Potential of natural biocides for biocontrolling phototrophic colonization on limestone

S. Sasso¹, A. Z. Miller^{2*}, M. A. Rogerio-Candelera², B. Cubero², M. L. Coutinho³, L. Scrano⁴, S.A. Bufo¹

¹ Department of Sciences, University of Basilicata, Via dell'Ateneo Lucano 10, 85100, Potenza Italy.

² Instituto de Recursos Naturales y Agrobiología de Sevilla (IRNAS-CSIC), Av. Reina Mercedes 10, 41012 Seville, Spain.

³ Vicarte, Departamento de Conservação e Restauro, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Monte de Caparica, 2829-516 Caparica, Portugal.

⁴ Department of European Cultures (DICEM), University of Basilicata, Via dell'Ateneo Lucano 10, 85100, Potenza Italy.

* Corresponding author: anamiller@irnas.csic.es

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 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 *Trichoderma 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.

Keywords: stone, biodeterioration, biocide, phototrophic colonization, digital image analysis

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 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 *Burkholderia 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 of 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 antifungal and antibacterial activities (Elshafie et al. 2012a).

Trichoderma 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 koniginine AE and G, viridine, anthraquinones or viridifungins. 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 disruption cell membranes than solasonine by a factor of 2 or 3 (Roddick et al. 1990).

GAs and CFF of *Trichoderma harzianum* and *Bga* were tested against a multi-species phototrophic culture developed on Hontoria limestone probes. Tests using multi-species phototrophic cultures, as occurs in nature, provide a better understanding of stone-microorganisms-biocide interactions (Miller et al. 2009a, 2010b). The efficiency of these natural biocides as biocleaning 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 and identified by 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 3L 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 concentration of the inoculum was 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 concentration was 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% with 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 15cm, 4 probes per dish), which were then sterilized at 120 °C and 1 atm, for 20 min.

After cooling, the upper surfaces of stone probes were inoculated with 200µL 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

Burkholderia gladioli pv. *agaricicola* ICMP11096 (*Bga*) and *Trichoderma 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 *Solanum 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 (0.2µm) after five days of growing in Minimal Medium broth (Elshafie et al. 2012a). CFF of *Trichoderma 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 *Solanum 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 experiments we used extracts from *Solanum nigrum*, containing two main GAs, solamargine and solasonine, and other less abundant components (Milner et al., 2011; Eldridge and

145 Hockridge 1983). These extracts, characterized by liquid chromatography-electrospray ionization-
146 tandem mass spectrometry (LC/ESI-MS), contain a similar quantity of two main GAs: 285.7 ± 22.5
147 μM of solamargine (considering the percent content of water in berries of 84.8%) and 303.0 ± 23.2
148 μM of solasonine.

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

152

153 *2.4 Monitoring of biocides efficiency*

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

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

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

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

177 Images were recorded using a 488-nm Ar/ArKr laser and a 20 $\times$ objective with emission signals
178 being collected at $690 \pm 60 \text{ nm}$ for chlorophyll autofluorescence. Images were acquired at 1,25 μm
179 intervals along 100 μm in the Z axis, at three-frame averaging and analysed with the FluoView 2.1
180 software (Olympus). Image acquisition parameters including PMT settings were optimized initially
181 and not changed during the acquisition of subsequent images.

182

183 *2.5 Assessment of chromatic variations of the substrate after biocide application*

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

194 treatment); $\Delta a^* = a^*(\text{after treatment}) - a^*(\text{before treatment})$; $\Delta b^* = b^*(\text{after treatment}) - b^*(\text{before}$
195 treatment).

196
197

198 3. Results

199 3.1 Visual inspection of phototrophic growth on stone probes before and after biocidal treatments

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

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

210

211 3.2 Monitoring of phototrophic growth

212 3.2.1 Digital Image Analysis

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

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

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

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

229

230 3.2.2 Confocal laser scanning microscopy

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

233 Abundant filaments of phototrophic microorganisms were observed on the CA stone probes, as
234 revealed by the intense *in vivo* pigment fluorescence (Fig. 4a). In contrast and interestingly, on the
235 treated stone probes (BA, GA and TA) non-filamentous phototrophic microorganisms were
236 abundant; in general, only unicellular phototrophic microorganisms, displayed in green colour, were
237 observed for the treated stones (Fig. 4b,c,d). Photosynthetic-based cell clusters were specially
238 evidenced on BA-treated stone probes. However, filamentous structures depicting very low
239 fluorescence intensity were also detected on these samples (Fig. 4b). The lowest chlorophyll
240 fluorescence intensity, both from filamentous or unicellular microorganisms, was obtained for TA-
241 treated samples, where almost no cells were observed (Fig. 4d).

242

243 3.2.3 Photosynthetic biomass quantification

244 Photosynthetic biomass estimation (Fig. 5), performed by means of chlorophyll *a* extraction,
245 showed that the highest chlorophyll *a* content was obtained for control stone probes (CA), as
246 expected. GA-treated stone probes almost reached the same chlorophyll *a* concentration values as
247 CA. The value decreased for limestone probes treated with BA and TA. A significant decrease of
248 chlorophyll *a* content was observed for TA-treated stone probes, which strongly agrees with the
249 results obtained by DIA and CLSM.

250

251 3.2.4 Colour variation after biocide application

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

260

261 4. Discussion

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

274 DIA allowed establishing the extent of the microbial colonization along time. PCA-based DIA
275 protocols have proven to be useful for the delimitation of elements enclosed in highly correlated
276 images, as in the case of rock art paintings (Rogerio-Candelera et al. 2011; Rogerio-Candelera
277 2015), or the extent of microbial mats (Miller et al. 2010b; Coutinho et al. 2013). The common
278 point lies in the small amount of the total variance attributable to these features; for this reason, the
279 use of minority Principal Components becomes a tool fitted to this goal. In this context, the
280 combination of PCA and segmentation algorithms revealed that BA and GA treatments were not
281 effective to control the growth of phototrophic microorganisms tested in this work. On the contrary,
282 the colonized area of TA probes initially decreased until reaching a steady state at the end of the
283 experiment. This pointed out that this last treatment was the most effective out of three natural
284 biocides tested in this work.

285 CLSM provided valuable information not only on the complex structures of photosynthetic biofilms
286 developed on the stone probes, but also on their degree of resistance to biocide treatments, based on
287 the intensity of *in vivo* chlorophyll autofluorescence. This technique has demonstrated to be a useful
288 tool for direct investigation of the biocide activity within biofilms as it allows recognizing resistant
289 microorganisms (Davison et al. 2010; Bridier et al. 2011a,b). According to several authors, this

290 resistance to biocides is closely related to the organization of cells in a biofilm and its exopolymeric
 291 matrix (Xu et al. 2000; Mah et al. 2003; Izano et al. 2009; Bridier et al. 2011b). CLSM revealed that
 292 the application of cells free filtrates and plant extract on the stone samples caused changes in the
 293 microbial composition of the inoculated phototrophic community. These treatments suppressed the
 294 growth of filamentous phototrophic microorganisms, but promoted the growth of unicellular
 295 phototrophic microorganisms, probably *Chlorella* sp. and *Pleurocapsa* sp., originally present in the
 296 inoculum.

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

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

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

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

338 It should be noted that in a previous study, sound stone probes were impregnated with GAs and
 339 CFF of *Bga* and *T. harzianum*, which were further inoculated with phototrophic microorganisms, in
 340 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.

353

354 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 colourimetry were applied in order to evaluate a new procedure of stone bio-cleaning consisting of the application of cells free filtrates of *Trichoderma harzianum* and *Burkholderia 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 *Trichoderma 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 to choose 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.

381

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578

579 CAPTION FOR TABLES

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

582

583 CAPTION FOR FIGURES

584

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

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

590 **Figure 3.** Stone green covered areas measured by Digital Image Analysis during 45 days of
591 incubation on the non-treated (CA) and treated stone probes with: CFF of *Bga* (BA); CFF of
592 *Trichoderma harzianum* (TA) and Glycoalkaloids (GA). Vertical dashed lines indicate the
593 incubation days when biocidal treatments were applied on the stone probes.

594 **Figure 4.** CLSM images of photosynthetic-based biofilms developed on the stone probes after 45
595 days of incubation. A) green biofilm on CA samples; B) green biofilm on BA-treated stone probes;
596 C) green biofilm on GA-treated stone probes; and D) green biofilm on TA-treated stone probes.
597 Bars = 50 μm .

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

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602 **FIGURE 1**

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605 FIGURE 2

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614 **FIGURE 5**

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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