Study on the biological hazards and control measures of the Dunhuang manuscripts

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Abstract. The Dunhuang manuscripts are world-renowned precious paper cultural relics, and it is necessary to conduct research on the prevention and control of harmful biological hazards. Taking 6 Dunhuang manuscripts as the research object, through appearance observation, data measurement and literature review, the paper material characteristics are analyzed, and the types of microorganisms on its surface are collected, cultivated and identified, and the cellulolytic ability of the Penicillium strains in them is determined. The spore germination rate of Penicillium at different temperatures was measured, and the effect of low temperature on Penicillium harming paper relics was analyzed. The results showed that the 6 Dunhuang manuscripts were made of hemp paper and thin white paper from the Tang Dynasty to the Song Dynasty. A total of 12 bacteria species and 1 fungus species were isolated and identified. Among them, Penicillium has the ability to decompose cellulose on paper, which is the main harmful microorganism to the Dunhuang manuscripts, low temperature can significantly reduce the germination rate of Penicillium, so low-temperature refrigeration technology can be used to effectively inhibit the damage of Penicillium to paper cultural relics.

1. Introduction

The Dunhuang manuscripts belong to organic paper cultural relics. Researches on the protection of ancient books, paintings and archives show that the paper cultural relics are seriously damaged by microorganisms in the environment of temperature adaptation, humidity and unsealed preservation.

Microorganisms include bacteria, viruses, fungi, protozoa and a few algae. Among them, the main group of paper cultural relics is fungi. According to literature reports, more than 100 genera and 200 species of mold were isolated from paper. Among them, Trichoderma, Penicillium and Chaetomium are the most harmful to paper. The propagation process of mold is accompanied by the destruction of paper [1]. The process is as follows: first, in the early stage of mildew, the mold attached to the paper has a slight odor, material moisture and other phenomena; second, in the breeding period of mold, when the mold enters the stable growth period, the downy or hairy colonies appear in the moldy parts, and the color changes from white to gray; third, in the moldy period, the paper of cultural relics is most damaged; Serious, the physical and chemical stability of the paper are significantly changed, until the damage can not be used.
The damage of mold to paper cultural relics is mainly manifested in two aspects: first, the mold generally has pigments, such as gray green, yellow green, light yellow, yellow brown and other colors of *Aspergillus, Penicillium*, which form various colors on the surface of paper cultural relics that are difficult to remove, damaging the quality of paper cultural relics, second, the mold will secrete pigments and acid substances in the process of metabolism, which will pollute the paper. The acid substances secreted from the paper will hydrolyze the paper cellulose, reduce its physical and chemical stability and shorten its service life. In summary, the paper cultural relics are seriously damaged by harmful microorganisms.

At present, there are few researches on the microbial damage of paper cultural relics. The Dunhuang manuscripts are not only an important carrier of Dunhuang studies, but also an important cultural heritage of mankind. In recent years, cultural relic protection experts have accumulated rich experience in the protection and research of Dunhuang Grottoes murals and painted sculptures, but the scientific protection of Dunhuang relics, especially the biological protection, is rarely involved. This paper hopes to study and analyze the current situation of microbial biological damage of Dunhuang relics through the interdisciplinary of cultural heritage and biology, so as to accumulate the biological value of precious paper relics. Based on the experience of prevention and control, this paper puts forward new ideas and methods for the protection of the Dunhuang manuscripts.

2. Materials and methods

2.1. Test materials
Total 6 Dunhuang manuscripts collected in the Museum of Northwest Normal University are studied in this research institute, which are: Prajna paramita Sutra Volume 1, Mahayana sutra's self-understanding of the realm Volume 7 (2), Mahayana Wuliang Shouzong. The Buddha's Dharma practice proves the Sutra of all Bodhisattvas Volume 4, Nanwu Buddhist scroll, the Heart Sutra of the eleven side Mantra.

2.2. Main reagents and instruments
Beijing Adlay bacterial DNA extraction kit, Beijing Aoke 16S universal primer (27F / 1492r), Beijing Aoke 18S universal primer (ITS1 / its4), Shanghai bio engineering DNA extraction kit Polymerase premix, SGG penicillin, SGG streptomycin, glucose (analytically pure), glacial acetic acid (analytically pure), sodium hydroxide (analytically pure), sodium acetate (analytically pure), sodium carboxymethyl cellulose (analytically pure), 3,5 dinitrosalicylic acid (analytically pure), Difco TSB medium, Difco R2A medium, Difco PDA medium, Qingdao Haibo bio cellulose Congo red medium.

Shenzhen Shengli vc1010a illuminance meter, Wenzhou th20r-ex temperature and humidity meter, Beijing saiduoli SQP analytical balance, Shanghai spectral element double beam UV visible spectrophotometer alpha-1900s, Beijing tormor ultra clean worktable skjh-2109, Qingdao Haier ultra low temperature refrigerator dw-86l288, Shanghai Jinghong electric constant temperature blast drying oven dhg-9076a, Shanghai Yuejin hpf-270 water proof electric constant temperature cultivation machine. In addition, it also includes the following items: incubator, Shanghai Yuejin yjy-211b constant temperature oscillation incubator, Qingdao Haier hr1200-ia2 biosafety cabinet, Singapore thermo verititm 96 gene amplification instrument, Japan Olympus cx23 microscope, American labnet centrifuge and German GFL 1005 water bath pot.

2.3. The paper characteristics of the Dunhuang manuscripts
Through the appearance observation, data measurement, literature review and other ways, the paper of the Dunhuang manuscripts is analyzed and studied to further clarify the paper material and characteristics of the manuscripts.
2.4. Identification of harmful microorganisms in the Dunhuang manuscripts

Microorganisms on the surface of the Dunhuang manuscripts were scraped with aseptic apparatus, cultured in medium, amplified by PCR, and sent to biological testing company for DNA sequencing to determine the species of bacteria and fungi.

2.5. Detection of cellulose decomposing strains

The purified strain was put into cellulose Congo red medium plate and cultured at 28 °C for 10 days. The strain with hydrolysis circle was cellulose decomposing strain. Definition of cellulase activity unit: at 50 °C, 1 mg enzyme catalyzes hydrolysis of cellulose to 1 μg glucose per minute, which is defined as an activity unit (U).

2.6. Effect of low temperature on harmful microorganisms

The germination rate of fungal spores at different temperatures was studied to analyze the effect of low temperature on harmful microorganisms of paper relics. After elution, filtration and centrifugation (1000rpm/min), discard the supernatant, add deionized water to resuspend to 1×10^5 ~ 1×10^7/ml, add a small amount of 0.5% glucose solution, mix well, drop it onto the glass slide with depression, place it in -20, 4, 8, 16 °C and room temperature (25 °C) for cultivation (keeping certain humidity), and repeat each treatment for three times. The germination rate of spores cultured at room temperature was more than 90%. Germination rate (%) = number of spores germinated / number of spores examined × 100%.

2.7. Data processing and analysis

Microsoft Excel 2016 was used to collect the test data, and one-way ANOVA in graphpad prism 8.0 was used for univariate analysis and mapping. All the data were mean ± SD, and P < 0.05 was used as the standard of difference significance.

3. Results

3.1. The paper features of the Dunhuang manuscripts

A total of 6 Dunhuang manuscripts were sampled. Through literature review, interpretation of the book content and analysis of paper material, the following results were obtained (Table 1).

| ID  | Years                  | Material              | Details                                                                                                                                                                                                                                                                                                                                 |
|-----|------------------------|-----------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1   | Late Tang Dynasty      | Hemp paper            | Basically in good condition. There is no wood shaft. There are black silk bars and the paper color is beige. The height is 26cm and the total length is 890.5cm.                                                                                                                                                                                  |
| 2   | Late Tang Dynasty      | Hemp paper            | Basically in good condition. There is no wood shaft. There are black silk bars and the paper color is beige. The height is 26cm and the total length is 890.5cm.                                                                                                                                                                                                                   |
| 3   | Tang Dynasty           | Hemp paper            | Installed on a reel, without wood reel, and the head and tail are complete. The height is 31cm, the total length is 134cm, the paper is 3.5 papers, each paper has 19 rows, and the row height is 1.5cm.                                                                                                                    |
| 4   | Tang Dynasty           | Hemp paper            | Light yellow in color. The height is 28.6cm and the total length is 147.5cm.                                                                                                                                                                                                                                                                                   |
| 5   | Late Tang Dynasty - Five Dynasties | Hemp paper | The color is beige. The total length is 695.5cm, the height is 29.5cm, 18 pieces of paper are used, each paper is 49cm, column width is 2.4cm, and one column is 16 words.                                                                                                                                                      |
| 6   | Dynasty-Song Dynasty   | Thin white paper       | Installed on a reel, and the wooden reel is missing. The head of the scroll is incomplete. The name of the scroll can be seen at the end of the scroll.                                                                                                                                                                                                                   |
3.2. Identification of harmful microorganisms in the Dunhuang manuscripts

In this study, 19 strains belonging to 12 bacterial species and 1 fungal species were identified from 6 Dunhuang manuscripts. They were *Bacillus aryabhattai*, *Bacillus velezensis*, *Psychrobacter pulmonis*, *Bacillus subtilis* sub *sp*. Stercoris, *Acinetobacter lwofii*, *Exiguobacterium mexicanum*, *Brevundimonas vesicularis*, *Rhodococcus pyridinivorans*, *Bacillus halosaccharovorans*, *Achromobacter marplatensis*, *Acinetobacter johnsonii*, *Brevibacillus centrosporus* and *Penicillium sp*. (Table 2). Most of these bacteria and fungi can secrete pigments that pollute paper, and many species have the ability to decompose paper cellulose [2-7]. Harmful bacteria and fungi have been found in the environment of the Dunhuang manuscripts preservation, indicating that there are great hidden dangers in this preservation environment, it is necessary to further clarify the harmful microorganisms.

Table 2. Determination of microbial decomposing cellulose in the Dunhuang manuscripts

| Species name                        | Sample | Cellulase activity (U) |
|------------------------------------|--------|------------------------|
| *Bacillus aryabhattai*             | 3      | -                      |
| *Bacillus velezensis*              | 5      | -                      |
| *Psychrobacter pulmonis*           | 2      | -                      |
| *Bacillus subtilis* sub *sp*. Stercoris | 1      | -                      |
| *Acinetobacter lwofii*             | 2      | -                      |
| *Exiguobacterium mexicanum*        | 2      | -                      |
| *Brevundimonas vesicularis*        | 2      | -                      |
| *Rhodococcus pyridinivorans*       | 1      | -                      |
| *Bacillus halosaccharovorans*      | 1      | -                      |
| *Achromobacter marplatensis*       | 6      | -                      |
| *Acinetobacter johnsonii*          | 5      | -                      |
| *Brevibacillus centrosporus*       | 3      | -                      |
| *Penicillium* sp.                  | 2      | 5.90±0.21              |
|                                    | 6      | 5.36±0.54              |

3.3. Detection of cellulose decomposing strains

The damage of microorganism to paper cultural relics is secret and serious. In order to identify the harm of 13 bacteria and fungi to the Dunhuang manuscripts, the paper fiber decomposition ability test was set up. It can be seen from Table 2 that none of the 12 kinds of bacteria isolated from the Dunhuang manuscripts can decompose cellulose. *Penicillium sp.* (2 and 6) has the ability to decompose cellulose. The cellulase activity is 7.34 ± 0.21u and 5.37 ± 0.54u respectively. It has strong ability to decompose paper fiber and is harmful to the paper safety of the Dunhuang manuscripts.

3.4. Effect of low temperature on harmful microorganisms

There are mainly physical and chemical methods for the protection of paper-based cultural relics, among which the physical method of low-temperature freezing has attracted much attention because of its less damage to paper-based cultural relics and no harm to personnel.

Combined with the research results in the previous section, from the perspective of spore germination rate of *Penicillium*, the low temperature cold storage test of *Penicillium* which has the ability to decompose cellulose was carried out. The results showed that the germination rate of *Penicillium* spores was more than 90% at 25 °C, 7.30-8.70% at 16 °C, 0.30-0.50% at 8 °C and 0.10-0.20% at 4 °C (Fig. 1).

It can be seen from Table 3 that low temperature has a significant effect on the germination rate of *Penicillium* (*F*=8116, *df*= 3, *P* < 0.05). With the decrease of environmental temperature, the germination rate of *Penicillium* spores decreased significantly. Low temperature cold storage can effectively inhibit the harm of *Penicillium* to paper cultural relics.
Figure 1. Effect of low temperature on germination rate of Penicillium sp. Note: The lower case letters on different data indicated that there were significant differences in spore germination rate between different temperatures at $P < 0.05$ level.

Table 3. One-way ANOVA results for effects of low temperature on germination rate of Penicillium sp.

| Variable            | Temperature | $F$  | $df$ | $P$   |
|---------------------|-------------|------|------|-------|
| Germination rate    | 8116        | 3    |      | <0.05 |

4. Discussion

The research data show that the paper materials of the Dunhuang manuscripts are mainly hemp, Broussonetia papyrifera and mulberry, which are mainly from different places. The paper materials and production technology of the Dunhuang manuscripts in different periods are obviously different. 6 samples of the Dunhuang manuscripts were selected. No. 1-4 samples are Tang Dynasty hemp paper, with phloem fiber type. The width and length of the paper are relatively fixed, and the curtain pattern is obvious, which conforms to the Tang Dynasty system. Among them, the paper of No. 1 and No. 2 samples is fine and hard yellow paper after yellow dyeing, waxing and calendering. Sample 3 is white linen paper. The paper color of sample 4 is light yellow and the paper quality is poor; sample 5 is hemp paper from the late Tang Dynasty to the Five Dynasties, the paper quality is poor and the curtain pattern is obvious. Sample No. 6 is thin white hemp paper from late Tang Dynasty to Song Dynasty.

Mold is the most vulnerable biological hazard under normal temperature and humidity, so mold is the most harmful to paper cultural relics. Penicillium is the most widely distributed and easily occurring fungus species, which is the most harmful to cultural relics. Penicillium belongs to ascomycete class, sacriforme order, sacriforme family. It is widely distributed in nature and has many kinds. The vegetative mycelium of Penicillium is colorless, light or bright color. There are transverse septa and conidiophores, which are smooth or rough. There are no podocytes at the base. The apical sac does not expand, but forms broom like branches, which are called broom like branches. The conidia are spherical, elliptic or short columnar, smooth or rough, and most of them are blue-green.

Harmful microorganisms can cause mildew of paper cultural relics and decrease the structural strength of paper. The main reasons for the damage of cultural relics by mildew are as follows:

First, the material of cultural relics is decomposed and utilized by microorganisms as nutrients, which directly causes damage. In order to reproduce, microorganisms need to absorb nutrients from the
external environment and obtain energy to synthesize cell substances and metabolism. Some small molecular substances, such as ions, monosaccharides, amino acids, glycerol and organic acids, can be directly utilized by microorganisms. For the high molecular materials in paper cultural relics, such as starch, protein, cellulose, pectin, fat, etc., microorganisms will secrete corresponding enzymes, such as amylase, protease, cellulase, pectinase, lipase, etc., the macromolecular materials will be hydrolyzed into water-soluble small molecular materials, which will be absorbed and utilized by microorganisms. The process of microbial decomposition and absorption of these high molecular substances is the process of paper cultural relics being destroyed by mildew. It is the result of the assimilation of starch and animal glue by molds.

The second is the organic acid produced by microorganisms in the process of metabolism, which makes the paper cultural relics suffer from acid corrosion. Organic acids produced in microbial metabolism include citric acid, gluconic acid (mainly some species of *Penicillium* and *Aspergillus*), kojic acid, lactic acid, fumaric acid, propionic acid, gallate, etc. These acidic metabolites increase the acidity of the surrounding environment and corrode the paper relics. Acid can reduce the activation energy of cellulose sugar bond breaking, accelerate the hydrolysis speed, which is more obvious under the condition of high humidity, so that the long chain of cellulose breaks and becomes fragile hydrolyzed cellulose, which makes the paper cultural relics become old and brittle.

Third, the pigment produced by mold colony pollutes the paper cultural relics. Some molds can produce pigment during metabolism. Different kinds of mold also produce different colors, such as red, yellow, orange, green, blue, purple, brown, black and so on. Pigment forms unsightly mildew on paper relics. Due to the accumulation of bacteria or the viscous substances it produces, the polluted parts are highly hygroscopic. These parts of cultural relics become soft, damp and sticky, and emit the smell of mildew. After a long time, the paper will stick together.

Fourth, the new aging of mold produces heat, which speeds up the destruction of paper cultural relics. Mold is an aerobic microbe, which can completely oxidize some organic matter through the tricarboxylic acid cycle, and then produce dioxygen and water, and release energy. One part of the energy is used to meet the needs of mold life activities, and the other part is emitted in the form of heat, which makes the paper cultural relics heat and damp. With the increase of temperature and humidity, mold can grow faster and produce greater destructive effect [8-12].

The paper of the Dunhuang manuscripts is mainly composed of plant fiber, which can be damaged by microorganisms with the ability to decompose cellulose. It is reported that *Penicillium*, *Fusarium*, *Aspergillus*, *Pythium fulvum* and *Trichoderma* have strong ability to decompose cellulose [1-7]. The common fungi in the air are *Penicillium*, *Aspergillus*, *Trichoderma* and *Mucor* [13, 14] Wen-xia Ma of Lanzhou University studied the brick murals and ambient air in the tomb, and found that *Penicillium* and *Aspergillus* were the dominant genera, other fungi, including *Fusarium*, *Chaetomium* and *Cladosporium*, are also common [15]. According to Xu Ma's research, *Cladosporium*, *Fusarium*, *Penicillium*, *Alternaria* and *Aspergillus* are the main fungi in the air of Dunhuang Mogao Grottoes [16].

*Penicillium* can survive on the surface of the Dunhuang manuscripts. Two strains of *Penicillium* isolated from the Dunhuang manuscripts have the ability to decompose cellulose. Therefore, the protection of the Dunhuang scriptures is mainly to prevent fungi, especially *Penicillium*.

The spore germination of *Penicillium* was inhibited by low temperature. Therefore, the warp should be preserved in a low temperature environment. In addition, the germination and growth of fungal spores need certain humidity, so dry air is more suitable for the needs of warp preservation.

5. Conclusion

The six Dunhuang manuscripts studied in this paper are made of hemp paper and thin white paper from Tang Dynasty to Song Dynasty. Among the 13 kinds of microorganisms identified on the surface of the Dunhuang manuscripts, *Penicillium* has the ability to decompose cellulose on paper, which is the main harmful microorganism of the Dunhuang manuscripts. It can be used to protect the Dunhuang manuscripts by inhibiting the germination rate of *Penicillium* by means of cold storage.
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