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Biocidal effect of lichen secondary metabolites against rock-dwelling microcolonial fungi, cyanobacteria and green algae

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ABSTRACT

The use of commercial biocides in outdoor environments is increasingly discouraged because of their ecotoxicity, new methods being thus invoked to control patinas of biological origin on the stone cultural heritage. The effects of secondary metabolites (usnic acid, norstictic acid, parietin) produced by saxicolous lichens, natural competitors of rock dwelling microorganisms, were investigated in vitro against microcolonial fungi (MCF: *Coniosporium apollinis, Coniosporium perforans, Coniosporium uncinatum, Phaeococcomyces*-like sp.), coccoid cyanobacteria (*Chroococcus minutus*) and green algae (*Scenedesmus ecornis*) which commonly occur on stonework. An acetone/water 10/90 vol/vol mixture was screened as suitable to solubilise the lichen metabolites and to not affect the bioassay results. Benzalkonium chloride 1% was used as positive control.

All the three metabolites (approx. 10^{-2} mM) inhibited the growth of the assayed MCF species, displaying the same effect of benzalkonium chloride. *Chroococcus* and *Scenedesmus* exhibited sensibility to the lichen metabolites when exposed to high incubation temperatures (35 °C), chemicals and temperature synergically yielding percentage decreases of intact cells with red chlorophyll epifluorescence. These findings suggest lichen secondary metabolites as allelopathic agents against rock dwelling microorganisms and as potential natural sources for their control on stone materials in restoration and conservation programmes. In this perspective, the detection of a negligible chromatic alteration ($\Delta E<0.5$) caused by LSM to the white Carrara marble is reported as the first step of the necessary extensive evaluation of the LSM-stone material interactions.

1. Introduction

Black patinas are a common biodeterioration phenomenon affecting the stone cultural heritage (Brimblecombe and Grossi, 2005; Caneva et al., 2008). Although the blackening of buildings and monuments can be also related to anthropogenic causes, as the deposition of gases and particles or sulphation mechanisms (Prieto et al., 2007), microorganisms as dematiaceous meristematic fungi, including microcolonial fungi (MCF), filamentous and coccoid cyanobacteria and, subordinately, green algae are also recognized as common agents of such threat to conservation (Gorbushina and Broughton, 2009; Macedo et al., 2009; Sterflinger, 2010). These organisms determine anaesthetic discoloration because of their dark pigments (Scheerer et al., 2009) and are often associated to physical and chemical deterioration processes because of their mechanical penetration and the release of acidic and chelating compounds (Macedo et al., 2009; Sterflinger, 2010; Favero-Longo et al., 2011).

The removal of these patinas is invoked in conservation programmes (Delgado Rodrigues and Valero, 2003), but biochemical and structural characteristics (e.g. EPS and/or mineral coatings, pigments) which make rock-inhabiting fungi, cyanobacteria and green algae adapted to the extreme microclimate conditions, including high temperatures, desiccation and osmotic stress (Sterflinger, 1998; Gorbushina and Broughton, 2009), also support a peculiar resistance to biocides (Gorbushina et al., 2003; Nugari et al., 2009). Anyway, the ecotoxicity of commercial biocides increasingly discourages their use in outdoor environments, where black patinas prevail (Scheerer et al., 2009).

On the other hand, natural products represent a huge potential source of compounds with biological activity, including phytotoxicity, which may be directly used as “pesticides” or represent a model to develop natural product-based pesticides (Duke et al., 2002). In this context, lichen secondary metabolites (LSM) have been suggested as potential natural herbicides because their chemical simplicity makes their synthesis potentially easy in the

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laboratory (Dayan and Romagni, 2001). LSM are a group of more than 800 compounds, in part exclusively synthesized by lichen-forming fungi, which include aliphatic, cycloaliphatic, aromatic and terpenic components (Huneck and Yoshimura, 1996; Elix and Stocker- Wörgötter, 2008). Many LSM are well known for determining allelopathic effects on bryophytes and vascular plants (Lawrey, 1984; Favero-Longo and Piervittori, 2010). Antibiotic, antiviral and anti-proliferative functions have been also recognized, suggesting their potential use for therapeutic applications (Oksanen, 2006; Boustie et al., 2011). The antimicrobial activity of LSM has been assessed against a wide set of bacteria and filamentous fungi, mainly of medical interest (Lawrey, 1984; Molnár and Farkas, 2010; Mitrović et al., 2011) or plant pathogens (Halama and Van Haluwin, 2004), but researches overlooked their effects against rock-dwelling organisms, thus preventing the evaluation of their potential use for the control of the biological colonization on stone materials.

This work aims to evaluate the effects of LSM on MCF, cyanobacteria and green algae which occur in black patinas and other biological patinas of different colours on the stone cultural heritage. The effects of usnic acid, norstictic acid and parietin on the fungal growth in axenic conditions and on the chlorophyll integrity in the photosynthetic organisms were assayed. These three LSM commonly occur in saxicolous lichens, in both silicicolous and calcicolous species (Smith et al., 2009). Usnic acid is a yellowish dibenzofuran, exclusive to lichen-forming fungi and widely distributed in the vegetative and reproductive structures of several species (e.g., species of genera *Lecanora, Ramalina*) (Ingólfsdóttir, 2002; Liao et al., 2010). It is well known for antibacterial and antifungal properties (and for antiprotozoal, antiviral, anti-proliferative, antiinflammatory, analgesic, antipyretic, antitumor activities) which have been mostly studied for potential therapeutic applications (Cocchietto et al., 2002; Ingólfsdóttir, 2002), and is also known to play UV protection of thalli (McEvoy et al., 2006). Norstictic acid is a depsidone, exclusive to lichen-forming fungi (e.g., in species of genera *Buellia, Lecanora, Porpidia, Rhizocarpon*), which is mostly deposited as crystals in the apoplast and soluble in water in a small proportion, being likely involved in the metal uptake and homeostasis of lichens (Hauck et al., 2010). Parietin is an orange antraquinone, non exclusive to lichens, located as extracellular crystals in the top layer of the upper cortex of Teloschistaceae (e.g., in species of genera *Caloplaca, Xanthoria*), protecting the photobionts from excessive solar radiation (Gauslaa and McEvoy, 2005). The antimicrobial activity of both norstictic acid and parietin has been demonstrated against a small set of bacteria and fungi of medical interest (Tay et al., 2004; Manojlovic et al., 2005).

In order to assay the potential applicability of the investigated LSM to control biological patinas on the stone cultural heritage, their interaction with a historical and culturally significant stone material (the white Carrara marble) was analysed with regard to the chromatic alteration, having a high relevance in stone conservation (Prieto et al., 2007; Tretiach et al., 2007). The necessity to perform further tests on the effects of LSM on other physico-chemical properties of stone materials and to assess their biocide effects in situ, i.e. on microorganisms growing on rock substrates, is finally addressed.

2. Material and methods

2.1. Tested organisms and lichen secondary metabolites

Strains of the MCF *Coniosporium perforans* Sterflinger (CBS885.95), *Coniosporiumuncinatum* De Leo, Urzì, De Hoog (CBS100212) and *Coniosporium apollinis* Sterflinger (CBS109867) were purchased from Centralbureau von Schimmelcultures (CBS, The Netherlands) and maintained on Malt Agar Extracts (MEA) at 15 °C. Another MCF isolated from the travertine blocks of the Roman Theatre of Aosta, Italy (MCF-AO), whose ITS sequence (JF809604) matched most closely to GenBank accession sequences of the dematiaceous microcolonial fungus *Phaeococcomyces chersonesos* (AJ507323.4) (Gazzano, 2010), was also maintained in the same culture conditions (hereafter indicated as *Phaeococcomyces* sp.).

Strains of the green alga *Scenedesmus ecornis* (Ehrenberg) Chodat (SAG2332) and the cyanobacterium *Chroococcus minutus* (Kützing) Nägeli (SAG41.79) were purchased from Sammlung für Algenkulturen von Göttingen (SAG) and maintained on agarized Trebouxia Medium (TB; Ahmadjian, 1993) and BG11 (Castenholz, 1988), respectively, at 20 °C in the light.

Fungal, algal and cyanobacterial colonies were at first treated with mixtures having different acetone/H_2O vol/vol ratios (0/100, 10/90, 50/50, 100/0) to select a suitable solvent for the solubilisation of the lichen metabolites which are poorly soluble in water (Elix and Stocker- Wörgötter, 2008). Three lichen metabolites were thereafter assayed (Fig. 1): (+)-usnic acid, in some cases reported as the more active enantiomer (Cocchietto et al., 2002; Ingólfsdóttir, 2002), was purchased by Sigma–Aldrich (St. Luis, MO, USA); norstictic acid and parietin were extracted from lichen thalli. For all experiments purified water (ELGA Purelab, Elga, UK) was used.

2.2. Extraction and purification of lichen metabolites

Norstictic acid and parietin were extracted from *Pleurosticta acetabulum* (Neck.) Elix & Lumbsch and *Xanthoria parietina* (L.) Th. Fr., respectively. Thallus fragments of 20–40 mg were incubated for 1 h within 1 ml of acetone. The obtained solutions were filtered (0.45 μm; Albet, Barcelona, Spain) and the target metabolites were purified and collected using a HPLC system composed of Waters (Milford, MA, USA) components, including Waters 1525 binary HPLC Pump, Waters 2389 UV–Vis detector and Waters Fraction Collector-III. The column was a dC18 Atlantis ® reversed-phase column (150 × 4.6 mm, 5 μm). The mobile phase was 1 ml min^{-1} methanol (99.8%, Fluka, Buchs, Switzerland). According to Huneck and Yoshimura (1996), norstictic acid was detected on the basis of its UV absorption maxima at 212 nm, 239 nm, 270 nm and 317 nm; parietin was detected on the basis of UV–Vis absorption at 266 nm, 288 nm and 431 nm. The collected purified fractions were dried using a rotary vacuum evaporator (RE300, Stuart, UK).

2.3. Solubilisation of lichen metabolites

According to the preliminary assays on the acetone effects on the tested organisms, usnic acid and the obtained precipitates of norstictic acid and parietin were solubilised in an acetone/water 10/90 vol/vol mixture. The concentrations of the lichen metabolites were assessed using a DU530 Life Science UV–Vis Spectrophotometer (Beckman, Pasadena, CA, USA) on the basis of UV absorption at 270 ($\log_\varepsilon = 4.02$) and 317 nm (3.70) for norstictic acid, at 266 nm (4.36) and 431 nm (4.20) for parietin, and at 290 nm (4.45) and 325 (3.85) for usnic acid. As acetone avoided the measure of UV absorption of water/acetone mixtures, sub-samples of these latter were dried with the evaporator and re-suspended in methanol for the analyses. The final concentrations of norstictic acid, parietin and usnic acid in the acetone/water 10/90 vol/vol mixture were 0.05 mM, 0.05 mM and 0.02 mM.

2.4. Evaluation of the effects of lichen metabolites on the tested organisms

Three mm diameter discs of mycelium were cut from the edge of young colonies of the MCF strains and inoculated on plastic Petri

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