vascular epiphyte flora (Ribeiro et al., 1999). However, most knowledge about this hyper-diverse functional group in Amazonia originates from single, small-scale inventories (ter Steege and Cornellisen, 1989; Nieder et al., 2000; Benavides et al., 2011; Obermüller et al., 2012; Irume et al., 2013; Boelter et al., 2014; Quaresma and Jardim, 2014; Mari et al., 2016; Quaresma et al., 2017, 2018), which complicates the interpretation of biodiversity patterns at larger geographic scales. To date, no compilation of studies addressing the Amazonian continental scale was carried out.

Because epiphytes by definition are exposed in tree canopies and lack underground root systems, they are thought to be particularly vulnerable to local climate conditions (Nadkarni and Solano, 2002; Zotz and Bader, 2009; Zotz, 2016) and potentially respond more strongly to environmental gradients than most terrestrial plants (Taylor et al., 2021). Epiphytes, in addition, provide important ecosystem services as they play key roles in water retention, nutrient cycling, and provision of habitat shelter and food for several animals (Lowman and Schowalter, 2012; Gotsch et al., 2016; Nakamura et al., 2017). Therefore, we consider it fundamental to characterize baseline biogeographic patterns underlying vascular epiphyte assemblages (hereafter referred to as VEA) for future reference in light of climate change and to provide a first handle toward conservation planning, as changes in their communities can potentially negatively affect the ecological role they play and the ecosystem services provided.

Amazonia is characterized by a South to North-West gradient of increasing moisture, and an East to West gradient in soil fertility (ter Steege et al., 2006). This huge forest territory is far from uniform, containing different forest types like upland forests (*terra firme*), seasonally inundated floodplains (*várzeas* and *igapós*), coastal forest (*restingas*) and savanas (white-sand forests, *campinaranas*), among others (Junk et al., 2011). Earlier studies have indicated that these forest types differ in their structure and species composition (Oliveira-Filho et al., 2021). The Andes-Amazonia interface is one of the most important speciation centers for epiphytes (Kreft et al., 2004), and Western Amazonian forests have been assumed to host a substantially richer epiphyte flora compared to other Amazonian regions (Gentry and Dodson, 1987; Kreft et al., 2004; Küper et al., 2004). This prediction, however, has not yet been adequately tested due to lack of data.

Here, using information of 32 inventories across the region yielding a total of 518 vascular epiphytic species, 96,448 epiphyte individuals on 10,907 phorophytes, we highlight emerging patterns and provide: (1) the geographic distribution of the study sites and highlight regions deemed of high priority for future quantitative inventories; (2) the geographic structure of Vascular Epiphyte Assemblages (VEAs) at the region scale and (3) a quantification of community attributes across major Amazonian habitat types.

MATERIALS AND METHODS

Description of the Epiphyte Inventories in the Amazon Basin

The Amazon Epiphyte Network (AEN) consists of 32 inventories of vascular epiphytes conducted in the Amazon basin (**Figure 1**; dd. February 2022). The network was formed with the aim of specialists and knowledge about epiphytes in the Amazon that allow the integrated analysis of this plant assembly on a basin scale. The work combines results from different independent initiatives in four countries (Brazil, Colombia, Guiana, and

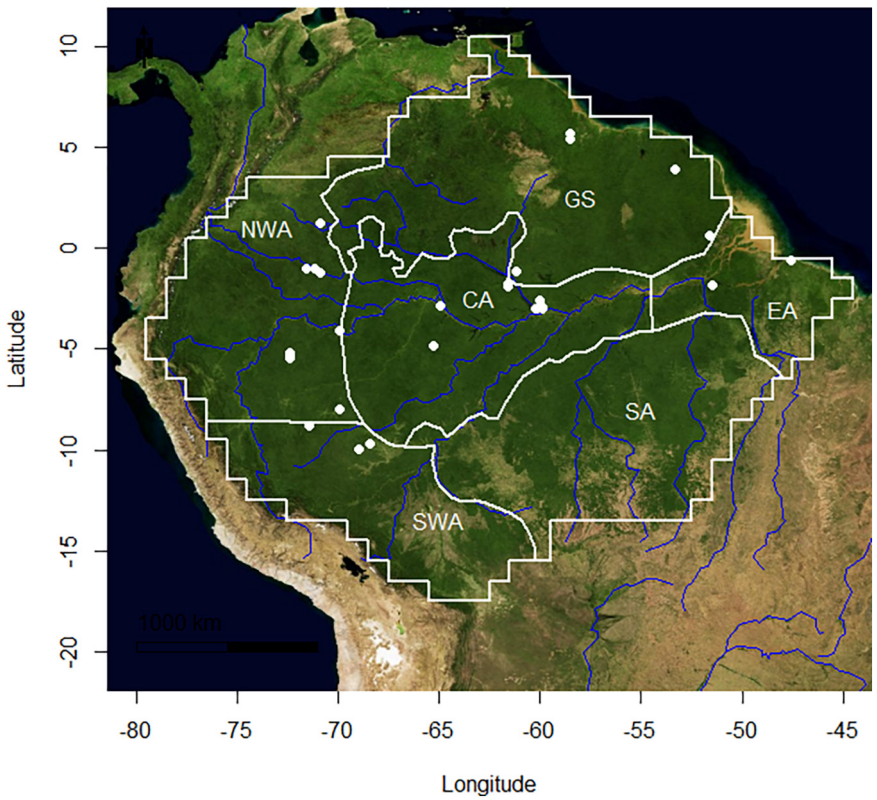

FIGURE 1 | Map showing epiphytes floristic inventories in Amazon biome and its six biogeographic regions. CA, central Amazonia; EA, eastern Amazonia; GS, Guiana Shield; SA, southern Amazonia, NWA, northern part of western Amazonia; SWA, southern part of western Amazonia.

French Guiana). To our knowledge, based on the number of published manuscripts on epiphytes in Amazonia, the AEN represents 94.1% of the total inventories with vascular epiphytes in the Amazon basin. We divided the biome in six biogeographic regions: CA, central Amazonia; EA, eastern Amazonia; GS, Guiana Shield; SA, southern Amazonia, WAN, northern part of western Amazonia; WAS, southern part of western Amazonia (sensu ter Steege et al., 2013). Study sites were plotted geographically to identify those areas well represented but also those still remaining underrepresented and where future endeavors for inventories should be focused (**Figure 1**).

Data Gathering

The 32 studies used several different sampling methods working with epiphytes in Amazonia, particularly relating to the selection of minimum tree sizes and the definition of the sampling unit itself. The sampling method in 78% of the datasets included a combination of crown access and ground observations utilizing binoculars, while 22% were entirely restricted to single-rope climbing techniques or other methods for actual crown access. In 51% of the cases epiphytes were inventoried in trees with DBH (diameter at breast height) $\geq$ 10 cm, and in 24.2% all trees inside the plots were inventoried (but without access to all tree canopies); in another 18% only big trees (DBH $\geq$ 20 and 30 cm) were inventoried, and in 6% sampled trees were $\geq$ 5 cm. The definition of sampling unit varied among studies: it can correspond to the entire phorophyte – i.e., a single list of species per phorophyte, or to the different height zones in one phorophyte – i.e., a number of species lists per phorophyte, according to a pre-determined subdivision of the sampling. Either way, compositional data can be easily lumped and brought down to phorophyte level to ensure comparability among datasets. The details of the methods are presented in **Appendix 1**.

Taxonomic Vetting

Spelling and synonymy of all names gathered in this preliminary epiphyte species list was checked using with the Taxonomic Name Resolution Service,[1] the Plant List,[2] and the Brazilian flora checklist.[3] When inconsistencies were encountered, we chose to use the most current database as our reference. For example, all *Codonanthes*, present in TROPICOS and the Plant List, were changed to *Codonanthopsis* present in

[1]http://tnrs.iplantcollaborative.org/
[2]http://www.theplantlist.org/
[3]http://reflora.jbrj.gov.br

Brazilian Flora List, as recent morphological analysis has revealed that all Amazonian species can be considered *Codonanthopsis* (Chautems and Perret, 2013).

All lianas (no loss of soil connection), parasites (fully dependent on the phorophyte), and tree species (accidental germination in the epiphytic environment) were excluded from the database. Varietal and subspecies status were ignored (i.e., all individuals were assigned to the species level). All epiphyte species, as defined by Benzing (1990), were included, i.e., holoepiphytes (always germinating and growing on other plants), facultative holoepiphytes (growing either as epiphyte or terrestrial); accidental holoepiphytes (terrestrial with occasional presence as an epiphyte); primary hemiepiphytes (germinating on plants with subsequent soil connection); secondary hemiepiphytes (soil germination with subsequent establishment on the phorophyte, and possible loss of the basal portion of the stem/root system). Here we chose to follow Benzing's (1990) classification for a better comparison of our dataset with previous studies, which mostly used Benzing's classification or adaptation of it.

Compatibility Among Datasets to Avoid Taxonomic and Ontogenetic Entanglements

For our analysis, we only used species observations that were validated for their taxonomic identification as described above. All morphospecies were removed from the database so that comparisons between environments and biogeographic regions were possible. To minimize the discrepancy between different sampling efforts we performed a rarefaction analysis for each inventory and reduced the sample units to a minimum number that still represented the species richness present in the data. In order to evaluate differences in epiphytic composition between environments and regions, we used non-metric multidimensional scaling (NMDS) with the Bray-Curtis distance metric to represent compositional differences. Relative abundance distributions were used to investigate the species abundances in each Amazonian environment, defined by ter Steege et al. (2013). We conducted all analyses in R (R Development Core Team, 2011). NMDS was conducted using the vegan package (Oksanen et al., 2016), using the metaMDS function. Rarefaction and extrapolation curves were performed using the iNEX library (Hsieh et al., 2020).

RESULTS

All Amazonian biogeographic regions showed large gaps of completed inventories of vascular epiphytes (**Figure 1**). The northern part of western Amazonia and central Amazonia had the largest number of inventories, but still extensive unsampled areas. The largest sampling gap is shown to be in the southern Amazon region, which does not have any epiphyte inventory. Most of the inventories are located in the Brazilian (60.6%) and Colombian Amazon (27.2%), which together comprise 87.8% of all data. The inventories were established in six different forest-ecosystem types (white-water floodplain *várzea* (VA), black-water floodplain *igapó* (IG); upland *terra firme* of the Guiana Shield (TFGS) and upland *terra firme* of the Pebas Formation (TFPF); white or brown sand forests *campinaranas* on podzols (PZ) and coastal sand forests (CF) (**Appendix 1**). *Terra firme* forests (TFGS) had the greatest species richness. This pattern is consistent even when using rarefaction as explained above (**Figure 2**).

Composition

Our raw data consisted in a total of 833 epiphytic taxa (including taxa identified only at the family, genus or morphospecies level), 70% of these were indentified to species, 26% to genus and 3.2% to the family level, while 1.2% remained unidentified. After filtering and taxonomic cleaning, approximately 9% of the identified species names were considered synonyms and corrected. The final number of 518 valid epiphyte species belonged to 36 families, 154 genera and 60,467 individuals. Most species were registered in Brazilian inventories (204) followed by Colombia (132; **Figure 3A**), and the most species rich environment was TFGS (387), followed by PZ (177), IG (170), and VA (156; **Figure 3B**). The family with the highest species richness was Orchidaceae (168 species, 32.3%), followed by Araceae (105 species, 20.1%), Bromeliaceae (42 species, 8%), Polypodiaceae (27 species, 5.2%), Clusiaceae (21 species, 4%), and Dryopteridaceae (20 species, 4%). Together, these families represented nearly three-quarters of all species in the dataset. But the representation of families changed according to the environment (**Supplementary Material, 3**). The dominant ecological category was holoepiphytes with 342 species (65.9%), followed by primary hemiepiphytes with 128 species

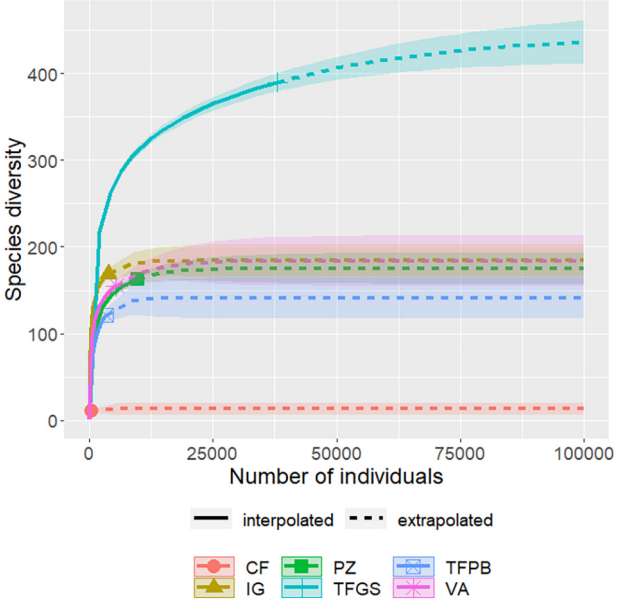

FIGURE 2 | Rarefaction and extrapolation curves for the six environments sampled in the Amazon. Colors represent environments. Dotted lines represent the richness found and shaded lines represent the extrapolation of species richness.

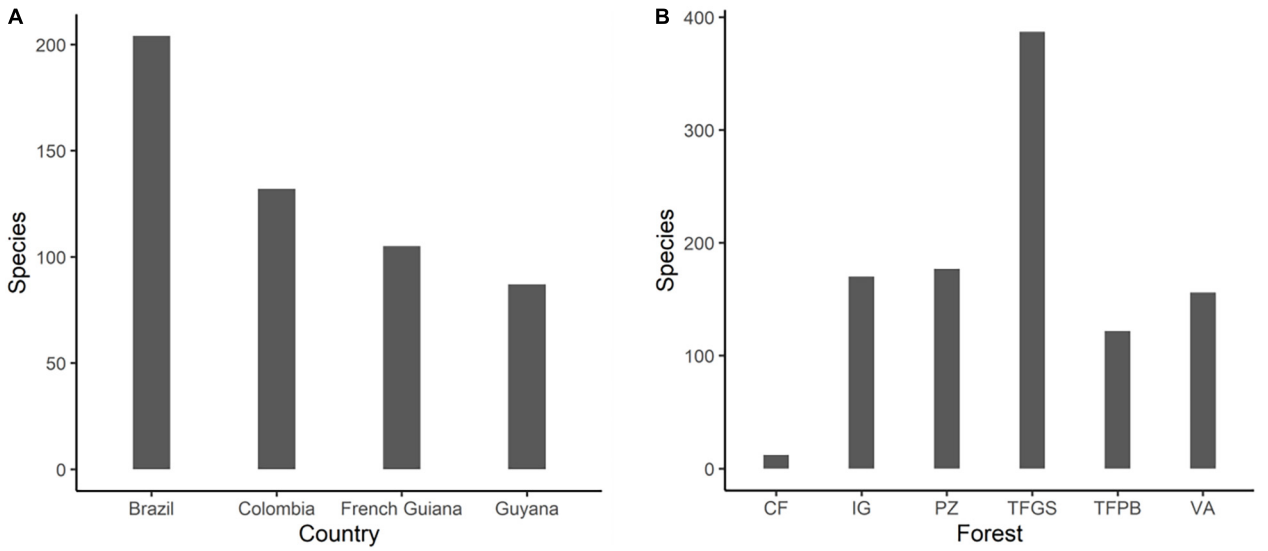

FIGURE 3 | Species richness by country **(A)** and by environment **(B)** in Amazonia. There are clear differences in the number of species by environment and by country, however, this may be a reflection of the sampling effort for each location VA, Várzea; IG, Igapó; TFGS, Terra Firme Guiana Shield and TFPB, Terra Firme from the Pebas region; PZ, White Sand Forests on Podzols and CF, Coastal Sand Forests.

(24.8%) and secondary hemiepiphytes with 48 species (9.1%), (**Supplementary Material, 1**).

Philodendron was the most species-rich genus with 54 species recorded, followed by *Anthurium* (23 spp.), *Clusia* (21 spp.), *Maxillaria* and *Trichomanes* (19 spp., each). The most abundant species were *Maxillaria camaridii* (7,392 individuals), *Prosthechea fragrans* (2,026), *Codonanthopsis crassifolia* (1,879), *Philodendron linnaei* (1,694) and *Ornithidium rigidum* (1,651) with a combined total representing nearly one fourth of all individuals sampled.

Rank Abundance Distribution of Epiphyte Species

The twenty-two most abundant species represented half of all sampled individuals. Likewise, 103 species were either represented as singletons or doubletons (i.e., occurring only once or twice), and 198 species were represented by less than 10 individuals. Consequently, whereas less than three percent of the species represented half of all individuals sampled, half of the species sampled were represented fewer than 10 times in the data (**Figure 4**).

Compositional Variation

Variation in composition of VEAs was shown to be mostly spatially and not environmentally structured with floristic similarity generally decaying with geographic distance (i.e., plots in the same biogeographic region had higher similarity than plots in different regions independent of the habitat type). One of the most visible result was that different habitat types from the same region shared higher floristic similarities than did similar habitat types among regions. For example, seasonally inundated and podzol-based forests from Central Amazonia share a much greater composition of VEA than each of these habitat types share with their counterparts in other regions, as can be seen in the NMDS analysis, which shows a sharp distinction between regions (**Figure 5**).

DISCUSSION

Epiphytes grow detached from the ground (at least at some stage of their life cycle), and are dependent on the structural support of trees for survival (Zotz, 2016). These unique characters make them exceptionally sensitive to climate change, habitat alterations

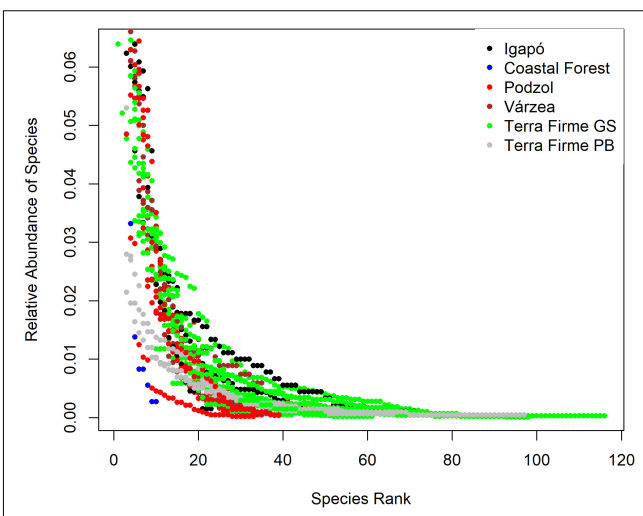

FIGURE 4 | Abundance ranking of vascular epiphyte species for the different environments in the Amazon basin.

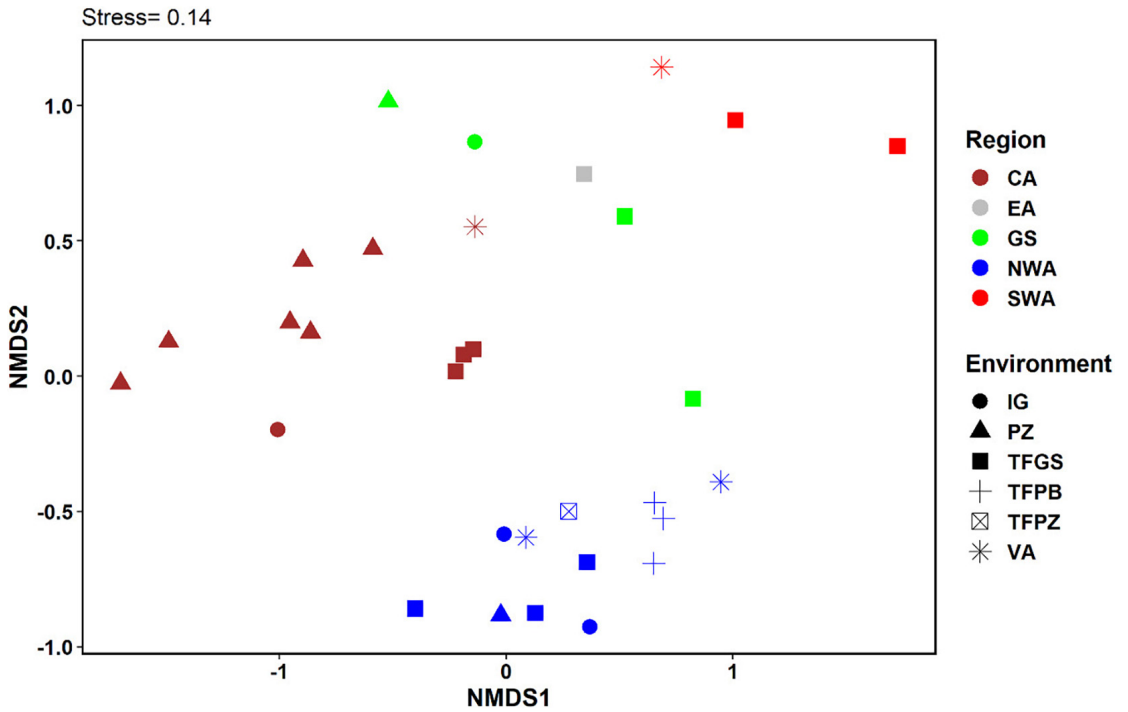

FIGURE 5 | The first two axes of a Non-metric Multidimensional Scaling (NMDS) illustrating VEA compositional structure across the Amazon Basin. Colors represent biogeographical regions and symbols represent environments.

via land-use change and any other related anthropogenic activities (Zotz and Bader, 2009; Zotz, 2016). Despite continued threats to their survival (i.e., Barthlott et al., 2001; Krömer et al., 2014, Leao et al., 2014), our limited knowledge regarding Amazonian epiphyte assemblages has not allowed us to establish fundamental baselines such as estimates for basin-wide species number, general compositional patterns and patterns of rarity and commonality. Here, we explore the general patterns in VEAs across Amazonia. To our knowledge, this is the first study which presents data at the Amazonian scale. The vascular epiphyte distribution reported here is a first step to help us unveiling priority regions for the future sampling of epiphyte inventories.

Our results reinforce the richness pattern frequently found in tree species diversity wherein uplands forest has higher species richness than other environments (Oliveira-Filho et al., 2021). However, our result can be biased in our knowledge between environments because most epiphytes inventories were in *terra firme* forests with other environments being less represented. The greater arboreal richness in *terra firme* is explained mainly by edaphic and hydrological differences, but the reasons for these differences in epiphyte assemblages are still not clear. In contrast, it could have been expected that seasonally flooded ecosystems (*várzeas* and *igapós*) potentially might present higher species richness, due to their proximity to water-bodies and consequent increases in air humidity (i.e., Flores-Palacios and García-Franco, 2006), but this was not found. This might be because long and high flooding (manly in central Amazonia) results in the scarce or even absent colonization of epiphytes on flooded tree stems and twigs (Quaresma et al., 2017). In turn, trees grow taller (vertically) and slower (laterally), but more evenly, throughout the year in *terra firme* forests (Bredin et al., 2020). Slow lateral growth results in more structural matter per unit volume wood, and thus greater stability, supportive of higher stems (Chave et al., 2009; Bredin et al., 2020). Generally, terra firme forests present taller canopies with higher levels of stratification, which is associated to a higher diversity of phorophytes, which in turn contributes to a higher diversity of epiphytes. The greater height of the trees provides a greater amount of substrate (which is also more stable) and vertical niches to be colonized, fundamental characteristics for the occurrence of epiphytes (Ruiz-Cordova et al., 2014; Zotz, 2016).

The number of species recorded was below our expectations, even considering the low number of inventories relative to the extent of the study area, because some local studies in Amazonia registered a high number of species. For reference, nearly half the total number of species tallied for this study (256) was recorded only at the Tiputini Biodiversity Station, Ecuador (Kreft et al., 2004), in an area of 650 ha. The species number calculated from this entire study is also an order of magnitude lower than that recorded for other Neotropical forests phytogeographic domains such as the Atlantic Forest (2,095 species; Ramos et al., 2019). The large number of unidentified taxa or identified to the genus or family level may have contributed substantially to the low species numbers, considering that across all databases, between 10 and 20% of the samples were not identified at the

specific level. On the other hand, considering that the most tropical plant species have an aggregated distribution, another factor for the low number of species, recorded in this study, was probably the large inventory gaps in our sampling, for example, an entire biogeographic region (southeast Amazonia) did not have any registered inventory. Therefore, despite recent advances in the studies of epiphytes in Amazonia, improving our understanding of biogeographic questions, it is urgent that more inventories are carried out; because the lack of information is clearly problematic as it obscures representative characterizations of species diversity and distribution patterns at broad spatial scales (Küper et al., 2004; Engemann et al., 2015; Mendieta-Leiva et al., 2020).

In general, the systematic composition of Amazonian epiphyte communities is highly predictable at the family level with Orchidaceae, Araceae and Bromeliaceae being the most species-rich families (Kreft et al., 2004). This pattern was reported earlier by Taylor et al. (2021), using inventories and distribution information for 27,850 epiphyte species derived from literature sources, where Orchidaceae comprises 67% of the epiphytic flora, Bromeliaceae (11%) and Araceae (4%) being the dominant plant families. Marcusso et al. (2022) it also reported this same pattern for the Neotropics and our results confirm this for Amazonia. Noteworthy, most of Araceae species (60%) recorded in our inventories are excluded in the new classification of vascular epiphytes and are now considered vines by Zotz (2013) and Zotz et al. (2021). Future efforts should focus on in-depth knowledge of species ecology to confirm this new classification, mainly for Amazonia, where in some places aroids are more important than orchids (Leimbeck and Balslev, 2001; Benavides et al., 2005). Another example derived of the personal observation on monitoring permanent plots (Quaresma pers. obs.) in black-water floodplain ecosystems (Igapó), where some secondary hemiepiphytes (i.e., *Philodendron solimoesense* A.C.Sm. and *Philodendron billietiae* Croat) never were observed growing on the forest floor during 6 years of monitoring, likely due to annual floods which in some areas may persevere for up to 300 days per year (Junk et al., 2011).

Results furthermore showed that a core group of 22 oligarchic taxa accounted for more than a half of all epiphyte individuals. In Amazonian tree communities it is well established that a small proportion of species form so called oligarchies and are hyperdominant across huge geographical areas, but these results were shown only for tree species (Pitman et al., 2001; ter Steege et al., 2013). For epiphytes, in contrast, this oligarchy is geographically structured as the species dominance changes with habitat type. For instance, *M. camaridii* has 97.1% of individuals occurring in the TFGS and PZ; and *P. fragrans* has 98.3% of individuals occurring only in PZ, suggesting that the dominance of species may be related to the type of environment (or region).

Hyperdominance, however, can also be explained without invoking the role of the habitat type. Using a density-dependent speciation model and the framework of neutral theory, Janzen et al. (2020) hypothesized that very abundant epiphytic species are either less prone to speciation or have a competitive advantage over rare species. Fitting the models on field data, the authors showed that, in the case of lower speciation rates, species would reach higher number of individuals in the assemblage and become more plastic, which leads to hyperdominance and in turn to lower speciation rates. This is corroborated by epiphyte population structure data that show that more abundant species tend to have a higher number of juveniles (Zotz, 2007) and can help to explain our results for few species with many individuals (hyperdominant) and many species with low number of individuals. Future collection efforts are needed to have a clearer view of this pattern.

Amazonia VEA characterized from inventories of a similar region, even when sampled from different environments, are more floristically similar when compared among regions. A similar pattern was reported by Marcusso et al. (2022) where some distant areas in Amazonia Basin were grouped in the same cluster. Such as pattern would suggest a "spillover-effect" where habitat types in proximity literally "share" species through constant propagule rain. Specifically, higher levels of extinction of epiphytes recruited into suboptimal habitat type are buffered by the elevated rates of nearby dispersal events from their nearby highly abundant sources. In summary, this broad-scale partitioning of VEA floristic similarity which comparatively washes out local compositional distinctions among habitat types could suggest that either region wide historical and/or dispersal related factors influence the distributional factors of the more than half of the epiphyte species which were deemed rare ($<$ 10 individuals).

Interestingly, we found clear evidence for floristic discontinuity and heterogeneity in species composition among biogeographic regions of Amazonia. Janzen et al. (2020) hypothesized (but their results were inconclusive) that VEA in the Amazonian basin are not limited by dispersal, as the region does not present many dispersal barriers (such as the mountainous regions of the Andes and Central America) and have a higher prevalence of wind-dispersed species (e.g., orchids and ferns), which may attain long-tail dispersal kernels. Others also suggested that epiphytes have a wider distribution range than terrestrial plants (Ibisch et al., 1996; Nieder et al., 1999; Kessler, 2000), our results, however, do not support these hypotheses as we observe a clear geographic structure of epiphyte composition.

We hypothesize that the revealed large-scale patterns for vascular epiphytes in the Amazon are similar to those found for trees because epiphyte composition follows the unique phorophyte composition in different biogeographic regions. It is already well known that vascular epiphytes do not occur randomly on their phorophytes and show preferences for specific tree species (ter Steege and Cornellisen, 1989; Burns and Zotz, 2010; Zotz, 2016), also shown in Amazonian igapó forests (Quaresma et al., 2018), supporting this view.

In addition, Amazonia presents a wide variation of local environmental conditions resulting in an arboreal flora of specific hosts for epiphytes, causing compositional differences across the basin. Even in relatively close areas, where soil gradients cause changes in tree composition, marked differences are found in the

composition of vascular epiphytes (Boelter et al., 2014; Quaresma et al., 2017). On the other hand, recent studies with vascular epiphytes on a global scale strongly suggest that epiphytes vary relative to trees in their responses to environmental gradients, with epiphytes responding more quickly to the atmospheric gradient due to their air and water-limited growth habit (Taylor et al., 2021).

We hope that future efforts make these aspects clearer and help us to understand the diversity and compositional patterns for Amazon vascular epiphytes. For that, it is necessary increase of inventories of VEA in biogeographic regions (i.e., Southern Amazonia) and ecosystems (i.e., *buritizais*) improving the species identification. Further it is necessary to improve techniques for more efficient inventories of VEA, for instances, using drones with high-resolution cameras, as conventional sampling techniques climbing trees are time-consuming and limited. Concurrent to this, we need to model species occurrence and phenology of VEA (vs. tree community), verify relationships between local and regional diversity, start studies analyzing functional traits of epiphytes (i.e., hyperdominant vs. rare species) in the background of climate change (increasing temperature) and land-use change affecting tree species communities, verify relationships between VEA and phorophytes on species level (tree age, physiochemical characteristics of bark) and community level (succession, disturbances).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

AQ and CZ had the original idea and under the guidance of HTS started the formation and organization of Amazonia Epiphyte Network. AQ, CZ, and MP wrote the first version of the manuscript. HTS, FW, MJ, and JT contributed with many good comments and analyses. VK, MI, AB, LF, CB, FO, AD, MM, JS, EA, EP, RE, JD, KB, and IB improved the manuscript. FP improved the manuscript in the final revision. All authors contributed data and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ffgc.2022.828759/full#supplementary-material

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APPENDIX

TABLE A1 | Detailed description of sampling methods for Amazon vascular epiphytes in 32 different inventories.

Full name	Habitat/forest type	Country	Abundance method	Observation method	Minimum tree DBH
Algodoal Protection Area	Coastal forest	Brazil	Stands	Binoculars/climbing	≥10 cm
Humaita	Terra firme	Brazil	Individuals	Climbing	≥30 cm
Caqueta River	Terciary forest	Colombia	Stands	Binoculars/climbing	All trees
Amapá National Forest	Terra firme	Brazil	Individuals	Binoculars/climbing	≥5 cm
Caxiuana National Forest	Terra firme	Brazil	Individuals	Climbing	≥10 cm
Caqueta River	Terra firme	Colombia	Stands	Binoculars/climbing	All trees
Chiribiquete National Park	Terra firme	Colombia	Stands	Binoculars/climbing	All trees
Porongaba	Terra firme	Brazil	Individuals	Climbing	≥30 cm
Florestal Reserve Adolpho Ducke	Terra firme	Brazil	Stands	Binoculars/climbing	≥10 cm
Florestal Reserve Adolpho Ducke	Terra firme	Brazil	Stands	Binoculars/climbing	≥10 cm
Florestal Reserve Adolpho Ducke	Terra firme	Brazil	Stands	Binoculars/climbing	≥10 cm
Saul	Terra firme	French Guiana	Individuals	Climbing	≥10cm
Urucu River	Terra firme	Brazil	Stands	Binoculars/climbing	≥10 cm
Urucu River	Terra firme	Brazil	Stands	Binoculars/climbing	≥10 cm
Urucu River	Terra firme	Brazil	Stands	Binoculars/climbing	≥10 cm
Zafire	Terra firme	Colombia	Individuals	Climbing	≥10 cm
Jaú National Park	Igapó	Brazil	Stands	Binoculars/climbing	≥10 cm
Mora	Igapó	Guiana	Individuals	Climbing	≥10 cm
Caqueta River	Igapó	Colombia	Stands	Binoculars/climbing	All trees
Chiribiquete National Park	Igapó	Colombia	Stands	Binoculars/climbing	All trees
Andira	Várzea	Brazil	Individuals	Climbing	≥30 cm
Caqueta River	Várzea	Colombia	Stands	Binoculars/climbing	All trees
Chiribiquete National Park	Várzea	Colombia	Stands	Binoculars/climbing	All trees
Mamirauá Development Reserve	Várzea	Brazil	Stands	Binoculars/climbing	≥10 cm
AgroVila Comunity	Podzois	Brazil	Stands	Binoculars/climbing	≥20 cm
Caqueta River	Podzois	Colombia	Stands	Binoculars/climbing	All trees
Jaú National Park	Podzois	Brazil	Stands	Binoculars/climbing	≥10 cm
Jaú National Park	Podzois	Brazil	Stands	Binoculars/climbing	≥10 cm
Florestal Reserve AdolphoDucke	Podzois	Brazil	Stands	Binoculars/climbing	≥10 cm
Campina Biological Reserve	Podzois	Brazil	Stands	Binoculars/climbing	≥20 cm
Tarumã River	Podzois	Brazil	Stands	Binoculars/climbing	≥20 cm
Wallaba	Podzois	Guiana	Individuals	Climbing	≥10 cm

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Life-stage dependent response of the epiphytic lichen *Lobaria pulmonaria* to climate

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Lichens are poikilohydric organisms, whose internal water content tends to reflect external humidity conditions. After drying, they can reactivate their metabolic activity through water vapor uptake or liquid water input. Thus, lichen water-related functional traits are important as they are involved in the duration of the hydrated period. Models predicting the effect of environmental conditions on lichens are based mainly on the presence or absence of adult thalli. Nevertheless, ecological conditions required by lichens might vary during their life cycle, for example during propagule establishment or in the first stages of thallus development. Little is known about the different ecological requirements at the different development stages in lichens. In this work, we measured water holding capacity (WHC) and specific thallus mass (STM) of adult and juvenile thalli of the model species *Lobaria pulmonaria* along a climatic gradient to constrain the process-based model LiBry. The LiBry model allows accounting for the productivity of lichens with different physiological strategies under various environmental conditions. We simulated the activity and performance of adult and juvenile thalli in 9 regions of Italy and Corsica. The model was used to test if adult thalli of *L. pulmonaria* have a higher survival probability due to their higher aerodynamic resistance. In the current climatic condition, the LiBry model predicts a higher survival probability of adults with decreasing absolute survival rates of both life stages with increasing temperature. Adult thalli also result in having higher active time, STM, and relative growth rate (RGR). We discuss the main implications of our simulation outputs, provide future perspectives and possible implementations of the LiBry model.

KEYWORDS

lichens, LiBry, mechanistic models, growth, *Lobaria*

Introduction

Human-induced atmospheric CO_2 rising has led to temperature increase together with more frequent and intense drought events (Astigarraga et al., 2020). Numerous negative effects have already been reported to potentially affect biodiversity, for instance species distributional shift, phenological changes, and variation in population dynamics (Bellard et al., 2012). However, not all organisms are affected in the same way: different ecological and physiological characteristics determine their sensitivity to warming (Pörtner and Farrell, 2008; Paaijmans et al., 2013). Lichens, for example, being poikilohydric organisms, are not able to regulate their water content, which tends to reflect external conditions (Proctor and Tuba, 2002). Consequently, both water availability and temperature are fundamental in determining the duration of their hydrated periods, influencing their physiology and, as a consequence, their growth (Gauslaa, 2014). In general, lichen ecophysiological performance depends on external environmental conditions that regulate their photosynthetic and respiratory activities (Green and Lange, 1995; Green et al., 2008). This strong dependence on external climatic factors makes them susceptible to climate change (Nash and Olafsen, 1995). This effect is exacerbated in the Mediterranean region, where a reduction of precipitation and warming are expected (Giorgi and Lionello, 2008), thus determining a climate change hotspot (Tuel and Eltahir, 2020).

Lichens cannot actively control water loss but they can recover their metabolic activity at low water potentials, through water vapor uptake or liquid water input *via* dew and fog (Gauslaa, 2014). In this framework, identification of key water-related traits in lichens could help determine their susceptibility to environmental factors mainly related to water uptake and loss (Phinney et al., 2018; Phinney, 2019). Water-related traits are involved in the duration of hydrated periods and mediate the response to different sources of water (Gauslaa and Coxson, 2011; Gauslaa, 2014; Di Nuzzo et al., 2022a). A high intraspecific variability has been observed in such traits, reflecting their fundamental role in acclimation to local conditions of water availability (Merinero et al., 2014; Longinotti et al., 2017; Wan and Ellis, 2020). Increasing specific thallus mass (STM), for example, which strongly drives water holding capacity (WHC), has been observed to increase in dry and high light conditions or in the drier part of the year (Larsson et al., 2012). Intraspecific variability in STM and WHC is also associated with thallus size and age (Merinero et al., 2014), leading to different ecological requirements at different life stages as observed, for example, for *Lobaria pulmonaria* Hoffm (Benesperi et al., 2018; Ignatenko et al., 2020). The model species *L. pulmonaria* is a foliose green-algal lichen (**Figure 1**) with cyanobacteria in small internal cephalodia. It is mainly restricted to humid old-growth forests characterized by ecological continuity, and it is often associated with other rare species. During the last centuries, its presence has declined due to forest management and air pollution (Scheidegger and Werth, 2009; Paoli et al., 2019). This negative trend is expected to be worsened by both direct and indirect effects of climate change. Directly, by a reduction of its climatic suitability in the geographical space (Nascimbene et al., 2016) and indirectly through climatic-induced loss of suitable substrates, such as host trees (Nascimbene et al., 2020). Thus, different environmental filters will shape the adult population, to such an extent that even the fine-scale location of the juvenile thalli may determine their survival and development success (Benesperi et al., 2018). It is, therefore, crucial to consider population structure and development stages when testing the effect of climate change on lichens (Benesperi et al., 2018; Bianchi et al., 2020; Di Nuzzo et al., 2022b).

Predicting species responses to climate change can be a challenging task, yet it is fundamental to provide effective management policies to counteract the negative effects of rising temperature (Bellard et al., 2012). Correlative models use the currently recognize niche of a species, comparing it with future climatic conditions (Pacifici et al., 2015). By contrast, mechanistic simulation models can be valuable complementary tools because they allow projections to be made in time and space by analyzing underlying physiological processes that lead to the observed effects of climate change on organisms (Pacifici et al., 2015). Mechanistic modeling through detailed small-scale information offers the possibility to define niche estimates to reflect the specific niche characteristics of locally adapted individuals (Peterson et al., 2015). Moreover, this approach allows for accounting for both population structure and possible differences in ecological requirements at different life stages. So far, this has mostly been done for vascular vegetation, such as forest stands, for instance (Fisher et al., 2018). As mechanistic models are often more time-consuming in their development and require a large amount of observational data for evaluation, in ecology, they are less used than correlative models. One of the advantages that we want to highlight of a mechanistic model, is the possibility of switching processes on and off, and examining them separately, to gain an understanding of their role and relevance. Such a modeling approach indeed requires making basic assumptions that facilitate the link between environmental conditions, available resources, and ecological interactions (Pontarp et al., 2019).

Regarding lichens, previous studies mainly modeled ecological niches and patterns taking into account adult thalli (Nascimbene et al., 2020; Ellis and Eaton, 2021; Vallese et al., 2021). Thus, less is known on the effects of climatic conditions on different life stages in lichens. Moreover, different ecological requirements at different life stages are fundamental to determining the survival of a population. Some steps of the life cycle are more affected by climatic conditions and are extremely critical for population development, for instance, dispersion and establishment of propagules. In fact, some traits of the organisms could change during development.

FIGURE 1
Life stages of Lobaria pulmonaria as considered in this study: **(A)** adult thallus with downward growing lobes that tend to overlap leaving an air space between the different levels; **(B)** juvenile thalli at the beginning of development. The differentiation between ascending and descending lobes is still in the early stages, but the lobes are not yet overlapping; **(C)** adult thallus in the dehydrated state with convoluted apex of the lobes; **(D)** detail of an adult thallus in the hydrated state in a natural situation after heavy rain. The accumulation of external water favored by the spoon-shaped conformation of the lobes is visible.

For example, the WHC could be different at different life cycle points determining a non-homogeneous probability of occurrence of a thallus along the whole life cycle.

This study aims at testing the hypothesis that adult *Lobaria pulmonaria* thalli better survive under given climatic conditions than juveniles, due to slower water loss rate related to higher aerodynamic resistance. This advantage may overcompensate the benefit of a low STM in juveniles, which means that juveniles may require less carbon assimilation to achieve the same given relative growth rate (RGR).

We tested this hypothesis using the process-based non-vascular vegetation model LiBry (Porada et al., 2013). The LiBry model accounts for the productivity of lichens in many physiological strategies under a broad range of environmental conditions (Porada et al., 2013).

Materials and methods

Study area and sampling design

For the purpose of this work, we have established some operational definitions, which are described below:

- Region: each of the 9 ERA5 grid cells located in different areas of Italy and selected for this study. Each region contains a number of sites ranging from 1 to 14, depending on the local abundance of *L. pulmonaria*.
- Sites: areas within the regions where biological populations of *L. pulmonaria* are present.
- Plot: each of the 30 × 30 m areas located within each site and used to statistically select samples of *L. pulmonaria*.
- Population: the biological population of *L. pulmonaria* present at each of the selected sites.

Sites were selected randomly among all localities known to host *L. pulmonaria* in Italy and Corsica (Nascimbene et al., 2016). The selection was done trying to represent all different ecological conditions (climate, substrata, habitat) in which *L. pulmonaria* is present in Mediterranean environments. We selected 50 sites in 9 regions across Italy and Corsica (**Figure 2**). In accordance with Benesperi et al. (2018), we selected 2 life stages of the thalli: (i) juvenile thalli (with an area $\leq$ 1 cm^2, lacking sexual or vegetative diaspores) and (ii) adult thalli (with sexual or vegetative diaspores). In each site, we randomly selected a 30 × 30 m plot in which at least 3 trees with a minimum of 10 adult thalli of *L. pulmonaria* occurred. In each plot, the number of adult thalli was counted. We considered a thallus an individual if it was completely separated from the closest thallus (> 2 cm). We then randomly collected 5–15 lobes, proportionally to the overall number of adult thalli. To avoid damage to *L. pulmonaria* populations, we collected only the last 5 cm of each lobe. In order to have a variable range of measurements without harming the population, we also collected 4–5 samples of juvenile thalli of *L. pulmonaria* in a subset of populations where it was possible (i.e., where the abundance of juveniles was higher enough so that the collection would not cause a threat to the population).

Water-related traits

The measurement of water-related traits was performed according to Longinotti et al. (2017). The upper surface of collected thalli was sprayed with deionized water reaching full hydration, i.e., continuing spraying would not induce wet mass gain. The water mass (WM) was then measured in three subsequent steps. Thalli were measured when (i) fully water-saturated (WM_{max}), (ii) after being gently shaken until no more drops were falling ($WM_{shaking}$), and after being blotted with dry filter paper ($WM_{blotted}$). The hydrated thalli were then scanned with a Canon i-SENSYS MF4320d (Canon Inc., Tokyo, Japan). Images obtained from scansion were processed digitizing the thallus outline using Photoshop CS6 Extended (Adobe Systems, San Jose, CA, USA) to measure the projected area (A_{wet}). Thalli were dried at environmental conditions and weighted to measure dry mass (DM).

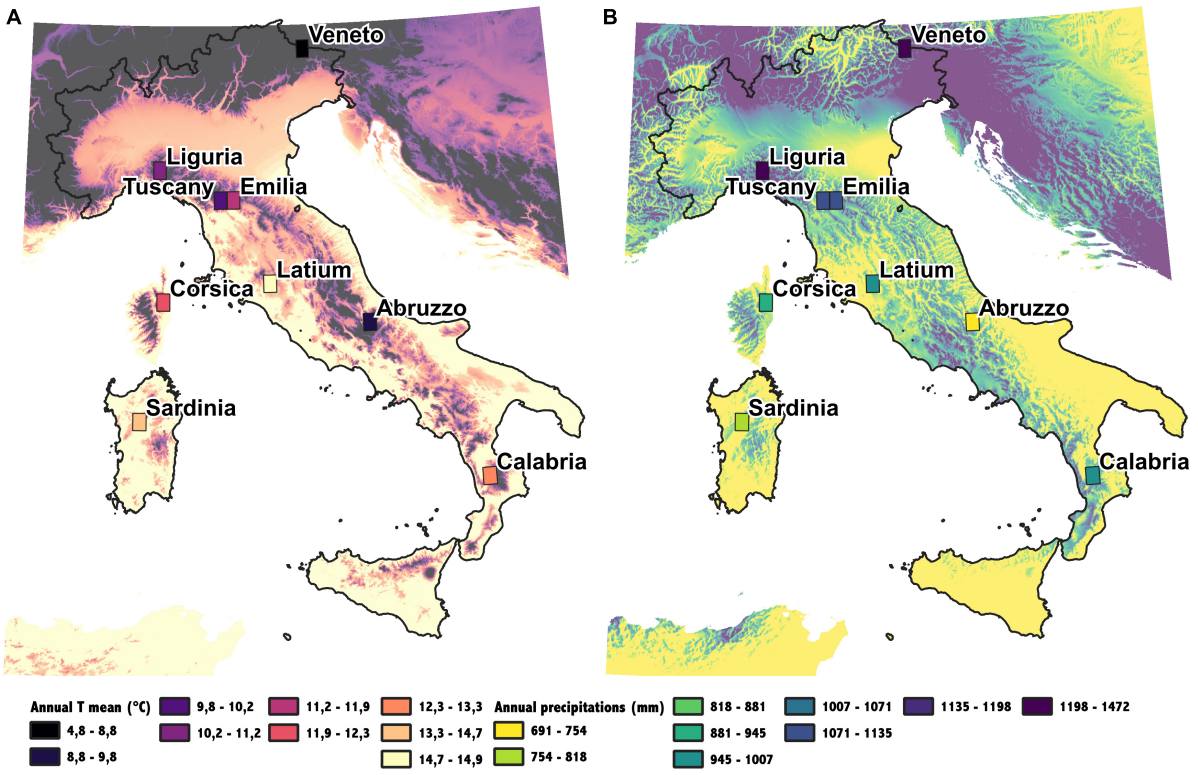

FIGURE 2
Climatic conditions in the studied area. Annual mean temperature **(A)** and Annual precipitation **(B)**. The rectangles indicate ERA5 pixels used for each site, the color inside rectangles is the mean value of temperature and precipitation.

STM was calculated as DM/A_{wet}. In order to calculate WHC_{max}, $WHC_{shaking}$ and $WHC_{blotting}$ we used respectively, WM_{max}, $WM_{shaking}$ and $WM_{blotting}$ as WM in the equation $(WM\text{-}DM)/A_{wet}$. Finally, we calculated the external WHC (WHC_{ext}) as $WHC_{shaking}$ -$WHC_{blotting}$, while WHC internal (WHC_{int}) was assumed to be equal to $WHC_{blotting}$. Moreover, we calculated percent water content (WC) as (WM-DM)/DM both after shaking and blotting.

Thallus thickness was estimated by 3 measures on a thin section of the thallus under a standard light microscope.

For a subset of lobes, we measured the $WM_{blotting}$ loss halftime (T50). All lobes were hydrated for 24 h in distilled water. Lobes were then left dehydrated at air temperature and humidity and weighted with a time interval of 5 min on an analytical scale until the desiccation at room temperature was reached. The evaporation process could be described as,

$$\frac{dRWC_{\%}}{dt} = -k_{wl}t_{1/2}$$

Where kwl is water loss rate $t_{1/2}$ and water loss halftime. Samples k_{wl} and $t_{1/2}$ were calculated following the exponential function which better approximates the experimental points. The dehydration curve was constructed interpolating 10 subsequent weights.

The model: LiBry

To test our hypothesis, we apply here a mechanistic computer simulation model of lichens, bryophytes, terrestrial algae, and cyanobacteria, called LiBry. This model was developed as a dynamic global vegetation model (DGVM) which, in contrast to most other DGVMs, focuses on non-vascular vegetation, while trees, shrubs, and grasses are only included as a relatively static environment which influences the growth of non-vascular organisms (Porada et al., 2013). The original purpose of the LiBry model was to assess the impact of non-vascular vegetation on global biogeochemical cycles, such as cycles of carbon, water, and nitrogen, and it has been applied to various research questions in this regard (Porada et al., 2013, 2014, 2018, 2019).

The model, however, is not limited to biogeochemical processes alone, it also accounts for factors that shape the physiological diversity of non-vascular communities. Hence, impacts of environmental factors, such as climate, on community composition or on individual species can be assessed using LiBry (e.g., Porada et al., 2019; Baldauf et al., 2021). This is possible through the explicit representation of the large physiological and morphological diversity of non-vascular communities in the model. To this end, LiBry simulates a large

number (thousands) of different strategies at the same time in a given location. Each of these strategies is characterized by a unique combination of values of 11 physiological and morphological traits. This means that real non-vascular species can be described by the model to the level of the phenotype. Thereby, one strategy may match individuals from more than one species in the real world, since these may be highly similar in their trait values. Moreover, individuals from the same species may correspond to different strategies in the model, in case of high intra-specific variation in trait values. The traits which are considered in LiBry include STM, WHC, thallus thickness, optimum temperature of photosynthesis, or photosynthetic capacity.

At the beginning of a LiBry simulation, the ranges of possible trait values, which are based on literature, are randomly sampled to create a set of initial physiological strategies (see Porada et al., 2013 for details). Subsequently, the long-term carbon balance of each strategy under given climatic conditions is computed by the model. Differences in trait values usually result in a divergence of the carbon balance values of the simulated non-vascular community at a given location. Consequently, the carbon balance is used as a selection criterion for the success of each strategy: Those strategies which show a negative carbon balance in the long term are removed from the simulation, and the remaining ones are weighted according to a scheme, which is based on growth and other properties, and which determines their relative abundance and, thus, the community composition at the respective location. It should be noted that WHC values reported here (WHC_{LiBry}) do not correspond to the WHC of a single individual, but represent the community mean WHC weighted by relative abundances of the simulated strategies. Hence, to compare WHC_{LiBry} to observations, the measured samples should be representative of the entire community in a given location.

The biomass of each strategy in the LiBry model corresponds to their accumulated long-term carbon balance. Biomass dynamics depend on Net Primary Production (NPP) and mortality, where the latter is based on tissue turnover and disturbance. Moreover, biomass translates into the relative cover fraction of a strategy in the community, depending on its STM. Thereby, the RGR of the cover of a strategy corresponds to the difference between NPP and tissue turnover. In a steady state of the cover, the RGR is balanced by disturbance. It should be pointed out that RGR is a spatially averaged property that integrates processes such as dispersal and establishment. It cannot be compared to the growth rate of an individual thallus, it is rather the average expansion of a population of thalli which share the same trait values. NPP is computed as the difference between photosynthesis and respiration. The former is estimated as a function of light, CO_2, and temperature according to Farquhar and von Caemmerer (1982), and the latter is derived from a Q10-relation to temperature. While these basic processes are similar in vascular and non-vascular organisms, the LiBry model also explicitly accounts for physiological properties which only occur in non-vascular vegetation. These include poikilohydry, which means a lack of active control on water loss, the exchange of water *via* the thallus surface, due to the absence of roots and stomata, the ability to deactivate metabolism upon desiccation, and the reduction of CO_2 diffusion at high water saturation of the thallus. Thereby, simulated metabolic activity of the strategies in LiBry is based on the (dark) respiration rate which increases with water saturation. Usually, full activity is reached before the thallus is saturated with water.

Furthermore, several physiological processes in LiBry are connected *via* trade-offs, which are characteristic for non-vascular organisms and which have a large influence on carbon balance, and, consequently, success in the simulated selection. These trade-offs include a positive correlation between photosynthetic capacity and specific respiration rate, or the negative correlation between metabolic activity and CO_2 diffusivity, mediated by water content (see Porada et al., 2013; and Porada and Giordani, 2021 for a more detailed description).

Adaptation of LiBry to *Lobaria pulmonaria*

To represent both adult and juvenile thalli of *L. pulmonaria* in the LiBry model, the representation of (a) water pools in the lichen thallus and (b) the morphology of the thallus was extended for this study, compared to the latest published LiBry version (Porada and Giordani, 2021). Furthermore, the initial strategies in LiBry were constrained by observational data.

In LiBry, water in any part of the thallus can be stored in two pools: (1) inside the cells (internal), which expand upon water uptake, or (2) between the cells (external), either in the pore space or attached to the surface of the thallus (see **Figure 3**). The remainder of the thallus part is filled with air. Under natural conditions, a certain fraction of the thallus (free pore space) always remains air-filled due to either hydrophobicity or pore size effects. In lichens the free pore space usually corresponds to parts of the medulla. Since LiBry simulates a large variety of different physiological strategies, the relative fractions of dry matter of the cells, storage capacities for internal and external water, and free pore space vary largely between the strategies with which the model is initialized. Thereby, however, a fixed relation between the amount of cell dry matter and the amount of internal water is assumed, which means that the cells are limited in their capacity to expand. This is supported by various studies on the relation of STM to WHC_{int} (e.g., Gauslaa and Arsenault, 2020) (see **Figure 4**). Furthermore, in the LiBry version that is applied here, the transition point to full metabolic activity corresponds to the saturation of the internal water pool.

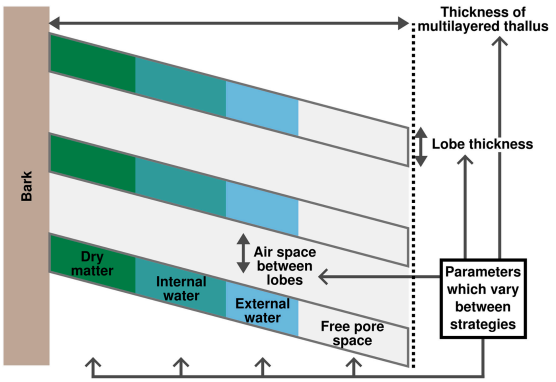

FIGURE 3
Overview on the morphological representation of L. pulmonaria: in the LiBry model. The individual morphological properties (fractions of dry matter, internal/external water, air in the free pore space inside lobes and air between lobes, lobe thickness, and total thallus height) vary between the strategies in the model. The ratio of dry matter to internal water, however, is constant across strategies.

Once all cells are fully turgid, activity is assumed to remain constant.

To account for the characteristic lobe structure of *L. pulmonaria*, an additional scaling relation was introduced in LiBry, which prescribes a minimum of air space between lobes, and which leads to relatively more air space in multilayered thalli with a higher thickness. Furthermore, thicker thalli are slightly more efficient in storing water in this model version due to the more complex surface structure (**Figure 3**).

Several further constraints were applied to LiBry in order to represent *L. pulmonaria*: First, the value of the fixed ratio between cell dry matter and internal water was set similar to that found for *L. pulmonaria* by Gauslaa et al. (2021). It was, however, adapted subsequently to observations from the sites used in this study (see **Figure 2** and **Table 1**). Thereby, adult thalli have a higher fraction of WHC_{int} compared to juvenile thalli for the same STM. This is due to the prescribed lower efficiency of water storage per STM for smaller thalli in the model, which was motivated by the observed lower ratio of internal to WHC_{ext} in juvenile thalli (**Table 1**). In this simplified scheme, it is possible that juvenile thalli have STM values similar to adult thalli in the LiBry model, since a small strategy (low overall thickness) may have a high fraction of dry matter, and a tall strategy a substantially lower dry matter fraction. While it is likely that juvenile thalli have a lower STM on average than adult ones, the low number of measurements of STM of juveniles does not allow for a definite distinction between juveniles and adults in this regard. To test the implications of this simplification, we run a sensitivity analysis (see below).

Secondly, the thickness of the lobes (**Figure 3**) was constrained to the range of 200–1,000 μm, based on observations from the sites (**Table 1**), and the maximum thickness of the total multilayered thallus, meaning the distance between the bark surface and the outer tips of the lobes, was set to 10 cm, which means that all strategies differed in their thickness, but could not exceed 10 cm. Finally, initial strategies which had an STM outside the range of 4–40 mg/cm^2 were excluded from the model, and those strategies which had a ratio of external/internal WHC outside the range of 0.21–2.7 were excluded, too. These ranges were based on Median values of observations from the sites, using half the minimum and twice the maximum value, except for max. STM, which would have increased the average STM of the initial strategies too strongly.

According to our hypothesis (see above), the taller structure of adult thalli may lead to an increased resistance against evaporation of water compared to juvenile thalli. This was tested and confirmed in the laboratory using a subset of sampled *L. pulmonaria* thalli. Subsequently, we used the evaporation scheme built into the LiBry model to reproduce the observed drying dynamics. For the adult and juvenile thalli, we set WHC to 14 and 7.6 mg/cm^2, respectively, and the observed T50 time was 24 min for adults and 12 min for juvenile thalli (median values). Environmental conditions for the evaporation model were set to those of the laboratory (20°C and 60% RH, and the surface resistance of the thalli was used as the calibration parameter to match the observed difference in T50 time.

It was found that the difference in WHC alone could not explain the longer T50 time of adult thalli. Instead, it was required to set the resistance of the juvenile thalli to 0.3 times that of the adult thalli, all other conditions being equal, to match the observations. Therefore, we then used the thallus height Z of a strategy in the LiBry model to define its surface resistance, prescribing an exponential scaling from the minimum height z_{MIN} of 5 mm to the maximum z_{MAX} of 10 cm:

$$r_s = \frac{log\left(\frac{Z}{Z_{MIN}}\right)}{log\left(\frac{Z_{MAX}}{Z_{MIN}}\right)} r$$

with r_{S_MAX} = 200 s/m. This simple relation resulted in median values of the juvenile initial strategies which were roughly 0.3 times that of the adult initial strategies.

Simulation setup

To differentiate between adult and juvenile individuals of *L. pulmonaria* strategies in the LiBry model, we used only the thickness of the multilayered thallus. Individuals which were smaller than 1 cm were assigned to the juvenile group, and the remainder to the adult group. **Figure 5** shows the distributions of STM, WHC_{int}, and the $WHC_{ext/int}$ ratio for the initial strategies in the LiBry model, and also for the observational data from **Table 1**.

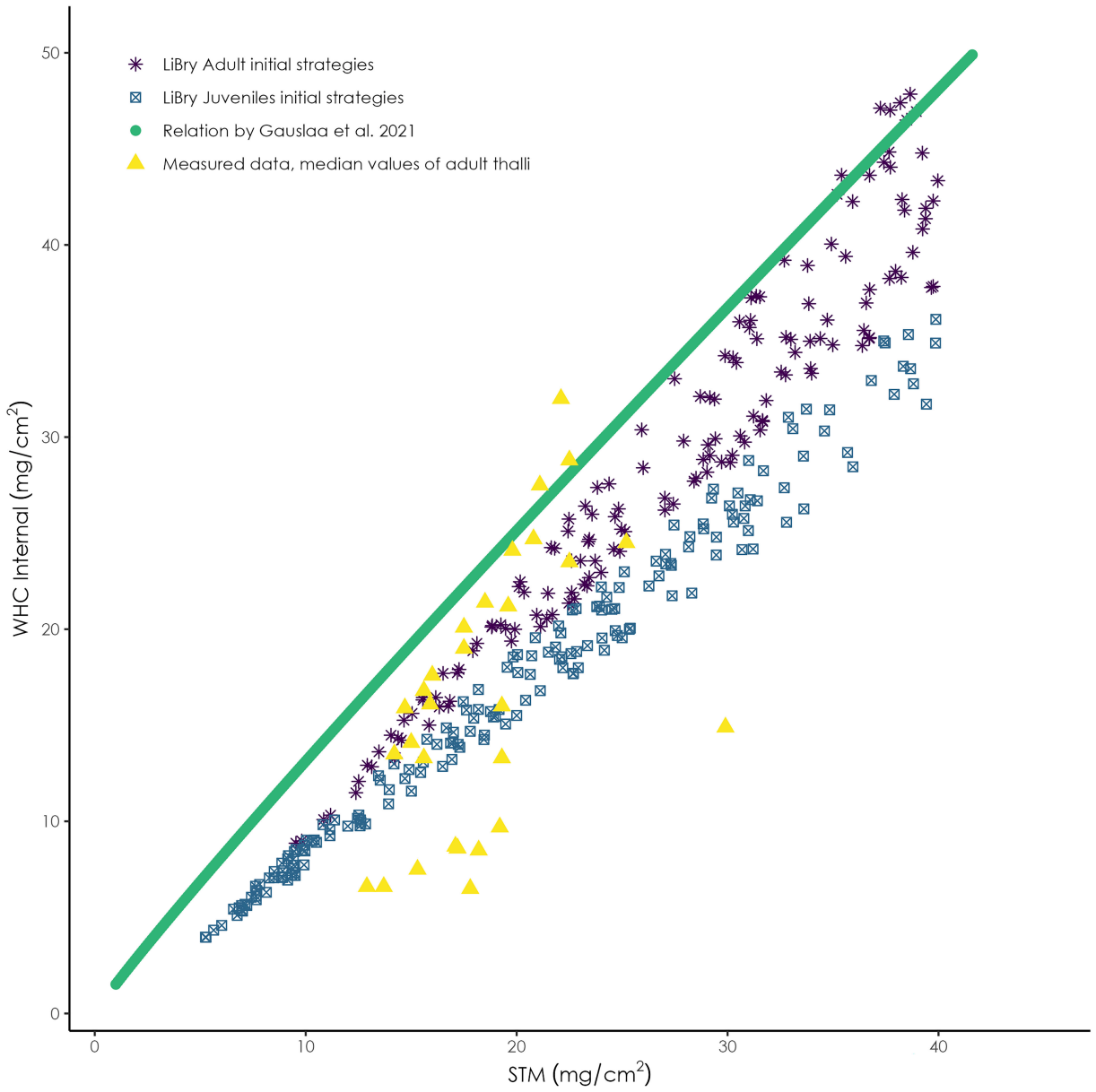

FIGURE 4
Relation between STM and internal WHC for the initial adult and juvenile strategies in the LiBry model and of measured adult thalli. The scatter in the strategies results from the free variation of several morphological parameters in the model, such as thallus height and the relative fractions of water and air in different parts of the thallus.

Two simulations were run with the LiBry model, which only differed with regard to the group of initial strategies (juvenile vs. adult). The simulations were carried out for 9 regions across Italy (**Figure 2**) and the model was driven by climate data (years 1979–2019) from the ERA5 data set (Hersbach et al., 2020). Those grid cells were cut out from the global data set which corresponds roughly to the center of the individual sites in the 9 respective regions.

Leaf area index was derived from site observations, by imposing a seasonal variation (monthly resolution) on median LAI values for each region. The extent of the variation was based on a previous study carried out in Sardinia (Porada and Giordani, 2021). Stem area index was set to a constant value of 0.1, also based on the study by Porada and Giordani (2021). Note that the SAI in the LiBry model is used to obtain correct estimates for ecosystem level extensive properties, such as biomass or productivity, since per-area growth needs

TABLE 1 Median of measured values for each region.

Region	STM adult (mg/cm^2)	WHC_{int} adult (mg/cm^2)	Ratio WCext/int adult	Ratio WCext/int juvenile	Thallus thickness adult (μ m)	Thallus thickness juvenile (μ m)	Annual precipitation (mm)	Annual mean temperature (°C)
Abruzzo [1]	20.8	24.7	0.55	–	500	–	691	9.3
Calabria [14]	18.2	16.0	0.69	–	490	–	983	12.9
Corsica [2]	18.4	20.4	0.64	–	536	–	925	12.0
Emilia Romagna [2]	22.3	30.4	0.62	–	650	–	1,134	11.9
Liguria [10]	14.8	11.1	0.64	0.81	602	325	1,295	10.3
Sardegna [14]	13.8	14.9	0.67	0.79	455	340	770	14.7
Latium [2]	18.0	24.9	0.40	–	–	–	967	14.9
Toscana [3]	18.5	21.4	0.45	–	582	–	1,111	9.9
Veneto [2]	14.7	14.5	0.84	–	507	–	1,472	4.8

In brackets the number of sample populations in each region.

to be scaled up spatially. Since we focus in this study on differences in the energy and water balance between juvenile and adult individuals of *L. pulmonaria*, the exact area which is available for growth on the stems is not of crucial relevance here.

The simulations were run using 3,000 initial strategies, out of which 632 remained after constraining their properties to characteristics of *L. pulmonaria*. The juvenile and adult groups consisted of 359 and 273 strategies, respectively. The simulation was run for 300 years to ensure equilibrium with regard to community composition, thereby repeating the 41-year climate data. At the end of each simulation, the number of surviving strategies and their community-averaged properties were analyzed. Thereby, all strategies were assigned equal weights in the averaging. Finally, a sensitivity analysis was carried out to test the effects of different uncertain morphological and physiological properties and environmental conditions on the outcome of our study. This run is carried out for one site only with the same setup as used in a preceding publication (Porada and Giordani, 2021), but applying the new model version.

We want to point out here that LiBry is a deterministic model, which means that each of the strategies is simulated as an individual, consisting of dynamic pools of carbon and water, which are changing based on the state of the individual and the driving environmental conditions. Hence, for the same set of initial strategies and climate data, the model will always calculate the same output values. When comparing differences between the simulations for adult and juvenile strategies, it is thus not appropriate to test if these differences are significant, since the model estimates cannot be interpreted as draws from a statistical population. It is rather the generation of the initial strategies itself which corresponds to statistical sampling. Here, it is important to test if the sample size of strategies is large enough to ensure consistent results. This is done in the last part of our sensitivity analysis.

Results

Simulation output

The results of our simulations with the LiBry model confirmed our overall hypothesis. Adult strategies of *L. pulmonaria* had a 32% higher survival rate than juveniles (comparison of the median values of all regions, see also Figure 6). Adult strategies show a higher fraction of active time compared to juveniles (Act.Frac.). They also have a higher (WHC_LiBry), and a higher RGR, see also Table 2.

When the 9 regions are sorted along climatic gradients, certain patterns in the ratios of survival rates and physiological functioning of adult vs. juvenile strategies can be noted (Figure 7 top panels). Increasing total amount of rainfall (range: 691–1,472 mm/a) does not seem to result in a clear pattern in the ratios of survival rate or the physiological functions (WHC, Act.Frac., RGR), and also absolute survival rates of adults and juveniles show no clear trend (Figure 7 middle panels). Instead, survival rates seem to be lowest at intermediate amounts of rainfall. Increasing mean air temperature (range: 4.8–14.9°C), however, seems to lead to an increase in the relative survival rate of the adult strategies compared to juvenile ones, and to a decrease in the difference in WHC. RGR seems to be connected to survival rate, while active time fraction does not show a clear pattern or a strong correlation to other ratios. For high air temperature, absolute survival rates show a decrease for both adult and juvenile thalli. Moreover, it should be pointed out that the ratio of adult to juvenile survival is always larger than one, which means that the simulated advantage of adult thalli is consistent throughout the study region.

When considering adult and juvenile thalli separately, and comparing the shift in their mean properties (due to the selection in the LiBry model), it becomes clear that the ratio of external to internal WHC in juveniles exhibits the strongest shift from initial to final mean properties (Figure 7 bottom

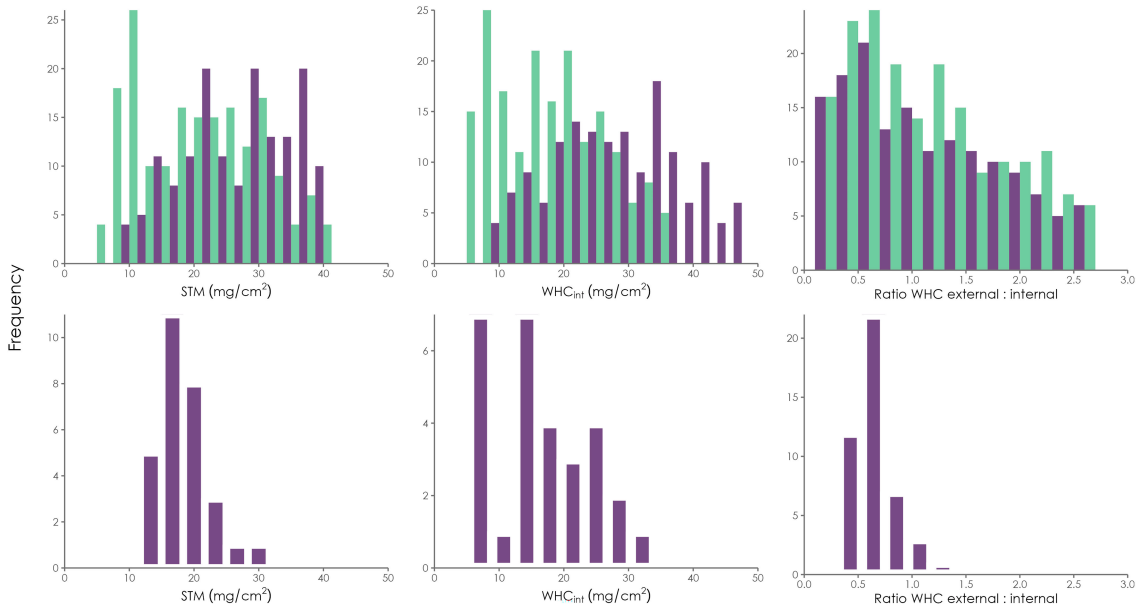

FIGURE 5
The initial LiBry strategies are separated into juvenile thalli (light green) and adult ones (dark purple). Frequency distributions of STM, internal WHC, and the ratio of external to internal WHC, both of the initial strategies in the LiBry model (Upper row), and also of the observed adult thalli (Lower row) of which only median values are shown.

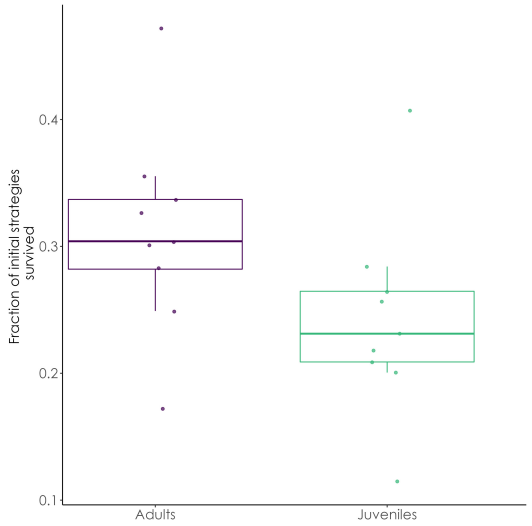

FIGURE 6
Boxplots of the fraction of initial strategies which survived the simulation, adult (Left) vs. juvenile thalli (Right). The boxplots contain 9 values each, corresponding to the different regions of Italy analyzed here. Colored bars represent the density of values.

panels). This means that juvenile strategies are selected for higher external: internal WHC ratio during the simulation, but not adult thalli. Another consistent pattern is the selection for slightly higher resistance against evaporation in adult thalli, but not in juveniles. Other properties, such as STM and WHC_{LiBry}, show a slight decrease in the mean values, but no clear pattern along the climatic gradients. Another feature seems to be the increase in WHC_{LiBry} for juveniles in regions with the lowest survival rates.

TABLE 2 Median values of the 9 simulated regions.

	Adult	Juvenile
%S	30.4	23.1
WHC_{LiBry} (mg/cm^2)	56.564	35.470
Act.Frac.	0.68	0.64
RGR [y*(-1)]	0.33	0.23

%S corresponds to the percentage of surviving strategies at the end of the simulation, Act.Frac., corresponds to the fraction of hours of the simulation in which the strategies showed metabolic activity. RGR corresponds to the relative growth rate per year at the end of the simulation.

Sensitivity analysis

The sensitivity analysis confirmed our assumptions about the direction of the impacts of physiological and environmental properties on the survival of strategies in the LiBry model:

(1) When the physiological disadvantage of juvenile strategies due to the reduced resistance against evaporation is switched off in the model, and the maximum resistance value r_{S_MAX} is used for every strategy, the survival rates

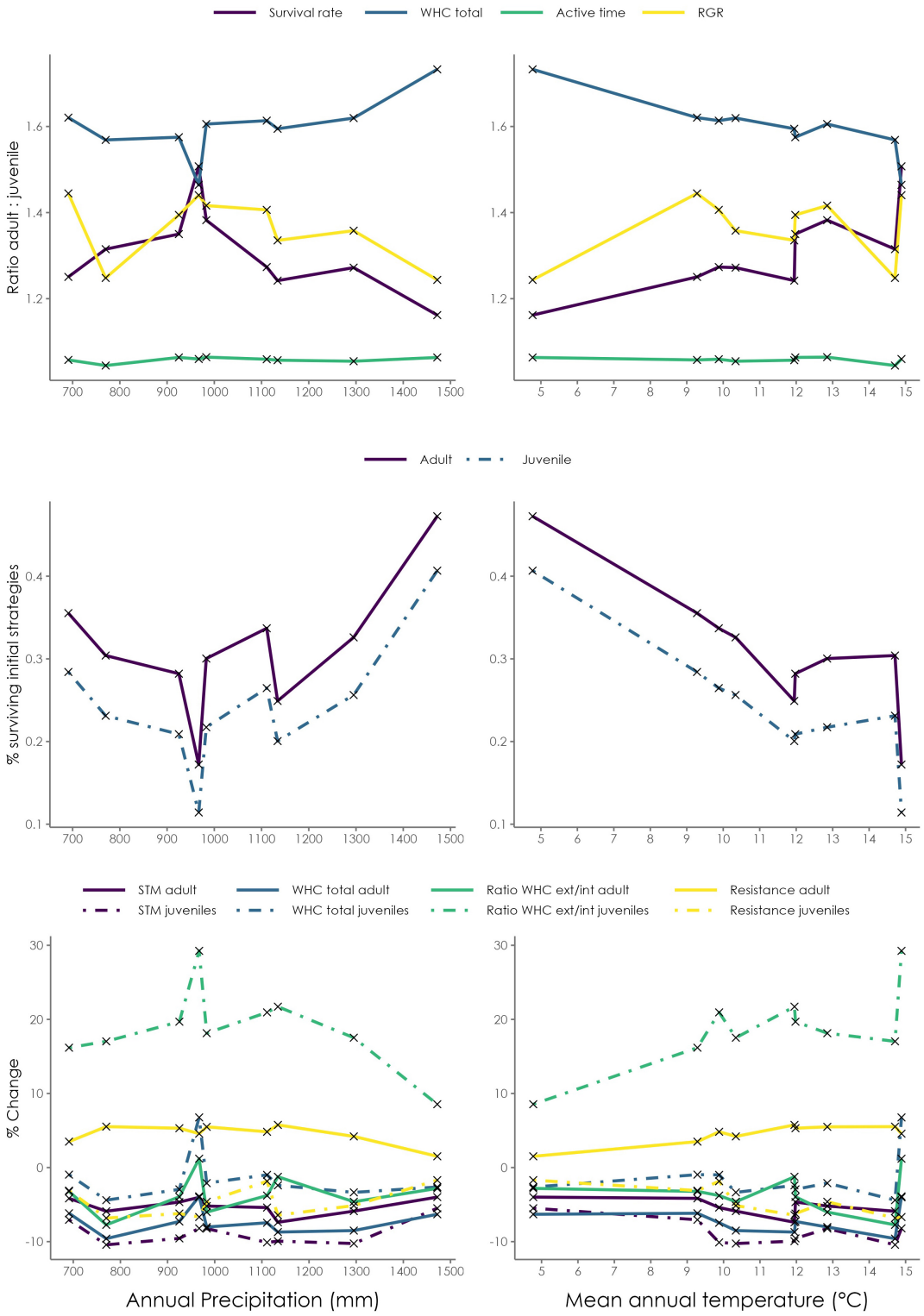

FIGURE 7

(Top) Simulated ratios of adult to juvenile survival rate (purple), Water holding capacity—WHC_{LiBry} (blue), Active fraction—Act.Frac. (yellow) and Relative Growth Rate—RGR (yellow) for the 9 regions in Italy examined here. The climate variable increases from left to right, total rainfall **(Left)**, and average air temperature **(Right)**. **(Middle)** survival rates of adult (purple) and juvenile (blue) thalli. **(Bottom)** Shift in the community mean values of four hydration traits from the initial strategies to those surviving at the end of the simulation; STM (purple), WHC_{LiBry} (blue), the ratio of external to internal WHC (green), and resistance against evaporation (yellow) are shown separately for adult thalli (solid) and juvenile ones (dashed).

TABLE 3 Summary of sensitivity analysis.

		Adult	Juvenile
Control			
	%S	20.9	15.6
	WHC_{LiBry} (mg/cm^2)	39.7	25.0
	Act.Frac.	0.83	0.7
	RGR[y˙(−1)]	0.23	0.18
(1) No difference in surface resistance			
	%S	24.2	21.5
	WHC_{LiBry} (mg/cm^2)	37.6	24.4
	Act.Frac.	0.87	0.84
	RGR[y˙(−1)]	l0.27	0.26
(2) With resaturation respiration			
	%S	17.6	11.4
	WHC_{LiBry} (mg/cm^2)	39.7	27.9
	Act.Frac.	0.83	0.78
	RGR[y˙(−1)]	0.23	0.15
(3) With bark water supply?			
	%S	24.5	23.1
	WHC_{LiBry} (mg/cm^2)	36.9	23.0
	Act.Frac.	0.9	0.85
	RGR[y˙(−1)]	0.34	0.42
(4) With constrained dry matter fraction			
	%S	21.5	18.8
	WHC_{LiBry} (mg/cm^2)	39.7	16.7
	Act.Frac.	0.83	0.78
	RGR[y˙(−1)]	0.24	0.18

TABLE 4 Percentage of surviving strategies for 5 different drawings of 3,000 initial strategies from the pool of potential strategies, and the ratio of adult: juvenile survival.

	Control	#2	#3	#4	#5
Adult	20.9	23.7	26.2	23.1	24.8
Juvenile	15.6	13.5	14.8	15.4	14.3
Ratio A: B	1.34	1.76	1.77	1.5	1.73

increase overall and the adult strategies have only a 13% higher survival rate than the juvenile ones. This suggests that more than half of the advantage in the survival of adult vs. juvenile thalli depends on their higher resistance against water loss, and the remainder may be due to their higher WHC per area.

(2) It has been observed that lichens show a burst of respiration upon rewetting from the dry state, with magnitude varying between individuals and species (Smith and Molesworth, 1973). In the standard version of LiBry, this resaturation respiration is not included, due to a lack of data on potential trade-offs which may explain the variation in the magnitude of respiration. Hence, we here test the effect of a simple parameterization of resaturation respiration, where a 50% increase in respiration is assumed for 2 h after each rewetting event. As expected, overall survival rates declined compared to the control run. Moreover, the advantage of the adult strategies increases and they show 54% more survival than the juvenile ones.

(3) It has been shown that the bark may act as an important water reservoir for epiphytic lichens (Porada and Giordani, 2021), and utilizing a bark water reservoir in the LiBry model increases survival rates, as expected. The conditions improve to an extent that almost removes the difference between adult and juvenile strategies (6%).

(4) We additionally constrained the dry matter fraction in the model to 5% or less. In this way, juvenile thalli cannot compensate their lower stature (less than 1 cm in the model) by a high fraction of dry matter in the thallus, and thus automatically have a lower STM on average. We thereby account for a potential systematic lower STM of juveniles thalli which is not captured by our simplified model. The modified set of initial strategies does not change substantially the outcome of our simulation (see **Tables 3**, **4**). The WHC of the juveniles is lower, which can be explained by the now prescribed low amount of dry matter that stores water for each strategy. Also, the survival rate of the juveniles is slightly higher now, since those strategies with high STM values were already removed in the initialization of the model, due to the new constraint. In the control version, the average STM of juveniles declined only during the simulation due to selection, leading to fewer survivors compared to the number of initial strategies.

(5) The advantage of adult thalli compared to juvenile ones regarding survival rate is consistent across 5 drawings of 3,000 initial random strategies in the LiBry model, which suggests that this effect is not a random result due to insufficient sample size.

Discussion

The results of our simulations depict for the first time a different survival probability of life stages of *L. pulmonaria*. The LiBry model highlights a consistently higher probability of adult thalli to survive and more active time. Our simulations set the basis for a better understanding of how climatic gradients affect different age stages of *L. pulmonaria*, revealing complex population dynamics. *L. pulmonaria* is known to be influenced by both spatial (Eaton and Ellis, 2012) and seasonal climatic gradients (Larsson et al., 2012). More oceanic climates seem to enhance biomass gain and reduce the age-at-reproduction (Eaton and Ellis, 2014 and references therein). Nevertheless, also microclimatic and microhabitat conditions, nested inside macroclimatic gradients, influence the growth rate and the overall survival of *L. pulmonaria* populations (Benesperi et al., 2018; Ellis, 2020; Di Nuzzo et al., 2022b).

For example, the same distance from watercourses in two different areas could lead to different growth rates (Ellis, 2020).

A consistent outcome of the simulation of *L. pulmonaria* along climatic gradients is that the advantage of adult thalli compared to juvenile ones, regarding survival, increases with decreasing absolute survival rates of both life stages, which can be interpreted as stressful conditions. It is interesting that adult and juvenile thalli seem to become more similar with respect to WHC_{LiBry} under these conditions (**Figure 7** top panels). A more detailed analysis shows that the relative increase in WHC_{LiBry} in juvenile thalli compared to adult ones seems to be accompanied by a selection of juvenile strategies toward higher ratios of external to internal WHC (**Figure 7** bottom panels). This increase in external WHC can be interpreted as a means to maintain active time and, thus, growth under stressful conditions, at lower carbon cost facilitated by a lower fraction of dry matter (which is related to internal WHC). Alternatively, the reduced fraction of internal WHC compared to external one may be driven by the advantage of earlier activation, since only internal WHC is relevant for the level of metabolic activity in LiBry. Usually, the increased external WHC would also have a negative effect on growth *via* the associated decrease in CO_2 diffusivity (Lange and Green, 1996; Lange et al., 1999). However, it could be possible that this effect is not relevant in warm and dry climatic conditions which seldom lead to full saturation, or where evaporation rapidly decreases the external water. In contrast to juvenile strategies, adults do not show a shift toward higher WHC, but they instead have slightly increased their resistance against evaporation. A plausible explanation for these contrasting patterns is the limitation of juvenile thalli to a height of 1 cm in the model. Since we parameterized resistance against evaporation as a function of height, juvenile strategies are constrained in their ability to increase surface resistance. The lack of simulated increase in external: internal WHC ratio in adult thalli as an additional means to increase active time may be explained by their larger overall WHC. Due to this, periods of higher water saturation after rainfall may be prolonged compared to juveniles and they may be more often limited by CO_2 diffusion under these conditions if water is stored externally. This may also explain why the external: internal ratio only increases at high temperature for adult thalli, since they may be more frequently dry then. Merinero et al. (2014) demonstrated that the preferred source of water between younger and adult thalli shift from air humidity to liquid water. The increment of STM in adult thalli increases the amount of water necessary to achieve full saturation, making them more dependent on liquid water. At the same time, thicker thalli could prolong the hydrated periods prolonging the photosynthetic activity.

Finally, the slight decrease in STM and WHC_{LiBry} for both adult and juvenile strategies may be explained by the general disadvantage of high STM under stressful conditions in the model. This is due to the fact that, for the same amount of lateral expansion, a strategy with a high STM has to assimilate more carbon than one with a low STM. Expansion, however, is crucial for survival in LiBry since it needs to compensate for the loss of cover due to disturbance and turnover. In the standard version of LiBry, thallus height, which is associated with high STM under otherwise equal trait values, is connected to a competitive advantage, and thus results in higher relative cover of the surviving tall strategies in the simulated community (Porada et al., 2019). Here, however, we treated all strategies as equally competitive since we focused on ecophysiological processes, and not on potential interactions between different life stages in the same population. The latter process is poorly known, since data on competition between adults and juveniles on single trees are currently not available. We thus wanted to avoid an overestimation of the advantage of adults based on their growth height in the model.

The simulation outputs obtained from LiBry led to two main implications: (1) Warming due to climate change may be harmful to the survival of juvenile thalli. In fact, harsher conditions could exacerbate the difference in terms of survival between adults and juveniles. Moreover, an overall lower active time could lead to delaying the age-at-reproduction, reducing the dispersal capability of such a population. Such effects of warming on *L. pulmonaria* may be studied through forcing LiBry by climate change scenarios. (2) A monitoring of the metabolic activity of *L. pulmonaria* thalli of different ages under field conditions along a climatic gradient, combined with laboratory analysis of their water relationships could be used to evaluate in better detail the findings predicted by the LiBry model. In particular, analysis of mean morphological traits at the community level for different locations along the climate gradient could be used to assess the selection process in LiBry.

The present implementation of LiBry does not account for either intra-and interspecific competition and facilitation processes. Nevertheless, these dynamics could be extremely important over long time periods. In the case of *L. pulmonaria*, interaction with bryophytes could play a facilitation role at an early stage of development, as bryophytes could provide supplementary water enhancing photosynthetic active periods (Benesperi et al., 2018). This is supported by our sensitivity analysis with regard to the bark water reservoir, which may have a similar effect. A possible further application of the LiBry model could be to test the competition and facilitation dynamics at a community level, by setting different interaction schemes and then comparing them to observed community compositions of both lichens and bryophytes. These interactions are probably mediated by different functional traits. For instance, foliose aerohygrophytic chlorolichens, such as *L. pulmonaria* (Larsson et al., 2012) could benefit from bryophytes species with a growth form that reduces evaporation of water but at the same time could be overgrown by these latter. Crustose lichen species

could be outcompeted by fast-growing bryophyte or other lichen species. As demonstrated for Alpine plant communities, complex interactions of facilitation and competition might support higher biodiversity (Losapio et al., 2021).

Populations of *L. pulmonaria* have strongly declined during the last century, mainly due to anthropogenic disturbance. Moreover, future climate change is predicted to worsen and reduce suitable conditions (Nascimbene et al., 2016, 2020). The results discussed here strengthen the importance of management practices focused on the preservation of specific conditions aimed at enhancing the buffer potential of forests (De Frenne et al., 2021). The temperature under the forest canopy could be up to 2 degrees lower compared to free-air temperature. At the same time, forest structure also influences the quantity and time of different types of water sources (Gauslaa, 2014; Di Nuzzo et al., 2022a). Also, topography could play an important role in buffering harsher climatic conditions, for example protecting forests close to watercourses could be important for providing more humidity (Ellis, 2020). In harsher conditions, where juveniles seem to have a lower possibility of survival, preserving forests with favorable temperature and water conditions could enhance the development of larger populations of *L. pulmonaria*.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

PP and PG designed the study concept together. PP developed the model code and ran the simulations. LD, GC, PG, RB, JN, EB, and AP provided data for model evaluation. LD, GC, PP, and PG wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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What makes a good phorophyte? Predicting occupancy, species richness and abundance of vascular epiphytes in a lowland seasonal tropical forest

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Epiphytes typically exhibit clustered distribution patterns, but predicting the spatial variation of their distribution at fine scales has long been a challenge. Taking advantage of a canopy crane giving access to 1.1 ha of lowland seasonal rainforest in Yunnan (China), we assess here which factors promote the probability that a given tree hosts epiphytes, and the variation of species richness and abundance of epiphytic spermatophytes and ferns among trees. Variation in epiphyte species richness as a function of host tree size, characteristics of its surrounding environment, topography and microclimatic conditions, were analyzed by Random Forest. Epiphytic spermatophytes and ferns occupied 2.3 and 10.8% of the available host trees, respectively. Significant models predicting which trees are more likely to host epiphytes than others were obtained, indicating that host tree characteristics and their local environment play a significant role in determining which host tree is most likely to be colonized. These models, as well as models for species richness and abundance, however, exhibited a moderate to low accuracy (r^2 0.28 and 0.24 and of 0.12 and 0.14 for spermatophyte and fern richness and abundance, respectively). The best predictor of the presence of epiphytes on a tree, of its epiphytic species richness and abundance, was its DBH. In ferns, however, two peaks of species richness were observed, representing shade-loving ferns on small trees and sun-loving ferns on large trees. Microclimatic conditions and light intensity were the second best factor accounting for variation in species

richness and abundance among trees. The contribution of liana infestation, host tree identity, and characteristics of neighboring trees were marginal. Our inclusion of a large number of host-tree characteristics and their local environment did not allow for an apparent improvement of model accuracy over studies with a more limited number of predictors, pointing to the role of chance upon tree colonization. Our results confirm the utmost importance of large trees with emergent canopies for the conservation of the epiphytic flora, but also indicate that epiphytic diversity assessments in tropical forests must also include small understorey trees, which should be further considered for conservation. The importance of the micro-climatic conditions that prevail at the level of each individual host tree further points to the necessity of maintaining a buffer zone around large host trees targeted for conservation.

KEYWORDS

vascular epiphytes, colonization, richness, abundance, microclimate, conservation, forest canopy

Introduction

Forest canopy harbors 40–50% of the global terrestrial biodiversity, of which nearly 10% is restricted to this specific environment (Ozanne et al., 2003; Basset et al., 2012). The so-called "last biological frontier" (Lowman and Nadkarni, 1995; Stork et al., 1997; Lowman and Rinker, 2004) has been considered as one of the most diverse, but little-known habitats in the biosphere (Lowman and Schowalter, 2012). If forest canopy science has indeed been an active discipline since the nineteenth century, its progress has been slow, partly due to the limited accessibility of canopies. Since 1990, the development of a canopy crane network, presently operating at 22 locations around the globe, has substantially boosted research on the biodiversity and functioning of forest canopies (Nakamura et al., 2017).

Among the wide array of organisms occurring in canopies, epiphytes represent approximately 10% of vascular plant diversity (Zotz et al., 2021). At the interface between atmosphere and forest canopy, they are exposed to harsh conditions of temperature and humidity, are physiologically dependent on rainfall for water and nutrient supply, and are, hence, extremely sensitive to climatic conditions (Nadkarni, 2010).

Vascular epiphytes comprise about 28,000 species, of which 68% are orchids and 10% are ferns. They typically occur on different parts of their host tree, with orchids prevailing in the outer canopy, whereas hygrophilous ferns dominate in the lower strata (Zotz, 2016). These patterns reflect the sharp gradients in light, micro-climatic conditions, and physical properties of the substrate, such as bark texture and physico-chemistry, branch orientation and diameter, which prevail from the base to the uppermost canopy (ter Steege and Cornelissen, 1989). These conditions further vary along horizontal gradients due to both extrinsic and intrinsic factors. Intrinsic factors include the successive ontogenetic stages of development of the host tree, during which variation in tree architecture, bark characteristics, canopy soil chemistry, microclimate conditions and host tree size occur (Taylor and Burns, 2015), but also among tree species with different branching architecture, bark texture and physico-chemistry (Hidasi-Neto et al., 2019). Therefore, host specificity has been reported in many instances (Sáyago et al., 2013; Zhao et al., 2015; Hayward et al., 2017; Wang et al., 2017; Adhikari et al., 2021). Determining the degree of host specificity is important in a conservation context because specialist species are generally more vulnerable to habitat alterations and climate change than generalist species, and host specialists, in particular, are threatened by coextinction with their hosts (Wagner et al., 2015). Host specificity is, however, complex to demonstrate and, if applicable, control for, especially in tropical rainforests, which typically host hundreds of tree species. Furthermore, host tree identity cannot be analyzed independently from all other factors that jointly shape epiphyte distributions (Wagner et al., 2015).

Extrinsic factors include the direct environment of the host tree, which may further contribute to account for epiphytic distribution patterns. Such factors include local light and micro-climatic conditions at the focal tree, which may be influenced by tree height and size (Baker et al., 2014), tree density (Von Arx et al., 2013), tree species (Kovács et al., 2017), the distance to neighbor trees, and topography (Bramer et al., 2018). If the impact of climatic variation on epiphytes has been evidenced at regional scales through analyses of elevational diversity patterns (Nadyeina et al., 2014; Reina-Rodríguez et al., 2016; Eaton et al., 2018; Flores-Tolentino et al., 2020), and at local scales through analyses of the vertical stratification of epiphytic species distributions within trees (Krömer et al., 2007; Woods et al., 2015; Murakami et al., 2022), relatively

little is known about the importance of fine-scale variation in climatic conditions between individual host trees on the distribution of epiphytes (Toivonen et al., 2017). As a matter of fact, predicting whether a given tree is likely to host epiphytes, and the factors promoting epiphytic species richness remains challenging. In many instances, even large, old trees lack any epiphytes. Johansson (1974), Zotz et al. (1999) and Zotz and Vollrath (2003) reported epiphytic occupancy rates of about 50% in tropical forests, raising the question of the factors driving host tree selection by epiphytes. While epiphytes typically exhibit non-random, clustered distribution patterns (Nieder et al., 2000; Seto et al., 2020; but see Hirata et al., 2008), models attempting at predicting which trees are more likely to be colonized than others, and how epiphytes species richness varies, typically exhibit low predicting power, which has been interpreted in terms of the role of chance during epiphytic dispersal and tree colonization (Zotz and Vollrath, 2003; Zotz and Schultz, 2008).

Here, we took advantage of a tropical canopy crane facility to conduct a comprehensive census of vascular epiphytes and record detailed information on both the intrinsic factors of each individual tree and extrinsic factors describing their environment. In particular, the prime importance of microclimates actually experienced by organisms has been increasingly acknowledged (De Frenne et al., 2021), but it is only recently that microclimatic conditions have been monitored, modeled and used to explain the spatial variation of epiphyte distributions (Murakami et al., 2022; Shen et al., 2022). In this framework, we address the following questions: (1) if epiphytes are not randomly distributed among trees, to what extent can we, using a comprehensive description of the characteristics of individual trees and their local environment, predict which trees are likely to host epiphytes and which trees are not, and how epiphytic species richness and abundance vary among individual trees? What are the variables involved? (2) How do these patterns vary between epiphytic ferns and epiphytic spermatophytes? (3) Which management strategies can be accordingly proposed to promote the conservation of vascular epiphytes?

Material and methods

Study site and sampling design

This study took place in a pristine lowland seasonal rainforest within the core area of Mengla subdistrict (101°35'E, 21°37'N), Xishuangbanna National Natural Reserve in Yunnan, SW China. Mean monthly relative humidity and mean monthly temperature recorded by 12 dataloggers at 2 m during 2017–2019 were 95.3% (minimum of 90.3% in June and maximum of 98.3% in July) and 20.8°C (minimum of 15.8°C in January and maximum of 25.2°C in June), respectively. This site offers the unique opportunity to explore epiphyte diversity along entire trees, up to 70 m, thanks to a canopy crane. The crane provides access to 1.1 ha, wherein 8,477 healthy individuals of 297 tree species were reported by the Xishuangbanna Station for Tropical Rainforest Ecosystem Studies (XTRES) in 2019. The emergent tree layer (30–70 m high) is dominated by *Parashorea chinensis,* which reaches 45–70 m, and a layer of 30–45 m high trees, such as *Canarium album*, *Pometia tomentosa*, *Sloanea tomentosa, Semecarpus reticulata* and *Nephelium chryseum*. The canopy layer (18–30 m high) is mainly comprised of *Barringtonia fusicarpa*, *Diospyros hasseltii*, *Drypetes hoaensis,* and *Pseuduvaria indochinensis*. The understorey layer (6–18 m) high is composed of *Cleidion brevipetiolatum*, *Dichapetalum gelonioides*, *Diospyros xishuangbannaensis*, *Garcinia cowa,* and *Pittosporopsis kerrii*.

We focused on 1,334 individual trees (excluding tree ferns) belonging to 47 species with a diameter at breast height (DBH) greater than 5 cm. Each individual (or selected leaves or flowers in the case of rare species) of vascular epiphyte was collected and identified in the herbarium of Restoration Ecology Group, CAS Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden.

The vertical location of epiphytes was measured using a tape from the gondola of the canopy crane. An occupancy rate was computed as the number of trees occupied by at least one individual fern or one individual spermatophyte, respectively, divided by the total number of trees (1,334). We also partitioned this occupancy rate by DBH class, using three DBH classes as defined by Bradford and Murphy (2019): small (DBH $<$ 30 cm), medium-sized (DBH $\geq$ 30 and $<$ 70 cm), and large (DBH $\geq$ 70 cm).

Environmental variables

Thirty-eight variables, including 6 intrinsic tree variables and 32 extrinsic variables describing the local environment of each tree, were recorded (**Table 1**). Intrinsic factors included size (DBH, height (Z), and tree layer (1) emergent (30–70 m high), (2) canopy (18–30 m high), (3) understorey (6–18 m high), canopy width (W1: largest length of canopy crown; W2: canopy length perpendicular to W1), and canopy crown area (π/4·W1·W2), taxonomic identity, and relative abundance of individual host-tree species (**Supplementary Table 2**). Extrinsic factors included 32 variables describing the environment of each host tree. The mangling index (M) defines the probability that the focal tree belongs to the same species as its four nearest neighbors. The dominance index (U) defines the relationship between the DBH of the focal tree and its four nearest neighbors, describing whether a focal tree is larger or smaller than its neighbors (Zhang et al., 2018). The average distance between the focal tree and its four nearest-neighbor trees (meanDist) was computed to characterize geographic isolation. These variables

TABLE 1 Environmental variables used to predict the probability of occurrence, species richness and abundance of epiphytic spermatophytes and ferns on individual trees in a 1.1 ha tropical rainforest (Yunnan, SW China).

Environmental variables	Definition
DBH	Diameter at breast height (m) at 1.3 m above ground
Z	Tree height (m)
Canopy area	Canopy crown area (m2), measured as $\pi/4 \cdot W1 \cdot W2$. W1: largest length of canopy crown. W2: canopy length perpendicular to W1
Proportion	Proportion of each tree species among all tree individuals
Tree layer	Categorical variable describing whether an individual tree is an emergent (30–70 m high), canopy (18–30 m high) or understorey (6–18 m high) tree
Tree species	Taxonomic identity of host tree species
M	Mangling index (probability that the focal tree belongs to the same species as its four nearest neighbors)
U	Dominance index (relationship between the size of the focal tree and its four nearest neighbors, describing whether a focal tree is larger or smaller than its neighbors)
meanDist	Average distance (m) between the focal tree and its four nearest-neighbor trees
X	x-coordinate (m)
Y	y-coordinate (m)
Illumination index	1: no direct light, 2: $<$ 10% of lateral light, 3: 10–90% of overhead light, 4: $\geq$ 90% overhead light, 5: crown completely exposed
Liana infestation	Categorical index (0–5) describing whether a tree is liana-free to fully invaded
Elevation	Elevation (m)
TPI	Topographic position index (relative topographic position of a focal tree as the difference between its elevation and the mean elevation of all other trees)
Slope	Slope (°) of the ground at the level of each focal tree
Eastwest	East/west orientation of the ground at the level of each focal tree; value from -1 (West) to 1 (East)
Northsouth	North/south orientation of the ground at the level of each focal tree; value from -1 (South) to 1 (North)
MeanT	Annual average temperature
MeanRH	Annual average relative humidity
MeanL	Annual average light intensity
MeanPAR	Annual average photosynthetic active radiation
MaxT	Annual maximum of temperature
MaxRH	Annual maximum of relative humidity
MaxL	Annual maximum of light intensity
MaxPAR	Annual maximum of photosynthetic active radiation
MinT	Annual minimum of temperature
MinRH	Annual minimum of relative humidity
MinL	Annual minimum of light intensity
MinPAR	Annual minimum of photosynthetic active radiation
RangeT	Annual temperature range
RangeRH	Annual range of relative humidity
RangeL	Annual range of light intensity
RangePAR	Annual range of photosynthetic active radiation
SDT	Standard deviation of annual temperature
SDRH	Standard deviation of annual relative humidity
SDL	Standard deviation of annual light intensity
SDPAR	Standard deviation of annual photosynthetic active radiation

were computed using the nnIndex and fsasN4 functions from the forestSAS package (Chai, 2021). The crown illumination index was divided into 5 scales (1: no direct light, 2: $<$ 10% of lateral light, 3: 10–90% of overhead light, 4: $\geq$ 90% overhead light, 5: crown completely exposed, Dawkins and Field, 1978). Liana infestation was evaluated using Rutishauser et al. (2011) index (0–5). The X-Y coordinates of each tree were obtained from the XTRES. A topographic map (Elevation) was produced with the raster package (Hijmans, 2021) from measurements made by the autopilot vehicle (LiAIR VUX-1350, Beijing, China) equipped with VUX-1UAV Laser (RIEGL Laser Measurement Systems GmbH, Horn, Austria) at 10 m intervals. This 10

m resolution topographic map was employed to derive the topographic position index (TPI, characterizing the relative topographic position of each focal tree as the difference between its elevation and the mean elevation of all other trees (Gallant and Wilson, 2000), the slope (in degrees), and the orientation (Eastwest and Northsouth, in radiant) by SAGA-GIS v7.9.1. Light intensity ('L', W/m^2), air temperature ('T', °C), relative humidity ('RH', %) and photosynthetic active radiation ('PAR', $\mu mol \cdot m^{-2} \cdot s^{-1}$) were recorded at 1-hr intervals from 12 trees at five height zones (tree base, middle trunk, inner canopy, middle canopy and outer canopy) from July 2017 to December 2019. To predict the spatial variation of light and microclimatic conditions from the data collected by 54 dataloggers, we modeled hourly variation in T, L, RH and PAR in an X-Y-Z space (thus including tree height and elevation) using Random Forest (Shen et al., 2022) as implemented by the randomForest package (Liaw and Wiener, 2002) in R v4.0.4 (R Core Team, 2021). The microclimatic conditions that prevail on each tree substantially vary from the base to the canopy. Between 2 and 62 m above ground, day (8 a.m.–7 p.m.) relative humidity ranged between 53.6 and 99.9% and day temperature between 12.0 and 31.7°C (Shen et al., 2022), challenging the description of the global microclimatic conditions that prevail at each host tree. Each epiphytic fern and spermatophyte community was, however, restricted to a specific height zone on a tree (**Figure 1B**). To best characterize the microclimatic conditions that prevail at the level of each community, we determined the "centroid" point, i.e., the average height, at which epiphytic spermatophytes and ferns, respectively, were recorded. The climatic conditions prevailing at the "centroid" of each of the 1,344 trees were summarized using several statistics, including the annual average (meanT, meanRH, meanPAR, meanL), maximum (maxT, maxRH, maxPAR, maxL), minimum (minT, minRH, minPAR, minL), range (difference of maximum and minimum rangeT, rangeRH, rangePAR, rangeL), and standard deviation (SDT, SDRH, SDPAR, SDL).

Statistical analyses

All statistical analyses were conducted in R v 4.0.4 (R Core Team, 2021). To test the null hypothesis that epiphytes are randomly distributed among trees, we randomized 1,000 times the distribution of epiphyte individuals across the 1,334 host trees and computed 1,000 random occupancy rates. Based on this, we determined whether the observed occupancy rate significantly differed from values expected by chance, i.e., whether the observed occupancy rate was lower than 95% of the 1,000 random occupancy rates. To control for DBH, we assigned each individual tree to one of three DBH classes and repeated the above procedure for each DBH class.

To determine whether a given tree is likely to be occupied by epiphytes, how many epiphytic species it is likely to host and at which abundance, we applied classification and regression Random Forest, respectively, using the randomForest package (Liaw and Wiener, 2002). Predictors included the 9 intrinsic and 32 extrinsic factors listed above, respectively. We computed Pearson's correlation coefficients (r) among each pair of predictors (**Supplementary Figure 1**). To avoid multicollinearity issues, one predictor in a pair correlated at r higher than |0.7| was kept (Dormann et al., 2013). We took advantage of the ability of Random Forest to handle large numbers of predictors (Speiser et al., 2019). As our goal was to find which factors affect variation in species richness for interpretation purposes rather than to develop a prediction model, we kept all variables (except the correlated ones to avoid multicollinearity) in the model, and ranked them by importance, i.e., their contribution to the model. The contribution of the retained predictors to the model was measured by the mean decrease in accuracy for classification and the mean decrease in node impurity for regression models, respectively (Liaw and Wiener, 2002). These metrics characterize the difference in accuracy between full models and models, wherein individual variables are successively removed. High, positive values characterize variables that substantially contribute to the model, whereas negative values characterize variables that do not contribute to the model.

To evaluate the models, we applied 100 repeated split-sampling cross-validation, where 70% of the data are used to calibrate the models and the remaining 30% to compute the different evaluation metrics from the "train" function of the caret package (Kuhn, 2021). We set the number of trees to 1,000. We computed the average, across the 100 cross-validation replicates, of the following statistics to evaluate model accuracy: sensitivity (true positive rate, ranging between 0 and 1), specificity (true negative rate, ranging between 0 and 1) and two statistics derived from sensitivity and specificity (Area Under the ROC Curve, AUC and True Skill Statistic, TSS), for classification-type models and RMSE (Root Mean Square Error), MAE (Mean Absolute Error) and R^2 for regression-type models. AUC ranges between 0 and 1, with a value of 0.5 characterizing a model with no discriminatory power and values of 0.7–0.8 characterizing acceptable models. TSS ranges between -1 and 1, with a negative value characterizing a model with no discriminatory power and values > 0.6 characterizing useful models (Guisan et al., 2017). The best value of the mtry (number of variables available for splitting at each tree node) parameter was selected, during the cross-validation procedure, via the AUC and RMSE, for classification and regression-type models, respectively.

Results

Fifty-six species of vascular epiphytes were recorded on trees with a DBH > 5 cm, including 44 spermatophytes (36 orchids,

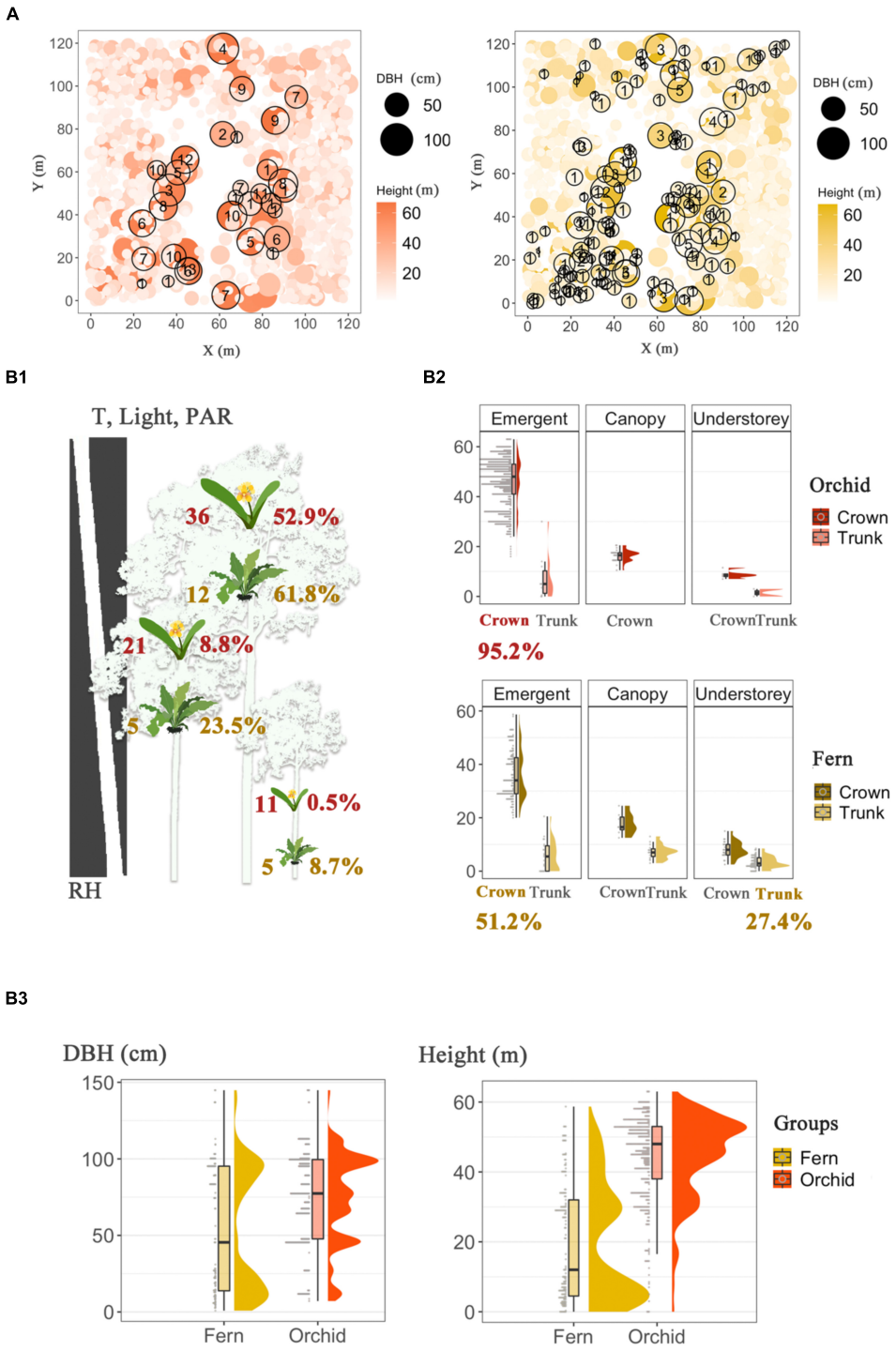

FIGURE 1
Horizontal and vertical distribution of epiphytic spermatophyte and fern richness and abundance on individual trees in a 1.1 ha tropical canopy crane facility, Yunnan, SW China. **(A)** Horizontal distribution of epiphyte spermatophytes (left) and fern (right) richness on individual trees in a x-y space (120 m*120 m) depending on DBH and tree height. **(B1)** Vertical distribution of epiphyte richness and the ratio of epiphytic individuals on canopies or trunks to the total number of epiphytic individuals controlling for tree size. **(B2)** Box plots [showing the 1st and 3rd quartiles (upper and lower bounds), 2nd quartile (center), 1.5* interquartile range (whiskers) and minima-maxima beyond the whiskers] of the number of individuals controlling for tree layers and tree structures. **(B3)** Histogram of species abundance of two groups depending on DBH and height, respectively. Gray triangles represent relative humidity (RH), temperature (T), light intensity (Light) and photosynthetically active radiation (PAR) along a tree.

3 *Hoya*, 1 *Dischidia,* and 4 *Aeschynanthus*) and 12 fern species (**Supplementary Table 1**, see also https://doi.org/10.6084/m9.figshare.21186049.v3 for comprehensive information on individual trees). The most predominant spermatophytes were orchid species (*Oberonia jenkinsian*, *Phalaenopsis marriottiana* var. *parishii*, *Pinalia amica*, *Cleisostoma fuerstenbergianum,* and *Luisia morsei*). *Asplenium nidus* and *Microsorum punctatum* were the most frequent epiphytic ferns. Eleven species are listed in the IUCN red list of threatened species, including the fern *Goniophlebium subauriculatum* (CR) and 10 orchid species [*Coelogyne suaveolens* (EN), *Cymbidium dayanum* (VU), *Dendrobium aphyllum* (VU), *D. densiflorum* (VU), *D. devonianum* (EN), *D. exile* (VU), *D. fimbriatum* (VU), *Oberonia rufilabris* (EN), *Pelatantheria rivesii* (VU), *Sarcoglyphis smithiana* (VU)]. Epiphytes were not randomly distributed across host trees, but were instead significantly clustered on specific tree individuals. In fact, the observed occupancy rates of 2.3 and 10.8% in epiphytic spermatophytes and ferns, respectively, were substantially and significantly lower than the occupancy rate expected if epiphyte individuals would randomly colonize any available tree (**Table 2**). This pattern prevailed for small, medium and large trees, except for ferns on small trees (**Table 2**). This clustered pattern can be visualized by the distribution of epiphytic spermatophytes and ferns in a two/three-dimensional space (**Figure 1A** and **Supplementary Figure 2**). Both epiphytic spermatophytes and ferns exhibited a higher abundance along a central ridge and a higher richness on large and tall trees (**Figures 1A,B**). Globally, the average fern and spermatophyte species richness per occupied tree was 1.32 ± 0.87 and 5.77 ± 3.79, respectively. Species richness was higher on large trees, with an average of 2.67 ± 1.43 and 6.56 ± 2.94 fern and spermatophyte species, respectively, than on small trees, with an average of 1.04 ± 0.23 and 2 ± 2.45 fern and spermatophyte species, respectively. In ferns, however, the distribution of species richness depending on DBH exhibited a bimodal response. The two peaks of species richness represent shade-loving ferns on small trees and sun-loving ferns on large trees (**Figure 1B**). *Davallia trichomanoides*, *Drynaria roosii*, *Nephrolepis cordifolia*, *Drynaria coronans*, *Pyrrosia nummulariifolia*, and *Goniophlebium subauriculatum* were strictly restricted to large trees.

Among occupied host trees, 95.2% of epiphytic spermatophyte individuals and 51.2% of epiphytic fern individuals were restricted to the canopy crown of emergent trees. Among them, 10 red-listed species were completely constrained to the emergent canopy crown. 27.4% of epiphytic fern individuals occurred on the trunk of understorey trees (**Figure 1B2**).

Based on the correlation matrix among variables (**Supplementary Figure 1**), DBH, canopy area, tree species, proportion, M, U, meanDist, elevation, slope, Eastwest, Northsouth, illumination index, maxT, minRH, maxPAR, minPAR, maxL, minL, liana infestation, tree layers and the taxonomic identity of tree species were retained as predictors of species richness.

The Random Forest models predicting whether a given tree is likely to host epiphytes exhibited a higher accuracy for epiphytic spermatophytes than for epiphytic ferns due to a lower model specificity in the latter (**Table 3**). The models describing variation in epiphytic spermatophyte and fern species richness and abundance exhibited a cross-validated r-square of 0.28 and 0.24, and of 0.12 and 0.14, respectively. For all models, features of tree size, (DBH and canopy area) were the most important variables followed, with an almost similar contribution, by microclimatic conditions (maxT, minRH, maxPAR, maxL) (**Figures 2**, **3**). The contribution of liana infestation, host tree identity, and characteristics of neighboring trees (M, U) were marginal.

Discussion

Vascular epiphytes were not randomly distributed but tended to cluster on specific host trees. Significant models predicting whether a tree is likely to be colonized in a landscape dominated by non-colonized ones were obtained, indicating

TABLE 2 Spatial patterns of epiphytic spermatophytes and ferns in a 1.1 ha tropical rainforest (Yunnan, SW China).

Groups	Host tree size	Host tree individuals	Epiphyte richness	Epiphyte abundance	Epiphytic occupancy rate	Randomized occupancy rate (mean ± SD)
Spermatophytes	All	1,334	40	801	2.30%	12.2 ± 0.22%
	Small (< 30 cm)	1,232	11	39	0.50%	0.96 ± 0.02%
	Medium (≥ 30 and < 70 cm)	68	21	223	8.80%	48.40 ± 3.23%
	Large (≥ 70 cm)	34	33	539	52.90%	97.89 ± 0.02%
Ferns	All	1,334	12	362	10.80%	13.64 ± 0.19%
	Small (< 30 cm)	1,232	5	170	8.70%	0.90 ± 0.06%
	Medium (≥ 30 and < 70 cm)	68	5	39	23.50%	30.50 ± 1.83%
	Large (≥ 70 cm)	34	12	153	61.80%	84.81 ± 4.66%

For each group, the observed occupancy rate is compared with a randomized occupancy rate, wherein individual epiphytes are reshuffled across all individual trees. All *p*-values (proportion of randomized occupancy rates that are higher than the observed occupancy rate across 100 replicates) are < 0.001.

TABLE 3 AUC, TSS, sensitivity and specificity of Random Forest models predicting the probability that a tree is colonized by epiphytic spermatophytes and ferns in a lowland tropical rain forest (Xishuangbanna, Yunnan, SW China).

Group	AUC	TSS	Sensitivity	Specificity
Spermatophyte	0.94 ± 0.04	0.78 ± 0.10	0.82 ± 0.10	0.96 ± 0.03
Fern	0.79 ± 0.03	0.48 ± 0.05	0.72 ± 0.07	0.76 ± 0.07

Values are average ± SD across 100-fold cross-validation replicates.

that, at the local scale, host tree colonization is not random as host tree characteristics and their specific environment play a significant role in determining which host tree is most likely to be colonized (Zotz and Schultz, 2008). Bypassing the random effect associated with dispersal and establishment, Callaway et al. (2002) showed that transplanted epiphytes grow faster on trees hosting massive loads of epiphytes than on trees with no or few epiphytes, evidencing that the former exhibit key properties that are responsible for their suitability as hosts. Our models exhibited, however, a moderate (AUC of 0.93, TSS of 0.78) to low (AUC of 0.79, TSS of 0.48) accuracy for spermatophytes and ferns, respectively. These values are in the range of similar studies aiming at modeling epiphytic species distributions (mean AUC in epiphytic lichens of 0.79 ± 0.08 (Dymytrova et al., 2016), AUC range of 0.67–0.96 in epiphytic orchids in Colombia (Reina-Rodríguez et al., 2016), AUC of 0.97 in the epiphytic orchid *Laelia speciosa* in Mexico (Flores-Tolentino et al., 2020), median AUC of 0.50–0.90 in epiphytic lichens in temperate forests (Eaton et al., 2018). Similarly, the models describing variation in epiphytic fern and spermatophytes richness exhibited r-squares of 0.28 and 0.24, respectively, in the range to slightly lower than similar reports for epiphytic ferns and spermatophytes (e.g., r^2 = 0.44 and 0.32 for orchid and fern species richness; Adhikari et al., 2017). This suggests that our inclusion of a large number of host-tree characteristics, including host tree identity, features of tree size, light and microclimatic conditions, characteristics of the neighbor trees, and liana abundance, did not allow for an apparent improvement of model accuracy over studies with a more limited number of predictors. Additional variables characterizing microhabitat conditions, such as bark texture and chemistry, branch diameter, or percentage cover of canopy humus (Woods et al., 2015), would possibly increase model accuracy. Although not necessarily contributing more than other variables such as DBH, tree age, and hence, growth rate, is another important variable to consider as DBH is an imperfect proxy for tree age, so that trees of similar DBH may exhibit very different areas and age (Wagner and Zotz, 2019). The relatively low explanatory power of the models reported here and in previous studies suggests, however, that the distribution of epiphytes among trees is largely stochastic.

Epiphytes need to track patches of suitable trees in a dynamic landscape for persistence (Snäll et al., 2005) and are, at first sight, expected to display high dispersal capacities. This is especially true in ferns and orchids, whose dust-like seeds are the smallest among spermatophytes, and hence, display a large potential for long-distance dispersal (Einzmann and Zotz, 2017). Accordingly, the composition of epiphyte communities is better explained by host-tree characteristics than by the distance among trees (Mota de Oliveira and ter Steege, 2015; Mendieta-Leiva et al., 2022), suggesting that niche-based mechanisms prevail over dispersal limitations. Mounting evidence points, however, to substantial limitations in the capacities of epiphytes to successfully disperse and colonize new trees. Epiphytes distribution patterns are spatially aggregated (Zotz and Schultz, 2008; Zhang et al., 2010), in line with the dependence of epiphyte occupancy rates on tree density, and hence, connectivity among trees (Francisco et al., 2021), and accessibility (distance to major population sources). In dry forests, these factors were shown to be the most important for explaining the distribution of epiphytic orchids (Reina-Rodríguez et al., 2016).

Colonization may further be hampered upon establishment. Spicer et al. (2022) in fact reported that, if substrates with a high rugosity initially host more epiphytes than smooth substrates, seedling mortality was eventually very high, regardless of substrate texture, due to severe climatic conditions, and seed or seedling removal by rain or animals, especially ants (Vergara-Torres et al., 2018). Furthermore, the establishment of some "late-successional" epiphytes depends upon the accumulation of sufficient canopy soil (Victoriano-Romero et al., 2020). First colonizers (bryophytes and lichens) initiate the process of soil formation, subsequently allowing late-succession vascular epiphytes to establish, thereby participating in the clustering of epiphytes on specific host trees. Altogether, these results suggest that colonization of new trees is compromised during the establishment phase, contributing to the role of chance upon tree colonization, and accounting for the comparatively low proportion of the variation in epiphytic species richness among trees explained by environmental variables. This was particularly the case in the present study, where occupancy rates of 2.3 and 10.8% in orchids and ferns, respectively, pale by comparison with other studies in tropical rainforests (48%, Zotz and Vollrath, 2003; 30%, Zotz and Schultz, 2008; 56–100%, Zhao et al., 2015), which may be explained by the length of the dry season (May-October) in the study area.

If chance associated with dispersal limitations plays such an important role in epiphytic distributions, models with higher accuracy would be expected in good than in poor dispersers. In contrast, despite higher occupancy rates in ferns than in spermatophytes, our models displayed a lower accuracy in the former due to a lower specificity, pointing to suitable, but unoccupied trees. Although zoochory might play a more important role in fern dispersal than previously thought (Boch et al., 2013, 2016), fern spores are typically dispersed by wind. Fern spores are smaller than orchid seeds and exhibit

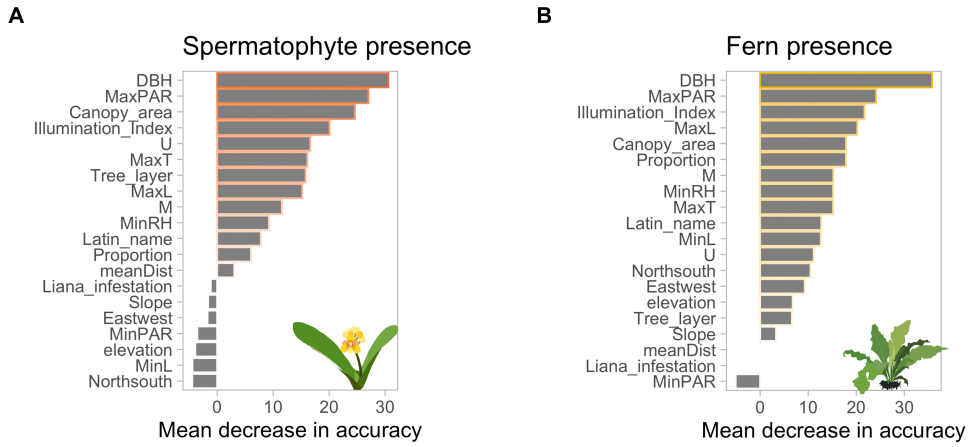

FIGURE 2
Importance (as measured by the mean decrease in accuracy) of the factors accounting for the probability that a given tree hosts epiphytes in classification random forest in a lowland tropical rainforest (Xishuangbanna, Yunnan, SW China). **(A)** Epiphytic spermatophytes; **(B)** epiphytic ferns. See **Table 1** for abbreviations.

a lower settling velocity (0.06 m/s) than orchid seeds (0.09–0.4), enhancing long-distance wind dispersal (Zotz et al., 2016). Analyses of population genetic structure in tropical epiphytic ferns further revealed that most of the genetic diversity was distributed within populations and failed to evidence any significant clustering, pointing to strong migration rates among populations (Winkler et al., 2011). Analyses of epiphytic tropical bryophytes, which produce smaller spores than those of ferns, revealed, however, spatial genetic structures comparable to those documented for spermatophytes, whose diaspores are orders of magnitude larger (Ledent et al., 2020). In fact, anemochorous plants in dense tropical rainforests typically exhibit tighter clusters than animal-dispersed species because of the barriers imposed by the dense forest canopy on wind speed (Seidler and Plotkin, 2006).

Globally, epiphytic spermatophyte and fern species richness was described by the same predictors. The best predictors of the presence of epiphytes on a tree, but also its epiphytic species richness and abundance, included features of tree size, mostly DBH and, to a lesser extent, canopy area. Tree size has indeed been invariably identified as the main driver of epiphytic species richness and abundance (Zotz and Vollrath, 2003; Zotz and Schultz, 2008; Francisco et al., 2021). Tree size is a complex factor that integrates several ecological processes relevant to epiphyte community assembly (Zhao et al., 2015). It is linked to the exposure time of the host to epiphyte seed rain, but also the greater available space for epiphytes and the increased number of microhabitats available on the tree (Paillet et al., 2019).

Microclimatic conditions were precisely the second factor after DBH most accounting for variation in species richness and abundance among trees. In fact, epiphytes are constantly exposed to light, water and nutrient stress, whose intensity increases from the base to the canopy, leading to a succession of communities with increasing levels of stress tolerance (Dias-Pereira et al., 2022, and references therein). Microclimate is thus a major determinant of the local distribution of vascular epiphytes, as can be deduced from the vertical stratification of species recurrently reported (ter Steege and Cornelissen, 1989; Krömer et al., 2007; Zotz and Schultz, 2008; Dias-Pereira et al., 2022). Our results suggest that microclimatic variations among trees, caused by a series of factors including topography, tree height and the relative position of each tree as compared to its neighbors, must hence be taken into account in analyses of epiphytic species distributions. Although our analyses did not include an index of canopy openness *per se*, they included information on the neighboring environment of focal trees (M, U. . .), which did not prevail over microclimatic and light variables in our models, questioning the use of easy-to-measure variables such as canopy openness, as suggested by Toivonen et al. (2017) in such analyses.

The importance of light and PAR in the models accounts for the high light demand of epiphytic orchids to photosynthesize and reproduce (Tremblay, 2008), but also for a substantial proportion of the fern community, which was restricted to the inner canopy. The inclusion of the illumination index, which was negatively correlated with the dominance index (U), likely explains why the latter, and other potentially important variables describing the surrounding environment of each host tree (Fardhani et al., 2021), did not or marginally contribute to the present models.

Other factors, such as host tree identity, played a marginal role in the models. While host trees may differ in their branching architecture, bark texture and pH (Zotz and Schultz, 2008; Francisco et al., 2021), the impact of host tree specificity on epiphytes has been challenged based on the fact that upper canopy branches, where the bulk of orchids occur, may

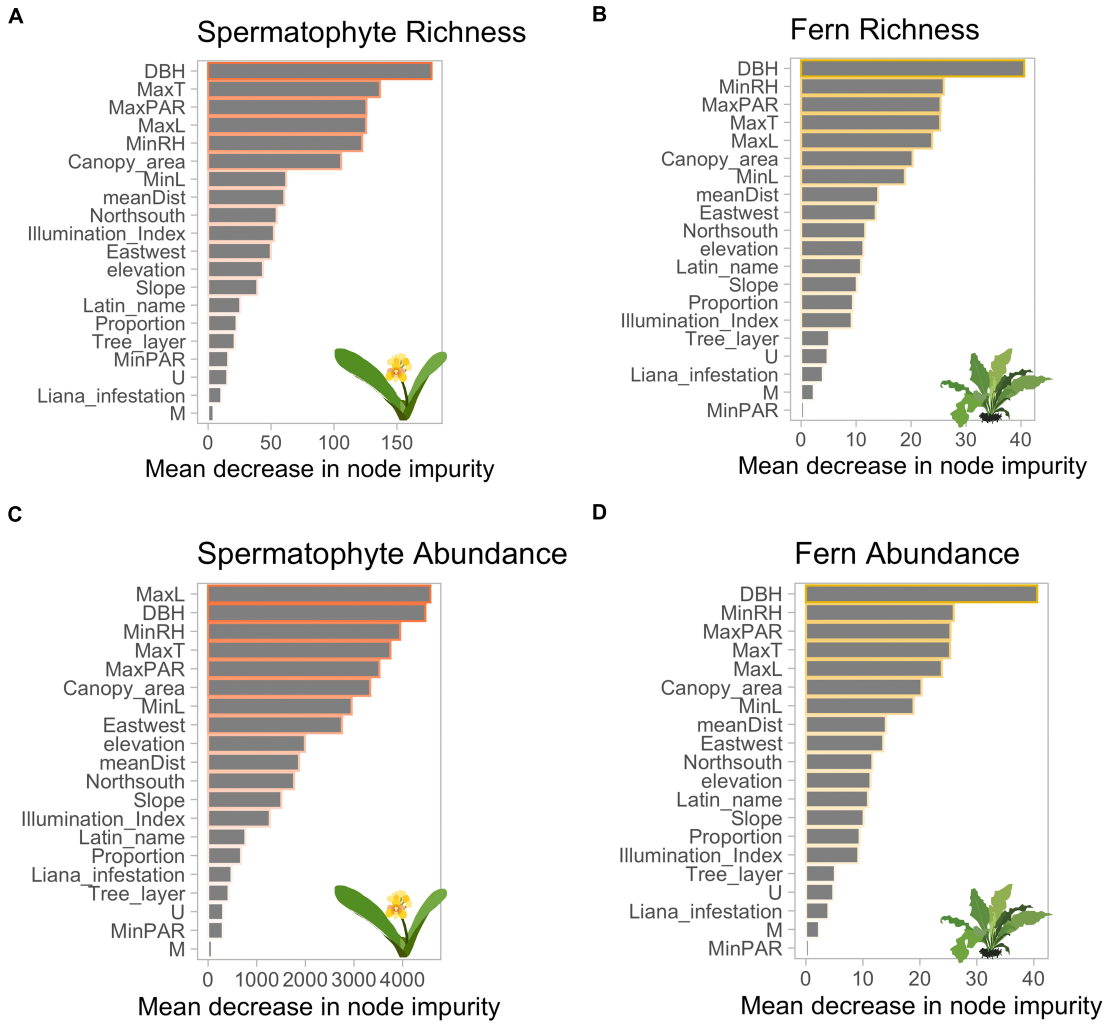

FIGURE 3
Mean decrease in node impurity of the factors accounting for epiphytic spermatophyte **(A)** and epiphytic fern **(B)** species richness and for epiphytic spermatophyte **(C)** and epiphytic fern **(D)** abundance per tree. See **Table 1** for abbreviations.

accumulate bryophytes, lichens and dead organic material (Zotz and Vollrath, 2003), potentially homogenizing habitat structure among host tree species. Furthermore, host tree identity may impact species composition, but not necessarily species richness, as in temperate forests at least, the range of microhabitats across host-tree taxa is very similar (Paillet et al., 2019).

Our results have several implications in terms of conservation. In fact, if the total species richness (56 species) is not higher, and even somewhat lower than that reported in other areas (21–48 species in 1ha mountain Asian tropical forest plots, Zhao et al., 2015; 66–85 species in 2 ha Neotropical cloud forest and inselberg, Francisco et al., 2021, 37–188 species in ca 1 ha plots of Neotropical rainforest, Zotz and Vollrath, 2003; Zotz and Schultz, 2008, and references therein), the proportion of almost 19.6% of threatened species of high conservation relevance is remarkable. Remarkably, 51.2% of epiphytic fern individuals and 95.2% of epiphytic spermatophyte individuals were only limited to the canopy crown of large trees. Given the substantial contribution of DBH to explain variation in species richness, we confirm the utmost importance of large trees with emergent canopies for the conservation of the epiphytic flora (Shen et al., 2018; Adhikari et al., 2021; Francisco et al., 2021). In ferns, six species were strictly associated with large trees. 81.3% of tropical dominant fern, *Asplenium nidus*, which can offer a cool and moist microhabitat for arboreal fauna, prefers growing on small trees. Species richness of liverworts was also maximum on small trees (Shen et al., 2022). Altogether, these observations support the idea that epiphytic diversity assessments in tropical forests must also include small understorey trees (Sporn et al., 2010), which should be further considered for conservation.

The low occupancy rates reported here further suggest that, for optimal epiphyte conservation, a much higher proportion of large trees than the ones that are actually occupied would need to be kept outside of protected areas to maintain the colonization dynamics of new host trees. This is especially true for trees located in ridges, which experience suitable micro-climatic conditions for the epiphytic flora. Given the importance of the micro-climatic conditions that prevail at the level of each individual host tree, however, the impact of the harvesting of trees in the vicinity of conserved individuals raises the question of the maintenance of suitable conditions at the level of isolated trees. In fact, occupancy rates vary depending on the density of available trees due to increased connectivity among them (Francisco et al., 2021), but also likely due to differences in micro-climatic conditions, further pointing to the necessity to maintain a buffer zone around large host trees targeted for conservation.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding authors.

Author contributions

LS, AV, and TS conceived and designed the research. LS, YW, and J-LD conducted field work. TS and FC performed the analyses. FC, AG, and YS provided suggestions on data analysis. TS, AV, and LS wrote the manuscript with the assistance of all co-authors. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ffgc.2022.1007473/full#supplementary-material

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Surviving in a new host: Eight years of monitoring translocated aroids, bromeliads, and orchids in the Andean forests in Colombia

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Vascular epiphytes are extraordinarily diverse in the tropical Andean region. Compared to trees and terrestrial herbs, epiphytes are more vulnerable to forest alteration due to their structural dependence on trees and environmental requirements. Based on experimental approaches for ecological purposes, monitoring air pollutants, and seeking propagation alternatives, the rescue and translocation of vascular epiphytes (mainly bromeliads and orchids) from a threatened forest to a safer forest has been recently conducted in Colombia. Preliminary assessments indicate that epiphytes benefit from such well-planned measures, and their mortality and survival might be associated with extrinsic and intrinsic factors, which remain to be understood. We evaluated the survival of 16 vascular epiphyte species after translocation into a secondary forest in Antioquia (Colombia) for 8 years. We assessed the role of intrinsic (foliar area, number of leaves, initial pseudobulbs, stems or rosettes, functional group, and epiphyte species) and extrinsic factors (host tree species, bark water-holding capacity, type of substrate, location on the host tree, nutrients, and hormone addition) and the effect of climatic variables on plant survival. The overall mortality rate in this study ranked 1–7% per year, and survival decreased annually, reaching 44% by the end of the 8th year. Host tree species and intrinsic factors such as the functional group and epiphyte species significantly affected the probability of survival. Bromeliads, in particular, exhibited high mortality, which their monocarpic growth form could explain. Another group of species showing high mortality were the miniature orchids, *Masdevalia amanda* and *M. platyglossa*, and are associated with short life cycles. Five host tree species appear to affect the survival of translocated epiphytes; however, the factors or characteristics involved remain unclear. A higher seasonality of precipitation was related to the percentage of overall mortality. This result indicates that extreme precipitation events or drought reduce epiphyte longevity. In conclusion, our study suggests that a wide range of epiphytes may be successfully translocated to secondary forests in the Colombian Andes and demonstrates that the effective introduction of epiphyte assemblages may be useful for ecological restoration efforts in Andean forests.

KEYWORDS

Andean forests, aroids, bromeliads, conservation actions, mortality rate, orchids, survival rate

1. Introduction

Vascular epiphytes are extraordinarily diverse in the tropical Andean region, reaching up to 50% of vascular plant species diversity at a local scale (Gentry and Dodson, 1987a,b). Compared to trees and terrestrial herbs, epiphytes are more vulnerable to forest alteration due to their environmental requirements such as higher humidity and substrate accumulation (Nadkarni, 2000; Köster et al., 2013; Barrancos et al., 2020). Seeds and spores of epiphytes arrive and colonize host trees, increasing species biomass and abundance in a well-established

forest (Benavides et al., 2006). During the last few decades, land-use change has dramatically impacted tropical ecosystems, resulting in degraded landscapes with fragmented forests in asymmetric conservation states and submerged in a mosaic of agricultural and livestock patches. This condition affects the dispersion and natural regeneration processes of overall plant communities. It reduces dispersal opportunities for epiphytes, which require well-established and interconnected tree communities (Köster et al., 2009, 2013). However, fragmented forests can recover ecological attributes obtained by the epiphyte assemblage by introducing and maintaining populations of different species (Duarte and Gandolfi, 2013, 2017).

Andean forests in Colombia are home to one of the most extraordinary, highly endemic, and globally threatened diversities of epiphytes. Research on this enormous epiphytic species diversity has significantly progressed in Colombia. However, protecting this unique diversity is restricted to natural reserves and complemented by rescues and translocations of plants growing in forests, which will be destroyed to establish new infrastructure, agriculture, and livestock. Rescues and translocations of plants from forests threatened by extractive activities and land-use change are an alternative for saving epiphytes in Colombia, Brazil, and Peru (Ávila et al., 2017; Fernandez Barrancos et al., 2017). However, increasing pressure from the infrastructure sector requires a deep understanding of the factors associated with epiphyte survival after translocation.

Based on experimental approaches for ecological purposes, monitoring air pollutants, and seeking propagation alternatives, rescue, and translocation of vascular epiphytes (mainly bromeliads and orchids) from a threatened forest to a safer forest have been recently conducted (Malm, 1998; Callaway et al., 2002; Rapp and Silman, 2014). Surveys after 3 years of monitoring indicated that epiphyte survival was associated with both intrinsic and extrinsic factors, such as functional traits and spatial distribution (niche partition; Zotz, 2000; Petter et al., 2015; Duarte and Gandolfi, 2017; Izuddin et al., 2018; Agudelo et al., 2019; Domene, 2019; Faleiro et al., 2020). Studies have also indicated that an epiphyte spatial distribution on the host tree might respond to a niche partition (Wolf, 2005; Reyes-García et al., 2008; Petter et al., 2015; Agudelo et al., 2019). Deep knowledge of factors associated with the establishment and survival of epiphytes after translocation will contribute to maintaining epiphytes, which otherwise might move toward local or even global extinction scenarios.

This study provides the most comprehensive monitoring of translocated vascular epiphytes in Andean forests in Colombia. We evaluated the survival of 16 vascular epiphyte species after translocation into a secondary forest in Antioquia (Colombia) for 8 years. We assessed the role of intrinsic (foliar area, number of leaves, initial pseudobulbs, stems or rosettes, functional group, and epiphyte species) and extrinsic factors (host tree species, bark water-holding capacity, type of substrate, location on the host tree, nutrients, and hormone addition) and the effect of bioclimatic variables on plant survival.

2. Material and methods

Research activities were conducted in northwestern Colombia at 2,300 m in the municipality of Medellin (75° 30′8.04"W, 6°16′54.39"N). The area has an average temperature of 15°C, varying from 5 to 25°C, annual precipitation of 2,000 mm/year, and relative humidity of 89% (SIATA, 2011). The area corresponds to fragmented forests scattered and extensive coverage of cypress pine plantations (*Cupressus lusitanica*). These plantations offer conditions for well-established bryophyte mats on the ground and vascular epiphytes to grow profusely (Morales-Morales et al., 2015; Carmona Higuita et al., 2017).

We collected healthy epiphyte individuals of 16 species corresponding to aroids, bromeliads, and orchids from cypress plantation grounds in November 2013 (Table 1). We assigned functional groups to each species, according to Agudelo et al. (2018, Table 1). Orchids were selected rhizomatous plants with pseudobulbs or stems (corresponding to functional group 7) and ramicuals (FG 3). The bromeliads (FG 4) in this study exhibit sympodial growth; ramets mature by producing a terminal inflorescence, and after flowering, the rosette dies and produces one or two offshoots (rosettes). Aroids, nomadic vines, correspond to functional group 6. We sought initial size conditions to be homogenous among species (Table 1). For aroids, we obtained stem fragments of at least four internodes. Between 33 and 42 individuals per species were attached to the trunk of 70 selected host trees. Host trees were selected adjacent to the nearest tree with a diameter at breast height (DBH) >9 cm, with a straight trunk, and a height of the first branch >4 m. Epiphyte individuals were positioned every 0.5 m (starting at 0.5 m) until reaching 4 m along host trees. The designation of the position and host tree for each individual was randomly selected, except for nomadic vines (aroids), which we located at the base of host trees. Relocated individuals in 35 trees of the 70 trees were irrigated with water during the first 2 months (November and December 2013) and fertilized with phosphorus (Master® 13-40-13), and a synthetic plant hormone from the auxin group, which stimulates root production (Superthrive®), was added. During the same period, watering with sprinklers was conducted after more than 72 h without rain. Half of the individuals of each species were attached to host trees with a substrate made of fique (Sisal) fiber (*Furcraea andina)* or coconut mesocarps fiber (*Cocos nucifera*). We designed a pocket with the fique fiber and added ∼40 g of peat. The second substrate consisted of coconut fibers and mesocarp fragments of an average granulometry between 2.5 and 10 mm. We fastened both substrates with strips of cotton and lycra. We registered each individual's initial number of leaves, pseudobulbs, rosettes, or stems. Annually, we registered the survival and the number of leaves until 2021.

A total of five fully expanded and healthy leaves were photographed *in situ* or sampled from three individuals. When it was impossible to get five leaves per individual, we collected additional individuals until we obtained a minimum of 15 leaves per species. We calculated the area from the foliar area mean quantile and multiplied it by the number of leaves registered each year. We considered an individual alive when photosynthetic tissue (green) was evident, and meristems or lateral shoots were present. We reported the formation of flowers or fruits and dry peduncles as evidence of flower formation. Pseudobulbs were recorded at the beginning, but later measurements were not considered due to a high error in the observations because they were hidden by the substrate.

We identified the host tree species, and we determined their maximum water-holding capacity (WHC) at saturation per area. WHC was calculated based on three to five random samples (of ∼2 cm^2) that were chiseled from the bark at 1.3 m above ground (three tree species presented only one individual in the area). At the laboratory, each bark sample was cleaned; area and thickness were

TABLE 1 Species functional group [*sensu* Agudelo et al., 2019], initial number of leaves or/and pseudobulbs (p), stems (s) or rosette (r), survival probability z-score, and percentage of mortality at the end of the 8th year*.

Functional group	Family/species	The initial number of leaves ± SD	The initial number of pseudobulbs (p), stems (s), or rosette (r)	Foliar area cm^2 ± SD	Survival probability z-score	Mortality%
	Orchidaceae					
FG 3	*Masdevalia amanda* Rchb.	6.10 ± 4.79		2.02 ± 0.74		46
	Masdevallia platyglossa Rchb.	13.00 ± 8.85		1.04 ± 0.42	−3.36***	47
	Pleurothallis lindenii Lindl.	4.35 ± 1.73		25.17 ± 12.95	−7.41***	12
	Stelis crassilabia Schltr.	7.07 ± 4.40		16.28 ± 13.18	−7.285	15
FG 7	*Cyrtochilum divaricatum* (Lindl.) Dalström	5.38 ± 2.47	2.60 ± 2.47 (p)	17.32 ± 17.72	−5.47**	28
	Dichaea moritzii Rchb. f.	11.73 ± 6.50	4.38 ± 2.92 (s)	5.44 ± 2.08	−6.05***	22
	Maxillaria brevifolia (Lindl.) Rchb. f.	15.63 ± 9.41	4.00 ± 5.46 (s)	1.44 ± 0.88	−2.60**	35
	Odontoglossum sceptrum Rchb. & Warsz.	4.54 + 2.11	2.54 + 2.11 (s)	32.58 ± 19.62	−6.36***	19
	Oncidium cultratum Lindl.	4.49 ± 2.94	2.70 ± 1.08 (p)	8.21 ± 9.36	−5.20***	24
	Bromeliaceae					
FG 4	*Racinaea subalata* (André) M. A. Spencer & L. B. Sm.	10.54 ± 5.18	2.43 ± 0.65 (r)	28.39 ± 14.78		46
	Racinaea penlandii (L. B. Sm.) M. A. Spencer & L.B. Sm.	17.71 ± 11.54	2.18 ± 0.40 (r)	11.37 ± 11.37		44
	Racinaea sp.	9.60 ± 4.56	2.20 ± 0.42 (r)	92.52 ± 19.34		50
	Tillandsia archeri L. B. Sm.	13.63 ± 8.33	2.40 ± 0.84 (r)	36.05 ± 23.50		59
	Tillandsia tetrantha Ruiz & Pav.	13.63 ± 7.97	1.50 ± 0.71 (r)	34.64 ± 13.71		39
	Araceae					
FG 6	*Anthurium nigrescens* Engl.	1.33 ± 1.41		101.97 ±102.46		48
	Philodendron danielii Croat & Oberle	0.79 ± 1.12		102.30 ± 50.45	−6.83***	6

Probability < 0 "***", p < 0.001 "**", p < 0.01 "*".

measured and oven-dried at 60°C for 48 h; their dry weight was determined. Maximum water-holding capacity was determined after soaking the samples in water for 24 h. Excess water was shaken off in a consistent manner, and the samples were weighed again (Einzmann et al., 2015).

Given that epiphyte species seem to be most affected by drought events, four bioclimatic variables related to precipitation dynamics were calculated: annual precipitation amount, precipitation amount of the driest month, precipitation seasonality, and mean monthly precipitation amount of the driest quarter (Karger et al., 2017). These variables were derived from diary precipitation data between 2014 and 2021, obtained from a meteorological station [Santa Helena (27010810)] located at Santa Elena rural township from Medellin (latitude: 6.1969, longitude: 75.5167, elevation: 2550 m asl). This meteorological station belongs to the meteorological monitoring network of the "Colombian Institute of Hydrology, Meteorology, and Environmental Studies" (Instituto de Hidrología, Meteorología y Estudios Ambientaes de Colombia, IDEAM). First, we evaluated variable collinearity among bioclimatic variables and plant survival annual probability and species functional group mortality using the Pearson test. This test allows us to check the dependence among variables, and then, correlated variables were removed. To assess

the effect of precipitation bioclimatic variables on plant survival, we performed a multiple linear regression. These analyses were performed using *the stats* R package.

To estimate the probability of survival over time, we used the formula Surv (time, status) ~1 and the survfit() function to produce the Kaplan–Meier curve. We used the Cox proportional hazards (CoxPH) model to evaluate overall multifactor survival; we ran three models: (a) epiphyte functional groups, substrate, addition or not of fertilization, and height above the host tree; (b) tree species and media WHC; and (c) epiphyte species. The models were constructed using language R (R Development Core Team, 2021) and package survival version 3.2-13 (Therneau and Grambsch, 2000; Therneau, 2022).

3. Results

The initial number of leaves varied between the species at the beginning of the experiment (Table 1). Aroids, *Anthurium* and *Philodendron*, presented 1.1 ± 1.3 leaves (functional group 6). Orchids, rhizomatous with ramicaul stems (FG 3), presented 7.58 ± 6.34 leaves. Rhizomatous plants with pseudobulbs or stems (FG 7) presented 8.28 ± 7.02 leaves, 2.6 ± 2 pseudobulbs, and 4.1 ± 4.5 stems. Bromeliads (FG 4) initially presented 2.3 ± 0.54 rosettes and 13.75 ± 8.46 leaves (Table 1). *Masdevallia platiglossa* showed the mean smallest leaf area (1.02 ± 0.42 cm^2), in contrast to *Philodendron danielii*, the species with the highest mean leaf area (102.29 ± 50.45 cm^2). However, there were no significant differences in the leaf area between species ($F = 1.67$, $P = 0.058$, Table 1). In general, the total leaf area was stable over the years for all species, with the exception of *Philodendron danielii*, which formed new leaves every year (Figure 1).

The host tree's structure was relatively homogeneous (total host tree height 8.2 ± 1.7 m, first branch height 5.2 ± 1.09 m, and DBH 11.2 ± 2.3 cm), and the distance between host trees was, on average, 5.5 ± 4.2 m. Epiphytes were attached to 12 host tree species; *Clusia ducu* was the most abundant species, with 48% of host trees, followed by *Sciodaphyllum trianae* with 10%. Across host species, the maximum water-holding capacity (WHC) content ranged from 21 to 496 mg cm^2, and the statistical result suggests that there is no significant difference between the study tree species bark WHC records ($F = 1.714$, $p = 0.153$, Table 2 supported by Figure 2); however, there was substantial variability in bark WHC within and between tree species (Table 2). The bark thickness ranged, on average, from 1.25 to 3.11 mm (Table 2). Although the measurement of bark WHC has been based on the area, samples chiseled from the bark presented a thickness, giving a volume to the water retention capacity. We checked whether thickness affects water retention capacity in the samples; however, it was not significant ($p > 0.167$; Supplementary material 2).

By the end of the 8th year, 44% of epiphytes survived. Bromeliads, functional group 4, presented the highest mortality, with ~47% of the plants dying at the end of the 8th year (Figure 3C, Table 1). *Tillandsia archerii* was the species with the highest mortality, with 59%. Orchids presented ~28% of mortality at the end of the 8th year *with Masdevallia* (FG 3), and miniature orchids with ramicuals presented the highest mortality (46–47%). *Pleurothallis lindenii*, with 11%, was the orchid with the lowest net mortality. A*nthurium nigrescens (*FG 6*)* presented high mortality (48%). By contrast, *Philodendron danielii* showed low mortality, with just two plants perishing during the study. Individuals that appeared dead during monitoring (dry and without evidence of photosynthetic tissue) exhibited evidence of survival as the formation of new leaves and living tissue during subsequent monitoring. This phenomenon was observed among 10–16 plants annually, being more frequent in orchids (FG 3, 30%), followed by aroids (24%), and uncommon in bromeliads (3%). All orchid and bromeliad species formed flowers and fruits during the study; 51% (327 individuals) and 31% (195) of the individuals were flowered and fructified, respectively. *Philodendron danielii* was observed initiating flowers in November 2021.

The mortality events, dead plants, fluctuated over the years. A total of 4 years presented mortalities ranging over 5% (37–44 dead plants; 2015, 2017, 2018, and 2019; Supplementary material 1). The annual precipitation amount in the last 8 years was 2,653.37 ± 498.51 mm $year^{-1}$, where the least rainy years were 2014 and 2015; the precipitation amount of the driest month was 110.5 ± 51.36 mm $year^{-1}$, the precipitation seasonality was 0.44 ± 0.06 mm $year^{-1}$, and the mean monthly precipitation amount of the driest quarter was 3.91 ± 1.23 mm $year^{-1}$ (Supplementary material 1). Regarding correlations between bioclimatic variables and survival dynamics, it was found that only precipitation seasonality showed a significant negative effect on the cumulative number of annual dead plants ($r = 0.512$; $p < 0.05$; Figure 4).

In CoxPH, (a) model, type of substrate, location on the host tree, nutrients, and hormone addition showed no significant effect on the probability of survival ($p > 0.01$). By contrast, FG 4, bromeliads, had a significant effect on the probability of survival (Figure 3A). In model (b), five host tree species, and in model (c), nine epiphyte species showed a significant effect on the probability of survival (Tables 1, 2, Figures 3B, C, respectively).

4. Discussion

The overall mortality rate in this study ranked between 1 and 7% per year (Supplementary material 1), which is expected for annual epiphyte mortality (Matelson et al., 1993; Sarmento Cabral et al., 2015; Zuleta et al., 2016). In the Andes, non-mechanical factors, such as desiccation, accounted for a mortality rate of 1.9% per year, and mechanical factors, such as falling branches, accounted for a mortality rate of 5.6% per year (Zuleta et al., 2016). Our study had one of the highest first-year survival rates in the region, in particular for bromeliads and orchids (96 and 98%, respectively, Supplementary material 1). However, this high survival rate, which was within the expected range, did not prevent survival from declining in subsequent years. Enrichments and translocations of the same plant families conducted in forests in Brazil, Costa Rica, and Peru reported lower survivals of 60 and 80% for orchids and bromeliads, respectively (Duarte and Gandolfi, 2017). The particular conditions of the locality, such as high humidity and lower seasonality of precipitation, as well as irrigation during non-rainy periods during this first year, could have favored the survival of the species in this study.

In our study, species and functional groups responded differently over the 8 years of monitoring. Bromeliads (FG 4), in particular, exhibited high mortality, which could be explained by their monocarpic growth form; the plant dies after the fruit is developed. Although under natural conditions, new rosettes would form, and our observations indicated that a large number of the bromeliads detached or turned over, drying out on site, suggesting that no

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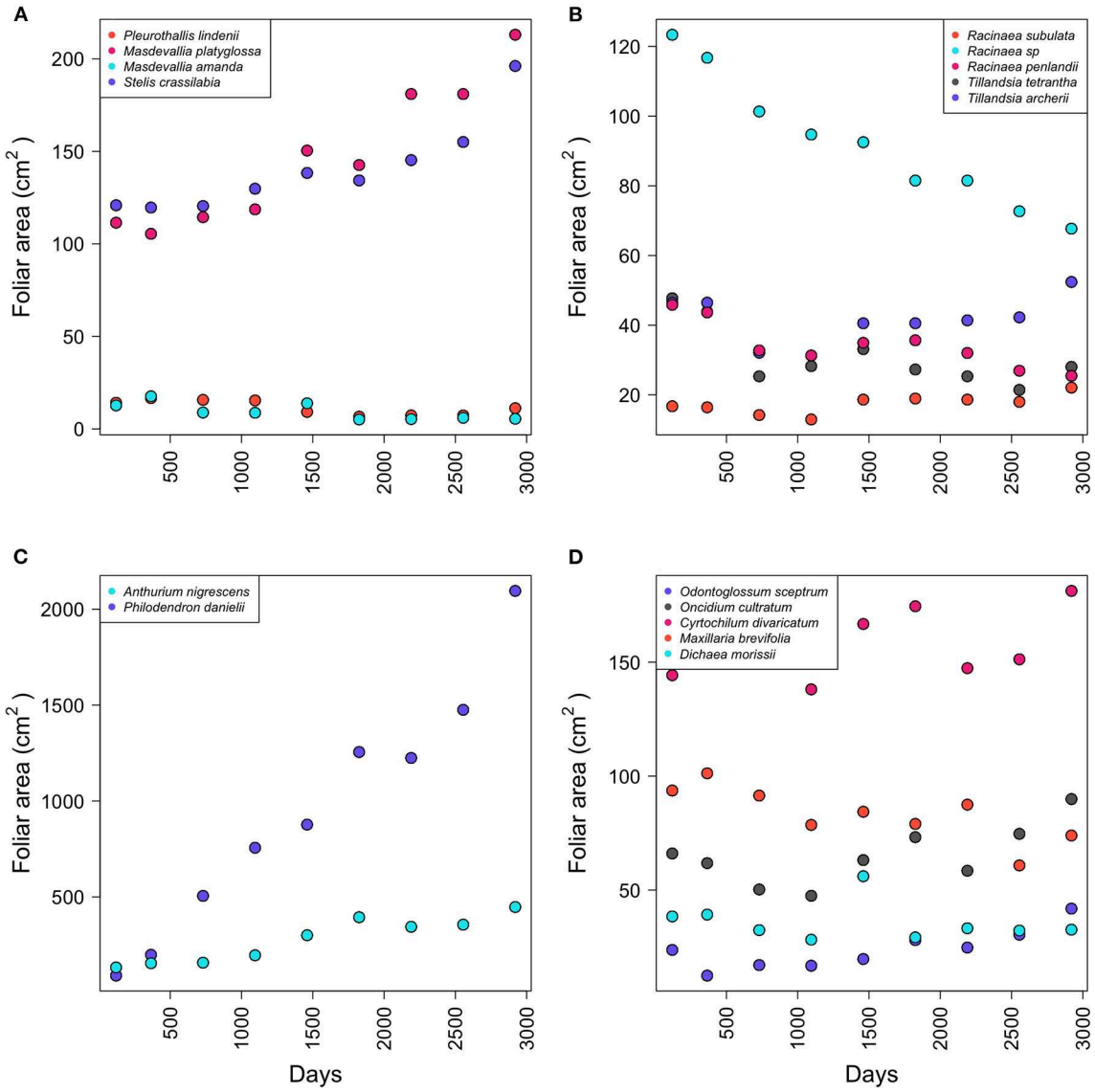

FIGURE 1
Total leaf area by species according to functional group. Epiphytic species correspond to the following functional groups (FG): **(A)** FG3, **(B)** FG4, **(C)** FG6, and **(D)** FG7.

supporting roots were formed, which would limit the establishment of adult plants. Another group of species showing high mortality was the miniature orchids with ramicuals of the FG 3, *Masdevalia amanda*, and *M. platyglossa*. These are associated with short life cycles. However, the presence of seedbeds a few centimeters from the mother plant after 3 years was a remarkable finding for these species. The other species of orchids, FG 3, had longer life cycles and persisted with a stable proportion. The orchids of FG 7, rhizomatous plants with pseudobulbs or stems, presented homogeneous survival percentages below 35% as storage organs for water and nutrients; stems and pseudobulbs play an important role in the survival of orchids. Araceae presented a high contrast between the two species, showing different responses to vegetative reproduction, while *Anthurium nigrescens* presented one of the highest percentages of mortality, which was concentrated in the early years. *Philodendron danielii* showed favorable survival over the years. The ability to reiterate over extended periods of time was surprising; the resilience of many individuals who seemed to be dead for months and had the capacity to reiterate was observed in species of aroids and orchids, which evidences the capacity to reiterate and propagate clonally, as observed in other studies (Lasso et al., 2009; Benavides, 2010), and draws our attention to mortality studies over short periods or isolated observations.

Differences in survival between epiphyte species and functional groups indicate that attention should be paid to the differences required by each species, and therefore, epiphyte adaptations and lifespan must facilitate sexual and asexual reproductive processes that are effective in short periods of time (Zuleta et al., 2016). Although we did not focus on flowering and fruiting, numerous individuals flowered and fructified during this study, indicating the importance of the availability of pollinators in the translocated sites (Phillips et al., 2020). Therefore, it is more likely that epiphyte

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TABLE 2 Descriptors of host tree species and the survival probability z-score*.

Tree host species	Average maximum water-holding capacity mg/cm^2 ± desvest	Bark thickness mm	Survival probability z-score
Myrcia cf. *Popayanensis* Hieron.[a]	21.71 ± NA	1.25	
Clethra fagifolia Kunth	98.73 ± 28.01	2.20	−2.739**
Alchornea acutifolia Müll. Arg.[a]	101.11 ± NA	1.59	
Ilex laurina Kunth	110.61 ± 42.42	2.41	
Sciodaphyllum trianae Planch. & Linden ex Marchal	115.70 ± 97.10	1.58	−3.084***
Ladenbergia macrocarpa (Vahl) Klotzsch	153.61 ± 61.74	3.02	
Clusia ducu Benth	173.51 ± 78.72	2.39	
Vismia guianensis (Aubl.) Choisy.	198.93 ± 98.84	1.61	−3.525***
Persea chrysophylla L. E. Kopp; k	265.23 ± 126.31	3.11	−4.058***
Myrcia cf. *Fallax* (Rich.) DC.[a]	267.92 ± NA	2.73	
Chrysochlamys myrcioides Planch. & Triana	296.28 ± 196.05	1.87	
Eschweilera antioquensis Dugand & Daniel	170.91 ± 104.91	1.16	−3.314**

Probability < 0 "***", p < 0.001 "**", p < 0.01 "*".
[a]Only a single data per species.

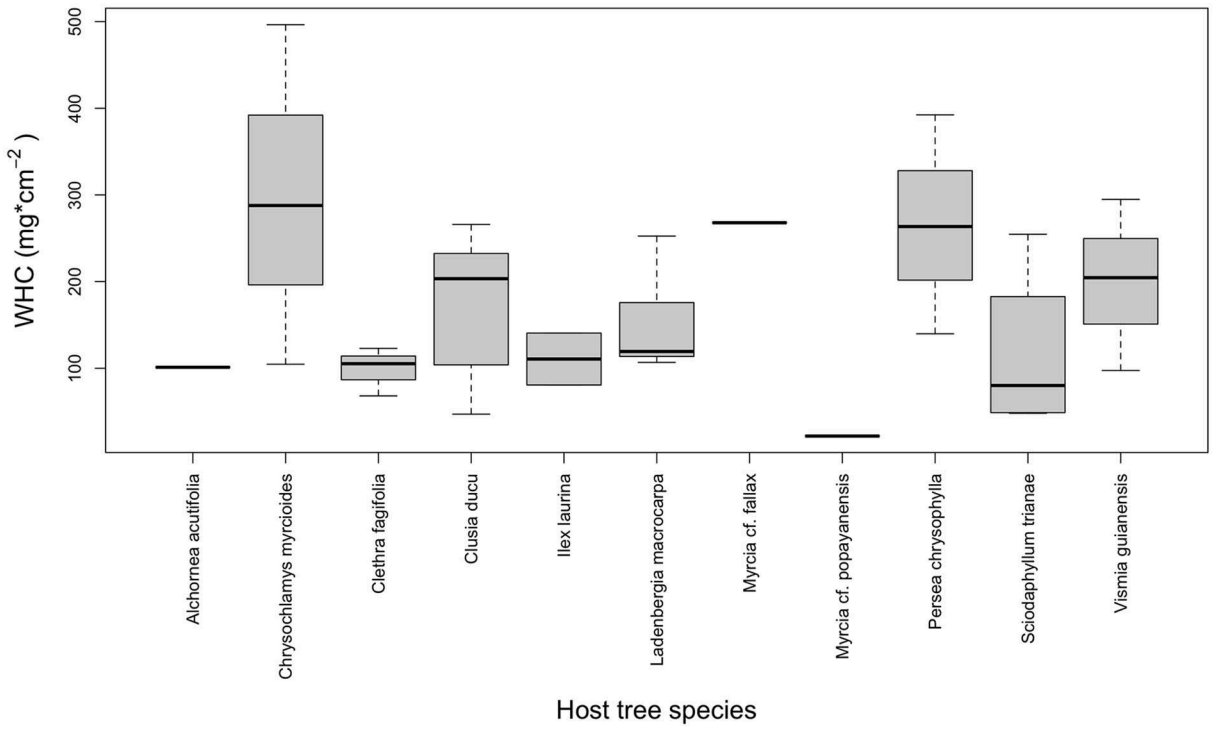

FIGURE 2
Boxplot of water-holding capacity content (WHC) among host tree species.

species producing seeds will increase populations within the forests (Duarte and Gandolfi, 2017). It is also recommendable to explore the viability of these seeds and their established processes to improve conservation outcomes. An unexpected result was the differential effect of survival according to host tree species; five species showed an effect on survival. However, it needs to be made clear which factors or characteristics of tree species affect survival; for example, there is no relationship between structural characteristics that were relatively homogeneous. Species-controlled experiments will be necessary to understand the mechanisms that might be favoring survival in certain species. Moreover, overall annual mortality was not related to other climatic variables associated with precipitation. However, a higher seasonality of precipitation was related to the percentage of overall mortality. This indicates that although epiphytic species are adapted to minimum levels of annual precipitation, they are more affected by extreme precipitation events or drought, indicating that the survival

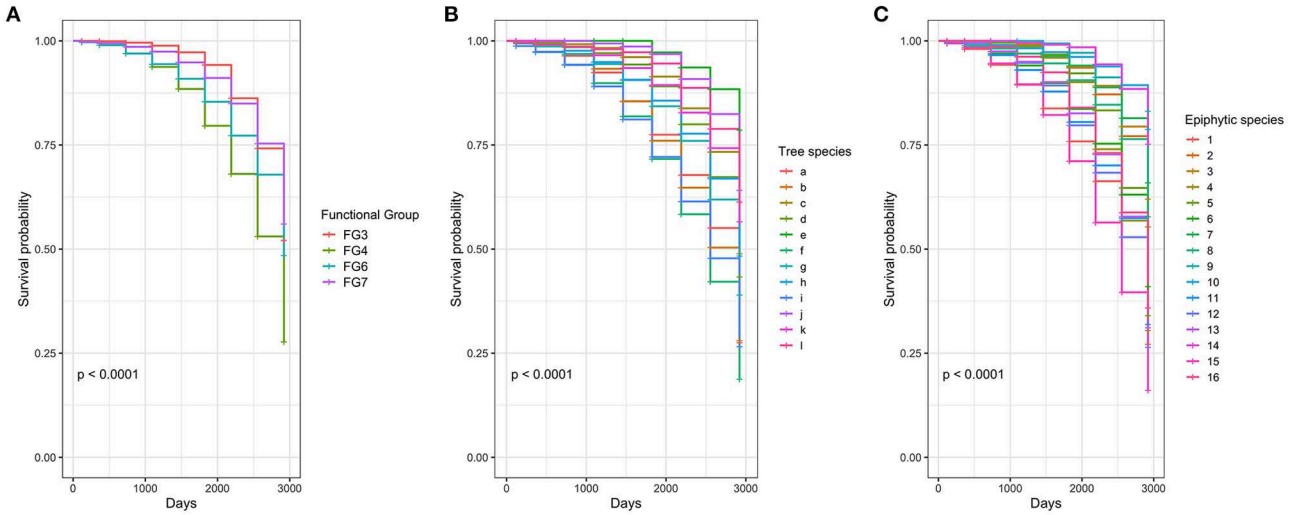

FIGURE 3
Survival probability according to **(A)** functional groups, **(B)** host tree species, and **(C)** epiphytic species. Letters in **(B)** are related to a, *Alchornea acutifolia* Müll. Arg.; b, *Chrysochlamys myrcioides* Planch. and Triana; c, *Clethra fagifolia* Kunth; d, *Clusia ducu* Benth.; e, *Eschweilera antioquensis* Dugand and Daniel; f, *Ilex laurina* Kunth; g, *Ladenbergia macrocarpa* (Vahl) Klotzsch; h, *Myrcia* cf. *fallax* (Rich.) DC.; i, *Myrcia* cf. *popayanensis* Hieron.; j, *Persea chrysophylla* L.E. Kopp; k, *Sciodaphyllum trianae* Planch. and Linden ex Marchal; l, *Vismia guianensis* (Aubl.) Choisy. Numbers in **(C)** correspond to 1, *Anthurium nigrescens*; 2, *Cyrtochilum divaricatum*; 3, *Dichaea morissii*; 4, *Masdevallia amanda*; 5, *Masdevallia platyglossa*; 6, *Maxillaria brevifolia*; 7, *Odontoglossum sceptrum*; 8, *Oncidium cultratum*; 9, *Philodendron danielii*; 10, *Pleurothallis lindenii*; 11, *Racinaea penlandii*; 12, *Racinaea* sp; 13, *Racinaea subulata*; 14, *Stelis crassilabia*; 15, *Tillandsia archerii*; and 16, *Tillandsia tetrantha*.

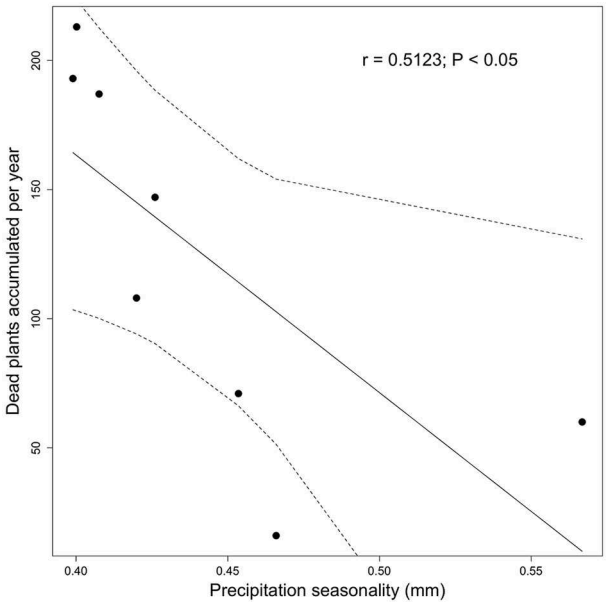

FIGURE 4
Precipitation seasonality vs. dead plants accumulated per year.

probability would be more favored by years with more homogeneous precipitation. This may be essential in order for epiphytes to continue surviving in a rapidly changing climate, as demonstrated by Nadkarni and Solano (2002), who found that increased climatic condition variability may reduce epiphyte assemblage longevity.

Epiphytes have shown a relationship with substrates that allow them to retain water (Dematte and Dematte, 1996; Ghosal et al., 1999). However, differences were not registered after using both substrates (one made of fique fiber and the other made of coconut mesocarp fibers) on individuals from the same species. Natural fibers used have effectively substituted substrates needed for the establishment, which under natural conditions might take years to accumulate (Nadkarni, 2000; Cobb et al., 2001). It is highly recommendable to evaluate the effectiveness of artificial substrates in further studies. Moreover, bark water-holding capacity varied widely among species, and we did not find a direct relationship with survival. In this study, we used plant-associated substrates that can minimize the direct effect of the bark. A similar effect may be occurring in cloud forests, like our site study, where it is common for soil and bryophytes to accumulate massively on top of branches and trunks, forming an interface between the bark and the plants. However, the effect of the bark water retention capacity could be more significant in other ecosystems, such as dry forests or lowland tropical forests with a low presence of fog. In addition, it is important to note that in this study, we only took a measure of the bark water-holding capacity at breast height (1.3 m), but the bark water-holding capacity can vary vertically, and it also depends on the age of the individual and the site conditions (Klamerus-Iwan et al., 2020).

Considering that microclimatic conditions in the first layers of a secondary forest are not expected to vary (Jucker et al., 2018), there was no effect on epiphyte survival within the first 4 m from the base of the host tree, where temperature and humidity facilitate their establishment. Selected host trees did not present branches or bifurcations below the first 4 m from their bases, and therefore, all individuals were positioned on the trunk. However, it is well-documented that branches of the host tree provide higher stability and an opportunity for natural accumulation and retention of substrate for epiphytes (Ingram and Nadkarni, 1993; Zuleta et al., 2016). In our experience, individuals weakly attached to the trunk are more likely to undergo death during the first 6 months after translocation. Therefore, we highly recommend firmly attaching

(without movement) individuals to the trunk for at least 6 months, a time in which the majority of them will develop new roots. Moreover, establishing new plants from seedlings or juvenile stages directly in the host tree could guarantee a better long-term establishment of epiphytes, especially for bromeliads (FG 4), allowing the development of holdfast roots, characteristic of these species.

Overall survival of 44% of translocated individuals represents a medium survival percentage. This result indicates that translocation may be an effective conservation action for maintaining individual epiphytes of the selected species in secondary and fragmented forests. Translocation can be a cost-effective measure considering proximity and accessibility to the selected secondary forest and people involved (translocation of 629 epiphytic individuals on 70 host trees took four people and 10 working days). The average cost for the translocation in 2013 for each individual was estimated to be 2.5 dollars (COP 3700), not including technical or professional expenses. As implemented in sites prone to be deforested, this action is an ultimate measure of giving a second chance to these species. Achieving an effective ecological restoration of the epiphyte community requires a deep understanding of the biological aspects of the species as well as their responses to translocation protocols. Colombia's National Development Plan (2018–2022) aims to better leverage natural resources in service to the energy industry, which may increase pressures on the epiphytes assemblages that rely on these same natural areas. However, this context also represents an exciting and promising opportunity to engage local environmental authorities and communities toward biodiversity protection. As local environmental authorities and communities rely on their environments, it is more likely that community-based conservation approaches (engaging local communities in the protection of biodiversity actions) can contribute to protecting forests and their associated epiphyte assemblages. Based on our results, these forests can recover ecological attributes obtained by the epiphyte assemblages by introducing and effectively maintaining populations of different species.

According to the Ministry of Environment and Sustainable Development of Colombia, compensation processes for environmental damage caused to epiphytes (including lichens, bryophytes, orchids, and bromeliads) have mainly focused on strategies of translocation of a determined percentage of individuals to nearby forests and to host trees with similar structural conditions. Details of these processes, such as methods used for plant selection, nursery conditions (or step houses), types of ties used, and type of fertilization, remain to be described. Therefore, documentation and publication of these processes, including successes and failures, are urgently needed. Although compensation is not restricted to reintroduction processes, most compensation processes have focused on it in Colombia. Other measures may include research to generate better management, addressing fundamental questions on epiphyte ecology, adaptation to climate change, and mitigation of species loss. Evaluation of strategies that include propagation from seeds using *in vitro* protocols or nurseries must be included to enhance management and translocation success (Phillips et al., 2020).

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

AB designed the study, monitored each year, and wrote the manuscript. JC-C proved and analyzed the data. All authors jointly discussed and agreed to the final version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ffgc.2023.834669/full#supplementary-material

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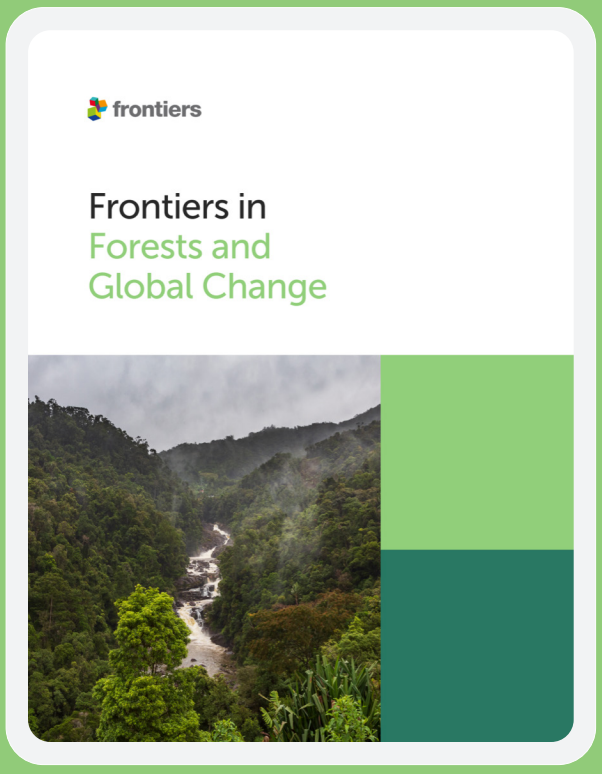

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