TRICHODERMA SPP. IN CULTURAL HERITAGE MURAL PAINTINGS OF ANCIENT EGYPTIAN TOMB, THEIR ANTIFUNGAL AND BIOACTIVITY

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ABSTRACT

[AR] **النشاط الحيوى وضد الفطرى للتريكوديرما فى التراث الثقافى للصور الجدارية فى مقبرة مصرية قديمة**

لقد تم عزل أجناس فطر التريكوديرما *Trichoderma* spp من أسطح الصور الجدارية بمقبرة «نفر-باو-بتاح». لقد تعرضت هذه المقبرة سابقاً فيعام 2007 لهجوم ميكروبى مُتمثل فى *A. niger* و*A. flavus* و*Alternaria alternata* فى عام 2019 تم عزل فطر الاسبرجلس نيجر فقط، بالاضافة إلى عزل أنواع من فطر التريكوديرما والتى لم يتم عزلها من قبل خلال عام 2007. لقد تم تحديد ثلاثة أجناس من فطر التريكوديرما وفقًا لتأثيرها الحيوى على *A.niger* و*A flavus* و*A. alternata* ومن خلال تسلسلها فى GenBank إلى *Trichoderma harzianum* و *T. hamatum*و*T. aureoviride*. و لقد تم تحليل اللوحات الجدارية فى حجرة الدفن بتقنية تحليل EDX الذى أظهر أن الطبقة الأرضية تتكون من الكوارتز والكالسيت والجبس، بينما أظهر تحليل المواد الملونة إستخدام الألوان المُتمثلة فى الأخضر المصرى والأزرق المصري والهيماتيت. ولقد أثبت تحليل الرامان أن الوسيط اللوني المُستخدم كان بمثابة وسيط صفار البيض. لقد تم قياس المواد الملونة باستخدام جهاز السبيكتروفتوميتر مرتين: الأولى خلال عام 2007م والثانية بعد نمو *Trichoderma* خلال عام 2019. ولقد أظهرت النتائج أن عملية التمثيل الغذائي خارج الخلية لفطر التريكوديرما اثر على اللون الأزرق المصري والأخضر المصري وتجدر الإشارة إلى أن نتائج التحليل قد أظهرت قدرة فطر التريكوديرما على تثبيط نمو *A. niger* و*A. flavus* و*Alternaria alternata* ، والتى تم العثور عليها سابقًا خلال عام 2007م. ولقد تم تحديد العوامل القياسية لتعزيز نمو فطر التريكوديرما،حيث أظهرت النتائج أن 5٪ من (1: 1) $NaCl: NaNO_3$ كان بمثابة أفضل تركيز للنشاط المضاد للفطريات لجميع أنواع *Trichoderma* عند درجة الحرارة الواقعة بين 30و 35 درجة مئوية في وسط حمضي. تجدر الإشارة إلى أن عملية الحفظ الحيوي للتراث الثقافى مازالت قليلة الاستخدام فى نطاق الشرق الأوسط، وهى تعتبر منهجية علمية وتقنية علاج صديقة للبيئة، وذلك مع الأخذ فى الإعتبار للمخاطر التى تُسبب التغير اللوني، بالإضافة إلى كونها تقنية سهلة التطبيق، حيث يُمكن تطبيقها في نطاق الحيز المكاني والبيئات المفتوحة أو في نطاق المتاحف.

[EN] *Trichoderma* spp. was isolated from the mural paintings' surfaces of the tomb of Nefer-Bau-Betah. This tomb was previously deteriorated in 2007 by *Aspergillus niger, A. flavus,* and *Alternaria alternata* but in 2019, the tomb was deteriorated by only *A. niger*, and *Trichoderma* species that had not been isolated before in 2007. Three species of *Trichoderma* were identified according to their bioactivity effect on these microorganisms and by their sequences in the GenBank to *Trichoderma harzianum, T. hamatum, and T. aureoviride.* Furthermore, mural paintings in the burial chamber were characterized by EDX analysis which indicated that the ground layer consisted of Quartz, Calcite and Gypsum, while the pigments were characterized as Egyptian green, Egyptian blue, and hematite (Fe_2O_3). The binding media was egg yolk according to the spectrum of Raman spectroscopy. The pigments were assayed by the spectrophotometer but it caused a little effect in the color change of the pigment inside the tomb especially the blue and green Egyptian pigments, while it caused less effect in the color change of red hematite pigment. The optimization factors for increasing the bioactivity of the *Trichoderma spp.* were 5% of sodium nitrate and sodium chlorine that crystallize in the tomb, where the average temperature is between 30:35°C in acidic pH (pH=5.5). These conditions helped *Trichoderma* species to grow and work as antifungal factors in the tomb *A. niger, A. flavus,* and *Alternaria alternata. Trichoderma spp*. can be used as a new methodology for controlling the deterioration of cultural heritage. It is an eco-friendly methodology, risk-free when controlling the color change, and easy to apply anywhere in *vivo* or *vitro* in cultural heritage found in open air or in the museums.

KEYWORDS: Applied microbiology, *Aspergillus*, Bioactivity, Biotreatments, biodeterioration, color changes, mural paintings, pharaonic tomb, *Trichoderma.*

I. INTRODUCTION

Biodeterioration of cultural heritage is considered as one of the most critical factors that may destroy the mural paintings, while other environmental factors like relative humidity, salts, organic matter, and high temperatures help in increasing the deterioration effect of the microorganisms[1].

Microorganisms in cultural heritage may cause biomechanical deterioration due to the growth or penetration of them or their parts such as hyphae, mycelium, fruiting bodies, and extensive root systems inside the pores of the stone and mural paintings which leads to cause cracks or micro-cracks[2]. Otherwise, it causes biochemical deterioration resulting from metabolic processes. Microorganisms can react with the mural painting minerals by their acids and enzymes or even toxic products. These acids decompose the minerals of pigments and shed layers by producing salt and chalets. When the soluble salts or chalets crystalize, they cause stresses in the mural paintings' pores resulting in cracks[3]. In addition to the aesthetic biodeterioration, the growth of microbes on the mural paintings' surfaces cover the pigments and destroy historical evidence[4].

Conservators around the world applied numerous methodologies to prevent the cultural heritage from biodeterioration, including physical treatments of radiation[5], fumigation[6], low-temperature helium[7], essential oils[8], chemical treatments by biocides[9], applied nanoparticles[10] and bio-control by valuable microbes cells[11].

Trichoderma strains were well known for their anti-microbial ability towards fungi by their bio-control agents. The production of secondary metabolites by *Trichoderma* strains showed high application potential in the cultural heritage field[12]. Although, *Trichoderma* species were well-known producers of enzymes essentially cellulases, Tanshinone IIA, Tanshinone I, and chitinases[13]. These enzymes could control the growth and activity of fungi[14].

Bio-cleaning treatment methodology is not widespread in Egyptian tombs and the cultural heritage field, it is still a new field in Egypt. In 2007, numerous fungi were isolated from the tomb Nefer Bou Betah, like *Aspergillus niger*, *Aspergillus flavus*,

[1] CRISPIM & GAYLARDE 2005: 1-9; FAVERO -LONGO & VILES 2020: 100.
[2] STERFINGER 2010: 47-55; BJELLAND & ET AL. 2011: 434-442; BARTOLI & ET AL. 2014: 157-165.
[3] ADAMIAK & ET AL. 2017: 2448; DYDA & ET AL. 2019: 416.
[4] EL HAGRASSY 2018: 43 – 50.
[5] DRÁBKOVÁ & ET AL. 2018: 75-80.
[6] KHAIRY& ET AL. 2019: 289-300.
[7] TURNAU& ET AL. 2020: 126485
[8] PALLA& ET AL. 2020: 730.
[9] FIDANZA & CANEVA 2019: 271-286; KAKAKHEL & ET AL. 2019: 104721.
[10] GALDIERO & ET AL. 2011: 8894-8918; REYES-ESTEBANEZ & ET AL. 2018:1-11.
[11] EL HAGRASSY & HAKEEM 2018: 43-50; TURNAU & ET AL. 2020: 126485
[12] KESZLER & ET AL. 2000: 421-424; HOELL & ET AL. 2005: 180-186.
[13] STERNBERG & DOVAL 1980: 181-192; DI PIETRO & ET AL. 1993: 308-313; MING & ET AL. 2012:330-333.
[14] LOEPPKY & ET AL. 1983: 798-799; SEGUIN & ET AL. 1995: 445-448; KLEIN & EVELEIGH 1998: 57-69; HARMAN & ET AL. 2004: 43-56.

Penicillium, Alternaria, Rhizopus[15]. The black fungi as *Aspergillus niger, Alternaria, and Rhizopus* were a problem and caused a color change in the tomb pigments. In 2020, species of *Trichoderma* were isolated from the same mural paintings in Nefer- Bau- Betah tomb, and a crystalline salt has deteriorated the mural paintings.

II. Material and Methods

1. Scanning Electron Microscope {SEM}

The scanning electron microscope was used for characterizing the Mural painting's structure. The model of the SEM was Philips XL 30, Jeol JSM (5600 LV) used for micrographs. The SEM microscope was associated with the EDX Unit, «30 KV. The image magnification started from (10x to 400.000x), with the resolution for W. about (3.5nm) ». The samples were examined without coating by gold, and the conductive layer had not been sputtered. The examination was applied on the whole sample as a map not as points.

2. Raman Spectroscopy

The spectroscopy was applied via a laser beam with an average wavelength of 785nm, the power was 25 MW while the aperture setting was 50*1000 μm. The cross-sections were scanned twice, and the spectrometer calibration was obtained from a silicon crystal in which the «Raman signal was at 520.5cm^{-1}».

3. Spectrophotometer

Color –E Y E- 3100- Spectrophotometer – operation manual, S D L Company. To essay the color change of the mineral pigments, primary points were selected. These points were measured both in 2007 and 2019 to indicate the ΔE according to this equation:

$$\Delta E^*_{ab} = \sqrt{(L^*_2 - L^*_1)^2 + (a^*_2 - a^*_1)^2 + (b^*_2 - b^*_1)^2}$$

, (L) indicates lightness, (a) is (green/ red) coordinate, and (b) is blue / yellow coordinate.

4. Fungal Strains

Fungi used in this study were obtained as follows:

A. *Aspergillus niger, Aspergillus flavus, A. alternate* that were previously isolated in summer 2007 and identified by (El Hagrassy; 2010). The identification of fungi genus was carried out according to their morphology and spore structures. (Richardson and Watling, 1997). Each single fungi was inoculated in 20 ml of YEA 01497, of YEM (Yeast extract media) from Sigma Aldrich and incubated for 84 h at 25°C.

B. *Trichoderma* species were isolated from the same mural paintings in summer and winter 2019 and cultured in Petri dish containing 20ml of Czapek's Dox broth medium and incubated for 7 days at 25°C. After this step the collecting mycelium and spores inoculated (vaccination) into 50ml of PDA for another 7 days at 30°C for optimal growth.

[15] El Hagrassy 2010: 163

5. Bioactivity Media

(PDA) Potato dextrose agar powder was used to determine the bioactivity of *Trichoderma sp.* against *Aspergillus niger, Aspergillus flavus,* and *Alternaria alternata*. The commercial PDA NutriSelect® Plus consist of 20g of Dextrose, 15 g Agar, 4g Potato extract in addition to Chloramphenicol, VETRANAL® about 25mg/L with a final pH of 5.6 +/- 0.2 at 25°C.

6. Antifungal Activity

The biculture test method was prepared according to (Soytong and Quimio; 1989). A growing mycelium plugs disc (0.5 cm-diameter) was taken from the edge of PDA plate for each *Trichoderma* spp. and transferred to one side of a PDA plate about 2.0 cm from the center of the 9 mm Petri dishes). An agar disc of *Aspergillus niger, Aspergillus flavus,* and *A. alternata* was placed on the other side of the plate.

For positive control treatment, an agar disc of *Aspergillus niger, Aspergillus flavus,* and *A. alternata* were placed in the center of the Petri dish (9mm diameter). All plates were incubated at 28±2°C until the colony of control grew to a full plate.

Colony diameter was measured using a measuring ruler. The percentage of the growth inhibition was calculated using the following formula:

inhibition = (A-B/A) × 100%

A = colony diameter in control, B = colony diameter in biculture.

7. Identification of *Trichoderma*

For the identification of bioactive *Trichoderma* spp. the mycelium plugs of *Trichoderma* growing on PDA were taken using a (0.5-cm-diameter) cork borer and transferred to Czapek's Dox broth according to (Allen, 1950) in 500 ml flask. All flasks were incubated in a shaken incubator at 25°C for 7-10 days, then the DNA was extracted and purified by the DNA PowerPlant® Pro Kit (Qiagen, Milan, Italy).

The identification was carried out by amplification and analysis of the regions of the (ITS) region of ribosomal DNA (RDNA) *Internal Transcribed Spacer*. The amplification was made by *Taq* DNA polymerase, recombinant (Invitrogen, Milan, Italy) with the universal primer pair ITS 5 and 4 regions of the ribosomal RNA gene cluster sequences according to the method described[16] as ITS 5 «(50-GGAAGTAAAAGTCGTAACAAGG-30)» and ITS-4 «(50-TCCTCCGCTTATTGATATGC-30)».

PCR amplification mixture reaction carried out using PCR buffer «(1X), dNTP mixture (0.2 mm), $MgCl_2$ (1.5 mm) », forward and reverse primers (0.5 mm), *Taq* DNA Polymerase (1 U), and 100 ng of DNA.

Thermo-cycler conditions were: 94° C for 3 min in a «C1000 Touch™ Thermal Cycler (Bio-Rad, Munich, Germany)» followed by 35 cycles of 0.5 min at 94°C, 0.5 min at 55°C, 0.5 min at 72°C, and ending with 10 min at 72°C. Amplified DNA products were analyzed using electrophoresis in 1% agarose gels run in Tris/borate/EDTA buffer and sequenced in the Scientific Research Center of the Biotechnology, Lebanon. Obtained sequences were analyzed using FinchTV[17] v.1.4.0[18]. The sequencing data were compared with the GenBank database[19].

[16] THE METHOD DESCRIBED BY WHITE & ET AL. 1990.

[17] FINCH: «FINCHTV 1.4», https://finchtv.software.informer.com/1.4/ Accessed on 13 /08 2020.

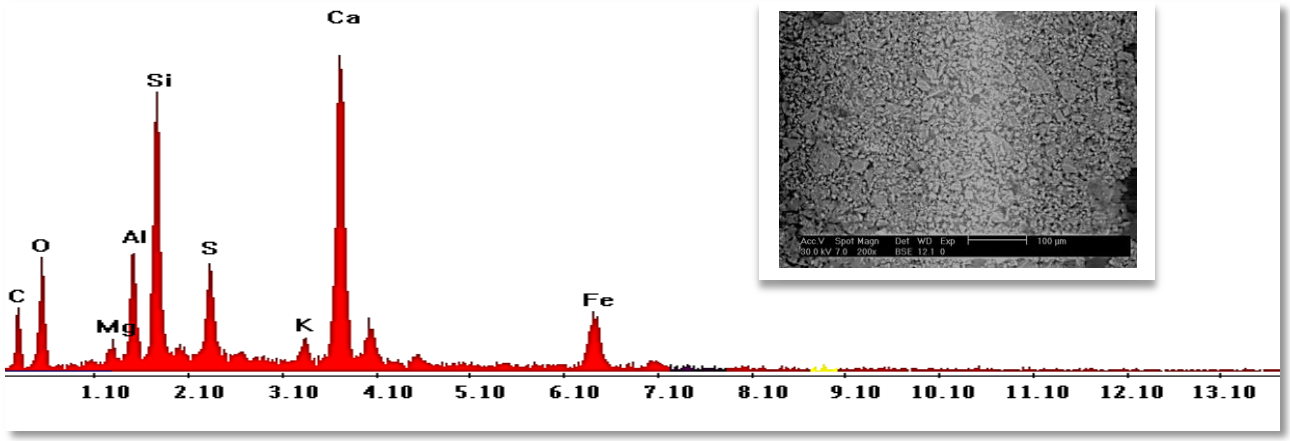
III. Results

1. Structure of Nefer Bau Betah Mural Painting

Fragments were taken from ground layer and pigments of the mural painting.

A. Ground Layer

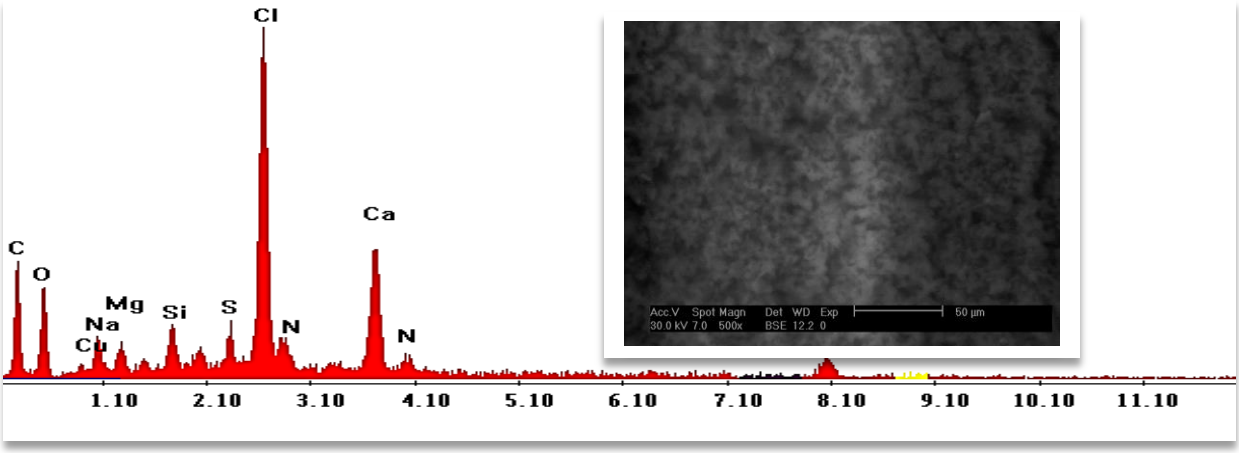
The SEM- EDX analysis showed that the ground layer consisted of Calcite "$CaCO_3$" therefore the presence of (Ca, O & C). In addition to the Quartz «SiO_2» due to the existence of (Si, O) element, as well Gypsum «$CaSO_4$» considering the presence of (Ca, S, O) elements **[Figure 1].**



[Figure 1]: EDX *spectrum* the component elements consisted the ground layer of the mural paintings 500x.Acc V =30KV, spot 7, Det: BSE, WD: 12.1, scale: 100µm

B. Pigments

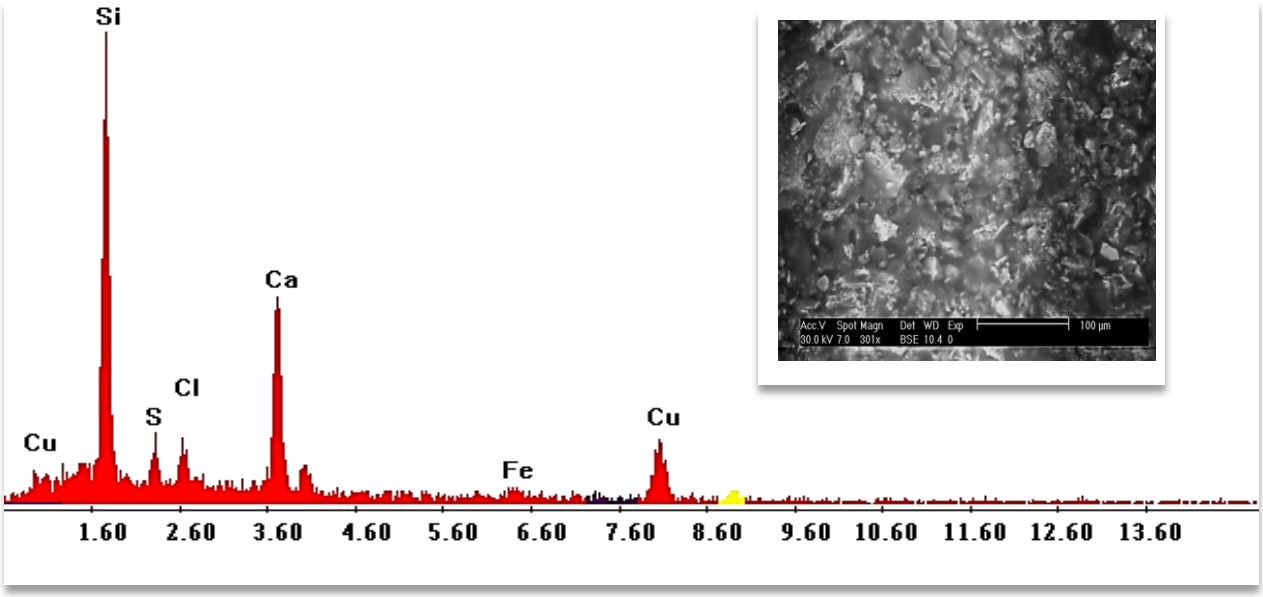
Small fragments of the pigments were investigated and analyzed by SEM-EDX. The analysis showed that, the green pigment was Egyptian green ($CaSiO_3$), **[Figure2]**, the blue pigment was Egyptian blue ($CaCuSi_4O_{10}$) as shown in **[Figure 3]**, and the red pigment is hematite (Fe_2O_3), **[Figure 4].**



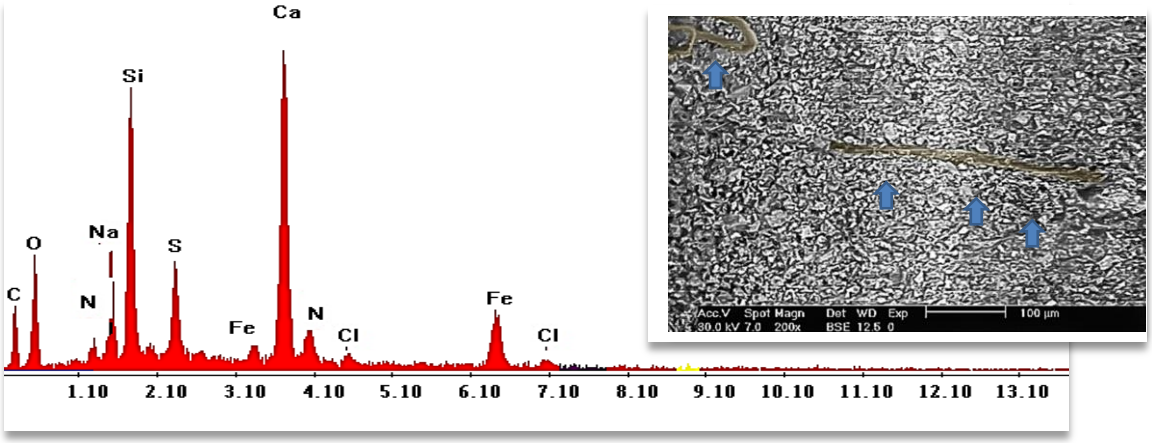
[Figure 2]: EDX *spectrum* the component elements consisted the Green pigment of the mural paintings 500x, Acc V =30KV, spot 7, Det: BSE, WD: 12.2, scale: 100µm.

[18] Finch: «FinchTV 1.4», (https://finchtv.software.informer.com/1.4 Accessed on 13 /08 2020.

[19] GeneBank: «Basic Local Alignment Search Tool», https://blast.ncbi.nlm.nih.gov/Blast.cgi Accessed on 13/08 2020.



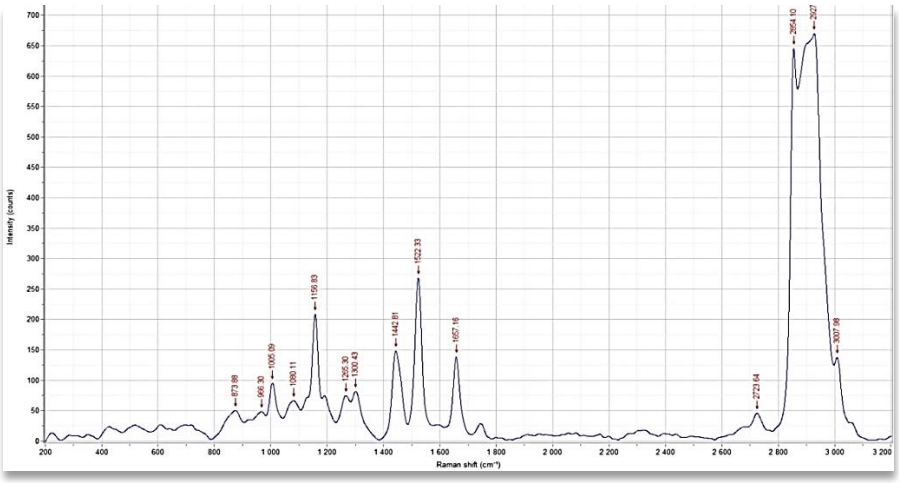
[FIGURE 3]: EDX *spectrum* the component elements consisted the Blue pigment of the mural paintings500x.Acc V =30KV, spot 7, Det: BSE, WD: 10.4, scale: 100µm.



[FIGURE 4]: EDX *spectrum* the component elements consisted the red pigment of the mural paintings with the presence of mycelium 200x, Acc V =30KV, spot 7, Det: BSE, WD: 12.5, scale: 100µm.

C. Binding Medium

Raman spectra showed a slight degree of egg yolk that was used as a binding media. The sample was characterized due to the presence of the functional groups as follows: (n=CH) in 3007, (n=CH) in 2927, (C=C) in 1667, (d- CH) in 1442, (C≡H) in 1265 and (C-O) in 1156 as presented in **[FIGURE 5]**.

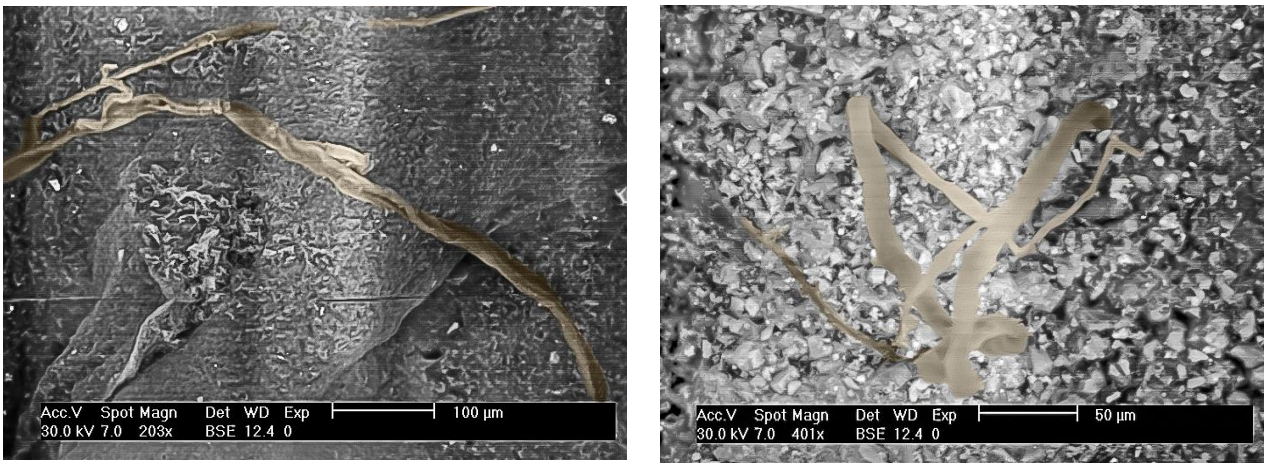


[FIGURE5]: The spectra presented the use of egg yolk as a binding media of the pigment.

D. Deterioration Phenomena

Three main factors should be studied to understand the deterioration factor; first; the environmental conditions inside the tomb, especially after it was closed for five years, second; the crystallization of salts inside the tomb **[FIGURE 6],** and third; the different species of microorganism affecting the mural painting's structure that changes with time **[FIGURE 7].**

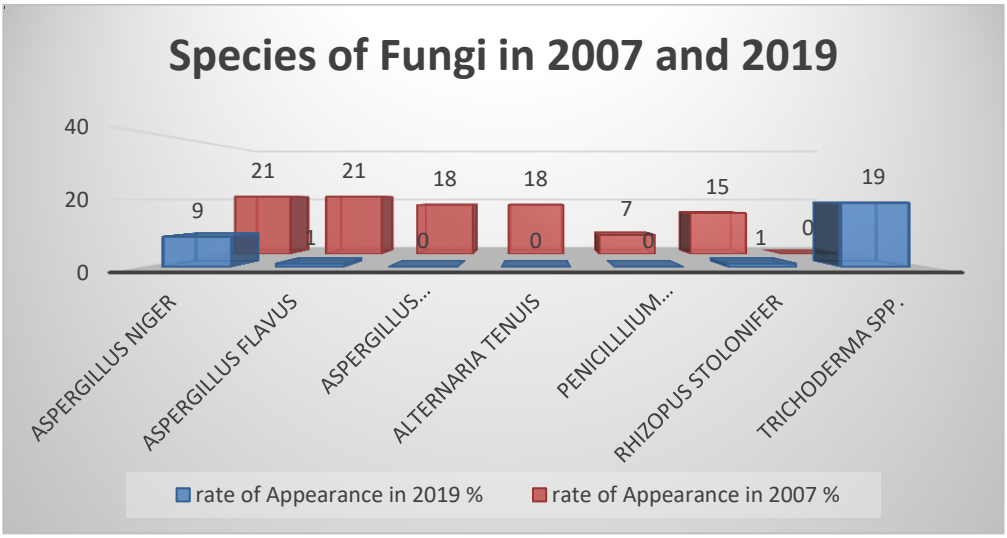
[FIGURE 6]: A: the crystallization of salts over the pigments, B: The SEM of the salts (200X).
© Photo taken by the researcher



[FIGURE 7]: *The mycelium* penetrate the mural paintings layer. (200x, 400x)

E. Identification of Microorganisms

During the four seasons in 2019 swabs analysis were taken to culture the microorganisms and compared with the microorganisms that had previously deteriorated the tomb in 2007 and in 2019 **[FIGURE 8].**



[FIGURE 8]: The species of fungi founded in 2007 and on 2019.
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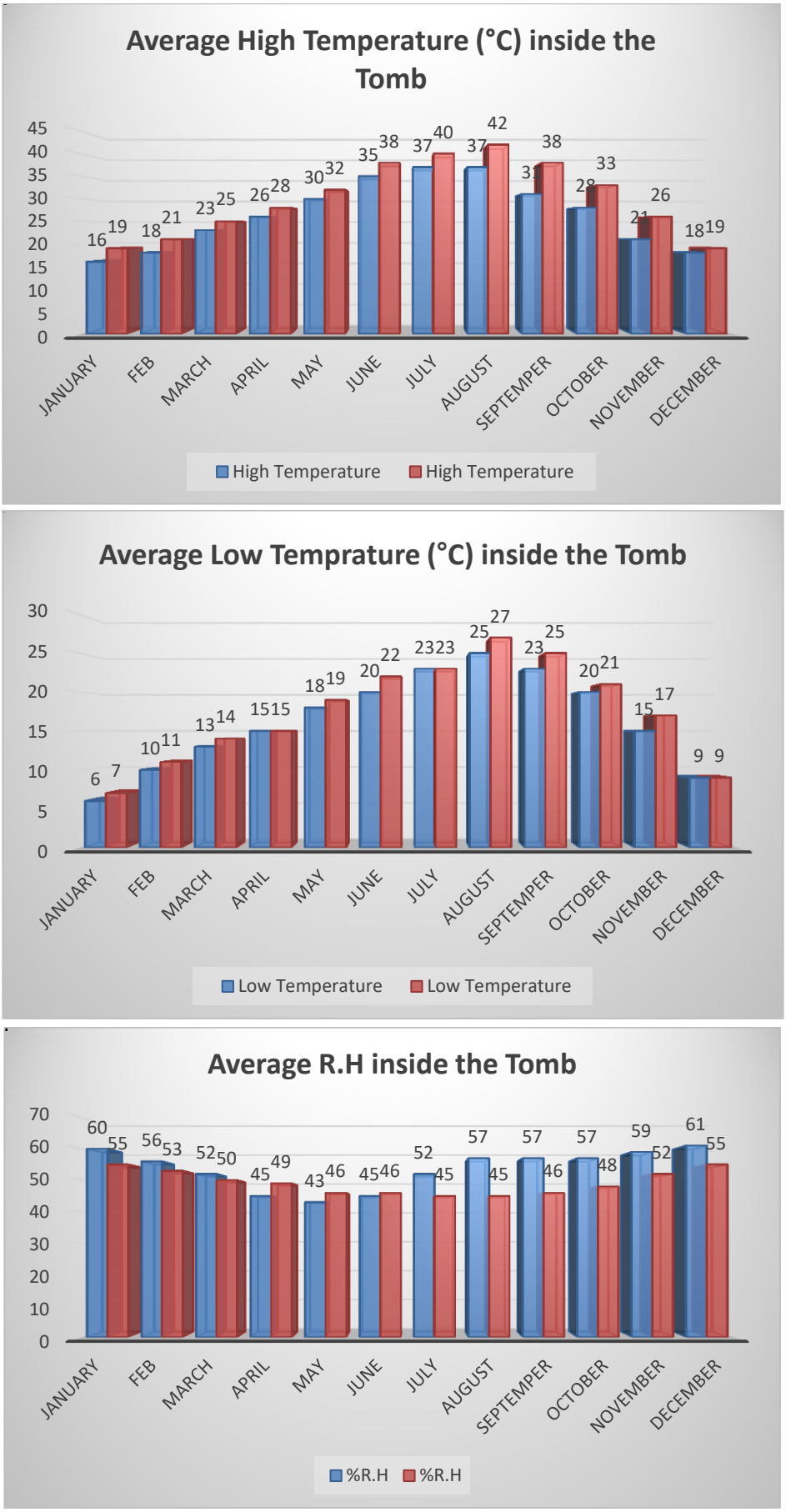
The tomb was closed particularly from 2011 to 2017, then re-opened for conservation in 2018 after crystallization of salts over the wall paintings. The swabs in 2019, showed that the tomb deteriorated with *Aspergillus niger* and species of *Trichoderma* **[FIGURE 9].**

[FIGURE 9]: The deterioration of the tomb by salts and Biodeterioration effects.
© Photo taken by the researcher

F. Environmental Condition

The environmental conditions inside the tomb in both 2007 and 2019 were with average high temperature from 18 to 35°C, and the average low temperature 8 to 11°C in 2007 and from 9 to 10 °C in 2019, while the average relative humidity from 45 to 59% in 2007 and from 46 to 61% in 2019 **[FIGURE 10].**



[FIGURE 10]: The environmental condition inside the tomb in 2007 and 2019.

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G. Color Change by Colorimeter

The data showed that the color change (ΔE) for the pigments in the tomb were less than 5 which is in the safe range as presented in **[TABLE 1]**.

Pigments	2007			2019			ΔE
	ΔL	Δa	Δb	ΔL	Δa	Δb	
Red	53.53	-1.98	13.47	52.98	-3.29	13.11	1.51
Blue	-54.53	-1.77	36.26	-53.51	-3.34	35.15	2.66
Green	-59.45	-45.98	55.03	-57.90	-43.59	54.79	2.86

[TABLE 1]: The color change for the main pigments inside the tomb in 2007 and 2019.
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H. Antifungal Activity and the Identification of Active *Trichoderma spp*

The positive control of *Aspergillus niger*, *Aspergillus flavus,* and *A. alternata* grew faster and significantly formed a large colony in the control plate; the growth diameter was about 8.89; 8.81; 8.71, respectively. According to the biculture plate, only three species of *Trichoderma* affected the growth rate of the three fungi *Aspergillus niger*, *Aspergillus flavus*, and *Alternaria alternata*. The effective *Trichoderma* species were identified according to the GenBank as «*Trichoderma harzianum, T. hamatum*, and *T. aureoviride*».

The antifungal activity of the three *Trichoderma* species were assayed as illustrated in **[TABLE 2].**

Fungi	*A. niger*		*A. flavus*		*Alternaria alternata*	
	Colony diameter (mm)	Inhibition %	Colony diameter (mm)	Inhibition %	Colony diameter (mm)	Inhibition %
Control	8.89	0	8.81	0	8.71	0
Trichodermah arzianum	4.52	65.12	3.23	63.33	2.11	75.77
T. aureoviride	4.16	48.14	3.35	61.97	2.24	74.28
T. hamatum	4.66	4.58	3.09	64.92	2.36	72.90

[TABLE 2]: Antifungal effects of *Trichoderma spp.* on *Aspergillus niger*, *Aspergillus flavus*, and *Alternaria alternata*. © Designed by the researcher

1. Optimisation of Antifungal Activity

The tomb was deteriorated by three main factors. First, salt crystallization. Second, the temperature inside the tomb. Third, poor ventilation which increases humidity, especially inside the burial chamber.

A. Salt Concentration

According to the EDX analysis, the tomb was deteriorated by two types of salts, sodium nitrate, and sodium chloride. To detect the effect of salt on growth and antifungal

activity, after incubation for 14 days in a shaker, different concentrations of NaCl: $NaNO_3$ (1: 1) from 0 to 5% were carried out. In the end, the biomass accumulations for each concentration were dried and weighed, the percentage of inhibition was calculated according to t Topps and Wain equation methodology[20], as represented in the **[TABLE 3]**

NaCl:$NaNO_3$ (1: 1) Salt consentration	*Trichoderma spp.* Dry weight		Growth Reduction (%)		
			A. niger	*A. flavus*	*Alternaria alternata*
0	*T. harzianum*	0.36± 0.01	23.1±0.4	31.6±0.26	36±0.3
	T. hamatum	0.37±0.1	18.6±0.2	20.12±0.1	18.5±0.36
	T. aureoviride	0.39±0.1	19.6±0.1	19.1±0.37	20.1±0.01
1	*T. harzianum*	0.38±0.02	30.4±0.1	32.2±0.26	36.9±0.006
	T. hamatum	0.40±0.001	19.2±0.34	22±0.001	19.14±0.01
	T. aureoviride	0.39±0.001	20.3±12	22.3±0.01	21.8±0.42
2	*T. harzianum*	0.38±0.02	32±0.42	33±0.01	41.3±0.24
	T. hamatum	0.41±0.1	23.53±0.23	24.2±0.04	23.75±0.3
	T. aureoviride	0.40±0.001	23.4±0.02	24.98±0.25	25.3±0.1
3	*T. harzianum*	0.39±0.002	45.8±0.54	49.2±0.002	48.9±0.36
	T. hamatum	0.43±0.2	31.82±0.65	29.86±0.14	31.56±0.001
	T. aureoviride	0.44±0.1	27.12±0.7	28.6±0.01	30.12±0.02
4	*T. harzianum*	0.44±0.001	51.3±0.2	53.21±0.1	53.28±0.003
	T. hamatum	0.43±0.02	33.1±2.3	33.6±14	33.9±0.1
	T. aureoviride	0.41±0.002	31.98±0.2	31.9±0.7	35.13±0.01
5	*T. harzianum*	0.46±0.002	52.12±0.5	52.4±0.32	53.6±0.12
	T. hamatum	0.45±0.001	33.69±0.1	36.12±45	35.9±21
	T. aureoviride	0.40±0.001	33.15±0.4	33.7±0.25	35.64±0.23

[TABLE 3]: Dry weights of *Trichoderma harzianum, T. hamatum, and T. aureoviride.* and its Antifungal activity expressed as % of the growth reduction of the *Aspergillus niger, Aspergillus flavus, and Alternaria alternata* at different salt concentration. © Designed by the researcher

B: Temperature

To assay, the effect of temperature, different degrees from 20°C to 50°C were applied and essayed its effect on the growth and antifungal activity after incubation for 14 days in a shaker. The biomass accumulations were dried and weighed, as represented in the **[TABLE 4].**

[20] TOPPS & WAIN 1957: 506-511.

Temperature (°C)	*Trichoderma spp.* Dry weight		Growth Reduction (%)		
			A. niger	*A. flavus*	*Alternaria alternata*
20	*T. harzianum*	0.21±0.1	24.16±0.3	20. 13±0.65	18.21±0.64
	T. hamatum	0.23±0.001	29.32±0.001	28.11±0.01	25.12±0.06
	T. aureoviride	0.25±0.1	31.22±0.11	34.24±0.25	37.23±0.007
25	*T. harzianum*	0.24±0.002	45.23±0.14	43.32±0.1	39.32±0.6
	T. hamatum	0.26±0.01	48.32±0.35	48.2±0.004	47.32±0.1
	T.aureoviride	0.25±0.06	45.21±0.17	46.98±0.3	47.98±0.1
30	*T. harzianum*	0.48±0.002	56.3±0.4	55.21±0.01	58.6±0.21
	T. hamatum	0.39±0.3	51.36±0.1	64.93±0.87	50.36±0.41
	T. aureoviride	0.40±0.6	48.37±0.1	50.33±0.12	52.69±0.005
35	*T. harzianum*	0.41±0.25	54.31±0.02	52.4±0.21	55.24±0.2
	T. hamatum	0.43±0.06	54.1±0.2	67.12±0.3	49.65±0.001
	T. aureoviride	0.45±0.004	50.71±08	52.3±0.04	55.36±0.01
40	*T. harzianum*	0.46±0.6	40.32±0.1	45.21±0.25	50.21±0.03
	T. hamatum	0.37±0.1	38.21±0.04	39.54±0.36	36.51±0.01
	T. aureoviride	0.40±0.7	36.65±0.1	39.74±0.42	37.62±0.69
45	*T. harzianum*	0.41±0.001	35.21±0.74	33.96±0.1	31.6±0.2
	T. hamatum	0.31±0.8	31.23±0.2	32.6±0.81	31.12±0.5
	T. aureoviride	0.35±0.01	30.6±12	31.21±0.4	30.61±0.03
50	*T. harzianum*	0.30±0.01	28.12±0.3	24.31±0.65	20.17±0.64
	T. hamatum	0.29±0.3	29.32±0.001	28.11±0.01	25.12±0.06
	T. aureoviride	0.29±0.01	25.21±0.18	24.54±0.32	20.69±0.23

[TABLE 4]: Dry weights of *Trichoderma harzianum, T. hamatum,* and *T. aureoviride."* and its Antifungal activity expressed as % of the growth reduction of the *Aspergillus niger, Aspergillus flavus, and Alternaria alterna*ta at different temperature. © Designed by the researcher

C. pH Value

The average pH from (5 to 8) was examined its effect after incubation for 14 days at 35°C by using 0.1 N HCL and 0.1 N NaOH. The biomass accumulations were dried and weighed, as represented in the **[TABLE 5].**

pH Value	*Trichoderma spp.* Dry weight		Growth Reduction (%)		
			A. niger	*A. flavus*	*Alternaria alternata*
5	*T. harzianum*	0.47±0.024	46.23±0.07	50.1±0.06	51.62±0.06
	T. hamatum	0.44±0.001	45.91±0.01	42.31±0.07	50.41±0.02
	T. aureoviride	0.40±0.32	41.23±0.06	51.01±0.001	51.36±0.9
5.5	*T. harzianum*	0.49±0.063	58.98±0.12	60.23±0.2	67.32±0.12
	T. hamatum	0.46±0.004	56.14±0.65	57.36±0.001	60.32±0.01
	T.aureoviride	0.44±0.17	52.13±0.1	53.62±0.22	57.32±0.23
6	*T. harzianum*	0.33±0.52	0.13±0.007	0.55±0.01	0.38±0.21
	T. hamatum	0.31±0.06	35.45±0.06	35.98±0.36	34.22±0.9
	T. aureoviride	0.30±0.14	36.2±0.01	38.62±0.11	40.98±0.07
6.5	*T. harzianum*	0.24±0.004	34.12±0.007	38.16±0.012	38.33±0.001
	T. hamatum	0.20±0.19	30.18±0.01	30.98±0.03	31.15±0.01
	T. aureoviride	0.19±0.001	29.26±0.07	29.88±0.045	30.19±0.07
7	*T. harzianum*	0.20±0.025	28.7±0.06	29.64±0.73	30.15±0.08
	T. hamatum	0.20±0.17	25.11±0.041	26.79±0.16	29.50±0.1
	T. aureoviride	0.19±0.1	21.33±0.4	22.14±0.01	20.36±0.41
7.5	*T. harzianum*	0.17±0.041	15.32±0.01	18.98±0.2	19.63±0.03
	T. hamatum	0.15±0.01	16.9±0.21	18.63±0.01	19.1±0.23
	T. aureoviride	0.15±0.021	14.32±0.01	14.82±0.31	14.66±0.5
8	*T. harzianum*	0.11±0.021	10.22±0.003	17.32±14	20.35±0.01
	T. hamatum	0.10±0.15	13.34±0.1	13.45±0.06	11.62±0.07
	T. aureoviride	0.08±0.021	14.23±0.21	13.98±0.01	14.25±0.03

[TABLE 5]: Dry weights of *Trichoderma harzianum, T. hamatum, and T. aureoviride.*" and its antifungal activity expressed as % of the growth reduction of the *Aspergillus niger, Aspergillus flavus, and Alternaria alternata* at different pH value. ©Designed by the researcher

IV. DISCUSSION

According to the swabs that were previously taken from the mural paintings of the Egyptian tomb (Nefer- Bau- Betah) in 2019, species of *Trichoderma* were detected. The EDX analysis of the ground layer of the paintings consisted of calcite and gypsum which is an evidence that the tomb had been prepared by tempera technique[21]. The EDX of the pigments showed that both the blue and green pigment were ancient manufactured pigments; Egyptian blue and Egyptian green, while, the red pigment was hematite. All these pigments were mixed with a binding media. The Raman spectroscopy analysis indicated that the binding media was egg yolk. According to Lucas & Harris[22], mixing the pigments with binding media was applied only when ancient Egyptians prepared tombs by tempera technique and in this case Nefer Bau Betah Tomb was decorated by the egg yolk tempera technique which is one of the most common technologies used in ancient Egypt.

The tomb of Nefer- Bau- Betah had been deteriorated in 2007 by *Aspergillus niger, Aspergillus flavus, and Alternaria alternata*. These three fungi were considered as the most deteriorating fungi that usually cause damage to the Egyptian mural paintings[23]. Therefore, the threats posed by the biodeterioration factor have been studied in several researches in different countries[24]. In 2019, the same tomb was badly affected by *Aspergillus niger* and species of *Trichoderma* and was deteriorated by salt crystallization.

According to the EDX analysis, two types of salts deteriorated the tomb, namely sodium chloride, and sodium nitrate. In 2007, the high temperature inside the tomb was on average from (31 to 39°C), but in 2019 the high-temperature average increased from (35 to 44°C). These temperatures were theorized as an optimized temperature for the growth of *Trichoderma*.[25]. On the other hand, the relative humidity inside the tomb ranged from 46 to 61 %. These three main factors inside the tomb (temperature, humidity, and salt) played a main role for the growth of *Trichoderma*.

The swabs that were taken in 2019 displayed that the tomb was deteriorated by only *Aspergillus niger* and 3 species of *Trichoderma*. The growth of *Trichoderma* helped to control the growth and the bioactivity of other species that previously deteriorated the same tomb in 2007. Numerous studies have been carried out on the effect of *Trichoderma* in the inhibition of fungi.[26]

Although *Trichoderma* saved the mural paintings from the effect of other microorganisms, it caused further effects by the color change in the natural and synthetic pigment. According to the spectrophotometer analysis, the ΔE was a little high in Egyptian blue and Egyptian green (the ΔE was about 2.5 which is easy to be noticed by the naked eye), while red hematite as a natural pigment was less deteriorated (the ΔE was

[21] NICHOLSON & SHAW 2000: 269.

[22] LUCAS & HARRIS 1948: 202.

[23] HELMI& ET AL. 2009: 306.

[24] CIFERRI 1999: 879-885; HOFFLAND & ET AL. 2004: 258-264; MILANESI & ET AL. 2006A: 168-173; MILANESI& ET AL. 2006B: 7-13; VENERANDA & ET AL. 2017: 19599–19608.

[25] ABOU-ZEID & ET AL. 2011: 233-244; DARYAEI & ET AL. 2016A: 999-1009; DARYAEI& ET AL. 2016B: 24-30.

[26] ZHOU & ET AL. 2017: 18-20; ZHANG & ET AL. 2018: 59-66; KHAN & ET AL. 2020: 817; ZHU & ET AL. 2020: 105161.

about 1.5 which can be neglected). This result needs special studies in the future to assay why the synthetic pigments are more sensitive than the natural pigments.

The preliminary examination for antifungal activity of all *Trichoderma spp.* showed that only three species have the ability to control the growth of "*Aspergillus niger, A. flavus, Alternaria alternata."* which were identified according to 16s DNA sequences to "*Trichoderma harzianum, T. hamatum, and T. aureoviride."* These three species of *Trichoderma* have the ability to control the growth of the other fungi.[27]

To assess the optimum conditions three main factors were studied, first, salt crystallization inside the tomb as presented in the EDX results, where two types of salts were found: NaCl and $NaNO_3$, second, the temperature inside the tomb and third, pH value.

The optimum salt concentration required for the production of antifungal activity was examined with different salt concentrations (0- 5%), the sodium chloride and sodium nitrate were prepared (1:1). The data showed that 4% of NaCl: $NaNO_3$ (1:1) was the optimum concentration of antifungal activity[28] for *T. harzianum* in inhibiting the growth of *A. flavus,* while 5 % of NaCl: $NaNO_3$ (1:1) was the best concentration for antifungal activity of all *Trichoderma* species to control the growth rate of other fungi. [29]

The experimental test represented that the antifungal activity of *T. harzianum* was found to be optimum at 30°C, while the 35°C was the optimum temperature for the antifungal activity for both *Trichoderma hamatum, and T. aureovirid.* These results corresponded with the results of Sterflinger[30], Hoell[31] and Hosseni & Soltani[32]. The pH value was essayed to indicate the optimum value for antifungal activity of the *Trichoderma* species, the acidic 5.5 pH was the optimum value of antifungal activity of all species of *Trichoderma* and controlled the growth rate of other fungi[33].

[27] LORITO & ET AL. 1993: 302-307; VITERBO & ET AL. 2001:169-174; KÜÇÜK& ET AL. 2003: 247-253; SHALINI & KOTASTHANE 2007: 2272-2281; SHARMA & ET AL. 2011: 19898-19907; BAAZEEM& ET AL. 2021: 331.

[28] AIT-LAHSEN & ET AL. 2001: 5833–5839.

[29] BUGNI & IRELAND 2004: 143-163; HARMAN 2006: 190-194.

[30] STERFLINGER 1998: 217-281; STERFLINGER 2018: 10-12.

[31] HOELL & ET AL. 2005: 180-186.

[32] HOSSENI & SOLTANI 2014: 753–761.

[33] STEYAERT& ET AL. 2010A: 198-208; STEYAERT & ET AL. 2010B: 179-188; DARYAEI & ET AL. 2016: 999-1009.

V. CONCLUSION

In this research, two swabs were taken from the same tomb Nefer Bau Betah in 2007 and 2019. The swab analysis in 2019 showed that the microorganisms that were previously found in 2007 had disappeared and new species of *Trichoderma* were isolated. Three species of *Trichoderma* were identified according to their bioactivity effect over *Aspergillus niger, Aspergillus flavus, and Alternaria alternata* and by their sequences in the GenBank to «*Trichoderma harzianum, T. hamatum,* and *T. aureoviride*» These three *Trichoderma* species have the ability to control the growth rate of the microbes that were found in 2007. The optimization factors for increasing the bioactivity of the *Trichoderma spp.* were 5% of sodium nitrate and sodium chlorine that crystallize in the tomb with an average temperature between 30:35°C in acidic pH (pH=5.5). These conditions helped *Trichoderma* species to grow and work as antifungal factors in the tomb *Aspergillus niger, Aspergillus flavus,* and *Alternaria alternata* but it caused a little effect in the color change of the pigment inside the tomb especially the blue and green Egyptian pigments, while it caused less effect in the color change of red hematite pigment.

Finally, the effect of the bioactivity of *Trichoderma* species on the ancient Egyptian pigments and media, and a biochemical studies for these pigments will be discussed in a future study.

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