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Effects of lichen symbiotic bacteria-derived indole-3-acetic acid on the stress responses of an algal–fungal symbiont

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Abstract

Lichens, comprising filamentous fungi and algae/cyanobacteria engaged in mutualistic symbiosis, exhibit remarkable adaptability to environmental challenges. While fungi safeguard algae from dry conditions, their ability to mitigate other stresses remains uncertain. Additionally, the functions of coexisting bacteria within lichen communities remain relatively unexplored. This study investigates the potential of indole-3-acetic acid (IAA) as a stress-response signaling molecule in lichen symbiosis. We subjected IAA-treated monocultures of algae and co-cultures of the fungal–algal complex to various stress conditions. IAA's role in bolstering resilience was evident, as demonstrated by the release of IAA (0–500 µM) by bacteria isolated from the lichen *Parmelia tinctorum*. This IAA was subsequently utilized by the lichen photobionts to alleviate oxidative stress. IAA acted as a communication signal, priming algal cells to defend against impending stressors. Further microscopic examinations unveiled that only the fibrous extensions were exposed in fungal cells that were in direct physical contact with viable algal cells. Co-cultivation and subsequent microscopic observations revealed that the algal cells were protected from diverse stressors by a barrier of fungal hyphae. Our findings underscore the significance of IAA in enhancing stress resistance within the context of lichen symbiosis, thereby advancing our understanding of the adaptability of these unique organisms. Further exploration of bacterial functions in lichen symbiosis holds promise for uncovering novel insights into their ecology and biology.

Keywords Holobiont · Lichen · Mutualism · Phytohormone · Symbiosis

Introduction

Symbiosis encompasses a diverse array of biological interactions among different organisms, including mutualism, commensalism, and parasitism. Lichen serves as a prime example of a classic mutualistic symbiosis, characterized by the close coexistence of numerous filamentous fungi alongside microorganisms such as algae, cyanobacteria, and yeasts [1, 2]. Lichens exhibit a wide range of colors, shapes, and sizes due to their diverse algal species combinations [3]. Notably, their mutualistic interactions enable lichens to thrive in challenging environments, such as rocks or barks, where nutrients and water are typically scarce. Photosynthetic algae supply carbohydrates through photosynthesis, while cyanobacteria contribute nitrogenous compounds. Fungal mycelium shields algae from dryness and excessive light, while root-like fungal structures, known as rhizines, anchor the lichen to the substrate. This mutualistic association allows both algae and fungi to thrive in harsh conditions. Previous studies have primarily focused on algal–fungal symbiosis for harvesting microalgae during wastewater treatment and comprehending internal lichen relationships through co-culturing. However, recent research emphasizes the presence of not only fungi and algae but also bacteria and yeast in lichen symbiosis, although the precise roles of bacteria and yeast remain largely unexplored [2, 4, 5].

Lichens can secrete a diverse array of lichen acids, and their physiological metabolism influences the weathering and decomposition of the rocks they inhabit, indirectly affecting the rate at which rocks become sand particles.

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These sand particles, along with decomposed lichens and other organic matter, become crucial sources of soil for plant growth. Therefore, lichens are important pioneer species in primitive ecosystems. The substances produced by lichens, including the antibiotic usnic acid known for its potent anticancer activity, have applications in daily life and scientific technology [6]. Additionally, the sensitivity of lichens to air pollution makes them valuable indicators for monitoring and assessing air quality. However, the factors influencing lichen growth are complex and not yet fully understood. These factors include climate change and anthropogenic stressors such as land pollution, deposition, deforestation, and human activities. Recent studies have predominantly focused on the effects of climate change and human-induced pressures on the morphology and distribution of lichens, with limited research on the response of the associated microbial communities to these environmental changes. In earlier studies, scientists used techniques such as FISH and comparative analysis of 16 S rRNA gene sequences to demonstrate the diversity of bacteria within lichens [7, 8]. However, understanding the roles and functions of these microbial communities has only started to emerge in recent years with the systematic exploration of microbial composition and functionality using various omics technologies [9]. For instance, in 2017, Cernava et al. used transcriptome sequencing to reconfirm the significance of bacterial diversity within lichens. The transcriptome data suggested potential roles of bacteria in providing vitamins and degrading phenolic compounds, such as phenylpropanoids, xylenols, and cresols [10]. Most studies on lichen-associated bacterial communities have focused on Lecanoromycetes lichens, particularly the large, foliose lung lichen (*Lobaria pulmonaria*), which is a symbiosis of an ascomycete fungus, a green alga (*Dictyochloropsis reticulata*), and a cyanobacterium (*Nostoc* sp.) [5]. Consequently, research on bacterial communities in other lichen species, especially those in the Asian region, is relatively scarce.

Indole-3-acetic acid (IAA), a crucial plant growth auxin, promotes cell expansion, differentiation, and elongation [3]. In addition to being secreted by plants, IAA is produced by plant-associated bacteria, playing a significant role in plant growth and development [11]. Algae, akin to plants, sense IAA signals released by competitors and, in response, adapt their physiology and optimize survival strategies by accumulating lipid droplets under high IAA concentrations [12]. A review highlights that IAA-producing bacteria use IAA as a communication signal between microbial species. Microorganisms utilize chemical signaling molecules to shape their interactions with the external environment [13].

Bacterial communities involved in lichen symbiosis have been well-documented, showing significantly greater diversity compared to the associated fungal or algal communities [14, 15]. However, the specific roles and benefits of these bacteria to other symbiotic organisms remain unexplored [16]. The addition of exogenous IAA to lichen photobionts resulted in increased water content, supporting the signaling role of phytohormones in lichen symbiosis [17]. The present study proposes that IAA serves as a pivotal signaling molecule within lichen symbiosis, influencing stress response mechanisms and enhancing adaptability. We demonstrate that IAA derived from lichen-associated bacteria serves as a pivotal regulator of stress resilience in both algal and fungal components of the symbiotic structure. Furthermore, we anticipate that manipulating these bacterial communities will affect IAA production and availability, thereby influencing the effectiveness of lichen symbiosis against environmental stressors. Through systematic experimentation across various stress contexts, our investigation aimed to elucidate the intricate pathways through which IAA regulates stress responses within lichen symbiosis, thereby enhancing our understanding of the interactions among these unique organisms.

However, the successful isolation of endosymbiotic algae and fungi from lichens remains a persistent challenge. Nevertheless, our efforts in bacteria-free cultivation of lichen symbiotic structures yielded promising results, leading to the development of what we term “lichen-like” spherical symbiotic structures within approximately 1–2 wks. This accomplishment was facilitated by employing purified lichenic fungus in combination with the photobiont extracted from *Parmelia tinctorum* (a foliose lichen species) and epiphytic corticolous lichens. Our laboratory’s cultivation environment, noted for its efficiency, is characterized by two crucial aspects: firstly, the ability to rapidly assess fungal and algal pairings conducive to symbiotic structure formation, and secondly, the proficiency in mitigating the impact of lichenic bacteria on the lichen formation process. Lichens produce various metabolites that are crucial for environmental adaptation, including responses to oxidative stress. However, these metabolites can also be influenced by the microbial community within the lichen. Since both the algal and fungal components of lichens are known to be affected by IAA, and bacteria are among the most complex and variable contributors to the lichen microbiome, this study aims to explore whether lichen-associated bacteria produce IAA at different levels compared to fungi and whether altering bacterial composition affects IAA concentrations. Additionally, we will investigate the impact of IAA on algae subjected to different stress treatments and its implications for the co-cultured fungal–algal symbiotic complex under various stress conditions.

Materials and methods

Lichen collection sites

The foliose lichen *P. tinctorum* (Fig. 1A) was carefully collected from the bark of a plum tree (*Prunus mume*) in Fuxing Township (25°02'00.6''N 121°33'50.8''E), Taoyuan County, Taiwan (average annual rainfall: 2,903 mm; average annual temperature: 20.6 °C; elevation: ~640 m). Similarly, the epiphytic corticolous lichens (Fig. 1B) were obtained from the bark of a plum tree (*P. mume*) in Yuchih Township (23°55'06.2''N 120°53'06.4''E), Nantou County, Taiwan (average annual rainfall: 2,285 mm; average annual temperature: 20.8 °C; elevation: ~600 m). Lichen identification will primarily rely on their morphological characteristics, with references to previous studies [18, 19]. After removing surrounding impurities, the entire lichen was gently scooped and placed in a Petri dish with absorbent paper. Subsequently, the lichen was cut into small pieces (1 cm^2) using a sterilized scalpel. Then, the surface of the lichen pieces was sterilized with 70% ethanol and washed three times with sterile water. Following this, the lichen pieces were homogenized in a microtube using a sterilized pestle. To obtain various concentrations, the homogenate was diluted with sterile water and added to different media.

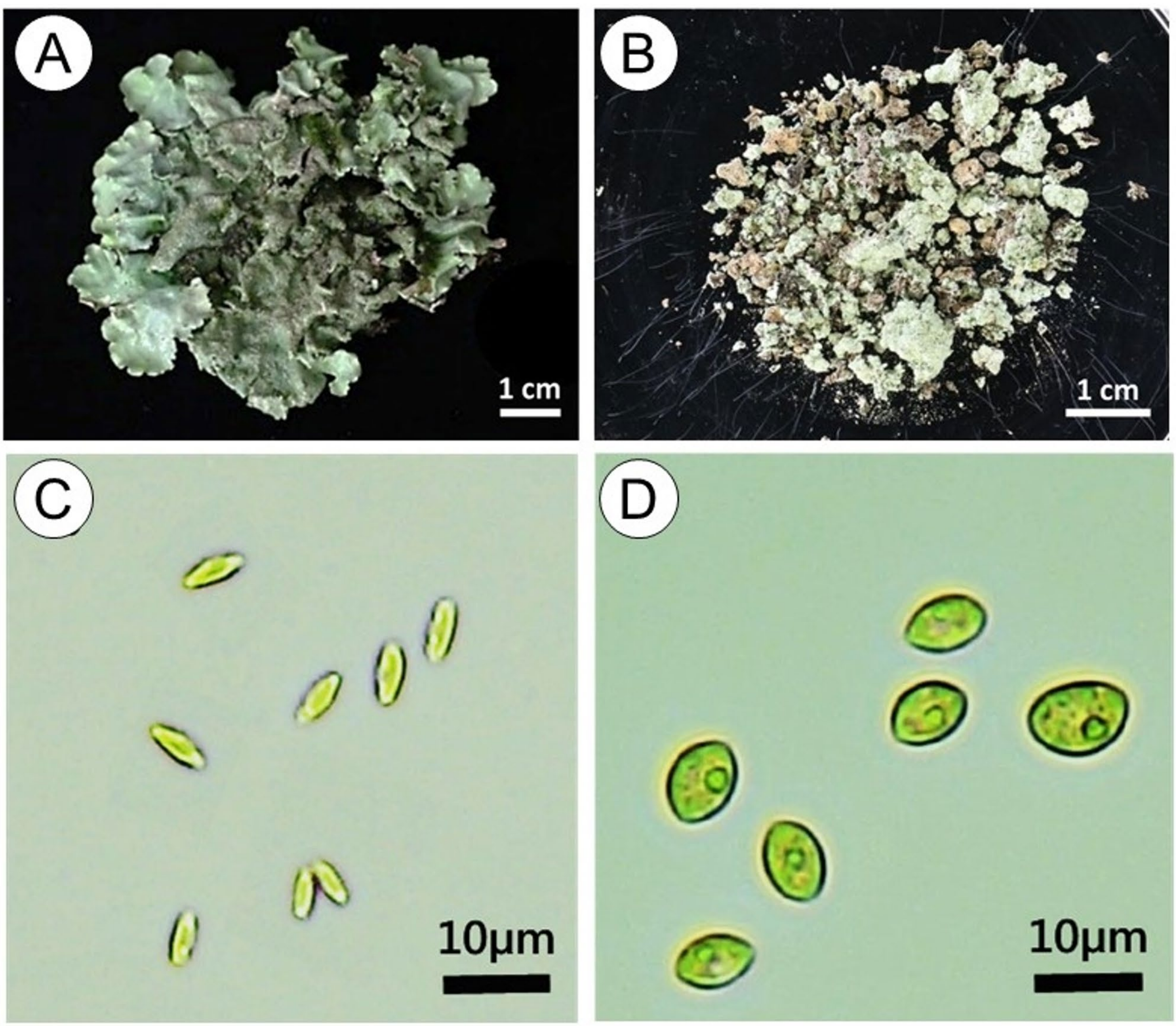

Fig. 1 Light microscopy images of lichen samples and associated algae after a 1-wk culture period. (**A**) *Parmelia tinctorum*, a common foliose lichen in Taiwan. (**B**) Epiphytic corticolous lichens collected from bark. (**C**) *Coccomyxa* sp. isolated from foliose lichen. (**D**) *Coelastrella* sp. isolated from epiphytic corticolous lichens

Microbes and culture conditions

Purified microbes isolated from the internal lichen were cultured under different conditions. Fungi were cultured in YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) [20], while bacterial strains were cultured in Luria–Bertani (LB) medium (tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L) [21]. This study focused exclusively on isolating bacteria and algae from the foliose lichen *P. tinctorum*. The cultures were maintained under continuous aeration conditions at 28 °C with shaking. Algae were cultured in CA medium (consisting of 2 mg/L $Ca(NO_3)_2.4H_2O$, 10 mg/L KNO_3, 5 mg/L NH_4NO_3, 3 mg/L β–Na_2glycerophosphate.$5H_2O$, 2 mg/L $MgSO_4.7H_2O$, 0.01 µg/L vitamin B12, 0.01 µg/L biotin, 1 µg/L thiamine HCl, and 0.1 mL/L PIV metals (1000 mg/L $Na_2EDTA.2H_2O$, 196 mg/L $FeCl_3.6H_2O$, 36 mg/L $MnCl_2.4H_2O$, 10.4 mg/L $ZnCl_2$, 4 mg/L of $CoCl_2.6H_2O$, and 2.5 mg/L of $Na_2MoO_4.2H_2O$), 0.1 mL/L Fe (as EDTA; 1:1 molar; 702 mg/L $Fe(NH_4)_2(SO_4).6H_2O$ and 660 mg/L $Na_2EDTA.2H_2O$), and 40 mg/L of HEPES; all compounds were directly mixed, the pH was then adjusted to 7.2) and autoclaved (15 min at 120 °C) and incubated at 25 °C under a 12-h light/12-h dark cycle [22]. In this experiment, the algal cell density (y × 10^4/mL) of *Coelastrella* sp. was estimated using a regression equation derived from the relationship between optical density at 680 nm (OD680, x) and cell density. Cell density was determined using a hemocytometer observed under an optical microscope (DMRB, Leica, Germany), and OD_{680} was measured using a spectrophotometer. Based on the calculated data, we constructed the following regression equation: $y = 1290.5x + 27.332$ ($r^2 = 99.75\%$).

Genomic DNA extraction

Fresh cultures of fungi, bacteria, and algae were individually transferred into 1.5 mL microtubes and centrifuged at 13,000 × g for 1 min. The supernatant was discarded and the cell pellet was resuspended in 200 µL of lysis buffer (comprising 2% Triton X-100, 1% sodium dodecyl sulfate, 100 mM sodium chloride, 10 mM Tris (pH 8.0), and 1 mM ethylenediaminetetraacetic acid), along with 200 µL of phenol-chloroform-isoamyl alcohol (in a 25:24:1 ratio; isoamyl alcohol is optional) and sterilized glass beads. The cells were disrupted by vortexing for 5 min and then centrifuged at 13,000 × g for 5 min. Subsequently, 160 µL of the aqueous layer from each sample was individually transferred to a clean microtube, and 400 µL of 95% ethanol and 16 µL of 3 M sodium acetate (pH 5.2) were added to it. The samples were vortexed and centrifuged at 13,000 × g for 5 min to pellet the DNA. Then, the supernatant was discarded, and the pellets were washed with 200 µL of 70% ethanol and centrifuged at 13,000 × g for 2 min. Subsequently, the microtubes were inverted and left overnight to air-dry the DNA pellets. Finally, the genomic DNA obtained from each sample was individually dissolved in 50 µL of sterile water [23].

Identification of fungi

Fungal genomic DNA was extracted from fungal hyphae, and the ribosomal DNA (rDNA) sequences were amplified using polymerase chain reaction (PCR) with the following primers: ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3'). For bacteria, the 16 S rRNA gene was amplified via PCR using the following primers: 8 F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1541R (5'-AAGGAGGTGATCCAGCCGCA-3'). The ITS1 region of algal genomic DNA was amplified using PCR with the following primers: ITS1F (5'-ACCTAGAGGAAGGAGAAGTCGTAA-3') and ITS1R (5'-TTCCTCCGCTTATTGATATGC-3'). The PCR process consisted of denaturation at 95 °C for 5 min, followed by 35–40 cycles of denaturation at 95 °C for 1 min, annealing at 48–55 °C for 45 s, and elongation at 72 °C for 2 min. A final elongation step was performed at 72 °C for 10 min. The DNA sequencing of these samples was performed at Genomics BioSci & Tech. Co., Ltd. The obtained nucleotide sequences were then compared to sequences in the National Center for Biotechnology Information (NCBI) homepage using BLAST for identification purposes.

Indole-3-acetic acid production by bacteria

The isolated bacteria were cultured in 3 mL of LB medium at 28 °C for 24 h with agitation (100 rpm). IAA biosynthesis in microbes is accomplished by both tryptophan-dependent and tryptophan-independent pathways. Subsequently, the cultures were transferred to another 3 mL of LB medium, with or without 0.1% tryptophan—an important precursor in the IAA synthesis pathway [24]. After 5 d of culture at 28 °C in the dark, the supernatant was collected through centrifugation (13,000 × g for 1 min). To determine the IAA concentration, the supernatant was mixed with the Salkowski reagent in a 1:1 ratio (Salkowski reagent comprised 0.5 M $FeCl_3$ and 35% $HClO_4$ in a 1:49 ratio), and the mixture was kept in the dark for 30 min [25]. Subsequently, color development (red) was quantified using a spectrophotometer (Unico 1200-Spectrophotometer, USA) at 530 nm. A calibration curve constructed using pure IAA was used to calculate the IAA concentration.

Stress treatment

The stress treatment involved three different stressors commonly experienced by terrestrial biota: oxidative stress, osmotic pressure stress, and pH variation. Oxidative stress was induced using three concentrations of H_2O_2 (4, 6, and 8 mM), with 0 mM H_2O_2 serving as the control treatment. Osmotic stress was achieved by exposing the algal cells to various concentrations of sodium chloride (25, 50, and 100 mM NaCl). Additionally, to examine the effects of different pH conditions, the pH of the CA medium was individually adjusted to pH 3 and 4. Before the stress experiment, algal cells were pre-treated with IAA (0, 50, 200, and 500 mM in CA medium) in 1.5 mL microtubes for 2 d. Subsequently, the supernatant, which contained IAA, was removed via centrifugation, and the algal cells were quantified and adjusted to the same cell number using fresh CA medium ($OD_{680} = 0.5$). Then, 50 µL aliquots of the algal suspension were subjected to the stress conditions mentioned above. Each treatment was performed in triplicate. The samples were then incubated at 25 °C for 9 d under a 12-h light/12-h dark regime. At the end of the incubation period, the algal cells were centrifuged, and their stress tolerance capacity was assessed by analyzing cell number (calculated based on the absorbance of the samples at 680 nm), chlorophyll content [26], or algal morphology (observed under a microscope).

Co-cultivation of fungi and algae

First, approximately 1 mm^2 of fungal hyphae was transferred from a YPD plate to 3 mL of YPD medium using sterilized tweezers and incubated for 3 d. During this period, the fungal hyphae formed a spherical structure. Concurrently, the algae were cultured in 3 mL of CA medium for 5 d, and their cell numbers were quantified through absorbance measurements ($OD_{680} = 0.5$). Next, a quantified suspension of algae (50 µL) was co-cultured with the spherical fungal structure in CA medium supplemented with four levels of IAA (0, 50, 200, and 500 µM) for 2 d as a pretreatment phase. During this period, the symbiotic complex was formed. Subsequently, the symbiont complex was transferred to stress conditions for 5 d. The entire co-culture complex was maintained for 1 wk at 22 °C temperature and a 12-h light/12-h dark regime. Subsequently, the stress tolerance of the co-culture complex was measured to assess its response to the imposed stress conditions.

Stress tolerance measurements

Stress tolerance measurements included the assessment of chlorophyll content. The co-cultured structures were initially disrupted using a mortar and pestle and then frozen at -80 °C for 1 d. Each sample was individually homogenized in a vortex for 30 min using glass beads (0.45–0.52 mm) and then centrifuged at 8,000 × *g* for 5 min. Subsequently, the supernatant was removed, and 1 mL of methanol was added to the pellet. The homogenization process was repeated in a vortex for 10 min, followed by freezing at -20 °C for 10 min; this process was repeated three times. The mixtures were kept at 4 °C for 24 h in the dark. Subsequently, they were centrifuged at 13,000 × *g* for 5 min. Each experiment was conducted in triplicate. Chlorophyll *a* and *b* were estimated by measuring the absorbance of the supernatant at 645 and 662 nm using a UV-VIS spectrophotometer (UNICO UV-1200). The concentrations of chlorophyll *a* and *b* were calculated using the following equations: chlorophyll *a* ($\mu g \cdot mL^{-1}$) = 11.75 OD662 − 2.35 OD645; chlorophyll *b* ($\mu g \cdot mL^{-1}$) = 18.61 OD645 − 3.96 OD662 [26].

Microscopic observation

The viability of the algae (*Coccomyxa* sp.) and fungi in co-culture was evaluated using a fluorescence microscope (Leica DM2500). To enhance visibility, 1–2 drops of Calcofluor White (CFW) and 10% KOH were added to the samples and incubated in the dark for 3 min. CFW, a fluorochrome, binds to chitin and cellulose present in the fungal cell wall, aiding in the delineation of fungal elements. CFW was excited at 347 nm, and emission was detected within the range of 300–412 nm. Algal chlorophyll was excited at 559 nm, and emission was detected within the range of 655–755 nm. Using ImageJ software, the fluorescence intensity of the algal cells (red) and fungal hyphae (blue) was measured to determine their viability in the co-culture complex.

Light microscopy was employed to quantify algal cells, observe the interaction between the alga and fungi, and compare the differences between mono-cultured and co-cultured complexes. Scanning electron microscopy (SEM) was used to investigate the physical interaction between co-cultured algae and fungi. The algal–fungal symbiosis complex was collected 6 d after co-culturing, fixed in a 2.5% or 4% (v/v) glutaraldehyde solution, and then dried using a freeze dryer or a critical point dryer. Subsequently, the samples were coated with gold–palladium using a sputter coater and observed using SEM (TM-3000 or S-4700, Hitachi, Japan). Transmission electron microscopy (TEM) was employed for a more detailed examination. The samples were first prefixed overnight in 2.5% glutaraldehyde/0.1 M sodium cacodylate buffer (pH 7.3) with 1% tannic acid at 4 °C. After washing and postfixing in 1% osmium tetroxide, the samples were stained with 2% aqueous uranyl acetate and then dehydrated in an ethanol graded concentration series, followed by two washes in 100% acetone. Then, the samples were embedded in Spurr resin. Ultrathin sections

Table 1 Catalog of bacterial species extracted from the foliose lichen *Parmelia tinctorum*

Bacterial species	Percent identity	Number of strains
Bacillus cereus	100%	4
Bacillus megaterium	100%	3
Bacillus marisflavi	100%	2
Bacillus aryabhattai	100%	2
Bacillus toyonensis	100%	2
Bacillus tequilensis	100%	1
Bacillus subtilis	99.79%	1
Bacillus sp.	100%	2

(70 nm thick) were cut using a Leica Ultracut R ultramicrotome with a diamond knife and collected on formvar-coated copper slot grids. The sections were examined using TEM (H-7500, Hitachi, Tokyo, Japan) at 80 kV. Images were captured using a Macrofire monochrome CCD camera (Optronics, Goleta, CA, USA) [12].

Results

Identification of the microorganisms in lichens

After conducting a BLAST search, the nucleotide sequences were processed through the NCBI homepage. The green alga *Coccomyxa* sp. (Fig. 1C) was isolated from the lichen *P. tinctorum*, while *Coelastrella* sp. (Fig. 1D) was obtained from epiphytic corticolous lichens. Five fungal genera were identified from the samples collected during the study: *Xylaria*, *Hypoxylon*, *Sarocladium*, *Chaetomium*, and *Diaporthe* (data not shown). *Xylaria* sp. was selected for the experiment not only because it is well-recognized as an endolichenic fungus [27, 28], but also because it is the most frequently isolated fungal species from various *P. tinctorum* samples. The majority of bacteria isolated from the foliose lichen *P. tinctorum* belonged to the *Bacillus* genus, comprising eight distinct species (Table 1).

IAA production capability of the different bacteria isolated from lichen

To evaluate the potential for IAA production, bacteria were cultivated in LB medium with and without added tryptophan. All bacterial strains collected from lichen displayed the ability to produce IAA in LB medium, irrespective of the presence of supplementary tryptophan (Fig. 2). After a 5-d incubation period, the bacteria were stratified into three tiers based on their IAA production levels when cultured with tryptophan: (i) bacteria producing the highest IAA concentration, approximately 500 µM; (ii) bacteria producing approximately 200 µM of IAA; (iii) bacteria producing approximately 50 µM of IAA. The spectrum of IAA production potential in bacteria ranged from 0 to 500 µM. Therefore, we employed three IAA concentrations (50, 200, and

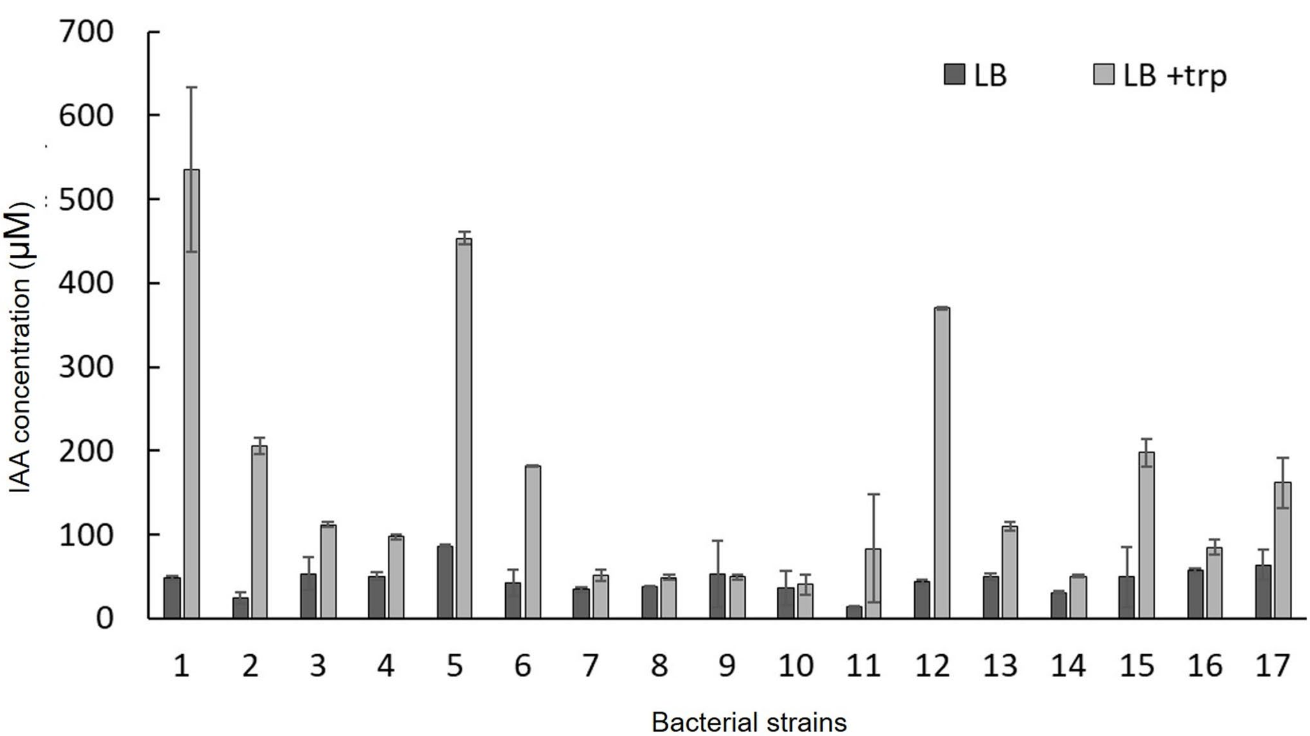

Fig. 2 Indole-3-acetic acid (IAA) production by lichen-isolated bacteria in the presence and absence of 0.1% tryptophan (Trp). Bars represent the mean and standard deviation of triplicate data

500 µM) to mimic the levels found within the internal lichen structure.

Impact of IAA on algal fitness under stressful conditions

In this study, the tested IAA concentrations had no impact on the growth of *Coelastrella* sp. algae under non-stressful conditions. However, following a 2-d IAA pretreatment, the algal doubling time exhibited variations across distinct stress conditions (Fig. 3). Under 4 mM H_2O_2-induced oxidative stress, higher concentrations of IAA were found to reduce algal doubling time (Fig. 3A). Notably, a lower IAA concentration (50 µM) further slowed down algal growth under 6 mM H_2O_2-induced oxidative stress (Fig. 3B). Conversely, higher IAA concentrations (200 and 500 µM) did not produce this effect. Furthermore, it is crucial to acknowledge that certain stress conditions exceeded IAA's protective capacity for algae. For instance, when exposed to conditions characterized by high H_2O_2 concentration (8 mM) and a pH of 3, algal growth was significantly inhibited, resulting in longer doubling times (data not shown). Conversely, IAA had no discernible impact on doubling time under the three levels of NaCl-induced osmotic stress (data were not shown). Interestingly, when algae were pre-treated with elevated IAA concentrations and subsequently cultivated in an acidic environment (pH 4), there was a marginal reduction in algal doubling time, although this effect was not statistically significant (Fig. 3C). This suggests that IAA pretreatment is effective in protecting algae against specific stressors, such as oxidative stress and low pH, but is ineffective against osmotic stress.

Algal–fungal interactions: attachment and connection of co-cultured *Coccomyxa* and *Coelastrella*

Both *Coccomyxa* sp. and *Coelastrella* sp. exhibited a remarkable ability to adopt a spherical structural form when in symbiotic association with the fungus *Xylaria* sp., as vividly depicted in Fig. 4. Meticulous microscopic examination revealed that *Coccomyxa* adheres to fungal hyphae (Fig. 4B). We discovered that algae are elliptical in shape and adhere vertically to the fungal hyphae along their long axis. This suggests that algae and fungi are not merely co-located but interact in a specific direction to form a symbiotic complex, facilitating information exchange. Continuing our exploration, we sought to determine whether *Xylaria* fungi could form similar symbiotic complexes even when the algae did not originate from the same lichen species. Surprisingly, in the presence of *Coelastrella*, co-cultured algal cells displayed a tendency to aggregate and were enveloped by fungal hyphae, as illustrated in Figs. 4C and D. In contrast to *Coccomyxa*, *Coelastrella* algae were enveloped by fungal hyphae and did not adhere to the fungal hyphae in a specific direction (Fig. 4D). The precise interaction sites between these algae and the fungus were determined through SEM analysis, as presented in Fig. 5. *Coccomyxa* algae exhibited a directional connection with fungal hyphae, a process facilitated by the secretion of adhesive substances leading to the formation of fibrous structures (Figs. 5A and B). However, the specific molecules and mechanisms responsible for this intriguing interaction remain unidentified. Unlike *Coccomyxa*, *Coelastrella* algae were found to exist in an aggregated state, enveloped by fungal hyphae. Consequently,

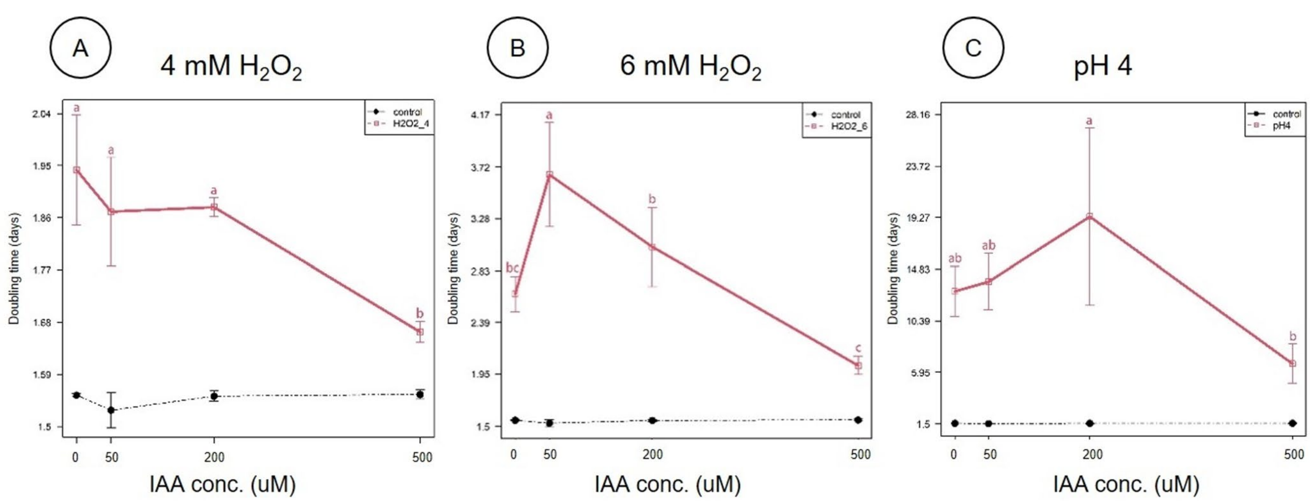

Fig. 3 Graphical representation of the doubling time of the algae *Coelastrella* sp. under various stress conditions following indole-3-acetic acid (IAA) pretreatment. (**A**) 4 mM H_2O_2 stress condition. (**B**) 6 mM H_2O_2 stress condition. (**C**) pH 4 stress condition. Distinct lowercase letters represent statistically significant differences in doubling time among the four IAA levels. The statistical analysis included a one-way ANOVA and Tukey's Post Hoc Test; statistical significance was set at $P < 0.05$. Means that do not share a letter are significantly different

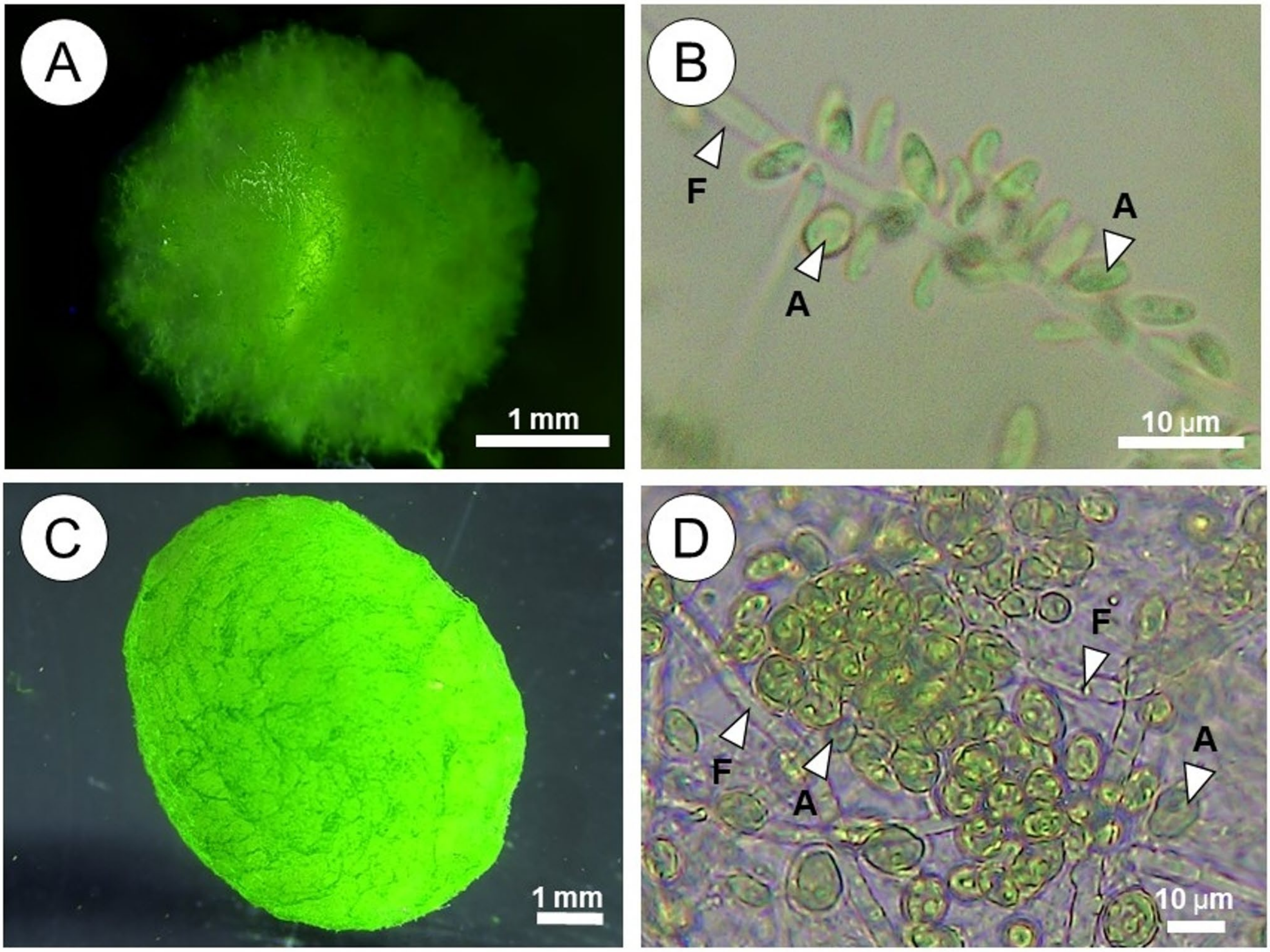

Fig. 4 Microscopic insights into algal–fungal interactions: green algae *Coccomyxa* and *Coelastrella* co-cultured with lichen fungi. (**A**) Dissecting microscope view of the co-culture of *Coccomyxa* sp. with lichen fungi. (**B**) Compound microscope observation revealing physical contact between *Coccomyxa* sp. and lichen fungi. (**C**) Dissecting microscope view of the co-culture of *Coelastrella* sp. with lichen fungi. (**D**) Microscopic view of the interaction of *Coelastrella* sp. with lichen fungi, characterized by algal cell clumping and attachment to fungal hyphae

determining whether the algae secrete specific substances to facilitate their attachment to the fungal hyphae was more challenging (Figs. 5C and D). These intriguing findings suggest that *Xylaria* fungi may possess a remarkable ability to communicate and form symbiotic associations with algae, regardless of whether they are their original partners from the same lichen species or not. This observation opens up exciting avenues for future research into the mechanisms and implications of such fungal–algal interactions. Additionally, a TEM analysis-based comparison between mono-cultured and co-cultured *Coccomyxa* revealed that septum formation was exclusively observed in the co-cultured algae, as depicted in Fig. 6. These findings clearly indicate that these two organisms, algae and fungi, indeed engage in communication, and the algae undergo physiological changes in response to their fungal partner. However, the biological significance of these septum structures remains an open question that warrants further investigation.

Dynamic changes in the chlorophyll content of algal–fungal co-cultures under stress conditions

After co-culturing for 1 wk, notable shifts in total chlorophyll content were observed across diverse stress conditions, as depicted in Fig. 7. When subjected to stressors (6 mM H_2O_2, 50 mM NaCl, or pH 4), the chlorophyll content of mono-cultured algae exhibited a decline. Interestingly, in the absence of stress, the chlorophyll content in co-cultured complexes was lower than that in mono-cultured algae (Fig. 7A). This discrepancy could be attributed to the nutrient-sharing dynamics between the algae and fungi within the co-cultured environment. Intriguingly, when

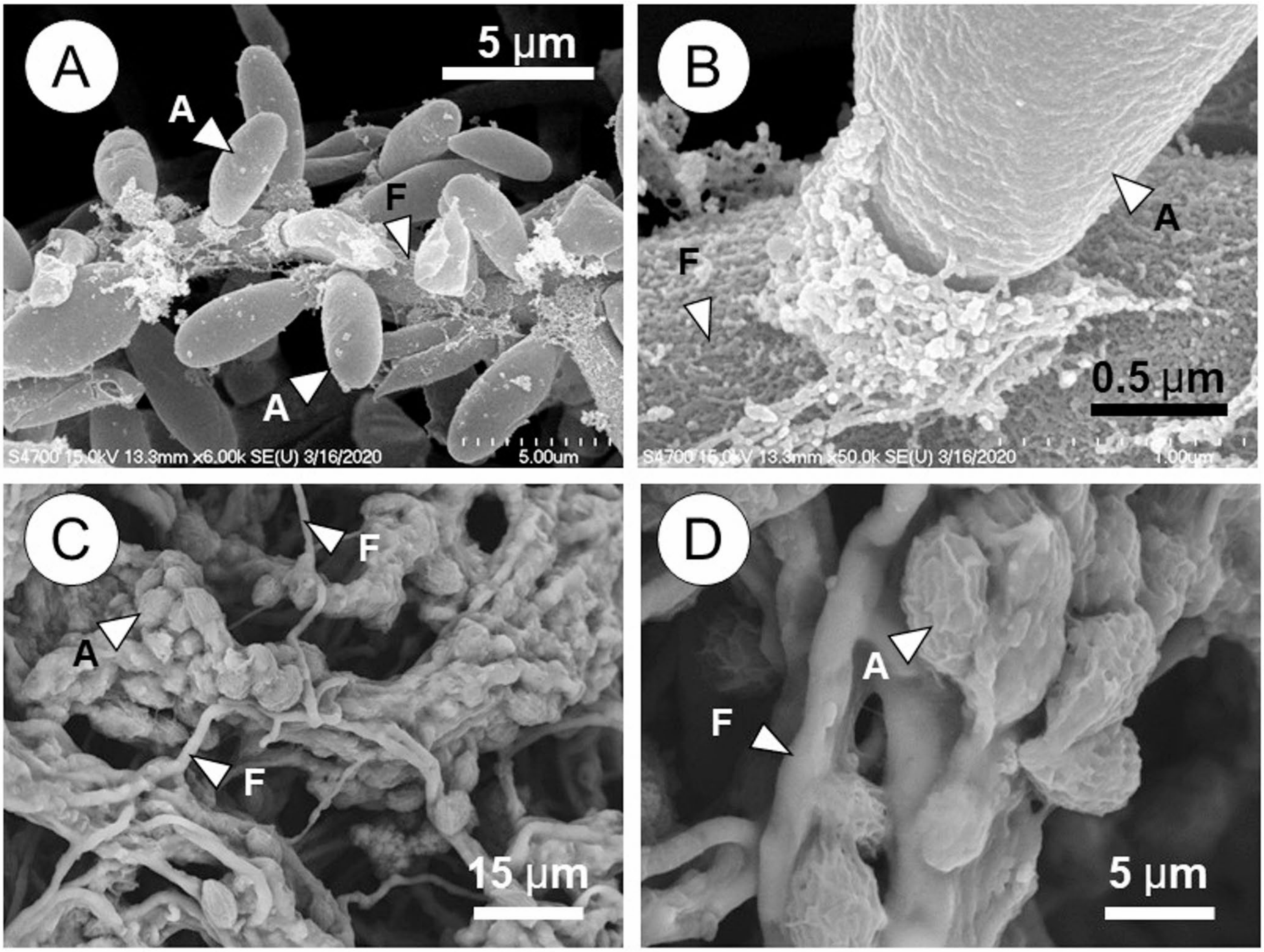

Fig. 5 Scanning electron microscopy (SEM) images depicting the interaction between algae and fungal hyphae following a 1-wk co-culture period. (**A**, **B**) *Coccomyxa* co-cultured with *Xylaria*, demonstrating the adhesion of algal cells to the fungal hyphae through sticky substrates. (**C**, **D**) *Coelastrella* co-cultured with *Xylaria*, illustrating the interaction between the two organisms. A: algae; F: fungal filaments

comparing the total chlorophyll levels of co-cultured and mono-cultured groups, a distinct trend emerged. Regardless of whether they were exposed to stressors or maintained under non-stressful conditions, the co-cultured group consistently maintained stable total chlorophyll levels, unlike their mono-cultured counterparts (Fig. 7B). This resilience in chlorophyll content in the co-cultured group could potentially be attributed to the protective influence of the fungal hyphae on algal cells. This direct defense mechanism, facilitated by the fungal component within the co-cultured environment, seems instrumental in maintaining chlorophyll levels even amidst challenging conditions.

In our investigation aimed at evaluating the health status of the algae within the intricate symbiotic complex, we embarked on a comprehensive assessment. To begin, we meticulously observed the autofluorescence emanating from algal chlorophyll, which manifested as distinctive red fluorescence. Simultaneously, we sought to elucidate the presence and status of fungal hyphae by employing a meticulous visualization technique involving blue fluorescence generated through CFW staining. Our experimental protocol involved subjecting the algal cells in the symbiotic complex to varying environmental conditions for 1 wk. Intriguingly, our observations unveiled a noteworthy discovery. Under the stress-inducing conditions of 6 mM H_2O_2, 50 mM NaCl, and pH 4, the autofluorescence exhibited by the algae within the symbiotic complex was similar to that observed under standard conditions (Fig. 8). Notably, our fluorescence imaging endeavors delivered striking visual evidence of the robust health of both the algal and the fungal counterparts within the co-cultured complex even under stress conditions. In summary, our comprehensive investigation yields compelling evidence suggesting that fungal hyphae play a pivotal role in shielding algae from the deleterious impacts of environmental stressors. This finding highlights the intricate and symbiotic nature of the relationship between these

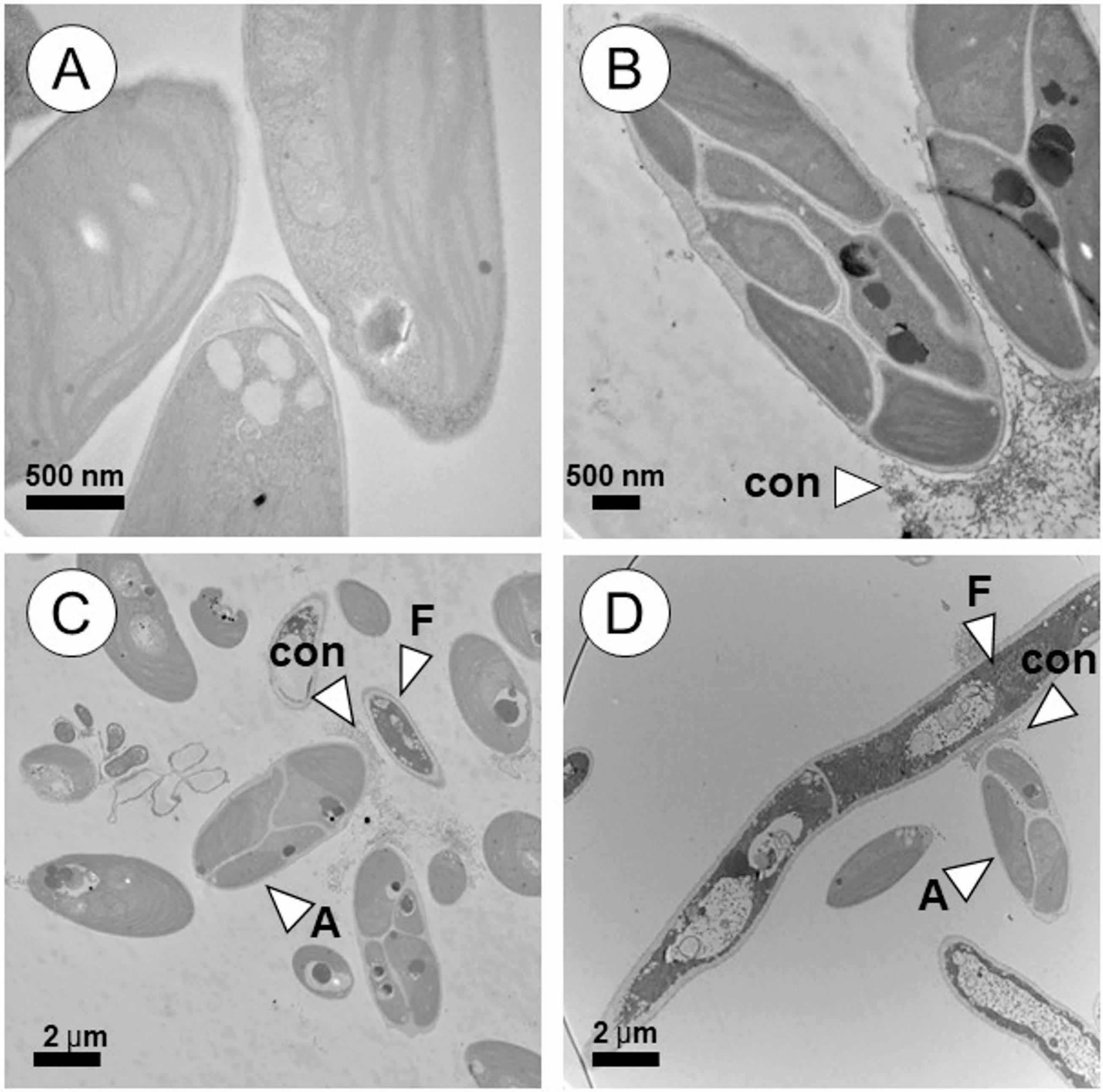

Fig. 6 Transmission electron microscopy (TEM) images of the algae following a 1-wk culture period. (**A**) Mono-cultured algae. (**B**–**D**) Algal–fungal co-culture of *Coccomyxa* and *Xylaria* for 1 wk, depicting various aspects of the interaction. A: algae, F: fungal hyphae, con: site of algal–fungal contact

organisms, underscoring their capacity to thrive even in challenging conditions.

Exploring IAA's potential in the growth dynamics of algae–fungi complexes under stressful conditions

Building upon our findings, it became evident that IAA possesses the capacity to mitigate stress for the algae. With this understanding in mind, we delved into the intriguing question of whether IAA could potentially exert a growth-stimulating influence when algae and fungi unite to form a complex through co-culturing. It is important to underscore that the stress concentrations for this investigation were meticulously selected to represent conditions where algal cells struggle to thrive but fungi exhibit greater resilience. Surprisingly, our efforts to implement IAA pretreatment

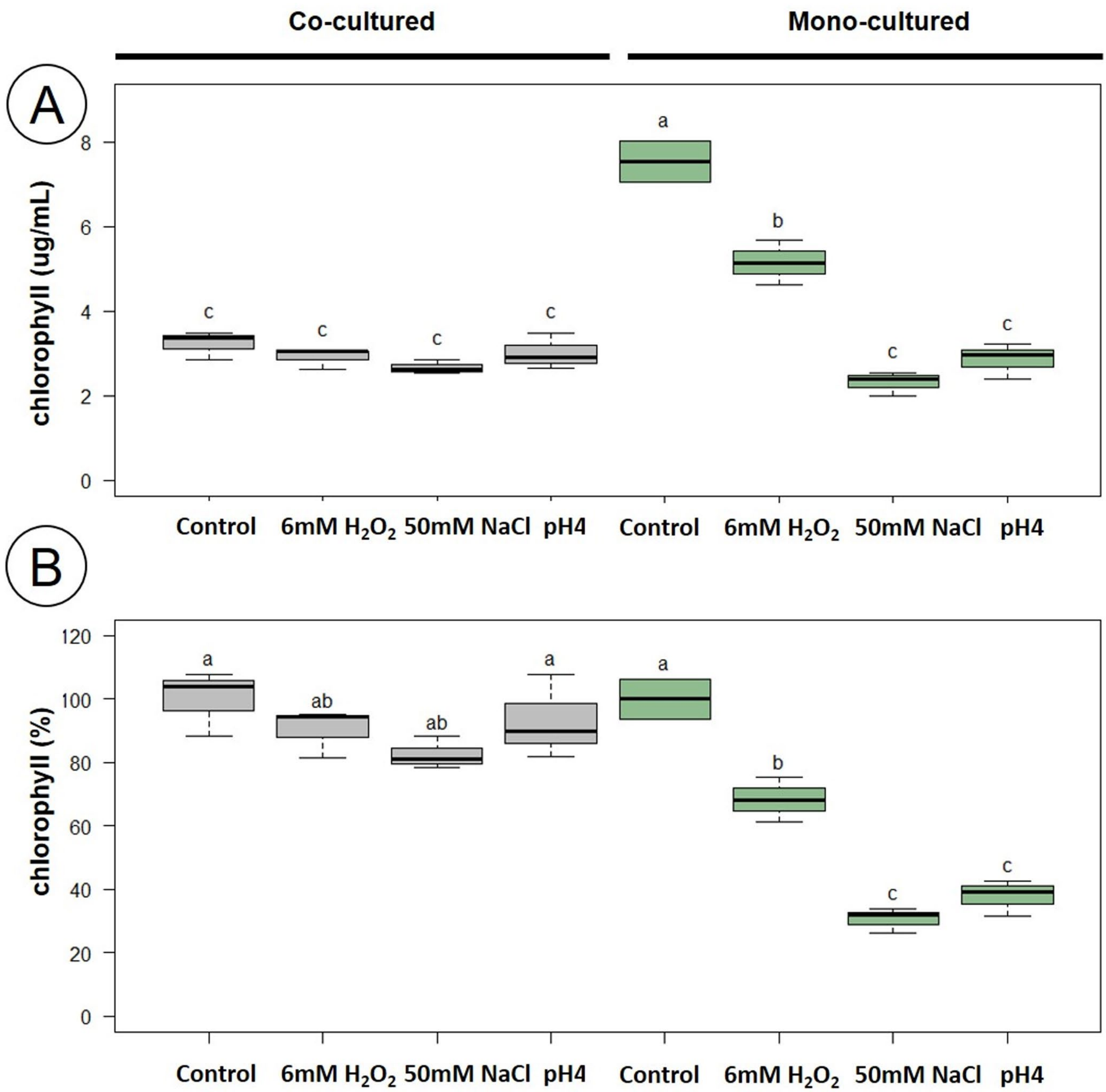

Fig. 7 Comparison of chlorophyll content in mono-cultured and co-cultured algae (*Coelastrella* sp.) under stress conditions. *Coelastrella* sp. were co-cultured with the fungus *Xylaria*. (**A**) Absolute chlorophyll content (μg/mL) under mono-cultured and co-cultured conditions. (**B**) Relative chlorophyll content compared to the control group under mono-cultured and co-cultured conditions. Different lowercase letters indicate significant differences. One-way ANOVA, followed by Tukey's Post Hoc Test, was employed; statistical significance was set at $P < 0.05$

on the co-cultured complex failed to produce the expected stress-alleviating effects or growth-stimulating impacts, as depicted in Fig. 9. This unexpected outcome challenges our initial hypothesis and underscores the complex interplay of factors governing the response of this symbiotic system to external treatments. This outcome can be attributed to the existing protection provided by the fungal hyphae, which effectively shielded the algae from the threat of stress-induced mortality. This defensive mechanism aligns with our earlier observations illustrated in Figs. 7 and 8, wherein the co-cultured complexes maintained their chlorophyll content more effectively. Moving forward, we intend to deepen our understanding of the intricate relationship between IAA and the symbiotic complex under stress conditions. To

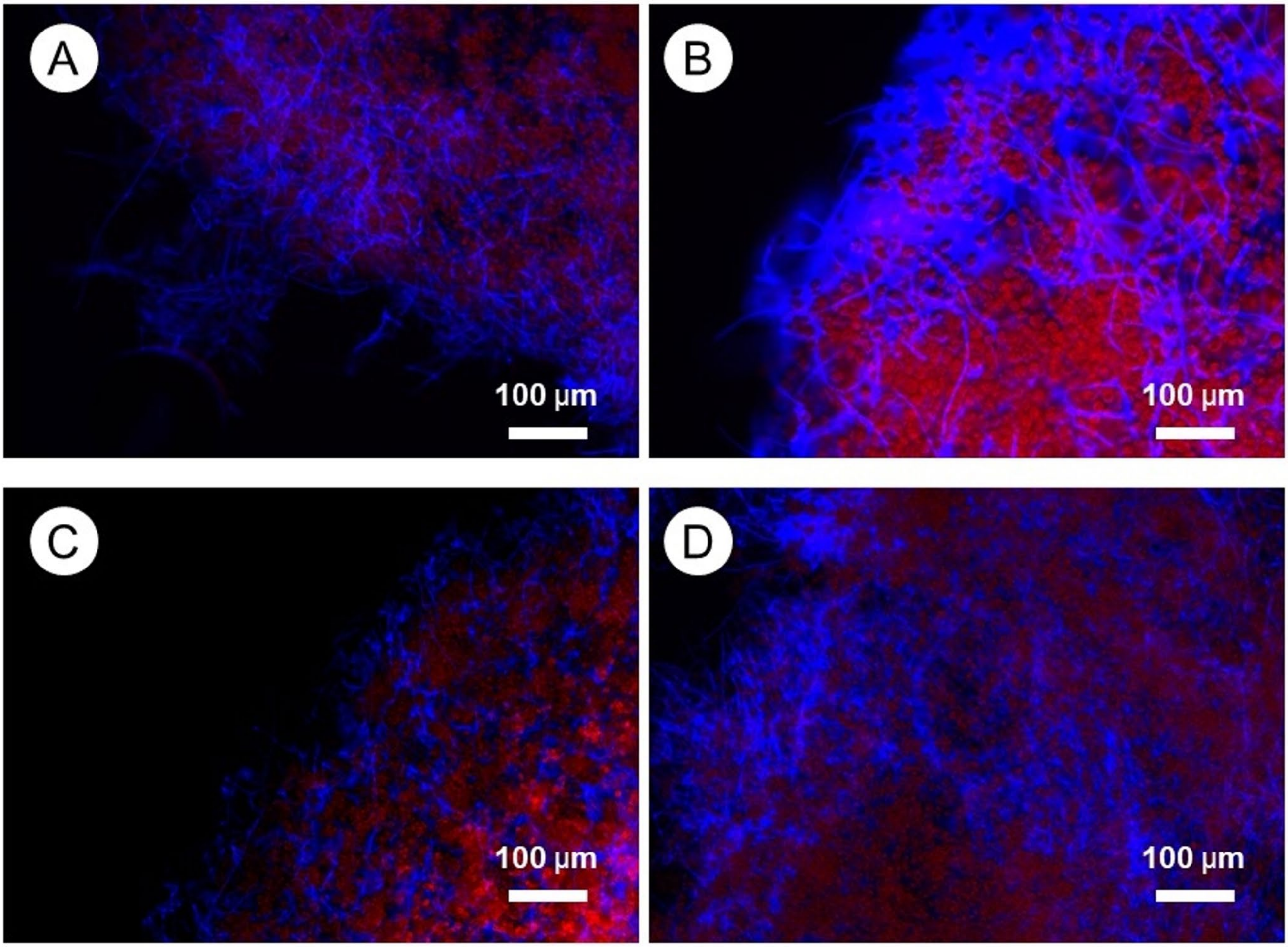

Fig. 8 Fluorescence microscopy images of co-cultured *Coelastrella* (algae) and *Xylaria* (fungus) subjected to various stress conditions for 1 wk. (**A**) Control condition. (**B**) 6 mM H_2O_2 stress condition. (**C**) 50 mM NaCl stress condition. (**D**) pH 4 stress condition. Fungal hyphae were stained with Calcofluor White (CFW), indicated in blue, while the autofluorescence of algal chlorophyll appeared in red

accomplish this objective, conducting further investigations aimed at intensifying the stress levels in the experiments is imperative. This additional research will enable us to gain deeper insights into the response of the symbiotic complex to varying stressors and potentially unveil more nuanced and intricate aspects of its behavior. This strategic approach aims to uncover the nuances of IAA's impact on the complex when subjected to heightened stress levels. By doing so, we hope to unravel the complex interplay between IAA, the co-cultured environment, and stress responses, thus contributing to a more comprehensive understandng of the dynamics of this symbiotic system.

Discussion

The role of bacteria in lichen symbiosis

Symbiotic relationships are ubiquitous in the evolution of life and play a crucial driving role. As we delve into solving many scientific problems using metagenomics technology, we gain deeper insights into the complex interactions and communication between the host and its symbiotic microbial community, consisting of multiple species. In traditional research, ecological data collection has been foundational, aiming to explain long-term interaction relationships between species at the individual level. However, recent breakthroughs in sequencing technology have prompted scientists to shift towards the concept of the holobiont—an inseparable and interdependent entity consisting of the host and its symbiotic microorganisms—to address scientific questions [29]. When the host and its symbiotic

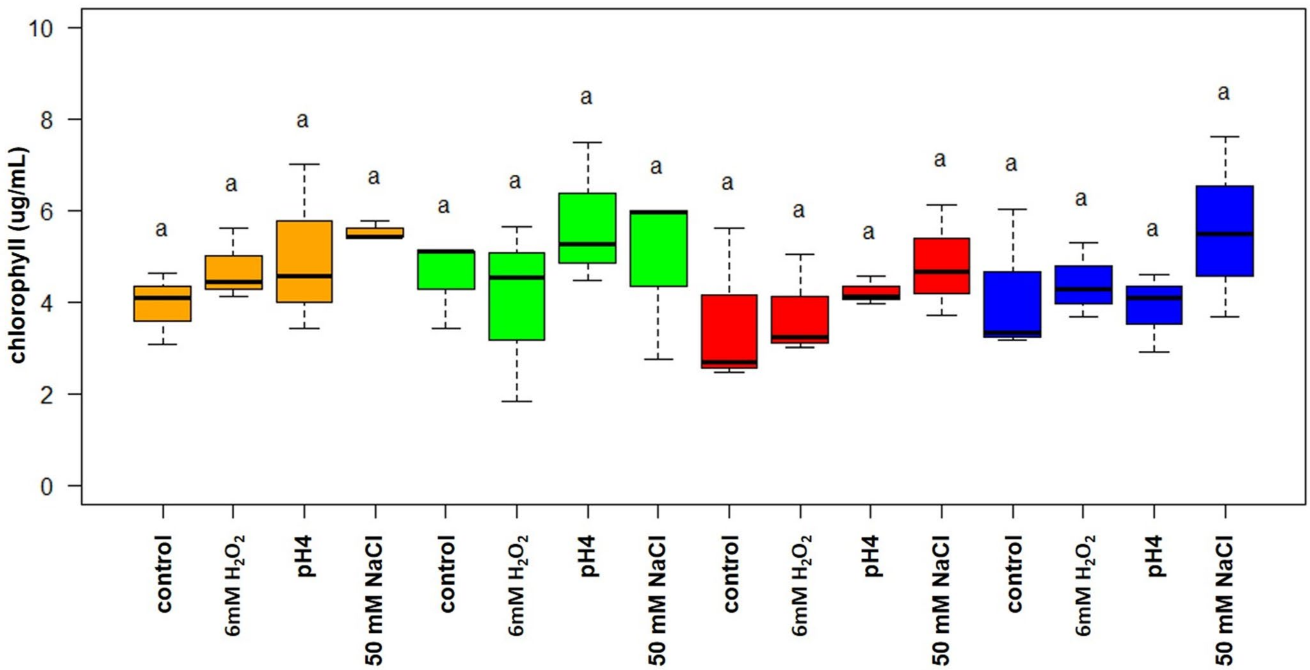

Fig. 9 Effect of indole-3-acetic acid (IAA) on the total chlorophyll of the co-cultured algal–fungal complex under stress conditions. After pre-treating the fungal–algal complex with different concentrations of IAA for 2 d, the symbiotic structures were transferred into stress conditions for 5 d. Each bar represents the mean of triplicate data. The stress conditions included control, 6 mM H_2O_2, 50 mM NaCl, and pH 4. The IAA concentrations used for pre-treatment are depicted in different colors: orange: 0 µM, green: 50 µM, red: 200 µM, blue: 500 µM. Statistical analysis included a one-way ANOVA, followed by Tukey's Post Hoc Test; statistical significance was set at $P < 0.05$

microorganisms are simultaneously exposed to environmental disturbances and selection pressures, the mode of communication between them changes. Adaptation to environmental changes necessitates alterations in both the host's physiological conditions and the composition of the microbial community. Consequently, variations in allelic loci of the microbiota's shared genes manifest as changes in the phenotype of the holobiont. This phenomenon is known as the concept of developmental symbiosis in ecological evolutionary developmental biology (eco-evo-devo) [30].

The phenotypical changes in the holobiont that facilitate its adaptation to environmental selection pressures include alterations in behavior, life history, and/or physiological responses (such as metabolic products). These changes further influence the ability of the holobiont to serve as a source of natural selection in both biotic and abiotic components within its ecological niche. According to the theory of niche construction, a fundamental core theory in current ecology, the mechanism involves feedback between the holobiont and the environment during evolution. This process shapes the co-construction of natural selection and ecological niche, with the interaction extending beyond the relationship between a single species population and the environment. The microbial community within the host plays an indispensable role in this dynamic interaction. This not only provides novel theoretical foundations for studying species adaptability within an evolutionary framework but also offers effective mechanistic models for explaining ecological phenomena across various scales. The holobiont's resilience, characterized by its ability to adapt to the environment, stems from its symbiotic relationship with the microbial community, which serves as an "external gene pool" distinct from the host's nuclear genetic factors [31]. Metaphorically, the communication between the host and symbiotic microorganisms through interspecies signaling is comparable to a symphony, where the conductor (host) and musicians (microbial community) mutually stimulate each other. This orchestration leads to changes in the composition and metabolic responses of microorganisms within the host, enabling the holobiont to perform different movements under varying selection pressures. This capacity allows the holobiont to explore and survive in diverse environments, potentially undergoing Lamarckian evolution, aligning with the concept of developmental plasticity proposed in the eco-evo-devo concept. The theory of coevolution between hosts and their symbiotic microbial communities provides a universal framework for exploring the fields of basic biology and species adaptation. This concept is frequently investigated across various organisms, including humans [32], cnidarians (corals and anemones) [33, 34], and poriferans (sponges) [35].

The exploration of internal bacterial communities within lichens has long been a focal point of research. Investigations into the structure of these bacterial communities have entailed comparisons across various lichen species, revealing intricate and well-organized patterns [36, 37]. Notably, the composition of bacterial communities varies significantly among different lichen species [4, 38, 39]. While many of these communities originate from the parental lichen, they can also be acquired from substrates [40]. It is important to acknowledge that the composition of lichen-associated bacterial communities can be influenced not only by the substrates where lichens thrive but also by climate change [41]. Given the intricate organization of these bacterial communities and their symbiotic relationships, scientific attention has shifted towards elucidating the role of bacteria within the lichen ecosystem. Notably, symbiotic bacteria have been postulated to contribute in various ways. For example, nitrogen fixation emerges as a potential functional role due to the presence of nitrogenase reductase (*nifH*) genes in lichen-associated bacteria [42, 43]. These bacteria also seem to provide micronutrients and cofactors, thereby furnishing essential nutrition and protection to the host [5]. Intriguingly, IAA production has been identified not only in lichens but also in lichen fungi and lichenized bacteria [17, 43, 44]. In a previous study, the application of 1 µM IAA, a concentration released by lichen fungi, led to an increase in the water content of algal cells [17, 45, 46]. Remarkably, our investigation revealed that bacteria isolated from lichen are capable of producing IAA at levels exceeding 50 µM (as depicted in Fig. 3). This heightened IAA production ability exhibited by the bacteria potentially holds benefits for their algal and fungal partners, expanding the spectrum of IAA concentrations. This adaptation might contribute to shaping the intricate dynamics of the lichen ecosystem. The field of symbiotic relationships has led to the construction of several models, such as the algal–fungal and the tripartite fungal–algal–bacterial models [47, 48]. These frameworks serve as valuable tools for delving deeper into the complexities of these interdependent relationships.

IAA's role in algal growth and stress response within lichen ecosystems

Indole and its derivatives, including IAA, are pivotal signaling compounds synthesized by various sources, including mycorrhizal fungi, bacteria, and lichen photobionts [49–53]. Notably, studies have unveiled IAA's influential role in modulating the growth dynamics of fungi, green algae, and plants [9, 54]. For instance, the fungal endophyte *Penicillium roqueforti*, which is capable of producing IAA, enhances stress tolerance and nutrient absorption in wheat plants cultivated in heavy metal-contaminated soils [55]. Similarly, in the context of algae, the exogenous release of IAA by plant growth-promoting bacteria (PGPB) has demonstrated the potential to augment algal growth [56–58]. Notably, the bacterium *Azospirillum brasilense* is capable of producing IAA in response to tryptophan and thiamine released by the alga *Chlorella sorokiniana* during their interaction, thereby fostering communication between bacteria and algae to promote algal growth [59]. The interaction among these three compounds serves as a foundation for enhancing algal growth, and *A. brasilense* further extends its impact by elevating chlorophyll content and mitigating oxidative stress in algae [60]. Interestingly, the growth dynamics of the green alga *Desmodesmus* have exhibited intricate responses to varying IAA concentrations. Paradoxically, higher IAA levels were found to hinder growth, leading to lipid droplet accumulation within algal cells. Notably, optimal growth stimulation was observed at lower IAA concentrations, revealing the nuanced relationship between IAA and algal growth dynamics [22].

In light of these observations, this study sought to elucidate the impact of IAA on algal growth under stress conditions. Our findings highlighted that IAA pretreatment can effectively ameliorate algal doubling time under oxidative stress, while its effects under other stressors remain limited. However, it is important to note that not all oxidative stress concentrations can be mitigated by IAA. Specifically, when algae are subjected to high levels of oxidative stress, the influence of IAA becomes negligible, although a stress-relieving effect is still discernible. IAA's effectiveness in pretreatment appears to be stress-specific, potentially aiding algal cells in early substance accumulation within their structures. The heightened sensitivity of algae to variations in IAA concentration within the lichen environment may prompt adjustments in cell composition or morphology, acting as a pre-emptive defense mechanism against impending stressors.

Exploring algae–fungi interactions and their potential stress resilience mechanisms through co-cultured models

In 2014, Dr. Andrew Murray's laboratory at Harvard University discovered that, under nutrient-limiting conditions, different strains of *Chlamydomonas* algae and Ascomycota fungi engage in mutualistic symbiosis by exchanging carbon and nitrogen sources. Electron microscopy observations revealed direct physical contact between the algae and fungi. Interestingly, the fungal cell wall appeared thinner at the points of contact. However, no intricate structural changes facilitating nutrient exchange between algae and fungi were observed [61]. In 2019, Dr. Christoph Benning's team at Michigan State University co-cultivated the microalga *Nannochloropsis oceanica* and the soil fungus *Mortierella elongata*. Electron micrographs showed algae cells clustering around and adhering to fungal cells. Isotope analysis provided conclusive evidence of carbon and nitrogen transfer between fungi and algae. Remarkably, both organisms remained physiologically active even after several months of co-cultivation. This led to the incorporation of photosynthetic algae into fungal cells, where the algae retained normal growth and division functions [47]. However, neither of these studies utilized lichenic fungi and lichenic algae as materials. In 2019, Dr. Daniele Armaleo's laboratory at Duke University utilized the lichenic fungus *Cladonia grayi* and the lichenic algae *Asterochloris glomerata*. In addition to analyzing changes in their genomes (e.g., contracted and expanded families), the researchers co-cultivated both organisms on a filter paper for 21 d. Transcriptomic analysis of the co-cultured and mono-cultured samples revealed increased expression of genes related to fungal secretion proteins, carrier proteins, signaling factors, extracellular hydrolases, ribitol transporters, and ammonium transport proteins in the co-cultured samples. In algae, genes related to DNA metabolism, signaling factors, and flagellar structure showed increased expression. Analysis of the genomic data further revealed commonly expanded protein families, such as signaling factors, ankyrin domain-containing proteins, and transcription factors associated with chromatin remodeling complexes and stress responses. On the contrary, contracted families comprised transport proteins and proteins associated with nitrate assimilation [62]. In 2020, Dr. Mieko Kono's laboratory at the Swedish Museum of Natural History used the lichen *Usnea hakonensis*, successfully co-cultivating its symbiotic fungi and algae (*Trebouxia* sp.) in vitro. After three months of co-cultivation, they observed the development of structures resembling leaves. Subsequent transcriptomic analysis to understand the gene expression in the fungal–algal structures revealed high expression levels of genes involved in modifying the cell wall at the contact site between fungi and algae, as well as genes responsible for producing a hydrophobic layer enveloping the fungal and algal cells. In terms of nutrient exchange, they observed high expression levels of genes related to photosynthesis, provided by algae to fungi, and genes related to phosphorus and nitrogen compounds, supplied by fungi to algae [63]. However, this study did not provide electron micrographs illustrating the physical contact and microstructure between fungi and algae in the leaf-like structures. Furthermore, the involvement of symbiotic bacteria in the symbiotic and nutrient synthesis processes was not clarified. Importantly, the transcriptomic analysis occurred weeks to three months after co-cultivation, potentially overlooking crucial genes involved in the initiation of symbiosis.

The establishment of a co-cultured model has paved the way for a comprehensive exploration of symbiotic interactions within lichen ecosystems. Through cultured experiments, lichenized structures were intentionally formed to delve into the preliminary stages of lichen development, offering insights into the nascent processes of lichen formation [64]. This approach has unveiled intriguing associations, such as the co-cultivation of various algae from lichen with the lichenized fungus *Schizoxylon*, and these associations have been examined through SEM [65]. In co-cultures with *Coccomyxa*-like algae, the fungus often enveloped the algal colonies with a delicate white mat of hyphae. Microscopic examination, specifically SEM analyses, revealed the close adherence of fungal hyphae to the algal cells. Conversely, *Schizoxylon albescens* did not exhibit any noticeable interaction with the other tested photobionts. Cultures containing *Trebouxia* sp., *Trentepohlia* sp., and *Chlorella*-like photobionts showed no evidence of interaction between the fungus and the algae. The current study represents an advancement, as algae and fungi isolated from filose lichen and lichen-like structures were united to form distinctive spherical structures, featuring unique attachments. The connection observed between *Coccomyxa* and fungi, facilitated by a sticky substance, mirrors the lichenoid structures previously identified [66]. Nevertheless, further investigations are necessary for comprehending the specific interactions between algal cells and fungal hyphae and their potential role in stress resilience. An upcoming strategy involves co-cultivating these components in diverse configurations, including pairing different free-living algal cells with lichenic fungi or various free-living fungi with lichenic algae, to discern the specificity involved in the formation of lichenoid structures. This avenue of investigation promises to unveil the exact dynamics that facilitate symbiotic partners to jointly combat stressors.

Conclusion

Our findings regarding the role of IAA in lichen symbiosis revealed that lichens are not merely passive bystanders in their environment but rather active participants capable of sophisticated responses to stressors. The symbiotic relationship between fungi and algae/cyanobacteria within lichens has long fascinated scientists. Our study adds depth to this understanding by highlighting the significance of chemical signaling, such as via IAA, in mediating responses to environmental challenges. Furthermore, the discovery of IAA's involvement in enhancing stress resistance underscores the potential for manipulating these signaling pathways to bolster the resilience of lichens in the face of environmental disturbances, such as climate change and pollution. This could have implications not only for the conservation of lichen diversity but also for broader ecological restoration efforts. Moreover, our study underscores the importance of considering the role of associated bacteria in lichen communities. While much attention has been focused on fungal–algal symbiosis, the contribution of bacteria to lichen ecology and biology is increasingly being recognized as significant. Further exploration of bacterial functions within lichens may help uncover novel mechanisms of symbiotic interaction and provide insights into the broader microbial ecology of these fascinating organisms. Looking ahead, interdisciplinary research integrating molecular biology, ecology, and biotechnology will be crucial for unlocking the full potential of lichens as model systems for studying symbiosis and developing innovative strategies for environmental stewardship. By delving deeper into the molecular mechanisms underpinning lichen resilience and exploring the intricate web of interactions within lichen communities, we can gain valuable insights into the adaptability of these organisms and their broader ecological significance.

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Declarations

Conflict of interest The authors declare no competing financial or non-financial interests.

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