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Potential of natural biocides for biocontrolling phototrophic colonization on limestone

S. Sasso [a], A.Z. Miller [b, *], M.A. Rogerio-Candelera [b], B. Cubero [b], M.L. Coutinho [c], L. Scrano [d], S.A. Bufo [a]

[a] Department of Sciences, University of Basilicata, Via dell'Ateneo Lucano 10, 85100, Potenza, Italy
[b] Instituto de Recursos Naturales y Agrobiologia de Sevilla (IRNAS-CSIC), Av. Reina Mercedes 10, 41012 Seville, Spain
[c] Vicarte, Departamento de Conservação e Restauro, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Monte de Caparica, 2829-516 Caparica, Portugal
[d] Department of European Cultures (DICEM), University of Basilicata, Via dell'Ateneo Lucano 10, 85100, Potenza, Italy

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ABSTRACT

Rocks, either in natural geological outcrops or in stone monuments, are common habitats for a wide variety of microorganisms which colonize both rock surfaces and cracks. Physical properties such as porosity and surface roughness make limestone susceptible to biological colonization, which may induces aesthetic and/or physical and chemical damages. Organisms causing biodeterioration on monuments have usually been controlled by chemical products (biocides). In order to overcome the impact of these substances on the environment, human health and stone substrates, alternative tools such as natural products from plants or microorganisms can be used as an innovative approach for stone conservation.

In this work, the efficiency of natural biocides (cells free culture filtrates of *Trichoderna harzianum* and *Burkholderia gladioli*, as well as glycoalkaloids from spontaneous *Solanaceae*) was tested under laboratory conditions against a multi-species phototrophic culture developing on Hontoria limestone. Their efficiency was assessed by digital image analysis, *in vitro* chlorophyll *a* quantification and confocal laser scanning microscopy. These techniques showed a good correlation, revealing that cells free filtrate of *Trichoderma harzianum* had an antagonistic action against the multi-species phototrophic culture tested in this work.

1. Introduction

Rocks, either in natural geological outcrops or in stone monuments, are common habitats for a wide variety of microorganisms. Due to their ubiquity and ability to produce metabolic products, microorganisms contribute to stone deterioration (Hirsch et al., 1995; Gaylarde and Morton, 1999; Dornieden et al., 2000; Miller et al., 2010a; Sterflinger and Piñar, 2013). Photoautotrophs, particularly cyanobacteria and microalgae, constitute the primary colonizers of building stones (Koestler et al., 1996; Bellinzoni et al., 2003; Miller et al., 2010b). Several investigations have demonstrated their importance on the physical and chemical biodeterioration of stone (Ortega-Calvo et al., 1991a, b; Urzì and Krumbein, 1994; Miller et al., 2010a). Therefore, cleaning is an important aspect for the conservation of stone buildings and structures.

Traditionally, the control of biodeterioration is based in the application of chemical products (biocides). Nevertheless, these products have limitations due to their negative impacts on the environment, human health and treated substrates. In order to minimize these impacts, the search of eco-friendly alternative tools, such as the rational use of natural products derived from plants or microorganisms, has recently increased. Although well established in agricultural science, the application of these procedures in stoneworks is still scarce (Salvadori, 2003; Szewczyk et al., 2006). Their use is particularly interesting due to their narrow specificity, effectiveness using small doses, fast decomposition, and low toxicity, which makes them harmless to people and wildlife (Ramírez et al., 2005; Szewczyk et al., 2006).

The goal of this work was to test the biocidal efficiency of glycoalkaloids extracted from unripe berries of *Solanum nigrum* and

* Corresponding author.
E-mail address: anamiller@irnas.csic.es (A.Z. Miller).

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cells free filtrates of the fungus *Trichoderma harzianum* T22 strain and the bacterium *Burkholderia gladioli* pv. *agaricicola* ICMP 11096 strain (*Bga*) against a phototrophic culture growing on limestone probes.

Burkholderia species are aerobic gram-negative rod-shaped bacteria occurring commonly in the soil, water and plant rhizosphere. Among these, strains of *B. gladioli* possess a great potential as plant pathogen antagonists and could be used for bioremediation of recalcitrant xenobiotics (Hu and Young, 1998). In particular, cells and cells free filtrate of *Bga* showed *in vitro* antagonistic activity against a wide range of fungal and bacterial species due to the secretion of secondary metabolites (Elshafie et al., 2012a, b; 2013). As shown in Elshafie et al. (2012a), five different active metabolites with high antimicrobial activity against gram positive and gram-negative bacteria were identified in an extract of broth culture. In addition, *Bga* showed a high ability to produce extracellular hydrolytic enzymes such as chitinase, protease and glucanase, and volatile organic compounds (VOCs) that exhibited anti-fungal and anti-bacterial activities (Elshafie et al., 2012a).

T. harzianum T22 strain is a filamentous fungus belonging to the Hypocreales order of the Ascomycota division found in the soil of all climatic zones. Due to the production of natural bioactive products such as extracellular enzymes, many volatile (e.g. pyrones, sesquiterpenes) and non-volatile secondary metabolites (e.g. peptaibols), this species is of great interest to the research community (Reino et al., 2008; Mukherjee et al., 2012). Enzymatic activity assays (Carder, 1986) performed on CFF broth confirmed the production of extracellular lytic enzymes (cellulase, protease, glucanase and chitinase) that play a basic role in the action of cell-wall degrading on host microbes (Mishra, 2010). Reino et al. (2008) identified some of the secondary metabolites produced by *Trichoderma*, which are active against a broad range of fungi and bacteria, such as koninginine AE and G, viridine, anthraquinones or viridiofungins. In addition, solid phase microextraction followed by gas chromatography-mass spectrometry showed the occurrence of several VOCs such as mono- and sesquiterpenes that have antibiotic activity (Siddiquee et al., 2012; Sivasithamparam and Ghisalberti, 1998).

Glycoalkaloids (GAs), a class of nitrogen-containing steroidal glycosides, are important bioactive secondary metabolites commonly produced by plants belonging to the *Solanaceae* family (Milner et al., 2011). GAs are present in all parts of the plant; they accumulate in cytoplasm and in vacuoles (Han et al., 1989). However, the highest concentrations are detected in active growing young tissues such as flowers, sprouts, unripe berries, or young leaves (Friedman and McDonald, 1997; Friedman, 2006). Structural variation of plant GAs is limited to two main groups, based on the skeletal type of the aglycone: the spirosolan- and solanidane-type (Friedman and McDonald, 1997). The two spirosolan-type GAs, solamargine and solasonine, showed anti-bacterial, anti-fungal and insecticidal activities (Milner et al., 2011; Ventrella et al., 2015). These activities are associated with their membrane-disruptive properties and their anti-acetylcholinesterase activity (Keukens et al., 1995). Solamargine was more potent in disrupting cell membranes than solasonine by a factor of 2 or 3 (Roddick et al., 1990).

GAs and CFF of *T. harzianum* and *Bga* were tested against a multi-species phototrophic culture developed on Hontoria limestone probes. Laboratory tests using multi-species communities, as occurs in nature, provide a better understanding of stone-microbe-biocide interactions (Miller et al., 2009a, 2010b). The efficiency of these natural biocides as bio-cleaning treatments was assessed by digital image analysis, *in vitro* chlorophyll *a* quantification and confocal laser scanning microscopy.

2. Materials and methods

2.1. Multi-species phototrophic culture enrichment

To promote the growth of a photosynthetic-based biofilm on the limestone probes, a multi-species phototrophic culture, previously collected from a limestone monument (Miller et al., 2008, 2009a), was used. The major components of the inoculum belonged to the genera *Chlorella* and *Stichococcus* among the Chlorophyta, and *Leptolyngbya* and *Pleurocapsa* among the Cyanobacteria.

Species enrichment culture was prepared by transferring stock cultures to an Erlenmeyer flask containing 3 L of BG-11 culture medium (Normal 9/88, 1988). This culture was thereafter incubated at 20 ± 2 °C in laboratory conditions under natural light/dark cycles for 30 days under continuous shaking. Growth was routinely checked by measuring the optical density at 600 nm (OD600) using a JENWAY 6315 spectrophotometer.

Chlorophyll *a* and pheophytin concentrations of the inoculum were determined by the extraction protocol used for phytoplankton as described by Shoaf and Lium (1976). The pigment was extracted in dimethyl-sulfoxide (DMSO) and measured by spectrophotometry before and after acidification with 1N HCl (Burnison, 1980). Their concentrations were calculated on the basis of Lorenzen (1967) equation, using the extinction coefficient of Jeffrey and Humphrey (1975).

2.2. Stone probes preparation

The lithotype used was Hontoria limestone, a white bioclastic limestone used in many Spanish monuments. It is composed almost exclusively of calcite with a porosity ranging from 20 to 25% and high surface roughness (Marcos et al., 1993). These physical properties make Hontoria limestone prone to the development of microorganisms on its surface (Miller et al., 2009b).

In this experiment, 16 stone probes of 3 × 3 × 0.5 cm were prepared by saw-cutting a limestone block. Later, they were washed with sterile water, dried and placed into 4 glass Petri dishes (Ø 15 cm, 4 probes per dish), which were then sterilized at 120 °C and 1 atm, for 20 min.

After cooling, the upper surfaces of the stone probes were inoculated with 200 μL of the multi-species phototrophic culture (0.6 g chlorophyll *a*/L) and incubated for 20 days under laboratory conditions near a window for natural light/dark cycles. Moisture was maintained by the periodical addition of sterile water in the bottom of the Petri dishes.

2.3. Preparation of biocidal treatments and application on stone probes

B. gladioli pv. *agaricicola* ICMP11096 (*Bga*) and *T. harzianum* T-22 strains were obtained from a collection of pure cultures maintained at the Department of Sciences of the University of Basilicata (Italy). Unripe berries of *S. nigrum*, a spontaneous *Solanaceae* plant, were collected in a greenhouse of Metaponto (40°23′37″ N–16°47′54″ E) in Matera, Italy.

Cells free filtrate (CFF) of *Bga* was obtained by filtration (0.2 μm) after five days of growing in Minimal Medium broth (Elshafie et al., 2012a). CFF of *T. harzianum* T-22 was also extracted by filtering the broth culture at 0.2 μm, after 15 days of growth in Potato Dextrose Broth (PDB).

Glycoalkaloids were extracted from unripe berries of *S. nigrum* by using the method of Cataldi et al. (2005). The extract was lyophilized and resuspended in water to obtain a stock solution of solamargine (principal component) at a concentration of 500 μM.

For our experiment we used extracts from *S. nigrum*, containing

two main GAs, solamargine and solasonine, and other less abundant components (Milner et al., 2011; Eldridge and Hockridge, 1983). These extracts, characterized by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC/ESI-MS), contain a similar quantity of two main GAs: 285.7 ± 22.5 μM of solamargine (considering the percent content of water in berries of 84.8%) and 303.0 ± 23.2 μM of solasonine.

A volume of 500 μl of each natural biocide was applied on the inoculated limestone surfaces every fifteen days (3 applications). Control probes (CA) consisted in stone probes without treatment, but inoculated with the multi-species phototrophic culture.

2.4. Monitoring of biocide efficiency

The development of photosynthetic-based biofilms on the treated probes was monitored by Digital Image Analysis (DIA), quantification of photosynthetic biomass, and Confocal Laser Scanning Microscopy (CLSM).

DIA was performed on photographs taken using a Canon IXUS 90 IS digital camera. The images obtained were processed by Principal Component Analysis (PCA), using the HyperCube v. 11.0 software (US Army Topographic Engineering Centre, Alexandria, Virginia, USA) as tested by Rogerio-Candelera et al. (2011). PCA was used to simplify the images, for later apply an iterative thresholding algorithm, based on Sezgin and Sankur (2004), for image segmentation avoiding the redundant data present in the different bands of the image. The detected particles within the selected area were measured using ImageJ v. 1.47 software (National Institutes of Health, Bethesda, Maryland, USA), following the protocols of Rogerio-Candelera et al. (2011).

The quantification of photosynthetic biomass on and within stone probes was performed according to the pigment extraction protocol for periphyton as described by Vollenweider et al. (1974). Inoculated stone probes were crushed (fragments no larger than 0.25 cm^3), added into 10 ml of DMSO and incubated at 65 °C for 1 h. Subsequently, the samples were filtered to remove stone particles, and the absorbance of the extract was measured at 665 and 750 nm. The extract was then acidified with HCl 1 M and the absorbance was read again at 664 and 750 nm in a JENWAY 6315 spectrophotometer. Chlorophyll *a* and pheophytin concentrations were calculated by the equation of Lorenzen (1967) using the extinction coefficient from Talling and Driver (1963).

The development of the phototrophic biofilm on Hontoria limestone probes was assessed by CLSM using a FluoView FV1000 Confocal microscope (Olympus) in fluorescence and reflection modes. CLSM allows the spatial semi-quantification of the micro-communities of the fully hydrated biofilms (Rodriguez and Bishop, 2007).

Images were recorded using a 488-nm Ar/ArKr laser and a 20 × objective with emission signals being collected at 690 ± 60 nm for chlorophyll autofluorescence. Images were acquired at 1.25 μm intervals along 100 μm in the Z axis, at three-frame averaging and analysed with the FluoView 2.1 software (Olympus). Image acquisition parameters including PMT settings were optimized initially and not changed during the acquisition of subsequent images.

2.5. Assessment of chromatic variations of the substrate after biocide application

Colour measurements were performed using a portable spectrophotometer (Minolta CM-508i). The results are the mean value of fourteen measurements per area according to the procedure of colour measurement for rock surfaces (Prieto et al., 2010). After biocide application on non-colonized stone probes, following the procedure described in Section 2.3, colour measurements were performed on the stones treated with the natural biocides (after 3 applications). CIELAB method was used in order to characterize the surface colour by three parameters: L* (lightness/darkness), a* (+a* indicating red and − a* green) and b*(+b* indicating yellow and − b* blue), defined by CIE (Commission Internationale de l'Éclairage). Total colour variation (ΔE*) was calculated as a spatial difference between two points, corresponding to the initial colour, before the treatment and to the colour after treatment: $\Delta E^* = \sqrt{((\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2)}$, being $\Delta L^* = L^*$(after treatment) - L*(before treatment); $\Delta a^* = a^*$(after treatment) - a* (before treatment); $\Delta b^* = b^*$ (after treatment) - b* (before treatment).

3. Results

3.1. Visual inspection of phototrophic growth on stone probes before and after biocidal treatments

After 20 days of biofilm incubation on non-treated stone probes, a green biofilm was clearly visible over the upper surfaces. The application of biocidal treatments conditioned the development of the biofilm on the inoculated stone probes. Fig. 1(in web version) shows the evolution in time of the green biofilms on one representative control stone probe (CA) and stone probes treated with: CFF of *Bga* (BA); CFF of *T. harzianum* (TA), and Glycoalkaloids (GA).

Visual inspection revealed that green stains tended to expand on CA stone surfaces in the course of the experiment. The same trend was observed for replicates treated with BA and GA. Particularly, an increasing of colour intensity was observed for these two treatments. In contrast, TA revealed a decrease of green covered area and a colour change from light green to yellow after 45 days of incubation (Fig. 1(in web version)).

3.2. Monitoring of phototrophic growth

3.2.1. Digital image analysis

PCA of the images allowed us to select the band corresponding to the second principal component (PC2) as the best for determining the shape and area of the colonized areas. After segmentation and scaling, the binary images were measured by counting the pixels classified as colonized (Fig. 2). Fig. 3 depicts the colonized area measured by DIA during 45 days of incubation. During the first 15 days of incubation, the photosynthetic colonization process on CA led to a gradual increase of green biofilms; in the following days, a significant increase was reported between 15 and 30 days-incubation. The growth reached a steady stage from 30 days-incubation until the end of the experiment.

On the stone probes treated with BA, the green covered area remained stable during the first 30 days. After this period, the green biofilm depicted an intense green colour (Fig. 3(in web version)).

GA-based treatment resulted to be efficient in the first instance (Fig. 3), as the area of the photosynthetic biomass decreased, but after the last biocidal application (30 days) the biofilm slightly increased until the end of the experiment (45 days).

TA-treated stone probes depicted a slight decrease of the stone surface covered area immediately after the first biocidal application. This trend was also observed during the following treatment applications (Fig. 3).

3.2.2. Confocal laser scanning microscopy

Biocide efficiency was also assessed by CLSM technique. This method enabled to observe variations in fluorescence intensity for each natural biocide tested on the stone probes.

Abundant filaments of phototrophic microorganisms were observed on the CA stone probes, as revealed by the intense *in vivo* pigment fluorescence (Fig. 4a). In contrast and interestingly, on the

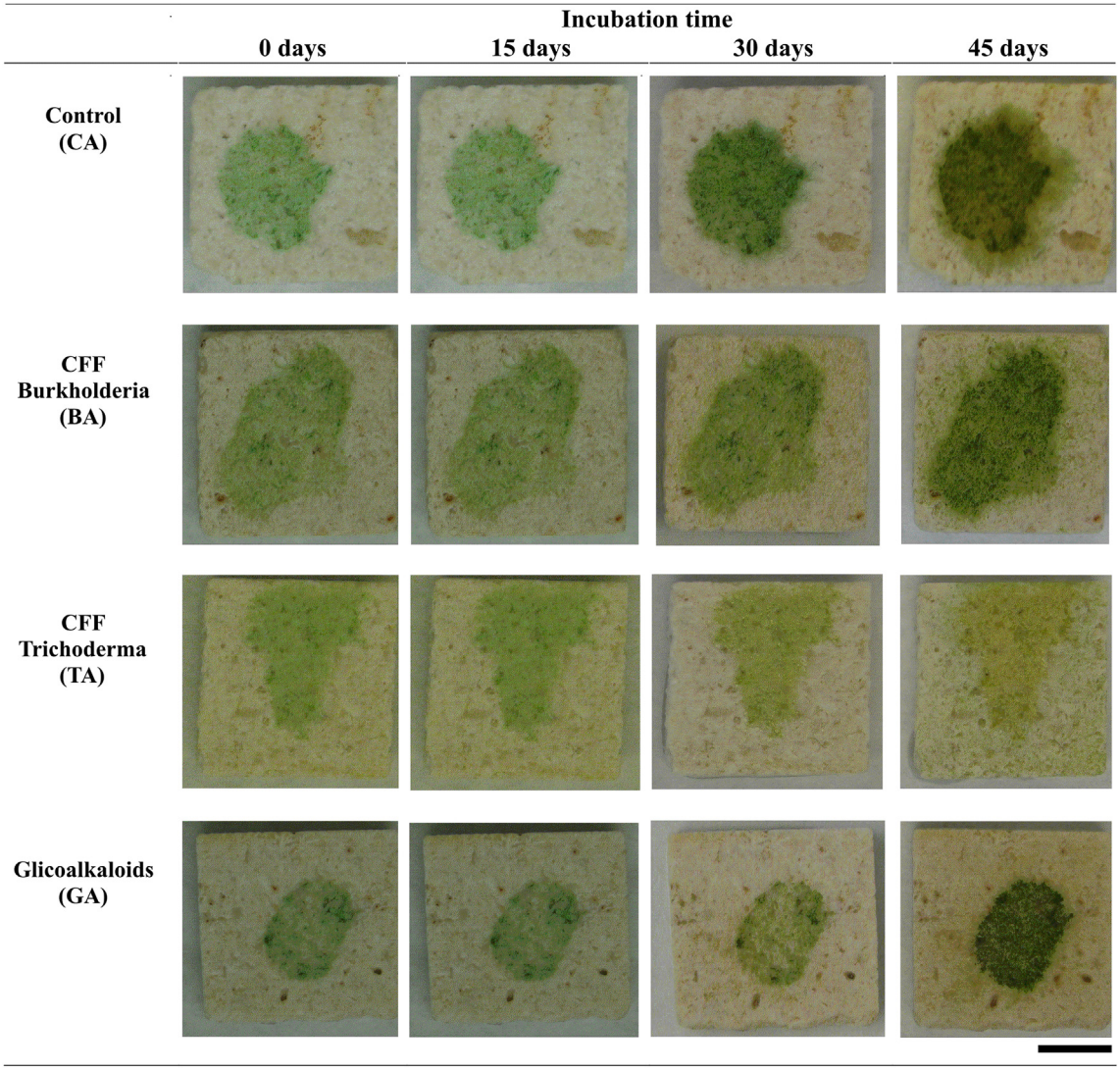

Fig. 1. Photosynthetic colonization on non-treated Hontoria limestone probes (CA) and treated stone probes with: CFF of *Bga* (BA); CFF of *Trichoderma harzianum* (TA) and Glycoalkaloids (GA). Bar = 1 cm.

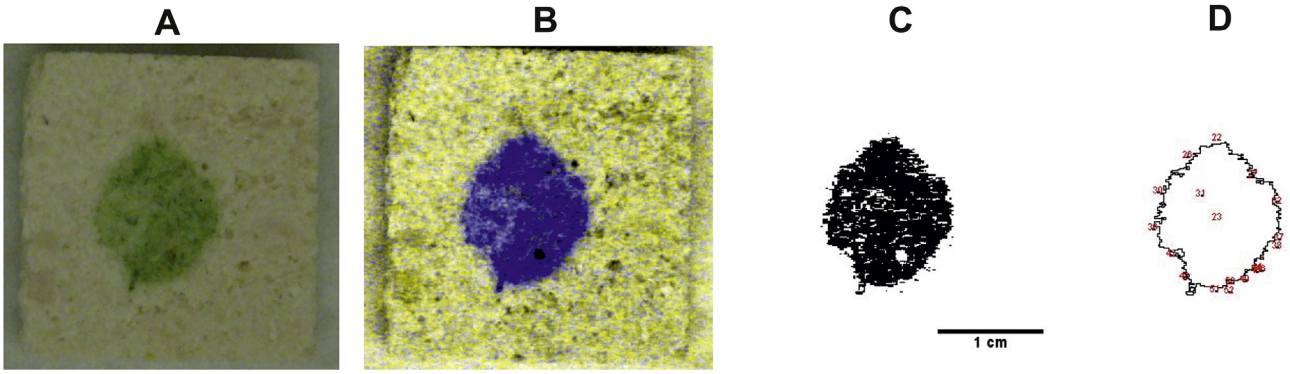

Fig. 2. Graphic results of the image processing approach. A) original RGB photograph; B) false coloured image elaborated from the PCA bands; C) segmented and scaled image; D) measured area.

treated stone probes (BA, GA and TA) non-filamentous phototrophic microorganisms were abundant; in general, only unicellular phototrophic microorganisms, displayed in green colour, were observed for the treated stones (Fig. 4b,c,d(in web version)). Photosynthetic-based cell clusters were particularly evident on BA-treated stone probes. However, filamentous structures depicting

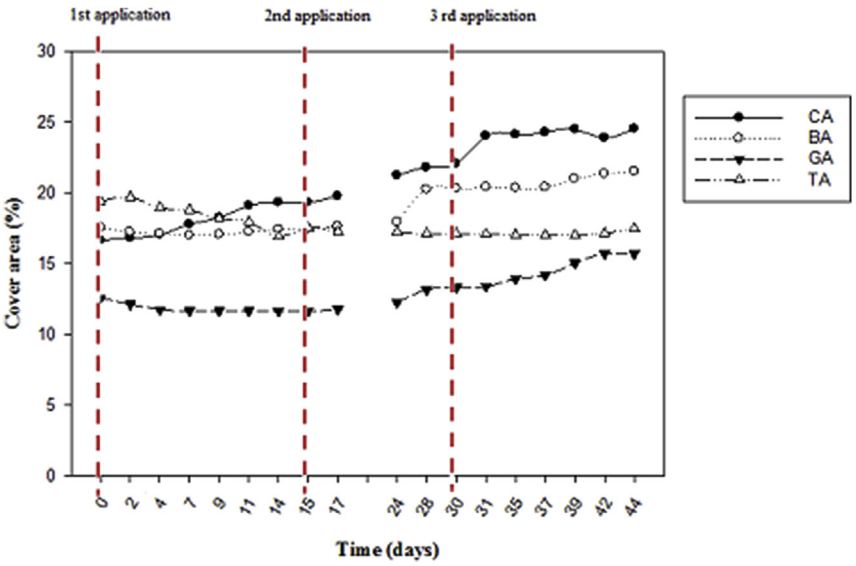

Fig. 3. Stone covered areas measured by Digital Image Analysis during 45 days of incubation on the non-treated (CA) and treated stone probes with: CFF of *Bga* (BA); CFF of *Trichoderma harzianum* (TA) and Glycoalkaloids (GA). Vertical dashed lines indicate the incubation days when biocidal treatments were applied on the stone probes (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

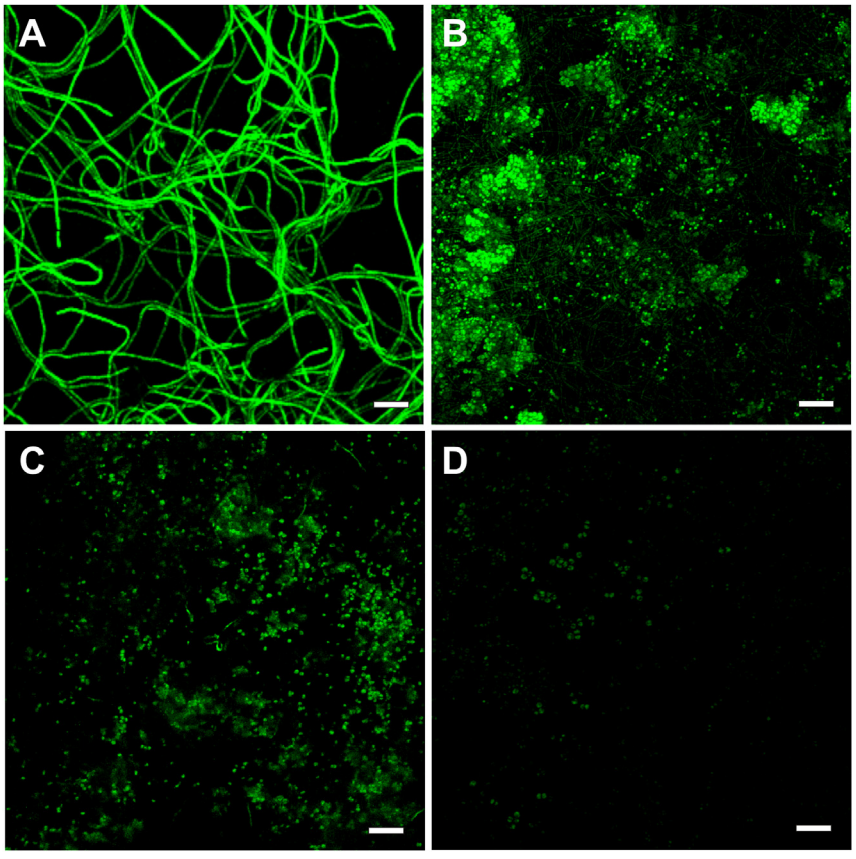

Fig. 4. CLSM images of photosynthetic-based biofilms developed on the stone probes after 45 days of incubation. A) green biofilm on CA samples; B) green biofilm on BA-treated stone probes; C) green biofilm on GA-treated stone probes; and D) green biofilm on TA-treated stone probes. Bars = 50 μm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

very low fluorescence intensity were also detected on these samples (Fig. 4b). The lowest chlorophyll fluorescence intensity, both from filamentous or unicellular microorganisms, was obtained for TA-treated samples, where almost no cells were observed (Fig. 4d).

3.2.3. *Photosynthetic biomass quantification*

Photosynthetic biomass estimation (Fig. 5), performed by means of chlorophyll *a* extraction, showed that the highest chlorophyll *a* content was obtained for control stone probes (CA), as expected.

GA-treated stone probes almost reached the same chlorophyll *a* concentration values as CA. The value decreased for limestone probes treated with BA and TA. A significant decrease of chlorophyll *a* content was observed for TA-treated stone probes, which strongly agrees with the results obtained by DIA and CLSM.

3.2.4. Colour variation after biocide application

Stone probes treated with each biocide were investigated in order to assess chromatic changes in comparison to untreated stone probes (before the treatment). The untreated probes corresponded to a light coloured stone, with values of L* varying between 92 and 88, a* varying between 1 and 3, and b* varying between 6 and 8. All tested treatments caused colour variation on the stone probes (Table 1). In average, the highest colour variation were observed for the stone probes treated with BA ($\Delta E^* = 13.88 \pm 0.94$) and the lowest variation, although very close to the TA-treated probes, was obtained for GA ($\Delta E^* = 8.28 \pm 1.48$) (Table 1). The parameters with the highest variation was ΔL^*, due to the darkening of the samples, and Δb^* with a yellow shift.

4. Discussion

Cells free filtrates of *T. harzianum* and *B. gladioli* pv. *agaricicola*, as well as glycoalkaloids from spontaneous *Solanaceae* were applied on Hontoria limestone probes in order to evaluate a new eco-friendly procedure for cleaning, removing and/or controlling the growth of phototrophic microorganisms on stone surfaces. Digital image analysis complemented with CLSM and chlorophyll *a* extraction method were employed for evaluating their potential biocide activity on a multi-species phototrophic culture inoculated on limestone probes. The anti-algal activity of these three natural biocides was tested against phototrophic microorganisms inoculated on stone probes because they are usually the first colonizers of inorganic building materials. Laboratory-based stone colonization experiments using phototrophic communities have long been used with great success (Prieto and Silva, 2005; Miller et al., 2008), and photoautotrophic biomass quantification methods are well-optimized for laboratory investigations with reproducible and statistically coherent data (Miller et al., 2010b; Fernández-Silva et al., 2011).

DIA allowed establishing the extent of the microbial colonization along time. PCA-based DIA protocols have proven to be useful for the delimitation of elements enclosed in highly correlated images, as in the case of rock art paintings (Rogerio-Candelera et al., 2011; Rogerio-Candelera, 2015), or the extent of microbial mats (Miller et al., 2010b; Coutinho et al., 2013). The common point lies in the small amount of the total variance attributable to these features; for this reason, the use of minority Principal Components becomes a tool fitted to this goal. In this context, the combination of PCA and segmentation algorithms revealed that BA and GA treatments were not effective to control the growth of phototrophic microorganisms tested in this work. On the contrary, the colonized area of TA probes initially decreased until reaching a steady state at the end of the experiment. This pointed out that this treatment was the most effective natural biocide tested in this work.

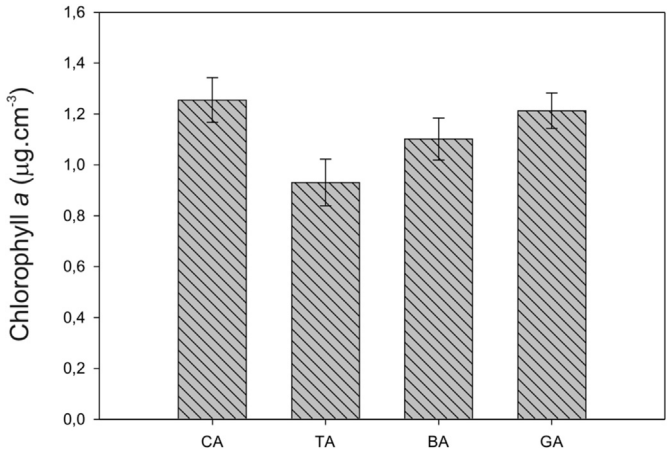

Fig. 5. Chlorophyll *a* content measured on control stone probes (CA) and on stone probes treated with CFF of *Bga* (BA), CFF of *Trichoderma harzianum* (TA) and Glycoalkaloids (GA) after 45 days of incubation.

Table 1
Average values and standard deviation ($n = 14$) of colour variation before and after treatments (BA, GA and TA).

Treatment	ΔL^*	Δa^*	Δb^*	ΔE^*
BA	−6.89 ± 1.05	1.28 ± 0.24	9.55 ± 0.64	13.88 ± 0.94
GA	−9.97 ± 0.83	0.61 ± 0.27	7.07 ± 1.29	8.28 ± 1.48
TA	−4.21 ± 0.97	2.17 ± 0.47	5.40 ± 0.81	9.06 ± 1.11

CLSM provided valuable information not only on the complex structures of photosynthetic biofilms developed on the stone probes, but also on their degree of resistance to biocide treatments, based on the intensity of *in vivo* chlorophyll autofluorescence. This technique has demonstrated to be a useful tool for direct investigation of the biocide activity within biofilms as it allows recognizing resistant microorganisms (Davison et al., 2010; Bridier et al., 2011a, b). According to several authors, this resistance to biocides is closely related to the organization of cells in a biofilm and its exopolymeric matrix (Xu et al., 2000; Mah et al., 2003; Izano et al., 2009; Bridier et al., 2011b). CLSM revealed that the application of cells free filtrates and plant extract on the stone samples caused changes in the microbial composition of the inoculated phototrophic community. These treatments suppressed the growth of filamentous phototrophic microorganisms, but promoted the growth of unicellular phototrophic microorganisms, probably *Chlorella* sp. and *Pleurocapsa* sp., originally present in the inoculum.

Regarding the quantification of photosynthetic biomass by the *in vitro* chlorophyll *a* method, these data showed significant differences among the TA-treated stone probes and the control samples, reinforcing the results obtained by DIA and CLSM. The chlorophyll *a* content obtained from GA- and BA-treated stones were quite similar to CA samples, which also agreed with DIA and CLSM data.

Gathering all the results together, BA treatment failed to effectively inhibit the growth of photosynthetic biomass on the stone surfaces. GA seemed to reduce the growth of microorganisms during the first month of incubation, but after the third application (30 days-incubation) it induced biofilm expansion as revealed by DIA. This also demonstrates that GA is ineffective as biocide for the consortium of microorganisms tested, particularly for unicellular phototrophs. Hence and instead of controlling biological colonization, these natural biocides could even act as nutrient sources for microbial growth. Several studies have shown that the application of biocides on stone surfaces may increase stone tertiary bioreceptivity (Guillitte, 1995; Miller et al., 2012). One of the most famous cases on microbial development after the application of biocides is the recolonization of the walls of Lascaux Cave (France) after several treatments with quaternary ammonium derivatives (Bastian et al., 2010; Martin-Sanchez et al., 2012). This recolonization was associated with the organic components of quaternary ammonium derivatives, which might be utilized by microorganisms as nitrogen and carbon sources (Pinna and Salvadori, 1999; Bastian et al., 2010).

In general, the cells free filtrates of *T. harzianum* and *B. gladioli*, and glycoalkaloids from spontaneous *Solanaceae* used in this study to control *in vitro* photosynthetic colonization on limestone probes, exhibited biocide activity solely against the filamentous phototrophic microorganisms of the inoculum, as revealed by CLSM. However, in nature microorganisms develop in more or less complex communities (Sand, 1997). On exposed stone surfaces they rarely grow as colonies comprising single species, and thus, selective treatments of a single type of organism are far to be the best approach, and may originate inaccurate conservation interventions. From the three natural biocides tested, TA was the only one that evidenced an antagonistic capacity against the photosynthetic-based consortium as a whole, developed on the limestone probes during 45 days-incubation.

The antimicrobial activity of the CFF of *T. harzianum* and *B. gladioli*, and glycoalkaloids from spontaneous *Solanaceae* was previously tested against bacterial and fungal strains by the agar diffusion test (Sasso et al., 2013; Caligine et al., 2013). It was demonstrated that glycoalkaloids extracts inhibited all bacterial strains tested, while CFF of *Bga* resulted more selective against bacteria belonging to Firmicutes. Regarding the biocontrol capacity of *T. harzianum*, it has been shown that *T. harzianum* significantly reduces plant diseases caused by fungi (Elad et al., 1980; Schuster and Schmoll, 2010). However, our data showed that the biocidal action of these three treatments is drastically reduced when applied to a consortium of phototrophic microorganisms growing on a lithic substrate. This can be due to the complex interactions among microbial species and the microorganisms and the lithic substrate, as well as the synergies generated. Moreover, as reported by Mukherjee and Raghu (1997), the biocide action of *Trichoderma* sp. is dependent on temperature, which could explain the low biocidal activity against the phototrophic consortium tested in this work.

It should be noted that in a previous study, sound stone probes were impregnated with GAs and CFF of *Bga* and *T. harzianum*, which were further inoculated with phototrophic microorganisms, in order to assess their efficiency as preventive treatments (before colonization). After 90 days of incubation, it was demonstrated that TA and BA in fact fostered the increment of photosynthetic biomass on the stone surfaces (Sasso et al., 2014). These former results, led us to test in this work the curative potential of these compounds on colonized stone surfaces performing several applications throughout the incubation time, instead of using them as preventive treatments. Repeated applications of biocide treatments is a common procedure in conservation interventions when using commercial biocides to efficiently mitigate biological colonization on outdoor cultural heritage assets to avoid rain water lixiviation (Russel and Chopra, 1990; Mandal and Rath, 2013).

The treatments were also assessed in regard to their effect on the colour variation which is an important parameter in the field of cultural heritage. It is generally accepted that treatments should not induce greater variation of ΔE^* than 5 (Vigliano, 2002). However, the applied treatments caused variation greater than this value due to the yellow tonality of the applied treatments that caused the dark- and yellowing of the treated stone probes.

5. Conclusions

The combination of several analysis techniques is compulsory in the field of cultural heritage in order to develop and design new and effective conservation strategies to prevent, control and minimize biodeterioration. In this study, digital image analysis, confocal laser scanning microscopy, *in vitro* chlorophyll *a* quantification and colorimetry were applied in order to evaluate a new procedure of stone bio-cleaning consisting of the application of cells free filtrates of *T. harzianum* and *B. gladioli* pv. *agaricicola*, and glycoalkaloids from spontaneous *Solanaceae* with biocide properties. These techniques have shown a good direct correlation with the data obtained from these approaches, revealing that cells free filtrate of *T. harzianum* has an antagonistic capacity against the multi-species phototrophic culture tested. Nevertheless, the efficiency of this compound on colonized stone surfaces needs further experiments to assess their mid- and long-term efficiency since efficient mitigation should inactivate the organisms and prevent their re-growth for an acceptable period of time. Moreover, colour variations greater than the generally accepted value represents a drawback scenario.

In spite of the vast literature on the successful application of these potential natural biocides for controlling plant pathogens, their application on stone cultural heritage as an alternative approach to conventional biocides is still in its infancy. In fact, there is still a paucity of knowledge on natural products for biocontrolling purposes in the field of cultural heritage, and consequently, conservation interventions do not always obtain the expected result, and sometimes they even hasten the biodeterioration process. Thus, with a view towards the future conservation of deteriorated stone monuments, laboratory-based experiments should be frequently developed since they allow the management of preventive conservation strategies and help choosing the appropriate treatments and conservation strategies. Laboratory experiments present the advantage of controlling environmental variables which simplifies the answering of important questions, particularly in the field of stone biodeterioration. These experiments are prerequisite in the diagnosis of monuments and in the design of effective treatments for eliminating active microbial communities, since they allow an affordable evaluation of the efficacy of biocides, as showed in this work.

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