Characterization of an Early 20th Century Chinese Manuscript with Foxing Stains

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Foxing spots are reddish-brown, brown, or yellowish spots in irregular shapes that are commonly discovered on paper materials. Effects of such foxing spots on degradation of Chinese papers have rarely been reported. In this study, a 20th century Chinese manuscript with few foxing stains was examined to explore the cause of stain formation. The paper areas with foxing stains were more acidic than those without the stains, while no obvious differences in cellulose crystallinity and iron and copper contents were observed when comparing paper areas with and without foxing spots *via* X-ray diffraction (XRD) and energy-dispersive X-ray fluorescence spectroscopy (ED-XRF), respectively. For further exploration, few fungal hyphae and spores in various sizes were observed using SEM, leading to increased mean roughness of the paper surface for the foxed area. This is further supported by the presence of amide II in the foxed area only, as detected *via* attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy. Fungal culture was then carried out to demonstrate that fungi belonging to the genii *Alternaria tenuissima* and *Alternaria solani* were present. This research provides an improved understanding of the effects of foxing spots on Chinese archives and informs of further conservation efforts.

Keywords: Foxing spots; Chinese paper; Fungi; Paper conservation

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INTRODUCTION

The term "foxing" originated from the rusty red color of foxes, and its use was first recorded in 1848 (Meynell and Newsam 1978; Manso *et al.* 2009). Foxing spots in various sizes and shapes range from yellow to black in color, and they are commonly found on Chinese books, paintings, and calligraphy (Chen and Xie 2002; Chen and Zhang 2012). Chen and Xie (2002) found that foxing spots on Xuan paper (made of blue sandalwood and straw) (Mullock 1995; Chen and Xie 2002) were due to the growth of fungi, *i.e.*, *Aspergillus* and *Penicillium*. However, the fungal activity on other types of Chinese paper remains unclear.

Since the 1930s (Iiams and Beckwith 1935), attempts have been made to understand and describe color changes caused by foxing spots on western papers, and three major explanations have been proposed. First, transition metals were detected from these spots, such as iron (Tang 1978; Tang and Troyer 1981; Cain and Miller 1982; Kenjo *et al.* 1987), tin (Kenjo *et al.* 1987), and copper (Tang 1978; Tang and Troyer 1981; Rebrikova and

Manturovskaya 2000), which act as catalysts for cellulose oxidation (Rebrikova and Manturovskaya 2000; Bicchieri Marina *et al.* 2001; Bicchieri M. *et al.* 2002).

Fungal infection is another possible cause of the foxing spot (Rebrikova and Manturovskaya 2000). It leads to acid-catalyzed hydrolysis of paper due to the generation of extracellular enzymes and metabolites from fungi, such as butyric, lactic, citric, and oxalic acids (Rebrikova and Manturovskaya 2000; Bicchieri *et al.* 2002). Other organic acids, such as amino acids and malic acid, yielded melanoidins in papers (Arai 1987, 2000). The fungal species isolated from foxing spots have been identified as *Penicillium*, *Cladosporium*, *Aspergillus*, and *Eurotium* (Wei 1979; Arai 2000; Zotti *et al.* 2008; Manente *et al.* 2012; Nunes *et al.* 2015). Other fungal species, such as *C. globosum*, *E. purpurascens*, *Peziza ostracoderma*, *P. pomorum*, *Trichoderma* spp., and *U. botrytis*, were found to have intense cellulolytic activities (Montemartini Corte *et al.* 2003). Florian (1996) and Florian and Manning (2000) suggested that autoxidation of lipids from conidia could also explain the discoloration of papers.

Third, a dual activity between fungi and iron was proposed by Iiams and Beckwith (1935), which suggested that the acids and metabolic substances produced by fungi could react with iron in paper and yield an unstable organic ferric substance (Iiams and Beckwith 1935). Cain and Miller (1982) found that snowflake-like foxing spots contain higher concentration of iron than surrounding paper areas where hyphae and occasional fruiting bodies were detected.

Traditional Chinese paper is made manually with non-woody fibers, such as hemp, ramie, jute, paper mulberry, bamboo, rice, and wheat straw fibers (Pan 1998, 2002). Papers made of kraft pulps, sulfate pulps, cotton pulps, and wood pulps are commonly used in Europe (Hubbe and Bowden 2009). In the 19th century, machine-made papers became widely used in China, and wood pulp started to predominate the market (Wang 2006). Chen and Zhang (2012) showed that the proportion of books with foxing spots (produced from 1659 to 1982) reached 60% in the Shanghai History Museum, and the occurrence rates of the spots on machine-made papers were higher than those of handmade papers.

Certain sizing and fillers can promote fungal growth and paper degradation (Beckwith *et al.* 1940). During the eastern Han dynasty (25 CE to 220 CE), sizing began to be applied to Chinese papers using starch, gelatin, and gum. Occasionally, the gelatin has been replaced by acidic rosin/aluminum in modern Chinese machine-papermaking (Wang 2006). However, due to the catalytic ability of alum (Erhardt and Tumosa 2005), alkaline and neutral sizing agents were employed in the 1980s, such as alkyl ketene dimer (AKD), alkenyl succinic anhydride (ASA) (Espy 1990; Pinzari *et al.* 2006; Pinzari *et al.* 2010; Area and Cheradame 2011; Song *et al.* 2011). In addition, various fillers make papers smoother, flatter, and less transparent (Casoli *et al.* 2013). Kaolin filler (Al_2O_3•SiO_2•$2H_2O$) was found in most of 19th-century printing papers (Beazley 1991). From the 20th century on, other fillers such as calcite ($CaCO_3$), gypsum ($CaSO_4$), and white zinc oxide (ZnO) were mostly used, and talc ($3MgO$•$4SiO_2$•H_2O), titanium dioxide (TiO_2), and barium sulfate ($BaSO_4$) were also employed (Beazley 1991; Manso *et al.* 2011; Manente *et al.* 2012).

To improve the understanding of foxing spots, their chemical and biological properties were explored on a 20th century Chinese manuscript *via* a case study. Due to sample availability, non-invasive instruments and tests that required small sample quantities were used. Herzberg staining was employed for fiber identification, and paper fillers were analyzed by scanning electron microscopy coupled energy dispersive spectroscopy (SEM-EDS) and attenuated total reflection Fourier transform infrared

spectroscopy (ATR-FTIR). Multiple analytical methods were used for the morphological and chemical characterization of foxed and un-foxed areas of the investigated archival paper, and they were combined with the results of microbiological study.

EXPERIMENTAL

Sample Description

A handmade manuscript (made in 1938) was used for all analyses (Archives Centre of Xi'an Jiaotong University, Xi'an, China). It has irregular yellowish-brown spots in the upper left corners, lower right corners, and in the central part of the paper (Fig. 1a). The spots penetrated the layers of paper beneath the top layer (Fig. 1b), which led to incomplete, fragile, and brittle pieces. The method of papermaking was judged based on the presence of 15 bamboo stick lines (Fig. 1c) of 1 cm and thread lines with 17-mm intervals (Fig. 1d).

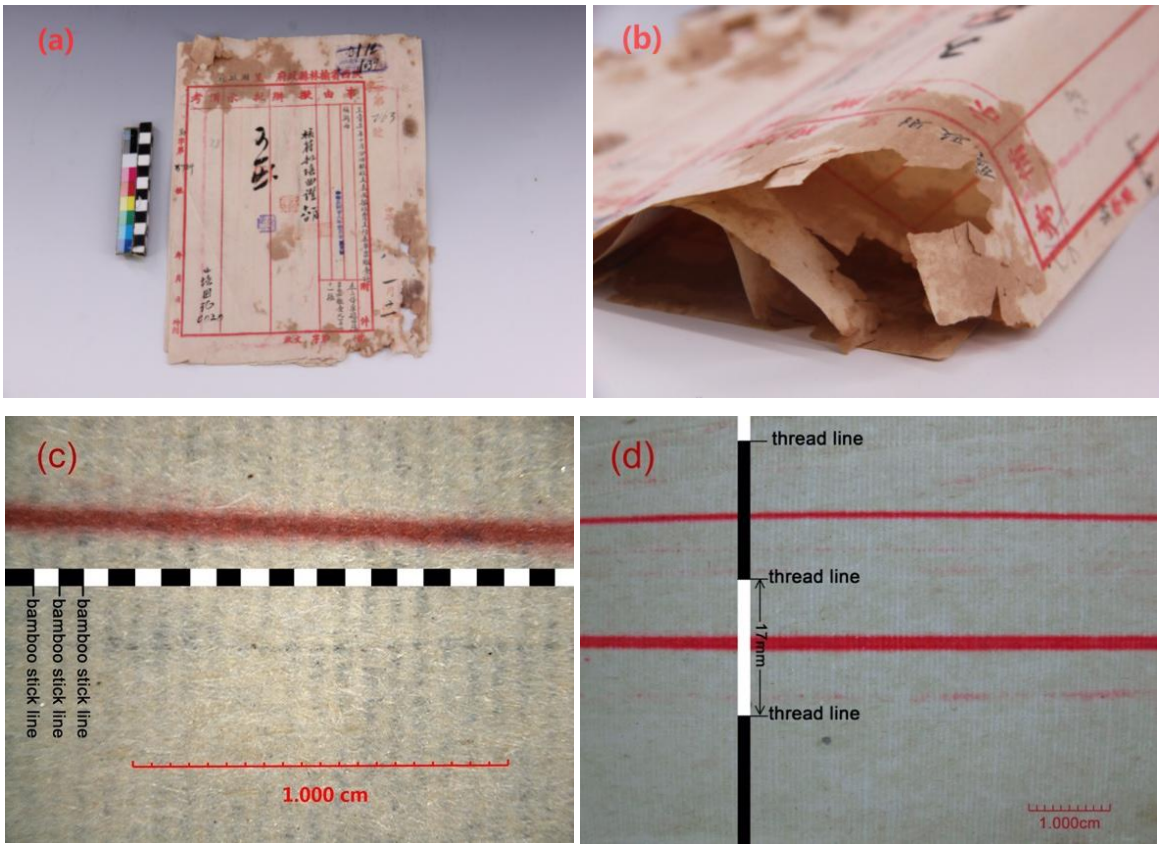

Fig. 1. A Chinese manuscript (a) with foxing spots: (b) details of foxing spots; (c) lines of bamboo sticks; and (d) thread lines

UV Examination

Colors and morphology of the spots were observed in images taken with a camera (Leica D-LUX, Type 109; Leica Camera AG, Weztlar, Germany), under natural light. Ultraviolet filters were used to observe the spots at 254 nm (Philips TUV 6W/G6T5; Royal Dutch Philips Electronics Ltd., Amsterdam, Poland) and 365 nm (Hitachi F6T5 6WATT; Hitachi Ltd., Tokyo, Japan).

Herzberg Stain Test

In accordance with the standard TAPPI T401 om-03 (2008), the Herzberg stain was made by mixing 25 mL of a saturated solution of zinc chloride in distilled water with iodine solution (0.25 g of iodine and 5.25 of g potassium iodide dissolved in 12.5 mL of distilled water). The resulting solution was kept still for over 6 h, and supernatant was then decanted into a dark bottle to avoid excessive exposure to light and air. A small piece of iodine was added during storage (Clark 1920).

Samples were taken from paper pieces with foxing spots (approximately 2 mm × 2 mm), stained with Herzberg solution (China National Pulp and Paper Research Institute, Beijing, China), and observed and photographed using a XWY-VI paper fiber analyzer (Zhuhai Hualun Papermaking Technology Co., Ltd., Zhuhai, China) with 4× and 10× magnifications.

pH Test

According to the cold extraction method described in TAPPI T529 om-14 (2009), the pH values of five random points on foxed and un-foxed areas on the first page were measured using a pH meter (Mettler Toledo FE28; Mettler Toledo International Trading (Shanghai) Co., Ltd., Shanghai, China).

Optical Profilometer

A non-invasive optical profilometer (Sensofar S neox, Sensofar Metrology, Barcelona, Spain) was used for topographic observation and measurement of surface roughness (1.68×1.40 mm).

X-ray Diffractometer (XRD)

Two small detached pieces of foxed and un-foxed paper areas (approximately 5 mm^2 × 5 mm^2) were used for XRD analysis. An XRD spectrometer (PANalytical X'Pert^3 Powder; PANalytical B.V., Almelo, Netherlands) equipped with Cu target and Cu-Kα radiation was used. An angular range (2θ) was scanned from 10° to 50° at a step size of 0.013°, and the working voltage and current were 40 kV and 40 mA, respectively.

Scanning Electron Microscopy with Energy Dispersive X-ray Spectroscopy (SEM-EDS)

The two samples used for XRD analysis were placed on SEM sample holders using double-sided sticky tape, and then a SEM-EDS (Hitachi SU3500; Hitachi Ltd., Tokyo, Japan) was used. High vacuum (the pressure of 60 Pa) in the chamber and the accelerating voltage of 5 kV was used for the analyses.

Attenuated Total Reflection-Fourier Transform Infrared (ATR-FTIR) Spectroscopy

An ATR-FTIR spectrometer (PerkinElmer Spectrum Two; Perkinelmer Inc., Waltham, MA, USA) was used coupled with middle and far infrared diamond ATR modules for filler analysis. Spectra were acquired in the transmittance mode over the range of 4000 to 450 cm^{-1} for 4 scans at 4 cm^{-1} resolution.

Energy-Dispersive X-ray Fluorescence Spectroscopy (ED-XRF)

XRF analyses were carried out on randomly selected foxed and un-foxed areas on the first page with an energy dispersive X-ray fluorescence Spectrometer (SHIMADZU EDX-7000: Shimadzu Corporation, Kyoto, Japan). Operating conditions of 15kV,1000μA and testing range of 1mm in diameter were used for the elemental analysis.

Fungal Culture and Identification

A sample was taken with a sterile inoculating loop from the foxed paper areas of the first page, and it was then was inoculated in the CYA medium (Containing Sucrose 30 g/L, $NaNO_3$ 3 g/L, K_2HPO_4 1.0 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, KCl 0.5 g/L, $FeSO_4$ 0.01 g/L, yeast extract 1 g/L, and agar 15 g/L) (Montemartini Corte *et al.* 2003) for fungal growth at 28 °C and 80% relative humidity (RH) for 4 to 5 days, and the fungi were isolated in the Sabouraud dextrose agar (SDA) (Ezekwueche *et al.* 2018). Then, all different colonies were picked and inoculated in Sabouraud dextrose broth (SDB) to obtain pure cultures under the same conditions. The fungi were stained with lactophenol cotton blue, observed with the microscope (Olympus BX41; Olympus Corporation, Tokyo, Japan), and digitally recorded with a camera (Leica D-LUX, Type 109, Leica Camera AG, Weztlar, Germany).

The pure cultures were inoculated in SDB and shaken mechanically at 28 ℃ to obtain the fungal solution for DNA sequencing. The fungal strains were identified at the genus level by observing the macroscopic features of the colonies (texture and color) and the hyphae and reproductive structures. The results were compared with Internal transcribed spacer (ITS) sequences in the National Center of Biotechnology Information (NCBI) database.

RESULTS AND DISCUSSION

Descriptive Information of Foxing Spots

A clear rim of florescence surrounded each yellowish-brown spot under UV illumination at 365 nm (Fig. 2a to Fig. 2d). The formation of these fluorescent species is an intermediate stage of paper degradation, which eventually leads to the brown discoloration of paper (Rebrikova and Manturovskaya 2000; Pedersoli *et al.* 2001) due to increases in the number of conjugated double bonds (C=C, C=O, or C=N) in cellulose molecules (Brandt *et al.* 2009).

Figure 2e shows that the fibers from the un-foxed area were purple-blue (approximately 80%) and yellow (approximately 20%) from the Hertzberg stain. The fibers presented ribbon-like structures with pointed ends and pits in cross-fields. In addition, vessels were not found (Fig. 2f). These characteristics indicated that the raw materials of this paper consisted of 80% chemical and 20% mechanical softwood pulp (Wang 1999).

For filler identification, the main elements detected in the manuscript were C, O, Al, Si, Ca, S, and Mg, and the EDS mapping of the elements is presented in Fig. 3. The high Ca amount suggested that calcium carbonate ($CaCO_3$) or/and calcium sulfate ($CaSO_4$) may have been added. The presence of Al and Si, and Mg and Si likely correspond to kaolin ($Al_2O_3 \cdot 2SiO_2 \cdot 2H_2O$) and talc ($3MgO \cdot 4SiO_2 \cdot H_2O$).

Fig. 2. The fiber morphology of the foxed area of the manuscript under different light illuminations (a and c: standard light; b and d: 365 nm UV light). The morphological features of the fibers from the un-foxed area of the manuscript were 40× (e) and 100× (f)

Comparison between Paper Areas with and Without Foxing Spots

The un-foxed areas were slightly acidic with an average pH of 5.68 (uncertainty 2%, n = 5), while the pH of the foxed areas was much lower at 4.73 (uncertainty 3%, n = 5), which might due to metals or acids from fungal metabolites accelerating the oxidation of the cellulose chain, as suggested by Manso *et al.* (2009). For further verification, a series of characterizations was carried out.

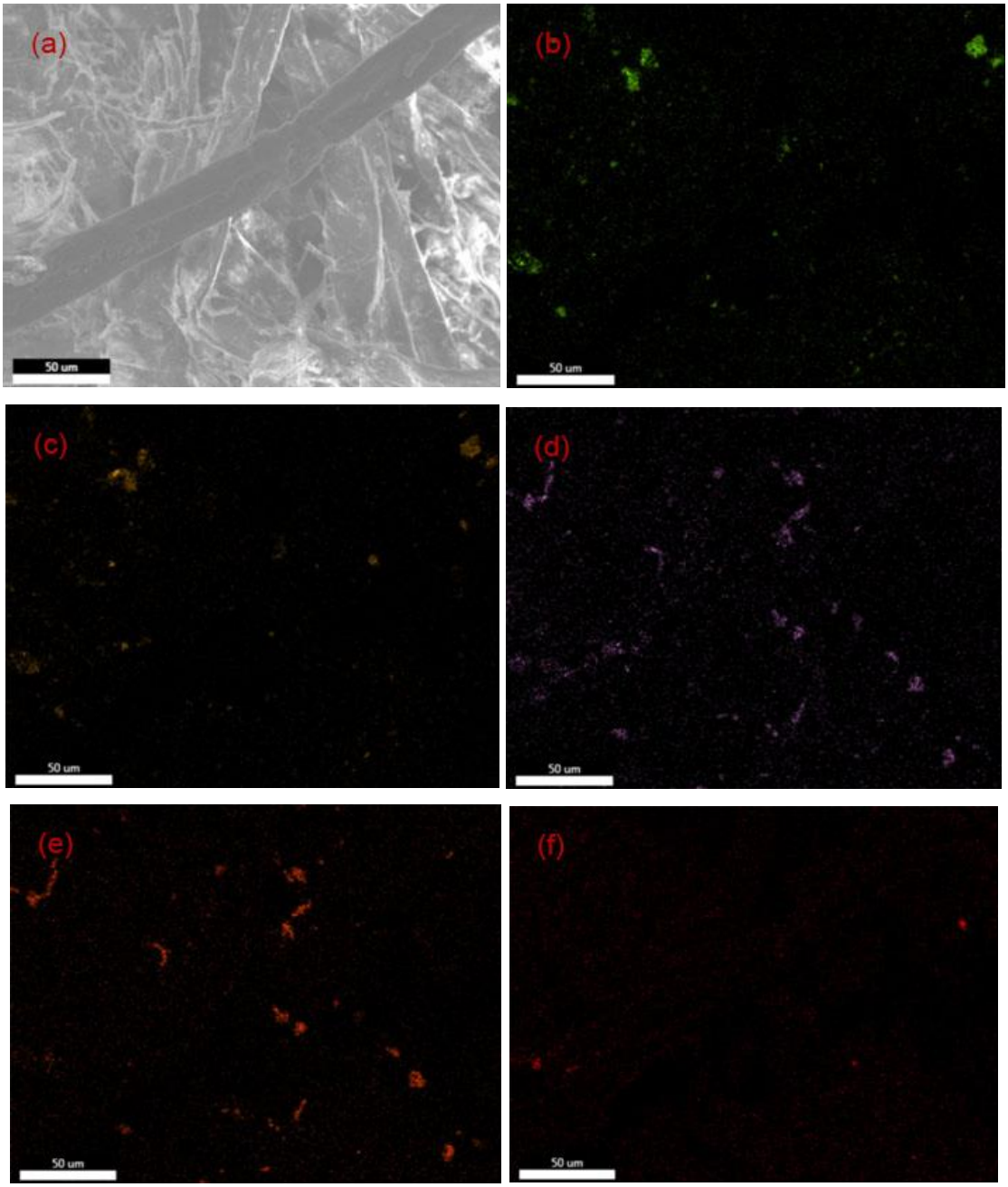

Fig. 3. The SEM images and elemental mapping of the un-foxed areas of the manuscript: SEM image of (a) the paper; the distribution of elemental (b) Al; (c) Si; (d) Ca; (e) S, and (f) Mg in the map

X-ray diffractometry can determine the crystallinity and orientation degree of cellulose (Ma *et al*. 2012). The XRD pattern of cellulose I was characterized by a well-defined principal peak at 22.5° (2θ) and two secondary peaks at 14.5° and 16.3° (2θ). However, as cellulose degradation occurred, two secondary peaks merged initially and the disappeared, leading to a smaller principal peak (Foner and Adan 1983) at 22.7° (2θ) that was attributed to the (002) crystal plane (Fig. 4) (Moropoulou and Zervos 2003). A discernible difference in cellulosic crystalline structure was not found between the samples with and without foxing stains.

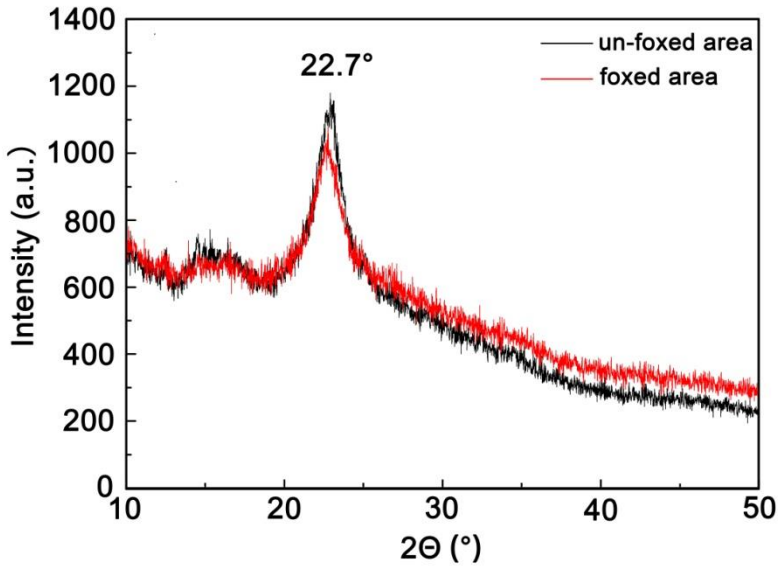

Fig. 4. The XRD pattern of the un-foxed area and the foxed area of the manuscript

As described in the standard ISO 25178-2 (2012), the parameter – arithmetical mean height (S_a) is used generally to evaluate surface roughness. By the observation with the optical profilometer in Fig. 5, although fiber surfaces of both un-foxed and foxed paper areas look similarly, S_a values of the two spots were 4.14 μm and 5.24 μm respectively, indicating that the roughness of the foxed area might be increased by additional substances.

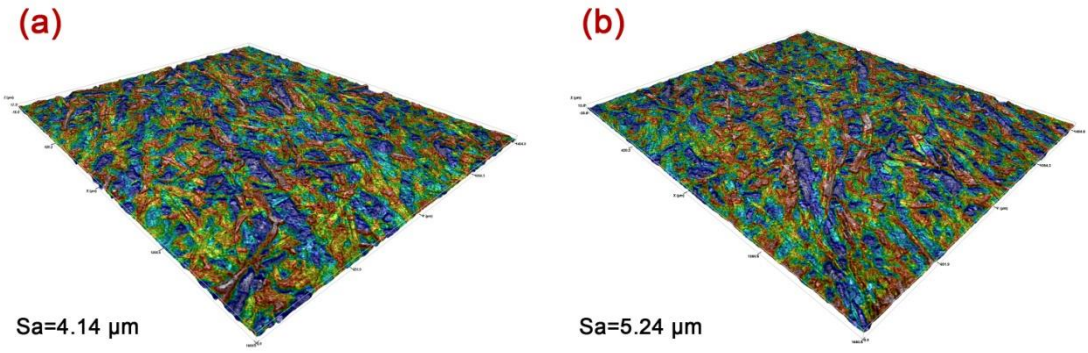

Fig. 5. 3D images taken by optical profilometer for the un-foxed area at 10 × (a) and the foxed area of the manuscript at 10 × (b)

For further exploration, SEM images (Fig. 6a) show that the surface of un-foxed area were structurally organized with intact fibers with several additives (fillers or impurities) spreading evenly, while some broken fibers (red circles in Fig. 6b) were found in foxed area, and it can be clearly seen that and fungal hyphae and spores of different sizes and morphology are present (Fig. 6c).

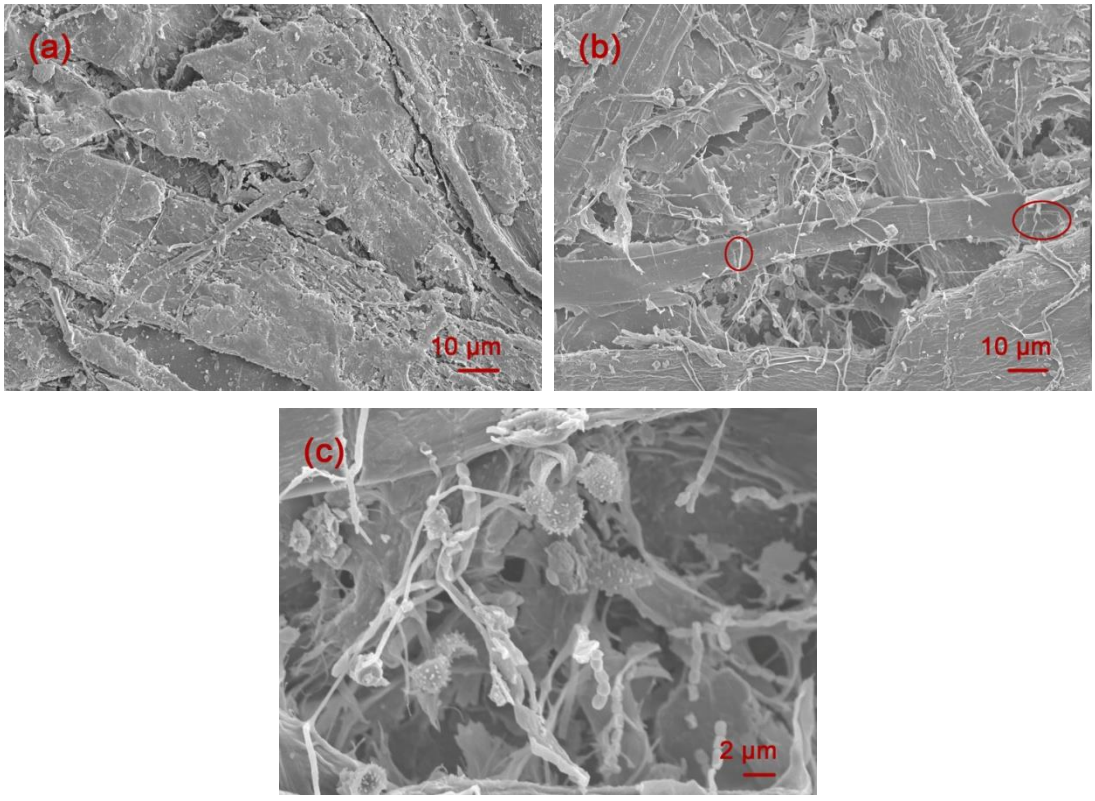

Fig. 6. SEM images of the un-foxed area at 1000 ×(a) and the foxed area of the manuscript at 1000 ×(b) and 4000 ×(c)

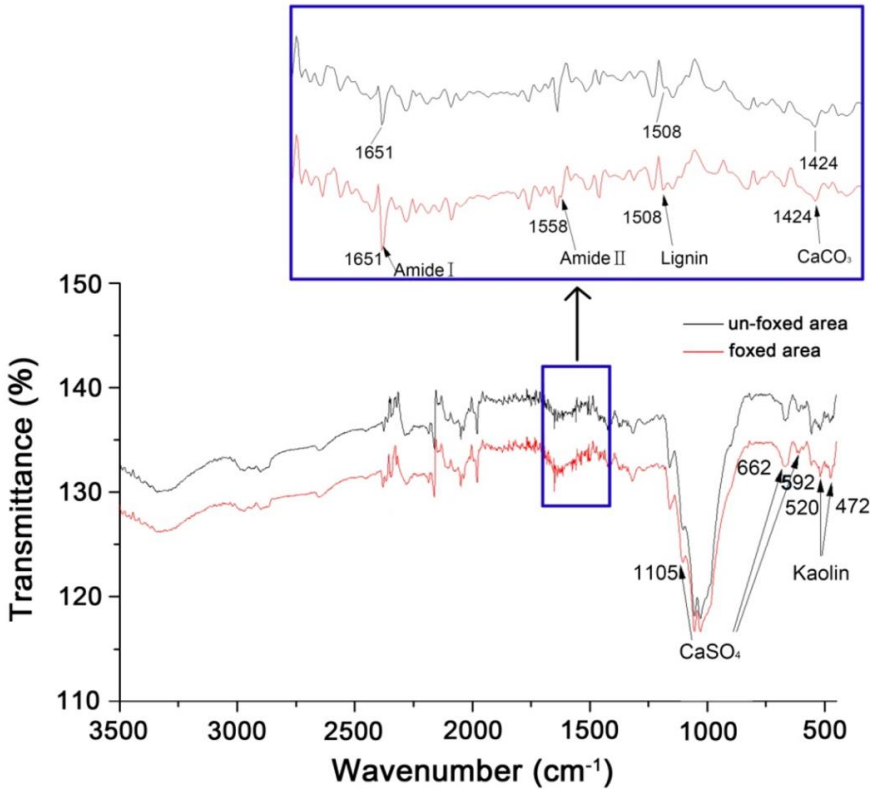

Fig. 7. The ATR-FTIR spectra of the un-foxed and foxed areas of the manuscript

As shown in ATR-FTIR spectra (Fig. 7), the peak at 1651 cm^{-1} was attributed to amide I (1600 cm^{-1} to 1700 cm^{-1}), suggesting the presence of proteinaceous materials (Derrick *et al.* 1999; Manente *et al.* 2012), which might be attributed to the sizing agent for both un-foxed and foxed areas; however, the peak of amide II at 1558 cm^{-1} was detected on the foxed area only, which further confirms the presence of fungi in this area (Nunes *et al.* 2015) .

Table 1. Attribution of Characteristic Peaks and Interpretation of ATR-FTIR Spectra for Un-foxed Area and Foxed Areas of the Investigated Manuscript (Manso *et al.* 2009; Nunes *et al.* 2015)

Wavenumber (cm^{-1})	Interpretation
3334	O-H stretching vibration
2897	C-H, $C-H_2$ stretching vibration
1651	C=O stretching vibration
1558	C-N stretching vibration
1508	Aryl ring asymmetric stretching
1424	CO_3^{2-} stretching vibration
1105	C-O-C symmetric stretching vibration, SO_4^{2-} stretching vibration
662	SO_4^{2-} bending vibration
592	SO_4^{2-} bending vibration
520	Si-O-Al stretching vibration
472	Si-O-Si bending vibration

In Fig. 8, elemental analysis shows that Ca content increased from foxed to un-foxed areas. This was not directly related to foxing stains as Ca is often present in paper materials (Manso *et al.* 2009). Also, contents of transition metals (Fe and Cu) in both areas were quite similar (Table 2), suggesting that this might not be the main cause for the foxing spots.

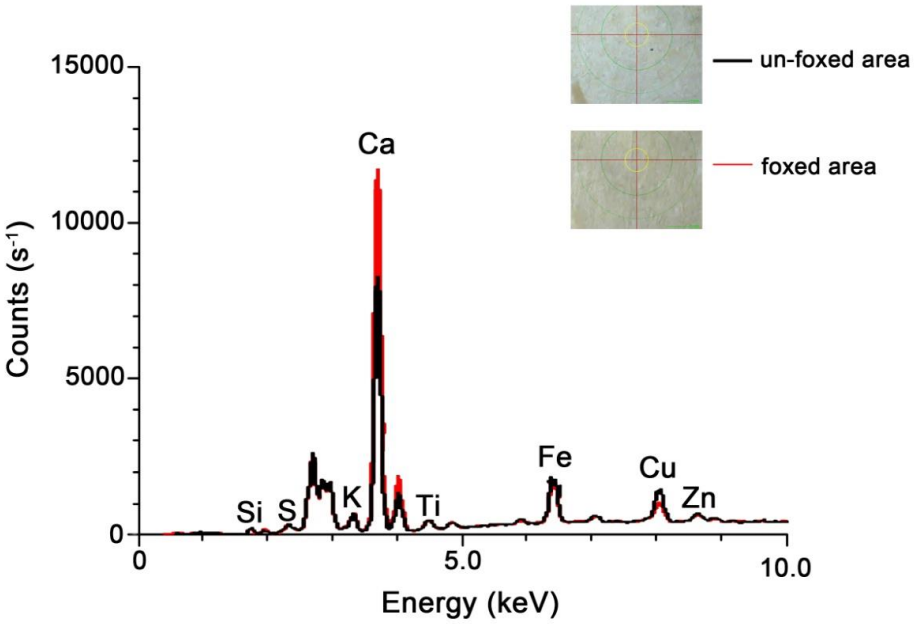

Fig. 8. The ED-XRF spectra of the un-foxed and foxed areas of the manuscript

Table 2. ED-XRF Result of Samples (Weight %)

Sample	Ca	Si	S	K	Fe	Ti	Cu	Zn
Un-foxed	53.770	30.008	9.297	3.678	1.464	1.194	0.404	0.185
Foxed	61.291	23.315	9.638	2.938	1.470	0.931	0.264	0.154

Isolation and Identification of the Fungi

Infections of fungi are widespread in libraries and archives, and the fungal species identified belong to four main genera, *i.e.*, *Aspergillus* sp. (approximately 30%), *Penicillium* sp. (approximately 30%), *Cladosporium* sp., and *Ulocladium* sp. (Nyuksha *et al.* 1993; Zyska 1997; Mesquita *et al.* 2009; Manente *et al.* 2012). Therefore, the foxing stains in this study may have been caused by one of these species.

To verify this hypothesis, three strains of fungi were selected, isolated, and purified from the foxing spots on the manuscript; they were labelled as No. 1, No. 2, and No. 3 according to morphological characteristics and cultural properties (Wei 1979; Zhang 2003). The isolated fungal colonies were flocculent and grew rapidly. At the beginning of the growth, the hyphae were colorless or dark white. However, they later turned brown (No. 1 and No. 2) or black brown (No. 3) (Fig. 9). Conidia mostly presented obclavate shapes that were divided by both horizontal and vertical septa (Fig. 10).

After DNA sequencing, the obtained ITS sequences were compared with the NCBI database. The results showed that all three strains (No. 1, No. 2, and No. 3) belonged to *Alternaria*, in which homologies of No. 1, No. 2, and No. 3 fungi are attributed to sequences of *Alternaria tenuissima* (KX783391.1), *Alternaria tenuissima* (KX783385.1), and *Alternaria solani* (LC339938.1), respectively. In addition, the homology of all fungi reached 99% in their nucleotide. Although both No. 1 and No. 2 were identified as *Alternaria tenuissima*, different sequences of ITS-DNA should be considered different species. Genus *Alternaria* is one of the most common fungal conidia with strong adaptability to environment and matrix. The hyphae are slender, and they range in color from pale to brown. Most of them can parasitize plants, which leads to plant diseases, such as leaf and steam stains or fruit rot. In addition, some are saprophytic fungi that live in soil. Cellulase can be produced from this species *via* a metabolism process (Zhang 2003) to hydrolyze the β-1, 4-glucoside bond of cellulose.

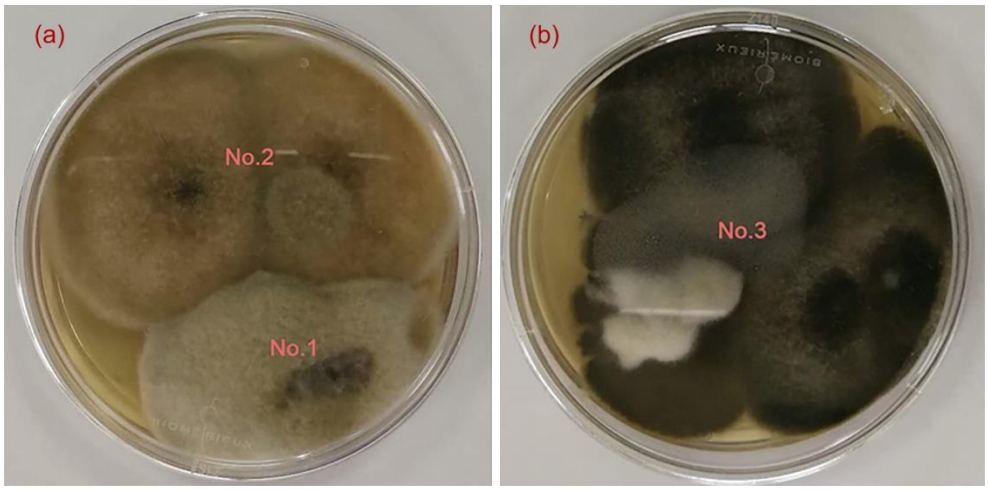

Fig. 9. The final morphological characteristics of the isolated fungal colonies labelled (a) No. 1, No. 2, and (b) No. 3

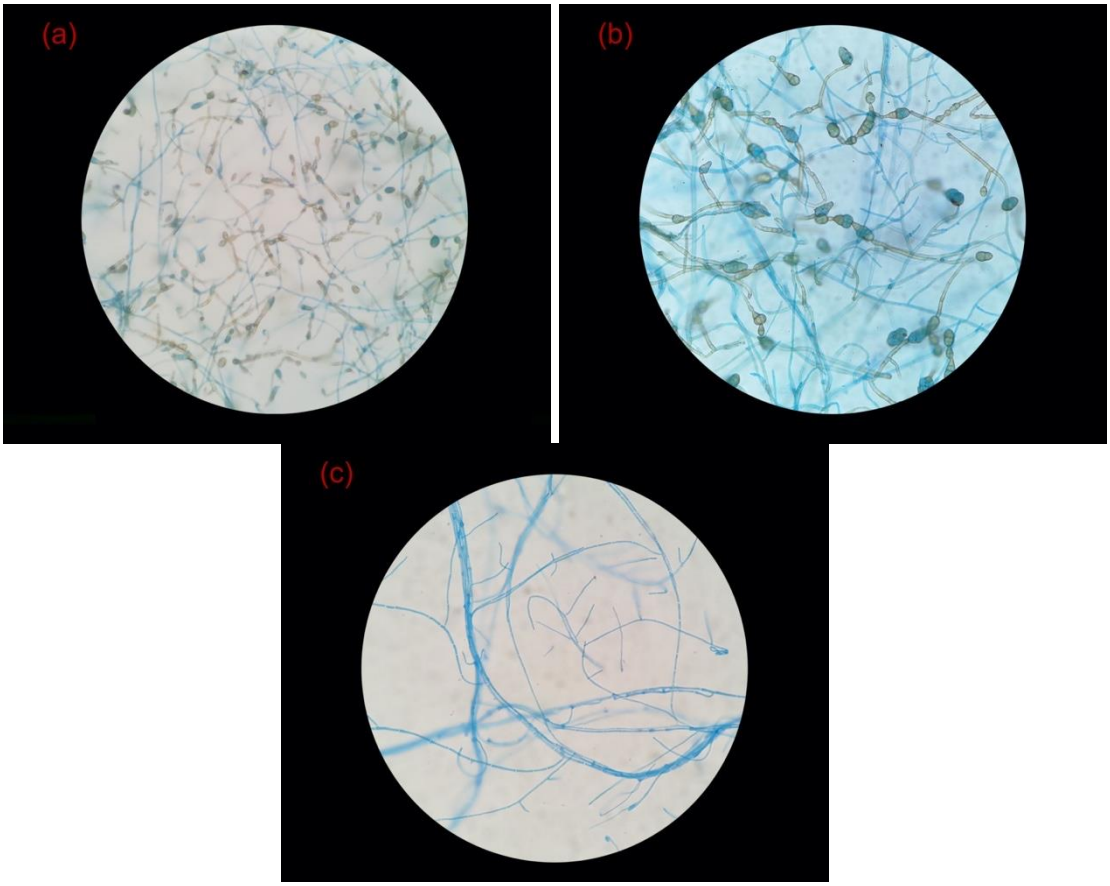

Fig. 10. The microscopic morphological characteristics of the isolated and purified strains: (a) No. 1, (b) No. 2, and (c) No. 3 at 20× magnification

CONCLUSIONS

1. Foxing spots were able to grow on the paper made of 80% chemical softwood pulp and 20% mechanical softwood pulp. Fillers, such as kaolin (Al_2O_3•$2SiO_2$•$2H_2O$), calcium carbonate ($CaCO_3$), calcium sulfate ($CaSO_4$), and talc ($3MgO$•$4SiO_2$•H_2O), were added during papermaking. Whether the paper pulp and additives have an impact on the generation of foxing spots should be further studied.
2. A clear rim of florescence surrounded each spot when irradiated by UV light, which presented bigger and more obvious foxed stains than the stains observed by the naked eye.
3. The pH test demonstrated that the paper area with foxing spots had lower pH than the un-foxed area. However, there were no obvious differences in crystallinity and iron and copper contents between paper areas with and without foxing stains as explored using XRD and ED-XRF.
4. Fungal hyphae and spores in different sizes and morphology were found on the surface of the foxed area, leading to the increased mean surface roughness of the paper on the

foxed area. Also, the peak of amide II was only present in ATR-FTIR spectrum of the foxed paper area, further indicating the presence of fungi .

5. *Alternaria tenuissima* and *Alternaria solani* were identified from fungi identification, which verified that fungi were the main cause for the foxing spots on this manuscript. Therefore, conservation strategies could be formulated to inhibit fungal growth.

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