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Dry-hot stress significantly reduced the nitrogenase activity of epiphytic cyanolichen

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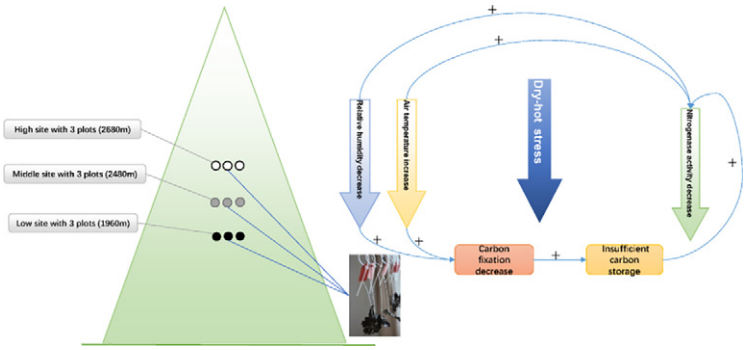
HIGHLIGHTS

- Nitrogen fixed by cyanolichens is an important resource in some forest ecosystems.
- Dry-hot stress reduced the nitrogenase activity of cyanolichens.
- Drought and warming is responsible for reduced nitrogenase activity in the early stage.
- Imbalances in the C budgets will be the ultimate arbiter in later stages.

GRAPHICAL ABSTRACT

We simulated climate change conditions by transplanting *Lobaria retigera*, a common cyanolichen in the area, to lower elevations, and measured nitrogenase activity in response to warmer and drier conditions. The nitrogenase activity of *L. retigera* decreased with a declining elevation all year long.



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ABSTRACT

Nitrogen (N) fixed by epiphytic cyanolichens (i.e. lichens that contain cyanobacterial symbionts) is thought to be the most important resource of this nutrient in some natural forest ecosystems. Although a great deal of work has been carried out to evaluate the biomass of this group as well as its contribution to ecosystem N budgets, empirical studies are needed to confirm the N input responses by cyanolichens under climate change conditions (dry-hot stress) as well as to determine the factors that control this process. We simulated climate change conditions by transplanting *Lobaria retigera*, a common cyanolichen in the area, to lower elevations, and measured nitrogenase activity in response to warmer and drier conditions. In addition, we conducted a series of laboratory and greenhouse experiments to determine the dominant factors influencing nitrogenase activity in this species. The results of this study show that mean annual nitrogenase activity at the higher site was 1.5 and 2.4 times that at the simulated warmer and drier (middle and lower) sites, respectively. Combining laboratory experimental conclusions, we show that thallus water content is a key factor determining the nitrogenase activity of *L. retigera* in early transplantation while insufficient carbon storage resulting from a combination of warming and desiccation was likely responsible for reducing nitrogenase activity in later months of the transplant experiment. The results of this study imply that the negative impact of climate change (dry-hot stress) on ecosystems not only impacts the distribution and growth of species, but also nutrient circles and budgets.

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1. Introduction

The element N most commonly limits net primary production in terrestrial ecosystems (Johnson and Turner, 2014). The main reactive N source in terrestrial ecosystems is inert N_2 gas, comprising 78% of the atmosphere (Marschner and Rengel, 2007). The conversion of N_2 to reactive N for uptake by plants can be due to biological and industrial processes (as well as lightning). Of these processes, biological N fixation (BNF) is responsible for a substantial input of N in many terrestrial ecosystems and has a major impact on its biogeochemical dynamics and ecosystem productivity (Thomas et al., 2006). Previous estimates of global N budgets have suggested that input via BNF accounts for 25% of total fixation, whereas industrial fixation and the combustion of fossil fuels together account for about 75% (Galloway et al., 1995). However, in the absence of industrial input and low deposition of N, the dominant source of reactive N into natural terrestrial ecosystems comes from BNF. In this context the Azotobacter bacteria—which occur in forest floor soils, in plant foliage, in legume root nodules, and in epiphytic lichens and bryophytes—can function as the primary participants in forest ecosystem N fixation. Available estimates, however, suggest that the contribution of free-living bacteria and cyanobacteria-bryophyte symbionts to BNF are small (Vitousek, 1994; Son, 2001; Matzek and Vitousek, 2003). Nonetheless, montane forests will often sustain relatively small numbers of legumes and actinorhizal fixers (Gentry et al., 1995; Crews, 1999) yet support a greater proportion of epiphytes than typically found in other forested ecosystems; the biomass of epiphytes in these forests can rival that of tree foliage (Coxson and Nadkarni, 1995). The biomass of the epiphytes can reach about 44,000 kg ha^{-1}, including 20.4 kg green tissue per tree (Bryophytes,14.77 kg; Lichens, 1.9 kg; Ferns, 2.8 kg; Vascular plants, 1.51 kg) (Hofstede et al., 1993). Meanwhile, the biomass of cyanolichens (*Lobaria oregana*) can reach about 10–15 kg per tree, or approximately 500 kg ha^{-1} in Douglas fir forests (Denison, 1979). Thus, while N fixation by symbiotic vegetation is probably not a major component in montane forests, the N fixed by cyanolichen may represent the major input in those forest ecosystems where they are most abundant (Denison, 1979; Pike et al., 1972; Benner et al., 2007). For example, in New Zealand forest, the N fixed by the cyanolichen genera *Pseudocyphellaria* and *Sticta* can reach 10 $kg \cdot N \cdot ha^{-1} \cdot yr^{-1}$, approximately 5–10-fold the N input from atmospheric deposition (Green et al., 1980). Data show that the N fixed by *Lobaria oregana* in old-growth Douglas fir forests in northeastern Oregon is between 3 $kg \cdot ha^{-1} \cdot yr^{-1}$ and 4 $kg \cdot ha^{-1} \cdot yr^{-1}$, and between 2 $kg \cdot ha^{-1} \cdot yr^{-1}$ and 17 $kg \cdot ha^{-1} \cdot yr^{-1}$ in the Pacific Northwest (Denison, 1979; Antoine, 2004). In addition, the decomposition of epiphytic lichens is faster than that of biological soil crusts and arboreal litter fall (Campbell et al., 2010). As such, these lichens can quickly release nutrients into the surrounding environment and contribute significantly to the N input into forest ecosystems (Pike, 1978; Knops et al., 1996). It follows that determining the rates and controls of cyanolichen BNF under changing ambient conditions is crucial to better understanding the role of these vital cyanolichens in forest ecosystem functioning and in future climate change impacts (Elbert et al., 2012).

While epiphytic cyanolichens may tolerate climatic changes, they are nevertheless sensitive to environmental fluctuations (Nadkarni and Solano, 2002). The nitrogenase activity of these lichens is particularly sensitive to changes in the environment (Belnap, 2001). In the last 100 years, a trend of warming and increasing temperature extremes has been recorded across most regions of Asia that is predicted to continue into the next millennium (Pachauri et al., 2014). In addition, local precipitation varies strongly among different regions and seasons, yet it is reportedly both increasing and decreasing (Pachauri et al., 2014). In Yunnan Province (southwestern China), for example, the mean annual temperature has increased by 1.2 °C over the last 40 years (Fan et al., 2011), whereas the mean annual precipitation is predicted to decrease by 130 mm by the 2050s when compared with current conditions in montane moist evergreen broad-leaved forests (Hijmans et al., 2005; Ramirez and Jarvis, 2008). Therefore, empirical research that monitors the influence of this ensuing 'dry-hot stress' environment on the nitrogenase activity of cyanolichens is necessary.

The available data suggest that water availability or thallus moisture content are the most important abiotic factors influencing N fixation by cyanolichens and cyanobacteria (Nash, 2008), because these organisms are only physiologically active in wet conditions. Ambient temperature is also an important factor that affects the rate of N fixation (Coxson and Kershaw, 1983a), in that BNF is inherently constrained by low temperature, in part due to the optimum of nitrogenase activity, which reaches its maximum efficiency at about 25 °C (Vitousek, 2002; Houlton et al., 2008). Conversely, high temperatures are also known to inhibit the nitrogenase activity of cyanolichens (Hitch and Stewart, 1973). In a study focused on a particular cyanolichen, Antoine (2004) showed that an approximate 10 °C increase in temperature led to a 3–4-fold increase in the *L. oregana* N_2-fixation rate. However, short-term increases in N fixation in response to warming may reflect kinetic responses (Gundale et al., 2012). Other researchers have suggested that long-term warming may result in decreased biomass, abundance and N_2-fixation rate (Lindo et al., 2013). In addition to these direct effects, factors including light, temperature and water supply can each limit nitrogenase activity in an indirect way by influencing both C fixation and consumption processes, as N fixation requires the availability of photosynthetic products (Belnap, 2001). At the same time, evidence suggests that plant productivity and biomass exert stronger controls on N_2-fixation (Lindo et al., 2013). A linear relationship has been demonstrated between biomass production and N fixation in vascular plants (Pimratch et al., 2008), and other studies have shown a similar trend between biomass accumulation and N fixation (Goh and Bruce, 2005). This is mainly because N_2-fixation requires an abundant supply of carbohydrates to meet its high-energy demands. Not surprisingly then, N-fixing organisms are reportedly often abundant in high light environments (Vitousek, 2002; Houlton et al., 2008) where the potential for C accumulation is greatest (Pimratch et al., 2008).

The Ailao Mountains in southwestern China contains one of the largest known tracts of natural evergreen broad-leaved forest, where epiphytic macro- and microlichens are abundant and widely distributed, consisting of 217 species in 76 genera (Li, 2013). Because previous surveys in our field study area have recorded 30 species of epiphytic cyanolichens dominated by *Lobaria retigera* (Li et al., 2011; Li, 2013), this species was selected for further investigation. Based on current research and the distribution of cyanolichens in this region, we hypothesized that (1) The nitrogenase activity of cyanolichens is strongly depressed by a warmer and drier environment, and (2) A decrease in C (energy) to cyanobionts, as driven by reduced lichen growth caused by 1-year warming and drought, should account for any negative impact on the nitrogenase activity of cyanolichens. To test these two hypotheses, we transplanted *L. retigera* at sites at different elevations, as a proxy for estimating the effect of dry-hot stress on N fixation, and conducted laboratory and greenhouse experiments to understand the interacting influences of water supply, temperature, and light on nitrogenase activity.

2. Materials and methods

2.1. Study site

This study was carried out in the Ailao Mountains (23°35′–24°44′N, 100°54′–101°30′E). This forest covers 50,400 ha across elevations that range between approximately 2000 m and 2600 m (Qiu and Xie, 1998; Liu et al., 2002a, 2002b). A mosaic of primary montane forest and secondary forests characterizes this landscape, and a decade of meteorological observations (1996 through 2005) indicated a mean precipitation of 1947 mm, most of which (85%) occurs in rainy season between May and October. The annual mean temperature of this region is 11.3 °C, with average annual evaporation of 1192 mm, and 85% annual

mean relative humidity. The frost-free period lasts for c. 200 days (Yang et al., 2008). The ambient N deposition (i.e., throughfall ammonium plus nitrate) at the site was c. 10–15 $kg \cdot ha^{-1} \cdot yr^{-1}$ (Liu et al., 2002a, 2002b), a rate much lower than found in other areas of China. This montane forest is co-dominated by *Lithocarpus hancei* (Benth.) Rehder, *L. xylocarpus* (Kurz) Markgr and *Castanopsis wattii* (King ex J. D. Hooker) *A. Camus* (You, 1983). The canopy contains abundant epiphytes, including 176 bryophyte species (Ma, 2009) and all 217 lichen species, c. 30 of which are cyanolichens (Li, 2013).

2.2. Field experiments

To induce dry-hot conditions, from 10th to 15th March 2015, we established a set of three plots at each elevation site—a 'high site' at 2680 m, a 'middle site' at 2480 m, and a 'lower site' at 1960 m altitude (Song et al., 2012; Nadkarni and Solano, 2002) (Fig. 1). The slope, aspect, and tree species composition and forest structure at each of these sites were similar to those studied by Qiu and Xie (1998), in that they were all dominated by *Lithocarpus hancei*. After the snowfall in January 2015, the canopy cover at each elevation site was about 75%. There are abundant macrolichens, such as cyanolichens, foliose chlorolichens and fruticose chlorolichens, at the high and middle elevation sites (Li et al., 2011), whereas the low site has only crustose lichens.

Transplantation was carried out during 16–25 March 2015. We prepared three groups of 90 thalli of *L. retigera*, that were placed at the three elevation sites. Each group was further divided in three subgroups of 30 thalli each, which were placed in three nearby plots. (Fig. 1). To ensure that the microclimate of the three plots within each elevation site stayed basically the same, the slope and aspect of three plots was kept the same. The distance between each plot was about 10 − 20 m. We used the pendant transplant method (McCune et al., 1996) to transplant thalli fragments of *L. retigera* cut from 270 separate lichen samples, collected from tree trunks at the high sites after being cleaned of debris and organic matter, other non-target species, and any thalli base plates. These fragments were then air-dried to a constant weight in the lab, and cut into sections 3–7 cm long and 2–5 cm wide (weighing 0.1–0.3 g). We then placed a small amount of silicone sealant (<0.1 g) on the ventral surface of each fragment, at the cut base or the edge of the thallus, and attached nylon monofilament loops to the single thallus pieces (McCune et al., 1996). Then, these affixed loops were suspended on coarse fishing line at distance intervals of about 20 cm, identified by using different marks; the line was tied horizontally and loosely between trees (to allow for swaying trunks), the distance between two trees was c. 6 m, and all the samples were c. 2 m above the ground (McCune et al., 1996).

Following transplantation, we measured the nitrogenase activity in May, August, and November of 2015, and in February 2016, Nine samples were selected from each elevation site (three samples from each plot) for the nitrogenase activity evaluation at the same day, in situ, for 24 h. At the experiment sites, the sample was directly placed in a glass culture tube to measure the nitrogen fixation rate without rehydration. Later the data were analyzed by comparing the nitrogenase activity among the different elevation sites and seasons. The biomass growth rate (BGR) was also evaluated at each site in each month (March, May, August and November 2015; February 2016). The BGR was calculated using Eq. (1) (McCune et al., 1996), as follows:

$$BGR = \frac{(B_{t+x} - B_t) * 100}{B_t} \quad (1)$$

B_t to the initial oven-dry mass of the thallus, and B_{t+x} to the oven-dry mass of the thallus after x transplantations.

2.3. Laboratory and greenhouse experiments

We conducted warming and light shading experiments in the shade shelters inside and outside of a greenhouse to study the effects of light and temperature on nitrogen fixation. To rule out the effect of moisture on nitrogenase activity, the water supply to the lichens was kept sufficient throughout the greenhouse experiment and the thalli were rehydrated to saturated water content before the data collection formally began. The greenhouse is 30 m from the forest edge, at 2480 m altitude, and temperature inside it was c. 2 °C higher than outside. Because this greenhouse is made of glass, the illumination intensity inside and outside of it was almost the same.

We use scaffolds and shade nets to create shade shelters that were 1.5 m in length, width and height, with the shade netting material covering the top and side of the frame. We established three shade treatments (i.e. 0%, 50%, and 100%) inside and outside the greenhouse, with corresponding light intensity of c. 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$, 500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and 0 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The light intensity in the shelters was recorded every 2 h, for ten days, by using a portable light quantum instrument. In the greenhouse experiment, lichen thalli were suspended under different light and temperature conditions

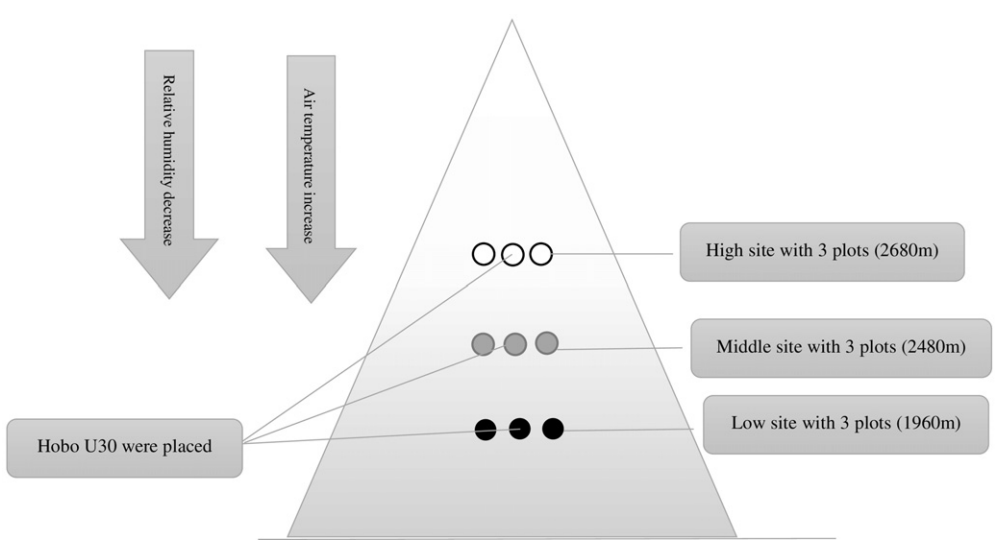

Fig. 1. Schematic of the field experiment.

throughout August 2015, and they were sprayed every 2 h with distilled water during the daytime to maintain their moisture content. Before measuring the nitrogenase activity of thalli after 1 month of the shading treatment, they were all re-hydrated to saturation.

The laboratory experiment studied the effects of water content on the nitrogenase activity and photosynthesis rate of *L. retigera*. To ensure that both temperature and illumination remained identical among the different samples for the given water contents, we established a known gradient moisture content using a series of samples. This was done during cloudy days in the rainy season, as thalli have a low rate of water loss during the incubation time. To do this, we weighed each thallus before and after incubation to evaluate its water content, after each sample was dried in an oven at 65 °C for 48 h and weighed to assess its water content. Effects of the different water contents and light intensity on the photosynthetic rate were also studied when determining the effect of different water contents on N fixation rate. Because nitrogenase activity is limited by thallus C acquisition, which is controlled by the lichen photosynthesis rate, we assessed this under different water content and light intensity conditions by removing fragments to the laboratory and making measurements using a portable photosynthesis system (LI-6400, Li-Cor, Inc., Lincoln, NE, USA) (Chen et al., 2014).

2.4. Measurements

We used the acetylene reduction assay (ARA) to measure nitrogenase activity in the field and laboratory experiments (Stewart et al., 1967; Hardy et al., 1968; DeLuca et al., 2002; Whiteley and Gonzalez, 2016). Each sample of *L. retigera* was placed into a 140-ml glass culture tube. Ten percent of the total headspace of the tube was evacuated and replaced with acetylene, and then the tube was settled back into the transplantation sites. Lichen thallus were incubated for 24 h; then 5 ml of gas samples were withdraw using a gas-tight syringes, and these samples were immediately injected to the vacuum blood tube and brought back to the laboratory. The ethylene content of each sample was determined by using a Hewlett Packard 6890 gas chromatograph equipped with a flame ionization detector and Porapak T column. We also measured the ethylene concentrations in the lichen-only and ethylene-only tubes to ensure that the ethylene detected in these sample tubes was derived via the nitrogenase enzyme. Next, each thallus was taken back to the laboratory and weighed, and its nitrogenase activity was measured using the ethylene concentration in tubes, and then converted to $\mu mol \cdot g^{-1} \cdot day^{-1}$—using the universal gas law—and lichen dry mass.

Samples were placed in a whole plant chamber equipped with a roof-mounted full spectrum cold light source (LI-6400-18), connected to a photosynthesis gas analyzer via a mounting plate (LI-6400-18; LI-COR, Lincoln, NE, USA). This set-up was used to determine the photosynthetic water and light response curves. Lichen light response curves were determined using an incremental set of 12 illumination intensities (i.e. 0, 20, 50, 80, 100, 200, 400, 600, 800, 1000, 1200, and 1400 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at saturated water content under ambient environmental conditions (i.e., temperatures ranging between 16 °C and 20 °C, and an RH ranging between 85% and 95%). To determine the photosynthetic water response curve, we set the illumination intensity to 800 $\mu mol \cdot m^{-2} \cdot s^{-1}$, a level that is saturating for *L. retigera* (Chen et al., 2014). Data encompass saturated water content to constant weight, with the latter recorded before, and after, each measurement to evaluate the lichen water content.

To measure water availability, air temperature, and relative humidity (RH), an automatic weather station (Hobo U30, Onset Computer Corporation) with an air temperature and humidity sensor was placed at each elevation site, at a height of about 1.5 m beneath the forest canopy. Microclimate data were recorded every 30 min for the first ten days in February, May, August, and November.

2.5. Statistical analyses

We subjected all data to normality and homoscedasticity tests before analysis the difference of nitrogenase activity, temperature and RH between different elevation sites using one-way ANOVA with LSD's or Tukey's post hoc tests. Two-way ANOVA was used to analysis the impact of warming, shading, and their interactions on nitrogenase activity. Before analysis, we convert the data through the square root of the original data to satisfy the normality and variance homogeneity test. We conducted all analyses using SPSS 20.0 (SPSS, Inc., Chicago, IL, USA). All graphs were drawn using Sigmaplot 12.5. The photosynthetic data were fitted using a correction of the rectangular hyperbolic model in Eq. (2) (Ye, 2007) as follows:

$$P_n = \alpha \frac{1-\beta I}{1+\gamma I} I - Rd \quad (2)$$

Where P_n denotes the net photosynthesis rate, while I refer to the illumination intensity, Rd to the dark respiration rate.

3. Results

3.1. Mean annual nitrogenase activity and microclimate at different elevation sites

The mean annual nitrogenase activity decreased from the high to low sites, by 67.9% and 41.0% at the middle and low sites when compared with the higher site (Table 1). These sharp reductions in N fixation can be attributed to changes in the microclimate. Over the course of 1 year, the recorded RH decreased in concert with declining elevation in all seasons; average annual RH at the high site was 87.6%, higher than that of the middle (80.8%) and low sites (73.0%), while the temperature showed a reverse trend. Average annual temperature at the high site was 10.0 °C, which was significantly lower than that at middle (11.6 °C) and low sites (14.9 °C) (Fig. 1). The seasonal variation of air temperature and RH of the three elevation sites was similar (Fig. 2).

3.2. Nitrogenase activity and biomass growth rate after transplantation

The nitrogenase activity of *L. retigera* decreased with a declining elevation all year long (Fig. 3a). Average annual values (±SE) for the nitrogenase activity at the high, middle, and low sites were 7.8 ± 1.2 $\mu mol \cdot g^{-1} \cdot day^{-1}$, 5.3 ± 0.8 $\mu mol \cdot g^{-1} \cdot day^{-1}$, and 3.2 ± 0.4 $\mu mol \cdot g^{-1} \cdot day^{-1}$, respectively, while values for the rainy season (August and November) were significantly higher than those for the dry season (May and February).

Accumulated biomass can reflect a C sink or fixation of the thallus. The data revealed a close relationship between biomass growth rate (BGR) and nitrogenase activity, in that between March and May the BGR of *L. retigera* decreased at all three sites. The low site (−16.2% ± 3.0) showed a more rapid decline than did the high (−5.8% ± 0.4) and middle sites (−6.6% ± 0.5), whereas between May and November, the BGR at the high and middle sites continued to increase, reaching 23.2% ± 3.8 and 10.3% ± 2.0, respectively (mean ± SE). By contrast, at the low site, following some temporary growth, the BGR fell to

Table 1
Values of mean annual nitrogenase activity (MANA, $\mu mol \cdot g^{-1} \cdot day^{-1}$), relative humidity (%), and air temperature (°C) for the three elevation sites (means ± SE). Bold numbers inside the parentheses are the percentages of nitrogenase activity (NA) compared with the high site. For MANA, n = 36; for Relative Humidity and air temperature, n = 40.

	MANA ($\mu mol \cdot g^{-1} \cdot day^{-1}$)	Relative humidity (%)	Air temperature (°C)
High site	7.8 ± 1.2 (**100%**)	87.6 ± 2.4	10.0 ± 0.5
Middle site	5.3 ± 0.8 (**67.9%**)	80.8 ± 2.3	11.6 ± 0.5
Low site	3.3 ± 0.5 (**42.3%**)	73.0 ± 2.8	14.9 ± 0.8

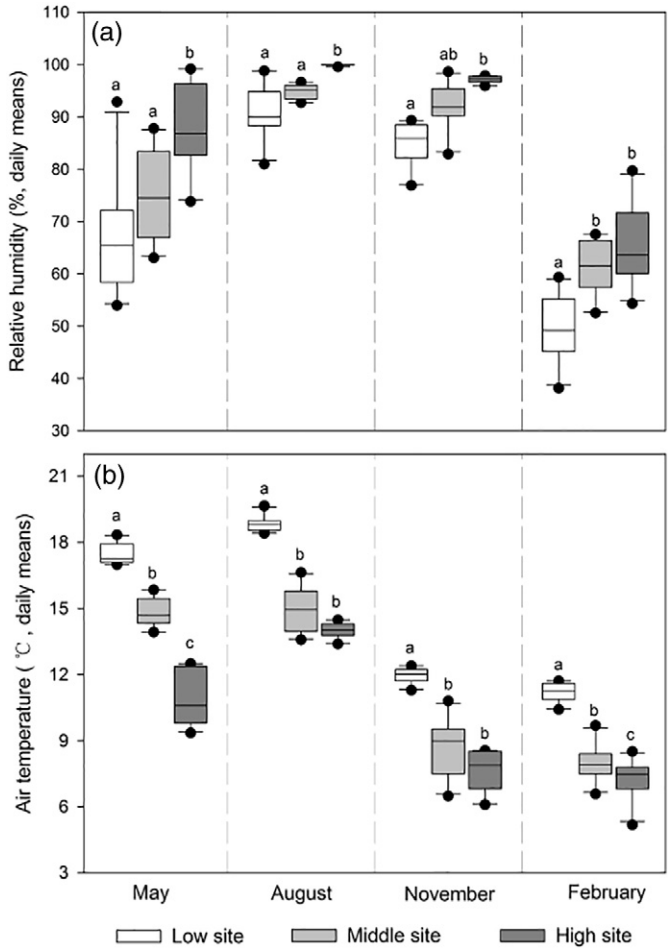

Fig. 2. The relative humidity (a) and air temperature (b) at the different elevation sites (means ± SE, n = 10). Boxes with different letters are significantly different from each other (P < 0.05).

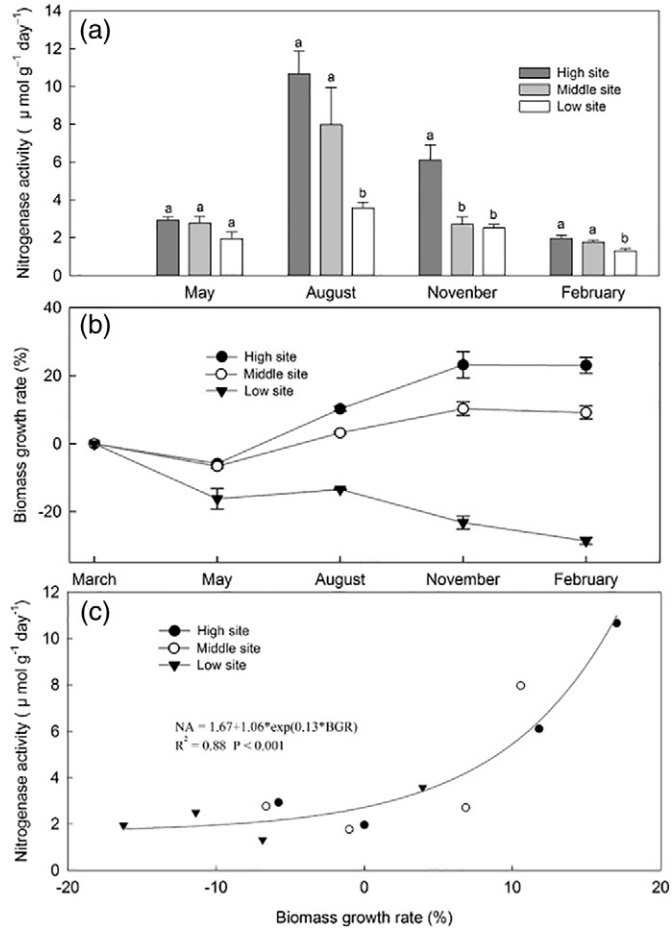

Fig. 3. The (a) nitrogenase activity in the different months (means ± SE, n = 3) (P < 0.05). The (b) biomass growth rate compare to the original biomass at each month after transplantation (means ± SE, n = 5). The (c) relationship between the biomass growth rate and nitrogenase activity data for *L. retigera*; the biomass growth rate here was calculated to compare with the biomass of *L. retigera* in the last month.

−23.3% ± 1.9. One full year after transplantation, the BGR of the high and middle sites had respectively increased to 23.0% ± 2.4 and 9.2% ± 1.9, whereas that of the low site had decreased to −28.6% ± 1.1 (Fig. 3b). Moreover, nitrogenase activity was maintained at a low level when the BGR was <0%, but it increased significantly with an increasing BGR at between 0% and 20% (Fig. 3c).

3.3. The influence of thallus water content on nitrogenase activity

The nitrogenase activity of *L. retigera* increased in concert with its thallus water content (Fig. 4a). Indeed, the level of N fixation was lowest when water content was <100%; but when the thallus water content was between 100% and 200%, nitrogenase activity also increased significantly. Above 300%, nitrogenase activity was maintained at a high level and it did not change significantly.

We measured photosynthesis rates under different water content conditions. We found that the nitrogenase activity in *L. retigera* was not linked to photosynthetic activity, as the latter increased significantly as the water content increased from 100% to 180%, but then it dropped rapidly between 180% and 300% (Fig. 4b).

3.4. Nitrogenase activity responses under different light and temperature conditions

The nitrogenase activity of *L. retigera* was highest when subjected to 50% shading, and both warming and non-warming treatments. This activity was also significantly limited by both low and high light conditions (Fig. 5). Our use of GLMs shows that there was a significant effect of shading, but not of warming; however, the interaction between these two main factors was significant (P < 0.05) (Table 2). Hence, the effect of the warming treatment on the N fixation of *L. retigera* depended on a change in light availability (i.e., shading). Due to the differences between the effect of shading (P < 0.001) and warming (P = 0.09) on nitrogen fixation, we suggest that the effect of light is more important than the temperature increase of 2 degrees (Table 2).

We also measured the photosynthetic rate of *L. retigera* under different light conditions. This rate increased rapidly with a greater light intensity up to 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$, after which, up to 1400 $\mu mol \cdot m^{-2} \cdot s^{-1}$, it slightly decreased (Fig. 5). Because the light saturation and compensation points were 406 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and 22 $\mu mol \cdot m^{-2} \cdot s^{-1}$, respectively, these results suggest that moderate light intensities are most suitable for both photosynthesis and nitrogenase activity in *L. retigera*.

4. Discussion

4.1. The influence of dry-hot stress on nitrogenase activity

Our experiments show that nitrogenase activity decreases with a declining elevation; Given the similar tree species composition, forest structure, and canopy cover among the elevation sites (Qiu and Xie, 1998), we therefore attribute the factors that affected nitrogenase activity to water supply and temperature. In the early transplantation stages,

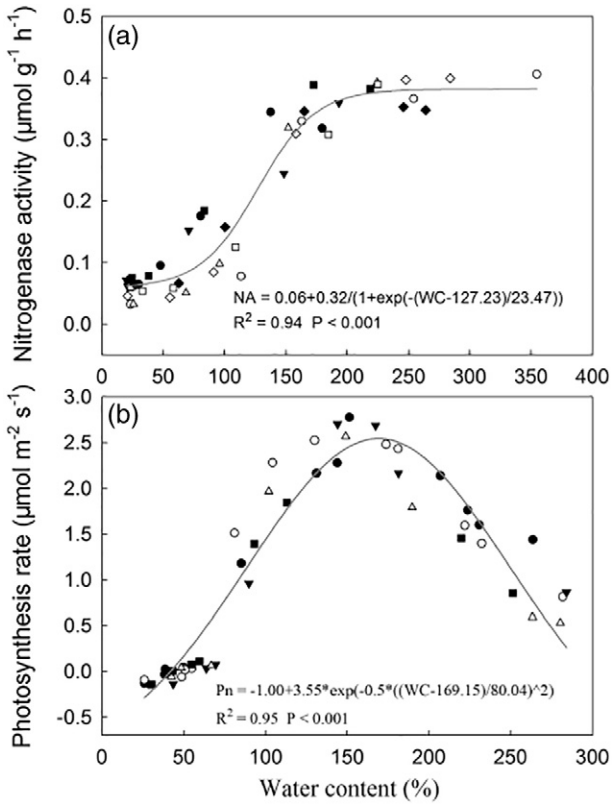

Fig. 4. Graphs showing the responses of nitrogenase activity (a) (n = 8), and of photosynthesis rate to the different water contents (b) (n = 5). The symbols within the graph having different shapes represent the different samples.

decreases in nitrogenase activity between elevation sites can be driven by a reduced water supply to the thallus, as reflected by the relative humidity. Our laboratory study corroborates this plausible explanation; under suitable conditions, nitrogenase activity remains at a low level when the thallus water content is <100% (Fig. 4). At the same time, however, other studies have suggested that thallus water content is just one of the major factors limiting cyanobacterial activity in the field (Coxson and Kershaw, 1983a, 1983b). Compared with the thallus water supply, there is evidence that temperature has a direct effect on N fixation in other cyanolichens. For example, *Peltigera* exhibits

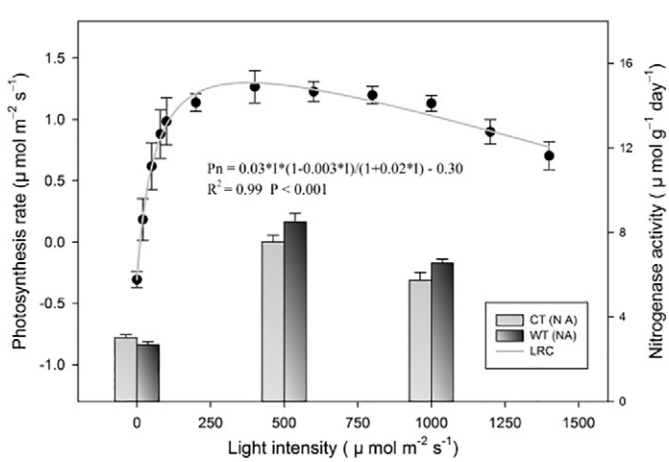

Fig. 5. Response of thallus nitrogenise activity to the different shading rates and warming treatments, and relationship between light intensity and photosynthetic rate. Abbreviations: WT, warming treatment at 16.81 °C; CT, contrast treatment at 14.85 °C; LRC, light response curve). The symbols in the light response curve and the vertical bars for the nitrogenase activity in warming and shading treatment denote the means ± SE, each with n = 5.

Table 2
Comparison nitrogenase activity between different shading and warming treatments and their interactions (warming treatment, n = 15; shading rate, n = 10).

Source	df value	F value	P value
Warming	1	3.14	0.089
Shading rate	2	229.07	<0.001
Warming × shading rate	2	4.05	0.031

nitrogenase activity even at subzero ground temperatures (Hitch and Stewart, 1973), but high temperatures, those above 32.5 °C and 20 °C will often inhibit activity in this species and in *Lichina* (Hitch and Stewart, 1973), respectively. It has also been demonstrated that nitrogenase activity in *L. oregana* increases as the temperature is raised from 2 °C to 14 °C (Antoine, 2004). Meanwhile, the mean nitrogenase activity of *L. retigera* in our field experiment during the rainy season—especially at the high elevation site in August 2015—was higher than the value measured in the laboratory experiment at saturated water content (Fig. 4). The nitrogenase activity may have been overestimated due to several high values at each elevation sites. These higher values may be due to the unavoidable excess heating of the samples caused by the sunlight penetrating through the canopy, as warming accelerates nitrogen fixation under sufficient water and light conditions. Our study also shows that a warming of just 2 °C facilitates the process of N fixation under 0% and 50% shading, while limiting this process under complete shade (i.e., 100% shading). Indeed, our results suggest that under 0% and 50% shading, C fixation can persist at a high level, especially as there was sufficient C compounds present in the thallus (Fig. 5). However, the C sink in the thallus may become deficient because C fixation stops in darkness of 100% shading (Fig. 5). Thus, our results suggest that warming can both promote and limit N fixation, given sufficient and deficient C storage, respectively. On the basis of our increased BGR data, we further suggest that, in the early stages, C storage in the thallus is sufficient for N fixation (Fig. 3). In this situation, a warming trend should accelerate the N fixation process, while a decreased water supply along the elevation gradient could offset this promotional effect. Some other studies have also suggested that BNF is inhibited more by drought than by warming (Whiteley and Gonzalez, 2016).

4.2. Influence of biomass accumulation on nitrogenase activity

The mycobiont hyphae make up the dominant part of the lichen biomass (Büdel and Scheidegger, 1996), which arise from interactions of individual hyphae that continue to grow at their tips (Wessels, 1993). In this process, N is obviously necessary for the synthesis of new proteins, membranes and DNA, as well as structuring the cell walls—which contain chitin, a nitrogenous carbohydrate—of the hyphae. It is also reported that the amount of chitin is higher in those species with access to an ample nitrogen supply via N fixation (Schlarmann et al., 1990). Therefore, cyanobacteria N fixation is likely very important for biomass accumulation in these organisms. To the contrary, nutrient assimilation (including N fixation) is also energized by the substances which are converted from photo-assimilate by lichen respiration (Palmqvist, 2000). So, we suggest that there must be an intimate relationship between cyanobacteria N fixation and C acquisition, as well as a link to lichen biomass accumulation. Specifically, we regard the imbalances in C acquisition and expenditure, as caused by the warming interacting with the reduced precipitation, which led to the reduced biomass of the thallus, to be the main reason underlying the decreased N fixation seen in our later field experiments. Data show that C acquisition is most affected by water supply and air temperature (Lange, 2002). In our study, the photosynthesis of *L. retigera* was maintained at a low level before 100% water content, beyond which C fixation really started (Fig. 4). Lange et al. (2004) confirmed that lichen *Pseudocyphellaria aurata* had a strong net photosynthetic rate under the condition of 100–200% water contents. Our results showed that the maximum net

photosynthetic rate of *L. retigera* occurs at about 150–180% water contents. But the photosynthesis in this cyanolichen obviously decreased after 180% (Fig. 4); hence, it is possible that C acquisition is limited by both low and saturated thallus water contents (Lange et al., 1993). High water content inhibits the photosynthesis of lichens. Some lichens cannot tolerate prolonged flooding, which can lead thallus death (Nash, 2008). However, while a saturated water content depressed photosynthesis, but it showed no signs of limiting N fixation in our experiment (Fig. 4). The negative impact of saturated water content on photosynthesis is not a common occurrence in the field in the dry season from November to April, as water saturation can only happen after rainfall and will rapidly revert to a low water content in a very short time (Antoine, 2004). While in rainy season, due to frequent rainfall and higher air humidity, the thalli often experience water saturation, which may affect photosynthesis and N fixation to a certain extent. In addition to the thallus water supply, temperature is also an important factor influencing C acquisition and expenditure. Research has suggested that short-term increases in N fixation in response to warming may occur due to kinetic responses, with the majority of studies implying that long-term warming, resulting in reductions in biomass, has a negative impact on N fixation (Lindo et al., 2013). In our present study, a C imbalance was demonstrated by a decreased biomass at the low site (Fig. 3); this lack of C, as driven by desiccation and the warming of the environment, should negatively affect the process of N fixation. In addition, because the limited water supply at the low site resulted in a reduced nitrogenase duration and photosynthetic activity, our experiment suggests that 1 year of warming restrained, rather than facilitated, nitrogenase activity by accelerating the C expenditure (Fig. 3). At the same time, while the nitrogenase activity was maintained at a low level when the BGR was between −20% and 0%, it increased significantly in our field experiment when BGR was between 0% and 20% (Fig. 3). This observation, when coupled with the results of our shading experiment, together suggest that cyanolichen nitrogenase activity under dark conditions is significantly lower than nitrogenase activity under 50% and 0% shading, even under saturated water conditions. As such, we conclude that it was the C budgets of thalli which dominated N fixation in the middle and later transplantation experiments.

5. Conclusion

This study is first to assess the possible impacts of climate change via so-called dry-hot stress on the nitrogenase activity of cyanolichens in a subtropical montane forest. We achieved this using careful transplantation experiments, and have shown that the nitrogenase activity or the estimated mean annual nitrogenase activity of *L. retigera* is sensitive to simulated climate change conditions, which are predicted to become warmer and drier by the 2050s in montane moist evergreen broad-leaved forests (Hijmans et al., 2005; Ramirez and Jarvis, 2008). The N fixed by this community is likely to decrease, however, following the predictions of climate change, even though it is presently the main N input into these ecosystems, at least in the epiphyte communities. Based on our experimental laboratory and greenhouse results, we suggest that the imbalances in the C budgets caused by dry-hot stress will be the ultimate arbiter in affecting nitrogenase activity in cyanolichens. Climate warming promotes nitrogenase activity when both water and C storage are sufficient, but it suppresses N fixation when these inputs are insufficient. In conclusion, we suggest that dry-hot stress or future climate change will have a negative impact on N and C fixation by epiphytic cyanolichens as well as their biomass accumulation.

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