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Review

Stains *versus* colourants produced by fungi colonising paper cultural heritage: A review

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ABSTRACT

Books, prints, drawings, watercolours, engravings, as well as all other works of art based on paper, are very susceptible to fungal development. The excreted substances and the fungal structures themselves are often coloured and interfere with the readability of the artefacts, diminishing their artistic and monetary value. In order to direct cleaning methods for specific fungal stains on paper, the colourants (molecules) responsible for those stains need to be assessed. However, the literature regarding fungal stains on paper and colourants produced by those fungi is very dispersed and scarce. Therefore, the main objectives of this work were surveying the most common stains on paper, the colourants present on those stains and the main fungi responsible for these colourants' production. To achieve these goals, two different but complementary literature reviews were made: one on paper conservation literature, where fungal stains observed on paper cultural heritage are reported; and another survey on the chemical/food/pharmaceutical fields where colourants' molecules produced by fungi, that can colonise paper cultural heritage, are identified and studied in greater detail. This paper presents the first literature review on this subject. The results show that the fungal genera more frequently related with fungal stains on paper cultural heritage are *Aspergillus* (29%) and *Penicillium* (13%). The most common colour of the stains is brown (54%), caused by foxing in most of the cases. However, in the paper conservation literature, no consistent correlation has been observed between stain colour on paper/specific fungal species and colourants/chemical compounds. On the literature review regarding the use of fungal colourants for industrial/commercial purposes, the referred colourants can be mostly classified chemically as carotenoids and polyketides. Biosynthetically, most colourants produced by fungi are polyketide-based and representative classes may include chemical structures such as azaphilones, anthraquinones, hydroxyanthraquinones, naphthoquinones and other structures. From a total of 80 different colourants, the ones mostly produced by paper colonising fungi were polyketide quinones, namely the hydroxyanthraquinoid (HAQN) colourants. This review showed that the most commonly studied colours are yellow and red, followed by orange. The production of these colourants is often associated with the genera *Aspergillus* sp. and *Penicillium* sp. which are frequently found on stained paper. Overall, there is no doubt that colourants producing fungi are a serious problem to paper conservators, since there is a great variety of colourants produced by different species of fungi colonising paper. This review catalogues the fungal genera, species and excreted colourants mentioned in the literature as being responsible for staining paper cultural heritage.

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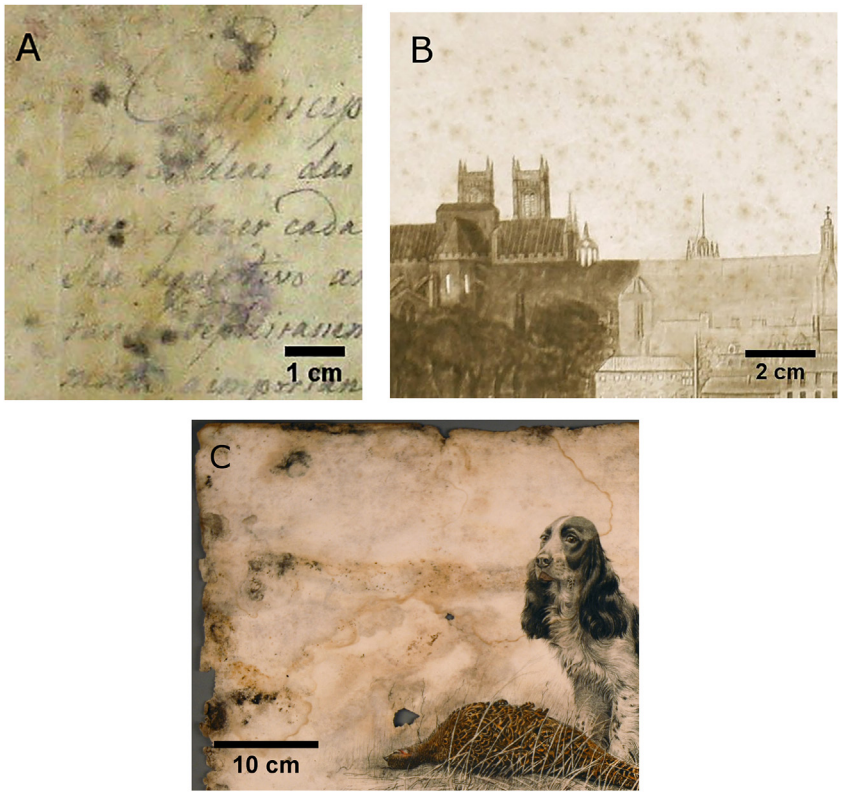

Fig. 1. Three examples of different stains on paper. A. Black, brown and purple fungal stains on an archival document (AHU-DGLAB, Portugal) [12]. B. Foxing stains on an etching (private collection) [12]. C. Black and brown stains on an etching – CleanART case study (private collection).

1. Research aim

The main goal of this work was to perform a comprehensive review on the most common fungal stains appearing on cultural heritage paper, the colourants (molecules) present on those stains and the main fungi responsible for these colourants production. All this knowledge is essential for the development of new and safer cleaning methods for the removal of stains produced by fungi on paper, without damaging the substrate, by targeting the specific molecules (colourants) causing those stains. By analysing the current literature, this work also highlights the significant gaps in knowledge on this issue, bringing to light future research.

2. Introduction

Books, documents, maps and works of art on paper are the carriers of a precious heritage left by our ancestors. These special items are of great significance, since they ensure the link between present and future by sending a set of values from one generation to another [1]. To ensure the passage of such legacy to future generations, knowing how to preserve paper materials is a matter of the utmost importance.

Paper can be deteriorated due to physical, chemical and biological agents. This material is particularly susceptible to biodeterioration processes due to its organic composition and hygroscopicity [2]. Biodeterioration can be defined as any unwanted alteration in a material caused by the vital activities of organisms [3].

The main microorganisms that deteriorate collections based on paper are filamentous fungi [1], affecting collections in museums, archives, and libraries all over the world [4–6].

Filamentous fungi growing on paper induce several chemical and physical decomposing processes, due to the excretion of metabolic substances that interact with the substrate, and the development of fungal structures that alter the structural organisation of the paper [7,8]. The excreted metabolites include colourants, enzymes (e.g., cellulases and proteases), organic and inorganic acids, chelating agents and other biochemical substances [8].

The excreted substances and the fungal structures themselves are often coloured [9,10] producing stains on paper. Accordingly to the literature, these fungal stains on paper present a great variety of colours from black to brown, red, yellow and purple [7,9] (Fig. 1). Fungal stains might migrate through successive pages and the number of stained areas increases with time. These stains can ultimately deem the document unreadable, even when fungi are already dead [11]. Consequently, the successful cleaning of fungal stains from paper is a mandatory conservation task, and the development of new cleaning methods is considered a priority by paper conservators [12].

To better achieve a successful removal of the fungal stains from paper, knowing the chemical composition of the colourant, or colourants, which produce a given stain, would be ideal. In this study, we present a compilation and a critical review of the most common stains on paper, the colourants present on those stains and the main fungi responsible for these colourants' production. In order to achieve these goals, it was necessary:

- to determine the most common colours of fungal stains reported on paper cultural heritage;
- to gather all literature, concerning the identification of fungi that produce those stains on paper;
- to ascertain what kind of colourants are produced by fungi, by collecting information in distinct fields of knowledge, such as food and pharmaceutical.

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Colourants can be classified as pigments or dyes [13]. Despite the reference to pigments throughout the literature, by definition, a pigment is insoluble in the given medium, whereas a dye dissolves during application, losing their crystal or particulate structure in the process [13]. For instance, carotenoids (terpenoid colourants) are dyes in oil, but pigments in water [14]. Thus, the term colourants will be used throughout this text in general, to refer to either pigments or dyes, since the discussion on the solubility of each molecule in different media is outside the scope of this article.

3. Material and methods

In this work, we started by doing a literature review on stains produced by fungi within the context of the paper conservation area. A second literature review, regarding fungal colourants and species commonly used by the chemical, food and pharmaceutical industries was also made. In this second literature review, only fungi known to be found colonising paper cultural heritage were taken into consideration. A compilation and a critical review of these two literature reviews were accomplished.

4. Results and discussion

4.1. Stains produced by fungi on paper

Fungi can produce organic colourants of different colours during their development. The colourants are characteristic of different species, but the colour of stains arises not only from the chemical composition of colourants but also from many other factors. These factors include the reaction of colourants with the substrate (paper, in this case), which can have different chemical compositions; the presence and possible reaction with metallic trace elements (iron, zinc, manganese and copper); nutrients availability; the acidity or alkalinity of the paper; the presence of other microbiotic species; and environmental conditions [15].

Many fungal strains produce a variety of colourants (e.g., black, green, blue, purple, violet) with different chemical structures, often belonging to the anthraquinonoid or carotenoid groups. Some colourants are known to be enzyme inhibitors or antibiotics, while other colourants' functions are not yet entirely clear [15].

Fungal colourants, defined as secondary metabolites, are typically composed of many complex chemical substances that are formed during metabolic processes; these colourants may be encrusted in spores, present in mycelium (the mass of filaments constituting the body of the fungus), or secreted to a substrate such as paper [16]. The release of colourants into the substrate, or the presence of coloured microorganisms' structures, causes the appearance of different colour stains or patches on many works of art [15].

For the literature review on the paper conservation area, 29 distinct research articles were analysed and are presented in chronological order (Table 1). From the different articles a total of 49 case studies arise, since, in some cases [16,17], diverse types of paper objects were considered.

The studies presented in Table 1 are focused on paper objects dated from the 13th to the 21st centuries, particularly from the 19th and 20th centuries (Fig. 2). This higher incidence of analysed papers dating from the last couple of centuries may be related to the decreasing of paper's material quality on that period, or simply because there is a higher quantity/availability of this kind of papers for studies to be carried out.

In Table 1 we can observe that most of the reviewed studies were made in Italy and in the USA. Table 1 also shows that the most commonly addressed type of stain is foxing (more than 50% of the articles reported) and that most of the studies (around 83%) used paper already colonised by fungi. Furthermore, the use of molecular biology for fungal identification on stains appears to be more frequent after 2006, as expected, since this technique, only recently became broadly used in the area of cultural heritage.

4.2. Fungi that stain paper

The literature review regarding the stains caused by fungi on paper (Table 2) gives information on the genera and/or species responsible for the stain or stains occurring in the paper substrate, together with the colourant, fungal identification method and the corresponding bibliographic references. In this literature review, a total of 28 genera and 59 species of fungi were identified as causing stains on paper (Table 2).

Fig. 3 presents the percentage of the main genera of fungi reported in the literature as causing stains on paper. The staining fungi that are most frequently identified on paper belong to the genera *Aspergillus* (29% in red) and *Penicillium* (13% in green) (Fig. 3 and Table 2). The genera *Chaetomium*, *Cladosporium* and *Eurotium* were found in a percentage of 5–7%, whilst *Alternaria* and *Trichoderma* were both reported in about 4% of the studies. The remaining 32% of the genera mentioned in the literature represents less than 3% and correspond to the remaining 21 fungal genera that can be consulted in Table 2.

It has been reported that most filamentous fungi can grow under conditions of 0.6 to 0.98 water activity (a_w) [15]. This property, ranging from 0 to 1, can be defined as the water that is available to support microbial life and is, therefore, a crucial factor in determining whether or not fungal growth can be initiated [47]. There is no definitive limit value of a_w for general fungal growth as this is very species-dependent. Caneva et al. [15] stated that *Aspergillus* and *Penicillium* are particularly harmful to paper because they are able to grow on substrates having 7–8% moisture content, which can be reached in some types of paper under relative humidity (RH) conditions as low as 62–65%. The fact that *Aspergillus* sp. and *Penicillium* sp. were detected in a great percentage in this literature review is probably due to the fact that both these genera have species able to develop on paper under low water activity. *Aspergillus* and *Penicillium* also present a rapid growth in culture media, as well as in the paper. Moreover, they produce air-borne conidia, which facilitate material contamination [23]. Micro-climates inducing very high a_w can be generated in a room with an otherwise low RH. For this reason, a measurement of indoor RH alone can be a poor predictor of mould problems [47] and merely decreasing RH might not be enough. Besides, many fungi are resistant to changes in RH and temperature [48].

4.2.1. Fungal identification methods

In order to analyse the results from the literature review presented in Table 2, it is important to consider the fungal identification methodology. Traditional culture methods with morphological identification are advantageous giving us the results of viable and cultivable fungi and allowing the study of fungal physiology. However, it is generally accepted that traditional culture methods cover less than 1% of the total microorganisms present in environmental samples [49], and therefore, the real culprit of fungal stains may be left unidentified.

From the reviewed literature (Table 2 and Fig. 4) it can be noticed that more than 50% of the fungi (in green), said to be responsible for the reported stains, were identified by culture methods and only 14% of the authors use both culture and molecular biology methods (in yellow) (Fig. 4). Circa 25% of the reported studies used molecular biology methods alone (in blue).

Molecular biology methods are a reproducible and powerful technique for the identification not only of viable fungi but also of formerly active fungi, which could hence be responsible for the

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Table 1
Case studies reviewed in this work together with the type of paper, type of study, location and respective reference are presented chronologically.

Publication number	Paper type (century)	Study description	Paper already colonised by fungi	Local and/or country	Reference
1	Weathered cardboard (20th century)	Fungal identification by culture	Yes	New England, Coastal area (USA)	[17]
2	Saunders Company medium-weight, pliable, rag paper – no heavy sizing (20th century)	Production of fungal stains on paper. Analyses of stains by SEM	No	(USA)	[16]
3	a) Flemish etching (19th century)	Effectiveness of stain laser removal from prints, drawings, and artworks on paper. Analyses of stains by SEM	Yes	New York (USA)	[18]
	b) Wove paper (medium weight), - Rives and Arche		No		
4	Rag paper (16th-20th century)	Investigation about the role of fungi in foxing	Yes	Libraries in England, South Africa, Brazil, Canada and the USA	[19]
5	Paper documents (18th-19th century)	Isolation/microscopic examination of fungi colonising the documents	Yes	Easton, Maryland (USA)	[20]
6	a) Untreated linen rag paper (19th century) b) Alum-rosin-treated paper (18-19th century)	Examination of fungal fox spots by EDX and SEM	Yes	New York (USA)	[21]
7	a) Hemp paper (20th century)	Identification of "foxing-causing fungi"	Yes	Hoodo of Byodoin Temple (Japan)	[22]
	b) Paper (20th century)	Isolation and culturing of fungi that can produce the foxing effect	No		
8	Israeli stamps (20th century)	Investigation on fungi responsible for the foxing phenomena. Analyses of stains by SEM	Yes	(Israel)	[23]
9	a) Samples of model-paper inoculated (20th century)	Molecular methods to identify fungal communities colonising paper samples of different composition and age	No	–	[24]
	b) Paper samples (20th century) c) Paper samples with 20-years-old inocula and naturally occurring infections (20th century)		Yes		
10	Book - linen and hemp (19th century)	Identification of isolates from brownish areas by MB	Yes	Paris (France)	[11]
11	a) Topographic map (18th century)	Investigate the fungistatic properties of the commercial 4-HB spray and calcium propionate on fungal strains isolated from foxing	Yes	Museo di Sant'Agostino in Genoa (Italy)	[9]
	b) Topographic map N° 17 (19th century) c) Cardboard backing Lignin-containing (20th century)				
12	a) Topographic map (18th century)	Verifying the presence of fungi in biodeteriorated paper; characterizing the paper surface by FTIR spectroscopy and fluorescence under UV radiation	Yes	Genoa (Italy)	[25]
	b) Topographic map (19th century) c) Cardboard backing - Lignin-containing (20th century) d) Ancient print GE1 (19th century) e) Ancient print GE2 (18th century) f) Ancient print GE3 (19th century)				
13	Book (cotton linters fibres) (16th century)	Study of microbiological damage	Yes	Rome (Italy)	[26]
14	Italian Manuscript (13th century)	MB analysis and microscopy	Yes	Italy	[4]
15	a) Passe-partout (20th century)	FTIR-ATR spectroscopy - understanding the controversial nature of foxing	Yes	Genoa (Italy)	[27]
	b) Passe-partout (21st century) c) Backing cardboard (21st century)				
16	Printed book and inoculation on sample paper	Procedures required for a conservative approach to the evaluation and description of the damage and the organisms. SEM	Yes	Rome (Italy)	[28]
17	a) Atlantic Codex set of drawings by Leonardo da Vinci (15-16th century) - Codex folio b) Atlantic Codex set of drawings by Leonardo da Vinci (15-16th century) - Modern paper frames	Molecular biology methods used to assess the current microbiological risk to stained pages of the manuscript	Yes	Milan (Italy)	[29]

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Table 1 (*Continued*)

Publication number	Paper type (century)	Study description	Paper already colonised by fungi	Local and/or country	Reference
18	Historic paper (18-20th century)	Evaluation, through a combination of culture and molecular methods, of the microbial diversity of different kinds of stains	Yes	Martin (Slovakia)	[30]
19	a) 17th-century paper b) 20th-century paper	Focused studies on black stains which are prevalent on art rendered on paper	Yes	–	[31]
20	Three paper photos (F1, F2 and F4), one book (L1) and two maps (M1 and M3), two paper notarial acts (P1a, P1b and P2) (19th Century)	Study microbial contamination of the environment and its influence on biodeterioration by the biofilm formation; analyse the relationship between environment microbiota and biofilm formation in materials stored in archives	Yes	(HAMLP), (AHCRD), (AN), (PL) and (ML) of the (NARC)[a]	[32]
21	General information on the main fungi and bacteria that attack paper	Highlight the role played by biocides in the destruction of microorganisms that attack irreversibly the paper, but also in the processes of preservation, restoration and conservation	Yes	Iaşi (Romania)	[1]
22	Engraving by G.B. Piranesi entitled *Veduta del Ponte e Castello Sant'Angelo* (18th century)	Laser removal of fungal growths and foxing stains from old paper artefacts	Yes	Italy	[33]
23	Japanese paper in the underlining of a painted folding screen and paper envelope	On-site investigations and culture-based analysis carried out to determine the extent of the microbial deterioration of paper-based objects	Yes	Japan	[34]
24	a) Ancient paper (19th century)	Identification and characterization of the microflora damaging historical manuscripts	Yes	Medina of Fez (Morocco)	[35]
	b) Sterile Whatman paper (21st century)	Effect of fungal contamination in paper artificially attacked for 18 months at 25 °C was examined	No		
25	a) Handmade–cotton (17th century) b) Engraving - Machine-made -cotton (20th century)	Explores black stains on paper attributed to Dematiaceous, meristematic fungi and their interactions with the paper matrix	Yes	Collection of the Maltese Archives (Malta)	[36]
26	Leonardo da Vinci's self-portrait (16th century)	Investigation on the possible causes of a very famous foxing through molecular and microscopic techniques	Yes	Royal Library in Turin 'Biblioteca Reale' (Italy)	[37]
27	Six foxed paper samples (from José de Figueiredo Conservation Laboratory in Lisbon) (20th century)	Characterization of foxed paper samples, regarding their cellulose matrix, fillers, and sizing materials, and possible paper degradation	Yes	Évora (Portugal)	[38]
28	Biodeteriorated paper from Kasr Abdin, Cairo, Egypt	Optimization of the conditions of laccase enzyme activity and its use for decolorizing the fungal pigment on ancient paper and parchment	Yes	Cairo (Egypt)	[39]
29	a) Whatman No. 1056	Cleaning paper samples by the application of pulsed light	No	Athens (Greece)	[40]

Information regarding if the paper was already colonised or if it was inoculated under laboratory conditions is also given.

[a] Historical Archive of Museum of La Plata (HAMLP), Archive of Historical and Cartographic Research Department from the Geodesy Direction (AHCRD), Archive of Notaries of Buenos Aires Province (AN), Argentina; at two repositories: Photo Library (PL) and Map Library (ML) of the National Archive of the Republic of Cuba (NARC).

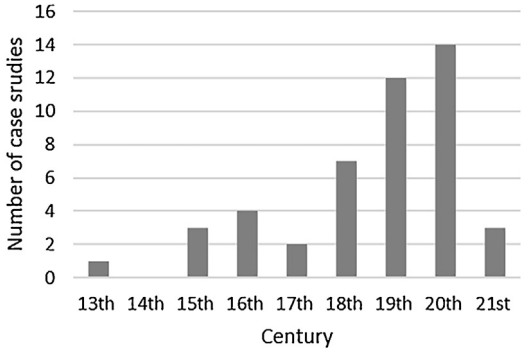

Fig. 2. Dating of paper objects analysed in the revised literature, by century.

biodeterioration processes [24]. Nevertheless, this method is still hampered by the costs and technical expertise needed to apply the DNA based methods. The ideal approach is, therefore, to use both methods (culture and molecular biology) as a complement to each other [19].

As far as other identification methods are concerned, Scanning Electron Microscopy (SEM) may be a useful tool for direct observation of fungal structures in the samples [28]. This approach can help in gaining knowledge on the association of specific microorganisms with particular types of biodeterioration. SEM, although not an accurate method for fungal identification, allows an observation of the fungal structures *in loco* comparing them with the species identified by, for e.g., molecular biology, enabling a better approximation to the actual culprit of the observed deterioration [29].

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Table 2
Fungal genera or species found staining on paper, together with their stain colour, colourants, fungal identification method and respective references.

Fungi	Stain colour	Colourants	Fungal identification method	References
Alternaria sp.	Red, purple, yellow, brown, black, etc.	–	–	[1,32]
Alternaria solani	Black	–	C	[18]
	Black stain that adheres very strongly to the paper	Black pigments	C	[16]
Aureobasidium pullulans	Brownish-red (foxing)	–	C	[9,25]
Aspergillus	Brown spot (foxing)	–	C	[9,25]
Aspergillus carneus	Foxing (light yellow to reddish brown)	–	C	[23]
	Brown spot (foxing)	–	C	[25]
Aspergillus flavus	Foxing (light yellow to reddish brown)	–	C	[23]
	Brown spot (foxing)	–	C	[25]
Aspergillus fumigatus	Black-brown	–	C and MB	[30]
	Black which is			
Diffused in the whole surface		–	C and MB	[30]
	Black and pink	–	C and MB	[30]
	Foxing (light yellow to reddish brown)	–	C	[23]
Aspergillus japonicus	Brown (foxing)	–	MB	[11]
Aspergillus melleus	Brown (foxing)	–	C	[27]
	Green yellow	–	C	[35]
Aspergillus nidulans (Emericella)	Purple	Purple-brown	C and MB	[26]
Aspergillus niger	Foxing (light yellow to reddish brown)	–	C	[23,32,41]
	Black	–	MB	[35]
Aspergillus oryzae	Brown (foxing)	–	MB	[11]
	Green yellow	–	MB	[35]
Aspergillus penicilloides	Brown (Foxing)	Melanoidines	C	[42]
Aspergillus sclerotiorum	Pale brown (foxing)	–	C	[27]
Aspergillus tamarii	Foxing (light yellow to reddish brown)	–	C	[23]
Aspergillus terreus var. aureus	Foxing; Brown pigmentation; Yellow spots	–	C	[23]
	Yellow pigments	–	C; Microscopy	[39]
Aspergillus ustus	Brown (Foxing)	–	MB	[11]
Aspergillus versicolor	Purple	Pink-orange (Versicolorine)	C and MB	[26]
	Foxing-light yellowish coating	Secreted yellowish pigments to the culture media	C	[43]
Bjerkandera adusta	Brown (foxing)	–	MB	[11]
Chaetomium (Dematiaceous fungi)	Dark pigmented individual fruiting bodies attached	Melanin	(CLSM) (LCA) (SEM-VP) (TLM)	[36]
	Black	Melanized fruiting structures	(SEM)(CLSM)	[44]
Chaetomium globosum	Brownish grey	–	C	[18]
	Black	–	C	[4]
	Brown (foxing)	–	MB	[11]
	Yellow (3 days); becomes greyish-brown in 2 days	Tomichaedin	C	[16]
Cladosporium cladosporioides	Black	Melanoidines (melanized cell walls)	C	[17]
	Brown spot (foxing)	–	C	[25]
Cladosporium sphaerospermum	Pale brown (foxing)	–	C	[27]
Doratomyces stemonitis	Brown spot (foxing)	–	C	[9,25]
Engyodontium album	Green–black staining	–	MB	[29]
Eurotium spp. (repens or *rubrum*)	Foxing	–	SEM	[21]
Eurotium amstelodami	Foxing	–	SEM	[21]
Eurotium halophilicum	Foxing	–	MB, SEM	[37]
Eurotium herbariorum	Brown (Foxing)	Melanoidines	C	[42]
Eurotium repens	Foxing - light yellowish coating	Secreted yellowish pigments	C	[43]
Eurotium rubrum	Foxing - light yellowish coating	Secreted yellowish pigments	C	[43]
Fusarium sp.	Red, purple, brown, black, etc.	–	–	[1]
Fusarium oxysporum	Pinkish	–	C	[18]
	Purple-pink	Fusarubin	C	[16]
Geomyces pannorum	Brownish-red (foxing)	–	C	[9,25]
Geosmithia putterillii	Brownish-red (foxing)	–	C	[9,25]
Gliocladium roseum	Foxing (light yellow to reddish brown)	–	C	[23]
Gloeotinia temulenta	Brown (Foxing)	–	MB	[11]
Hypocrea lixii	Green	–	MB	[35]
Mucor racemosus	Dark grey	–	MB	[35]
Mucor spinosus	Black	–	C and MB	[30]

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Table 2 (*Continued*)

Fungi	Stain colour	Colourants	Fungal identification method	References
	Black and pink	–	C and MB	[30]
Myxotricum deflexum	Light-red	Red pigmentation	Light microscopy; LV-SEM; MB	[34]
Paecilomyces variotii	Brown spot (foxing)	–	C	[25]
Penicillium spp.	Foxing	–	C; Microscopy	[38]
Penicillium atrovenetum	Green–black staining	–	MB	[29]
Penicillium chrysogenum	Yellow-green	–	C	[46]
Penicillium citrinum	Brown (foxing)	–	MB	[11]
Penicillium commune	Black-brown	–	C and MB	[30,32]
Penicillium coralligerum	Green–black staining	–	MB	[29]
Penicillium funiculosum	Foxing (light yellow to reddish brown)	–	C	[23]
Penicillium islandicum	Brown	–	C; Microscopy	[39]
Penicillium minioluteum	Brown (foxing)	–	MB	[11]
Penicillium notatum	Light green	–	C	[18]
	Yellow-green stain	Yellow compounds secreted	C	[16]
Penicillium purpurogenum	Brown-yellowish (foxing)	–	C	[27]
	Red	–	C; Microscopy	[39]
Penicillium raistrickii	Red	–	C; Microscopy	[39]
Penicillium restrictum	Brownish-red (foxing)	–	C	[9,25]
Penicillium spinulosum	Brownish-red (foxing)	–	C	[9,25]
Phoma herbarum	Black-brown	–	C and MB	[30]
	Black	–	C and MB	[30]
Polyporus brumalis	Brown (foxing)	–	MB	[11]
Ramichoridium apiculatum	Green–black staining	–	MB	[29]
Saccharicola bicolor	Brown (Foxing)	–	MB	[11]
Stachybotrys chartarum	Black	–	Microscopy;SEM;MB	[34]
Taeniolella sp.	Black	Melanins	(SEM)(CLSM)	[44]
Trichoderma citrinoviride	Brown (Foxing)	–	MB	[11]
Trichoderma koningii	Brown (Foxing)	–	MB	[11]
Trichoderma pseudokoningii	Brownish-red (foxing)	–	C	[9,25]
Ulocladium chartarum	Brown (foxing)	–	MB	[11]
	Brown/black	–	C; Microscopy	[40]
Ulocladium cucurbitae	Brown (foxing)	–	MB	[11]

C: culture; MB: molecular biology.

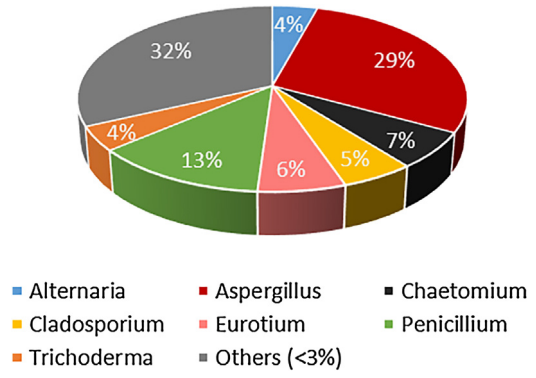

Fig. 3. Percentage of the main of fungi genera reported in the literature as causing stains on paper (data from Table 2).

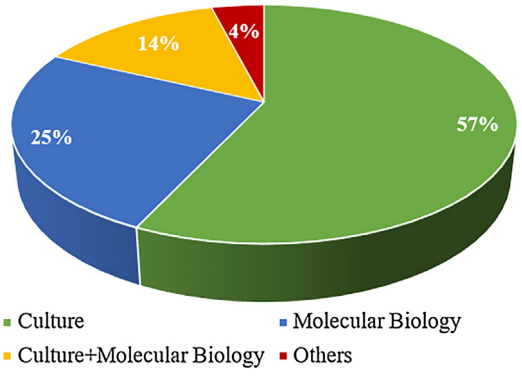

Fig. 4. Fungal identification methods (%) calculated from literature data from Table 2.

4.2.2. *Stain colours produced by fungi on paper*

The colours of fungal stains on paper identified in the literature are much diversified, ranging from brown, black, green, yellow, purple or pink (Table 2 and Fig. 5). Nevertheless, colour parameters were never reported in the review literature. Consequently, it is hard to confirm if different authors are referring to the exact same colour when using similar colour terminology.

By matching the various fungal genera with the reported colours (Table 2), it can be concluded that most fungal genera (54%) are related with brownish stains (Fig. 5). Also, 68% of the detected genera in brown stains were associated with the foxing phenomena (Fig. 6).

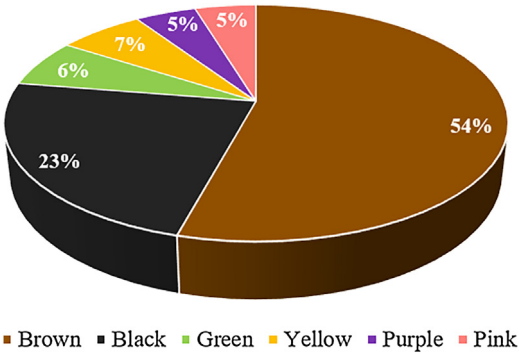

Fig. 5. Percentage of stain colours reported in the literature (data from Table 2).

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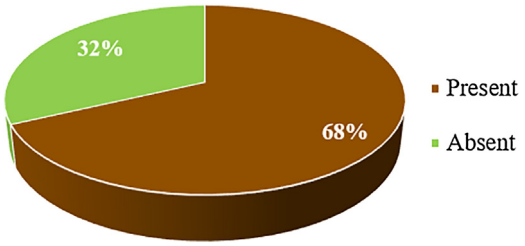

Fig. 6. Percentage of fungal genera associated with foxing, accordingly to the literature review on Table 2.

Foxing is a particular and very common chromatic alteration of paper, appearing as rust-coloured marks of different shapes, frequently as spots (Fig. 1C) [11,15,21,42,4].

The origin of foxing is still a matter of great discussion. In fact, there are two complementary theories for its occurrence [21,51]:

- the biotic theory — in which the stains are the result of the activity of microorganisms. These stains are usually circular in shape and, even when uncoloured, display a natural fluorescence that is yellow under UV light. This is likely due to the presence of organic compounds and fungal structures or to the release of melanoidines [21,42,52];
- the abiotic theory — in which the stains are the result of chemical phenomena, such as oxidizing and/or heavy metal deposits. In this case, the stains are more irregular, according to the shape of the contaminating substance (metals, fragments or crystals of chemicals incorporated in the paper during its manufacturing), and display a blue fluorescence under UV light [21].

According to Szczepanowska & Moomaw [18], fungal metabolism may change the oxidation state of trace metals present in the paper, such as iron, converting colourless areas into visible stains, observed as foxing. Metal and microorganisms contamination may come either from the papermaking and/or from airborne dust [33].

Other authors [11] refer that these foxed areas are a result of a dyeing structure and the chromatic changes induced in the paper are a result of these structures' ageing process. Nevertheless, it is thought that the majority of foxing stains are caused by fungi [11,42,51]. Arai [42] showed that, when the optimum environment exists, there is a possibility for foxing to appear on paper and other materials and suggested a mechanism for its formation. This author considers that foxing caused by fungi is composed by one of the melanoidines formed by the Maillard reaction between cello-oligosaccharides and γ-aminobutyric acid and other amino acids, which are produced by the growth of absolute tonophilic fungi on paper.

Although there are no species exclusively responsible for foxing, the species causing this type of stain have in common a leaning towards xerotolerance (can grow at low water activity levels) and osmotolerance (can grow in high osmotic pressure, e.g., concentrated solutions of nutrients). Some results appear to endorse the theory that foxing is mainly due to fungal agents which play a leading role in the complex dynamics of biodeterioration [9].

It was stated in a case study that the species of *Eurotium rubrum, Eurotium repens* and *Aspergillus versicolor* most probably participated in the formation of the foxing stains on "The Market in Gniew" drawing [43]. These species secreted large amounts of yellow, orange-brown or brown colourants into the culture medium, as well as to the paper samples placed on top of the colonies. These observations lead to the conclusion that the brown stains produced on the test papers, and most probably on the paper support of the studied drawing, were caused by those yellow, orange-brown or brown colourants [43]. However, the authors did not identify which fungi secreted each specific colourant.

The second main colour detected (23%) in the present literature review was black. Black colourants are frequently associated with the production of melanin by fungi. *Aspergillus niger* is an example of a melanin-producing fungus. One of the main features of this fungus is the production of black or dark brown conidia resulting from the combination of dark brown melanins with hexahydroxyl pentacyclic quinoid green colourants [54].

The four remaining colours (green, yellow, pink and purple), despite less frequent, comprise the remaining 23% of the mentioned colours. For instance, *Aspergillus nidulans (Emericella)* and *Aspergillus versicolor*, identified on purple stains, produce purple-brown pigments. These pinkish to purple-coloured spots were distributed throughout the 16th-century book pages [26], in a repetitive way, suggesting that these marks were caused by migration throughout the pages. These species are also an example of dangerous fungi to human health since they produce a carcinogenic mycotoxin (sterigmatocystin) [26].

The characteristics of the paper itself have a great influence on the intensity and results of the fungal activity. Colourants can change their shade depending on growth conditions and paper properties (e.g., pH, presence of starch or gelatine sizing, presence of metals) [15]. *Aspergillus carneus*, *Aspergillus terreus var. aureus* and *Penicillium funiculosum*, for instance, show a more intense foxing colour when in the presence of iron salts [23].

SEM observation of fungal structures showed that despite the apparent homogeneous growth, different physiological situations could be found, suggesting a sharpened and varied effect of the paper constituents in the different stages of the fungal life cycle [55]. This is noted for several species gathered in Table 2, where, for the same genera of fungi, different colours are mentioned, according to the author and the conditions in which they are identified.

Even in the cases where the colour is mentioned, this information is questionable, since, when the fungi are grown in culture media, their colour may diverge from the one observed on the paper substrate. The stains present in a paper document may arise from coloured fungal bodies or from fungal metabolic waste products that are excreted into the paper fibres [18]. For instance, in the case of the yellow-green stain produced by *Penicillium notatum* [16], the stain apparently penetrates the substrate, because its intensity is the same on both the back and front of the paper. Subsequently, it was found that the isolated stain, extracted from the growth medium, is bright yellow. The greenish appearance of the fungi corresponds to the colour of the spores, which are embedded on the paper surface and are difficult to remove mechanically [16].

Sometimes, the concomitant growth of two or more fungi causes stronger/different colour on the culture medium. Besides, when there are two or more species, it is possible that only the dominant one shows its colour on the medium [23]. Also, depending on the author mentioning the fungal genera/species, the colour denomination varies. A very interesting example is the case of *Aspergillus versicolour*, which is reported by one author as producing a yellow colourant [43], while another author refers the colourant by its specific name, versicolourine, attributing a pink-orange colour to it [26]. These authors may be referring to the same colourant, but their colours' interpretation could be different.

The literature shows (Table 2) that regardless of the colourants produced by fungi identified on different stains, only 10% of the reported species are associated with the colourant they produce. For instance: versicolourine (produced by *Aspergillus versicolour* [26]); tomichaedin (produced by *Chaetomium globosum* [16]); fusarubin (possibly produced by *Fusarium oxysporum* [16]); and melanoidines (produced by *Aspergillus penicilloides and Eurotium herbariorum* [42] and *Cladosporium cladosporioides* [17]). Melanoidines/melanins make about 62% of the 10% of the

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colourants that were identified in the literature and are always associated with black stains, but this is mostly a generic name for a series of different black colourants. However, no studies are carried out to assess if these colourants are indeed present in such stains.

Therefore, another literature review, outside the scope of the paper conservation field, was necessary to understand which colourant structures could be produced by fungi and also be responsible by paper staining. In the next section, literature from the chemical, food and pharmaceutical fields, where fungal colourants are identified regarding each fungal species, is compiled. For this review, only fungal species reported as paper colonisers were taken under consideration.

5. Identification of colourants produced by fungi

Fungi produce a wide range of secondary metabolites. Some of the metabolites are high-value products with pharmaceutical applications such as penicillins, a group of antibiotics isolated from *Penicillium chrysogenum*. Some common secondary metabolites produced commercially from fungi have several applications such as antibacterial, immunosuppressants, plant hormone or cattle growth promoter. Therefore, fungi can be used as micro-chemical industries for the production of biologically active secondary metabolites [56]. For instance, fungal colourants are of industrial interest because they are often more stable and soluble than those from plant or animal sources [57].

Since there is virtually no information concerning the colourants produced by fungi colonising paper substrates, other areas were taken into account, making this a broader research outside the conservation area. For instance, filamentous fungi are currently playing a crucial role as microbial cell factories for the production of food grade pigments due to their versatility in their colourant shade and chemical profile, amenability for easy large-scale cultivation, and a long-term history of well-studied production strains [58]. These fungi are known to produce an extraordinary variety of colourants (black, red, yellow, green, blue, purple, among others) that include several chemical classes such as carotenoids, melanins and quinones [15,58].

Here we gathered the information from 1934 to date, about colourants produced only by fungal species that were also detected in paper biodeteriorated by fungi [12].

On a preliminary phase, studies regarding the use of these colourants in the food industry were taken into account. Later, a broader search concerning their secondary metabolites, in general, was carried out. Some of these colourants were firstly a focus of investigation due to their antibiotic or inhibitory pharmaceutical properties and were subsequently targeted by the food industry. For instance, orevactaene, a novel oxopolyene, was isolated from *Epicoccum nigrum* WC47880 during the screening of microbial fermentation extracts for their ability to inhibit the binding between HIV-I regulatory protein and its viral RNA-binding site, in 1997 [59]. Most recently, it has shown potential to be a functional food colourant, exhibiting enhanced photostability over the commercially available red Monascus colourant [14].

5.1. Chemical classes of the identified colourants

Fungal colour is one of the major characterizing feature used in their microscopic identification. Furthermore, some fungi undergo distinctive colour changes when they are bruised or treated with alkali [60]. The colourants responsible by these colours generally play biologically important roles in fungi, like anti-bacterial activity, protection from bacterial attack and they are also light-absorbing molecules that can protect the organism from UV damage [60].

Colourants from fungi can be broadly classified chemically as carotenoids and polyketides [14]. Therefore, in order to make a clear assessment of which colourants are produced by fungi, a division was made into two different types of colourants: polyketide and carotenoids. Molecules responsible for the colour produced by specific fungi are presented in Tables 3–7, separated by their chemical classification: azaphilones, quinones, melanins, other polyketide colourants, and carotenoids.

5.1.1. Polyketide fungal colourants

Polyketides are important natural products that include numerous toxins, antibiotics, a variety of therapeutic compounds, fungal melanins, and other colourants. Polyketides have attracted great attention because of their biosynthetic complexity and importance in the pharmaceutical industry, for instance [61].

Polyketide synthases belong to a large enzyme family with diverse roles in the production of secondary metabolites such as colourants, toxins, antibiotics and signalling molecules [62]. Importantly, fungal polyketides are one of the largest and most structurally diverse classes of naturally occurring compounds, ranging from simple aromatic metabolites to complex macrocyclic lactones [63].

Biosynthetically, most colourants produced by fungi are polyketide-based [64]. Representative classes may include chemical structures such as azaphilones, anthraquinones, hydroxyanthraquinones, naphthoquinones and other structures, each of which exhibits an array of colour hues [14].

5.1.1.1. Azaphilones. Azaphilones are polyketide derivatives that can be defined as a structurally diverse class of fungal secondary metabolites [63,64], with a highly oxygenated pyranoquinone bicyclic core, usually known as isochromene, and a quaternary carbon centre [63]. These can be coloured or non-coloured and, when coloured, are responsible for the bright yellow, red or green colours of mycelia [63]. These are produced especially by numerous species of ascomycetes, including genera *Aspergillus, Penicillium, Chaetomium, Talaromyces, Pestalotiopsis, Phomopsis, Eremicella* and *Epicoccum*, as well as *Monascus* and *Hypoxylon* [63], which may colonise paper causing the appearance of coloured patches.

Table 3 shows the different azaphilone colourants that can be produced by fungi identified on paper.

5.1.1.2. Quinones. Many of the colourants of fungi are quinones or similar conjugated structures [60]. Numerous quinones that are biosynthesized by the polyketide pathway have been isolated from fungi [60]. Quinones display an array of colours: yellow, orange, or red according to the position of the keto groups [75]. The colour of the quinone that is isolated does not always reflect the colour of the fungus. This may be because the quinone is accompanied by its reduction products, forming a quinhydrone complex [60]. Table 4 shows the different quinoid colourants produced by fungi detected on colonised paper. The majority of quinones produced by these fungi are hydroxyanthraquinoid (HAQN) colourants, but there is also some naphthaquinoid colourants mentioned.

HAQN colourants are widespread in nature (plants, insects, lichens) and have also been found abundantly in microorganisms, particularly in filamentous fungi belonging to *Penicillium* sp. and *Aspergillus* sp., with different colour hues [58].

Naphthoquinone colourants are very common in fungi and have been the subject of many studies regarding their chemical structure, biosynthesis and biological significance [75].

5.1.1.3. Melanins. Melanins are macromolecules formed by oxidative polymerization of phenolic or indolic compounds [95]. The generally accepted [96] chemical criteria used to define a melanin

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Table 3
Azaphilone colourants chemical structure (base structure in bold), colour and the fungi responsible for its production.

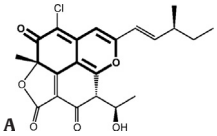
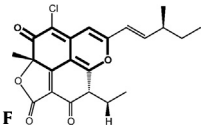
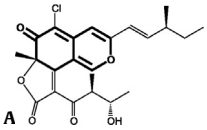
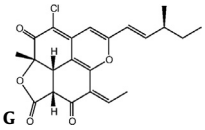
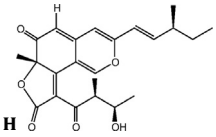
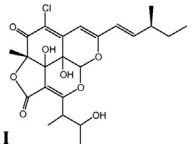
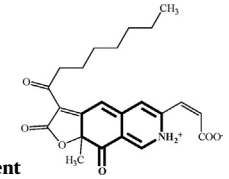
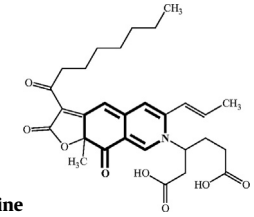
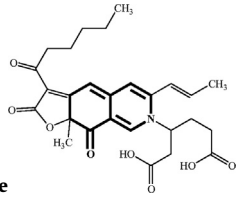
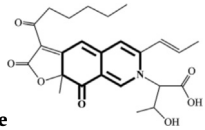
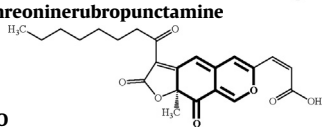
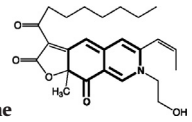
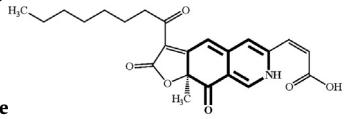
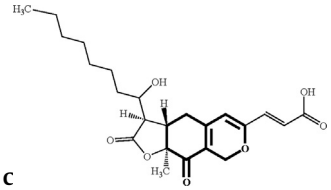
Colourant	Colour	Fungi	References
4'-Epichaetoviridin A 	Orange	*Chaetomium globosum*	[65]
4'-Epichaetoviridin F 	Orange	*Chaetomium globosum*	[65]
5'-Epichaetoviridin A 	Orange	*Chaetomium globosum*	[65]
Chaetoviridin G 	Orange	*Chaetomium globosum*	[65]
Chaetoviridin H 	Yellow	*Chaetomium globosum*	[65]
Chaetoviridin I 	Yellow	*Chaetomium globosum*	[65]
Monascus-like pigment 	–	*Penicillium*	[58]
N-glutarylmonascorubramine 	Red	*Penicillium purpurogenum*	[66]
N-glutarylrubropunctamine 	Red	*Penicillium purpurogenum*	[66]
***N*-threoninerubropunctamine** 	Red	*Talaromyces* spp.	[67]
PP-O 	Orange	*Penicillium purpurogenum*	[68]

Table 3 (*Continued*)

Colourant	Colour	Fungi	References
PP-R or 7-(2-hydroxyethyl)-monascorubramine 	Purple-red	*Penicillium purpurogenum*	[69,70]
PP-V or 12-Carboxyl-monascorubramine 	Purple-red	*Penicillium* sp. *Penicillium purpurogenum*	[14,68,71–74]
Sequoiamonascin C 	Yellow	*Penicillium* sp.	[14]

Note that only the genera that are also found on paper are reported here.

are as follows: black colour, insolubility in cold or boiling water and organic solvents, resistance to degradation by hot or cold concentrated acids, resistance to bleaching by oxidizing agents such as hydrogen peroxide, and solubilisation and degradation by hot alkali solutions [97]. Consequently, melanins are enigmatic colourants, found in all biological kingdoms, that have resisted atomic-level structural examination due to their insolubility and amorphous organisation [98].

Because they are amorphous and insoluble substances, melanins can only be broken down by oxidation and dissolved only in alkaline solvents [62]. This makes them very difficult to study since their purification requires harsh chemical methods, which can modify their structure [99].

Due to this chemical and physical endurance, the safe removal of melanised fungal stains from paper is very problematic [100], making them the hardest colourants to remove from paper substrata.

Part of the polymer subunits from melanin polymers have been identified in some studies; however, the exact arrangement of these subunits in the polymer chain remains unknown [62]. However, microscopic studies show that it has an overall granular structure. In fungi, melanin granules are localized on the cell wall where they are likely cross-linked to polysaccharides [62].

Often the resulting colourants are brown or black in colour, but many other colours have also been observed [62,95]. Melanins are also hydrophobic and negatively charged [95]. In fungi, several different types of melanin have been identified to date. The two most important types are DHN-melanin (named for one of the pathway intermediates, 1,8-dihydroxynaphthalene) and DOPA-melanin (named for one of the precursors, L-3,4- dihydroxyphenylalanine) [95].

In the Ascomycota fungi, where most of the fungi found in archives are represented [8], melanin colourant is generally synthesized from the pentaketide pathway in which DHN is the immediate precursor of the polymer [99]. DHN melanin is the fungal melanin on which research has concentrated, and it is the best characterized fungal melanin, otherwise known as polyketide melanin [97]. Even though, other fungi, like *A. nidulans*, produce DOPA-melanin instead [99].

Since this colourant itself is not yet well established and defined structurally, the information gathered about its production by fungi can often be erroneous and misleading. Numerous fungi that are now known to produce DHN melanin were previously reported to produce DOPA melanins [97]. Besides, in some species, such as *Aspergillus niger*, different loci have been described that affect conidial colour [54].

The fungal species that are known to produce melanins are presented in Table 5. The majority of the literature attributes the melanins production to three *Aspergillus* species (*A. fumigatus, A. nidulans, A. niger*).

5.1.1.4. Other polyketide colourants. Table 6 presents other polyketide colourants (yellow, brown, pink, purple, red) produced by fungi that also colonise paper. For instance, xanthoepocin belongs to the xanthomegnin class of fungal colourants, some members of which are known to be carcinogens or uncouplers of respiratory chain [106].

5.1.2. Carotenoids

Since fungi are non-photosynthetic organisms, carotenoids are not as widespread in fungi as they are in plants, where they play an important role in the photosynthetic process. Nevertheless, carotene hydrocarbons have been found in several fungi [60,126], and are widely accepted as protecting agents against oxidative stress or other different non-essential functions, related with the synthesis of physiologically active by-products [126]. Accordingly, on a study carried out by Han et al. [127] it was considered that in order to attain higher sclerotial biomass and carotenoid colourants yield, a strain of *Penicillium* sp. should be grown under high oxidative stress and in the absence of antioxidants.

Containing an aliphatic polyene chain usually composed of eight isoprene units that include light-absorbing conjugated double bonds, carotenoids provide characteristic yellow, orange or reddish colours [126]. Thus, as occurs with many flowers and fruits, carotenoids provide striking colours to some fungal species [126]. In the same way that the colours of flowers contribute to attracting pollinizing insects, the striking pigmentation of some fungi could play yet unknown ecological functions. The large amounts of conidia could easily adhere to any animal attracted to the mycelial surface, helping in the dispersion of the fungus [126].

5.1.2.1. β-carotene. β-carotene is one of the most abundant carotenoids in nature, being responsible for the yellow colour of many living organisms [126].

5.1.2.2. Neurosporaxanthin. Neurosporaxanthin is an amphipathic 35-carbon apocarotenoid, first discovered in the ascomycete *Neurospora crassa* [60,75,126,128–131]. Astaxanthin is more stable

Table 4
Quinone, HAQN and Naphthoquinone colourants chemical structure (base structure in bold), colour and the fungi responsible for its production.

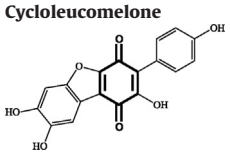
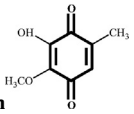
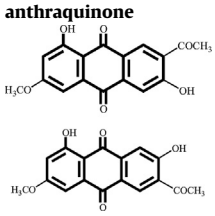
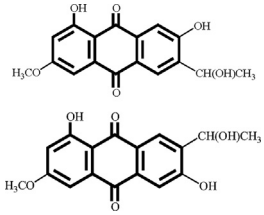
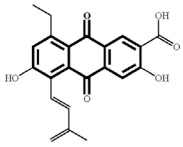
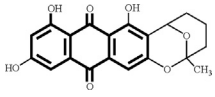
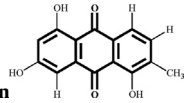
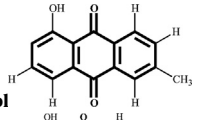
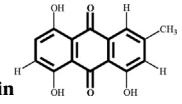
Colourant	Colour	Fungi	Reference
Cycloleucomelone 	Green	*Aspergillus niger*	[58,76,77]
Fumigatin 	Yellowish-brown	*Aspergillus fumigatus*	[60]
HAQN (Hydroxyanthraquinoid)			
2-Acetyl-3,8-dihydroxy-Gmethoxy or 3-acetyl-2,8-dihydroxy-6-methoxy-anthraquinone 	Yellow	*Fusarium oxysporum*	[78]
2-(1-Hydroxyethyl)-3,8-dihydroxy-6-methoxy or 3-(Z-hydroxyethyl)-2,8-dihydroxy-6-methoxy-anthraquinone 	Orange	*Fusarium oxysporum*	[78]
Arpink red™ 	Red	*Penicillium oxalicum*	[58,79,80]
Averufin 	Red	*Aspergillus versicolor*	[81–84]
Catenarin 	Red	*Aspergillus glaucus, A. cristatus and A. repens*	[58]
		Eurotium spp.,Fusarium spp.	[80]
Chrysophanol 	Red	*Eurotium spp., Fusarium spp.*	[80]
Cynodontin 	Bronze	*Eurotium spp., Fusarium spp.*	[80]

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Table 4 (*Continued*)

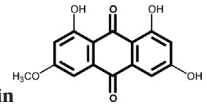
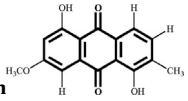
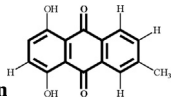
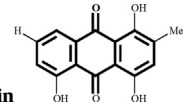
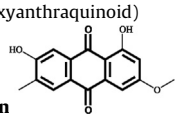
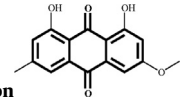
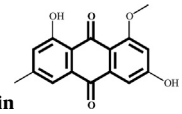
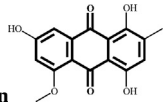
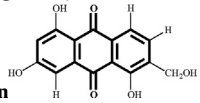
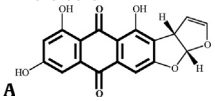
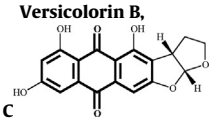
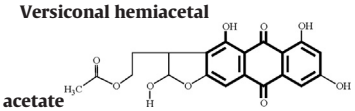
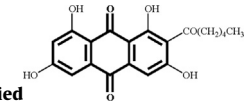
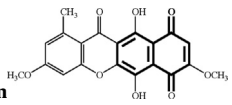
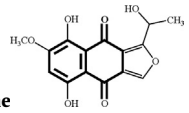
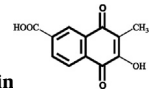
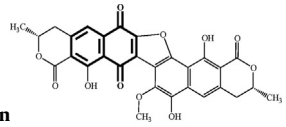
Colourant	Colour	Fungi	Reference
HAQN (Hydroxyanthraquinoid)			
 **Emodin**	Yellow	*Penicillium citrinum* *Penicillium islandicum* *Aspergillus glaucus,* *A. cristatus and* *A. repens*	[58,85]
	Red	*Aspergillus glaucus*	[75]
 **Erythroglaucin**			
		A. glaucus, A. cristatus *and A. repens*	[58]
		Eurotium/Fusarium spp.	[80]
	Maroon	*Eurotium spp., Fusarium* *spp.*	[80]
 **Helminthosporin**			
 **Islandicin**	Green/orange-red	*Penicillium islandicum*	[60]
HAQN (Hydroxyanthraquinoid)			
 **Macrosporin**	Yellow	*Alternaria* sp.	[58]
 **Physcion**	Yellow	*Aspergillus glaucus,* *A. cristatus and* *A. repens* *Alternaria* sp. *Eurotium* *herbariorum*	[58,70]
 **Questin**	Yellow to orange-brown	*Aspergillus glaucus,* *A. cristatus and* *A. repensEurotium* *rubrum*	[58,86]
 **Rubrocristin**	Red	*Aspergillus glaucus,* *A. cristatus and* *A. repens*	[58]
Rubroglaucin (mixture of Physcion and Erythroglaucin)	Red	*Aspergillus glaucus*	[60,87,88]
 **Tritisporin**	Red-brown	*Eurotium spp., Fusarium* *spp.*	[80]
Versicolorin	Pink-orange	*Aspergillus versicolor*	[26,83,89–91]
 **A**			
Versicolorin B,		*Aspergillus versicolor*	[26,89–91]
 **C**			

Table 4 (*Continued*)

Colourant	Colour	Fungi	Reference
Versiconal hemiacetal acetate 	Orange	*Aspergillus versicollor*	[83]
Unidentified Naphthoquinones 	Red	*Aspergillus versicolor*	[89]
Bikaverin 	Red	*Fusarium* sp.	[14,92]
Nectriafurone 	Yellow-brown	*Fusarium* sp.	[14]
Tomichaedin 	Yellow-brown Purple-black	*Chaetomium globosum* *Aspergillus* sp. *Aspergillus melleus*	[16,93] [14,94]
Viopurpurin 			

Note that only the genera that are also found on paper are reported here.

Table 5
Fungi known to produce melanins and to colonise paper.

Fungi	Reference
Alternaria alternata	[101]
Aspergillus fumigatus	[61,62,95,99,101,102]
Aspergillus nidulans	[62,95,99,101,103,104]
Aspergillus niger	[12,54,62,75,95]
Aspergillus penicilloides	[22]
Aureobasidium pullulans	[4]
Chaetomium sp.	[36]
Chaetomium globosum	[105]
Cladosporium cladosporioides	[17]
Eurotium herbariorum	[22]

when compared to other carotenoids, has a high antioxidant potential (10 times more than β-carotene) and high tinctorial property. The strong antioxidative activities of astaxanthin suggest its potential as photoprotector against UV irradiation [79].

Tables 3 to 7 present the colourants that can be produced by fungi identified on paper substrates. A total of 80 different colourants were gathered and described regarding their chemical structure, colour and fungal species responsible for their production.

From the over 80 colourants that can be produced by fungi identified as paper colonisers, the majority was comprised by polyketide colourants (about 96%) and only 4% of the colourants mentioned were carotenoids (Fig. 7).

There are only three different carotenoids that are produced by fungi identified on paper: neurosporaxanthin, β-carotene and sporopollenins. The latter are oxidative polymers of carotenoids and carotenoid esters [136].

The most diverse and major colourants produced by fungi colonising paper were polyketide colourants. From these 96%, about 20% were azaphilones and 33% were quinones. The remaining 46% were attributed to other types of colourants since they did not belong to the three chemical classes defined initially (Fig. 8).

Additionally, at least nine fungal species that are identified on paper are known to produce the polyketide colourant, namely melanin, which is structurally undefined. Melanin was here taken into account as a single colourant, although it is known that it can be produced by two biosynthetic pathways (DHN and DOPA).

The most studied colourants are the so-called *Monascus Pigments*, extracted from *Monascus* spp., since they have been used for a long time as meat colourants, disinfectants and in Chinese folk medicine [138]. These belong to the chemical class of the azaphilones, and despite there is no actual record of the fungal genera *Monascus* colonising a paper substrate, there are other fungi such as *Penicillium* sp., which can also produce this type of colourant given the right circumstances.

However, quinones were, in fact, the most predominant colourants identified in the literature which are also produced by fungi identified on paper (Table 4). These are produced mainly by *Aspergillus* sp. and *Penicillium* sp., which are the two genera that are mostly found colonising paper [15]. Among the quinones referred throughout the reviewed literature, the majority were HAQN colourants.

The most common colourants produced by fungi identified in the chemical/food/pharmaceutical literature have a yellow colour (44%), followed by red (19%) and orange (14%) (Fig. 9). About 23% of the colourants have other colours, such as bronze and maroon, and many times the colour is a mixture of hues (e.g. red-brown for tritisporin [80] or yellowish-brown for Fumigatin [60]).

However, these data must be considered in light of the bibliography they belong to, since traditionally, red and yellow hues have been the most extensively used food colourants [14].

According to the section in this manuscript focused on the conservation area (Table 1), the most common stains produced by fungi on paper substrates are brownish and black (Fig. 5). These stains may be a combination of the different colourants produced by the

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Table 6
Chemical structure, colour and fungal species responsible for the production of polyketide colourants besides melanin.

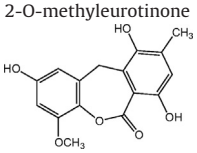
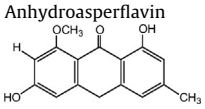
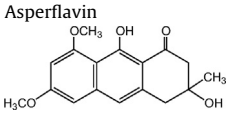
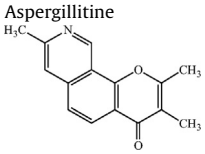
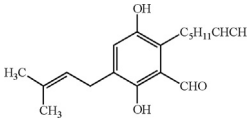
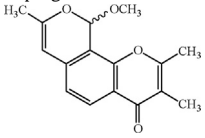
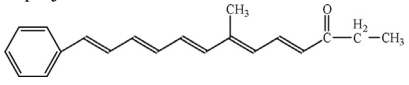
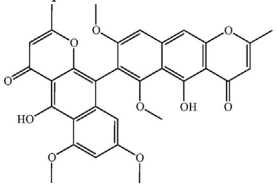
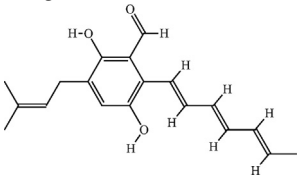
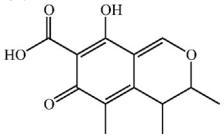
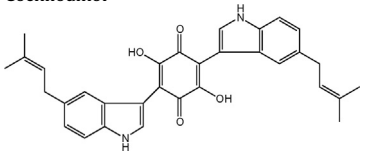
Colourant	Colour	Fungi	Reference
2-O-methyleurotinone 	Brown	*Eurotium rubrum*	[86]
Anhydroasperflavin 	Yellow	*Aspergillus flavus*	[107]
Asperflavin 	Yellow	*Aspergillus flavus* *Eurotium rubrum*	[86,107]
Aspergillin –hexahydroxyl pentacyclic quinoid (HPQ) and a melanin pigment	Brown	*Aspergillus niger*	Frey in [108][109]
Aspergillitine 	Yellow	*Aspergillus versicolor*	[110]
Aspergin 	Yellow	*Eurotium herbariorum*	[70,107]
Aspergione C 	Yellow	*Aspergillus versicolor*	[110]
Asperyellone 	Yellow	*Aspergillus niger*	[111,112]
Aurasperone 	Yellow	*Aspergillus niger*	[54,111,112]
Auroglaucin 	Orange-red	*Aspergillus glaucus* *Aspergillus repens* *Eurotium amstelodami,* *E. chevalieri and* *E. herbariorum*	[60,75,87,88]
Citrinin 	Yellow	*P. purpurogenum*	[113,114]
Cochliodinol 	Purple	*Chaetomium globosum*	[60,115,116]

Table 6 (*Continued*)

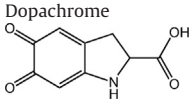
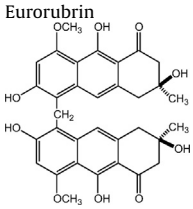
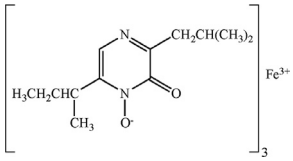
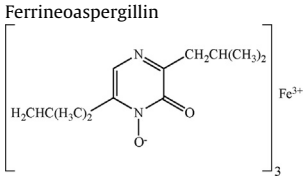
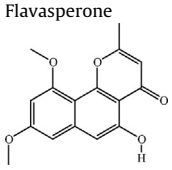
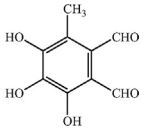
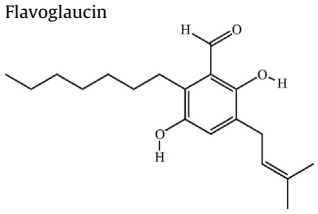
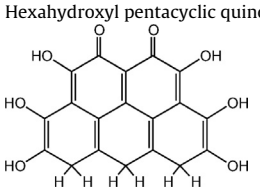
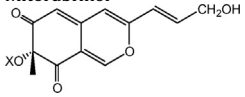
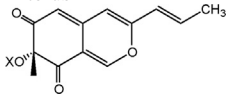
Colourant	Colour	Fungi	Reference
Dopachrome 	Pink	*Aspergillus nidulans*	[99]
Eurorubrin 	Brown	*Eurotium rubrum*	[86]
Ferriaspergillin 	Red	*Aspergillus flavus*	[117]
Ferrineoaspergillin 	Red	*Aspergillus melleus* *A. sclerotiorum Huber*	[117]
Flavasperone 	Yellow	*Aspergillus niger*	[54,111,112]
Flavipin 	Yellow	*Epicoccum purpurascens*	[80,118]
	Orange-Yellow	*Aspergillus flavipes* *Aspergillus terreus*	[119]
Flavoglaucin 	Yellow	*Aspergillus glaucus* *Aspergillus flavus* *Eurotium amstelodami,* *E. chevalieri and* *E. herbariorum*	[60,70,75,87,107]
Hexahydroxyl pentacyclic quinoid (HPQ) 	Green	*Aspergillus niger*	[54,109]
Melanochrome (intermediary in the formation of DHN-melanin)	Purple	*Aspergillus nidulans*	[99]
Mitorubrinol 	Orange-red	*Penicillium purpurogenum*	[63,70]
Mitorubrin 	Yellow	*Penicillium purpurogenum*	[70]

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Table 6 (*Continued*)

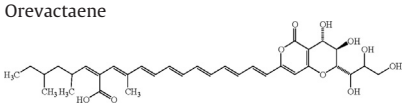
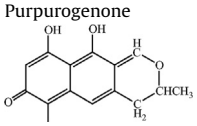
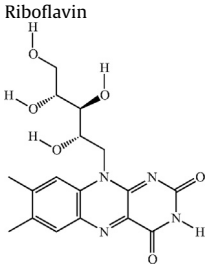
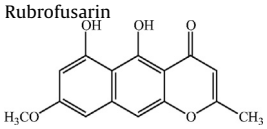
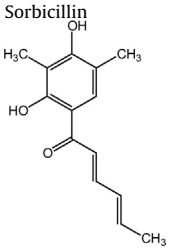
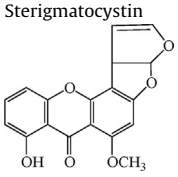
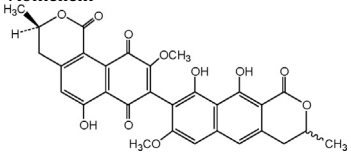
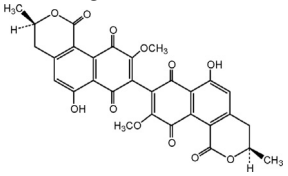
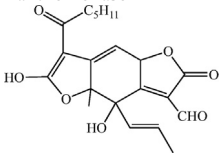
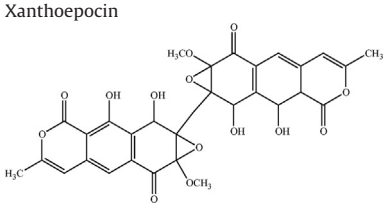
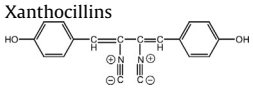
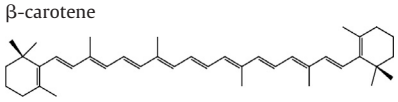
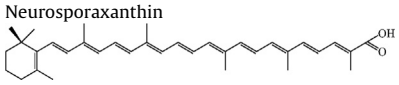
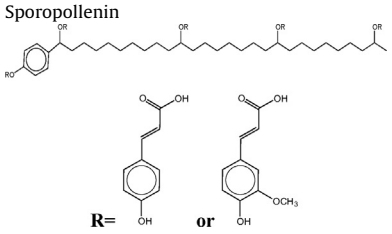
Colourant	Colour	Fungi	Reference
Orevactaene 	Yellow	*Epicoccum purpurascens* Ehrenb (formerly known as *Epicoccum nigrum* Link)	[58,14,120,121]
	Orange		[80,124]
Purpurogenone 	Yellow-orange	*Penicillium purpurogenum*	[70,71,113]
Riboflavin 	Yellow	*Aspergillus niger*	[108,112]
Rubrofusarin 	Red	*Fusarium* sp.	[60]
Sorbicillin 	Yellow	*Penicillium chrysogenum*	[122]
Sterigmatocystin 	Yellow	*Aspergillus versicolor*	[81,123]
Viomellein 	Reddish-brown	*Aspergillus* sp. *Aspergillus melleus* *Aspergillus ochraceus* *Penicillium* sp.	[70,75,94]
Xanthomegnin 	Orange	*Aspergillus* sp. *Aspergillus melleus* *Aspergillus ochraceus* *Penicillium* sp.	[70,75,94]
Xanthomonascin A 	Yellow	*Monascus* sp.	[124]

Table 6 (*Continued*)

Colourant	Colour	Fungi	Reference
Xanthoepocin 	Yellow	*Penicillium brevicompactum* *Penicillium simplicissimum*	[70,106]
Xanthocillins 	Yellow	*Penicillium chrysogenum*	[125] 25

Note that only the species that are also found on paper are reported here.

Table 7
Carotenoid colourants chemical structure, colour and the fungi responsible for its production.

Colourant	Colour	Fungi	References
β-carotene 	Yellow	*Aspergillus giganteus*	[127,130]
		Penicillium sp.	[127,132]
Neurosporaxanthin 	Orange	*Neurospora crassa*	[60,75,126,128–131]
		Fusarium sp.	[128,129,131,133,134]
Sporopollenin 	Brown	*Neurospora crassa*	[135–137]

Note that only the species that are also found on paper are reported here.

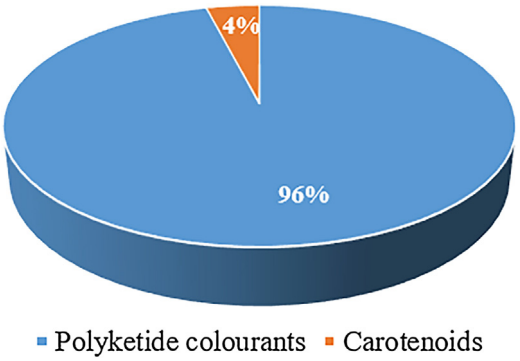


Fig. 7. Colourants produced by fungi identified on paper.

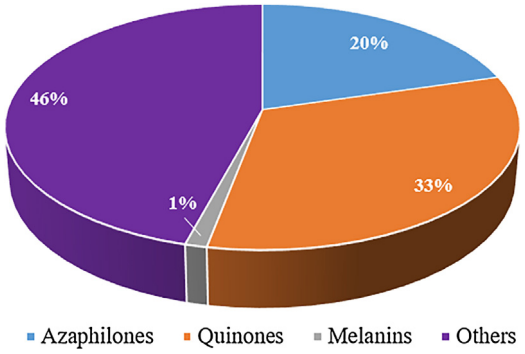


Fig. 8. Polyketide colourants produced by fungi identified on paper.

fungi present on paper. For instance, one of the main characteristics of *A. niger* is the production of black or dark brown conidia resulting from the combination of dark brown melanins with hexahydroxyl pentacyclic quinoid green pigments [54]. So, the apparent blackness of these fungi results from the combination of brown and green pigments which absorb light across the entire visible spectrum [109]. Also, when growing and extracting the colourant from *Aspergillus melleus*, it was verified that the brown solid contained viomellein – reddish-brown (11%), xanthomegnin – orange (50%), and viopurpurin – purple-black (1%) [94]. On the other hand, the colour of the stains produced by fungi on paper may as well be a result of the colourants' oxidation in the given medium, causing its darkening. For instance, sporopollenin is a brown product of oxidative polymerization of the yellow β-carotene [58].

Even when the colourant is defined, occasionally different authors mention different colours to characterize it. This might have to do with the colourant properties, like fumigatin, produced by *Aspergillus fumigatus*, which has a yellowish-brown colour but changes to a strong purple in alkaline medium [60].

Besides, many of the identified colourants are biosynthetic precursors of others, so depending on the development stage, a different colour may be observed. This is the case of 4C-labelled

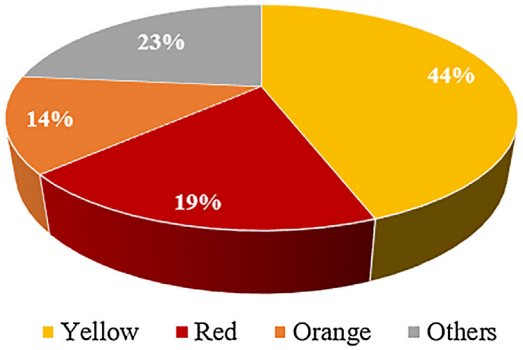


Fig. 9. Percentage of the colourants colours reported in the chemical/food/pharmaceutical literature, taken only under consideration the fungal species identified on paper.

red averufin, the orange versiconal hemiacetal acetate, and the pink-orange versicolourin A, which are converted to the yellow sterigmatocystin by *Aspergillus versicolour* [83]. Another example is the dopachrome and melanochrome, the pink and purple intermediates in the formation of the melanin colourant in *Aspergillus nidulans* by the L-DOPA pathway [99].

Finally, different colours may often be due to the modification of the same colourant, depending on different reactions of the medium. For instance, variation in copper levels among commercial fungal media (potato dextrose agar) correspond to variable colouration in multiple fungal species including *Trichoderma harzianum, Stachybotrys atra, Fusarium culmorum*, and *Cladosporium herbarum* [139].

6. Conclusions

The ultimate purpose of this review was gathering data that would be useful in studies regarding the removal of fungal stains from paper cultural heritage, namely on the targeting of stain responsible molecules. In order to achieve this goal, two different, but complementary literature reviews were made, compiled, connected and organised. First, a review on the paper conservation literature was made, where fungal stains observed on paper cultural heritage are reported together with the identified fungal species. The other survey was done on the chemical/food/pharmaceutical fields where colourants' molecules produced by fungi, that can colonise paper cultural heritage, are identified and studied in greater depth.

Regarding the survey on the paper conservation research field, it was concluded that the fungi most frequently related with paper staining belong to the genera *Aspergillus* (29%) and *Penicillium* (13%). These are amongst the most common genera found in air samples from archives and in outdoors worldwide. It was also reported that paper stains caused by fungi are mostly associated with a brownish colour (about 54%), and in about 68% of the literature reports this colour was related to foxing. The foxing phenomenon is still not fully understood, but it is a great matter of interest and discussion for conservators, since it is a very common stain that can be present in large areas of documents and works of art, greatly interfering with its readability and can also migrate through the sheets of a book. The second most detected colour is associated with black stains (23%), which is frequently related with melanins produced by fungi. The exact chemical structure of melanin is yet to be discovered. Nevertheless, the colours can change for the same microorganism depending on the conditions of growth and the properties of paper. Even when the colour is mentioned, it can vary from author to author, and when the fungi are isolated in culture media, the colour can be different from the one observed on the paper substrate. Therefore, a specific colour on a document cannot be associated with a specific fungus, because the same fungus can present different colours, and the same colour can be caused by different fungi. The paper conservation literature refers colours but does not indicate the colourimetric parameters. Consequently, different authors may attribute the same colour to very distinct stains. Also, there is a huge gap in this literature since most of the authors do not indicate the stain colourant. Therefore, another literature review was necessary to understand which colourant structures can be produced by fungi responsible by paper staining. It was noticed that the colourants produced by fungi were identified for only 10% of the species mentioned throughout the case studies in the conservation literature, and 62% of those 10% were associated with black stains and the production of melanins.

So, after gathering all the information from scientific publications concerning colourants produced by fungi, and relating it with the fungal species that have been identified as paper colonisers, it was possible to assess that the majority of colourants causing stains on paper are possibly quinoid colourants (33%), namely hydroxyanthraquinoid colourants.

The colourants produced by fungi identified on paper are quite diverse, but the most commonly studied ones are most definitely yellow and red colours. However, this may occur because these are the most appealing ones in the areas concerning the literature reviewed (namely food industry, for instance).

Overall there is no doubt that fungi producing colourants are a serious problem to conservators, since there is a great variety of colourants (over 80 different molecules, reviewed in the present study), produced by different species of paper colonisers. Additionally, the dominant species that produce colourants belong to the most common genera identified on paper substrates, namely *Aspergillus* sp. and *Penicillium* sp.

Each colourant, with different properties, may require diverse removal methods. Therefore, it is of the utmost importance that developments are carried out in this field of knowledge, so that targeted removal techniques can be developed without damaging our cultural records on paper.

The present study presents an important advance, since almost no information was published about the colourants produced by fungi on paper. By relating the information acquired in different areas, such as the chemical and food industry, with the information already acquired in the conservation field, we are one step closer to define which colourants may be present in specific stains on the paper substrate, which will have an impact on the conservation strategy. Likewise, it is also relevant to consider whether fungi produce colourants within their structure or as coloured products of their metabolism, which determines the extent of staining. Nevertheless, research is still needed regarding the correlation between fungal stains on paper and their culprit; about how the colourants are altered by different types of paper substrates; how the colourants change with ageing; and finally, about cleaning methodologies for the removal of this aesthetic and chemical damage, without damaging the paper substrate.

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