SECONDARY METABOLITES: APPLICATIONS ON CULTURAL HERITAGE

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SUMMARY

Biological sciences and related bio-technology play a very important role in research projects concerning protection and preservation of cultural heritage for future generations.
In this work secondary metabolites of *Burkholderia gladioli* pv. *agaricicola (Bga*) ICMP 11096 strain and crude extract of glycoalkaloids from Solanaceae plants, were tested against a panel of microorganisms isolated from calcarenite stones of two historical bridges located in Potenza and in Campomaggiore (Southern Italy). The isolated bacteria belong to *Bacillus cereus* and *Arthrobacter agilis* species, while fungi belong *to Aspergillus, Penicillium, Coprinellus, Fusarium, Rhizoctonia* and *Stemphylium* genera. Bga broth (unfiltered) and glycoalkaloids extracts were able to inhibit the growth of all bacterial isolates. Bga culture was active against fungal colonies, while Solanaceae extract exerted bio-activity against *Fusarium* and *Rhizoctonia* genera.

Key words: cultural heritage, calcarenite stones, bio cleaning, glycoalkaloids, *Burkholderia gladioli.*

INTRODUCTION

Bio deterioration of cultural heritage is mainly due to different processes: biofilm formation, bio corrosion caused by organic and inorganic acids, redox processes on cations from the mineral lattice, physical penetration by microbial communities etc. [Gómez-Alarcón *et al*. 1995; Warscheid and Braams 2000].
Microorganism are able to colonize surface and internal part of art stones causing serious degradation problems. Fungal colonies can penetrate deeply contributing to their mechanical deterioration.
These actions simultaneously allow the transport of water and nutrients through the porous medium, facilitating the colonization and concomitantly triggering biochemical deterioration of the interior of the stones [Gómez-Alarcón and De La Torre 1994]. The bio deterioration of artefacts commonly results from the complex interaction established among co-existing physico-chemical and biological activities [Warscheid and Braams 2000; McNamara and Mitchell 2005].
The maintenance and the conservation of cultural heritage can be carried out by using traditional chemicals, but many studies report that microorganisms are able to acquire chemical resistance [Bingaman and Willingham, 1994; McFeters *et al*., 1995]. For this reason, frequently, several chemicals need to be combined in order to achieve effective organisms' eradication.
Today, biological sciences and related bio-technology, can play a very important role in research projects concerning protection and preservation of cultural heritage for future generations. The study and knowledge of the complex interactions between colonizing organisms and chemical degradation processes represents a new and valuable tool for the preventive and the specific maintenance of artefacts.

Studies of microbial ecosystems on the artefacts have, only recently, put in a new light the bacterial world; these organisms, always confined to the role of deterioration agents, may specifically be useful as bioremediation agents [Fernandes, 2006].
Sulphate and nitrate reducers bacteria are the only microorganisms used for the removal of undesirable compounds (organic matter, crusts and mineral salts) from works of art: in anoxic conditions such bacteria are able to transform sulphate to hydrogen sulphide and nitrate to molecular nitrogen, which are both gaseous. Preliminary studies report the partial cleaning of black crusts after a *Desulfovibrio desulfuricans* application on movable objects, like marble statues [Gauri *et al*, 1989; Heselmeyer *et al*., 1991; Gauri *et al*., 1992; Cappitelli *et al*., 2006]. Using the selected strain *Pseudomonas stutzeri* A29, able to reduce nitrate in anaerobic conditions, 90% nitrate removal after 30 hours of application at 28°C was obtained [Ranalli *et al*., 1996, 2000]. More recently Ranalli *et al*.[2005] applied selected bacteria to the frescoes of Camposanto Monumentale of Pisa with some success.
The goal of this work was to test natural bio-cleaning products obtained from metabolism of some bacteria and of vegetal crops: these substances have a role in ecological function, including defence mechanism, are not harmful to human health, have a low environmental impact, highly selective and low cost.
There is an extensive literature concerning insecticidal, nematicidal, fungicidal and phytotoxic effects of active compounds produced by some bacteria and from Solanaceae plants [Brown and Morra 1997, Rosa and Rodrigues 1999, Elshafie *et al*., 2012, Ventrella *et al*., 2012].
Accordingly, in this work we tested the effects of secondary metabolites produced by *Burkholderia gladioli* pv. *agaricicola* ICMP 11096 strain (*Bga*), an aerobic gram-negative rod-shaped bacterium, which has the ability to produce in vitro secondary metabolites with relevant biological activities, and glycoalkaloids, important bioactive secondary metabolites commonly found in Solanaceae plants. As concerning *Burkholderia* the integral broth containing cells and the cells-free filtrate broth were used for assays.
The antagonist capability of these substances was investigated against a panel of microorganisms isolated from the calcarenite stones of two bridges located in Potenza and in Campomaggiore (Southern Italy) (Figure 1).

Figure 1. San Vito bridge in Potenza (a) and Della Vecchia bridge in Campomaggiore (b)

MATERIALS AND METHODS

Sampling, isolation and growth conditions

Samples were taken by carefully scraping off stone material using sterile swabs and scalpel in according to the *Italian Cultural Heritage Ministry Recommendation 3/80,* and were re-suspended in saline solution buffer (0.85% NaCl). Samples were duplicate and isolated by spread plating on PCA medium. For each sample, different colonies were selected and purified by streaking on PCA added with tetracycline hydrochloride (0.005 g/L) for fungi growth, and on PCA added with cicloxiamide (70-100 mg/L)) for bacteria growth. Colonies were examined for morphology, Gram reaction and catalase. Isolates were routinely cultivated in PCB and maintained frozen (-80°C) in skim milk.

DNA extraction

The total DNA was extracted from bacterial isolates by using the Marmur method [1961] modified. The total DNA was extracted from fungal isolates by using the Raeder and Broda method [1985] modified. 25 ng of DNA were used for PCR amplification.

16S rDNA amplification and sequencing

Synthetic oligonucleotide primers fD1 (AGAGTTTGATCCTGGCTCAG) and rD1 (AAGGAGGTGATCCAGCC) were used to amplify the 16S rDNA. PCR mixture, and PCR amplification conditions were performed as previously reported [Bonomo *et al*., 2008]. The PCR products were sequenced and DNA similarity was performed with the Gene Bank and EMBL database. The Gene Bank accession numbers of the sequences are reported in Table 1.

Internal transcribed spacer (ITS) region amplification

As described previously [White *et al*., 1990], primers ITS1 and ITS4 were used to amplify specific ITS regions of fungal ribosomal genes. PCR mixture and amplification conditions were performed as described by White *et al*. [1990]. The PCR products were sequenced, and DNA similarity was performed with the Gene Bank and EMBL database.

Antibacterial assay

Burkholderia gladioli pv. *agaricicola* ICMP11096 (*Bga*), obtained from International Collection of Microorganisms from Plant (ICMP), was used as reference strain and grown in King Agar B (KB) medium for 24 h at 30°C.

The antibacterial activity of *Bga* and cell-free filtrates of *Bga* was tested against all bacterial isolates by agar well diffusion method. The cell-free filtrate of *Bga* was obtained by inoculum of 150 mL of liquid minimal mineral medium (MM) with 1.5 mL of bacterial suspension and, after incubation at 30°C for 5 days, the culture was filtered (Millipore, 0.20 µm) [Elshafie *et al*., 2012].

Moreover, the antimicrobial activity of glycoalkaloids was also evaluated. Glycoalkaloids were obtained by unripe berries of *Solanum nigrum* (European Black Nightshade) and extracted by the method of Cataldi *et al*. [2005]. The extract was lyophilized and re-suspended in water to

obtain the stock solution of solamargine (principal component) at concentration of 500 µM. The agar media were inoculated with 60 µL of glycoalkaloids' solution, *or Bga* culture broth or cell-free filtrates of *Bga,* and after incubation for 3 days at 30°C the inhibition zone diameters were measured in cm.

Antifungal assay

The antifungal activity of *Bga* and cell-free filtrates of *Bga* was evaluated by diffusion method. Either *Bga* culture broth or cell-free filtrate of *Bga* was inoculated in PCA plates containing 1 cm^2 of fungal disc. After 4-5 days of incubation at 30°C, the diameter of fungal colonies were scored and measured in cm.
The fungitoxicity was expressed as percentage of growth inhibition (PGI) and calculated according to Zygaldo *et al*. [1994] formula:

$$PGI\ (\%) = 100(Gc-Gt)/Gc$$

Where: Gc represents the average diameter of fungi grown in PCA (control); Gt represents the average diameter of fungi cultivated on the treated PCA dish containing the antagonistic bacteria or filtrate.

RESULTS AND DISCUSSION

Identified microorganisms

On the basis of amplification and partial sequencing of 16S rDNA, we found that the bacterial isolated strains belong to *Bacillus cereus* and *Arthrobacter agilis* species, while fungi belong to *Aspergillus*, *Penicillium, Coprinellus Fusarium, Rhizoctonia,* and *Stemphylium* genera.
Table 1 describes the isolated bacterial strains, their molecular identification and the Gene Bank accession numbers of sequences.

Table 1. Molecular identification of bacterial strains isolated from the two bridges

Strain	Identified as [a]	Accession number
A1I	*Bacillus cereus* (98%)	FJ763651.1
B3-1I	*Bacillus cereus* (96%)	EU857430.1
A22-2I	*Bacillus cereus* (98%)	EU661712.1
A15TI	*Bacillus cereus* (98%)	FJ435213.1
A3I	*Bacillus cereus* (97%)	EF382364.1
B3TIII	*Arthobacter agilis* (97%)	NR_026198.1

[a] % of similarity on the basis of partial sequencing of 16S rDNA

Inhibition activity

The ability of *Bga*, cell-free filtrate of *Bga* and glycoalkaloid extracts to inhibit the growth of bacteria and fungi isolated on the two bridges was evaluated in this study.
Results reported in Figure 2 proved that *Bga* broth and glycoalkaloids extracts were able to inhibit the growth of all bacterial isolates while cell-free filtrate broth of *Bga* inhibited only the growth of several strains of *Bacillus cereus*. It was not active against *Arthrobacter agilis.*

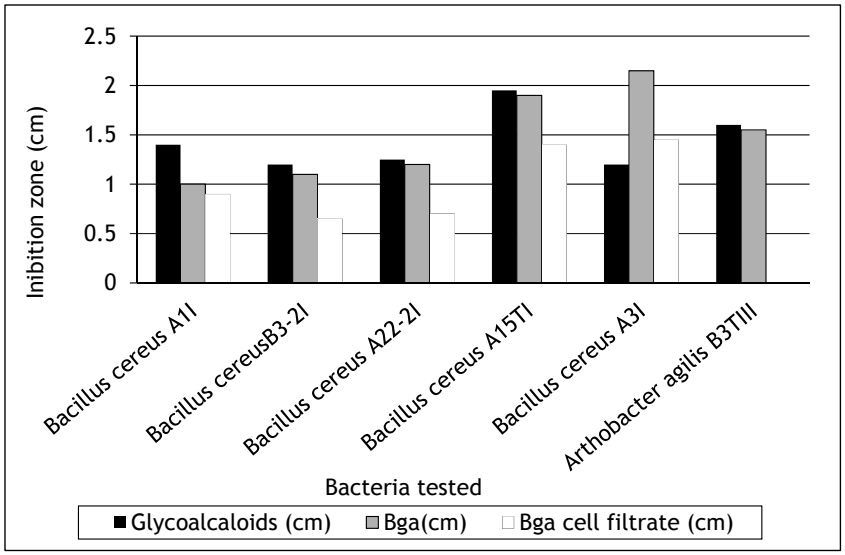

Figure 2. Antibacterial activity of glycoalkaloids, *Bga* and cell-free filtrate of *Bga*

As shown in Figure 3, *Bga* culture was more active against fungal colonies than the cell-free filtrate. The highest percentage of inhibition of *Bga* against fungal growth was observed versus *Penicillium* spp. (75%). The inhibition scale was *Penicillium > Stemphylium vesicarium> Coprinellus> Aspergillus*.

Solanaceae extracts tested against *Fusarium* and *Rhizoctonia* genera (Figure 4) showed a partial activity, confirming results obtained by previous research work [Ventrella *et al*, 2012]. Tests against other fungal colonies, isolated from the bridges, are in progress.

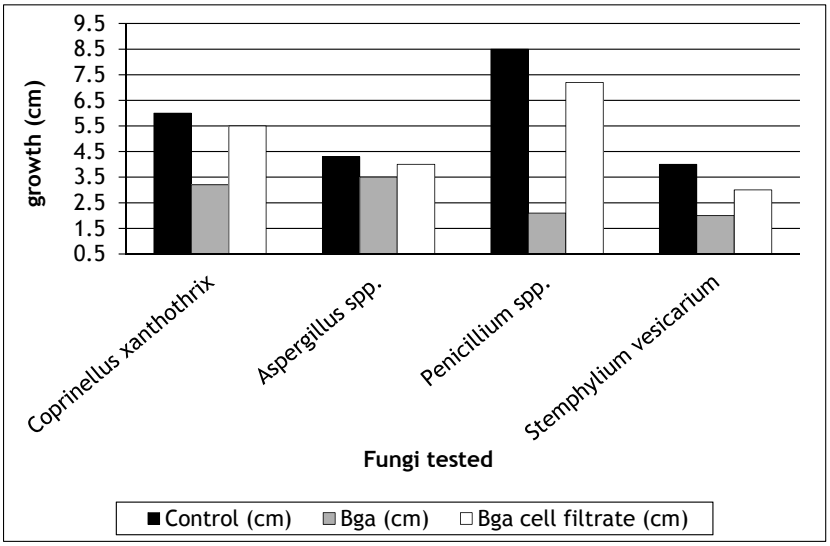

Figure 3: Antifungal activity of *Bga* and cell-free filtrate of *Bga*

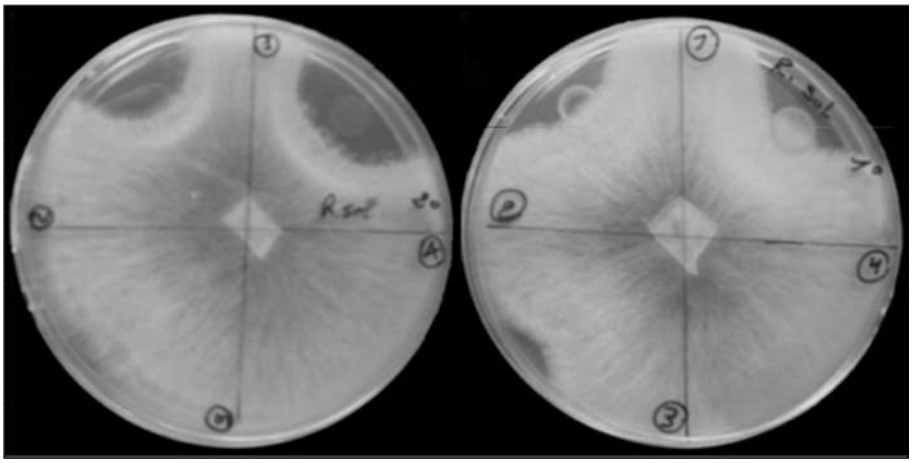

Figure 4. Inhibitory activity *of* glycoalkaloids against *Rhizoctonia solani strains*

CONCLUSION

Usually one of the first phases of the restoration of an artefact is the cleaning achievement, which allows the removal of the materials deposited on the surface. This action can cause irreversible damage on the structure, if it is not performed correctly. Then, it is necessary to carry out a selective procedure, through the deposit removal, in accordance with the surface zone features, by lightening or removing in depth, without direct contact with the original materials. Bio-cleaning by natural substances seems to meet these accuracy criteria.

Bga and glycoalkaloids showed an interesting activity against a panel of microorganisms isolated from two calcareous bridges. *Bga* and Solanaceae extracts were more selective especially against bacteria belonging to *Bacillus* and *Arthrobacter* genera while cell-filtrate of *Bga* showed a lower activity. These result could be due to the *Arthrobacter* genus resistance towards a variety of contaminants [Benyehuda *et al*., 2003; Margesin and Schinner, 1997].
Antifungal activity of these substances was less evident; probably, it could be due to the structural complexity of fungi.
The high activity of glycoalkaloids confirmed results of previous works that tested these substances for agricultural purposes [Jonasson and Olsson, 1994; Sinden *et al*. 1980, 1986].
The application of glycoalkaloids and metabolites of *Bga* on a cultural heritage could be an innovative challenge and an effective alternative to synthetic biocides for the cultural heritage preservation because it allows a homogeneous removal of the surface deposits preserving the substrate structure and favouring the maintenance of the dynamic equilibrium of the specific ecosystem.

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