ANA FÁVARO

EFFECTS OF CO_2 AND MICRONUTRIENT SUPPLY ON HETEROCYST PRODUCTION AND NITROGEN FIXATION IN CYANOLICHENS

LAVRAS – MG

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Profa. Dra. Flávia de Freitas Coelho

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EFFECTS OF CO_2 AND MICRONUTRIENT SUPPLY ON HETEROCYST PRODUCTION AND NITROGEN FIXATION IN CYANOLICHENS

EFEITOS DO CO_2 E DO FORNECIMENTO DE MICRONUTRIENTES NA PRODUÇÃO DE HETERÓCITOS E NA FIXAÇÃO DE NITROGÊNIO EM CIANOLIQUENS

Dissertação apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Ecologia Aplicada, área de concentração em Ecologia e monitoramento de ecossistemas sob interferência antrópica, para a obtenção do título de Mestre.

APROVADA em 29 de junho de 2021.

Dr. Guilherme Ramos Demétrio Ferreira (UFAL)

Dra. Natália Mossmann Koch (University of Minnesota)

Profa. Dra. Flávia de Freitas Coelho

Orientadora

LAVRAS – MG

2021

Aos meus pais, que sempre me incentivam a estudar,

Ao meu namorado, que há nove anos me inspira e me apoia,

E aos meus amigos, que acreditam em mim mesmo quando eu não acredito em mim mesma, que me dão forças e me proporcionam momentos que enchem o meu coração de gratidão

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"Não sei se realmente tenho a cabeça de um líquen, provavelmente não, mas invejo sua persistência. No grande mundo, o líquen não pede nada a ninguém, exceto um pedaço de pedra ou uma árvore. Pode-se dizer que floresce mal, que vegeta; mas eu gosto exatamente de sua escolha para ser ancorado, como uma alga ao ar livre."

— Antoine Emaz, Lichen lichen

RESUMO

A fixação de nitrogênio (N) é uma importante função do ecossistema por ser uma das principais fontes de N reativo para o ambiente. Apesar disso, os fatores que controlam esse processo em ambientes terrestres ainda são pouco conhecidos. A disponibilidade de micronutrientes, bem como o excesso de carbono, têm sido considerados reguladores chave da fixação de N, apesar de ainda existir grande incerteza sobre a forma como atuam na regulação desse processo. Projeções futuras preveem um aumento nas concentrações de dióxido de carbono (CO_2), que podem pressionar os organismos a fixarem N. Entretanto, uma limitação de micronutrientes pode gerar uma restrição na fixação de N, afetando o fornecimento de N reativo. Dessa forma, o objetivo deste trabalho foi verificar como o suprimento de molibdênio (Mo) e vanádio (V) e altas concentrações de CO_2 afetam a fixação de N e produção de heterócitos, células fixadoras, no cianolíquen *Leptogium cyanescens* (Rabenh.) Körb. Para tal, foi realizado um experimento de 27 dias, com 64 talos transplantados de uma mata ciliar na Reserva Biológica Boqueirão para uma câmara de crescimento com ambiente controlado. Os heterócitos foram contabilizados após cortes anatômicos dos talos serem feitos com um micrótomo de congelamento, e as taxas de fixação de N foram obtidas por meio do ensaio de redução de acetileno. Nossos resultados mostraram que a adição de micronutrientes e a exposição à altas concentrações de CO_2 aumentou a produção de heterócitos nos talos. Entretanto, os espécimes expostos ao Mo, V e à alta concentração de CO_2 simultaneamente não produziram mais heterócitos e não fixaram mais nitrogênio do que os talos expostos a essas condições separadamente, como era esperado. Além disso, também encontramos uma relação negativa entre o número de heterócitos e a fixação de N. A partir desses resultados, concluímos que o investimento em heterócitos é afetado pelos micronutrientes por eles participarem da síntese da nitrogenase, enzima fixadora de nitrogênio, e pela alta concentração de CO_2, por ela induzir a demanda por nitrogênio.

Palavras-chave: Dióxido de carbono. Molibdênio. Nitrogenase. Nitrogenase alternativa. Vanádio.

ABSTRACT

Nitrogen (N) fixation is an important ecosystem function as it is one of the main sources of N to the environment. Despite that, the factors that control this process in terrestrial environments are still poorly understood. Micronutrient availability and excess carbon have been considered key regulators of N fixation, but there is still great uncertainty about how they act in the regulation. Future projections predict an increase in carbon dioxide (CO_2) concentrations, which can pressure organisms to fix N. However, a micronutrient limitation may restrict N fixation, affecting the supply of reactive N to the ecosystem. Thus, this work aimed to verify how the supply of molybdenum (Mo) and vanadium (V) and high concentrations of CO_2 affect N fixation and heterocyst production, fixing cells, in the cyanolichen *Leptogium cyanescens* (Rabenh.) Körb. To do it, we performed a 27-day experiment, with 64 thalli transplanted from a riparian forest located within Reserva Biológica Boqueirão to a growth chamber with a controlled environment. Heterocysts were counted after we sectioned the thalli using a freezing microtome, and N fixation rates were obtained through an acetylene reduction assay. We found that the addition of micronutrients and exposure to high concentrations of CO_2 increased the thalli investment in heterocysts. However, specimens exposed to Mo, V, and high concentration of CO_2 simultaneously did not produce more heterocysts, and they did not fix more nitrogen than thalli exposed to these conditions separately, as expected. In addition, we also found a negative relationship between the number of heterocysts and N fixation. These results lead us to the conclusion that investment in heterocysts is affected by micronutrients because they participate in the synthesis of nitrogenase and by a high concentration of CO_2 because it induces the demand for nitrogen.

Keywords: Alternative nitrogenase. Carbon dioxide. Molybdenum. Nitrogenase. Vanadium.

SUMÁRIO

PRIMEIRA PARTE

INTRODUÇÃO GERAL

O nitrogênio (N) é um elemento essencial para a formação de biomoléculas que sustentam a vida, como aminoácidos, proteínas e nucleotídeos, e está amplamente disponível no planeta na forma de dinitrogênio gasoso (N_2) (HOFFMAN *et al*., 2014; JIA; QUADRELLI, 2014; THAMDRUP, 2012). No entanto, a maioria dos organismos é incapaz de metabolizar o N_2 por ele ser relativamente inerte (HOFFMAN *et al*., 2014; REED; CLEVELAND; TOWNSEND, 2011). Apenas alguns poucos organismos procariotos, bactérias e cianobactérias, de vida livre ou simbióticos, realizam um processo denominado fixação de N (GALLON, 1992; RAYMOND *et al*., 2004; ZHENG *et al*., 2019), sendo capazes de fazer o metabolismo do N_2

A fixação de N é a conversão do N_2 em amônia (NH_3) e outros compostos nitrogenados com auxílio da nitrogenase, enzima que catalisa a reação apenas na ausência de oxigênio (FOWLER *et al*., 2013; KUMAR; MELLA-HERRERA; GOLDEN, 2010). É um dos processos biológicos mais dispendiosos, pois gasta cerca de 15 a 16 ATPs por molécula de N_2 fixada (RAYMOND *et al*., 2004; REED; CLEVELAND; TOWNSEND, 2011; THOMPSON; ZEHR, 2013). Através deste processo, os organismos procariotos, além de metabolizar o N, acabam disponibilizando este elemento para os organismos não fixadores, ao incorporá-lo nas teias tróficas (CHENG, 2008; ROUSK; SORENSEN; MICHELSEN, 2017).

Além de ser um dos processos chave responsáveis pela sustentação da vida na Terra, a fixação de N é a principal fonte de N reativo para os ecossistemas terrestres (CHENG, 2008; GALLOWAY *et al*., 2004; HOFFMAN *et al*., 2014), podendo ser responsável por até 97% da entrada de N em ecossistemas terrestres não manejados (DARNAJOUX *et al*., 2014; WANG *et al*., 2010), afetando o ciclo global deste elemento (VITOUSEK et al., 2013). Além disso, a fixação de N influencia o crescimento de diversos organismos, a produtividade primária, a sucessão ecológica, o sequestro de carbono (C) e as respostas do ecossistemas frente as mudanças climáticas (BELLENGER *et al*., 2020; DYNARSKI; HOULTON, 2018; LÜSCHER *et al*., 2000; VITOUSEK *et al*., 2013). Porém, apesar da importância da fixação de N, os fatores que controlam esse processo permanecem pouco compreendidos (DYNARSKI; HOULTON, 2018; REED; CLEVELAND; TOWNSEND, 2011; ZHENG *et al*., 2019).

A disponibilidade de micronutrientes é considerada um fator regulador chave da fixação de N (REED; CLEVELAND; TOWNSEND, 2011; ZHENG *et al*., 2019). Dentre os micronutrientes, os que mais se destacam são o molibdênio (Mo), o ferro (Fe) e o vanádio (V)

(ZHENG *et al*., 2019). Esses micronutrientes são considerados importantes por participarem da síntese da enzima nitrogenase, responsável pela redução do N_2 (BOTHE *et al*., 2010; HOFFMAN *et al*., 2014; ZHENG *et al*., 2019).

A nitrogenase apresenta dois componentes: a dinitrogenase redutase e a dinitrogenase (HOFFMAN *et al*., 2014). A dinitrogenase redutase é formada por uma proteína de Fe que hidrolisa ATP e transfere elétrons para a dinitrogenase (HOFFMAN *et al*., 2014). Por sua vez, a dinitrogenase apresenta um aglomerado de fósforo (P) e um cofator (HOFFMAN *et al*., 2014). O aglomerado de P é responsável pela transferência de elétrons da dinitrogenase redutase para o sítio ativo do cofator desta enzima, onde ocorre a redução do N_2 (BOTHE *et al*., 2010; HOFFMAN *et al*., 2014; RAYMOND *et al*., 2004). Na grande maioria das vezes, o cofator é composto por Fe e Mo, mas também existem cofatores onde o Mo é substituído por Fe ou por V (BELLENGER *et al*., 2011, 2020; HOFFMAN *et al*., 2014; RAYMOND *et al*., 2004). Assim, são conhecidos três tipos de nitrogenase: nitrogenase Mo, nitrogenase Fe e nitrogenase V (BELLENGER *et al*., 2020).

Dentre os três tipos de nitrogenases existentes, a nitrogenase Mo é a nitrogenase mais estudada, provavelmente porque ocorre em todos os organismos fixadores de nitrogênio e por ser a única a participar de associações simbióticas com plantas superiores (BELLENGER *et al*., 2020; BOTHE *et al*., 2010). Toda essa atenção à nitrogenase Mo fez com que as nitrogenase V e Fe, também chamadas de nitrogenases alternativas, permanecessem negligenciadas até recentemente (BELLENGER *et al*., 2020). Nos últimos anos, muitos estudos têm demonstrado que a fixação de N feita por nitrogenases alternativas tem uma grande importância ecológica para os ecossistemas terrestres (BELLENGER *et al*., 2020; DARNAJOUX *et al*., 2019; ZHANG *et al*., 2016). Em um estudo feito por Darnajoux e colaboradores (2017) com o cianolíquen *Peltigera aphthosa* (L.) Willd, a nitrogenase V contribuiu significativamente para a fixação de N em laboratório, chegando a ser responsável por 60% da fixação. Em outro trabalho de Darnajoux e colaboradores (2019), a nitrogenase V chegou a contribuir em mais de 80% da fixação em cianoliquens do gênero *Peltigera*.

A distribuição filogenética das nitrogenases alternativas é pouco clara, mas já se sabe que determinados organismos podem apresentar todos os três tipos de nitrogenase, alguns apenas nitrogenase Mo e outros apenas Mo e V, mas não a nitrogenase Fe, ou as nitrogenases Mo e Fe, sem a nitrogenase V (BOTHE *et al*., 2010). As três nitrogenases são codificadas por genes diferentes (BELLENGER *et al*., 2020; BOTHE *et al*., 2010). Organismos com genes que codificam para mais de uma nitrogenase preferencialmente produzem a nitrogenase Mo

(BELLENGER *et al*., 2020). Além dos fatores genéticos, os fatores ambientais também determinam qual nitrogenase será sintetizada (REED; CLEVELAND; TOWNSEND, 2011). Quando as condições ambientais não permitem que o fixador de N produza uma nitrogenase Mo funcional, as nitrogenases alternativas acabam sendo sintetizadas (BELLENGER *et al*., 2020). Portanto, as nitrogenases Fe e V acabam funcionando como "backups" (BELLENGER *et al*., 2020). Um exemplo de organismo que possui os conjuntos de genes para os três tipos diferentes de nitrogenases é a bactéria diazotrófica *Azotobacter vinelandii* (BELLENGER *et al*., 2011; BOTHE *et al*., 2010). Quando o Mo apresenta disponibilidade suficiente no meio, *A. vinelandii* expressa o gene que codifica a nitrogenase Mo. Quando o Mo é limitante, mas a quantidade de V no meio é suficiente, a bactéria expressa o gene que codifica a nitrogenase V. Quando as concentrações de Mo e de V estão limitantes, *A. vinelandii* expressa o gene que codifica a nitrogenase Fe (BELLENGER *et al*., 2011; BOTHE *et al*., 2010). Dessa forma, os organismos que apresentam os genes que codificam as três nitrogenases demonstram uma vantagem adaptativa sobre aqueles que exibem genes que codificam apenas uma ou duas nitrogenases, já que eles se adaptam à disponibilidade de micronutrientes no meio (BELLENGER *et al*., 2011).

A fixação de N é comumente limitada pela disponibilidade de micronutrientes (BARRON *et al*., 2009; DYNARSKI; HOULTON, 2018; HARWOOD, 2020; REED; CLEVELAND; TOWNSEND, 2013; STANTON *et al*., 2019; WINBOURNE; BREWER; HOULTON, 2017). Em um estudo realizado no solo de florestas tropicais no Panamá, o fornecimento de Mo, isolado e combinado com fósforo (P), aumentou a fixação de N de bactérias heterotróficas de vida livre (BARRON *et al*., 2009). O mesmo resultado foi encontrado em um experimento conduzido no dossel de uma floresta tropical no mesmo país (STANTON *et al*., 2019). A fixação de N de microrganismos presentes em folhas e galhos desse microbioma foi co-limitada por P e por Mo (STANTON *et al*., 2019). Já o Fe limitou a fixação de N da serrapilheira presente em uma floresta tropical de calcário em Belize durante a estação chuvosa (WINBOURNE; BREWER; HOULTON, 2017). Entretanto, apesar de muitas pesquisas mostrarem um efeito positivo da adição de micronutrientes na fixação de N, alguns estudos também reportam uma ausência de restrição desses nutrientes na fixação (WONG *et al*., 2019, 2021). Wong e colaboradores (2021), por exemplo, reportaram que a fixação de N feita por procariotos de vida livre na floresta Amazônica não foi restringida por Mo. Portanto, ainda hoje, há muita incerteza sobre como a disponibilidade de nutrientes regula a fixação de N (ZHENG, 2019).

Além dos micronutrientes, a disponibilidade de carbono (C) pode influenciar a fixação de N (HUNGATE *et al*., 2004; HUTCHINS *et al*., 2015; TRIERWEILER; WINTER; HEDIN, 2018). Isso porque o metabolismo do C e do N está fortemente acoplado em diferentes organismos vivos (ZHANG *et al*., 2018). Em procariotos e plantas, esse acoplamento pode ser atribuído a dois fatores principais: primeiro, os dois elementos são os mais abundantes nas células, intensificando a necessidade de mecanismos de coordenação para evitar ineficiências metabólicas; segundo, a assimilação de nitrogênio depende da disponibilidade de um esqueleto de carbono para a biossíntese e, consequentemente, a limitação ou excesso de oferta de um elemento afeta fortemente o metabolismo do outro (ZHANG *et al*., 2018; ZHAO *et al*., 2010). Em cianobactérias, o excesso de C é compensando por mecanismos genéticos que aumentam a fixação de N (MURO-PASTOR; HESS, 2012; ZHANG *et al*., 2018).

Um grupo muito diverso de organismos é capaz de fixar N (VITOUSEK *et al.*, 2013). Dentre eles, os cianoliquens se destacam pelo grande fornecimento de N no ecossistema (ELBERT *et al*., 2012). Cianoliquens são associações simbióticas bipartidas entre fungos e cianobactérias ou tripartidas, quando a associação é formada por fungos, cianobactérias e algas verdes (RIKKINEN, 2015). Nos talos tripartidos, a cianobactéria permanece em uma estrutura denominada cefalódio ou em fotosimbiodemas (NASH, 2008; STOCKER-WÖRGÖTTER, 2002). As funções ecossistêmicas desenvolvidas pelos cianoliquens são diversas e muito importantes. Além de fornecer N para o ambiente e participar do ciclo biogeoquímico desse elemento, esses liquens fixam C, são fundamentais para a teia alimentar, aumentam a complexidade e diversidade dos ecossistemas (ASPLUND; WARDLE, 2016; ELBERT *et al*., 2012; ELLIS, 2012; GREEN; NASH; LANGE, 2008).

Nas cianobactérias, presentes nos cianoliquens, a fixação de N é realizada em células especializadas denominadas heterócitos (ADAMS; DUGGAN, 1999; KUMAR; MELLA-HERRERA; GOLDEN, 2010; PERNIL; SCHLEIFF, 2019). Essas células são, geralmente, maiores que as células vegetativas, têm um envelope celular espesso, apresentam nódulos polares e pouca pigmentação (ADAMS; DUGGAN, 1999; KUMAR; MELLA-HERRERA; GOLDEN, 2010; STAINIER; COHEN-BAZIRE, 1977). No heterócito, a nitrogenase converte o N_2 em NH_3 e esta é convertida em glutamina, que é repassada para as células vegetativas (NASH, 2008). Até o momento, apenas os genes que codificam nitrogenases Mo e V foram encontrados em cianobactérias que formam associações simbióticas com fungos (DARNAJOUX *et al*., 2017; GAGUNASHVILI; ANDRÉSSON, 2018; HODKINSON *et al*.,

2014; MARKS *et al*., 2015). O desbalanço entre as quantidades de C e N por um excesso de C reprime genes relacionados à aquisição de C e ativa genes que aumentam a produção de heterócitos e a fixação de N (MURO-PASTOR; HESS, 2012; ZHANG *et al*., 2018). Juntos, esses mecanismos atuam de forma a retomar o balanço entre C e N nas cianobactérias (ZHANG *et al*., 2018).

Considerando que um excesso de C pode afetar a fixação de N e que as projeções futuras mostram um aumento nas concentrações atmosféricas de CO_2 (950 ppm em 2100, no pior cenário) (PRATHER *et al*., 2013; SCHNEIDER, 2009), é crucial que novos estudos avaliem como os organismos fixadores de N respondem às altas concentrações de CO_2. Como os liquens não apresentam cutículas e estômatos, eles não conseguem selecionar o que absorvem ou excretar substâncias tóxicas e acabam armazenando essas substâncias no talo (HÄFFNER *et al*., 2001; KÄFFER *et al*., 2012). Portanto, caso as projeções futuras de elevação na concentração de CO_2 global se concretizem, os cianoliquens sofrerão com uma contínua exposição a esse gás. Essa exposição constante pode gerar um desbalanço na razão C:N e pressionar os organismos a fixarem N (ZHANG *et al*., 2018), mas existe a possibilidade de a fixação de N sofrer uma limitação devido a restrição de micronutrientes (HUNGATE *et al*., 2004; TRIERWEILER; WINTER; HEDIN, 2018).

Limitações na fixação de N causadas por baixa disponibilidade de Mo têm sido encontradas em ecossistemas tropicais, temperados e boreais (BARRON *et al*., 2009; ROUSK *et al*., 2017; WONG *et al*., 2020). A disponibilidade desse elemento é rara na maioria dos solos, ao contrário do V que é amplamente difundido no ambiente (HANUS-FAJERSKA; WISZNIEWSKA; KAMIŃSKA, 2021; TAYLOR; MCLENNAN, 1995; WONG *et al*., 2020). Em ambientes terrestres, a baixa disponibilidade de Mo favorece a formação da nitrogenase V, que, apesar de ser menos eficiente que a nitrogenase Mo, funciona como um backup na fixação de N (BELLENGER *et al*., 2014; 2020). Com o aumento das concentrações de CO_2 associados às mudanças climáticas, a biodisponibilidade de Mo pode diminuir e suprimir a fixação (HUNGATE *et al*., 2004).

Pouco se conhece a respeito dos efeitos do aumento na concentração de CO_2 na produção de heterócitos e na fixação de N de cianoliquens. Até onde sabemos, apenas dois trabalhos verificaram esses efeitos (FÁVARO; NASCIMENTO; COELHO, 2021; NORBY; SIGAL, 1989). Fávaro e colaboradores (2021) encontraram uma maior densidade de heterócitos nos talos de *Leptogium cyanescens* presentes em uma área com mais CO_2 (400 ppm, aproximadamente) do que em uma área com menos CO_2 (220 ppm, aproximadamente).

Norby e Sigal (1989) encontraram um aumento na atividade da nitrogenase em talos de *Lobaria pulmonaria* (L.) Hoffm após aumento da concentração de CO_2 atmosférico em câmaras com ambiente controlado. Além de mais estudos com CO_2 serem necessários, estudos comparativos sobre a importância de diferentes micronutrientes, como Mo versus V, na regulação da fixação de N também são fundamentais para que possamos prever a resposta dos cianoliquens frente a um cenário de mudanças climáticas (BELLENGER *et al*., 2020; ZHENG *et al*., 2019).

Neste sentido, esta dissertação de mestrado busca responder como altas concentrações de CO_2 e o fornecimento de Mo e V afetam o investimento em heterócitos e a fixação de N em cianoliquens da espécie *L. cyanescens*. Para isso, foi hipotetizado que: (1) O fornecimento de micronutrientes e a alta concentração de CO_2 aumentam a produção de heterócitos. (2) Talos expostos ao fornecimento de micronutrientes e à alta concentração de CO_2 simultaneamente produzem mais heterócitos do que talos expostos a essas condições separadamente. (3) Talos expostos ao fornecimento de micronutrientes e à alta concentração de CO_2 simultaneamente apresentam taxas maiores de fixação de N do que talos expostos a essas condições separadamente. (4) Quanto maior o número de heterócitos, maior é a fixação de N nos talos. Os dados coletados para este trabalho foram obtidos por meio de um experimento de curta duração onde os talos foram transplantados do ambiente natural para uma câmara de crescimento com ambiente controlado.

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SEGUNDA PARTE – ARTIGO

ARTIGO 1 - EFFECTS OF CO_2 AND MICRONUTRIENT SUPPLY ON HETEROCYST PRODUCTION AND NITROGEN FIXATION IN CYANOLICHEN

(Artigo elaborado de acordo com as normas do periódico)

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Effects of CO_2 and micronutrient supply on heterocyst production and nitrogen fixation in cyanolichens

Ana Fávaro[1*], *Daniel Eric Stanton*[2], *Natália Mossmann Koch*[2], *Lara Cecília Oliveira Lourenço* [3], *Flávia de Freitas Coelho*[3]

[1] Universidade Federal de Lavras, Departamento de Ecologia e Conservação, Lavras, Minas Gerais, Brasil

[2] University of Minnesota-Twin Cities, Department of Ecology, Evolution and Behavior, Saint Paul, Minnesota, USA

[3] Universidade Federal de Lavras, Departamento de Biologia, Lavras, Minas Gerais, Brasil

* Corresponding author: Ana Fávaro

E-mail: anafavarovga@gmail.com

ORCID

Ana Fávaro: 0000-0001-6430-9080

Daniel Eric Stanton: 0000-0002-6713-9328

Natália Mossmann Koch: 0000-0001-6958-4495

Lara Cecília Oliveira Lourenço: 0000-0001-6112-9551

Flávia de Freitas Coelho: 0000-0001-9338-8128

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ABSTRACT

Despite the importance of nitrogen (N) fixation as an ecosystem function, the factors that control this process in terrestrial environments remain poorly understood. Micronutrient availability and carbon excess have been considered key N fixation regulators, but the regulation mechanism is still uncertain. In this study, we aimed to verify how molybdenum (Mo) and vanadium (V) supply and high carbon dioxide (CO_2) concentration affect heterocyst production and N fixation in the bipartite cyanolichen *Leptogium cyanescens* (Rabenh.) Körb. To perform the 27-day experiment, we transplanted 64 thalli from a riparian forest to a growth chamber with a controlled environment. Heterocysts were counted after we sectioned the thalli using a freezing microtome, and N fixation rates were measured with an acetylene reduction assay. *Leptogium cyanescens* thalli from the control treatment decreased the production of heterocysts between the beginning and the end of the experiment. Investment in heterocysts increased in response to additions of Mo, V or CO_2, but these effects were not additive. Also, no significant differences in N fixation rates were found between the treatments. During this short-period experiment, we also observed a negative relationship between the number of heterocysts and N fixation. Micronutrients and CO_2 concentration may affect heterocyst production and N fixation because they participate in the synthesis of nitrogenase, and induced demand for nitrogen, respectively. Our findings present new evidence that Mo, V and, CO_2 can drive heterocysts production and, consequently, N fixation in a short period of cyanolichens exposition.

Keywords: Alternative nitrogenase; Carbon dioxide; Molybdenum; Nitrogenase; Vanadium

INTRODUCTION

Nitrogen (N) fixation is the principal source of new reactive N to unmanaged terrestrial environments [1]. It occurs when the nitrogenase enzyme reduces N_2 into NH_3 and other nitrogenous compounds [2–4]. This process is considered one of the most expensive biological processes since N-fixing organisms need to spend about 15 to 16 ATPs to fix each N_2 molecule [5–7]. Only a few free-living or symbiotic prokaryotic organisms, bacteria and cyanobacteria, can fix N [5, 8]. This important ecosystem function influences biogeochemical cycles, productivity, ecological succession, carbon fixation, and ecosystems' responses to climate change [3, 9, 10]. But, despite the importance of N fixation, the factors and mechanisms that control this process remain poorly understood [6, 8, 9].

Nutrient availability is considered a key driver of N fixation, and multiple types of nutrients have been discussed as potential regulators [6, 8]. Among them are iron (Fe), molybdenum (Mo), and vanadium (V) [8]: these micronutrients may affect N fixation because they act as co-factors in nitrogenase, which has three isoforms: Mo-nitrogenase, V-nitrogenase, and Fe-nitrogenase [3, 8, 11, 12]. Mo-nitrogenase is considered the main nitrogenase enzyme in N fixation and is the most studied, probably because it occurs in all N-fixing organisms and is the only isoform found in higher plant symbionts, whereas V-nitrogenase and Fe-nitrogenase, known as alternative nitrogenases, remained neglected until recently [3]. These three nitrogenase isoforms are encoded by separate genes and organisms that have more than one isoform appear to have a significant competitive advantage [3, 11]. When N-fixing organisms have more than one type of nitrogenase, micronutrient availability can favor a specific nitrogenase production [3]. For example, under Mo limitation, the alternative nitrogenases can be favored and assist N fixation, acting as backup enzymes [3, 13].

N fixation is commonly limited by the availability of micronutrients [9, 10, 14, 15]. Stanton and coauthors [14] found that the canopy microbiome of a tropical forest to be co-limited by phosphorus (P) and Mo. The study showed that Mo controlled the per-bacterial cell rate of fixation and increased fixation efficiency in P treatments [14]. Bellenger and coauthors [15] observed high N fixation rates by asymbiotic N-fixing community after V supply combined with sucrose, a carbon source. After being exposed to V, N-fixing organisms were able to use this micronutrient very quickly [15]. However, some studies also found no evidence of N fixation increase after micronutrient supply [16, 17]. So, there is still much uncertainty regarding how nutrient availability regulates N fixation [8].

Additionally to micronutrients, carbon (C) concentration can also influence N fixation [18–21]. The metabolism of C and N is strongly coupled in different living organisms, so a limitation or excess on the supply of one element strongly affects the metabolism of the other [22–24]. Under high CO_2 concentrations, photosynthesis rates increase [21, 25]. Consequently, N-fixing organisms may accumulate C, which causes demand for N and a rise in N fixation rates [21, 22]. In the worst scenario projected, CO_2 concentration can reach about 950 ppm in 2100 [26]. Considering that micronutrients and CO_2 may be involved in a co-limitation [20], new studies evaluating how they affect N fixation are critical to predict and manage the impacts of increases in CO_2 concentration on biogeochemical cycles [8, 10].

Among the highly diverse group of N-fixing organisms, cyanolichens stand out for their contribution to N-fixing [10, 27]. Elbert and coauthors [27] estimated that cryptogamic covers, of which cyanolichens are an important component, can uptake around 50% of the total terrestrial N fixation. Cyanolichens are obligate symbioses between fungi and cyanobacteria, and some of them are also associated with green algae forming a tripartite symbiosis [28]. In cyanolichens, N fixation occurs in specialized cells called heterocysts, present in the cyanobacteria [28]. Only Mo-nitrogenase and V-nitrogenase have been

documented in cyanolichen heterocysts [29–32]. These cells are generally larger than vegetative cells, have a thick wall, polar nodules, and lack chlorophyll [33–35]. Heterocysts differentiation and N fixation in cyanobacteria can be triggered by the amount of C and N available in the environment [22], but few studies verify how cyanobacteria associated with fungi respond to high CO_2 concentrations [36, 37]. In a study by Fávaro and coauthors [36], *Leptogium cyanescens* (Rabenh.) Körb thalli from an urban area had higher heterocyst investment than thalli from a forested area. The authors suggested that it could be a consequence of higher CO_2 concentration in the urban area [36]. In other research, Norby and Sigal [37] found an increase in nitrogenase activity under elevated CO_2 concentration in *Lobaria pulmonaria* (L.) Hoffm. Just as few studies evaluate the effects of CO_2 on cyanolichens, few published studies assess how they respond to the addition of micronutrients. One of them was made by Pérez et al. [38], in which the authors evaluated the effect of Mo addition in *Pseudocyphellaria berberina* (G. Forst.) D. J. Galloway & P. James and *P. coriifolia* (Müll. Arg.) Malme cyanolichens in a post-volcanic chronosequence from south-central Chile.

Thus, to better understand how micronutrients and CO_2 impact cyanolichen investment on heterocysts and N fixation, we tested the effects of Mo, V and CO_2 in *L. cyanescens* in a controlled environment experiment. We addressed the following hypotheses: (1) Micronutrient supply and high CO_2 concentration increase the production of heterocysts. (2) Thalli exposed to micronutrient supply and high CO_2 concentration simultaneously produce more heterocysts than thalli exposed to these conditions separately. (3) Thalli exposed to micronutrient supply and high CO_2 concentration simultaneously present higher N fixation rates than thalli exposed to these conditions separately. (4) The higher the number of heterocysts, the higher is thalli N fixation. So far, this is the first study to evaluate how the interaction between micronutrient addition and CO_2 affect cyanolichens.

MATERIAL AND METHODS

Specimen collection, fertilization and transplants

Leptogium cyanescens thalli (Fig. 1) were sampled in a riparian forest located within *Reserva Biológica* (Biological Reserve) *Boqueirão* (21°20′45″S and 44°59′37″W) in Minas Gerais, southeastern Brazil. The altitude within the reserve varies from 1100 to 1250 m, the average annual temperature is 19.3 °C, and the average annual rainfall is 1411 mm [39]. We collected the thalli in shaded microhabitats near a stream, in an area where they occurred abundantly, scattered on rocks and tree trunks.

Fig. 1 – Thallus of *Leptogium cyanescens* on the bark of a tree and associated with bryophytes at Reserva Biológica Boqueirão. Source: Fávaro et al. [39].

We sampled 64 *L. cyanescens* thalli from tree trunks to perform the experiment. Sampling events were made between June and September, 2020. Since we would need to section the specimens' margins to count the number of heterocysts twice, at the beginning and the end of the experiment, we collected large thalli with sizes between 6 and 10 cm in

diameter. The whole thalli were collected with part of the tree bark still attached, put on trays, and hydrated until getting into the lab. After that, we removed soil fragments and bryophytes from the thalli with deionized water and tweezers, put the specimens inside trays in a diagonal position to simulate their light conditions, and marked them with plastic plates to identify their number and treatment. The lichen transplants were transferred to a growth chamber with controlled environment (CONVIRON®, ATC60, Canada), with a relative humidity of approximately 75%, air temperature of 20°C during the day and 17°C during the night, photoperiod of 10 h and photosynthetic active radiation of 10 µmol m−2 s−1. To achieve this photosynthetic active radiation, we also installed manufactured shading nets with 70% shades over the trays inside the chamber (Fig. 2). These conditions were maintained until the end of the experiment. *Leptogium cyanescens* thalli sizes were homogeneously distributed among the treatments.

Fig. 2 – Thalli transplants of *Leptogium cyanescens* inside the growth chamber.

To evaluate how high CO_2 concentrations and micronutrient supply affect the number of heterocysts and the N fixation, we separated *L. cyanescens* thalli into seven treatments,

which were the control group, Mo, V, CO_2, CO_2Mo, CO_2V, and CO_2MoV. In treatments with high CO_2 concentrations, the thalli were sampled in June 2020 and exposed to 800 ppm of this gas. In treatments without high CO_2 concentrations, thalli were samples in July 2020 and exposed to the environmental levels of CO_2 of the lab, which were 450 ppm. Since CO_2 concentrations in the area of the collection are lower than concentrations in the lab, 239.4 ppm [36], we also had a treatment that we called natural. Thalli from this treatment had their heterocysts and N fixation measured right after the sampling, in September 2020, without exposition to the growth chamber conditions. This treatment was not considered as control group because the global average CO_2 concentrations are closer to laboratory concentrations than to the natural environment. Specimens from the control group were exposed to lab CO_2 concentrations without micronutrients supply. In treatments with micronutrient supply, we provided Mo as a 2.1 µM of $Na_2MoO_4.2H_2O$ solution and V as 12.1 µM solution of Na_3VO_4 solution to the thalli [32]. We sprayed approximately 2 mL of micronutrients every day.

Before being exposed to high CO_2 concentration and micronutrients, the thalli went through an acclimatization process of 4 days inside the chamber. After this period, we ran the experiment for 27 days. The number of heterocysts was counted on the first day (beginning) and at the last day (end) of the experiment and N fixation was measured only at the end. Each treatment had eight thalli, for a total of 64 thalli.

Heterocyst counting

To count the number of heterocysts, we adapted the method described by Fávaro et al. [36]. We removed fragments from the margins of *L. cyanescens* thalli, hydrated them with distilled water, and performed the sections using a LEICA 133 CM1850 freezing microtome with 8 µm thickness. The margins were used because, in foliose thalli, these areas are more actively growing compared to the central parts [40]. Therefore, heterocyst differentiation could be higher in the margins [41]. We did paradermal sections right below the upper cortex

to visualize the cyanobiont. After sectioning the specimens, we examined at least four fragments under an Olympus BX41 compound microscope at 1000x magnification, and photographed the regions with the greatest amount of heterocysts. From the images, we counted the cells using the Image J program. To determine the number of heterocysts produced in each thallus, we made a random selection of 10 images per thallus and added up the number of heterocysts found in these 10 images. The resulting value represents the number of heterocysts produced by the respective thallus considering the thallus sampling area that we defined.

N fixation

We used the standard assay for acetylene reduction assay (ARA) to estimate N fixation in *L. cyanescens* [42]. When exposed to acetylene, the nitrogenase enzyme reduces acetylene (C_2H_2) in ethylene (C_2H_4) instead of reducing N_2 to NH_3. So, taking advantage of this behavior, ARA measures the rate of acetylene reduction as a proxy for nitrogenase activity. To perform the ARA, we collected 0.005 g fragments from the margins of the thalli, hydrated these fragments with deionized water, and put them inside 250 mL jars. Inside the 250 mL jars, we also put 10 mL jars with 0.06 g of calcium carbide (CaC_2), and sealed the 250 mL jars with septa. After that, 2 mL of water was added with a syringe inside the 10 mL with CaC_2 to produce 10% atmosphere of acetylene and expose the thalli to it. Then, after three hours, a gas sample of 5 mL was taken and analyzed for ethylene using the CI-900 Portable Ethylene Analyzer (CID Bio Science, USA). To convert ARA to potential rates of N fixation, we used a ratio of 3 moles C_2H_4 produced to 1 mole N_2 fixed [42].

Statistical analyses

To test whether the period (beginning and end) and the treatments affected the number of heterocysts in *Leptogium cyanescens* thalli, we applied a General Linear Model (GLM) with quasiPoisson distribution. After seeing that both period and treatments impacted the

investment in heterocysts, we used GLM's with quasiPoisson distributions to evaluate if micronutrient additions and high CO_2 concentration increased the number of heterocysts throughout the experiment and verify if thalli exposed to these conditions simultaneously produced more heterocysts than thalli exposed to these conditions separately. We used GLM's with gaussian distributions to test if thalli exposed to micronutrient supply and high CO_2 concentration simultaneously had higher N fixation than thalli exposed to these conditions separately and verify if the higher the number of heterocysts, the higher was thalli N fixation. All analyses were carried on R Statistical Software [43]. We used the emmeans and multcomp packages to perform the pairwise comparisons and ggplot2 and hrbrthemes packages to plot the graphs [44–47].

RESULTS

Investment in heterocysts in *Leptogium cyanescens* thalli showed a clear response to period ($F = 39.68$; $df = 112$; $p < 2.20e^{-16}$) and to high CO_2 and micronutrient supply treatments ($F = 92.36$; $df = 111$; $p = 4.59e^{-16}$). The interaction between period and treatments was also significant ($F = 9.09$; $df = 105$; $p = 5.417e^{-08}$, Fig. 3).

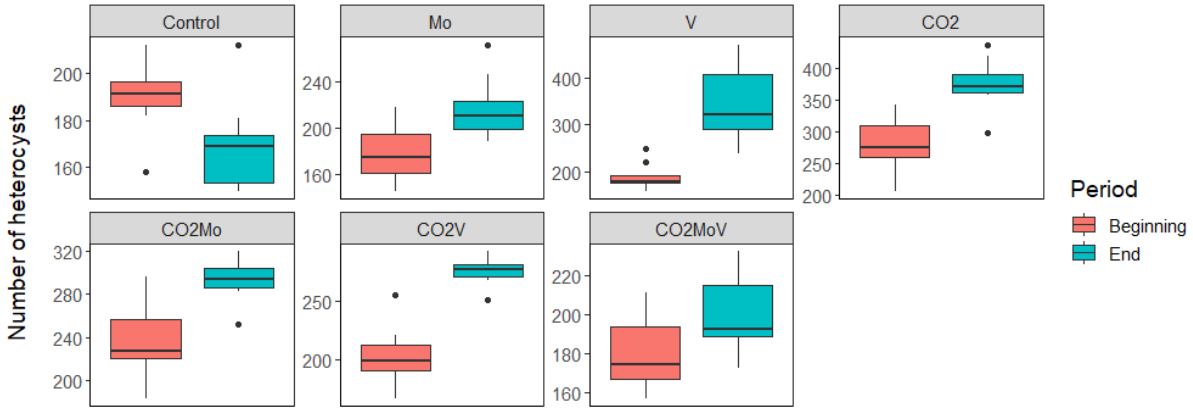

Fig. 3 – Number of heterocysts in thalli of *Leptogium cyanescens* at the beginning (red boxes) and at the end (blue boxes) of the experiment in each treatment. Thalli exposed to high carbon dioxide concentration (CO_2), molybdenum (Mo) and vanadium (V) had higher number of heterocysts at the end of the experiment than at the beginning, with exception of the CO_2MoV treatment. The experiment lasted 27 days. The boxes represent the 25th and 75th percentiles, the lines inside the boxes represent the median, the lines above and under the boxes represent the standard deviations, and the dots represent outliers.

Thalli exposed to CO_2, Mo, and V had an increase in the number of heterocysts from the beginning to the end of the experiment, except the CO_2MoV treatment (See Table 1 for more details; Fig. 3). Thalli that were exposed to micronutrients and high CO_2 concentration simultaneously had no significant difference between the beginning and the end of the experiment ($F = 3.9608$; $df = 14$; $p = 0.0665$). The investment on heterocysts decreased only in specimens from the control group ($t = 2.22$; $df = 14$; p-value = 0.04; Fig. 3). Only these thalli, without exposure to CO_2 and micronutrients, had fewer heterocysts at the ending of the experiment. Therefore, we partially accepted our first hypothesis that CO_2 and micronutrients increase the heterocysts production.

Table 1. Number of heterocysts produced by the thalli of *Leptogium cyanescens* at the beginning (B) and at the end (E) of the experiment and the difference between them (E - B). Thali were exposed to high carbon dioxide concentration (CO_2), molybdenum (Mo) and vanadium (V), simultaneously and separately.

Treatment	Heterocysts at the beginning (B)	Heterocysts at the end (E)	E-B
Control	1523	1358	-165
Mo	1419	1738	319
V	1509	2758	1249
CO_2	2249	3002	753
CO_2Mo	1908	2341	433
CO_2V	1618	2198	580
CO_2MoV	1441	1600	159

Treatments affected the number of heterocysts produced by the thalli at the end of the experiment ($F = 37.79$; $df = 56$; $p < 2.2e^{-16}$). Contradicting our second hypothesis, *L. cyanescens* thalli exposed to micronutrient supply and high CO_2 concentration simultaneously did not produce more heterocysts than thalli exposed to these conditions separately (Fig. 4). Thalli exposed to CO_2MoV produced fewer heterocysts compared to the thalli from CO_2, CO_2Mo, CO_2V, and V treatments, which had the highest heterocysts production. Thalli from the CO_2MoV treatment also had no significant difference with the other treatments (Fig. 4). We also found that heterocyst production in thalli from the control group had no significant difference when compared with thalli from the natural environment (Fig. 4).

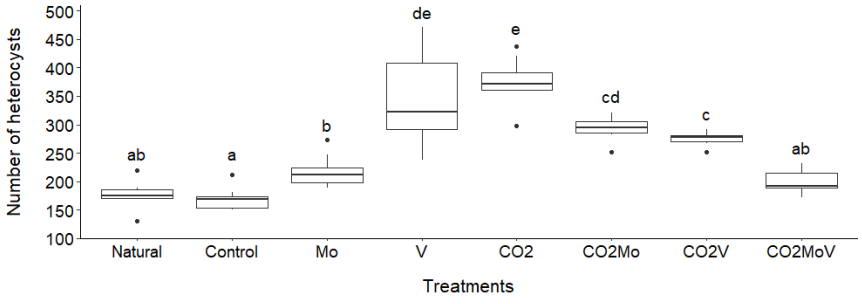

Fig. 4 – Number of heterocysts in the thalli of *Leptogium cyanescens* from the natural environment, from treatments with exposure to high carbon dioxide concentration (CO_2), molybdenum (Mo) and vanadium (V), simultaneously and separately, and from the control group. The boxes represent the 25th and 75th percentiles, the lines inside the boxes represent the median, the lines above and under the boxes represent the standard deviations, and the dots represent outliers.

High CO_2 concentration and micronutrient supply also affected the *L. cyanescens* N fixation (F = 3.42; df = 56; p = 0.004). However, thalli exposed to these conditions simultaneously did not have higher N fixation than thalli exposed to high CO_2 concentration and micronutrient supply separately (Fig. 5). So, we rejected our third hypothesis. Thalli exposed to high CO_2 concentration combined with micronutrients and thalli exposed to these conditions separately had no differences in N fixation, but they had lower N fixation compared to thalli from the natural environment (Fig. 5).

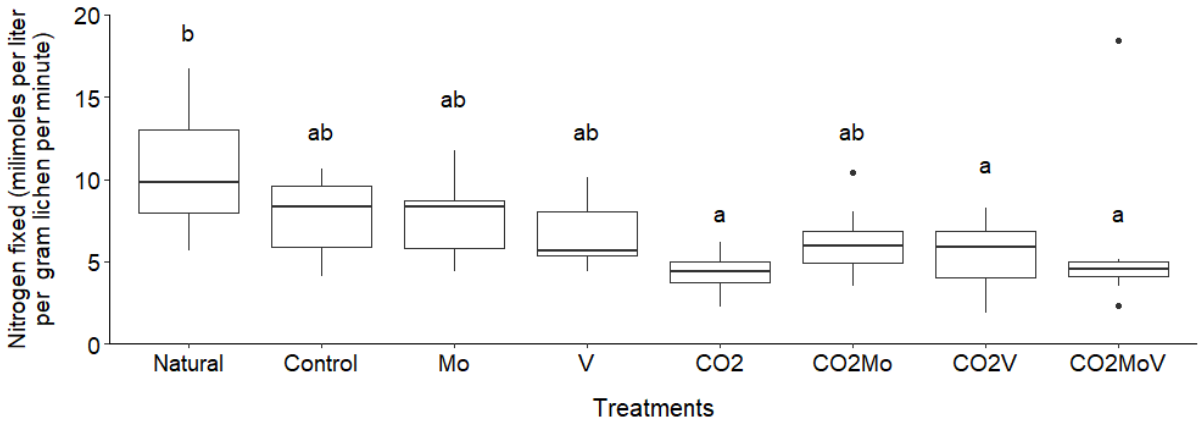

Fig. 5 – Nitrogen (N) fixed by thalli of *Leptogium cyanescens* from the natural environment, from treatments with exposure to high carbon dioxide concentration (CO_2), molybdenum (Mo) and vanadium (V), simultaneously and separately, and from the control group, where thalli did not receive CO_2 or micronutrients. Thalli exposed to CO_2 and micronutrients fixed similar quantities of N. However, their N fixation differed from thalli collected on the natural environment. The boxes represent the 25th and 75th percentiles, the lines inside the boxes represent the median, the lines above and under the boxes represent the standard deviations, and the dots represent outliers.

In addition, we found a relationship between the number of heterocysts and the N fixation in *L. cyanescens* thalli (F = 8.09; df = 62; p = 0.006). However, opposing to what we hypothesized, N fixation decreased with the investment in heterocysts during the experiment (Fig. 6).

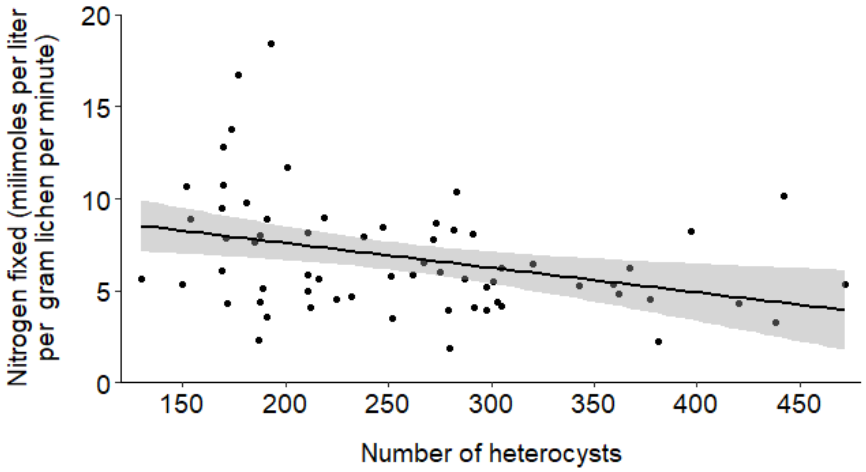

Fig. 6 – Nitrogen (N) fixation and heterocysts relationship in the thalli of *Leptogium cyanescens*. N fixation decreased with investment in heterocysts by the specimens. The circles represent the data collected during the experiment, the line represents the relationship between N fixation and the number of heterocysts, and the shaded area shows the confidence interval around the linear trend.

DISCUSSION

In our study, the investment in heterocysts in *L. cyanescens* thalli increased with high CO_2 concentration and micronutrient supply, but, contrary to expectations, N fixation decreased. The number of heterocysts only decreased in the control treatment from the beginning to the end of the experiment. This result did not surprise us because thalli from this treatment received only deionized water. Consequently, the specimens may have been nutrient-limited, constraining the production of heterocysts and N fixation. High CO_2 concentrations and micronutrient addition affected heterocysts production in *L. cyanescens* cyanolichens. After comparing the number of heterocysts between the beginning and the end of the experiment, we saw that thalli tended to increase the number of N-fixing cells in all treatments. This behavior can be explained by an imbalance, due to the increase of CO_2, between the amounts of carbon and nitrogen and by the supply of micronutrients, which are necessary for the synthesis of the nitrogenase enzyme [8, 11, 12, 20, 22, 24].

Carbon (C) and nitrogen (N) metabolism are linked in cyanobacteria [22, 24]. Consequently, limiting or excess amounts of one element affect the metabolism of the other [22]. Under carbon oversupply, cyanobacteria accumulate 2-oxoglutarate (2-OG), which is the

carbon skeleton for nitrogen assimilation and responsible for bonding carbon and nitrogen metabolism [22, 24]. 2-OG can bind to the NdhR and NtcA transcriptional regulators and form the 2-OG/NdhR and 2-OG/NtcA complexes [22]. The 2-OG/NdhR complex down-regulates genes involved in the CO_2-concentrating mechanism to decrease carbon uptake and the 2-OG/NtcA complex upregulates genes related to heterocyst differentiation and N fixation [22]. Together, the complexes help cyanobacteria cells to balance C and N metabolic networks [22]. In our study, high CO_2 concentrations probably caused an unbalance in C:N ratio of *L. cyanescens* thalli and trigger heterocyst differentiation to retake the balance.

In addition to Mo-nitrogenase, found in all N-fixing organisms, studies have found that cyanolichens are also able to produce V-nitrogenase [29–32]. These two nitrogenase isoforms are distinguished by the metal composition of their corresponding active-site cofactors: Mo-nitrogenase has FeMo cofactor, and V-nitrogenase has FeV cofactor [3, 5, 12] Considering that Mo and V are needed in the active site, a limitation of these micronutrients could decrease heterocysts differentiation and N fixation. Here, we observed an increase in the number of heterocysts in treatments with micronutrient addition, indicating that heterocysts production was favored by the availability of nutrients.

Heterocysts investment in thalli exposed to Mo, V, and high CO_2 together did not differ between the beginning and the end of the experiment. We also found that thalli from CO_2MoV treatment did not produce more heterocysts at the end of the experiment than thalli exposed to the other treatments, contradicting our expectations. These results can be explained by an increase in the efficiency of N fixation within heterocysts. Excess carbon increases the production of heterocysts, which only function is to fix N [22, 24]. But the differentiation of vegetative cells into heterocysts is an expensive process in terms of energy [21, 22, 41]. Considering this, thalli exposed to Mo and V simultaneously may have allocated resources to increase fixation efficiency instead of increasing heterocysts differentiation.

Therefore, *L. cyanescens* thalli could quickly retake the balance between C:N without expending too much energy.

The differential heterocysts production between Mo and V treatments may be explained by the fertilization regimens. Mo was supplied as a 2.1 µM of $Na_2MoO_4.2H_2O$ solution and V as 12.1 µM solution of Na_3VO_4 solution to the thalli, the same concentrations used by Marks and coauthors [32]. They studied how micronutrients and P affected lichen growth rates using *Lobaria pulmonaria* and *Usnea longissima* Ach. [32]. To determine the concentrations of Mo and V that would be used in the study, the authors looked at the natural Mo:P or V:P relationships in each species [32]. *Lobaria pulmonaria*, *U. longissima*, and *L. cyanescens* have a different symbiotic relationship. *L. cyanescens* thalli have only cyanobacteria as photobiont (in contrast to *U. longissima* that has green algae as photobiont) and they are abundantly spread throughout the medulla (in contrast to *L. pulmonaria* that limits cyanobacteria to the cephalodia). These differences in symbiotic relationships may lead species to require different amounts of micronutrients. Therefore, the V concentration used in our experiment may have been much higher than the V concentration available in thalli under natural conditions, and this V excess resulted in high heterocyst investment. The same situation did not occur in treatments with Mo, probably because the Mo concentration we used was just a little different from the Mo concentration available in thalli under natural conditions. Therefore, we believe that an increase in Mo concentration may cause an increase in heterocyst investment.

Contrary to expectation, specimens from the experiment in the growth chamber had similar N fixation rates and N fixation rates were negatively related with the number of heterocysts. These results could be due to the low-light conditions inside the growth chamber. Since cyanobacteria are photosynthetic organisms, their physiological properties are highly affected by light [21]. They invest large amounts of photosynthetic energy on N fixation [21,

48], so low light availability could limit N fixation rates. Other explanation for these results could be a limitation in our method. Since N fixation rates were measured only after 27 days, fixation may not have differed between treatments because the specimens responded faster than we expected and our method was not able to detect this response. Studying N fixation performed by moss-associated cyanobacteria, Rousk et al. [49] found a quick response in nitrogenase activity after Mo addition. However, the Mo supply did not affect nitrogenase activity after five weeks. Nitrogen fixation is an expensive biological process and, considering the resource allocation theory [50, 51], cyanolichens would not waste resources investing in heterocysts to not fix N. Therefore, *L. cyanescens* thalli may have responded faster to exposition to micronutrients and high CO_2 concentrations, differentiated heterocysts, synthetized the nitrogenase enzyme and increased N fixation in the beginning of the experiment, and, after some weeks, they may have adapted to the new conditions. After a long-period, cyanobacteria responses involve more complex feedbacks, such as nutrient recycling [49]. In our experiment, thalli were transplanted to the growth chamber on their substrates (tree cortices and bryophytes). The wood decomposing and bryophytes death may have affected N fixation. In addition, *L. cyanescens* cyanobacteria could have retaken the C:N balance and decreased the N fixation rates.

We also observed that thalli from CO_2, CO_2V, and CO_2MoV treatments had lower N fixation rates compared to thalli from the natural environment. It could be explained by a P limitation. CO_2Mo was the only treatment with high CO_2 that did not differ from the natural environment regarding N fixation rates. It happened because of the presence of an outliner in the analysis. So, without this outlier in CO_2Mo treatment, all thalli exposed to high CO_2 concentrations would have lower N fixation rates when compared to thalli from the natural environment. Considering that high CO_2 concentrations increase the pressure to differentiate heterocysts and fix N [22, 24], thalli would increase both heterocysts production and N

fixation to retake the balance between C:N. However, after a while, they could suffer a P-limitation. Stanton and coauthors [14] found that Mo and P co-limit N fixation in the canopy microbiome, but in a different manner. Mo has little biological importance outside of its role as a cofactor in the nitrogenase enzyme [14]. On the other way, P is the N fixation fuel and is also essential to other metabolic activities and cellular structures [14]. Therefore, the absence of P sources during the 27 days of the experiment may have caused a P-limitation that, consequently, decrease N fixation rates.

Our findings present new evidence that high CO_2 concentrations and micronutrients can drive heterocysts production in cyanolichens. Increases in CO_2 concentrations can cause an unbalance between C:N, trigger heterocysts differentiation and elevate N fixation. Availability of micronutrients can also increase heterocysts and N fixation because they participate in the synthesis of nitrogenase, the enzyme responsible for reducing N_2 in NH_3 and other reactive compounds. If cyanolichens keep being continuously exposed to high CO_2 concentrations in environments with low availability of micronutrients, it may decrease N fixation. Since cyanolichens have a huge contribution to N biogeochemical cycle, a decrease in N fixation may affect the whole ecosystem.

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