CONSERVAR PATRIMÓNIO

ARTICLE / ARTIGO

Evaluation of the antifungal efficiency of biocides currently applied in the Coimbra UNESCO area limestone monuments

Avaliação da eficiência antifúngica de biocidas atualmente aplicados em monumentos calcários da área UNESCO de Coimbra

JOÃO TROVÃO [1*]
ANTÓNIO PORTUGAL [1,2]

1. Centre for Functional Ecology, Department of Life Sciences, University of Coimbra, Calçada Martim de Freitas, 3000-456 Coimbra, Portugal
2. Fitolab-Laboratory for Phytopathology, Instituto Pedro Nunes, 3030-199 Coimbra, Portugal

*jtrovaosb@gmail.com

Abstract

The application of biocides to control microbial proliferation in stone materials is nowadays a rather common practice. In the Coimbra´s UNESCO World heritage monuments, biocide treatments rely on the application of Biotin T and Preventol R80. However, the application of these formulations often occurs without the complete knowledge of microbial communities' susceptibility to their application. The aim of this study was to evaluate the efficiency of these compounds and compare them with a commercial version of *Ocimum basilicum* essential oil, against various fungal species present in these areas. Through the application of *in vitro* antifungal activity assays, we were able to verify that, in general, Biotin T rather than Preventol R80 was more efficient against all tested fungi. In addition, the commercial version of the *Ocimum basilicum* essential oil also exhibited good results and might be an alternative option to be taken into consideration in future interventions in these monuments.

KEYWORDS
Biodeterioration
Biotin T
Fungi
O. basilicum essential oil
Limestones
Preventol R80

Resumo

A aplicação de biocidas para controlar a proliferação microbiana em materiais rochosos é hoje em dia uma prática bastante comum. Nos monumentos do património mundial da UNESCO de Coimbra, os tratamentos à base de biocidas têm consistido na aplicação de Biotin T e Preventol R80. No entanto, a aplicação dessas formulações ocorre muitas vezes sem o conhecimento completo da suscetibilidade das comunidades microbianas à sua aplicação. O objetivo deste estudo foi avaliar a eficiência destes compostos e compará-los com uma versão comercial do óleo essencial de *Ocimum basilicum*, contra várias espécies de fungos presentes nessas áreas. Por meio da aplicação de ensaios de atividade antifúngica *in vitro*, pudemos verificar que, em geral, o Biotin T, comparativamente ao Preventol R80, foi mais eficiente contra todos os fungos testados. Adicionalmente, a versão comercial do óleo essencial de *Ocimum basilicum* também apresentou bons resultados e pode ser uma opção alternativa a ser considerada em futuras intervenções nestes monumentos.

PALAVRAS-CHAVE
Biodeterioração
Biotin T
Fungos
Óleo essencial de *O. basilicum*
Calcários
Preventol R80

Introduction

Studies in cultural heritage monuments ultimately aim at providing, improving and evaluating practical solutions that can control microbial proliferation and consequently biodeterioration. The application of biocides to avoid microbial proliferation in stone materials is nowadays a rather common practice [1-3]. While the application of these molecules has long been reported to be highly efficient, their effective application relies on their putative negative impacts for humans, the environment and on the stone itself [1]. Moreover, recolonization after their application is also known to occur, thus often requiring their reapplication over time. In fact, the monitoring of microbial recolonization after the application of the treatment is still a topic being debated and a subject of interest in ongoing studies (e.g., [4]). For this reason, the continuous search for more efficient and greener biocides is still a widely researched topic today [2, 5]. For instance, in a recent study conducted by Marco et al. [6], the authors isolated various fungal species thriving in Portuguese mural paintings and evaluated the efficiency of several commercial biocides and natural essential oils against them. The authors verified that essential oils from *Ocimum basilicum* displayed a strong antifungal efficiency, thus highlighting these molecules as environmentally friendly green biocide alternatives [6].

Upon Coimbra´s application for the UNESCO World heritage site recognition, various *in situ* tests aiming for the evaluation of common chemical biocides against biological proliferation were performed. In 2007, the University tower was subjected to various cleaning attempts using both Biotin T and Preventol R80 (now Preventol RI80) at standard concentrations (3 % [w/v]). At the time of application for the UNESCO distinction, the staff responsible for such interventions had verified that no major differences between both products could be detected, although when considering color variations (ΔE), Biotin T was slightly closer to the controls considered. Moreover, they also noted that a trend was observable in areas with lichen colonization, where Preventol R80 was more efficient, while on the walls where fungi and algae were dominant, Biotin T was sometimes more effective (UNESCO executive summary). Both Biotin T (alkyl-benzyl-dimethylammonium chloride and isopropyl alcohol) and Preventol R80 (alkyl dimethyl benzyl ammonium chloride and isopropanol) have been widely and somewhat efficiently and safely used in the preservation of cultural heritage stone monuments in the past (Preventol R80 has, however, been reported as sometimes causing aesthetic modifications in the stone material) [1, 3]. Various additional interventions attempting the removal of the existing microbial populations with these formulations are still being conducted nowadays in monuments of the Coimbra´s UNESCO area. While necessary and welcomed, such tests are still being carried out without taking into account the complete characterization of the proliferating microbial communities and their resistance to such types of interventions. This entails a potential risk for the conservation of such monuments, since it can result in the incomplete removal of microbial populations, while also allowing future re-colonization events to take place [7-8]. This threat holds particular problems when considering that, for instance, in the beginning of 2021, full interventions have been scheduled to take place in various monuments in this area (for instance in the Old Cathedral of Coimbra).

From the diversified populations able to colonize stone relics, fungi play crucial roles on their sever and often irreparable biodeterioration [9]. In fact, fungi have nefarious effects on the typical lithotypes applied in these areas [10-13]. Nonetheless, their resistance to the commonly applied biocides in the Coimbra´s UNESCO areas remains so far uncharacterized. With this in mind, the aim of this study was to evaluate the efficiency of the above-mentioned Biotin T and Preventol R80 (at the currently standard concentrations applied) against various fungal species previously isolated from monuments in this area [10-11].

Materials and methods

Fungal cultivation, agar disc diffusion testing and statistical analysis

To evaluate the biocides efficiency on fungal proliferation, 13 fungal species were selected for *in vitro* testing, namely *Aspergillus glaucus*, *Aspergillus westerdijkiae*, *Parengyodontium album*, *Penicillium angulare*, *Penicillium brevicompactum*, *Penicillium chrysogenum*, *Penicillium crustosum*, *Penicillium glabrum*, *Penicillium scabrosum*, *Periconia byssoides*, *Stereum hirsutum*, *Trichoderma atroviride* and *Valsaria spartii*. These isolates were previously retrieved from dolomitic and/or Ançã limestone monuments in the Coimbra´s UNESCO site and identified through morphological and molecular methods [10-11]. Fresh fungal cultures were plated on potato dextrose agar (PDA) and grown in the dark, at room temperature (27±1 °C), for a period of 30 days. After this period, the fungal mycelium was scrapped with a sterile scalpel, placed in sterile deionized water, briefly homogenized with a sterile piston and the concentration adjusted to circa 10^5 cells/ml with the aid of a hematocytometer.

The biocide efficiency of the compounds under testing was evaluated by employing the Agar disc diffusion methodology [14]. For the preparation of both compounds, Biotin T and Preventol R80, were diluted to a concentration of 3 % (w/v) in sterile deionized water. Moreover, due to its extreme viscosity, Preventol R80 dilution was further performed by slightly heating the solution. The efficiency of both compounds was compared with a pure commercial version of *Ocimum basilicum* essential oil (methyl chavicol) (Biover, Belgium). The company produces totally organic oils, obtained by steam distillation, with their composition being characterized through gas chromatography.

Fresh PDA plates were prepared using Petri dishes of 90 mm diameter and the fungal inoculation was performed by the addition of 100 µl of the previous obtained spore solutions followed by uniform spreading using a microbiology plate spreader. A volume of 25 µl of each tested compound was inoculated on Whatman antibiotic assay paper discs of 9 mm diameter (Sigma-Aldrich, USA) and placed in the center of the Petri dishes, and the set up incubated at 27±1 °C for a period of fifteen days. Two replicas were considered for each treatment. Upon the ending of the incubation period, the verified halos around colonies were measured, the Petri dishes photographed and the obtained data further processed using the ImageJ software (National Institutes of Health, USA). Statistical analyses for the verified halo areas around fungal development were achieved with two-way ANOVA and Tukey´s multiple comparisons tests in GraphPad Prism 8.1 (California USA, www.graphpad.com).

Results

Tested biocides efficiency and statistical analysis

The results concerning the *in vitro* antifungal activity (halo diameters in cm^2) determined for each species tested can be verified in Figure 1. In general, Biotin T denoted the highest inhibitory action against all tested fungi. Additionally, examples of antifungal action can also be seen in Figure 2 and Figure 3.

Through the application of *in vitro* antifungal activity assays, using the Agar disc diffusion method, we were able to verify that, in general, Biotin T rather than Preventol R80, was much more efficient against all tested fungi. In addition, the commercial version of *Ocimum basilicum* (i.e., basil) essential oil (methyl chavicol) also revealed this compound to be generally more effective than Preventol R80. Thus, additional tests evaluating their current efficiency in stone samples are required and advised since, as verified by other authors, this compound might be a valuable, safe and green methodology to be taken into account in the future.

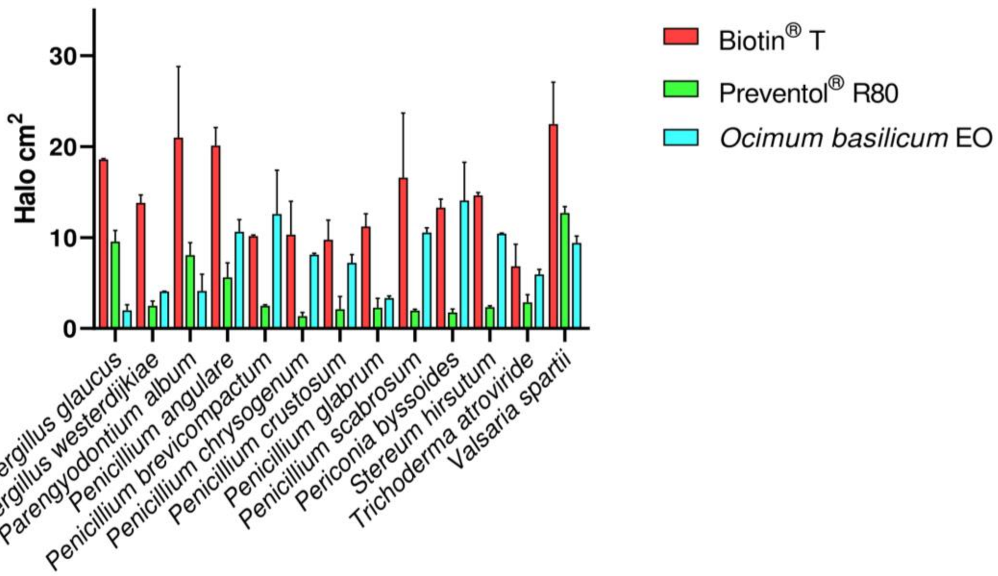

Figure 1. Overall results concerning the verified halo diameter (cm^2) for each species tested upon exposure to Biotin T, Preventol R80 and *Ocimum basilicum* essential oil.

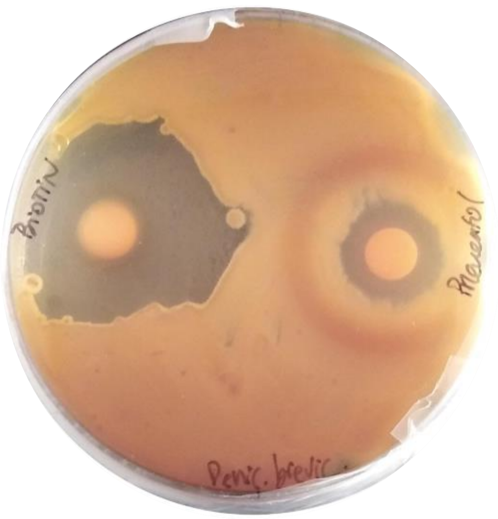

Figure 2. Example of a preliminary comparative evaluation of Biotin T and Preventol R80 efficiency against *Penicillium brevicompactum* evaluated through the Agar disc diffusion method.

Figure 3. Example of the efficiency of *O. basilicum* essential oil evaluated through the Agar disc diffusion method.

Particularly Biotin T sensitive fungi were *A. glaucus*, *P. album*, *P. angulare*, *P. scabrosum* and *V. spartti*. In parallel, and with only a few exceptions (e.g., *A. glaucus*, *P. album* and *V. spartti*), Preventol R80 exhibited the lowest inhibitory action. Complementarily, the *Ocimum basilicum* essential oil was somewhat highly efficient against almost all fungi. Particularly sensitive to this compound were *P. angulare*, *P. brevicompactum*, *P. scabrosum*, *P. byssoides*, *S. hirsutum* and *V. spartti*.

The results obtained for the statistical analysis can be verified in Table 1 to Table 3. The Two-way ANOVA results revealed highly significant statistical differences between each species (19.72 % of total variation), but also noteworthy variances among the three tested compounds (49.05 % of total variation). Moreover, a highly significant statistical difference could also be verified for the interaction analysis (the response of each species to each treatment) (23.39 % of total variation).

Table 1. Two-way ANOVA summary results obtained for the *in vitro* antifungal activity assays.

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	23.39	< 0.0001	****	Yes
Row Factor (Species)	19.72	< 0.0001	****	Yes
Column Factor (Compound)	49.05	< 0.0001	****	Yes

* level of significance.

Table 2. Two-way ANOVA table obtained for the *in vitro* antifungal activity assays.

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	Interaction	668.9	24	27.87	F (24.39) = 4.849
Row Factor	Row Factor	563.8	12	46.98	F (12.39) = 8.175
Column Factor	Column Factor	143	2	701.3	F (2.39) = 122.0
Residual	Residual	224.1	39	5.747	

Through the analysis of the Tukey´s multiple comparisons tests, further details concerning the impacts of each tested compound in each species could also be verified (Table 3). When considering the efficiency of Biotin T compared with Preventol R80, all species, with the exception to *T. atroviride*, exhibited statistical differences. Particularly, differences were mainly accentuated in *A. westerdijkiae*, *P. album*, *P. angulare*, *P. scabrosum*, *P. byssoides* and *S. hirsutum*. On the other hand, when considering the efficiency of Biotin T compared with the *Ocimum basilicum* essential oil, for six species (*P. brevicompactum*, *P. chrysogenum*, *P. crustosum*, *P. byssoides*, *S. hirsutum* and *T. atroviride*) no statistical differences could be detected. Nonetheless, particularly high statistical differences were observed for *A. glaucus*, *P.* album and *V. spartii*. Lastly, when considering the efficiency of Preventol R80 compared with *Ocimum basilicum*, for seven species (*A. westerdijkiae*, *P. album*, *P. angulare*, *P. crustosum*, *P. glabrum*, *T. atroviride*, and *V. spartii*) no statistical differences could be detected. For this case, statistical differences were particularly accentuated solely for the case of *P. byssoides*.

Discussion and Conclusion

This study aimed at evaluating the efficiency of the currently applied *in situ* preservation strategies at the Coimbra´s UNESCO World heritage site, under laboratory conditions. The data obtained during the course of this work provides updated information concerning the susceptibility against chemical compounds of fungal species present in these areas. As such, this work also provides valuable data that can help to improve, in a practical and applied manner, the conservation and preservation of these cultural heritage relics.

The pioneer study in this area was conducted in the University tower and employed Biotin T and Preventol R80 at standard concentrations (3 % [w/v]) against complex and diversified microbial proliferations (UNESCO executive summary). These procedures were conducted by initial brushing, and subsequently by through a tri-phasic approach: initial Preventol R80 application, followed by a second differentiated application using Biotin T, and a final re-application with Preventol R80. In a recent study, Favero-Longo et al. [15] demonstrated that in lichens, application of biocides (Preventol RI80) using cellulose poultices was more effective than brushing, thus highlighting that the application protocol impacts the effectiveness of biocides. Further studies are pending regarding the impacts that can occur when considering fungal communities. However, they are here highlighted as being of crucial importance, in order to further understand these organisms' responses to the treatments currently applied in the Coimbra´s UNESCO areas.

Table 3. Details of the Tukey's multiple comparisons tests.

Tukey's multiple comparisons test	Mean Diff	95,00% CI of diff	Significant?	Summary	Adjusted P Value
	Aspergillus glaucus				
Biotin T vs. Preventol R80	9.019	3.178 to 14.860	Yes	**	0.0016
Biotin T vs. *Ocimum basilicum* EO	16.580	74 to 22.420	Yes	****	<0.0001
Preventol R80 vs. *Ocimum basilicum* EO	7.560	1.719 to 13.40	Yes	**	0.0085
	Aspergillus westerdijkiae				
Biotin T vs. Preventol R80	11.310	5.470 to 17.150	Yes	****	<0.0001
Biotin T vs. *Ocimum basilicum* EO	9.743	3.902 to 15.580	Yes	***	0.0006
Preventol R80 vs. *Ocimum basilicum* EO	-1.569	-7.409 to 4.272	No	ns	0.7911
	Parengyodontium album				
Biotin T vs. Preventol R80	12.92	7.078 to 18.760	Yes	****	<0.0001
Biotin T vs. *Ocimum basilicum* EO	16.88	11.04 to 22.720	Yes	****	<0.0001
Preventol R80 vs. *Ocimum basilicum* EO	3.957	-1.884 to 9.798	No	ns	0.2371
	Penicillium angulare				
Biotin T vs. Preventol R80	14.50	8.659 to 20.340	Yes	****	<0.0001
Biotin T vs. *Ocimum basilicum* EO	9.489	3.648 to 15.330	Yes	***	0.0009
Preventol R80 vs. *Ocimum basilicum* EO	-5.011	-85.0 to 0.830	No	ns	0.1050
	Penicillium brevicompactum				
Biotin T vs. Preventol R80	7.685	1.844 to 13.530	Yes	**	0.0074
Biotin T vs. *Ocimum basilicum* EO	-2.401	-8.242 to 3.439	No	ns	0.5802
Preventol R80 vs. *Ocimum basilicum* EO	-10.090	-15.930 to -4.246	Yes	***	0.0004
	Penicillium chrysogenum				
Biotin T vs. Preventol R80	8.976	3.135 to 14.820	Yes	**	0.0017
Biotin T vs. *Ocimum basilicum* EO	2.187	-3.654 to 8.027	No	ns	0.6360
Preventol R80 vs. *Ocimum basilicum* EO	-6.789	-12.630 to -0.948	Yes	*	0.0195
	Penicillium crustosum				
Biotin T vs. Preventol R80	7.645	1.804 to 13.490	Yes	**	0.0078
Biotin T vs. *Ocimum basilicum* EO	2.544	-3.297 to 8.384	No	ns	0.5436
Preventol R80 vs. *Ocimum basilicum* EO	-5.101	-94.0 to 0.739	No	ns	0.0973
	Penicillium glabrum				
Biotin T vs. Preventol R80	8.906	3.065 to 14.750	Yes	**	0.0018
Biotin T vs. *Ocimum basilicum* EO	7.865	2.024 to 13.710	Yes	**	0.0061
Preventol R80 vs. *Ocimum basilicum* EO	-1.041	-6.882 to 4.800	No	ns	0.9016
	Penicillium scabrosum				
Biotin T vs. Preventol R80	14.600	8.763 to 20.440	Yes	****	<0.0001
Biotin T vs. *Ocimum basilicum* EO	6.036	0.195 to 11.880	Yes	*	0.0415
Preventol R80 vs. *Ocimum basilicum* EO	-8,568	-14.410 to -2.727	Yes	**	0.0027
	Periconia byssoides				
Biotin T vs. Preventol R80	11,520	5.684 to 17.370	Yes	****	<0.0001
Biotin T vs. *Ocimum basilicum* EO	-0.779	-6.620 to 5.061	No	ns	0.9435
Preventol R80 vs. *Ocimum basilicum* EO	-12.300	-18.140 to -6.463	Yes	****	<0.0001
	Stereum hirsutum				
Biotin T vs. Preventol R80	12.280	6.442 to 18.120	Yes	****	<0.0001
Biotin T vs. *Ocimum basilicum* EO	4.189	-1.652 to 10.030	No	ns	0.2009
Preventol R80 vs. *Ocimum basilicum* EO	-8.095	-13.940 to -2.254	Yes	**	0.0047
	Trichoderma atroviride				
Biotin T vs. Preventol R80	3.945	-1.896 to 9.785	No	ns	0.2391
Biotin T vs. *Ocimum basilicum* EO	0.895	-4.946 to 6.736	No	ns	0.9262
Preventol R80 vs. *Ocimum basilicum* EO	-3.049	-8.890 to 2.791	No	ns	0.4192
	Valsaria spartii				
Biotin T vs. Preventol R80	9.776	3.935 to 15.620	Yes	***	0.0006
Biotin T vs. *Ocimum basilicum* EO	13.060	7.220 to 18.900	Yes	****	<0.0001
Preventol R80 vs. *Ocimum basilicum* EO	3.285	-2.556 to 9.126	No	ns	0.3661

* level of significance.

When considering the results obtained during the course of this work, in general, Biotin T denoted the highest inhibitory action against all tested fungi. Nonetheless, when considering

T. atroviride no statistical differences were observed when compared with Preventol R80. Thus, similarities regarding this species susceptibility to both compounds should be expected. The overall results are in accordance with what the staff initially observed – i.e., higher Biotin T efficiency in zones where fungi and algae were dominant in the University tower. Moreover, these results are also in accordance with the currently available literature [1]. For instance, in a recent study, Li et al. [16] showed that isothiazolone (present in Biotin T) was more effective than benzalkonium chloride (present in Preventol R80) in the removal of *Aspergillus niger*, *Colletotrichum acutatum* and *Penicillium oxalicum* thriving in Feilaifeng limestones in Hangzhou, China. Moreover, Sanmartín and Carballeira [4], while working to evaluate the effects of similar biocidal treatments in heterotrophic microbial communities in the Monastery of San Martiño Pinario (Santiago de Compostela, Spain), also verified that Biotin T was the most effective treatment against fungal species. Additionally, Mateus et al. [17] also observed a good Biotin T efficiency against fungi retrieved from limestone walls of the unfinished Sacristy of the Convent of Christ (Tomar, Portugal). Nonetheless, it is also noteworthy to mention that while working on churches in Segovia (Spain), de los Ríos et al. [18] noticed that Biotin T application resulted in a cleaner stone surface, although unfortunately fungal hyphae were detected after two months of treatment application. Contrastingly, the works from Coutinho et al. [19] and of Vannini et al. [20], reported a higher efficiency of Preventol R80 rather than Biotin T [1]. As pointed by Li et al. [16], differences in the biocides additives and ratios may have a contribution for these differential results. Nonetheless, putative differences occurring due to differential species resistance mechanisms should also be taken into consideration, since they have, so far, barely been studied in detail. Nevertheless, taking into account the results obtained during the course of this work, *A. westerdijkiae*, *P. album*, *P. angulare*, *P. scabrosum*, *P. byssoides* and *S. hirsutum* were particularly susceptible to this compound, and thus good results might be expected if they are applied in areas where these fungal species are somewhat dominant. Complementarily, when considering the efficiency of Biotin T when compared to *Ocimum basilicum*, no statistical differences were detected for *P. brevicompactum*, *P. chrysogenum*, *P. crustosum*, *P. byssoides*, *S. hirsutum* and *T. atroviride* and similar antifungal spectrums might be expected. Nonetheless, due to the statistical differences verified, the application of Biotin T rather than *Ocimum basilicum* will likely be more efficient for cases with significant contamination by *A. glaucus*, *P. album* and *V. spartii*.

Regarding the usefulness of Preventol R80, the currently available literature points to various differential results [1]. Blazquez et al. [21] reported concentrations of 1 to 2 % as being efficient against microorganisms isolated from historic monuments. However, Monte et al. [22] noted that while working with microorganisms from frescoes, Preventol R80 concentrations of 10 % were the only ones able to effectively kill fungal species. Moreover, similar results were noted by Sanmartín and Carballeira [4], who verified that Preventol RI80 was ineffective for the removal of *Eutypa* sp. In addition, Marco et al. [6] also noted that Preventol R80 needed higher concentrations to reproduce similar results to the ones obtained with *Ocimum basilicum* essential oils. The results obtained during the course of this study highlight that for the species *A. westerdijkiae*, *P. album*, *P. angulare*, *P. crustosum*, *P. glabrum*, *T. atroviride*, and *V. spartii*, no statistical differences could be detected and, therefore, similarities in their antifungal spectrum might be somehow expected. Nonetheless, the obtained results also denoted that Preventol R80 exhibited the lowest antifungal activities. Nevertheless, some particularly Preventol R80 sensitive fungi could also be verified (*A. glaucus*, *P. album* and *V. spartti*). In parallel, the results also highlighted that, when compared, *Ocimum basilicum* rather than Preventol R80 will likely be more effective in dealing with cases of high contamination by *P. byssoides*.

Concerning the application of the *Ocimum basilicum* essential oil, the results revealed it to be somewhat highly efficient against almost all fungi. In addition, this compound might be particularly useful in cases of heavy *P. angulare*, *P. brevicompactum*, *P. scabrosum*, *P. byssoides*, *S. hirsutum* and *V. spartti* contamination. However, as pointed above, for the cases of *A. glaucus*, *P. album* and *V. spartti*, they might not display a high effectiveness. Nonetheless, the results obtained are in accordance with the findings of Marco et al. [6], who found a high antifungal efficiency for freshly extracted *Ocimum basilicum* essential oils. As such, additional studies aiming to further evaluate the most relevant chemical fractions (see for example [23]) and to understand possible differences between extracted and commercial version of such oils are needed. Moreover, in *Ocimum basilicum*, particular relevance should also be given to the extraction procedures conducted, the plant material origin and environmental factors, particularly considering that they are known to greatly affect the essential oil composition [24-25]. Nonetheless, the results obtained in this work demonstrate that the methyl chavicol extracts present in the commercial version of *Ocimum basilicum* essential oil might be a valuable and interesting greener alternative to be taken into consideration on further works aiming to protect stone monuments against fungal biodeteriorative action. Being a phenylpropene this compound can contribute to fungal oxidative stress [26] and plasma membrane alterations [27]. Nonetheless, further studies concerning their action mechanisms when considering, for instance, the tested species are also still needed and advised.

In parallel, and considering the results obtained, small changes in the currently tri-phasic biocides application can also be suggested. As such, in areas with high fungal contamination and/or notorious biodeterioration phenomena resulting from their action, the consideration to either increase Preventol R80 concentration or in parallel the workflow to include a possible forth phase with Biotin T might also be valuable hypothesis, requiring further analysis. On the other hand, if the application of essential oils is considered for these areas, alternative application methods should also be taken into account (in parallel to brushing or even the previously suggested application of cellulose poultices). For this purpose, protective coatings containing the encapsulated natural biocides might be a valuable alternative, since they have been recently demonstrated to be effective and compatible with various stone types [28]. Nonetheless, as pointed by Cappitelli et al. [2], if a biocide approach is planned to control microbial growth on a heritage surface, *in situ* pilot and small-scale testing are often necessary not only to attune the treatment but also to evaluate nefarious consequences of their application on the stone substrate. An additional aspect also requires attention and is related with the necessity of biocide reapplication. At first, this entails the systematic introduction of possible threats to the material, but might also contribute to the development of microorganism's biocide resistance or the development of more harmful populations (since they now have available niches often rich in micronutrients and lack competition) that can lead to future more severe, and difficult to control, outbreaks [5, 29]. Recolonization is generally expected and, as such, the selected biocide employed, the manner in which it is applied, as well as the material nature, its overall state of conservation and what alternative methodologies are available, should always be pondered in the process leading to the intervention. As such, additional studies concerning the putative negative impacts of all tested biocides on different lithotypes are also needed.

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