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Original Articles

Response strategies of N-fixation by epiphytic bryophytes to water change in a subtropical montane cloud forest

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ABSTRACT

Water changes are predicted to regulate physiological activities of bryophytes characterized by poikilohydric gametophytes. In montane forest ecosystems, nitrogen(N)–fixing bryophyte–cyanobacteria associations are main N resources. The aim of this study was to assess how bryophyte–associated microbiomes and their nitrogenase activity response to instant or long-term water content changes. We investigated the cyanobacterial colonization and nitrogenase activity of four epiphytic bryophyte species in a subtropical montane cloud forest during the dry and rainy seasons in Ailao Mountains, Yunnan, southwestern China. We also evaluated the nitrogenase activity of bryophyte–cyanobacteria associations in response to different water contents in laboratory experiment. The degree of cyanobacterial colonization was evaluated using ultraviolet fluorescence microscopy, and nitrogenase activity of bryophyte–cyanobacteria associations were measured using the acetylene reduction assay (ARA). Cyanobacteria showed an association with all four bryophyte species, with 1.04–3.37% area colonized of the shoot and 10.16–20.21 nmol $C_2H_4 \cdot g^{-1} \cdot h^{-1}$ average nitrogenase activity. Nitrogenase activity was positively related to cyanobacterial colonization ($R = 0.742$; $P = 0.0349$). The relationship between water content and nitrogenase activity was unimodal, and both water surplus and shortage inhibited N fixation. Furthermore, long–term drought conditions reduced the degree of cyanobacteria colonization on bryophyte shoots, resulting in decreased nitrogenase activity in the dry season. These results indicate that different response strategies of N fixation operate in bryophyte–cyanobacteria associations to cope with instant and long–term changes in water availability. Our data suggest that both extreme precipitation and drought have a negative impact on N fixation of cyanobacteria–bryophyte associations.

1. Introduction

Nitrogen (N) is one of the most important nutrients required for plant development and one of the main factors limiting net primary production in terrestrial ecosystems (Aber, 1992; Johnson and Turner, 2014). Because of the lack of industrial input, low wet and dry atmospheric N depositions, N-fixing bryophyte-cyanobacteria associations, cyanolichens, and soil surface microbes are the main sources of N in many natural terrestrial forest ecosystems, especially in montane forests that contain a small number of symbiotic N-fixing vascular plants (Crews, 1999) but support a large proportion of epiphytic cryptogams (Ma et al., 2009; Liu et al., 2018). Most studies focusing on bryophyte-cyanobacteria associations are conducted on the forest floor of boreal forests at high latitudes and *Pleurozium schreberi*, *Hylocomium splendens*, and *Sphagnum palustre* are the dominant species (DeLuca et al., 2002; Zackrisson et al., 2004; DeLuca et al., 2007; Bay et al., 2013; Stuiver et al., 2015), Cusack et al. (2009) revealed the importance of the biological N fixation by cyanobacteria associated with terrestrial mosses in humid tropical forests. N fixation by moss-cyanobacteria associations in the forest floor was closely constrained by N, phosphorous (P) addition or stoichiometry in a subtropical forest, southern China (Zheng et al., 2017; Zheng et al., 2018; Zheng et al., 2020b). Lindo and Whiteley (2011) were the first to show that epiphytic bryophytes in the canopy contribute to the vast majority of N fixation, rather than on the forest

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floor of temperate rain forests. The N-fixing ability of a wide variety of epiphytic plants were confirmed that serving as a new N source in the subtropical forest ecosystem (Han et al., 2010). Meta-analysis on a global scale revealed that terrestrial nitrogen fixation was stimulated by warming, elevated CO_2, and increased precipitation, but inhibited by increased drought, N deposition and multiple environmental change factors (Zheng et al., 2019; Zheng et al., 2020b). However, to the best of our knowledge, no further investigation about the N-fixing ability and response strategies to environment changes of epiphytic bryophyte-cyanobacteria associations has been carried out in the subtropical montane cloud forest, where epiphytic bryophytes were diverse and abundant (Ma et al., 2009).

However, physiological metabolic activity of bryophytes is likely to be inhibited as a result of global change, especially decreased precipitation, and more extreme weather events expected in the future (Rousk et al., 2014b; Rousk et al., 2021). Both bryophytes and cyanobacteria are poikilohydric and sensitive to water changes (Green and Lange, 1995). The physiological processes of these species highly depend on surrounding water availability (Proctor, 2008; Green et al., 2011). Most bryophytes, with high water retention capacity, could provide a relatively stable and favourable habitats for the growth and N–fixation activity of the associated cyanobacteria (Adams and Duggan, 2008). Furthermore, higher water content of host (or available moisture) promotes N fixation in bryophytes-cyanobacteria associations within a certain range and has a stronger effect than temperature, light and other environmental factors (Gundale et al., 2009; Stewart et al., 2011; Rousk et al., 2014b; Whiteley and Gonzalez, 2016; Rousk et al., 2017; Rousk et al., 2018). Whereas, excess water content would inhibit N fixation, suggesting an overall unimodal response of nitrogenase activity to water content changes (Whiteley and Gonzalez, 2016). N fixation is an energy consuming process and depends on photosynthesis for the carbonhydrates and ATP supply (Rousk et al., 2013). Nitrogenase enzyme activity depends on water availability, which directly affects the N fixation rate. Meanwhile, water content would affect photosynthesis, thus indirectly affecting the nitrogenase activity. Thus, we hypothesize that the nitrogenase activity dynamics under different water content conditions is similar to the photosynthesis–water content dynamics, and response strategies of N fixation by bryophytes under water surplus and shortage conditions strongly differ among species. The increasing frequency of extreme weather events and dry–hot spells caused by global warming threatens the physiological activity and survival of epiphytes in montane forests (Zotz and Bader, 2009; Liu et al., 2018).

The cyanobacteria present on bryophyte shoots can be quantified by ultraviolet (UV) fluorescence microscopy (DeLuca et al., 2007; Bay et al., 2013; Deane-Coe and Sparks, 2016). Bryophytes usually form epiphytic or endophytic associations with cyanobacteria, primarily belonging to the genera *Nostoc*, *Calothrix* and *Stigonema* (Gentili et al., 2005; DeLuca et al., 2007; Ininbergs et al., 2011). N fixation in cyanobacteria associated with bryophytes depends on numerous biotic and abiotic factors (moisture, temperature, nutrient availability, host species, microbial community composition, etc.), and the impact of these environment factors can be coupled and complex (Zheng et al., 2020a; Stuart et al., 2021). The results of previous studies on the role of host bryophyte species in N fixation are inconsistent (Gavazov et al., 2010; Stuart et al., 2021). Cyanobacteria are able to fix atmospheric N, as they possess heterocysts containing nitrogenase (Bay et al., 2013). The process of N fixation involves the digestion of chemical bonds, which requires energy. The carbon (C)–rich energy source required by heteromorphic cells is mainly derived from the surrounding vegetative cells. Bryophytes, as the host, provide a suitable environment for the attached cyanobacteria, which promotes N fixation (Bentley, 1987; Gundale et al., 2012a; Gundale et al., 2012b). Although many studies confirmed a positive correlation between the degree of cyanobacteria colonization and nitrogenase activity (DeLuca et al., 2007; Lindo and Whiteley, 2011), it is unclear whether the water changes (short–term water content changes vs. long–term drought in dry seasons) affects N fixation directly through a physiological process or indirectly through changes in cyanobacterial colonization is unknown.

The difference in relative humidity (RH) levels between dry and rainy seasons affects the nitrogenase activity not only by affecting the atmospheric water content but also by affecting cyanobacterial colonization. A better understanding of the relationship between N fixation and water content of epiphytic bryophytes in a subtropical cloud forest would provide important insights into their ecological function and predict their existence in the changing world. In this study, we determine the nitrogenase activity of four dominant epiphytic bryophyte species associated with cyanobacteria in a subtropical montane cloud forest under different water content conditions and in different seasons. The density of cyanobacteria on bryophytes estimated in different seasons, and the relationship of cyanobacterial colonization with nitrogenase activity was examined. The objectives of this study were as follows: (1) to assess the cyanobacterial colonization and nitrogenase activity in dry and rainy seasons; (2) to discuss the relation between cyanobacterial colonization and nitrogenase activity; and (3) to understand how water availibility affects cyanobacterial colonization and nitrogenase activity.

2. Materials and methods

2.1. Study area and species

This study was conducted in a subtropical, evergreen broad–leaved montane cloud forest in the Xujiaba region (23°32′N, 101°01′E) of the Ailao Mountain National Nature Reserve in Yunnan, China. In this region, the mean annual precipitation is ca. 1859 mm, (86% of which falls from May to October); mean annual relative humidity (RH) and temperature (based on the data recorded between September 2014 and August 2017) are 82.6% and 11.6 °C, respectively (Fan et al., 2020); and the N deposition rate is estimated at 10.5 kg ha^{-1} yr^{-1} (Liu et al., 2002). The study area is classified as a subtropical montane cloud forest. Because this region is affected by both the southern subcurrent of the western airflow (from India and Pakistan) and the southwest monsoon, it experiences a striking alternation of dry and wet conditions (Song et al., 2012b). The study area is rich in epiphytic cryptogams (Fan et al., 2020), including 176 bryophyte species belonging to 38 families and 83 genus, with an average biomass of 4.48 ± 0.29 t·ha^{-1}. Among these, four dominant epiphytic bryophyte species were selected, based on species frequency data (Ma et al., 2009), including *Homaliodendron montagneanum*, *H. scalpellifolium*, *Thuidium cymbifolium* and *Plagiochila assamica* (a liverwort). These bryophytes were mainly sampled from the trunks of dominant tree species (e.g., *Lithocarpus hancei*, *Castanopsis rufescens*, *Lithocarpus xylocarpus*), at a height 0.5–1.5 m above the ground.

2.2. Estimates of cyanobacterial colonization

The degree of cyanobacterial colonization was evaluated using ultraviolet fluorescence microscopy in April (dry season) and August (rainy season) 2017. According to Deane-Coe and Sparks (2016)'s study, a total of 6–10 shoots of each species were selected for microscopy analysis. Each shoot was immersed in deionized (DI) water and then mounted on a microscope slide. A total of 4–10 spots per shoot were randomly selected and examined under a UV–fluorescence microscope (Leica TCS LSI, Germany), fitted with a green filter, at 500 × magnification. Images were taken immediately after observation, and cyanobacterial colonization was analyzed using the ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA). The degree of cyanobacterial colonization was calculated as the percentage of shoot area colonized by cyanobacteria relative to the total shoot area (% area colonized).

2.3. Determination of the nitrogenase activity

The nitrogenase activity of the collected samples was measured with the ARA in the field (Stewart et al., 1967; DeLuca et al., 2002; Liu et al., 2018). These experiments were carried out in four replications (per bryophyte species), consisting of approximately 0.2–0.3 g dry weight shoots at the Ailao Mountain Ecosystem Research Station (Yunnan, China) in April and August of 2017. Healthy bryophyte samples were sprayed with DI water to saturation and placed in 60 ml glass culture tubes. Ten percent of the total headspace of each tube was evacuated and replaced with acetylene, and the tubes containing bryophyte thallus samples were settled back into the forest and incubated for 24 h. Then, 6 ml of gas was withdrawn from the headspace using an air–tight syringe and immediately injected into the vacuum blood tube. The tubes were brought back to the laboratory, and the ethylene content of each sample was determined using Agilent GC 6890 N gas chromatograph (Agilent Technologies Inc., Santa Clara, CA, USA), equipped with a flame ionization detector and Porapak T column. Then, the samples were oven–-dried at 65 °C for 48 h (to determine the dry mass). Nitrogenase enzyme activity in each sample was measured based on the ethylene concentration in the tube, and then converted to $nmol \cdot g^{-1} \cdot h^{-1}$.

2.4. Nitrogenase activity–water response curves

A short–term water control experiment was carried out in March and April 2017 to study the changes in the physiological indicators of epiphytic bryophytes under different water conditions. Four replications were performed for each species. Bryophyte samples in healthy growth conditions were selected from the subtropical montane cloud forest and submerged in a Petri dish filled with DI water for 60 mins. After reaching full saturation, the samples were removed from the petri dish and placed on an absorbent paper for 30 s to remove excess water. When moistened bryophytes are exposed to air, the water content decreases over time. The nitrogenase activity of each sample with different water content was measured during the drying process. Given the poikilohydric nature of bryophytes, it is impossible to control the water content at a fixed level. Therefore, each test sample was weighed using an electronic balance (with a precision of 0.001) before and after incubation, after each sample was dried in an oven at 65 °C for 48 h and weighed to assess its water content measuring the nitrogenase activity, and the average water content of each sample was calculated

2.5. Statistical analysis

One–way ANOVA was conducted to compare differences of air temperature and relative humidity at rainy and dry seasons. The normality of data was assessed using the Shapiro–Wilk's test, and the homogeneity of variance (i.e., homoscedasticity) was evaluated using the Levene's test. Independent samples t–test and the nonparametric Mann–Whitney test (when the normal assumption was not satisfied) were used to test the differences in the nitrogenase acticity and the degree of cyanobacterial colonization between dry season and rainy seasons across bryophyte species. One–way analysis of variance (ANOVA), followed by the least significant difference (LSD) test or Tukey's post hoc test were used to compare differences in nitrogenase activity and the degree of cyanobacterial colonization among different bryophyte species. Pearson correlation analysis was used to test the association between nitrogenase activity and the degree of cyanobacterial colonization. A log–normal function curve model was used to fit the relationship between the net nitrogenase activity and moisture content:

$$NF = y_0 + \frac{a}{WC} \cdot e\left[-0.5 \cdot \left(\frac{\ln(WC/x_0)}{b} \right)^2 \right]$$

where NF represents the nitrogenase activity; WC represents the water content; a and b represent the regression coefficients; and y_0 and x_0 are constants.

All data analyses were performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA), and effects were considered significant at $P \leq 0.05$. All graphs were drawn using SigmaPlot 12.5 (Systat Software Inc., San Jose, CA, USA).

3. Results

3.1. Cyanobacterial colonization of epiphytic bryophytes

Comparing with dry season, significantly higher air temperature and relative humidity was detected in rainy season (Table 1). Annual average degree of cyanobacterial colonization Cyanobacteria colonized the shoots of all four bryophyte species but annual average degree of cyanobacterial colonization is different (Fig. 1). *H. scalpellifolium* showed the highest % area colonized (3.37 ± 0.64%), followed by *T. cymbifolium* (2.31 ± 0.33%), *H. montagneanum* (1.46 ± 0.30%) and *P. assamica* (1.04 ± 0.17%). The % area colonized of *H. scalpellifolium* and *T. cymbifolium* were significantly higher than those of *H. montagneanum* and *P. assamica* ($P < 0.001$); however, the differences of % area colonized between *H. scalpellifolium* and *T. cymbifolium* and between *H. montagneanum* and *P. assamica* were non–significant (Fig. 2).

Significant differences were observed in the degree of cyanobacterial colonization on the four epiphytic bryophytes between dry and rainy seasons. In the dry season, *H. scalpellifolium* showed the highest % area colonized (1.82 ± 0.42%), followed by *T. cymbifolium* (1.58 ± 0.26%), *P. assamica* (0.83 ± 0.11%) and *H. montagneanum* (0.81 ± 0.31%). The % area colonized of *H. scalpellifolium* and *T. cymbifolium* were significantly higher than that of *H. montagneanum* within the dry season ($P < 0.05$), whereas the % area colonized of *P. assamica* differed non–significantly from the those of the other three species. In the rainy season, *H. scalpellifolium* showed the highest % area colonized (4.92 ± 1.06%), followed by *T. cymbifolium* (3.34 ± 0.60%), *H. montagneanum* (2.11 ± 0.45%) and *P. assamica* (1.17 ± 0.26%). Within the rainy season, the degree of cyanobacterial colonization of *P. assamica* was significantly lower than those of *H. scalpellifolium*, *H. montagneanum* and *T. cymbifolium*; among these three species, the degree of cyanobacterial colonization of *H. scalpellifolium* was significantly greater than that of *H. montagneanum*, whereas *T. cymbifolium* showed no significant difference in cyanobacterial colonization compared with *H. scalpellifolium* and *H. montagneanum*. Comparison between seasons showed that the degree of cyanobacterial colonization of *H. montagneanum*, *T. cymbifolium* and *H. scalpellifolium* in the rainy season were significantly higher than those in the dry season. Although the degree of cyanobacterial colonization of *P. assamica* in the rainy season was also higher than that in the dry season, the difference was non–significant (Fig. 2).

3.2. Nitrogenase activity of epiphytic bryophytes

The average annual nitrogenase activity was the lowest for *P. assamica* (10.16 ± 2.79 nmol $C_2H_4 \cdot g^{-1} \cdot h^{-1}$), followed by *H. montagneanum* (11.03 ± 4.39 nmol $C_2H_4 \cdot g^{-1} \cdot h^{-1}$), *T. cymbifolium* (20.21 ± 2.45 nmol $C_2H_4 \cdot g^{-1} \cdot h^{-1}$) and *H. scalpellifolium* (19.55 ± 6.89 nmol $C_2H_4 \cdot g^{-1} \cdot h^{-1}$). The average annual nitrogenase activity of *T. cymbifolium* was significantly higher than that of *P. assamica* and *H. montagneanum*, although differences between *P. assamica* and *H. montagneanum* and between *H. scalpellifolium* and the other three

Table 1
Analysis of meteorological parameters(mean ± SE) at the Ailao Mountain National Nature Reserve (Yunnan, China) during dry and rainy seasons.

Parameter	Dry season	Rainy season	F–statistic	*P*–value
Air temperature (°C)	13.20 ± 1.65	15.83 ± 0.52	13.945	0.000
Relative humidity (%)	76.44 ± 9.56	99.77 ± 0.44	30.568	0.000

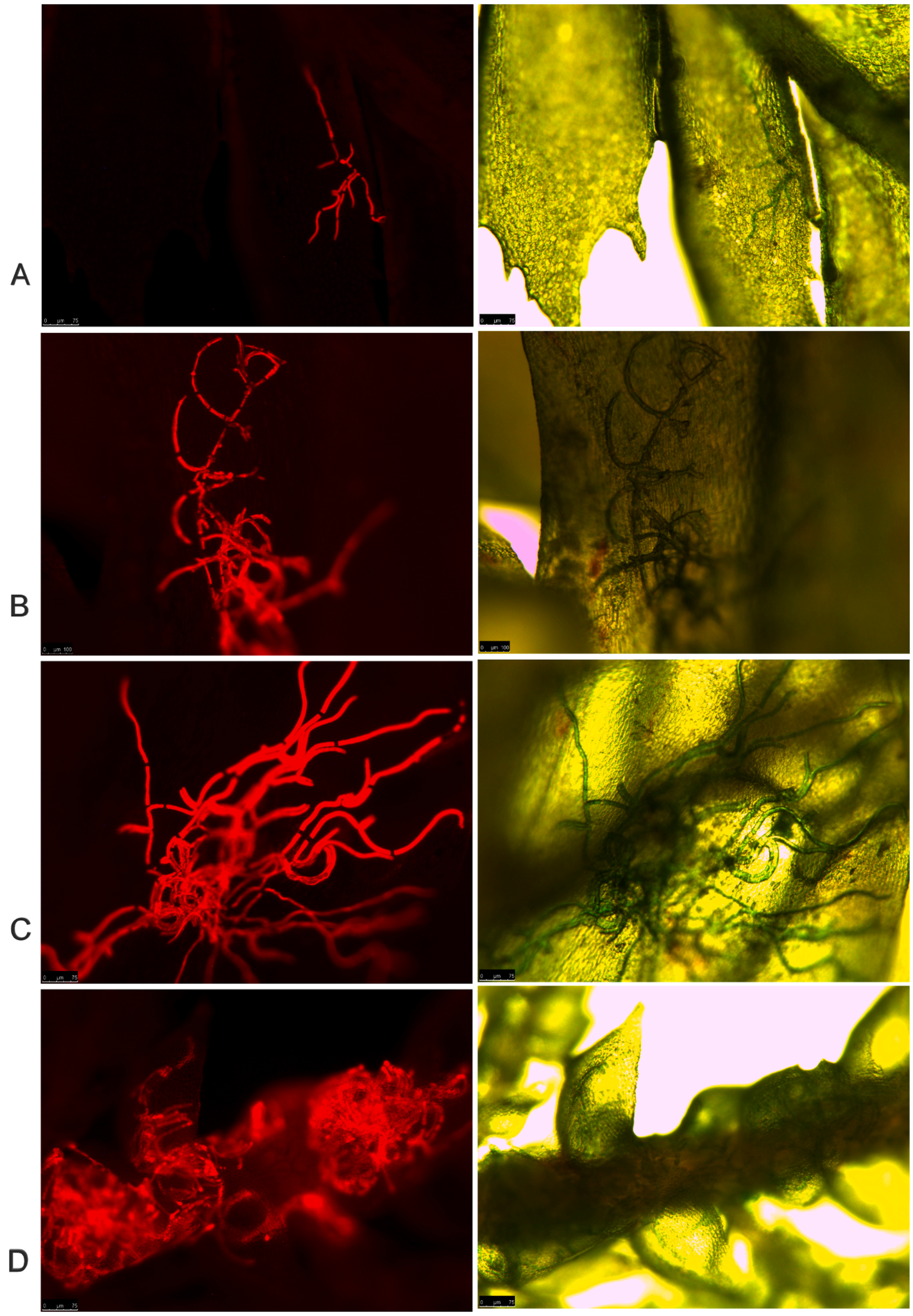

Fig. 1. Images showing cyanobacterial colonization of the shoots of four different bryophyte species. (A) *Plagiochila assamica*; (B) *Homaliodendron montagneanum*; (C) *Homaliodendron scalpellifolium*; (D) *Thuidium cymbifolium*. Images on the left were captured under a UV–fluorescence microscope fitted with a green filter, and images on the right were taken under a light microscope.

species were non–significant. In the dry season, *T. cymbifolium* showed the highest nitrogenase activity (25.43 ± 2.89 nmol $C_2H_4 \cdot g^{-1} \cdot h^{-1}$), which was significantly higher than that of the other three species, *P. assamica* (8.38 ± 2.21 nmol $C_2H_4 \cdot g^{-1} \cdot h^{-1}$), *H. scalpellifolium* (6.82 ± 1.85 nmol $C_2H_4 \cdot g^{-1} \cdot h^{-1}$) and *H. montagneanum* (3.99 ± 1.57 nmol $C_2H_4 \cdot g^{-1} \cdot h^{-1}$); however, no significant differences were observed among *P. assamica*, *H. montagneanum* and *H. scalpellifolium*. In the rainy season, the nitrogenase activity of *H. scalpellifolium* was the highest (32.29 ± 10.77 nmol $C_2H_4 \cdot g^{-1} \cdot h^{-1}$) followed by *H. montagneanum* (18.06 ± 7.38 nmol $C_2H_4 \cdot g^{-1} \cdot h^{-1}$), *T. cymbifolium* (15.00 ± 1.27 nmol $C_2H_4 \cdot g^{-1} \cdot h^{-1}$) and *P. assamica* (11.95 ± 5.42 nmol $C_2H_4 \cdot g^{-1} \cdot h^{-1}$); however, the differences among all four species were non–significant. Furthermore, the nitrogenase activity of *T. cymbifolium* in the rainy season was significantly lower than that in the dry season ($P < 0.05$). The nitrogenase activity of *P. assamica*, *H. montagneanum* and *H. scalpellifolium* were higher in the rainy season than in the dry season; however, there were no significant difference in the nitrogenase activity of the other three species between the dry and rainy seasons (Fig. 3). Importantly, a significant, positive relationship was detected between cyanobacterial colonization and the nitrogenase activity ($R = 0.742$; $P = 0.0349$) (Fig. 4).

3.3. The relationship between water content and nitrogenase activity

The nitrogenase activities of all four epiphytic bryophytes showed an upward and then a downward trend in response to changes in water

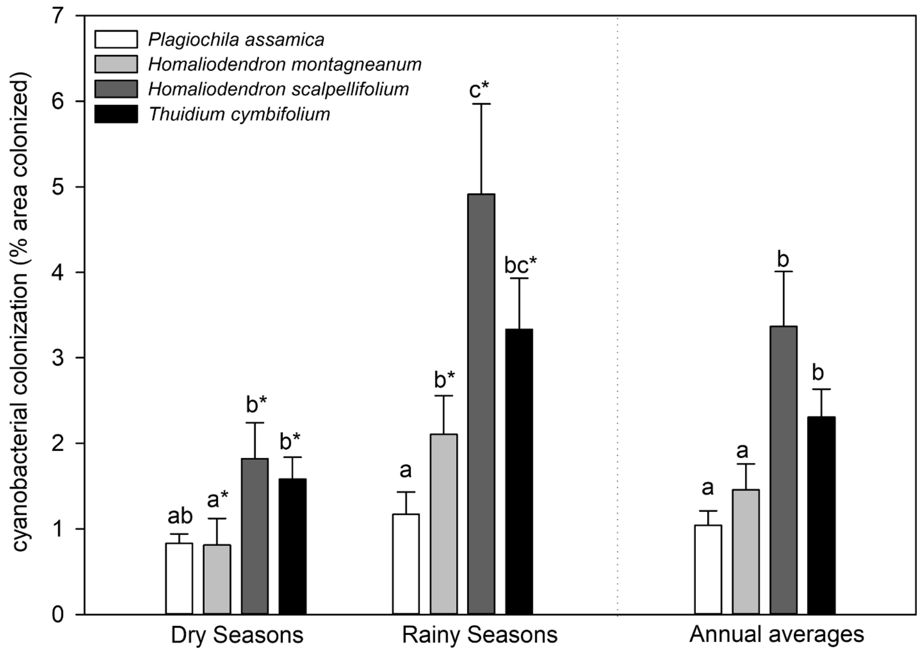

Fig. 2. Seasonal dynamics of the degree of cyanobacterial colonization on four epiphytic bryophytes. Different lowercase letters indicate significant differences among species within a season ($P < 0.05$), whereas the asterisk (*) indicates significant differences between dry and rainy seasons within a species ($p < 0.05$).

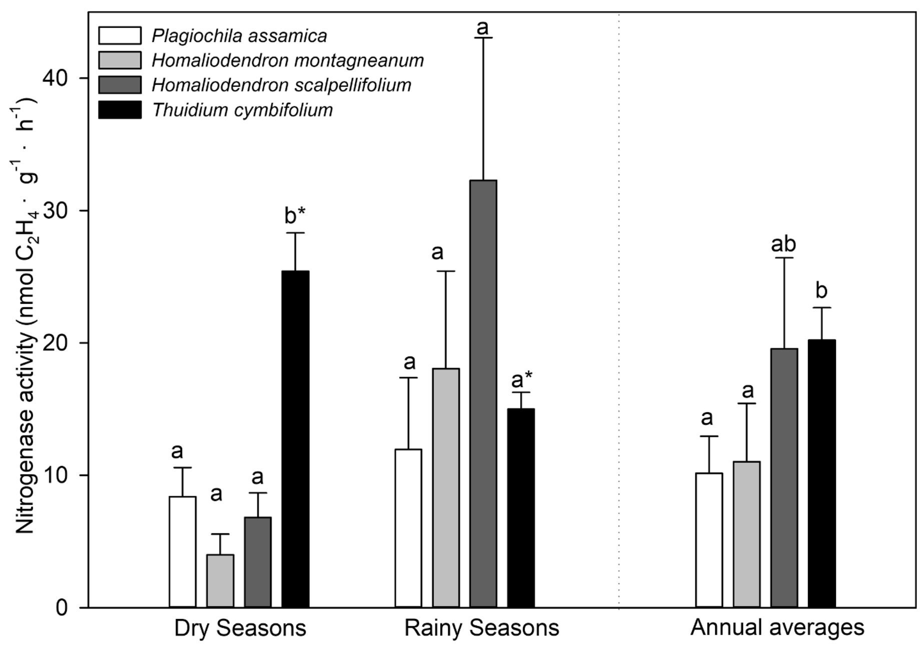

Fig. 3. Seasonal dynamics of the nitrogenase activity of four epiphytic bryophytes. Different lowercase letters indicate significant differences among species within a season ($P < 0.05$), whereas the asterisk (*) indicates significant differences between different seasons for the same species ($p < 0.05$).

content. The coefficient of determination (R^2) was greater than 0.90 for all four species, and the curve fitting was good (Fig. 5). The nitrogenase activities of all four bryophyte species were low at water content below 50% (Fig. 5). Upon a further increase in water content, the nitrogenase activities of *P. assamica* increased significantly (Fig. 5A), while that of *H. montagneanum* increased gradually (Fig. 5B). The nitrogenase activities of *P. assamica* peaked at a water content of 170% and then declined rapidly with further increase in water content (Fig. 5A). The nitrogenase activities of *H. montagneanum* increased until the water content reached 130% and then declined (Fig. 5B). The nitrogenase activities of *T. cymbifolium* (Fig. 5C) and *H. scalpellifolium* (Fig. 5D) also increased until the water content reached approximately 190% and 160%, respectively, and then decreased. The nitrogenase activities of all four epiphytic mosses was very low when the water content was <50%, and the optimal water content varied among species: *H. montagneanum*, 130% (lowest among the four species); *H. scalpellifolium*, 160%; *P. assamica*, 170%; *T. cymbifolium*, 190% (highest among the four species). Under the conditions of optimum water content, the nitrogenase activities of *T. cymbifolium*, *P. assamica*, *H. montagneanum* and *H. scalpellifolium* were 28.78, 24.49, 14.49 and 12.85 nmol $C_2H_4 \cdot g^{-1} \cdot h^{-1}$, respectively. The results of this study show that too high and too low water content inhibit the nitrogenase activities of epiphytic

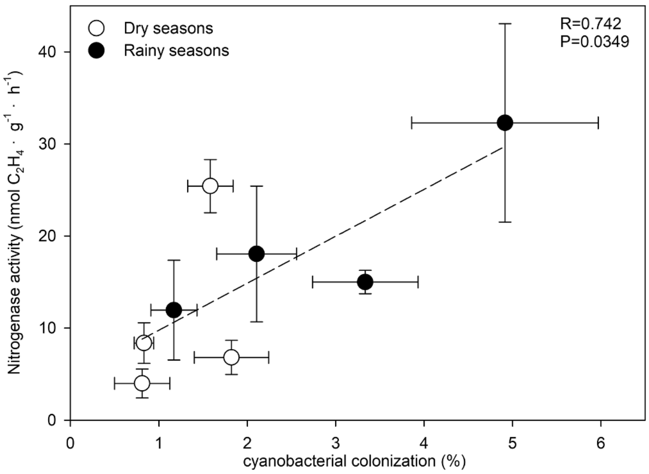

Fig. 4. Relationship between nitrogenase activity and cyanobacterial colonization of the epiphytic bryophytes in the dry and rainy seasons.

bryophytes and show that bryophytes need a suitable moisture content to maintain a high nitrogenase activities. The four species selected in this study showed the highest nitrogenase activity at 130–190% water content. Additionally, under the optimum water content, the nitrogenase activity of these species varied from 12.9 to 28.8 nmol $C_2H_4 \cdot g^{-1} \cdot h^{-1}$.

4. Discussion

4.1. Correlation between cyanobacterial colonization and nitrogenase activity

In this study, the nitrogenase activity of bryophytes showed a highly positive correlation with the degree of cyanobacterial colonization (Fig. 4), which is consistent with previous studies (DeLuca et al., 2007; Lindo and Whiteley, 2011; Rousk et al., 2014a; Jean et al., 2020). On a global level, the current research on biological N fixation in mosses is mainly focused on boreal forests. (DeLuca et al., 2002) reported that *P. schreberi* (a feather moss) associates with filamentous cyanobacteria shows a high N fixation capacity (1.5–2.0 kg $N \cdot ha^{-1} \cdot yr^{-1}$) and there is a close relationship between the N fixation rate and the number of cyanobacteria ($R^2 = 0.58$; $P = 0.01$) (DeLuca et al., 2007). Subsequently, Zackrisson et al. (2004) showed that the number of symbiotic cyanobacteria limited N fixation rate of *P. schreberi* at the early stage of secondary succession. Rousk et al. (2014a) studied the effects of N deposition on the N–fixing ability of *P. schreberi* in the coniferous forests of northern Sweden, and showed that the acetylene reduction after N deprivation was positively related to cyanobacterial colonization ($r = 0.94$; $P < 0.001$). Another study conducted in the coastal temperate rain forest of North America demonstrated a significant positive correlation between the N fixation rate and the total number of cyanobacteria ($R^2 = 0.268$; $P < 0.001$) (Lindo and Whiteley, 2011). Recent years, there are increasing studies that reported moss N fixation in tropical and subtropical forests (Cusack et al., 2009; Zheng et al., 2018; Zheng et al., 2020b). Although, subtropical or tropical forests are higher in soil N

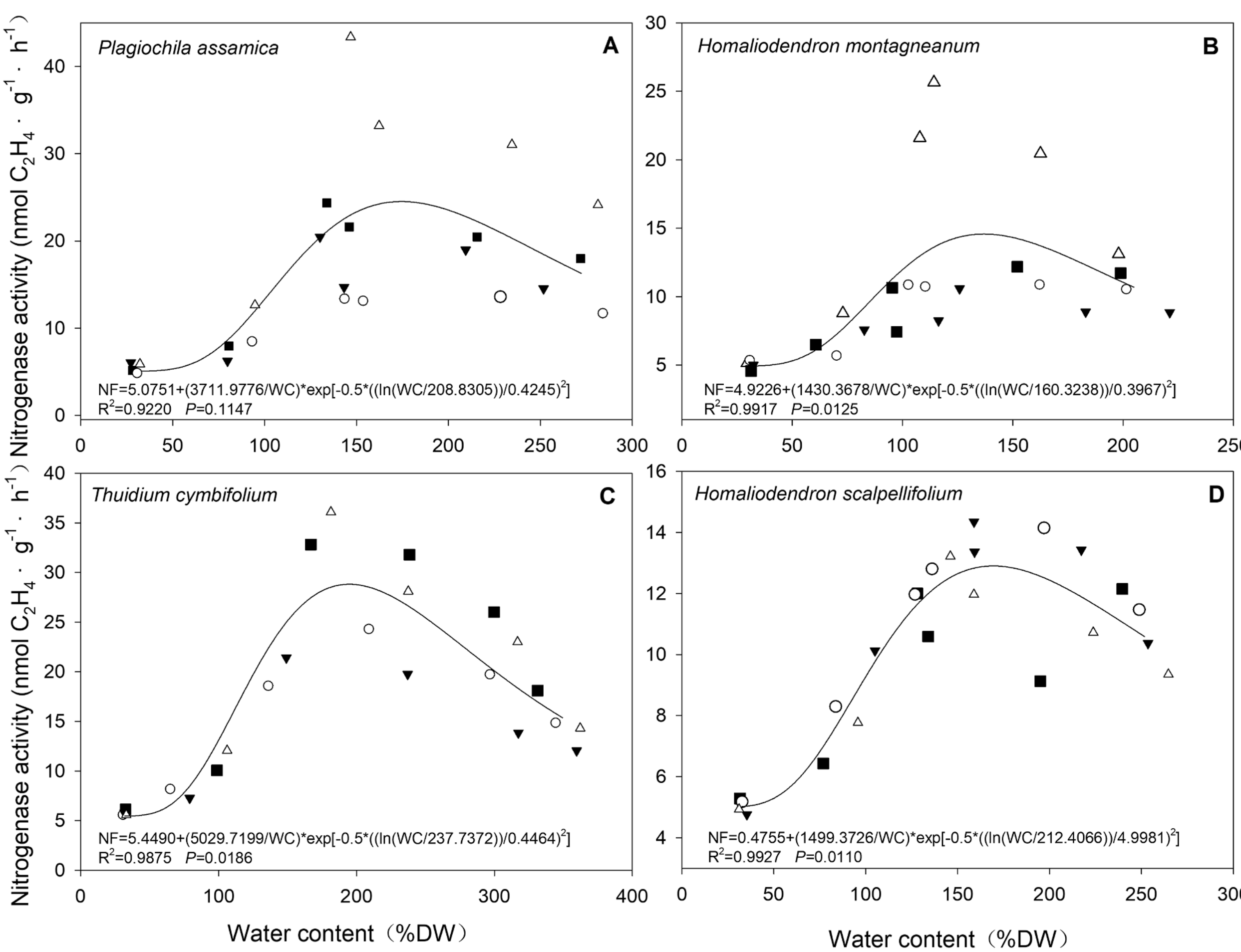

Fig. 5. Relationship between the nitrogenase activity and water content in all four epiphytic bryophytes. (A) *P. assamica*; (B) *H. montagneanum*; (C) *T. cymbifolium*; (D) *H. scalpellifolium*. Different symbols indicate different samples ($n = 4$).

availability than those of temperate and boreal forests, the stoichiometry of mosses as substrate drive associated cyanobacteria sustaining high rates of N fixation (Zheng et al., 2018; Zheng et al., 2020b). Cusack et al. (2009) reported soils and mosses provided the largest potential inputs of N via nitrogen fixation to tropical forests ecosystems in Puerto Rico. Warshan et al. (2016) showed that the expression of the *nifH* gene of bryophyte–associated cyanobacteria caused significant changes in the N fixation rate. The results of Deane-Coe and Sparks (2016) showed that compared with moss species that did not form a symbiotic association with cyanobacteria, the cyanobacteria–associated moss species not only had a significantly higher δ^{15}N but also showed a significantly higher tissue N content.

The degree of cyanobacterial colonization observed in the current study was lower than that obtained in other studies, probably because the degree of cyanobacterial colonization was estimated using different methods. We estimated the degree of cyanobacterial colonization as the percentage of the area colonized by cyanobacteria relative to the total area of bryophyte shoots, whereas Deane-Coe and Sparks (2016) estimated the degree of cyanobacterial colonization as the percentage of leaves displaying the presence of cyanobacteria in each sample. Another method that involves the disassociation of cyanobacteria from the moss, followed by the counting of cyanobacteria cells per moss shoot has also been frequently used to estimate the degree of cyanobacterial colonization (DeLuca et al., 2007; Lindo and Whiteley, 2011). While cyanobacteria often exist in the axillae or the flagellum, only observing the leaves removed from the shoots or disassociating cyanobacteria using sonication would result in an underestimation of the degree of cyanobacterial colonization. In this study, we explored a new calculation method for quantifying degree of cyanobacterial colonization on bryophyte shoots for the first time. Despite the use of different methods, the same conclusion was reached in different studies, i.e., the nitrogenase activity of bryophytes is positively affected by the degree of cyanobacterial colonization.

4.2. Response strategies to short-term water changes and long-term drought

In subtropical cloud forests, the nitrogenase activities of epiphytic bryophytes are sensitive and vulnerable to changes in water availability. The laboratory (control) experiment demonstrated that the nitrogenase activities of epiphytic bryophytes gradually increased with the increase in the water content of shoots. However, the nitrogenase activities gradually declined when the water content exceeded the saturation state. N fixation is an energy consuming process, which requires an abundant supply of carbohydrates and ATP (Belnap, 2002; Liu et al., 2018). Bryophytes fix C and N through leaf photosynthesis and associated cyanobacteria, respectively. As the host, bryophytes provide carbohydrates and energy to the associated cyanobacteria required for N fixation. Water content is the most important abiotic factor influencing N fixation by the bryophyte–associated cyanobacteria, because bryophytes are poikilohydric organisms and physiologically active only in wet conditions (Green et al., 2011). The water content of the bryophyte thallus is a major factor limiting the activity of cyanobacteria in the field (Coxson and Kershaw, 1983a; 1983b). Thus, water availability significantly affects the N fixation ability of bryophyte–cyanobacteria associations (Zielke et al., 2002; Zielke et al., 2005). Whiteley and Gonzalez (2016) investigated the effect of water content on the N fixation rate of moss, and showed that the N fixation rate was the lowest under low moisture conditions (<400–500%) and highest under intermediate moisture conditions (300–600%); however, under the highest moisture content (>600%), the N fixation rate decreased. The optimal water contents for bryophytes N fixation in our study is 150–200%, which is lower than the results of Whiteley and Gonzalez' study. We suggest two possible reasons fro this: First, Whiteley and Gonzalez focused on the effect of water content on bryophyte nitrogen fixation, but did not continuously measure the effect of water content on nitrogen fixation, so the optimal water content range is larger than the measurements in our study. Second, the water absorption capacity of *Pleurozium schreberi*, which is weft life-forms, was higher than the target species which are mostly fan life-forms living in a humid environment in our study (Song et al., 2012a,b). The results of our study indicated that the nitrogenase activity–water response curves were similar to the photosynthetic rate–water response curves, which showed a decline when the water content was surplus (Song et al., 2012a; Wang and Bader, 2018; Yuan et al., 2018). The optimum water content required for N fixation was generally lower than that required for photosynthesis in all four epiphytic bryophytes (*H. montagneanum*, 130% vs. 150–170%; *H. scalpellifolium*, 160% vs. 170–200%; *P. assamica*, 170% vs. 200–250%; *T. cymbifolium*, 190% vs. 200–300%) (Yuan et al., 2018). We assumed that a low level of photosynthesis would be sufficient for N fixation, and constant hydration may cause massive glucose efflux, leading to the depletion of energy reserves necessary for N fixation (Belnap, 2003). Given that N fixation by cyanobacteria is usually an anaerobic process (Bothe et al, 2010), the main reason why moss N fixation is down-regulated under very high water contents condition is that photosynthesis is inhibited (Bothe et al, 2010; Whiteley and Gonzalez, 2016). When bryophytes hold excess external water, the high resistance to air diffusion compromises CO_2 exchange, which reduces photosynthesis. Similar to cyano-lichens, the continuous high water content of bryophyte shoots can cause a large amount of non-crystalline glucose to be discharged causing energy storage for nitrogen fixation was consumed (Kershaw, 1985). Thus, superfluous water leads to a gradual decline in the nitrogenase activity. Furthermore, epiphytic bryophytes associated with cyanobacteria respond to short–term drought stress by reducing their N–fixing activity through physiological changes before changing their degree of cyanobacterial colonization.

For *H. scalpellifolium*, the N fixation activity in rainy season was the highest among four species. While in dry season, the N fixation activity was lower than *T. cymbifolium.* During short-term water-nitrogen fixation relation laboratory experiment, the peak value of fixation activity was lowest among four species. The results indicated that *H. scalpellifolium* has the highest potential to fix N, but it is more vulnerable to water loss, leading to restrictions on N fixation.

The Ailao Mountain area is controlled by the southwest monsoon, with distinct dry and wet seasons (Liu et al., 2018). During the rainy season, the microclimate of the subtropical montane cloud forest investigated in this study was characterized by high RH but low temperature and light intensity which is conducive for bryophytes and associations with N–fixing cyanobacteria (Lindo and Whiteley, 2011). Epiphytic bryophytes may remain continuously moist for days (Table 1; Song et al., 2015), leading to their successful growth and sporophyte production. Thus, N demand is very high during the rainy season, which requires N fixation and stimulates cyanobacterial colonization (Sprent and Meeks, 2013). Another reason why cyanobacteria exhibit high N fixation in areas with heavy rainfall is probably because the increase in water content results in the reduction in oxygen level, which protects the nitrogenase enzyme from oxidative damage (Zielke et al., 2002).

However, during the dry season, long–term drought may lead to an imbalance in the annual production of C in bryophytes as the host plant, thus decreasing cyanobacterial colonization. Bryophytes show little growth (Song et al., 2012b) and quickly lose water in dry seasons, which negatively affects their photosynthetic capacity, leading to a decline in N fixation. The cyanobacterial colonization and nitrogenase activities of epiphytic bryophytes were significantly higher in the rainy season than in the dry season. The N fixation rate and the number of symbiotic cyanobacteria of feather mosses increased upon the melting of snow because of higher water availability and temperature, followed by a rapid decline in boreal forest (DeLuca et al., 2002; Zackrisson et al., 2004; Gundale et al., 2012b). Zielke et al. (2002) studied the effect of soil water content and temperature on the N fixation rate of moss–associated cyanobacteria in the arctic region and reach the similar conclusion. The effect of water on the nitrogenase activity of bryophyte was greater than that of temperature. Drought can directly affect the activity and quantity of the associated cyanobacteria (Whiteley and Gonzalez, 2016). Long–term drought conditions during the dry season lead to the consumption of C reserves in the bryophyte, causing a decrease in the degree of associated cyanobacterial colonization. After rehydration, photosynthates are first used to build carbohydrates or energy reserves or to repair photodamage, and then used to rebuild the nitrogenase enzyme (Dodds et al., 1995; Lange et al., 1998; Belnap, 2003), thus causing lower nitrogenase activities during the dry season. We speculate that the decline in cyanobacterial colonization, together with the delayed initiation of N fixation compared with photosynthesis, leads to low nitrogenase activities in the dry season.

These results have several important implications for indicating climate changes and ecosystem dynamics. Biological nitrogen fixation performed by bryophyte-cyanobacteria associations was one of important N sources in many terrestrial pristine ecosystems (Calabria et al., 2020). Bryophytes are sensitive to climate changes. Biological nitrogen fixation by bryophytes is closely related to climate changes such as moisture change and drought. On the other hand, feedback mechanisms between organisms that regulate fundamental ecosystem processes is important to our ability to predict long-term results in natural and disturbed environments (DeLuca et al., 2008). There was a close relationship between biological nitrogen fixation and different organisms in the ecosystem (DeLuca et al., 2008). To a certain extent, changes in biological nitrogen fixation activity can be used to predict the impact of climate change on ecosystems, and also to predict the long-term outcomes in natural and disturbed environments (DeLuca et al., 2008). Thus, revealing the relationship between the variation in the colonization degree of cyanobacterial and nitrogen fixation activity and the water availability changes can be used to predict the impact of future environmental changes on ecosystems, especially precipitation and drought on the nitrogen cycle of the ecosystem.

5. Conclusions

The present results indicate that the nitrogenase activities of all four bryophyte species increased with the increase in water content but then gradually decreased with a further increase in the water content. This suggests that both excessive water and low water content inhibit the nitrogenase activity of epiphytic bryophytes. The nitrogenase activity of epiphytic bryophytes was positively affected by the degree of cyanobacterial colonization. When exposed to long–term drought conditions (e.g., during the dry season), the degree of cyanobacterial colonization associated with bryophytes declined, resulting in low nitrogenase activity. Bryophyte-cyanobacteria associations response to instant water changes by decreasing physiological activity of nitrogenase and to long-term drought by inhibiting cyanobacteria colonization. Overall, our results suggest that extreme precipitation and long–term drought have a negative impact on the cyanobacterial colonization and nitrogenase activity of epiphytic bryophytes.

CRediT authorship contribution statement

Xiaoyang Fan: Conceptualization, Methodology, Data curation, Formal analysis, Investigation, Writing – original draft, Software. **Guodi Yuan:** Data curation, Methodology, Formal analysis, Writing – original draft. **Wenyao Liu:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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