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ORIGINAL ARTICLE

Production of the bioactive pigment elsinochrome A by a cultured mycobiont strain of the lichen *Graphis elongata*

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Abstract

We report the production of the perylenequinone pigment elsinochrome A in aposymbiotic culture of the mycobiont of the crustose epiphytic lichen *Graphis elongata* Zenker (Lecanoromycetes), collected in Argentina (Buenos Aires). The substance was not detected in the lichenized thallus (using HPLC techniques) and is otherwise only known from one unrelated lichen and a few genera of non-lichenized, plant-pathogenic fungi in the class Dothideomycetes. The phylogenetic affinities of the lichen mycobiont and the cultured fungus were confirmed using DNA sequence data of the mitochondrial small subunit rDNA (mtSSU), which place the lichen fungus into the *Allographa* clade within Graphidaceae. The mycobiont pigment was purified and characterized by spectroscopic methods. This is the first case where a rare pigment, otherwise known from non-lichenized, plant-pathogenic fungi, is produced in aposymbiotic culture of a lichen mycobiont, while, at the same time, being absent from the lichen thallus itself. Based on this finding, we discuss the previously postulated hypothesis that lichen mycobionts maintain secondary metabolic pathways of non-lichenized ancestors in their genome, while gene expression and production of metabolites is suppressed in the lichenized state due to toxicity to the photobiont.

Keywords Perylenequinones · Axenic culture · Elsinochrome A · *Graphis elongata* · Lichen

Introduction

Lichens are symbiotic associations between a fungus and a photosynthetic partner (Mercado-Díaz et al. 2015). They frequently produce a diversity of fungal secondary metabolites. More than 800 substances have been described in detail (Culberson and Culberson 2001; Huneck 2001; Huneck and Yoshimura 1996) and probably more than 1000 are currently known (Stocker-Wörgötter et al. 2013). Many of these are unique to lichenized fungi, but about 60 have also been reported from other fungi or higher plants (Elix and Stocker-Wörgötter 2008; Stocker-Wörgötter 2008). Some of the functions that have been proposed for these secondary lichen compounds are to absorb UV light, protecting the photobionts against intensive irradiation, and to defend thalli from bacteria and viruses and against fungivores and herbivores (Lawrey 1984; Nimis and Skert 2006; Varol et al. 2015).

Lichens are considered a promising source of pharmaceutically useful chemicals because they produce a diverse array of secondary metabolites, many of them biologically active (Yamamoto 2000; Müller 2001; Takahashi et al. 2005; Niu et al. 2007; Studzińska et al. 2008; Muggia et al. 2009; Ranković 2015). Their potential applications, however, are

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limited by their finite sources and the slow-growing nature of lichens. Aposymbiotically cultured mycobionts frequently have the ability to produce diverse substances that are not detectable in the lichen voucher specimen but are structurally related to fungal metabolites (Takenaka et al. 2011). Aposymbiotic mycobiont cultures in the laboratory help to search for fast-growing strains producing high yields of bioactive metabolites, to obtain pure substances for biological activity assays or PKS genes for cloning, and to be expressed in well-defined cell factories, like *Aspergillus* spp. (Elix and Stocker-Wörgötter 2008).

Pigments, such as anthraquinones, dibenzofurans, and vulpinic acids, are amply represented in lichenized fungi; some of them, for example emodin, possess notable biological activities (Izhaki 2002; Lin et al. 2012). Pigments present in the lichen thallus have also been reported from aposymbiotic mycobionts in culture (Ahmadjian 1993; Brunauer et al. 2007; Fazio et al. 2012; Huneck and Yoshimura 1996).

Widespread and common lichen pigments, such as the anthraquinone parietin, graphenone, and graphisquinone, were produced by multisporic mycelia only and were not detected in the thalli of the lichens from which the cultures were derived (Miyagawa et al. 1994; Hamada et al. 2001). Hamada et al. (2001), therefore, postulated that these mycobionts preserve secondary metabolic pathways of free-living fungi from the pre-symbiotic age, which are then expressed in aposymbiotic culture, but suppressed in the lichenized state due to the toxicity of these compounds to the photobionts.

In the present study, we report the production of the perylenequinone toxin elsinochrome A (**1**) (Fig. 1) in aposymbiotic culture of a lichen mycobiont of the crustose epiphytic lichen *Graphis elongata* Zenker (Lecanoromycetes). Most members of the perylenequinone family fall into three classes: (a) C_{20} compounds without carbon substituents, (b) mold perylenequinones containing carbon substituents, and (c) perylenequinones from aphids. Since the discovery of cercosporin (**2**) (Fig. 1) more than 50 years ago (Kuyama and Tamura 1957), more than 20 perylenequinones have been isolated as red pigments from a variety of fungi within the Ascomycota (Weiss et al. 1987; Ahonsi et al. 2006). All of the fungal perylenequinone toxins that have been characterized are produced by Ascomycota, commonly by fungal plant pathogens, but also by some saprotrophic species (Daub and Chung 2009). They are believed to play a key role in the chemical interactions between plant-parasitic fungi and their hosts (Daub et al. 2005; Daub and Chung 2009). Fungal perylenequinone toxins are able to absorb light energy and react with oxygen molecules to produce highly toxic reactive oxygen species (ROS), such as superoxide and singlet oxygen, and cause oxidative damage (Daub et al. 2005). The best studied fungal perylenequinone toxins are the red pigments cercosporin and various hypocrellines and elsinochromes, obtained mostly from pathogenic fungi (Daub and Chung 2009). Photosensitizing has multiple biological effects and potential applications, as shown by the photodynamic inhibitory effects of elsinochrome A and hypocrellins A and B on carcinoma cells via inducing apoptosis (Ma et al. 2003, 2004; Li et al. 2006; Stergiopoulos et al. 2013).

Elsinochrome A is known from non-lichenized plant pathogenic fungi in the genera *Elsinoë* (Dothideomycetes: Myriangiales) and *Stagonospora* (Dothideomycetes: Pleosporales; Weiss et al. 1965; Chen et al. 1966; Ahonsi et al. 2006; Boss et al. 2007; Liao and Chung 2008a, b; Stergiopoulos et al. 2013), as well as from the lichen fungus *Astrothelium purpurascens* (Müll. Arg.) Aptroot & Lücking (syn.: *Cryptothelium rhodotitthon* R.C. Harris; Dothideomycetes: Trypetheliales; Mathey and Lukins 2001). Its structure and biosynthesis have been well studied (Weiss et al. 1965; Chen et al. 1966; Meille et al. 1989; Mebius et al. 1990; Lousberg et al. 1970; Cao et al. 2000), and its bioactive properties and potential applications are well documented, including experimental approaches (Weiss et al. 1957; Ahonsi et al. 2005, 2006; Chen et al. 2003; Zhang et al. 2009; Boss et al. 2007; Wang and Zhang 2005; Favilla et al. 2006; Li et al. 2015). The dark red pigment here produced in culture by the axenically grown aposymbiotic mycelia of *Graphis elongata* was purified and characterized by means of spectroscopic methods. We also obtained DNA sequences of the mitochondrial small subunit (mtSSU) and the nuclear large subunit (nuLSU) of both the mycobiont of the lichen thallus and the axenically grown culture. This enabled us to confirm the identity of the mycobiont culture and place the results in a phylogenetic context.

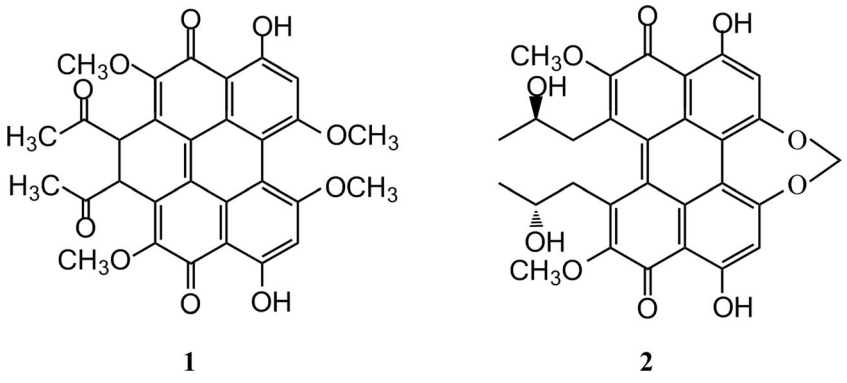

Fig. 1 Chemical structures of elsinochrome A (**1**) and cercosporin (**2**)

Materials and methods

Lichen sample collection

Thalli of *Graphis elongata* were collected on a single tree of *Pinus* sp. by A.T. Fazio and V. Avanzatto at Lucila del Mar, Buenos Aires Province, Argentina, on November 20, 2004. Reference specimens are preserved at BAFC (39315), with duplicate at F.

Isolation and culture of the aposymbiotic mycobiont strain

The isolation procedure followed that of Hamada (1989) to obtain polyspore colonies from discharged ascospores. Superficially clean lichen material was collected and processed after 3 days, cleaned under a dissecting microscope, washed with tap water (1 h) and subsequently with sterilized tap water with 2% Tween 20 (1 h). Finally, between 100 and 150 ascomata were selected under the dissecting microscope from four individual thalli with the anatomical and morphological characteristics typical of *Graphis elongata* (Lücking et al. 2009), cut off, blotted with sterilized filter paper, and fixed onto the top of a Petri dish by means of petroleum jelly, to let the spores discharge upwards onto the solid mineral Bold's basal medium, BBM (Deason and Bold 1960), after turning the dish upside down. The germinated spores were transferred onto fresh solid BBM until small (1 mm diameter) colonies were obtained after growing them in a growth chamber at 23 °C. The colonies were transferred onto fresh solid BBM several times over 2 months and preserved (BAFC cult. 3945) on this same medium.

After growth on BBM, 4-mm diameter pieces of colonies were transferred onto the following solid media (percentages refer to the final volume of each medium):

(1) BBM + glucose 1% (1% glucose, 1.8% agar, in liquid Bold's basal medium).
(2) LB, original Lilly and Barnett medium (Lilly and Barnett 1951; Ahmadjian 1993) with glucose 1% as carbon source and asparagine 0.2% as nitrogen source, agar 1.8%, in distilled water.
(3) MME, modified malt extract (Leuckert et al. 1990), malt extract 1.5%, glucose 2%, peptone 0.1%, agar 1.8% in distilled water.
(4) BMYE, 2% mannitol, 0.1% yeast extract, 1.8% agar in liquid Bold's basal medium.
(5) MEYE (Ahmadjian 1993), malt extract 2%, yeast extract 0.2%, agar 1.8% in distilled water.
(6) MY10 (Hamada 1989), malt extract 1%, yeast extract 0.4%, sucrose 10%, agar 1.8% in distilled water, final pH 7.
(7) BMRM, bold mannitol-rich medium (malt extract 1%, yeast extract 0.4%, mannitol 5.3%, agar 1.8% in liquid Bold's basal medium, final pH 7). This medium was developed in our lichenological laboratory, partly based on the composition of Hamada's MY10 medium, looking for the same molarity of the carbon sources (mannitol in BMRM and sucrose in MY10).

Morphological and microscopic observations

The morphology of axenically grown subcultures of the mycobionts developed over 5 months on these media was assessed. Growth and red pigment production of colonies was evaluated by examination under a dissecting microscope (ZEISS Stemi SR). Growth of 5-month-old mycelia was characterized using the following code: ± (poor growth), with most colonies expanded to less than 2 mm diameter during that time; + (low), expansion to 2–4 mm diameter; ++ (good), expansion to 5–9 mm diameter; and +++ (very good), expansion to 10–15 mm diameter. Observations showed that BMYE medium promoted red pigment production, as was confirmed by TLC. Therefore, mycelia grown on BMYE medium were harvested after 5 months of growth for chemical studies.

Pieces of the surface of colonies grown on BMYE were taken and mounted in distilled water to observe the red pigment in the hyphae and to obtain photographs with a ZEISS Axioscope optical microscope.

DNA extraction and molecular identity of the cultured mycobiont

New sequences were generated for this study from both the lichenized thallus and the isolated mycobiont using the Sigma-Aldrich REDExtract-N-Amp Plant PCR Kit (St. Louis, MO, SA) for DNA isolation following the manufacturer's instructions, except that 40 μL of extraction buffer and 40 μL of dilution buffer were used. DNA dilutions (5×) were used in PCRs of the genes coding for the nuLSU and mtSSU. Primers for amplification were: (a) for nuLSU: AL2R (Mangold et al. 2008) and nu-LSU-1125-3′ (= LR6) (Vilgalys and Hester 1990), and (b) for mtSSU: mr-SSU1 and Mr-SSU3R (Zoller et al. 1999). PCRs contained 5.0 μL of R4775 Sigma REDExtract-N-Amp™ PCR ReadyMix, 0.5 μL of each primer (10 μM), 2 μL of genomic DNA extract, and 2 μL of distilled water, for a total of 10 μL. The thermal cycling parameters were: (1) for nuLSU: initial denaturation for 5 min at 94 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 58 °C, 1 min at 72 °C, and a final elongation for 10 min at 72 °C; and (2) for mtSSU: initial denaturation for 5 min at 95 °C, followed by 35 cycles of 45 s at 94 °C, 1 min at 50 °C, 1 min 30 s at 72 °C, and a final elongation for 10 min at 72 °C. Samples were visualized on a 1% ethidium bromide-

stained agarose gel under UV light and bands were gel extracted, heated at 70 °C for 5 min, cooled to 45 °C for 10 min, treated with 1 μL of GELase (Epicenter Biotechnologies, Madison, WI), and incubated at 45 °C for at least 24 h. The 10-μL cycle sequencing reactions consisted of 1–1.5 μL of Big Dye version 3.1 (Applied Biosystems, Foster City, CA), 2.5–3 μL of Big Dye buffer, 6 μM primer, 0.75–2 μL gelased PCR product, and water. Samples were sequenced with PCR primers. The cycle sequencing conditions were as follows: 96 °C for 1 min, followed by 25 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min. Samples were precipitated and sequenced using an Applied Biosystems 3730 DNA Analyzer (Foster City, CA), and sequences were assembled in SeqMan 4.03 (DNASTAR) and submitted to GenBank [MG775653, MG775654, MG775657, MG775658].

Additional sequences of *Graphis* s. lat. (including *Allographa*) were downloaded from GenBank following the study by Rivas Plata et al. 2011). GenBank accession numbers are given in the tree figure (Fig. 2) and voucher information is available in Rivas Plata et al. (2011). All sequences were arranged into multiple sequence alignments (MSA) for each gene using BioEdit 7.09 (Hall 1999) and automatically aligned with MAFFT using the –auto option (Katoh et al. 2005). The unaligned MSA were also submitted to the GUIDANCE web server at http://guidance.tau.ac.il to assess alignment confidence scores for each site (Penn et al. 2010a, b). GUIDANCE uses an MAFFT alignment and returns a colored MSA that allows delimiting ambiguously aligned portions of the MSA. These were then excluded from further analysis. After testing for supported topological conflicts (Mason-Gamer and Kellogg 1996; Miadlikowska and Lutzoni 2000; Kauff and Lutzoni 2002), the two genes were combined into a single dataset. The combined dataset was subjected to maximum likelihood search using RAxML-HPC BlackBox 7.3.2 on the CIPRES Science Gateway server (Stamatakis 2006; Stamatakis et al. 2005; Miller et al. 2010), with parametric bootstrapping generating 350 replicates as automatically determined by RAxML using a saturation criterion. The universal GTR-Gamma model was chosen for the analysis.

Extraction of lichen and colonies

Air-dried lichen material (lirellae and pieces of scraped thallus) of *Graphis elongata* was extracted three times with acetone at room temperature, and then with ethyl acetate for 1 week. After evaporation to dryness under vacuum, the extract was analyzed by TLC using the following solvent systems: toluene–AcOH (170:30), toluene–EtOAc–formic acid (139:83:8), cyclohexane–diethyl ether–formic acid (26:16:1), and chloroform–MeOH (90:10) (Culberson and Ammann 1979; Orange et al. 2001).

In order to obtain the necessary amount of elsinochrome A for chemical studies, mycobiont mycelia from three cultures from the same strain grown on BMYE for 5 months were harvested. Each culture was extracted three times with acetone at room temperature, and then with ethyl acetate for 1 week. After evaporation to dryness under vacuum, the three extracts were analyzed by TLC using the same solvent systems as for the lichen extract, comparing them with the extract from the lichen.

Purification of colonies extracts and analysis by HPLC-DAD

Purification of the three acetonic/EtOAc extracts of the colonies was achieved by column chromatography (Sephadex LH-20, MeOH) and preparative TLC [polyamide, MeOH-formic acid (98-2)]. Pure red crystals of elsinochrome A were obtained from all the three extracts.

Lichen extract (1.8 mg) and pure elsinochrome A (0.3 mg) were dissolved in MeOH (0.5 and 0.1 mL, respectively) and analyzed by HPLC-DAD. Analytical HPLC was carried out on a Gilson 506C HPLC system using a Phenomenex Hypersil 5 μm column (25 cm × 4.6 mm i.d.). Compounds were detected using a 170 photodiode array detector set at 245 and 254 nm, operated in series with UniPoint System software, recording the absorption spectrum in the range 200–400 nm. Gradient elution was performed using two solvents: A: MeOH and B: 1% (v/v) aqueous orthophosphoric acid. The gradient started with 30% A for 5 min and was raised to 70% A within 15 min, then to 100% within 45 min, followed by 10 min at this condition. Solvents utilized in the HPLC were filtered through a 0.45-μm nylon filter prior to use. The samples solutions were filtered through 0.2-μm inorganic membrane filters prior to injection. The flow rate was 1.0 $mL.min^{-1}$.

Spectroscopic characterization of elsinochrome A

^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ on a Bruker AM-500 spectrometer. HR-ESIMS (positive ion mode) was obtained on a Bruker Daltonics micrOTOF-Q II mass spectrometer.

Elsinochrome A: red crystals. HR-ESIMS (positive mode): m/z 567.12695 $[M + Na]^+$, $C_{30}H_{24}O_{10}Na$ (567.12672 calcd.), EI-MS: m/z 544 (M^+, 100%), 501, 459, 444, 429, 415, 401. ^{1}H NMR (500 MHz, $CDCl_3$) δ = 2.04 (6H, s, CH_3-15, CH_3-16), 4.08 (6H, s, OCH_3-7, OCH_3-8), 4.36 (6H, s, OCH_3-3, OCH_3-12), 5.19 (2H, s, H-1, H-2), 6.63 (2H,s, H-6, H-9). ^{13}C NMR (125 MHz, $CDCl_3$) δ = 28.0, 48.7, 56.5, 61.1, 102.5, 107.8, 118.7, 121.6, 122.8, 130.3, 150.1, 167.5, 172.0, 179.9, 204.9.

Results

Phylogeny and taxonomy

The DNA phylogeny of both the lichen mycobiont (extracted from the thallus) and the cultured mycobiont showed that both were identical (Fig. 2) and represent a species of *Graphis* s. lat. falling into the *Allographa* clade (Rivas Plata et al. 2011). The morpho-anatomical features of this species agree with *G. elongata*, a species originally described from southern Brazil (Lücking et al. 2009) (Fig. 3a).

Growth and morphology of mycobiont colonies and pigment localization on hyphae

On BBM and BBM + glucose 1%, mycelia were cottony and white, with poor growth (±), whereas on MME, no growth could be observed. Development was satisfactory on LB, BMYE, MEYE (++ for all three of them), MY10, and BMRM (+++ for both of them). However, the red pigment was mainly produced on BMYE and LB media, although colonies grown on the first medium were of a darker red color (Fig. 3b, c). When grown on BMYE, 5-month-old mycobiont colonies were irregularly conical (Fig. 3c). Colonies grown on MEYE, MY10, and BMRM were light pink over the first 2 months, then turning yellowish ochre. The pigment production in these media was too low for purification.

BMYE was chosen to grow colonies for the chemical analysis because of better pigment production, as confirmed by TLC. The largest, most well-developed colonies on BMYE were mostly about 1.5 mm wide and 0.8–1.0 mm high (Fig. 3c). Sometimes, pigment crystals were observed on the medium surface (Fig. 3d). Microscope observations indicated that the red pigment accumulated inside the hyphae (Fig. 4), and, eventually, their walls became disrupted and the pigment crystals spread outside.

HPLC-DAD analysis and pigment characterization

TLC and HPLC analysis of the lichen extract did not detect any chemical compound. The red pigment present in mycelial extracts was analyzed by HPLC-DAD, showing one peak at R_t = 32.4 min whose UV spectrum (459, 528, and 568 nm) was coincident with that of elsinochrome A (Ma et al. 2003; Ahonsi et al. 2006). The red pigment, after purification of the three extracts of the colonies, was obtained with an average yield of 1.30%. The pure compound was identified by HR-ESIMS (positive ion mode) and comparison of their mass spectrum (EI) and ^{1}H and ^{13}C NMR spectra with bibliographic data (Drogies 1997).

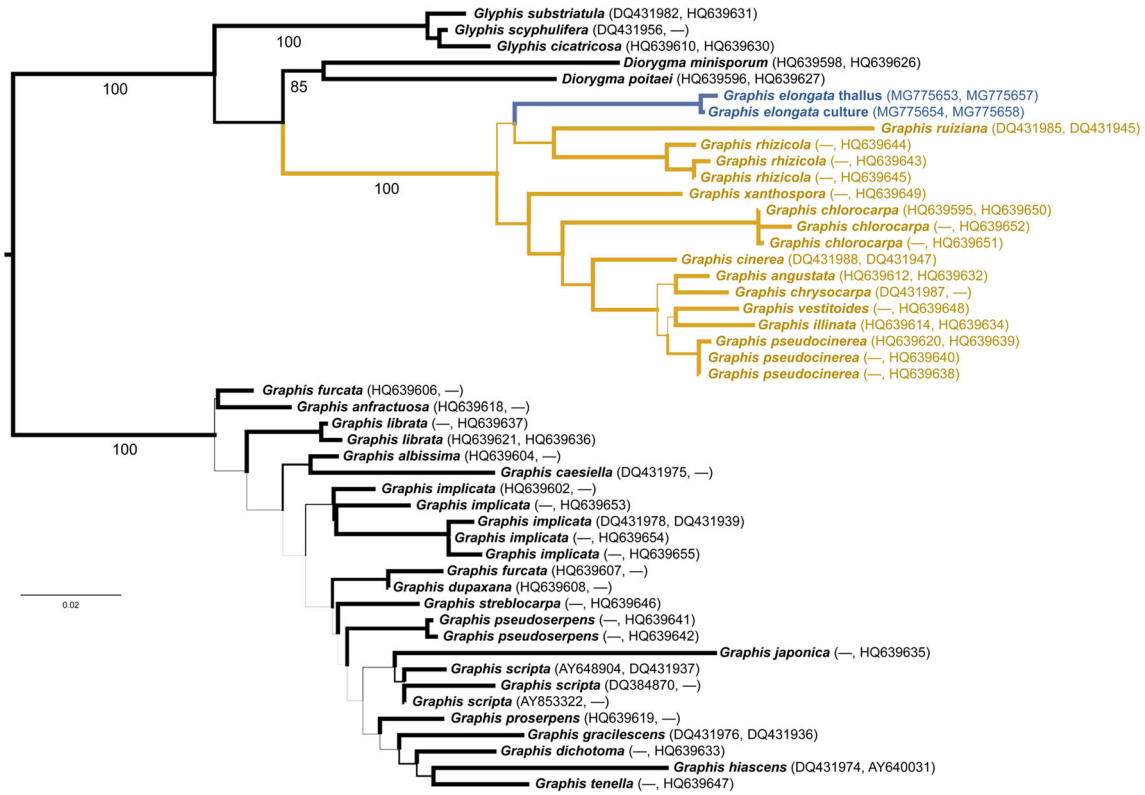

Fig. 2 Maximum likelihood tree of sequences of *Graphis* s. lat. (*Graphis* s. str. and *Allographa*), with *Allographa* (ochre) outgroups *Glyphis* and *Diorygma*, showing the phylogenetic placement of target sequences of the studied material (blue). Thickened branches indicate bootstrap support of 70% and higher, with exact values given for main branches

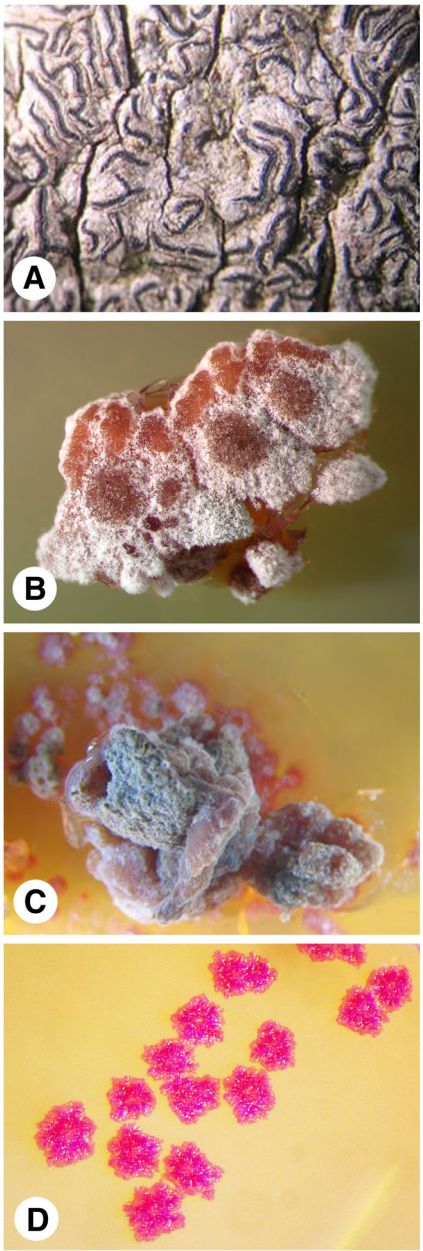

Fig. 3 **a** Thallus of the studied *Graphis elongata*. **b** Cultured mycobiont after 2 months' growth on LB, used for phylogenetic analysis. **c** Cultured mycobiont 5 months old, grown on BMYE, pigmented dark red. **d** Pigment crystals formed on BMYE medium (enlarged). Scale bars: **a** = 1 mm; **b**, **c** = 5 mm; **d** = 0.5 mm

Discussion

We report here for the first time the production of elsinochrome A by a cultured lichen mycobiont and the second report of this compound for lichenized fungi overall, the first being from the lichenized fungus *Astrothelium purpurascens* (Müll. Arg.) Aptroot & Lücking (syn.: *Cryptothelium rhodotitthon* R.C. Harris; Mathey and Lukins 2001). This is also the first report of elsinochrome A outside the class Dothideomycetes, in the unrelated class Lecanoromycetes. Red pigments are known

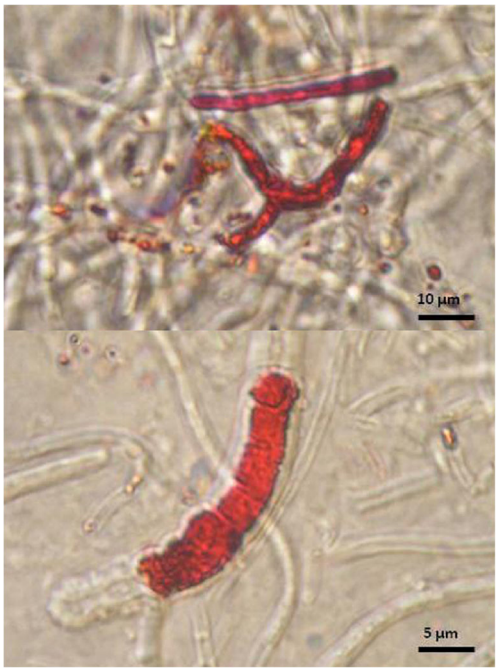

Fig. 4 Cultured mycobiont hyphae containing pigment internally

from several tropical crustose lichens of the families Graphidaceae, Pyrenulaceae, and Trypetheliaceae. However, perylenequinone pigments had not been included in the list of lichen substances until recently, when two of these compounds were analyzed for two species. Isohypocrellin was identified on the lirellae of the crustose lichen *Graphis haematites* Fée, now classified in the genus *Phaeographis* (Mathey 1986; Mathey et al. 1994; Mathey and Lukins 2001; Staiger 2002; Huneck and Yoshimura 1996), and elsinochrome A was found covering the tiny red warts emerging from vegetative parts in *Cryptothelium rhodotitthon* Harris (Mathey and Lukins 2001), now correctly named *Astrothelium purpurascens* (Müll. Arg) Aptroot & Lücking. Isohypocrellin has now been documented for several other species in the Graphidaceae, such as *Cruentotrema cruentatum*, *Graphis isohypocrellina*, *Graphis persicina*, and *Thecaria montagnei* (Staiger 2002; Lücking et al. 2008, 2009; Rivas Plata et al. 2012), while elsinochrome A has not yet been reported from any other lichen. In contrast to these species, in *Graphis elongata*, the pigment elsinochrome A was only detected in aposymbiotic culture, but not in the lichen thallus itself. Graphidaceae lichens are rather well studied chemically, including over 2000 species and thousands of specimens (e.g., Wirth and Hale 1963, 1978; Hale 1974, 1978, 1981; Staiger 2002; Frisch et al. 2006; Lumbsch et al. 2014), and in no instance was elsinochrome A detected in a lichenized thallus.

However, the presence of rare pigments in aposymbiotic culture was previously published for other species of *Graphis*: the novel compounds graphenone in several strains derived from *G. scripta* and graphisquinone in a strain derived from *G. desquamescens* (Miyagawa et al. 1994; Hamada et al.

2001). Although these authors did not perform molecular analysis to confirm the identity of their fungal cultures, our results suggest that the production of rare pigments in aposymbiotic culture of *Graphis* mycobionts may be a taxonomically widespread phenomenon in this group of lichenized fungi: *G. desquamescens* and *G. scripta* belong in the *Graphis* clade but are not closely related to each other, whereas *G. elongata* belongs in the *Allographa* clade (Rivas Plata et al. 2011). The occurrence of elsinochrome A, a well-studied substance with phytotoxic properties, both in plant-pathogenic fungi and in axenic culture of an unrelated lichen fungus poses questions about the biological capabilities of lichen fungi when occurring in the non-lichenized stage, such as immediately after ascospore germination.

The observation that certain rare or unique pigments detected in lichen mycobiont cultures are apparently suppressed in the lichenized condition was related to their potential toxicity to lichen photobionts (Hamada et al. 2001). Indeed, in cases where perylenequinones are found in lichens, such as elsinochrome A in *Cryptothelium rhodotitton* and isohypocrellin in *Cruentotrema cruentatum*, *Phaeographis haematites*, and *Thecaria montagnei*, the pigments are invariably restricted to ascomata, not in proximity to photobiont cells (Mathey and Lukins 2001; Staiger 2002; Rivas Plata et al. 2012). However, this phenomenon appears to be photobiont-specific, since all these lichens associate with *Trentepohlia* (Nelsen et al. 2011), whereas in a recently described species of *Sticta* with a *Dictyochloropsis* photobiont, the vegetative thallus produces the pigment isohypocrellin in low quantities in the thallus cortex close to the algal layer (Moncada and Lücking 2012).

The broad toxic effects of elsinochrome A, a compound known from pathogenic fungi in the genera *Elsinoë* and *Stagonospora*, has been well demonstrated (see also above): it exhibits photodynamic antibacterial effects (Towers et al. 1997), particularly against Gram-positive bacteria (Zhang et al. 2006), and it is also toxic to *Pseudomonas syringae*, cultured citrus and tobacco cells (Liao and Chung 2008a, b; Chung 2011), the protozoan *Tetrahymena pyriformis* (Skrobek et al. 2006), the crustacean *Artemia salina*, and the cladoceran *Daphnia magna* (Favilla et al. 2006). Additional bioactivities reported were a nematicidal effect (Dong et al. 2001) and antifungal activity against *Alternaria mali* (Xing et al. 2003). Theoretically, elsinochrome A and other perylenequinones, when present in lichens, could defend reproductive structures against herbivory, as proposed for other lichen substances (Lawrey 1984; Gauslaa 2009); however, such an effect would contradict a phytotoxic effect on the lichen photobiont itself. Nevertheless, such substances could be present in early stages of free hyphae to protect the germination mycelia prior to lichenization.

Considering the scarce reports of perylenequinones in lichens, the discovery of elsinochrome A in aposymbiotic culture of a randomly collected species of *Graphis*, one of the largest lichen genera known with close to 400 species (Lücking et al. 2009; Bárcenas Peña et al. 2014), suggests that a much larger number of lichen fungi might be capable of producing such substances exclusively in axenic culture under certain conditions. This is supported by the aforementioned report of novel pigments found in cultured mycobionts of other *Graphis* species (Miyagawa et al. 1994; Hamada et al. 2001) and similar reports for *Opegrapha* (Amano et al. 2000). Therefore, the commonly applied practice to test extracts from lichen thalli for biological activity might be misleading, as it misses the potential capabilities of the non-lichenized mycobiont in culture (Boustie and Grube 2005).

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