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To cite this article: L. Scrano *et al* 2020 *IOP Conf. Ser.: Mater. Sci. Eng.* **949** 012096

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**240th ECS Meeting** ORLANDO, FL

Orange County Convention Center Oct 10-14, 2021



Abstract submission due: April 9

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# Natural biocides and bio-calcite: innovative tools for cultural heritage

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**Abstract.** Lithic artworks as churches, historical buildings and every usage object are our precious cultural heritage, memory of our past history step needed to build present and future. These artistic heritages with morphological, chemical and physical properties totally dissimilar make them a “unicum”, characterized by a specific vulnerability. Their decay is unavoidable, but it is a challenge for humankind to protect and preserve them. Thus far, solutions for the safeguard of cultural heritage are usually based on chemical procedures to remove biodeteriogen agents, but these substances can be hazardous to the environment, to public health and to stone materials itself because it is not known about the consequences of repeated applications. Then the restoration methods require innovative approaches, eco-friendly and not harmful to human beings and the environment. Aiming at this objective a deeply study on two rupestrian churches located in Matera (Italy) was carried out in order to prevent and/or control microbial and biological evolution, and to exploit the potential of some bacterial species in the interventions of stone consolidation. Surface analyses of detached rocks to evaluate the (i) stone composition, (ii) isolation and characterization of biological and (iii) microbial colonizers present on the stone surfaces were formerly performed.

*In vitro* bioactivity test against autotrophic/heterotrophic species isolated was carried out using secondary metabolites extracted from cultivated crops and spontaneous herbs with a strong biocidal activity (extracts from *Solanum nigrum*, *Volutaria lippii*, *Moricandia arvensis*, *Pulicaria inuloides*). Finally, calcinogenic activity tests employing native bacteria (*Bacillus strains*) isolated on stone surfaces of the two churches constitute the conclusive research step. Glycoalkaloids inhibited all bacterial strains tested. Some strains of *Bacillus* to produce the greatest amount of crystals, coherent with the stone substrate, was evidenced. This research highlights the opportunity to change the way of thinking and performing recovery actions for cultural heritage. Not last-minute remediation but continuous monitoring and time by time actions to avoid the formation of aggressive colonisations.

**Keywords:** rupestrian churches, natural biocides, bioconsolidation

## 1. Introduction

The deterioration of lithic artworks, our precious, historical and cultural heritage, is a common problem of the everyday. It is a challenge for humankind to protect and preserve these artworks. The decay process already starts when stones from the quarry are moved to their final location, and still continues as they are exposed to weathering agents. Sunlight, temperature, moisture, as well as atmospheric contamination due to anthropogenic pollutants, undoubtedly, accelerate the stone deterioration, which is primarily related to dissolution of calcium carbonate induced by the solvent action of acid rainwater and, secondly, because it holds in solution substances (carbonaceous particles,



sulphur compounds, soluble salts) responsible for leaching of surfaces, pH reduction, dark crusts, presence of efflorescences and subflorescences [1-4]. In addition, biological degradation, for a long time underestimated and based on the fact that the role of organisms is considered only when their presence is macroscopically identifiable is an important problem because on the stone the microorganisms begin to settle, often not visually perceptible, since the time of extraction in the quarry, beginning to form a complex ecosystem similar to that of the soil [5-10]. Natural stone conservation is not a recent theme, but it was a well-known problem in antiquity: for example, Roman architects had used either specific construction measures such as coverings, or canopies, and protective treatments with a surficial layer of masonry stucco [11]. In time, climbing plants and chromatic alterations on stone surfaces caused by biological growth were considered to enhance the aesthetic value of ruins [12], but the neglect of historic sites and its consequent deterioration, have had aggravated the preservation of cultural heritage [13]. However, it was understood that bacteria perform also a positive consolidation work on the stone thanks to their ability of calcium carbonate deposition favouring the phenomenon of calcinogenesis [14-15]. Therefore, it is evident how the safeguarding and conservation practices have to be pursued in a continuous and effective manner.

The traditional cleaning of stone surfaces on historical buildings to reduce/eliminate the biological colonizers are based on the use of preservatives aiming at inhibiting biological attacks [16-17] together with remedial methods [18] aiming at eradicating the biological agents responsible of bio-deterioration. This is mostly performed by mechanical action and/or chemical methods. The second ones foresee commonly used compounds (or a mixture of compounds) able to kill microorganisms or inhibiting the microbial growth. Inorganic species such as borates, sulphates, nitrates, bleaching agents such as hypochlorite, hydrogen peroxide and native chlorine, and organic materials such as formaldehyde, esters, methyl-phosphates, chloramines, and many other substances are available in the market [19]. These chemicals can be harmful to humans and the environment and can have detrimental effects on the stones too [20], but, unfortunately, information about the consequences of repeated applications are few [21].

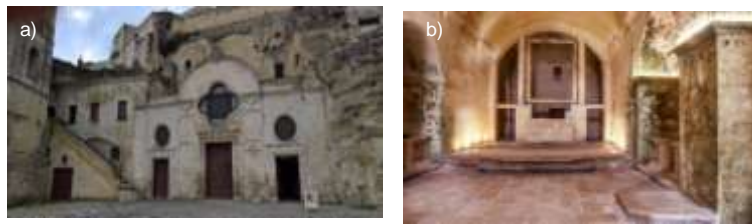
Hence, the recent trend is to search innovative approaches, eco-friendly and not harmful to human beings and environment like novel biocides based on natural compounds having the ability to interfere, at molecular level, with the microbial communication system, named “quorum sensing”, inhibiting the initial phase of biofilm formation [22], fundamental step for a subsequent biological colonization [23]. A promising biocontrol approach is the use of natural biocides based on secondary metabolites, i.e., substances obtained from the secondary metabolism of some microorganisms and/or plant extracts. *Solanum nigrum* (Solanaceae family), *Moricandia arvensis*, (Brassicaceae family), *Volutaria lippii* (L.) and *Pulicaria inuloides* (both belonging to Asteraceae family) were tested vs. a panel of autochthonous microorganisms collected, isolated and identified from two rupestrian churches, belonging to UNESCO World Heritage located in Matera (South Italy), chosen for the experimentation plan. The subsequent consolidation was carried out employing native bacteria isolated from the internal stone surfaces of the two churches: “San Pietro Barisano” (SPB) and “Santa Lucia alle Malve” (SLM).

## 2. Materials and methods

### 2.1. Investigated sites

The “San Pietro Barisano”, initially called “San Pietro in Veteribus”, is the largest rupestrian church in Matera, and it takes its current name from the Barisano district (where it is located), the most recent housing place in the Sassi.

The first building of the rupestrian church dates to around the year one thousand. It was restructured in the 13th century, while the masonry facade dates to 1755. From the outside one can see a bell tower erected on the left side of the church, which is placed on a rocky shelf with a higher level. At the entrance level 15th- and 16th-century frescoes of the Annunciation and a variety of saints can be found (Figure 1).



**Figure 1.** “San Pietro Barisano” church: a) the Main facade, b) biodeterioration internal status

The rupestrian church “Santa Lucia alle Malve” (Figure 2) is the first female monastic settlement of Benedictines, dating to VIII century, and the most important in the history of Matera.

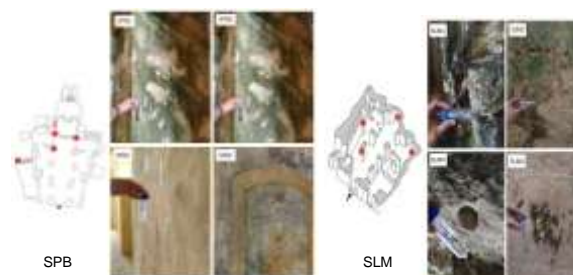
The external frontage is a part of a rocky slope. The entrance of the church, on its right side, is characterized by squared blocks of calcarenitic stone material very diffused in South Italy. On the internal walls of this church, located in the Sasso Caveoso – many mural paintings, mostly from the 13th century, are visible with a wide range of holy representations including the Nursing Madonna, the Archangel Saint Michael, Saint Gregory, Saint Benedict and Saint Scholastic.



**Figure 2.** “Santa Lucia alle Malve” church: a) the Main facade, b) detail of frescos and biodeterioration internal status

### 2.2. Sampling, biological degradation state assessment

Samples were collected from the inner surfaces of the two rupestrian churches based on the visual analysis according to the similarity of consistency and light and airflow exposure in both the churches. In the axonometric plans in figure 3, the sampling points are indicated with red dots in each church. Four samples were collected in each church according to their appearance and macrostructure: 1) mosses and bryophytes; 2) green algae; 3) lichens; 4) black crusts.



**Figure 3.** Sampling sites in “San Pietro Barisano” and in “Santa Lucia alle Malve”

Bryophytes and lichens were identified using light microscopy. Granulation of tissues was observed in polarized light. SEM (Zeiss Evo 40) observations were used to evaluate the shape of cells, spores and parasporal bodies of the isolated bacteria.

### 2.3. Petrographic Analysis

On small fragments of stones, detached from the walls of the rupestrian churches, petrographic analyses according to guidelines UNI 11176 were carried out. A petrographic microscope "ZEISS

Axioskop 40 POL" was used incorporating eyepieces of 2.5-10 magnifications by polarizing transmitted light, endowed with a micrometre for granulometric measurements and a digital camera.

X-ray powder diffraction (XRPD) analysis was performed by using a Philips PW1710 instrument equipped with a goniometric device with Bragg-Brentano geometry and Cu K $\alpha$  radiation (1.54178 Å) filtered on a curved graphite monochromator (X'Pert Pro). The analyses were conducted in analog/digital and were processed through software X'Pert Graphics, and phases and minerals were sought through database PDF2 plus. Stone fragment was previously coated with graphite and later observed in a LEO 50XVP Scanning Electronic Microscope (SEM), equipped with detector Penta FET with a Si (Li) operated at 25 KV for microanalysis in Energy Dispersive X-ray analytical system (EDS). The same analyses were carried out on the crystals produced from bacteria in order to verify the structure and the composition for the bio-consolidation purpose. These observations were performed after preparation of slides on coverslips and silver staining.

#### 2.4. Bacterial strains isolation and DNA extraction

In order to isolate bacterial strains and successive DNA extraction, both powder samples and cotton swabs were collected: powder samples by carefully scraping off material with a blade into sterile tubes according to the Italian Cultural Heritage Ministry Recommendation 3/80, swab samples by touching carefully surface with sterile dry swabs and storing them into sterile micro-tubes. All materials were stored at 4°C in controlled conditions until their use.

Samples were duplicate and isolated by spread plating on PCA (plate count agar) medium. For each sample, different colonies were selected and purified by streaking on PCA added with cicloxiamide (0.07-0.1 g/L) to only allow bacteria growth. The total DNA was extracted from bacterial isolates by using the Marmur method modified [24]. 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 [25]. The PCR products were sequenced using Genetic Analyzer 3130xl (Applied Biosystems), and DNA similarity was performed with the GenBank and EMBL database using the BLAST suite.

#### 2.5. Biocleaning: extraction from natural crops and antibacterial assay

*Solanum nigrum* (Solanaceae family), *Moricandia arvensis* (Brassicaceae family), *Volutaria lippii* (L.) and *Pulicaria inuloides* (both belonging to Asteraceae family) were cultivated crops and spontaneous herbs used for stone biocleaning. Glycoalkaloids (obtained from *Solanum nigrum*) were extracted by using Cataldi et al. (2005) method [26]. The extract was lyophilized and re-suspended in water to obtain the stock solution of solamargine (principal component) at a concentration of 500 µM. Extracts of *Moricandia arvensis*, *Volutaria lippii* (L.) and *Pulicaria inuloides* were obtained by using Milella et al. (2014) method [27]. Reagents were provided from Carlo Erba reagents S.r.l. (Cornaredo, MI, Italy) and Sigma-Aldrich (Steinheim, Germany).

The antimicrobial activity of seven plant extracts was tested using the modified Kirby-Bauer method [28] consisting in plating the bacterial strains on PCA medium, dispensing paper disks (Macherey-Nagel 640 d, GmbH & Co. KG) on the surface and adding 5 µl of the diluted 40% extract. Petri dishes were incubated at 30°C for 24 hours. The experiment was performed in triplicate and the antimicrobial activity, after incubation, was expressed as the average diameter of inhibition (mm).

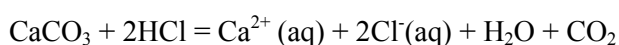
#### 2.6. Bioconsolidation: in vitro qualitative and quantitative determination of calcium-carbonate crystals

As the objective of this research is to proceed with bio-consolidation using native bacteria present on the stones and resistant to various climatic and anthropic conditions, bacteria strains collected on the walls and identified were tested for their capability to produce calcium-carbonate crystals *in vitro* growing on B4-agar medium (yeast extract 5 g/l, glucose 5 g/l, agar 15 g/l) with different concentration of calcium acetate: 0.25%; 0.5%; 1.0%; 1.5%. The medium was adjusted at pH using NaOH [29] and proper volumes of calcium acetate solution (25g/100ml), sterilized by filtering with

0.22 µm filter, were added after sterilization and cooling before pouring the medium in Petri dishes. Strains were plated using a pointed stick to ensure to have just a few colonies (only five per dish) for better growth and observation. Colony growth was observed using a stereoscope (ZEISS Stereo Lumar.V12) every 2-3 days for a total period of 20 days and crystal sizes were measured. The crystals produced were extracted from the agar medium, separated by precipitation, weighted and examined by using both X-ray diffraction (XRD) that Scanning Electronic Microscope (SEM).

In order to determine the amount of CaCO<sub>3</sub> produced by each bacterial species, after the qualitative analysis was carried out a quantitative analysis by titration with ethylenediaminetetraacetic acid (EDTA). This method exploits the complexation reaction between Ca<sup>2+</sup> ions in solution and EDTA a chelating agent. The CaCO<sub>3</sub> crystals collected were carefully transferred to a 100 mL volumetric flask and hydrochloric acid (HCl) diluted to 10% was added drop by drop. The volumetric flask was covered with a watch glass for twenty minutes until all solid carbonate has dissolved.

The obtained solution contains calcium ions according to the following reaction:



Distilled water and buffer solution were added, and pH value was measured. The solution was titrated with EDTA 0.005M.

### 2.7. Preparation of stone slabs

Bioconsolidation tests were conducted using a homogeneous limestone stone taken from a Matera quarry. This material selected and similar to the stones of the churches, was cut in slabs with 20 x 20 x 0.5 mm dimensions, washed with sterile water, covered with aluminium foil and autoclaved at 120 °C and 1 atm. for 20 min. Stone slabs, placed in a sterile test tube, were immersed in 20 mL of the B4 broth containing the bacterial suspension, grown overnight at 30°C in Plate Count Broth (PCB) supplemented with 1.5 % calcium acetate (v/v).

Control experiments identical to those indicated above were carried out without bacterial inoculation and B4 liquid medium alone. Both experiments were incubated at the same condition of 30°C for 40 days. Later, the stone slabs were removed and the calcinogenic activity of bacteria was evaluated by SEM-EDS.

## 3. Results

### 3.1. Biological degradation state assessment

Bryophytes and lichens, primary colonizers producing organic material and serving as a substrate for secondary colonists were identified on the internal walls of both churches. In particular, *Grimmia pulvinata* and *Tortula muralis* were identified. The first is a predominant species present on usually base-rich rocks and is a peculiar member of the wall community, while the second is the commonest moss on many mortared or base-rich walls – either brick or stone made – and can have different shades [30]. It also grows on concrete, roof tiles and other man-made structures, as well as outcrops of natural, base-rich rock. Both species are alongside and are also indirectly responsible for a small but significant input of nitrogen to the ecosystem, which is produced by cyanobacteria associated with the moss shoots [31].

From the samples collected in the churches, 18 colonies were isolated on the basis of their different morphology. Isolated strains were purified, and their DNA was used for 16S rDNA molecular identification, but only eight strains with the higher identity percentages were chosen (Table 1).

### 3.2. Petrographic analysis

The stones of two churches revealed to be a bio-lithoclastic packstones medium-grained, whitish in colour with a high porosity (Gravina limestone) mainly composed by calcite containing a low magnesium amount and minor constituents as kaolinite, hillite, chlorite, smectite and halloysite, gibbsite and goethite finely disseminated, quartz and feldspar present as individual grains.

**Table 1.** Identification of bacterial strains isolated from rupestrian churches

| Identified                          | Identity <sup>a</sup> | Colony form | Colony margin |
|-------------------------------------|-----------------------|-------------|---------------|
| <i>Staphylococcus warneri</i>       | 84%                   | circular    | smooth        |
| <i>Paenibacillus profundus</i>      | 91%                   | circular    | smooth        |
| <i>Bacillus cereus</i>              | 86%                   | irregular   | undulate      |
| <i>Bacillus sonorensis</i>          | 93%                   | complex     | lobate        |
| <i>Bacillus licheniformis</i>       | 80%                   | circular    | smooth        |
| <i>Bacillus thuringiensis</i>       | 91%                   | circular    | smooth        |
| <i>Planococcus massiliensis</i>     | 82%                   | irregular   | undulate      |
| <i>Paenibacillus dendritiformis</i> | 96%                   | circular    | smooth        |

<sup>a</sup> % of similarity on the basis of partial sequencing of 16S rDNA

### 3.3. Bacterial strains isolation and DNA extraction

Table 2 reports the antimicrobial activity of the extracts of *Moricandia arvensis*, *Volutaria lippii*, *Pulicaria inuloides* and glycoalkaloids towards the isolated bacterial colonies collected from the walls of the two churches.

**Table 2.** Antimicrobial activity of *Moricandia arvensis* (A), *Volutaria lippii* (V), *Pulicaria inuloides* (P) and glycoalkaloids (GAs) scored as the mean diameter of inhibition zone (mm) obtained on three replicates  $\pm$  standard deviation

| Bacterial strain tested             | A     | V     | P     | GAs                     |
|-------------------------------------|-------|-------|-------|-------------------------|
| <i>Staphylococcus warneri</i>       | 0.6 a | 0.6 a | 0.6 a | 11.01 $\pm$ 1.73 a,b    |
| <i>Paenibacillus profundus</i>      | 0.6 a | 0.6 a | 1.1 a | 14.83 $\pm$ 1.26 a,b,c  |
| <i>Bacillus cereus</i>              | 0.8 a | 0.9 a | 1.1 a | 15.33 $\pm$ 0.58 a,b,c, |
| <i>Bacillus sonorensis</i>          | 0.8 a | 0.8 a | 1.1 a | 13.17 $\pm$ 0.76 a,b    |
| <i>Bacillus licheniformis</i>       | 0.8 a | 1.0 a | 1.2 a | 10.83 $\pm$ 1.04 a      |
| <i>Bacillus thuringiensis</i>       | 0.8 a | 1.0 a | 1.2 a | 14.17 $\pm$ 6.01 a,b,c  |
| <i>Planococcus massiliensis</i>     | 0.8 a | 1.4 a | 1.2 a | 13.17 $\pm$ 2.75 a,b    |
| <i>Paenibacillus dendritiformis</i> | 0.7 a | 0.8 a | 1.2 a | 13.50 $\pm$ 0.50 a,b,c  |

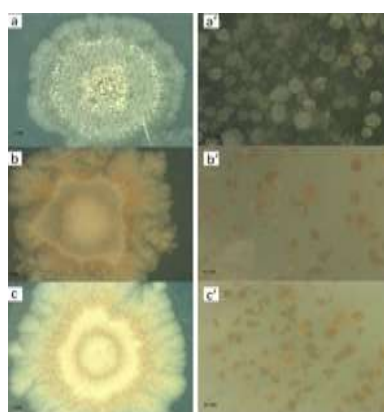
Strangely results obtained from other researchers about the antimicrobial activity of essential oils against Gram+ and Gram- bacteria are much more interesting respect this study case. In particular, *Pulicaria inuloides* extracts showed activity against *Staphylococcus aureus* and *Streptococcus pneumoniae* (Gram+) and no effect on *Salmonella typhimurium* and *Shigella dysenteriae* (Gram-). Same results were reported for *Volutaria lippii* (L.) and *Moricandia arvensis* [32]. Researchers involved in these studies have reported that these results could be explained by the fact that Gram-positive strains have only a peptidoglycan layer, which is not a selective barrier to plant extracts, and that the antibacterial activity could be due to the presence of a high concentration of phenolic compounds [32-34].

In this study, instead, the antimicrobial activity of tested substances was lower than glycoalkaloids that inhibited all isolated bacterial strains confirming results obtained by Sasso et al. [35, 36], who applied crude extracts of glycoalkaloids from Solanaceae plants against a panel of microorganisms (bacteria and fungi) isolated from calcarenite stones of two historic bridges located in Southern Italy: *Bacillus cereus* and *Arthrobacter agilis*, the isolated bacterial species, *Aspergillus*, *Penicillium*, *Coprinellus*, *Fusarium*, *Rhizoctonia* and *Stemphylium*, the fungal genera colonies. Glycoalkaloids extracts were able to inhibit the growth of all bacterial isolates and *Fusarium* and *Rhizoctonia* fungi. In the agricultural field, glycoalkaloids extracted from potato leaves were demonstrated to exert, also, negative effects on the hatching success of *Spodoptera exigua* eggs, and on the heart contractile activity of three beetle species *Zophobas atratus*, *Tenebrio molitor*, and *Leptinotarsa decemlineata* [37-39]. Moreover, leaf extracts and single pure glycoalkaloids have been demonstrated to affect the development and reproduction of *Drosophila melanogaster* [40].

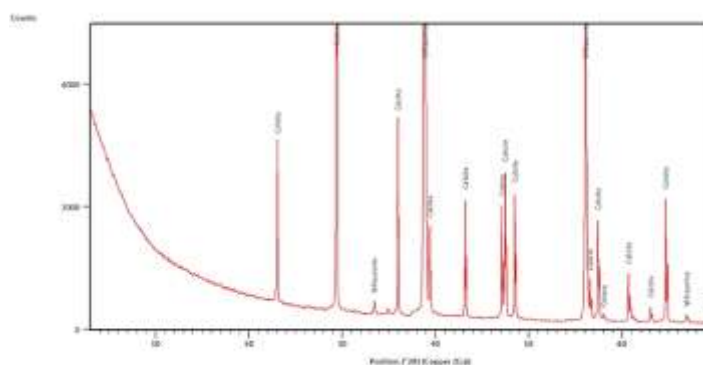
### 3.4. Bioconsolidation: *in vitro* qualitative and quantitative determination of calcium-carbonate crystals

Petri plates were examined periodically by optical microscopy for monitoring the formation of crystals. After 20 days of incubation at 30°C (Figure 4) all autochthonous selected strains produced large and visible to the naked eye crystals included in their colonies grown on the B4 solid medium supplemented with calcium-acetate). No carbonate crystals were visible as precipitates in the controls.

XRPD analysis conducted on crystals precipitated inside the bacterial colonies confirmed their identity as calcite (Figure 5).

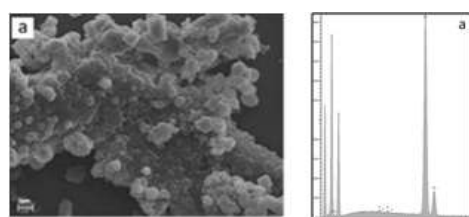


**Figure 4.** Stereoscopic observation of strains' colonies grown on the B4-agar medium supplemented with calcium acetate for biomineralization and details of the produced crystals: *Paenibacillus profundus* (a-a'); *Bacillus cereus* (b-b'); *Bacillus sonorensis* (c-c')

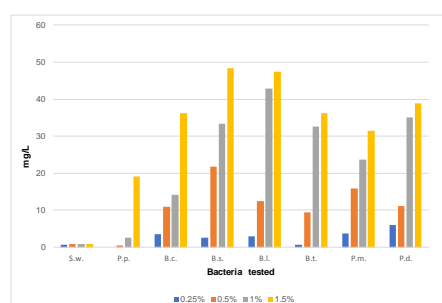


**Figure 5.** XRD spectrum of  $\text{CaCO}_3$  crystal products by *Bacillus cereus*

Further confirmation was obtained from the Scanning Electron Microscope (SEM) observation and EDS analysis (Figure 6).



**Figure 6.** SEM images of  $\text{CaCO}_3$  crystal products by *Bacillus cereus* and related EDS analysis



**Figure 7.** Results of EDTA titration. On the abscissa axis are indicated the bacteria tested, on the ordinate axis the amount ( $\text{mmol L}^{-1}$ ) of  $\text{CaCO}_3$  produced in different concentration (in g) of calcium acetate. S.w. = *Staphylococcus warneri*; P.p. = *Paenibacillus profundus*; B.c. = *Bacillus cereus*; B.s. = *Bacillus sonorensis*; B.l. = *Bacillus licheniformis*; B.t. = *Bacillus thuringiensis*; P.m. = *Planococcus massiliensis*; P.d. = *Paenibacillus dendritiformis*

It is evident that the maximum concentration of calcium acetate dissolved in the agar medium triggers the production of crystals (except for *Staphylococcus warneri*). *Bacillus licheniformis*, *Bacillus thuringiensis* and *Paenibacillus dendritiformis* show the same behaviour when the calcium



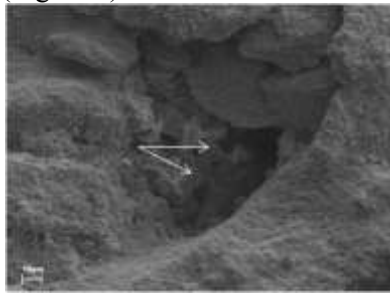
content in the agar medium is 1% and 2%. The other bacteria produce a major quantity of crystals adding 2% of calcium acetate in the nutritive solution (Figure 7).

### 3.5. Stone bioconsolidation under controlled conditions

As previously stated, the cultivable native bacteria collected from the walls of the churches and selected on the basis of calcium carbonate production have been used for *in vitro* stone bioconsolidation test.

Only *Staphylococcus warneri* and *Paenibacillus profundus* have not been used as they did not show an interesting biomineralizing activity in Petri dishes. All isolates of *Bacillus* strains have produced a good amount of calcite crystals adding 1.0 and 1.5 g of calcium acetate in the medium and showed in the stone the same behaviour obtained on Petri dishes.

In general, all bacteria produced rhombohedral crystals developed epitaxially in the pores of limestone, but it is necessary to precise the best activity of *Bacillus sonorensis* and *B. licheniformis* (Figure 8).



**Figure 8.** SEM images of calcite crystals precipitate by *B. licheniformis* on limestone Gravina slabs. Rhombohedral crystals developed epitaxially in the pores of limestone slabs immersed in B4 media with 1.5 g of calcium acetate.

## 4. Discussion

Results obtained with this research work have highlighted that the knowledge of the relationship among stone material, environment and microbial ecology is the starting point for development of the innovative suitable bio-cleaning and bio-consolidation methods confirming the previous studies on this topic [41-43]. Sampling and isolation of autochthonous bacteria, use of the best of those (in terms of calcinogenic activity) in stone bio-consolidation, stone surface cleaning by using secondary metabolites obtained from vegetables, follows the nowadays spread trend of using natural and eco-friendly products [44]. Considering that the rock churches belong to the UNESCO heritage and that many historical structures located in inhabited sites, unfortunately, need urgent cleaning and restoration actions, it seems almost essential to intervene promptly and green to safeguard heritage, environment and health. The application of microbial calcium carbonate precipitation (MCCP) as a stone reconsolidation methodology, furthermore, resulted to be a valid alternative to the common usage of concrete upon the historical monuments and buildings.

Results have confirmed that  $\text{CaCO}_3$  precipitation is bound by the amount of  $\text{Ca}^{2+}$  ions present within the B4 medium. In most cases, increasing the amount of calcium acetate an increased production of carbonates is ascertained. Better results have occurred by cultivating bacteria on B4-agar medium supplemented with 1.5% of calcium acetate solution (25g/100mL). The deposition of calcite crystals, geometrically perfect, was detected strongly adhered on the pores without plugging it on samples immersed in the culture medium. This phenomenon is important because it is necessary that the pores of the stones are not occluded but free to “breathe” i.e., to allow vapour transfer [45].

In a future perspective, it is necessary to specify that in particular fields like bioremediation/bioconsolidation generalization is not recommended, but each case study must be evaluated individually.

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