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Hornwort-cyanobacteria interactions: exploring ontogeny in *Nothoceros vincentianus* and *Dendroceros crispus* growing together with *Nostoc* cyanobacteria

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Abstract: In hornworts, the infection process by *Nostoc* begins early in gametophyte development, with multiple cyanobacterial strains often present in the same thallus, suggesting varying levels of specificity in symbiosis. We tested the effect of a *Nostoc* strain from the rupicolous hornwort *Nothoceros vincentianus* on spore germination and sporeling development of *N. vincentianus* and epiphytic *Dendroceros crispus*. Over 154 days, *D. crispus* outperformed *N. vincentianus*, which showed lower survival and growth when associated with *Nostoc*. These results highlight the complexity of *Nostoc*-hornwort interactions and suggest *Nostoc* strains naturally occurring within a species' gametophyte may not perform well in vitro.

Key words: bryophyte, plant-microbe interaction, spore germination, sporeling growth, symbiosis, specificity.

INTRODUCTION

Symbiotic relationships between cyanobacteria and plants have been crucial in various ecological contexts, especially due to their role in biological nitrogen fixation, which benefits the host plant by providing essential nutrients (Adams & Duggan 2008, Bustos-Díaz et al. 2019, Rasmussen & Nilsson 2002). These associations are particularly diverse, encompassing two well-documented types: 1) epiphytic associations in certain moss species like *Pleurozium schreberi* (Willd. ex Brid.) Mitt. and *Hylocomium splendens* (Hedw.) Br. et Sch. (Adams & Duggan 2008, Gentili et al. 2005, Rousk et al. 2013, Solheim & Zielke 2002), and 2) endophytic associations in liverworts (*Blasia* and *Cavicularia*) and all hornwort species (Adams 2000, Bustos-Diaz et al. 2019, Meeks 2005, Renzaglia et al. 2000, 2009). Notably, the genus *Sphagnum* can establish both epiphytic and endophytic interactions, depending on environmental and physiological conditions (Carrell et al. 2022, Kostka et al. 2016).

Several genera of cyanobacteria, including *Nostoc*, *Stigonema*, *Calothrix*, and *Cylindrospermum*, have been identified in symbiosis with bryophytes, with *Nostoc* being the most frequently documented symbionts (Adams 2000, Meeks 2007, Warshan et al. 2018, Rasmussen & Nilsson 2002). *Nostoc* is characterized by a complex life cycle, including gelatinous colonies containing many filaments, akinetes, and hormogonia—motile filaments responsible for infecting the host (Rasmussen & Nilsson 2002, Meeks 2007, Paulsrud 2001). Heterocysts, specialized nitrogen-fixing cells, also play a crucial role in these endosymbiotic relationships, increasing in frequency when

Nostoc is in land plants such as bryophytes, cycads, the fern *Azolla*, and the angiosperm *Gunnera*, highlighting its role in nitrogen fixation while benefiting from the host's protective and nutrient-rich environment (Adams et al. 2013, Meeks 2003, Zhang et al. 2006, Meeks 2007, Salzman et al. 2025).

The establishment of symbiosis between hornworts and *Nostoc* begins in the early stages of gametophyte development in early thallus, although it can also occur in adult plants, as observed *in vitro* (Frangedakis et al. 2021, Adams 2002, Renzaglia et al. 2009). This symbiosis is generally considered mutualistic, with the hornwort benefiting from biologically fixed nitrogen supplied by *Nostoc*, and the cyanobacterium receiving a protected environment and photosynthates or organic acids from the host (Meeks 2007, Adams & Duggan 2008). Morphological studies have identified two distinct forms of cyanobacterial colonies are observed in hornworts: an elongate central strand colony, found only in *Leiosporoceros*, and a dark, small, globose colony in all other genera (Renzaglia et al. 2009, Villarreal & Renzaglia 2006).

The morphology of hornwort gametophytes also facilitates this symbiotic interaction. Mucilaginous clefts commonly appear on the ventral gametophyte surface (i.e., the side in contact with the substrate), with two cells resembling stomatal guard cells surrounding a pore. Although these structures cannot open or close, they provide an entry point for endosymbionts (Frangedakis et al. 2021, Renzaglia et al. 2009). Since multiple clefts may be present on a single gametophyte, a single plant can host different strains of cyanobacteria. However, this phenomenon has only been investigated genetically in three hornwort species: *Anthoceros fusiformis* Austin, *Phaeoceros laevis* (L.) Prosk., and *Leiosporoceros dussii* (Steph.) Hässel (Bouchard et al. 2020, Costa et al. 2001, West & Adams 1997).

Sympatric hornwort species such as *Notothylas orbicularis* (Schwein.) Sull., *Phaeoceros carolinianus* (Michx.) Prosk., and *Anthoceros agrestis* Paton exhibit similarities in their cyanobionts, albeit with low specificity (Nelson et al. 2020). As a result, multiple cyanobacterial strains may coexist within the same hornwort, suggesting a low of selectivity and specificity in the cyanobacteria-hornwort symbiosis (Bouchard et al. 2020, Villarreal & Renzaglia 2006). This low specificity is comparable to that seen in mosses and liverworts, and contrasts with the high specificity found in lichens, the fern *Azolla-Anabaena*, and cyanobacteria-diatom symbiosis (Rasmussen & Nilsson 2002, Adams 2002, Magain et al. 2016, Raven 2002).

In addition to low partner specificity, the timing and morphological context of symbiosis initiation in hornworts is also key to understanding their interaction with cyanobacteria. In hornworts, spore germination typically gives rise to a globular sporeling, from which a thallus develops through the activity of a single apical cell, bypassing a filamentous protonemal stage as observed in mosses (Renzaglia & Vaughn 2000, Frangedakis et al. 2021, Renzaglia et al. 2000). The early development of the gametophyte is marked by the rapid formation of mucilage clefts on the ventral surface, which function as entry points for *Nostoc* hormogonia (Renzaglia et al. 2009, Adams 2002). Colonization has been observed to occur very shortly after spore germination, sometimes even before the full differentiation of internal tissues, suggesting that symbiosis is initiated at the onset of gametophyte establishment (Frangedakis et al. 2021, Renzaglia et al. 2009). Therefore, it is plausible that the presence of *Nostoc* at this early stage may

influence sporeling survival, morphology, or developmental dynamics.

Given the potential variability in symbiotic interactions between *Nostoc* and different hornwort taxa, we aimed to investigate the effect of a *Nostoc* strain in two species of hornworts. First, we isolated a *Nostoc* strain from the saxicolous *Nothoceros vincentianus* (Lehm. &Lindenb.) J.C. Villarreal, cultivated it, and established a healthy colony. Subsequently, we focused on the sporeling development of both *N. vincentianus* and the epiphytic *Dendroceros crispus* (Sw.) Nees, using two treatments: one where the spores grew in association with the *Nostoc* strain, and another where the spores grew independently. We hypothesized that the presence of *Nostoc* would influence the survival and development of sporelings, potentially enhancing these processes compared to non-*Nostoc* conditions. Additionally, we sought to examine the relationship between the cyanobacterial life cycle and the developmental stages of hornwort gametophytes, to better understand the dynamics of this symbiotic interaction.

MATERIALS AND METHODS

Isolation and culture of the cyanobacteria

A clonal culture of *Nostoc* sp. (Figure 1a-b) was obtained using the traditional micropipette isolation method (Andersen & Kawachi 2005) from a *N. vincentianus* gametophyte (Figure 1c) collected in May 2017 in the Atlantic Forest at Itatiaia National Park (22°27'06'' S, 44°36'44'' W, 837 m a.s.l.), Rio de Janeiro state, Brazil. This strain was maintained in Erlenmeyer flasks containing 300 mL of CHU10 medium under a 12:12 h light/dark regime. In the culture chamber, light intensity was 30 µmol photons m^{-2} s^{-1} and temperature was maintained at 20(±1) °C. Experiments were conducted using cultures in the exponential growth phase.

Sampling and cultures

We studied two hornwort species: *Dendroceros crispus* (Figure 1d), an epiphytic species with multicellular green spores, typically found in forests at 800-2000 m a.s.l. (Peñaloza-Bojacá et al. 2019, Duff et al. 2007), and *Nothoceros vincentianus* (Figure 1e), a terricolous or saxicolous species with unicellular green spores, common in Neotropical forests above 400 m a.s.l. (Renzaglia et al. 2009, Villarreal & Renner 2014). Field collections were conducted in October 2018 in the Atlantic Forest, São Paulo state, Brazil. We collected samples (~8 cm^2) from the Núcleo Santa Virgínia, Parque Estadual da Serra do Mar (23°20'-23°25'S, 45°08'-45°15'W), at elevations of 800-1000 m a.s.l., with temperatures ranging from 20-24°C and an annual rainfall of 1500-4000 mm (Fundação Florestal 2008). Collected plants were kept hydrated in a culture room (21°C, 74 µmol photons m^{-2} s^{-1}) until laboratory assays.

Given the asynchronous sporogenesis in hornworts, we collected only fully developed and mature spores from the apex of the capsules. Spores from ten capsules of each species were pooled and cleaned with 0.05% calcium hypochlorite for 40-50 seconds, followed by three rinses with autoclaved ionized water for 15 seconds each. A 0.5 mL spore suspension was plated in Petri dishes (30 mm diameter) on solid culture medium (0.5% agar, 50% Knop II; Nakazato et al. 1999). We opted to use a nutrient solution containing nitrogen (at half concentration) in the medium to ensure the spores could germinate and the sporelings would have sufficient energy to grow before the cyanobacterium infection. Spore density varied from 160,000 mL^{-1} in *D. crispus* to 405,000 mL^{-1} in *N. vincentianus*. Cultures were maintained under

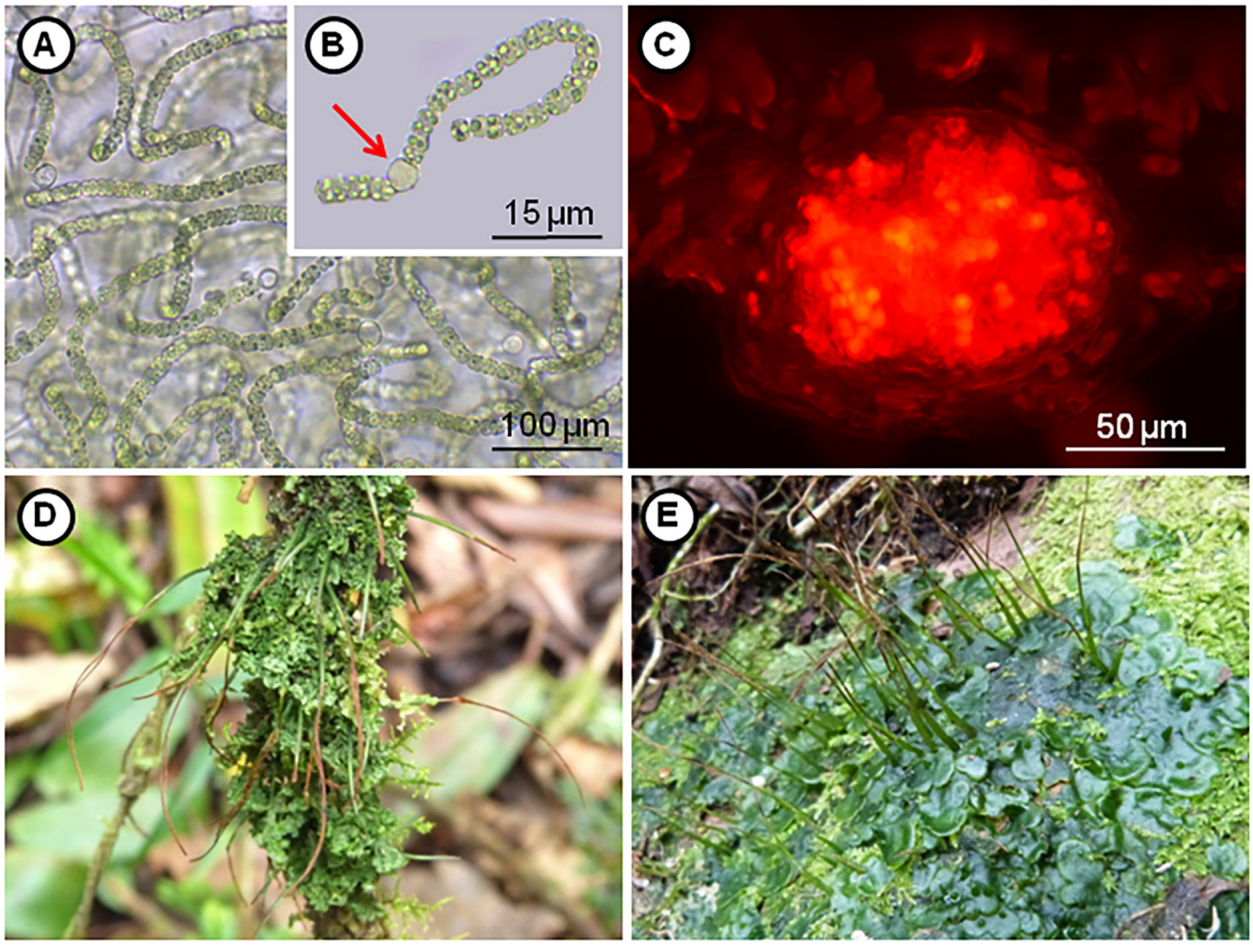

Figure 1. Cyanobacteria and hornworts used in the experiment. a: Details of *Nostoc* sp.; b: *Nostoc* sp. filament with heterocyst (red arrow); c: Epifluorescence image of a cross section of *N. vincentianus* showing a *Nostoc* sp. colony. The chlorophyll of the cyanobacteria appears in red using an excitation filter of 450-490 nm and an emission filter >600 nm; d: *Dendroceros crispus*; e: *Nothoceros vincentianus*.

constant conditions (21°C, 74 µmol photons m^{-2} s^{-1}) with a 12-hour photoperiod.

Experimental design

Spores of *D. crispus* and *N. vincentianus* were cultured as above described to non-*Nostoc* and *Nostoc* treatments. Fourteen independent observations with three replicates each were performed along 154 culture days. For *Nostoc* treatment, the Petri dishes containing spores were also inoculated with previously cultivated colonies of *Nostoc* that were passed through a mesh with porosity of 1 µm. An autoclaved glass syringe was used to break down the mucilage of colonies to release the filaments and 0.5 mL of this *Nostoc* solution was added to each plate. *Nostoc* addition occurred simultaneously to hornwort spores in media.

Survival was measured as germinated and viable spores, i.e., spores remaining the capacity to progress with mitosis, showing size increasing, green chloroplasts, development of rhizoid or germ tube, oppositely to dead spores (no signs of development, being hyaline or dark brown spores, no green chloroplasts, no rhizoids). Sporeling growth (*length* and *width* in micrometers) was analyzed using the BEL Capture Application software (400x magnification). 100 hornwort spores and sporelings regarding

survival and 50 to growth were measured at each observation time and in each replicate. Survival was recorded as percentage of viable and germinated spores in each replicate.

Ontogeny of hornworts and *Nostoc* sp.

We recorded the ontogenetic phases for both the plants and the cyanobacteria. The sporeling development of *D. crispus* and *N. vincentianus* was classified into seven phases (Table I; Figure 2) based on previous studies on hornwort sporelings (Campbell 1907, Oliveira et al. 2017, Renzaglia 1978, Schuette & Renzaglia 2010). Similarly, we described seven phases of the *Nostoc* life cycle (Table I; Figure 2; Supplementary Material - Video S1) following established classifications (Becerra-Absalón & Tavera 2009, Paulsrud 2001). Given the importance of N_2 fixation in the symbiosis, we also recorded the presence of heterocysts throughout the experiment.

Statistical analyses

To verify our early prediction of low hornwort-cyanobacteria specificity, we performed generalized linear models (GLMs) to test the

Table I. Gametophyte development phases in hornworts species (*Dendroceros crispus* and *Nothoceros vincentianus*) and development phases in *Nostoc* sp. life cycle.

Phase	*Dendroceros crispus*	*Nothoceros vincentianus*	Phase	*Nostoc* sp.
P1	Multicellular spores	Unicellular spores	C1	Hormogonia that are the motile filaments
P2	Sporelings are small globose mass two or three times larger than phase one and with a rhizoid beginning its development	Sporelings are small globose mass with more than two cells	C2	Hormogonia with enlargement of the cells and formation of a sheath around the filament
P3	Globose masses and large rhizoid filled with pieces of the chloroplast	Globose masses and large rhizoid filled with pieces of the chloroplast	C3	Vegetative filament long, contorted and wrinkled in a sheath
P4	Globose masses with develop wedge-shaped apical cells and pore	Globose mass two to three times larger than the previous phase; with one or two bifurcated rhizoids	C4	Abundant development of vegetative fragments that break up in the sheath and begin to form colonies and proliferate
P5	Globose masses elongate, differentiation of cells with hemidiscoid shape (apical growth) and beginning of the formation of the midrib	Globose mass with oval shape and several rhizoids; start of differentiation of wedge-shaped apical cells	C5	Colonies larger than the previous phase and a hardened sheath of mucilage
P6	Globose masses well elongate (two to three times longer than wide) and beginning of the sporeling bifurcation	Globose mass bifurcating and wedge-shaped apical cells dividing longitudinally and transversely	C6	Colonies with several vegetative filaments emerging from the broken sheath
P7	Distinction of the wing cells of the gametophyte with lateral expansion and bifurcations present a midrib	Sporeling two or three times larger than the previous phase, with elongated bifurcations and several long rhizoids	C7	Long vegetative filaments extremely contorted and wrinkled. That later can be divided forming the phase 1

influence of cyanobacteria on survival and growth of spores and sporelings of the two hornwort species. Additionally, to the "*Nostoc*" effect, we also included in our models the main and interaction effects of "*hornwort species*" and "*observation time*". GLMs with gaussian distribution and identity link function were used for "*survival*" and "*growth*" response variables (model details in Appendix S1). Additionally, when necessary, *post hoc* comparisons (Tukey test, α = 0.05) were applied. Furthermore, we tested a potential relationship between sporeling ontogeny and *Nostoc* phases with Spearman correlations for both species. Since we recorded hornwort sporelings at different phases at each observation time, we used the formula: *phase: (n1.C1+n2.C2+...n7.C7)/(C1+C2+...C7),* where *n* = number of sporelings at a phase and *C* = ontogenetic phase for each replicate. For cyanobacteria, since we were not able to

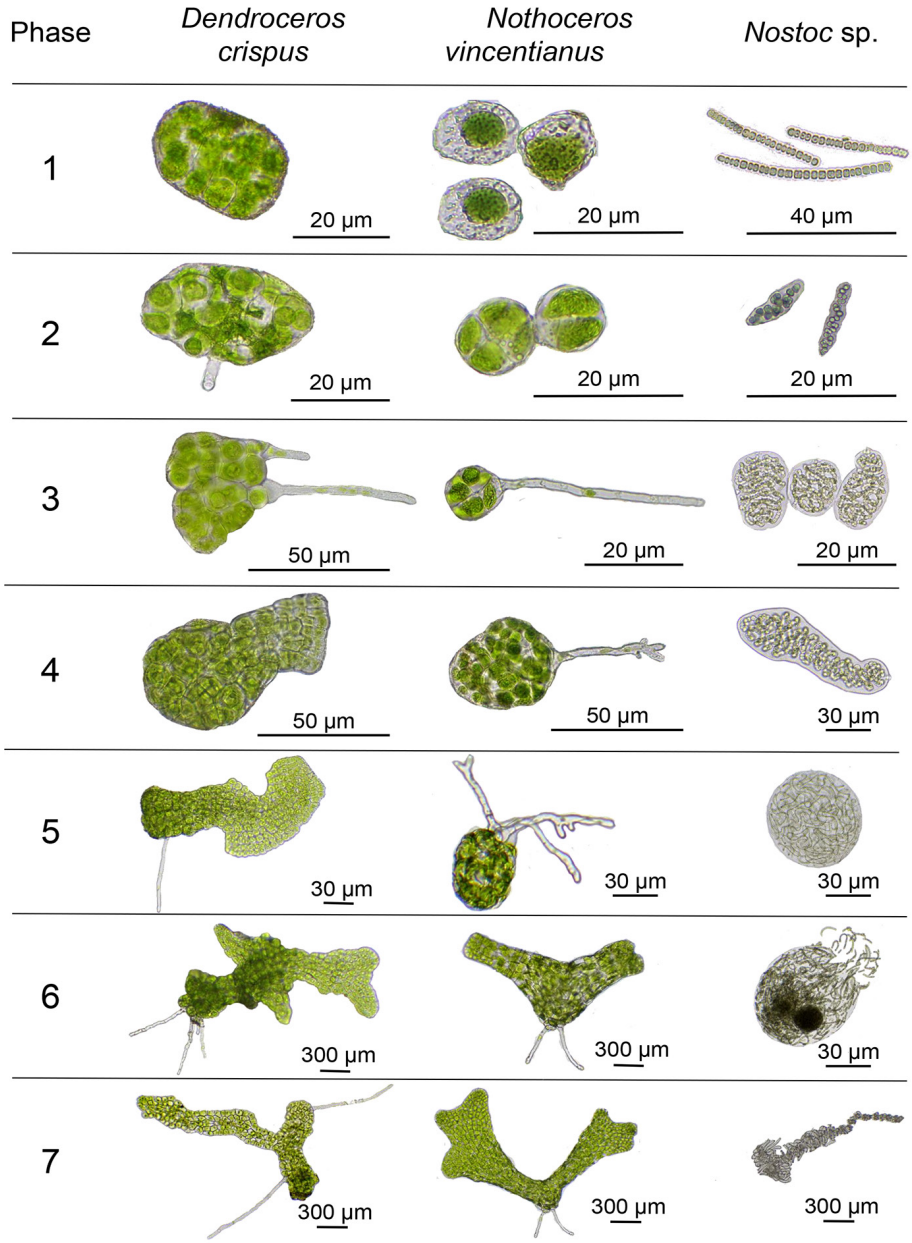

Figure 2. Representation of the ontogeny sporelings in seven phases for *Dendroceros crispus*, *Nothoceros vincentianus* and the life cycle of *Nostoc* sp.

count individuals in each phase, we recorded the presence of each phase as 1 (with no heterocysts) or 2 (with heterocysts). We applied a similar formula to quantify a phase single-value in each replicate. All analyses and graphs were performed in R v.4.0.4 (R Core Team 2024) using RStudio v.1.3.959 software (RStudio Team 2020).

RESULTS

Nostoc had a positive effect on sporeling survival and growth in *D. crispus* (e.g., 63.8% ± 1.2 survival), whereas its impact on *N. vincentianus* was limited and associated with a significant decline after day 56 (interaction β = 20.5, $p < 0.001$; Table II; Appendix S1). Within each species, sporelings in the *Nostoc* treatment performed better than

Table II. Generalized linear model of the survival and growth in sporelings of *Dendroceros crispus* and *Nothoceros vincentianus*. The GLM presents Gaussian distribution and identity link function. *Bold* indicates statistical significance.

Source	*Degree of freedom (d.f.)*	*Deviance*	*Resid. Df*	*Resid.Dev*	*F*	*Pr(>F)*
Survival parameters of Hornworts Sporelings						
Null model			167	81584		
Species	1	3447.1	166	78136	139.983	**<0.001**
Treatments	1	7968.1	165	70168	323.574	**<0.001**
Time	13	25501.9	152	44666	79.661	**<0.001**
Species:Time	1	4412.6	151	40254	179.190	**<0.001**
Treatments:Time	13	6270.6	138	33983	19.588	**<0.01**
Model: AIC: 1430.8; Residual deviance: 33983; 138 degrees of freedom (df)						
Growth (length) parameters of Hornworts Sporelings						
Null model			164	11187176		
Species	1	2410938	163	8776238	883.651	**<0.001**
Treatments	1	1268946	162	7507292	465.091	**<0.001**
Time	13	2887826	149	4619466	81.418	**<0.001**
Species:Time	1	193403	148	4426063	70.886	**<0.001**
Treatments:Time	13	742747	135	3683315	20.941	**<0.01**
Model: AIC: 2182.5; Residual deviance: 3683315; 135 degrees of freedom (df)						
Growth (width) parameters of Hornworts Sporelings						
Null model			164	935395		
Species	1	99982	163	835413	374.804	**<0.001**
Treatments	1	122582	162	712831	459.524	**<0.001**
Time	13	225120	149	487712	64.916	**<0.001**
Species:Time	1	30015	148	457697	112.517	**<0.001**
Treatments:Time	13	97574	135	360123	28.137	**<0.001**
Model: AIC: 1798.8; Residual deviance: 360123; 135 degrees of freedom (df)						

those without cyanobacteria in *D. crispus*, whereas the opposite trend was observed in *N. vincentianus*, where growth and survival were lower in the presence of *Nostoc*. Although the two species differ in ecology, they were cultured under identical controlled conditions, allowing for a comparative assessment of their symbiotic response. Quantitatively, *D. crispus* exhibited greater survival (63.84% ± 1.20), length (463.51 µm ± 25.80), and width (156.91 µm ± 6.73) than *N. vincentianus* (54.77% ± 3.21; 238.88 µm ± 26.24; 113.48 µm ± 9.24, respectively; Table II; Appendix S1; Figure 3).Despite the presence of motile hormogonia in the medium, the reconstitution of endosymbiosis was not fully achieved (Video S1).

Only weak positive correlations were detected between the ontogenetic phases of hornworts and *Nostoc* (*D. crispus*: rs = 0.37, P = 0.015; *N. vincentianus*: rs = 0.38, P = 0.013; Figure S1). *Nostoc* phases developed similarly in both hornwort species (Figure 4A). However, we observed a higher number of heterocysts in the *Nostoc* treatment among *N. vincentianus* sporelings compared to those in *D. crispus* (Figure 4B). *Dendroceros crispus* sporelings developed well in both *Nostoc* and non-*Nostoc* treatments over the 154 culture days. In contrast, various developmental phases of *N. vincentianus* sporelings were observed only in the non-*Nostoc* treatment (Figure 5).

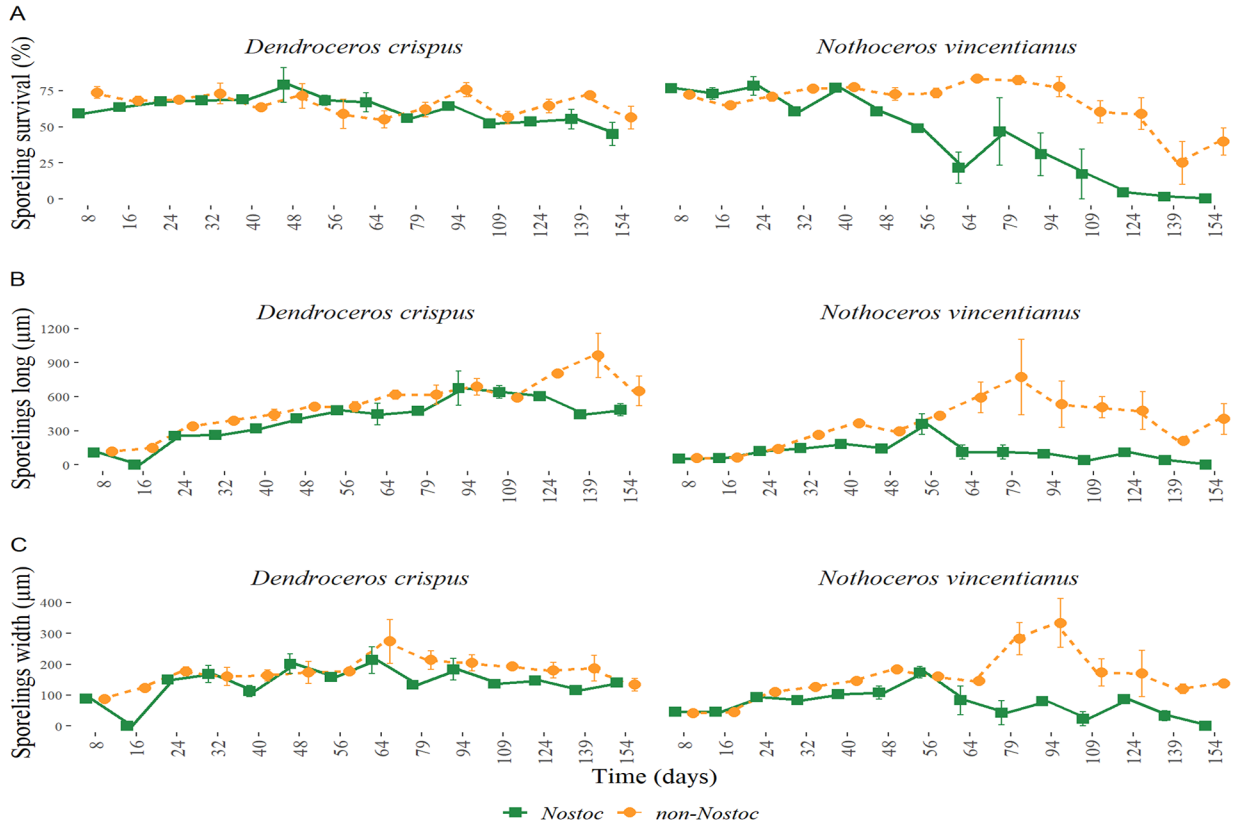

Figure 3. Spore survival percentages and sporelings growth of *Dendroceros crispus* and *Nothoceros vincentianus* overtime. a: Survival; b: growth in length and c: growth in width. Green square: treatment with sporelings and *Nostoc* sp.; Orange circles: treatment only with sporelings (non-*Nostoc* sp.). Error bars represent ± 1 SE (Standard Error).

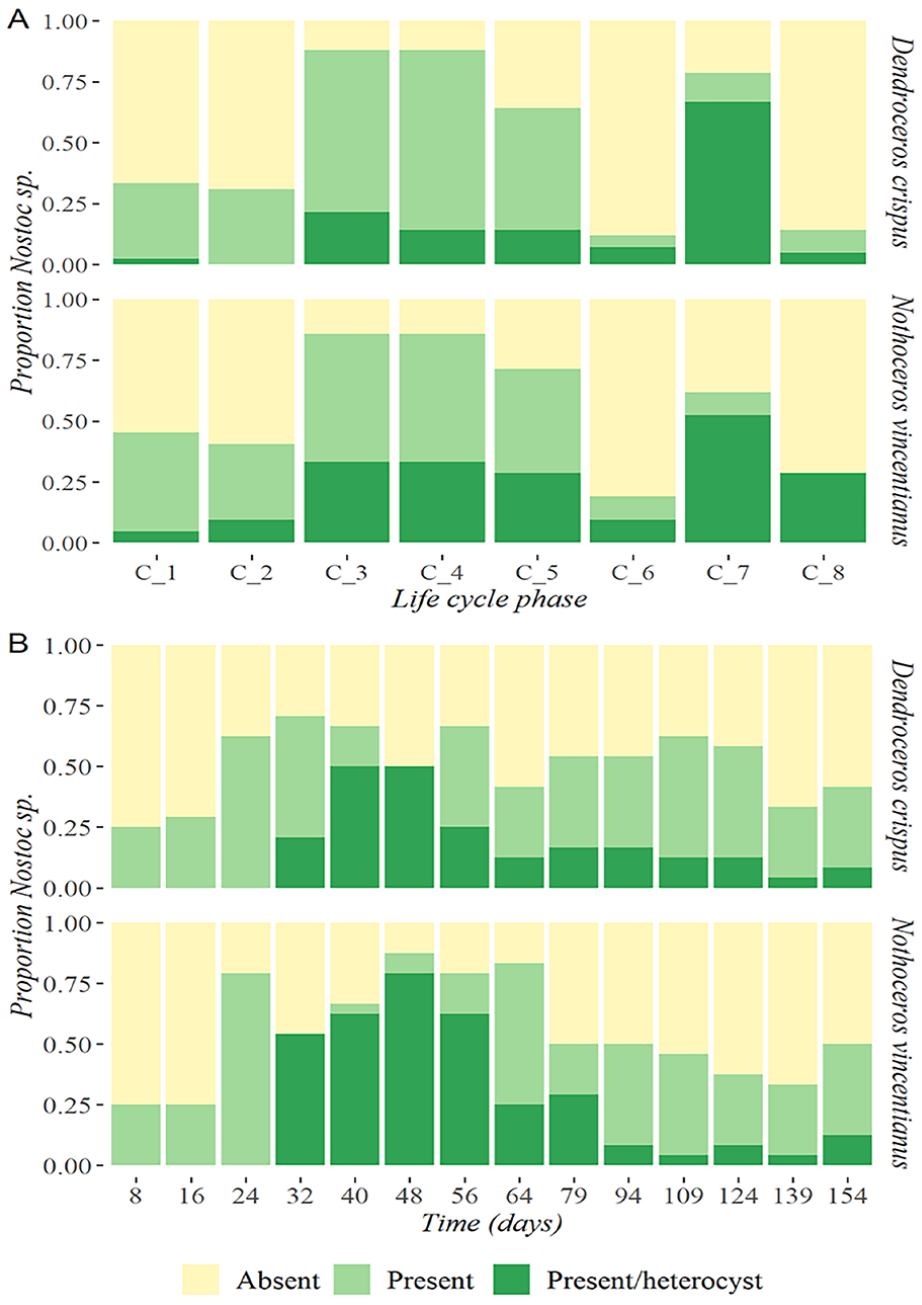

Figure 4. Proportion of the phases of the life cycle of *Nostoc* sp. in the treatments with spores of *Dendroceros crispus* and *Nothoceros vincentianus*. a: Life Cycle *Nostoc* sp. in seven phases (C1-C7; details in Table I); b: *Nostoc* sp. throughout 154 days of observation.

DISCUSSION

Our data indicate complex interactions between *Nostoc* and hornwort taxa. *Dendroceros crispus* outperformed *N. vincentianus* in both spore survival and sporeling growth, with similar performance across treatments (with and without *Nostoc*). Although previous studies have documented *Nostoc* colonization during the early stages of hornwort development (Renzaglia et al. 2009), the lack of colonization throughout our 154-day experiment raises questions about the factors influencing cyanobacteria-hornwort interactions. This is particularly intriguing given that *D. crispus* developed vigorous gametophytes with pores that typically serve as gateways for cyanobionts, and hormogonia were present in

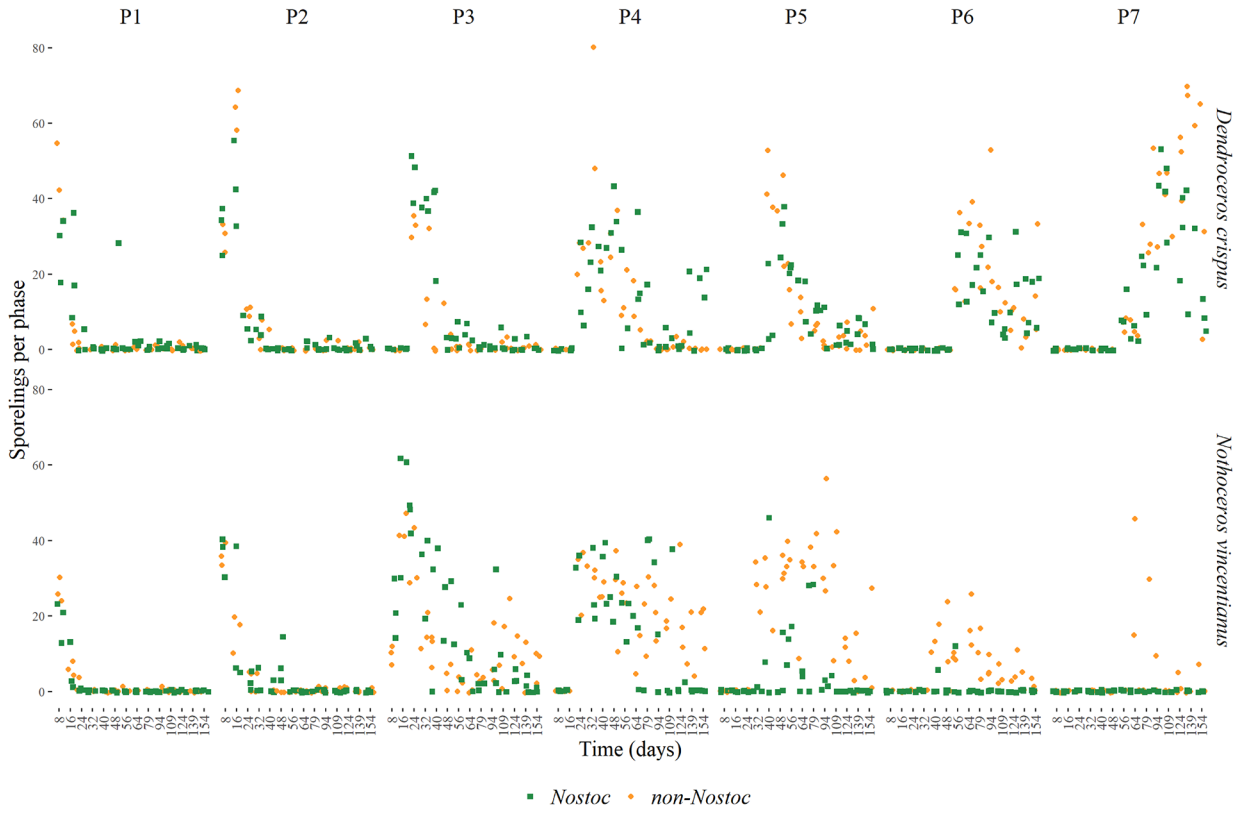

Figure 5. Sporelings development into seven phases (P1-P7; details in Table I) to *Dendroceros crispus* and *Nothoceros vincentianus* along the 14 observations within 154 days. Green square: treatment with sporelings and *Nostoc* sp.; Orange circles: treatment only with sporelings (non-*Nostoc* sp.).

the medium, which would generally suggest a high likelihood of colonization.

Previous research has demonstrated low specificity between *Nostoc* and *Anthoceros punctatus*. For example, a successful association was reconstituted between this hornwort species and *Nostoc punctiforme* isolated from the cycads *Macrozamia* sp. (Meeks 2003, 2007). However, external factors such as soil origin and microbial composition may strongly influence these interactions. Even sympatric hornwort species such as *N. orbicularis*, *P. carolinianus*, and *A. agrestis* exhibit similarly low specificity but show different preferences toward cyanobionts present in the soil (Nelson et al. 2020). In our study, *Nostoc* colonies isolated from *N. vincentianus* were collected from a different natural population than the one used for culture experiments, which could explain the lower performance of this species when growing with *Nostoc* in the laboratory and corroborates the findings of Nelson et al. (2020) regarding low specificity and high selectivity in the hornwort-cyanobacteria symbiosis.

The poor performance of *N. vincentianus* spores and sporelings in the presence of *Nostoc* suggests that the interaction might occur later in gametophyte development, rather than at the sporeling stage. The early presence of *Nostoc* in the culture medium (i.e., during unicellular germination) may have created competition for resources, negatively impacting the survival and growth of *N. vincentianus*. In contrast, *D. crispus*, which began as multicellular and chlorophyllous spores, appeared to be unaffected by competition with *Nostoc* colonies. Our studied species have

distinct spore traits (Ligrone & Renzaglia 1990, Renzaglia et al. 2020, 2009); spore germination and plant establishment differ in their sensitivity to environmental factors (Peñaloza-Bojacá et al. 2023), with the multicellular sporelings of *D. crispus* demonstrating greater resistance compared to the unicellular sporelings of *N. vincentianus*, enabling them to better withstand competition with cyanobacteria in the culture medium.

We observed hormogonia in cultures of both hornwort species (Video S1), excluding the possibility that symbiosis failure was due to lack of colonization-competent cells. Furthermore, the sporelings developed to the point of having pores (young plants) through which the hormogonia can infect. The high presence of *Nostoc* heterocysts—nitrogen-fixing cells (Meeks 2007)—in cultures of both hornwort species, combined with the weak correlation between hornwort and cyanobacterial life stages, suggests an independent development of hornworts and *Nostoc* in our study. This possibility is reinforced by the fact that a functional symbiotic association typically occurs when *Nostoc* filaments fragment and lose their heterocysts, generating hormogonia that serve as colonization units upon entering the hornwort host (Adams 2002, Adams & Duggan 2008, Meeks 2007). Unfortunately, this synchronization between cyanobacterial and hornwort development was not observed, despite the persistent presence of hormogonia being at all growth stages in *D. crispus* and *N. vincentianus*.

In conclusion, although we cannot completely rule out the possibility of plant-cyanobacteria specificity and selectivity, the contrasting responses of spores, sporelings and young plants of *D. crispus* and *N. vincentianus*—two species from distinct ecological backgrounds—underscore the complexity of *Nostoc*–hornwort symbiosis. Our findings suggest that both intrinsic plant traits (e.g., spore structure, developmental timing) and extrinsic factors (e.g., source of cyanobacteria, competition influence the establishment and success of this association. This highlights the need to examine symbiotic dynamics across developmental stages and environmental gradients. In particular, species from highly diverse ecosystems such as the Brazilian Atlantic Forest represent valuable systems for exploring how ecological complexity shapes symbiotic dynamics.

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SUPPLEMENTARY MATERIAL

Video S1.
Appendix S1.
Figure S1.

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Data availability

Data will be made available upon reasonable request.