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RESEARCH ARTICLE

High nitrogen-fixing rates associated with ground-covering mosses in a tropical mountain cloud forest will decrease drastically in a future climate

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Abstract

1. Tropical mountain cloud forests (TMCF) harbour a high bryophyte (mosses and liverworts) biomass and diversity. Furthermore, the high air humidity makes these forests well suited for bryophyte-associated nitrogen (N_2) fixation by cyanobacteria, providing a potentially important source of N input to the ecosystem. However, few studies have assessed bryophyte-associated N input in these ecosystems, and these have focused on epiphytic bryophytes, whereas abundant ground-covering bryophytes have not been included.
2. In this study, we quantified N_2 fixation rates associated with bryophytes, focusing on ground-covering mosses in a neotropical mountain cloud forest. Furthermore, we identified the effects of climate change (higher temperature 10 vs. 20° and lower bryophyte moisture level 50% vs. 100%) on N_2 fixation across bryophyte species and groups (mosses and liverworts).
3. Nitrogen fixation rates associated with ground-covering moss species were up to 2 kg N ha^{-1} $year^{-1}$, which is comparable to other N inputs (e.g. N deposition) in tropical cloud forests. Furthermore, changes in temperature showed little effect on N_2 fixation, but low moisture levels significantly suppressed N_2 fixation activity. We found low N_2 fixation activity associated with the investigated liverworts.
4. Our results demonstrate the importance of ground-covering, moss-associated N_2 fixation as a N source in tropical cloud forests and suggest that predicted future declines in precipitation in these systems will reduce N inputs from bryophyte-associated cyanobacteria.

KEYWORDS
bryophytes, climate change, cyanobacteria, liverworts, mosses, nitrogen fixation, nitrogen input, tropical mountain cloud forest

1 | INTRODUCTION

Large expanses of tropical mountain cloud forests (TMCF) and boreal forests receive low amounts of nitrogen (N) via deposition (Peñuelas et al., 2013; Vet et al., 2014), making biological nitrogen fixation (BNF) the main 'new' N input in these systems. Bryophytes are an abundant part of the vegetation in these forests and play a key role in ecosystem functioning (Benzing, 1998; Köhler et al., 2007). For instance, they host N_2-fixing bacteria (diazotrophs) (DeLuca et al., 2002; Markham & Fernández Otárola, 2021; Rousk, Jones, et al., 2013) capable of converting atmospheric N_2 into bioavailable N forms. In boreal forests, N_2-fixing bacteria associated with mosses are responsible for up to 50% of total ecosystem N input, sustaining plant productivity (Gundale, Nilsson, et al., 2012; Rousk, Jones, et al., 2013). Moss-associated N_2 fixation has been reported across boreal forests in Scandinavia as well as in North America (e.g. Jean et al., 2018; Rousk, Jones, & DeLuca, 2013) and in several moss species mainly belonging to the group of feathermosses. Yet, how widespread associations between mosses and cyanobacteria are in other forests, such as TMCF, remains unknown. However, the high bryophyte abundance in combination with high humidity levels as well as constant air temperatures throughout the year makes these forests suitable for high N_2 fixation rates associated with bryophytes (Cusack et al., 2009).

Biological nitrogen fixation (BNF) has been well documented in many lowland tropical forests (Brookshire et al., 2019; Van Langenhove et al., 2021). Here, symbiotic BNF is often the main BNF input pathway; however, the contribution of free-living N_2 fixation is also recognized as substantial (Cusack et al., 2009; Van Langenhove et al., 2020). Furthermore, free-living N_2 fixation can remain active in several N-rich tropical forests (Hedin et al., 2009; Reed et al., 2008; Zheng et al., 2018, 2020), and can contribute >10 kg N ha^{-1} $year^{-1}$ of new N into Asian tropical forests (Zheng et al., 2018). Also, in tropical moist forests across Latin America, free-living N_2 fixation activity contributes significantly to total N input, with average N input rates of 6 kg N ha^{-1} $year^{-1}$ (Reis et al., 2020), exceeding symbiotic BNF (3 Kg ha^{-1} $year^{-1}$). This is within the same order of magnitude as N deposition in these areas (2 to 10 kg N ha^{-1} $year^{-1}$) (Vet et al., 2014). Nitrogen fixation by free-living bacteria often includes N_2 fixation by bacteria associated with bryophytes (mosses and liverworts). Yet, studies on bryophyte-associated N_2 fixation in tropical forests are sparse (Cusack et al., 2009; Reis et al., 2020; Zheng et al., 2020), and in particular, studies in TMCF are, to our knowledge, even rarer (Markham & Fernández Otárola, 2021; Matzek & Vitousek, 2003).

Epiphytic bryophytes growing on trees are a major component of the vegetation in TMCFs (Horwath et al., 2019; Köhler et al., 2007) and most studies on bryophyte-associated N_2 fixation in tropical forests focus on epiphytic bryophytes. However, N_2 fixation associated with bryophytes growing on the forest floor is a potentially large, but overlooked N source for plants via the soil (Rousk, Sorensen, et al., 2017). Bryophytes growing as epiphytes on trees are more disconnected from the forest floor and may not have the same ecological role as a source of soil N, as it is seen in, for example, daily photosynthesis pattern between epiphytic and soil-covering bryophytes (Wagner et al., 2014). Furthermore, most topical studies focus on liverworts, but the type of association between diazotrophs and liverworts and mosses is different, which likely has consequences for the magnitude and climatic sensitivity of N_2 fixation between the bryophyte groups. Specifically, some liverworts form 'true' symbioses with diazotrophs, where the colonizers are hosted in specific structures (auricles) and bacterial morphology and physiology are impacted by the host (Adams & Duggan, 2008). In contrast, in most mosses, the association is epiphytic and often characterized as 'loose', with the diazotrophic bacteria colonizing the surface of moss leaves and stems (Figure 1b). But, little is known about the magnitude and physiological drivers of N_2 fixation associated with ground-covering bryophytes and in particularly ground-covering mosses in TMCFs.

Nitrogen fixation activity of bacteria hosted by bryophytes is affected by abiotic factors such as temperature and moisture availability (Cusack et al., 2009; Gundale, Nilsson, et al., 2012; Permin et al., 2022; Rousk, Jones, et al., 2013), with desiccation inhibiting activity (Rousk et al., 2014). Also increased N availability can inhibit N_2 fixation in mosses (Rousk, Rousk, et al., 2013; Wang et al., 2021) and moss pH can affect N_2 fixation activity (Alvarenga & Rousk, 2021; Liu & Rousk, 2021). Furthermore, specific morphology traits linked to bryophyte hydration rate (e.g. shoot length and leaf width) likewise affect diazotroph colonization and N_2 fixation activity (Liu & Rousk, 2021). The climate in TMCF is characterized by relatively cool temperatures and high air humidity (due to constant cloud immersion) throughout the year, suitable for bryophyte-associated N_2 fixation. But these factors are predicted to change in a future climate, for example, a decline in cloud formation (Helmer et al., 2019; Still et al., 1999) will likely decrease bryophyte-associated N_2 fixation in TCMFs. On the other hand, the expected increase in temperature in the relatively cool tropical mountain forests (IPCC, 2014; Metcalfe & Ahlstrand, 2019) can promote N_2 fixation activity by increasing enzymatic activity (Houlton et al., 2015). But increasing temperatures will also lead to higher evaporation rates (Foster, 2001) and since bryophytes lack mechanisms to regulate water loss (Elumeeva et al., 2011), the temperature rise may also lead to drier bryophytes, which again inhibits N_2 fixation activity (Gundale, Wardle, et al., 2012; Rousk et al., 2014). Thus, even if N_2 fixation associated with bryophytes in cloud forests occurs now, future climate changes are likely to affect the activity negatively, with consequences for ecosystem N input in TCMFs. Hence, the interaction between temperature and moisture is potentially an important driver of N_2 fixation activity in tropical bryophytes, but this has not yet been tested. Thus, large gaps remain in our understanding of whether bryophytes in TMCFs host diazotrophs, if N_2 fixation activity differs between ground-covering and epiphytic species, how important this association is in terms of N input rates, and how N_2 fixation will be affected by climate change.

The aims of this study were to (1) determine whether bryophytes, and in particularly ground-covering mosses, in a neotropical mountain cloud forest are colonized by diazotrophs, assessed with N_2 fixation

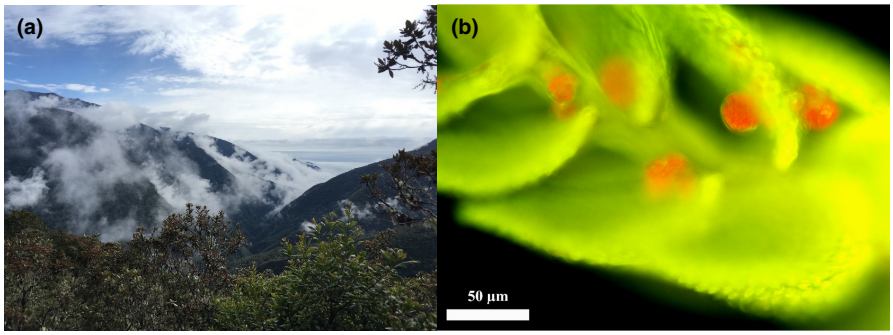

FIGURE 1 (a) View on a tropical mountain cloud forest (TMCF), Peru. Photo by Aline B. Horwath. (b) *Thuidium* sp. shoot at ×200 magnification under UV-fluorescence microscope. *Nostoc* sp. colonies are seen in bright red between moss leaf and stem in green. Photo by Aya Permin

activity, (2) quantify the N input mediated by N_2 fixation associated with bryophytes in these systems, and (3) assess how N input rates are affected by changes in temperature and humidity. For this, we collected eight different bryophyte species (four ground-covering mosses and four epiphytic liverworts) in a TMCF in Peru, exposed these to different temperature and moisture levels in a laboratory experiment and measured N_2 fixation activity. Specifically, we hypothesized that (H1) N_2 fixation rates associated with the bryophyte community are comparable to N deposition in TMCF's, and to bryophyte-associated N_2 fixation in other ecosystems, though with interspecies variation due to differences in bryophyte microenvironment. (H2) N_2 fixation activity in liverwort–diazotroph associations is higher than the more loose associations that mosses and diazotrophs share. (H3) Decreases in moisture content inhibit bryophyte-associated N_2 fixation activity, whereas increasing temperatures enhance N_2-fixing activity only when moisture levels are high.

2 | MATERIALS AND METHODS

2.1 | Sampling

Bryophyte samples were collected in a TMCF near the Wayqecha Research Station (latitude: 13°2′56″S, longitude 71°32′13″W), southeastern Peru (Figure 1a). The field sampling was performed with the full cooperation and approval of the landowner (Conservación Amazónica—ACCA). A permit for sampling outside of protected areas was secured from the national agency responsible prior to fieldwork (Servicio Nacional Forestal y de Fauna Silvestre, permit number: 064-2017-SERFOR-DGGSPFFS). The site is located about 3,000 m above sea level, with mean annual air temperature of 10.9°C and relative air humidity of 90.4% (2018 mean values from Wayqecha metrological station), and mean annual rainfall of 1,776 mm (Horwath et al., 2019; Metcalfe & Ahlstrand, 2019). Bryophyte collection was performed in November 2018 and each replicate sample (n = 6) consisted of composite bryophyte shoots collected within 2 m and with *c*. 10 m between replicates and epiphytes were collected on different trees. Four liverwort species were collected; *Bazzania* sp. (up to 20% field cover), *Herbertus* sp. (up to 30% field cover), *Plagiochila* sp. (up to 30% field cover) *and Riccardia* sp. (up to 5% field cover); and four moss species were collected; *Campylopus* sp. (up to 90% field cover), *Dicranum* sp. (up to 3% field cover), *Rhodobryum* sp. (up to 3% field cover) and *Thuidium* sp. (up to 50% field cover). Only mosses growing on the forest floor were collected, whereas the liverworts were collected from tree trunks and branches 1–2 m above the ground. Samples were air-dried after collection, shipped to the University of Copenhagen and kept dry until initiation of the experiment (*c*. 1 week).

2.2 | Nitrogen deposition

To measure the N deposition in the forest sites, ion exchange resin capsules (Unibest International, Walla Walla, USA) were placed at the soil surface (n = 12) and in the top soil (3 cm depth, n = 9) within a few 100 meters of the moss sampling locations. The resin capsules were collected after 3 months and sent to Unibest International, where they were extracted and analysed for NH_4^+ and NO_3^-.

2.3 | Climate change treatments

All bryophytes were soaked in double distilled (dd) water to rehydrate and recover activity for *c*. 30 min (Rousk et al., 2014), after which they were placed in transparent plastic containers, and kept in climate chambers until the start of the experiment. Initially, samples were kept with a daily cycle of 12 h full light with ca. 200 µmol m^{-2} s^{-1} incoming photosynthetic active radiation (PAR) at 12°C, followed by 12 h darkness at 8°C. To prevent the bryophytes from drying out, we regularly sprayed the samples with dd water. The bryophytes were kept at these initial settings for 1 week to acclimatize, before they were randomly assigned to the treatments. For this, samples were divided, transferred to a transparent, 50-ml Falcon tube and assigned to one of the four climate treatments consisting of two temperature levels and two moisture levels. The climate conditions were selected to mimic the effects of projected climate change on temperature and (air) humidity in tropical cloud forests, including settings mimicking present natural conditions in a full factorial design. For each factor (temperature/humidity), two levels were included, with daily temperatures at 10°C (6°C at night) and 20°C (16°C at night) and 50% and 100% bryophyte humidity, where 10°C at 100% humidity mimics present conditions at the sampling site. In total, we had 192 experimental units (8 species*6 replicates*4 treatments). The

daily light cycle was 12 h light with ca. 200 µmol m^{-2} s^{-1} incoming PAR light and 12 h darkness. The bryophytes were kept like this throughout the experiment (35 days).

The moisture levels were controlled by weight, where the weight of each sample at full hydration (soaked in dd water for >30 min) equals 100% moisture and 50% moisture was obtained by letting the samples air-dry to half the fully hydrated weight. The moisture levels were controlled throughout the experiment by monitoring and adjusting the weight accordingly with dd water.

2.4 | Nitrogen fixation rates measured as acetylene reduction and ^{15}N-N_2 assimilation

To assess N_2 fixation rates associated with the bryophytes, we used the acetylene reduction assay (ARA) to measure the activity of the nitrogenase enzyme that catalyses N_2 assimilation (Rousk, Pedersen, et al., 2017). This was measured 3, 6, 18, and 35 days after initiation of the treatments. For this, the 50-ml Falcon tubes containing the bryophytes were sealed with a rubber septum and 5 ml of the headspace was replaced with 5 ml acetylene gas (Acetylene gas, technical grade, Air Liquide) using a syringe (final headspace concentration: 10% acetylene). Samples kept at the higher temperature (20°C) were incubated for 3.5 h, whereas samples kept at the low temperature (10°C) were incubated for 7 h, assuming a Q_{10} of 2 for N_2 fixation (Rousk, Pedersen, et al., 2017). After the incubation period, a 6-ml gas sample was extracted through the septum and transferred to a pre-evacuated 6-ml Exetainer vial (Labco). Background ethylene in the acetylene gas was measured on three samples containing 10% acetylene gas without bryophytes to account for any ethylene residue in the acetylene gas. These background values (mean area [±*SE*] = 2. 07 ± 0.1) were subtracted from all samples before further calculations were performed. The gas samples were analysed for ethylene production with a gas chromatograph equipped with a flame ionization detector (SRI 310C, SRI Instruments). To assess N_2 fixation activity over the whole course of the experiment, we calculated the cumulative N_2 fixation over the 35 days duration of the experiment. For the cumulative N_2 fixation activity across the experiment, we assumed 12 h of activity per day (= light hours) and multiplied the N_2 fixation activity measured at each time point with the numbers of days since the last measurement.

To calculate the conversion factor between produced ethylene (ARA) and fixed N_2 (Bellenger et al., 2020), we performed a ^{15}N assimilation assay (as in Rousk & Michelsen, 2017). This enabled us to convert the rates measured as ethylene produced to N_2 fixed across the samples to be able to compare with N inputs via N deposition and to other moss-associated N_2 fixation rates reported in the literature. Due to the low N_2 fixation activity in liverworts measured with the ARA, this was only done for the mosses. For this, we first measured the activity with ARA (24 h incubation with 10% acetylene as above) followed by 24-h incubation with 7.5 ml of 98% enriched ^{15}N-N_2 gas (Eurisotop, Cambridge Isotope Laboratories), corresponding to 15% of the headspace. Mosses were kept at 20°C during the day and 16°C during the night for both assays (ARA and ^{15}N-N_2). The mosses were hereafter dried at 70°C for 24 h, ground and analysed for isotopes of N, C, and total N on an Isoprime isotope ratio mass spectrometer (Isoprime Ltd, Cheadle Hulme). The conversion factor between ethylene production and N_2 fixation was calculated with the formula from Liengen (1999).

2.5 | Ecosystem-level nitrogen fixation

To estimate N_2 fixation activity per ground area (Table S1) based on the moss species recorded, we used the cumulative N_2 fixation activity across the experiment and calculated the biomass (dry weight) per ground area for each moss species, multiplied with the ground cover percent in the forest sites. The ground cover percentages were estimated visually. It was not possible to estimate the biomass of the moss *Campylopus* sp. since this moss was too attached to the underlying soil and any attempt to separate the samples from the soil would have damaged the moss. Hence, the average biomass per ground area of the other mosses was used.

2.6 | pH of mosses

The pH of the mosses was measured by adding 50 ml dd water to 3 g (fresh weight) sample in a Falcon tube, shaking for 1 h on a table shaker before pH was measured with a pH meter (PHM240 pH/ION Meter, MeterLab, Radiometer Copenhagen).

2.7 | Statistical analyses

All statistical analyses were carried out in R (RStudio Team, 2020). To test for differences in moss characteristics (pH, Total N) among species, linear models (lm) were run with species as factor. Linear models (lm) were also run to test for differences in cumulative N_2 fixation rates between bryophyte groups (moss and liverworts), and within species and climate change treatments. To test for differences in N_2 fixation (as measured with ARA) between species and climate change treatments over time, linear mixed models (lmer) were performed with sampling unit as a random effect, to account for repeated measurements. These tests were run both with all species included and with each species separately. ARA data were log transformed to meet the model assumption of normal distribution. Tukey post-hoc test was employed.

3 | RESULTS

3.1 | Nitrogen deposition

The mean N deposition that reach the forest floor measured with resin capsules at the sampling site was 4.8 (1.1) kg N ha^{-1} $year^{-1}$ and the bioavailable N in the top soil was 1.6 (1) kg N ha^{-1}.

3.2 | Conversion factor

The conversion factor between N_2 fixation activity as reduced ethylene (measured with ARA) and fixed N_2 was similar in the mosses *Dicranum* sp., *Rhodobryum* sp. and *Thuidium* sp., ranging between 2.9 and 3.1 (Table 1), similar to the theoretical conversion factor of 3 (Hardy et al., 1968). Due to very low N_2 fixation activity in the moss *Campylopus* sp., which made the calculation for this species not possible, the average conversion factor for the other three moss species was used (2.96).

3.3 | Cumulative N_2 fixation across moss species and climate treatments

All bryophytes (liverworts and mosses) were colonized by N_2-fixing bacteria, though with much lower N_2 fixation activity associated with the investigated liverworts compared to the mosses. There was little variation in N_2 fixation activity over time for the moss species, for example, rates associated with *Dicranum* sp. differed between day 18 and day 3 and 6 at 50% moisture level and 10°C, but the 100% treatment did not differ, whereas there were no differences over time detected for the liverworts. As we found no clear trend in N_2 fixation activity over time for either the moss (Figure 2) or the liverwort species (Figure 3), we calculated the cumulative values for better interpretation of treatment effects. Thus, N_2 fixation activity is expressed and discussed as cumulative values throughout the manuscript. We detected N_2 fixation activity associated with all four moss species assessed in this study, but markedly different levels among species (Figure 4). Cumulative N_2 fixation activity over the course of the experiment (*c*. 1 month) was ~100 times higher in *Dicranum* sp. and *Thuidium* sp. than activity associated with *Campylopus* sp. and *Rhodobryum* sp., across all treatments ($p < 0.0001$). The highest activity was detected in association with *Dicranum* sp. and *Thuidium* sp. in treatments with the high moisture level (100% moisture). Here, cumulative N_2 fixation up to 923 $\mu g\, N\, g\, dw^{-1}$ was detected in *Thuidium* sp. in the climate treatment with 20°C and 100% moisture. In contrast, cumulative values associated with *Campylopus* sp. and *Rhodobryum* sp., were low, and close to zero in all treatments (Figure 2). The low moss moisture treatment (50% moisture) negatively affected N_2 fixation activity overall ($p < 0.0001$), but there was again substantial interspecies variation. Thus, mean activity associated with *Dicranum* sp. and *Thuidium* sp. declined up to 97% when exposed to the reduced moisture level, whereas for *Campylopus* sp. and *Rhodobryum* sp., there was only a tendency of reduced activity with low moisture level (Figure 4). The temperature treatment had no effect on N_2-fixing activity for any of the assessed mosses (Figure 4). However, interestingly, there was a tendency for reduced activity associated with *Rhodobryum* sp. when exposed to the high temperature, but this was not significant.

3.4 | Cumulative N_2 fixation across liverwort species and climate treatments

Nitrogen fixation activity in the liverworts was generally lower compared to the activity associated with the mosses ($p < 0.0001$), with mean cumulative values ranging from 0.003 N2 fixation activity to 4.2 µmol ethylene g/dw (Figure 5). As for the moss species, N_2 fixation activity differed among the four liverwort species ($p < 0.0001$, Figure 5), with the highest activity associated with *Herbertus* sp. at 100% moisture. Nitrogen fixation was negatively impacted by the low moisture level ($p < 0.0001$, Figure 5). The negative impact of low moisture was driven by the large response to low moisture level in N_2 fixation activity associated with *Herbertus* sp. compared to the other liverwort species. Like the moss species, temperature did not affect N_2-fixing activity for any of the assessed liverwort species (Figure 5).

3.5 | Moss characteristics

The pH of the mosses ranged between 4.37 and 5.75 and differed among species ($p < 0.001$), with higher pH of the moss *Thuidium* sp. compared to *Campylopus* sp. (Table 1). Nitrogen content differed among the species ($p < 0.01$) with higher N content in *Thuidium* sp. compared to *Dicranum* sp. Likewise, the C:N ratio among species differed ($p < 0.01$), with higher C:N ratio in *Dicranum* sp. compared to *Thuidium* sp. The mean density of the mosses ($g\, m^{-2}$) did not differ among species (Table 1).

4 | DISCUSSION

We detected N_2 fixation activity in all of the assessed bryophytes, though with different rates between the groups (liverworts and

TABLE 1 Characteristics of moss species collected in a tropical mountain cloud forest, Peru. $n = 1$–3 (pH), $n = 4,6$ (C and N), $n = 6$ (biomass) and $n = 3$ (conversion factor ethylene reduced to N_2 fixed). Superscript letters indicate significant differences between moss species (Tukey). Biomass and conversion factor values for the moss *Campylopus* sp. are mean of the other three mosses (*Dicranum* sp., *Rhodobryum* sp. and *Thuidium* sp.)

Species	pH mean	*SE*	Total N (%) mean	*SE*	C/N mean	*SE*	Biomass (g/m^2)	*SE*	Conversion factor (ethylene to N_2)
Campylopus sp.	4.37^{a}	0.1	1.8^{ab}	0.1	23.5^{ab}	2.4	245	35	3.0
Dicranum sp.	4.96^{ab}	0.1	1.4^{a}	0.1	29.3^{b}	1.9	425	31	2.9
Rhodobryum sp.	5.62^{bc}	na	1.8^{ab}	0.1	22.9^{ab}	1.2	164	44	3.1
Thuidium sp.	5.75^{c}	0.2	1.9^{b}	0.1	21.8^{a}	0.8	146	12	2.9

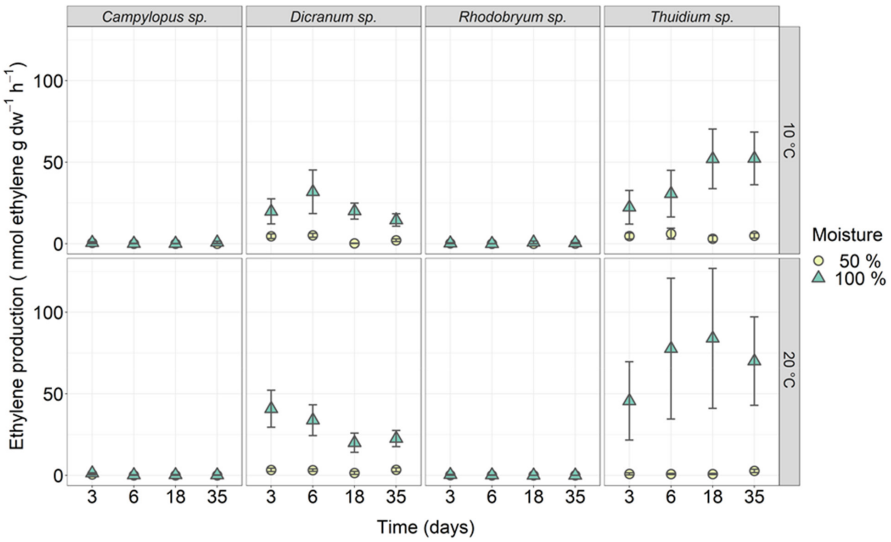

FIGURE 2 Ethylene production (nmol ethylene g dw^{-1} h^{-1}) in the four mosses *Campylopus* sp., *Dicranum* sp., *Rhodobryum* sp. and *Thuidium* sp. collected in a tropical mountain cloud forest, Peru. Given are mean values ±*SE* for each time point (3, 6, 18 and 35 days) in the climate treatments, temperature (10°C and 20°C) and moisture (50% and 100%). $n = 6$

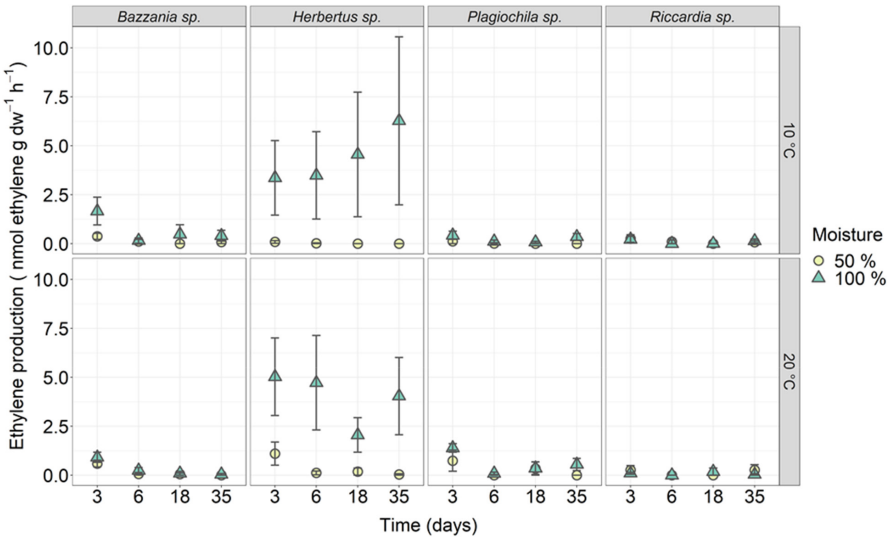

FIGURE 3 Ethylene production (nmol ethylene g dw^{-1} h^{-1}) in the four liverworts *Bazzania* sp., *Herbertus* sp., *Plagiochila* sp. and *Riccardia* sp. collected in a tropical mountain cloud forest, Peru. Given are mean values ±*SE*, in each time point (3, 6, 18 and 35 days) in the climate treatments, temperature (10°C and 20°C) and moisture (50% and 100%). $n = 6$

mosses) and among species within each group. This confirms only partly our first hypothesis (H1) as we found only N_2 fixation activity associated with mosses comparable with N deposition in TMCFs and to bryophyte-associated rates in other ecosystems, and not with liverworts. This is, to our knowledge, one of the first studies that reports N_2 fixation associated with a range of bryophyte species in TMCFs, especially associated with ground-covering mosses. Our results demonstrate that N_2 fixation associated with moss species on the ground potentially contributes significantly to ecosystem N input in TMCF, though it varies greatly among species.

4.1 | Nitrogen fixation activity across bryophyte groups and species

Large differences in N_2 fixation rates among species within each group (liverworts and mosses) were detected. Nitrogen fixation activity in mosses seems to be much more widespread and with higher rates, compared to the liverworts. This finding is contrary to our expectation of a closer association between liverworts and their N_2-fixing bacterial community leading to higher N_2 fixation activity (H2). However, none of the liverworts assessed in this study belong to genera already known to form 'true' symbioses with diazotrophs (Adams & Duggan, 2008). Specific bryophyte traits are a driving factor of associated N_2 fixation, for example, by controlling bryophyte moisture level and thereby diazotroph colonization and activity (Liu & Rousk, 2021). The liverwort associated with the highest N_2 fixing activity in our study, *Herbertus* sp., is a 'leafy' liverwort, with morphology similar to mosses in terms of size and structure, separated into stem and leaves, which could explain the similar high N_2 fixation activity associated with this liverwort species. In fact, differences in moss traits could likewise explain the differences in N_2 fixation activity among moss species found in this study that either promote or inhibit the bacterial community in terms of colonization and activity. However, when comparing the moss characteristics (Table 1) with the N_2 fixation pattern among species, there was no clear link between higher N_2

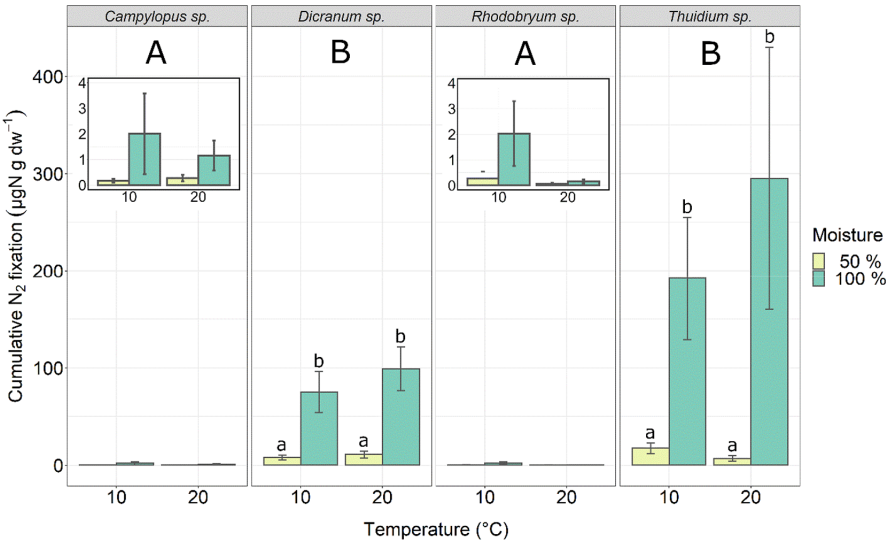

FIGURE 4 Cumulative N_2 fixation in the four mosses *Campylopus* sp., *Dicranum* sp., *Rhodobryum* sp. and *Thuidium* sp. collected in a tropical mountain cloud forest, Peru. Given are mean cumulative values ±*SE*, over the course of the experiment (1 month) in the four climate treatments, temperature (10°C and 20°C) and moisture (50% and 100%). Uppercase letters indicate significant differences among moss species and lowercase letters indicate differences among treatments within the given moss species based on Tukey post-hoc tests. Values for the moss species *Campylopus* sp. and *Rhodobryum* sp. are also given in the inserts to enable easier read of the values. $n = 6$

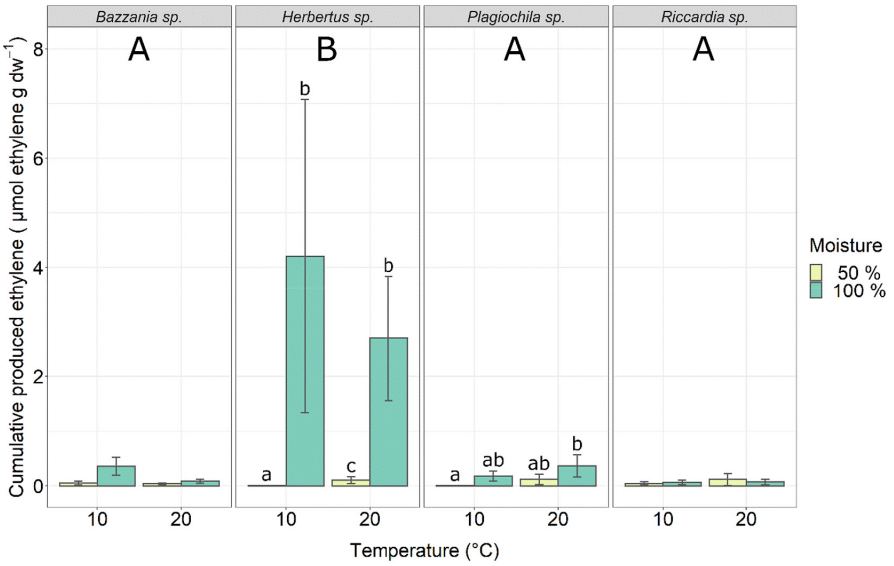

FIGURE 5 Cumulative produced ethylene as measure of N_2 fixation in the four liverworts *Bazzania* sp., *Herbertus* sp., *Plagiochila* sp. and *Riccardia* sp. collected in a tropical mountain cloud forest, Peru. Given are mean cumulative values ±*SE*, over the course of the experiment (1 month) in the four climate treatments, temperature (10°C and 20°C) and moisture (50% and 100%). Uppercase letters indicate significant differences among moss species and lowercase letter indicates differences among treatments within the given moss species based on Tukey post-hoc tests. $n = 6$

fixation activity found associated with the mosses *Dicranum* sp. and *Thuidium* sp. and the traits measured in this study (pH, total N, C:N ratio and biomass).

Our finding of major differences in N_2 fixation rates between bryophyte groups (liverworts and mosses) and among species within the groups underlines the importance of recording functional group and species level activity, which is also true for boreal forest mosses, where large differences in N_2 fixation activity among moss species are also found (Jean et al., 2020; Rousk, Pedersen, et al., 2017).

We found interesting differences in N_2 fixation activity between *Dicranum* sp. reported from boreal forests and the estimates in this study. While studies on *Dicranum* sp. in boreal forests report no activity associated with the species in these systems (e.g. Bay et al., 2013), we found that *Dicranum* sp. has one of the highest N_2 fixation rates in this study. This suggests that other factors than host identity (assuming closely related species form similar microhabitat and support similar bacterial colonizers) are important for controlling N_2 fixation activity. One such alternative factor could be the substantial differences in physical environment between tropical and boreal forests, such as nutrient availability (Du et al., 2020), temperature and precipitation (Jean et al., 2018; Metcalfe & Ahlstrand, 2019) and/or diazotroph community composition, as

an important driver for the bryophyte–bacterial association in *Dicranum* sp., but further investigation is needed.

4.2 | Effects of climate treatments

Our results demonstrate the strong control of bryophyte moisture level on N_2 fixation activity associated with bryophytes in TMCF, with a steep decline in activity in samples exposed to the low moisture level, confirming our hypothesis of reduced bryophyte-associated N_2 fixation activity with decreased moisture content (H3). Tropical mountain cloud forests are characterized by high air humidity due to frequent fog immersion (Foster, 2001). However, climate change is expected to decrease air humidity in TMCFs due to decreasing cloud formation and cover (Helmer et al., 2019; Still et al., 1999). This, together with higher evaporation rates imposed by higher temperatures, will lead to drier bryophytes. Thus, our findings on decreased N_2 fixation rates in less humid bryophytes suggest a major decline in N_2 fixation activity in the future, thereby reducing bryophyte-associated N input into these ecosystems. Interestingly, our hypothesis regarding the control of temperature (also H3) was not confirmed with our experimental setup, as we did not detect an effect of temperature on N_2 fixation activity in any of the investigated bryophytes in our study. Since temperature has been found to impact moss-associated N_2 fixation in other studies (e.g. Gundale, Nilsson, et al., 2012), this is somewhat surprising but underlies the importance of moisture, and the adaption to these conditions in these constantly moist tropical systems. However, we cannot exclude that the temperature levels we used were not extreme enough to cause a response. Only the moss *Rhodobryum* sp. had a tendency for lower N_2 fixation activity when exposed to the higher temperature. The clear control of moisture over temperature found in our study highlights the importance of humidity in shaping N_2 fixation activity and/or community structure of the N_2-fixing bacteria hosted by bryophytes in TMCFs. Furthermore, our results suggest that the cyanobacterial communities hosted by the bryophytes are adapted to the high and stable moisture level in the cloud forest, and are not capable of adjusting their N_2-fixing activity to the lowered moisture and higher temperature levels we exposed them to over *c*. 1 month.

4.3 | Conversion factor

The recent meta-analysis by Soper et al. (2021) shows a bimodal distribution of R ratio (conversion factors between ARA and fixed N_2) in mosses, likely due to different nitrogenase forms, with different enzyme efficiencies (Darnajoux et al., 2019). The most common nitrogenase form requires Molybdenum (Mo), but in areas that are Mo-limited or where the host lacks roots (like mosses), alternative nitrogenases can contribute to BNF (Darnajoux et al., 2019). Interestingly, the R ratios we obtained for the diazothrophs associated with the mosses in this study, were not bimodal, but similar to the theoretically R ratio of 3 (Hardy et al., 1968), suggesting that the diazotrophs on the mosses here expressed dominantly the common Mo-nitrogenase form unlike, for example, the alternative forms in mosses in the arctic (Rousk, Degboe, et al., 2017).

4.4 | Upscaling moss-associated nitrogen fixation

When scaling N_2 fixation up, yearly N_2 fixation rate associated with the ground-covering mosses per ground area (summing up the rates per species recorded and applying their ground cover %) in the treatments similar to the current conditions in the cloud forest (10°C, 100% moisture) was ~2 $kg\,N\,ha^{-1}\,year^{-1}$. If the rates measured in our study were found to be similar to in situ rates at the scale of the ecosystem and over the growing season, then we could expect rates in the same order of magnitude as N deposition rates in the same cloud forest sites (4.8 ±1.0 $kg\,N\,ha^{-1}\,year^{-1}$), bioavailable N in the top soil (1.6 ±1 kg N/ha) and estimated N deposition in this region (~4.5 $Kg\,N\,ha^{-1}\,year^{-1}$; Phoenix et al., 2006). Furthermore, the N_2 fixation rates are similar to the ones reported from boreal forest ecosystems, 1.5–2.0 $kg\,N\,ha^{-1}\,year^{-1}$ (DeLuca et al., 2002). This highlights the importance of N input via the ground-covering mosses in TMCFs. Yet, N_2 fixation rate associated with the ground-covering mosses is reduced to ~0.07 $kg\,N\,ha^{-1}\,year^{-1}$ when exposing the mosses to the climate change treatment (20°C, 50% moisture).

To our knowledge, this is the first time such relatively high N_2 fixation rates have been reported in association with ground-covering mosses in TMCFs. Only few studies have focused on N_2 fixation activity associated with ground-covering mosses, and even fewer compare a range of different moss species. One study that recorded N_2 fixation activity from ground-covering mosses in a cloud forest found much lower rates ca. 0.04 $kg\,N\,ha^{-1}\,year^{-1}$ (Markham & Fernández Otárola, 2021), compared to *c*. 1.4 $Kg\,N\,ha^{-1}\,year^{-1}$ associated with epiphytic mosses. Likewise, another study from a tropical montane rain forest site reported similar low rates between 0.08–0.29 $kg\,N\,ha^{-1}\,year^{-1}$ associated with mosses collected on the ground or lower parts of tree trunks (Matzek & Vitousek, 2003). Differences in bryophyte abundance and species composition between the investigated forests can explain the differences in N_2 fixation activity rates on area basis. This is further supported by the large differences between moss species' contribution to ecosystem N_2 input in our study, where, for example, *Thuidium* sp. contributes with 50-fold higher rates than *Campylopus* sp. and *Rhodobryum* sp. which is not directly linked to differences in moss cover but likely due to differences in moss traits as discussed above. Interestingly, the large difference in N_2 fixation activity between liverworts and mosses that we found in our study are not as clear in these studies (Markham & Fernández Otárola, 2021; Matzek & Vitousek, 2003). Furthermore, Markham and Fernández Otárola (2021) estimated the total amount of N_2 fixed from all cryptogams (soil covering and epiphytic) to 6 $kg\,ha^{-1}\,year^{-1}$, with the liverworts accounting for the major part of the total amount of fixed N. This discrepancy underlines the need for further research the role of bryophytes and in particularly ground-covering mosses in TMCFs and other wet forests, to

better understand the magnitude, variability and drivers of BNF in these biomes across the globe.

Even though it is the epiphytic bryophytes that dominate TMCF (Horwath et al., 2019), the high N_2 fixation activity associated with the ground-covering mosses detected in this study suggests substantial N input to the TMCFs from this bryophyte group, that will likely change in a future climate. Furthermore, predicted decline in bryophyte biomass and fitness due to environmental change (Horwath et al., 2019; Metcalfe & Ahlstrand, 2019) will likewise have an indirect negative effect on bryophyte associated N input in TMCFs. Hence, under future climate scenarios bryophyte-associated N_2 fixation in tropical mountain cloud forests may contribute less N to sustain plant growth, and thereby soil carbon sequestration (Lu et al., 2021).

AUTHORS' CONTRIBUTION

A.Pe., A.Pr. and K.R. designed and planned the experiment; A.B.H. performed the fieldwork; A.Pe. conducted the experiment and analysed the data. A.Pe. wrote the article and all authors contributed to the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data available from the UCPH ERDA digital repository: https://doi.org/10.17894/ucph.a6973e53-616e-46cf-836b-8fa88ff77c78 (Permin, 2022).

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