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Research concerning utilisation of some essential oils for the antifungal bioprotection of wood - opportunities and limits in the context of efficiency *versus* eco-impact

Cercetări privind utilizarea unor uleiuri esențiale pentru bioprotecția antifungică a lemnului - oportunități și limite în contextul eficiență *versus* eco-impact

SUMMARY

Doctoral Field: Forest Engineering

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Contents

Summary/Thesis

List of abbreviations

No.	Abbreviation	Full name	Notes
1	Ex.1.....3	Domain-specific examination	Original
2	R.1.....3	Scientific Report	Original
3	P1.....P9	Personal Scientific Publications	Original
4	O1.....O5	Thesis Objectives	Original
5	R-O1.....R-O5	Results of the Thesis Objectives	Original
6	EO	Essential oil	Common
7	B-EO	Basil Essential Oil	Original
8	C-EO	Clove Essential Oil	Original
9	O-EO	Oregano Essential Oil	Original
10	S-EO	Cinnamon Essential Oil	Original
11	T-EO	Savory Essential Oil	Original
12	LO	Linseed oil	Common
13	BRE	Classic biocides with proven effectiveness	Original
14	RN	Romalit N	Original
15	FTIR	Fourier Transform Infrared Spectroscopy	Common
16	GC-MS	Gas chromatography with mass spectrometry	Common
17	SEM	Scanning electron microscopy	Common
18	TV	*Trametes versicolor* Fungus	Common
19	CP	*Coniophora puteana* Fungus	Common
20	PP	*Postia placenta* Fungus	Common
21	GT	*Gloeophyllum trabeum* Fungus	Common
22	SL	*Serpula lacrymans* Fungus	Common
23	FC1-B, FC1-W, SW	Fungi isolated from heritage objects	Original
24	HP, HPI, CT, DH, R, RS, VM	Screening tests	Original
25	TF	Phytotoxicity test	Original
26	TPr	Preventive treatment test	Original
27	Tcu	Curative treatment test	Original
28	TCuP	Test curative treatment - heritage objects	Original
29	I_{sol}	Soil inhibition index	Adopted
30	I_{paper}	Paper inhibition index	Adopted
31	PM	Weight loss	Original
32	RPM	Weight loss reduction	Original
33	U	Humidity	Common
34	Abs	Product absorption	Common
35	WPG	Product retention	Common
36	PG	Germination percentage	Common
37	LMG	Average length of sprouts	Common
38	IG	Germination index	Common
39	E1.....E10	Wash-off waters	Common
40	FCA	Fungal coverage area	Original

Introduction

Wood represents an invaluable resource for humanity, but despite its numerous qualities, its structure makes it vulnerable to attack by biological degradation agents (Eriksson et al., 1990; Zabel & Morrell, 2020). Synthetic biocidal products have been long considered the ideal solution for protecting wood against biological degradation, but subsequent research has revealed concerning side effects of these products on both human health and the environment (Jadon et al., 2022; Reinprecht, 2010). Faced with this situation, competent authorities have had to impose strict regulations on the use of synthetic biocidal products. In this context, the attention of researchers and specialists has turned to natural solutions, including essential oils (EOs). Essential oils have started to be considered a promising option, due to their chemical composition, which includes compounds with remarkable properties against a wide range of microorganisms, including fungi, bacteria and viruses. As natural products that support the life and immunity of the plants from which they originate, these oils are expected to represent a much more environmentally friendly alternative compared to traditional synthetic biocides. However, aspects related to their possible ecological impact due to components with phytotoxic activity have been little studied and require attention and investigation (Ferraz et al., 2022). In addition to potential ecological benefits, the use of essential oils in cultural heritage conservation could bring significant advantages from an occupational health perspective. Conservation specialists, restorers and other professionals in the field are frequently and long-term exposed to substances used in conservation treatments (Varnai et al., 2011).

Therefore, the transition to using essential oils in wood preservation is challenging. Thorough research is needed to determine the specific effectiveness of various essential oils against different types of wood degradation agents. Clear application and dosage protocols must also be established to ensure treatment efficacy with minimal side effects on the artefact, environment, and human health and safety.

The doctoral thesis starts with a literature review on essential oils (Chapter 1), covering their history, properties, extraction methods, composition and antifungal characteristics, areas of application with emphasis on wood treatment, toxicity and ecotoxicity. The research objectives and phases (Chapter 2) were based on the current state of knowledge and the aim of the thesis. An original methodological concept was developed and applied (Chapter 3). Based on the literature review, various types of tests were selected, adapted and practically applied to be included in an analytical protocol (Chapter 4 - O1), which has been subsequently employed throughout the thesis to achieve the other objectives. Also as a result of the literature review, five essential oils were identified: **Basil (*Ocimum basilicum*) B-EO**, **Clove (*Eugenia caryophyllata)* C-EO**, **Oregano (*Origanum vulgare)* O-EO**, **Cinnamon (*Cinnamomum***

***verum)* S-EO** and **Savory (*Satureja hortensis)* T-EO**, which were analysed, characterised and compared (Chapter 5 - 02). The antifungal potential of these five essential oils was tested (Chapter 6-03) through screening tests from the analytical protocol, and as a result, two essential oils were selected for further research: **Clove (*Eugenia caryophyllata*) C-EO** and **Savory (*Satureja hortensis)* T-EO.** For these two oils, the antifungal effect on wood was determined through the miniblock test from the analytical protocol, and the potential eco-impact was evaluated through the phytotoxicity test from the same analytical protocol (Chapter 7-04). According to the scope pursued in the thesis, these two essential oils were used in several original tests for their implementation in the field of wood conservation-restoration (Chapter 8-05). These developed tests complement the previously elaborated analytical protocol. The results presented in this thesis make a significant contribution to existing knowledge regarding the use of essential oils in protecting wood against xylophagous fungi. The study provides a detailed analysis of the efficacy and ecological impact of these oils, as well as their potential for use in the field of wood conservation-restoration. This is well reflected in the conclusions of the thesis and future research directions (Chapter 9). The information provided constitutes a valuable database, with direct applications in scientific wood conservation, as well as in other related fields.

The thesis itself is structured into 9 chapters (193 pages), 46 tables, 92 figures and 162 references. The appendices of the thesis (177 pages) represent important databases for the studied subject.

Chapter 1 - The current state of knowledge regarding the nature, composition, biocidal effect and applications of essential oils in wood bioprotection

The term "essential oils" has a fascinating origin, derived from the expression "the most important oil". This name has its roots in Aristotelian philosophy, which proposed that all matter is composed of four fundamental elements: fire, air, earth and water. In this conception, it was believed that there was a fifth element, called "quinta essentia", which represented the spirit or life force of matter (Başer & Buchbauer, 2010).

The History of Essential Oils

The importance and use of essential oils in human history are remarkable, with origins dating back to antiquity. Historical evidence indicates that the use of aromatic plants and essential oils was a common practice in ancient civilisations such as China, India (5000 BC), Mesopotamia, Egypt and Greece (3000 BC). There are records from around 4500 BC describing the use of aromatic balsamic substances in religious rituals and medical applications. (*Essential Oils pocket reference*, 2019) Since their discovery, essential oils have been used for

flavours and food additives, as aphrodisiacs, perfumes, in cosmetics, medicine and religious and esoteric rituals (Dima & Dima, 2015).

Definition and sources

According to the 7th edition of the European Pharmacopoeia, an essential oil is "*An odorous product, generally of complex composition, obtained from a botanically defined plant raw material, either by steam distillation, by dry distillation, or by a suitable mechanical process without heating. An essential oil is usually separated from the aqueous phase by a physical method that does not lead to significant changes in its chemical composition*." (Asbahani et al., 2015).

Approximately 3,000 essential oils are known, of which 300 are commercially important, particularly for the pharmaceutical, agronomic, food, sanitary, cosmetic and perfume industries (Bakkali et al., 2008; Bhavaniramya et al., 2019; Butnariu & Sarac, 2018). The European Pharmacopoeia lists 28 essential oils as safe. (Sadgrove et al., 2021).

Physical properties

Essential oils are volatile compounds, transparent, colourless or lightly coloured, ranging from shades of blue (specific to chamomile) to brown tones (associated with cloves) or yellow-orange (characteristic of orange), which are distinguished by their unmistakable aroma (Dhifi et al., 2016; Laur, 2022). They have excellent solubility in fats and solvents. It is important to note that essential oils have a very high refractive index and optical activity (Dhifi et al., 2016; Laur, 2022).

Extracting methods

The extraction of essential oils is a complex process, and there is a wide range of techniques that can be used, as illustrated in Fig. 1.1. Choosing the optimal extraction method depends on several critical factors, including the type of plant being processed, the composition of the oils, the desired quantity of essential oil, the final product quality, as well as economic and efficiency considerations.

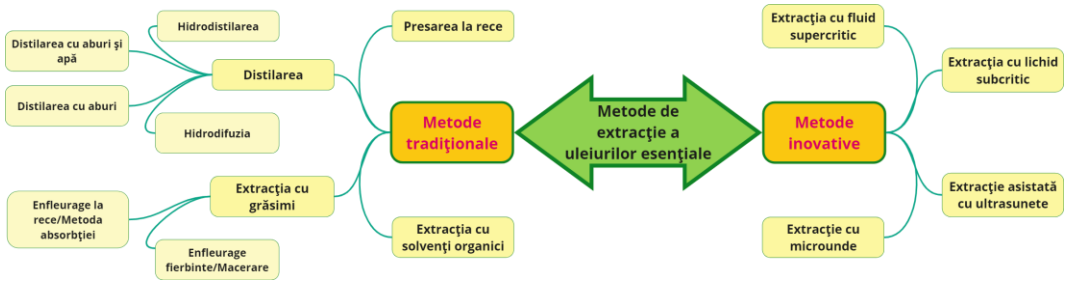


Fig. 1.1 Synthesis of essential oil extraction techniques

The chemical composition of essential oils

Essential oils are complex natural products characterised by remarkable diversity in their chemical composition, exhibiting significant variability in both qualitative and quantitative terms (Dhifi et al., 2016; Jürgens & Viljoen, 2010). The chemical composition of essential oils can vary significantly depending on factors such as plant genotype, chemotype, plant organ, geographical origin, season, environmental conditions, agronomic practices, extraction methods and storage conditions (Butta et al., 2023; Shirzad et al., 2011). Volatile compounds are primarily derived from three biosynthetic pathways: the mevalonate pathway leading to sesquiterpenes, the methyl erythritol pathway leading to mono- and diterpenes, and the shikimic acid pathway resulting in phenylpropenes (Başer & Buchbauer, 2010). These groups contain cyclic and acyclic compounds from various classes, such as hydrocarbons, alcohols, esters, phenols, ketones, lactones, aldehydes and oxides (Fig. 1.2) (Turek & Stintzing, 2013).

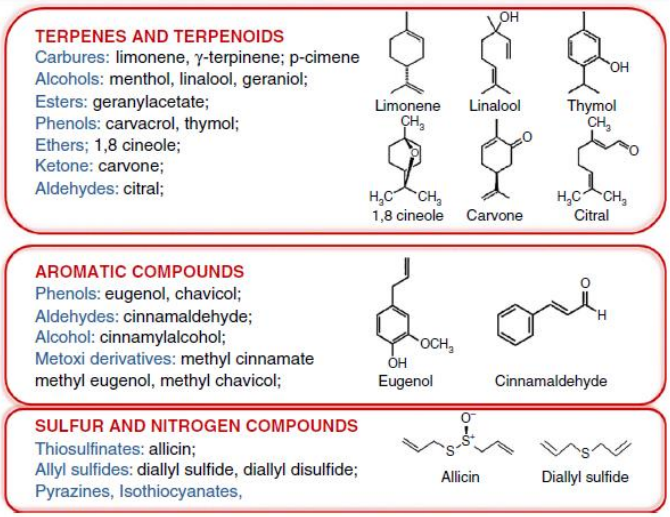


Fig. 1.2 The main chemical compounds of essential oils [1]

Areas of application

Essential oils have a wide range of applications in various industries due to their complex chemical composition and biological activity. They have been widely used for their properties already observed in nature, namely their antibacterial, antifungal and insecticidal activities. The natural mixture of monoterpenes, sesquiterpenes, diterpenes and aromatic compounds, with a variety of functional groups, gives essential oils antibacterial, antifungal, anti-termite and insecticidal properties (Mohareb et al., 2013).

In the pharmaceutical, medical, therapeutic and veterinary sectors, promising approaches have been reported using essential oils or their components in medicines, utilised for their potential as treatments for a range of conditions, including respiratory problems, infections and dermatological issues (Bakkali et al., 2008; Dhifi et al., 2016; Fokou et al., 2020; Jerry

[1] (Dima & Dima, 2015)

Atoche Medrano, 2020; Laur, 2022; Mohammed et al., 2024). In the food industry, essential oils are used as natural preservatives due to their antimicrobial and antioxidant properties (Bhavaniramya et al., 2019), which help extend the shelf life of food products. They are utilised in packaging and food preservation (Bakkali et al., 2008; Dima & Dima, 2015; Gatto et al., 2016; Juárez et al., 2015; Khalili et al., 2015; Nedorostova et al., 2009; Varona et al., 2013). The agricultural sector benefits from the insecticidal, fungicidal and bactericidal properties of oils, which are used to protect crops and food stocks from pests, without the adverse effects associated with synthetic chemicals (Bakkali et al., 2008; Butta et al., 2023; Gatto et al., 2016; Kumar et al., 2022; Righi-Assia et al., 2020). Essential oils have potential for use in wood bioprotection against mould fungi and wood-decay fungi attacks due to their antifungal properties (Bahmani & Schmidt, 2018; Brischke, 2020; Cheng et al., 2008; Marcias et al., 2005; Mohareb et al., 2013; Pánek et al., 2014a; Šimůnková et al., 2022; Singh & Chittenden, 2008; Timar et al., 2022; Vettraino et al., 2022; S.-Y. Wang et al., 2005; Yang & Clausen, 2007; Zhang et al., 2016; Zyani et al., 2011). The application of essential oils in cultural heritage conservation seems promising, particularly due to their antimicrobial properties and low toxicity (Varnai et al., 2011). For example, oregano essential oil has been shown to be extremely effective against fungi isolated from biodegraded archaeological mummified skin, including *Aspergillus tabacinus*, *Aspergillus tennesseensis* and *Trichoderma longibrachiatum*, both in in vitro tests and parchment tests (Sanchis et al., 2023). In the wooden heritage area, essential oils of oregano, cloves and basil demonstrated a strong antifungal effect against certain types of fungi isolated from wood sourced from an old house (Zyani et al., 2011). Oregano, in another study against fungi isolated from wooden heritage objects in Serbia, demonstrated antifungal effects comparable to a biocide (benzalkonium chloride) used for comparison (Stupar et al., 2014).

The antifungal effect of essential oils

The effect of essential oils on pathogenic fungi can be observed both at a macromorphological level and at a cellular level. Some of the macromorphological changes include: lack of sporulation or pigmentation, alteration in the number of conidia, increased branching of hyphae or changes in their size. These modifications are a consequence of the activities of oil components on the enzymatic process of cell wall formation, which affects fungal growth and morphogenesis, as well as the retraction of cytoplasm from hyphae, resulting in mycelium death (Butta et al., 2023; Plavsic et al., 2017). The cytotoxic properties of essential oils, often attributed to phenols, aldehydes and alcohols, are crucial for their antifungal effects, as these compounds can penetrate fungal cells more effectively during active growth phases (Bakkali et al., 2008).

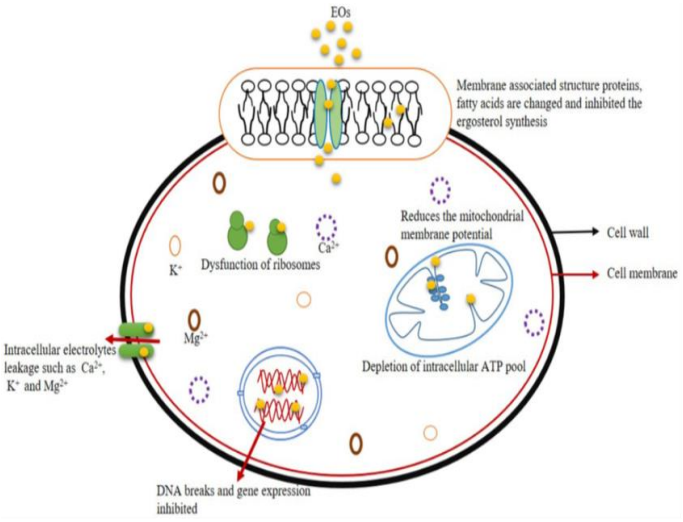


Fig. 1.3 The antifungal mechanism of essential oils [2]

Following the bibliographic research of 15 articles, 43 essential oils with antifungal potential were identified, synthesised in tabular form in the database (DB 1) attached at the end of the thesis.

Research on the use of essential oils in wood bioprotection

Given the characteristics of the chemical compounds of essential oils, these have been the subject of several studies regarding their use for protecting wood against lignivorous fungi, including mould fungi, brown rot fungi, white rot fungi and soft rot fungi (Cheng et al., 2008; Chittenden & Singh, 2011; Juárez et al., 2015; Mohareb et al., 2013; S.-Y. Wang et al., 2005; Zyani et al., 2011).

Research on the effects of essential oils against white and brown rot fungi has yielded promising and significant results. The analysis revealed the following aspects:

- A range of essential oils, including oregano, thyme, savory, clove, basil and cinnamon, consistently demonstrated efficacy against both types of fungi, featuring in multiple studies.
- These findings are supported by investigations examining pure compounds, where cinnamaldehyde (the main component of cinnamon oil) and eugenol (the predominant compound in clove oil) stood out as the most frequently tested and remarkably effective. Additionally, carvacrol, a key component of oregano oil, and methylchavicol (estragole), a primary constituent of basil oil, showed notable results in combating these pathogens.

[2] (Maurya et al., 2021)

- Comparative analyses revealed that, generally, the efficacy of essential oils proved superior against brown rot fungi compared to white rot fungi.
- These discoveries offer valuable insights for developing new strategies to protect wood and other materials susceptible to fungal attack, highlighting the significant potential of essential oils as a natural and environmentally friendly alternative to conventional treatments.

The toxicity and ecotoxicity of essential oils

The ecotoxicity of essential oils is a multifaceted subject, encompassing their effects on various organisms and ecosystems. Essential oils, derived from different parts of plants, are rich in compounds such as monoterpenes, sesquiterpenes and phenolic compounds, which confer diverse biological activities, including insecticidal, repellent and fungicidal properties (Kumar et al., 2022).

Despite their potent biological activities, essential oils generally exhibit low toxicity to mammals and fish due to the absence of specific targets in these organisms, making them suitable for use as "green pesticides" in agriculture and organic food production (Kumar et al., 2022). However, some essential oils can cause adverse effects; for example, *Melaleuca alternifolia* oil has been linked to toxicosis in pets (Kumar et al., 2022).

Essential oils are generally considered quite safe when usage recommendations and concentrations are followed. However, there are essential oils containing components which may cause adverse effects; for example, *Melaleuca alternifolia* oil has been linked to contact dermatitis (Kumar et al., 2022).

Conclusions

Essential oils represent a category of natural substances with remarkable diversity and complexity. Scientific research has confirmed that essential oils possess a wide range of beneficial properties, among which their antifungal effects are particularly noteworthy.

In the field of wood protection against brown and white rot fungal attack, basil, oregano, clove, cinnamon, thyme and summer savory oils have stood out. These oils have demonstrated promising efficacy in inhibiting the growth and development of these pathogens, offering a potential, more environmentally friendly alternative to conventional biocides for wood protection treatments.

Although essential oils are often, *a priori*, considered to be merely beneficial natural products without risks of toxicity or environmental impact, it is important to recognise that they may also present certain risks. It is essential, therefore, to conduct thorough research on the long-term efficacy of essential oils.in wood protection and the potential ecological impact of their use.

The results obtained can contribute to the development of alternative wood bio-protection methods that are both effective and sustainable. The extent to which essential oils represent a viable and responsible solution for wood bio-protection, as well as their applicability in the field of wooden cultural heritage. conservation, can be correctly assessed only through in-depth research looking to both the opportunities and limitations of their potential utilisation.

Chapter 2 - Objectives of experimental research

Aim of the Thesis

The aim of the thesis is to identify and test the applicability of essential oils in the antifungal protection of wood, whilst simultaneously assessing their effectiveness and impact on the environment. The central idea of the study is to identify essential oils that demonstrate adequate efficiency in the antifungal protection of wood, whilst having low toxicity for humans and the environment. The intended field of application is specifically the conservation and restoration of historic wood and furniture.

Overview of objectives

To achieve the proposed aim and considering the conclusions of the literature review, the following research objectives were assumed. (Tab. 2.1).

Tab. 2.1 Content and Experimental Objectives of the Thesis

Code	Objectives
O1	Establishing an analytical laboratory protocol for identifying products with antifungal biocidal activity (screening tests), testing efficacy (mini-block tests) and ecological impact (phytotoxicity tests); validating the protocol through tests on conventional biocidal substances with known efficacy (BRE)
O2	Identification and characterisation of essential oils with potential antifungal biocidal effects for wood protection
O3	Testing the antifungal biocidal effect through screening tests and selection of products with potential
O4	Determining the antifungal protection efficiency of wood and the potential ecological impact for selected products
O5	Opportunities and Limitations in Implementing in Wood and Historic Furniture Conservation and Restoration domain

Chapter 3 - Research methodology

Methodological concept

The methodological concept is an essential component of the overall thesis structure, integrated within it (Fig. 3.1 - black frame). It encompasses the process of selecting, adapting and practically implementing various types of tests to choose suitable variants for developing the analytical protocol specific to the thesis objectives, applying it throughout the entire research, as well as selecting appropriate materials and investigation methods to characterise them. The development of the methodological concept was based on literature research and adapted to the specific research needs, in order to fulfil the thesis objectives.

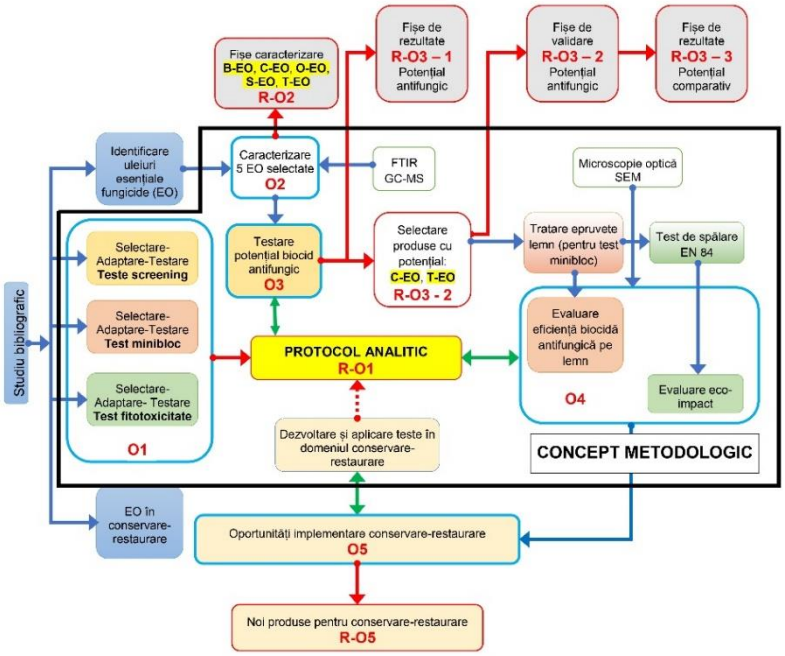


Fig. 3.1 General outline of the thesis highlighting the complex methodological concept specific to the thesis

Materials

Support paper for biological screening tests

Whatman chromatography filter paper (No. 1 and No. 3) and Fisher Scientific Antibiotic test paper (13 mm) were used throughout the entire research.

Wood

The wood material used in the mini-block tests (method adapted from EN 113:1995) and the original evaluations of potential use in conservation-restoration was unsteamed solid beech wood (***Fagus sylvatica*** L.) and pine sapwood (***Pinus sylvestris*** L.), these being the non-durable

reference species according to SR-EN 335:2002. Samples measuring 20x20x5 mm (L x R x T) were used, cut from healthy material without defects.

Essential oils

The research was conducted using five pure essential oils (100% concentration), packaged in 10 ml bottles and marketed under the Divine Star brand [3] (Tab. 3.1). Throughout the experiments, these essential oils were used both in their pure state (C=100%) and diluted in two different solvents: ethyl alcohol and linseed oil. The aim of this approach was to evaluate the efficacy and practical potential of essential oils under various conditions and concentrations, allowing for a comparative analysis of the results obtained.

Tab. 3.1 Experimentally used essential oils (EOs).

No.	EO	Scientific name	EO suplier	Code
1.	Basil	*Ocimum basilicum*	Steaua divină	B-EO
2.	Clove	*Eugenia caryophyllata*	Steaua divină	C-EO
3.	Oregano	*Origanum vulgare*	Steaua divină	O-EO
4.	Cinnamon	*Cinnamomum verum*	Steaua divină	S-EO
5.	Savory	*Satureja hortensis*	Steaua divină	T-EO

Classic biocidal products

In the research, some classic biocidal substances with recognised effectiveness (BRE) were also used, such as copper sulphate ($CuSO_4$), Romalit N (55% $CuSO_4$/ 45% $K_2Cr_2O_7$), Biotin T[4] și Diffusit S[5]. Some of these were used to validate the analytical protocol, while others served as references for evaluating the effectiveness of the essential oils investigated in the field of conservation-restoration. These substances were selected due to their current use and demonstrated efficacy in this domain.

Biological material - Wood-decaying fungi

During the biological tests, five types of wood-decaying fungi from the Basidiomycetes family, available in the mycological collection of the C11-ICDT laboratory, were used at various stages. (Tab. 3.2).

[3] https://www.steauadivina.ro/catalog/uleiuri-esentiale-10ml-71
[4] https://ctsconservation.com/en/solvents-and-chemical-products/6080-biotin-t-pack-size-1-kg.html
[5] https://www.pannon-protect.eu/files/roman/M_Diffusit%20S%20ro.pdf

Tab. 3.2 Types of fungi, names and experimental codes used in the thesis [6]

No.	Type	Scientific name	Code
1	White rot	***Trametes versicolor***	**TV**
2	Brown rot	***Coniophora puteana***	**CP**
3	Brown rot	***Postia placenta***	**PP**
4	Brown rot	***Gloeophyllum trabeum***	**GT**
5	Brown rot	***Serpula lacrymans***	**SL**

Culture medium - biological tests

In all biological tests, a MEA (malt extract agar) culture medium was used.

Seeds for phytotoxicity tests

In the phytotoxicity study, two seed species were initially used, namely lettuce (***Lactuca sativa*** L.) and cress (***Lepidium sativum*** L.)

Equipment used for preparing biological samples and tests

The experiments for the doctoral thesis were conducted in the Biological Testing and Ageing Laboratory within Research Centre C11: Eco-design of Furniture, Restoration and Certification in the Wood Industry, part of the ICDT at Transilvania University of Brașov, using the available equipment.

Investigation methods and equipment

Tab. 3.3 briefly presents the investigative methods used throughout this research for characterising essential oils, identifying/highlighting the biodegradation undergone by samples exposed to fungi, certain characteristics of rot fungi, and the analysis of sprouts within phytotoxicity tests.

[6] https://www.indexfungorum.org/names/Names.asp

Tab. 3.3 Research methods used in the study

Research methods	**Wood samples**	**Essential oils**	**Treated wood samples**	**Fungi**	**Phyto test**	**Equipment used**	Equipment Location
Macroscopic - Visual assessment - Photo	Yes	Yes	Yes	Yes	Yes	Canon 400D Camera Samsung Galaxy Note 10 Plus Mobile Phone	ICDT Brașov
Optical microscopy	Yes	No	Yes	Yes	Yes	Nikon SMZ25/SMZ18 Stereomicroscope Portable Microscope	ICDT Brașov
FT-IR Spectrometry	No	Yes	Yes	No	No	BRUKER ALPHA FT-IR Spectrometer	ICDT Brașov
Gas chromatography-mass spectrometry GC-MS	No	Yes	No	No	No	GC Focus equipped with a DSQII Mass Spectrometer and a TriPlus autosampler (Thermo Electron Corporation)	FSIM UBB Cluj-Napoca
Scanning electron microscopy SEM	Yes	No	Yes	No	No	FEI, Quanta 250	Ecole Supérieure du Bois from Nantes-France

Software

Throughout the research and development of this paper, various software applications were used to process different types of data. These are presented in Tab. 3.4

Tab. 3.4 Software used throughout the thesis

No.	**Name**	**Scope**
1	Microsoft Word	Document editing
2	Microsoft Excel	Spreadsheets
3	Opus	FTIR spectrum processing
4	CorelDraw; Inkscape;	Schemes, mycelium growth measurements on images
5	ImageJ	Photo processing, mycelium growth measurements on images
6	Photoshop, Paint	Photo processing
7	Zotero	Bibliographic reference management software

Biological tests

Within this research, various types of standardised biological tests or those established through previous research were carefully identified/selected, adapted and implemented. Additionally, original tests were devised and utilised to meet the specific needs of the research objectives. A summary of the tests is presented în Tab. 3.5.

Tab. 3.5 Types of tests conducted during the research

No.	Test type	Test name	Code
1	Testing biocidal potential	Humar and Pohleven Method	HP
2		Humar and Pohleven Method	HPI
3		Reinprecht Test	R
4		CT Test (test adapted from the Reinprecht test)	CT
5		ICWSE Test	DH
6		RS Test (Reinprecht)	RS
7		Agar dilution method	VM
8	Evaluation of biocidal efficacy	Test miniblock	R
9	Eco-impact assessment	Phytotoxicity test	TF
10	Evaluation of biocidal efficacy	Preventive treatment test	TPr
11	Evaluation of biocidal efficacy	Curative treatment test	TCu
12	Evaluation of biocidal efficacy	Curative Treatment Test - Practical Study on Heritage Objects	TCuP

Chapter 4 - O1 Establishing and validating an analytical laboratory protocol

This chapter aims to achieve the first objective of the thesis, namely establishing an analytical protocol useful for attaining all proposed objectives.

Fig. 4.1 presents a schematic of the analytical protocol, which includes three types of testing methods. The first phase examines the antifungal potential of the products used. Thus, it is necessary to establish a testing methodology that allows for determining the antifungal biocidal effect. After this initial phase, in which the products' effectiveness against fungi has been demonstrated, the next stage can begin. This stage comprises two other types of tests: one evaluating the antifungal effect demonstrated by testing the product on wood, and in parallel, studying the ecological impact.

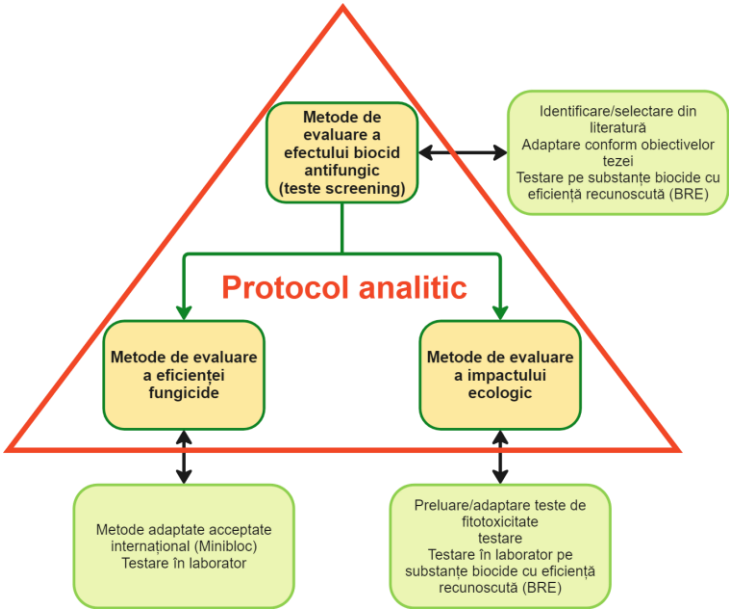


Fig. 4.1 Analytical protocol scheme

Screening tests

Testing the effectiveness of a wood protection product using the classic EN 113 method is laborious and time-consuming, thus creating a need for screening tests for an initial selection of substances with biocidal potential. The literature presents various screening tests, which have general applicability or are specific to fields other than wood protection: protection of documentary heritage (Borrego et al., 2012; GÓMEZ DE SARAVIA et al., 2008), agriculture (Ürgeová et al., 2013), nutrition (Gatto et al., 2016; Gurnani et al., 2016; Juárez et al., 2015; Varona et al., 2013; Viuda-Martos et al., 2008), medicine (Ullah et al., 2016).

Primarily, screening tests aim to rapidly identify substances with biocidal potential, evidenced in the test by inhibiting growth or causing lethal effects on fungi that have been carefully inoculated onto a sterile culture medium.

Identifying the most suitable methodology for preliminary testing of the biocidal effect of essential oils required several experimental stages in which 7 types of screening tests were carried out (Fig. 4.2), selected from literature and adapted. To validate the screening tests, biocidal substances with recognised efficacy (BRE- Copper sulphate ($CuSO_4$) și Romalit N (55% Copper sulphate Cu SO_4 + 45% Potassium dichromate $K_2Cr_2O_7$) were used, as well as an initial selection of essential oils, chosen after bibliographic study.

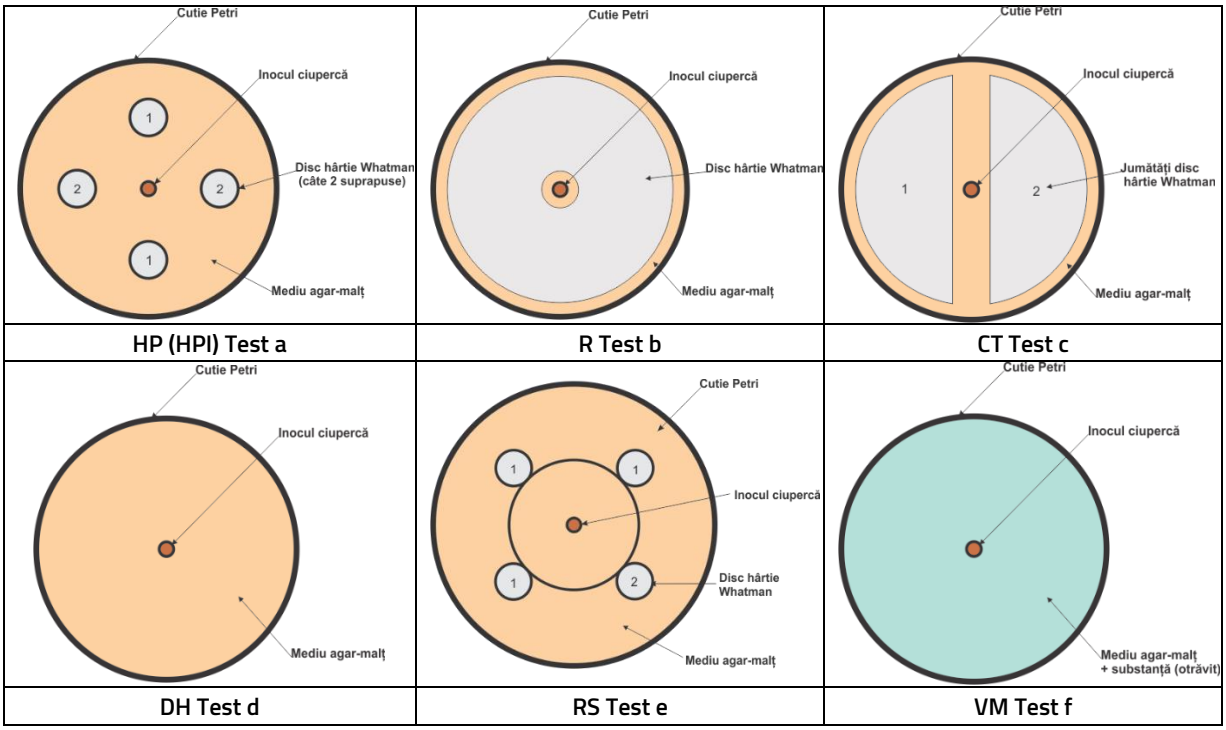


Fig. 4.2 General principles of screening tests

Qualitative and quantitative assessment

The qualitative assessment was carried out through visual analysis of the fungal growth from the initial inoculum and photographic documentation at various intervals after inoculation. The degree of mycelial development/expansion was monitored, as well as any preferential orientation (control vs. biocide). This was necessary both to track the evolution of fungal growth according to testing conditions and to establish relevant examination periods and maximum testing durations.

The quantification of visual results (quantitative evaluation) was carried out as follows:

For the HP, HPI, R, CT, DH and VM tests, a method was developed to measure the fungal mycelium growth using photographs in CorelDraw software and to calculate a Development Index (I) representing the ratio between the fungal growth towards/on the test solution and its growth towards/on the control (initial) solution.

For the RS Test, the quantitative evaluation method from the mycological testing laboratory at the Technical University of Zvolen was adopted, namely measuring the mycelium growth on medium and paper with a ruler. This allows for the subsequent calculation of the fungal growth inhibition index on medium (I_{sol}, %) and the fungal growth inhibition index on paper (I_{paper}, %).

Miniblock tests

Miniblock tests aim to verify the biocidal (fungicidal) effect on wood of products with potential biocidal properties previously identified through screening tests. Two types of tests were conducted (Fig. 4.3, Fig. 4.4)

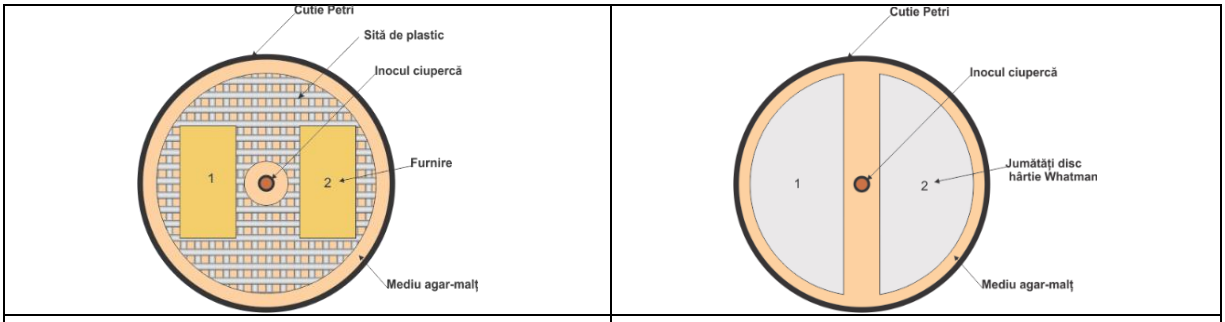


Fig. 4.3 Comparative aspects regarding the arrangement of test samples between the miniblock A test (a) and the CT screening test (b)

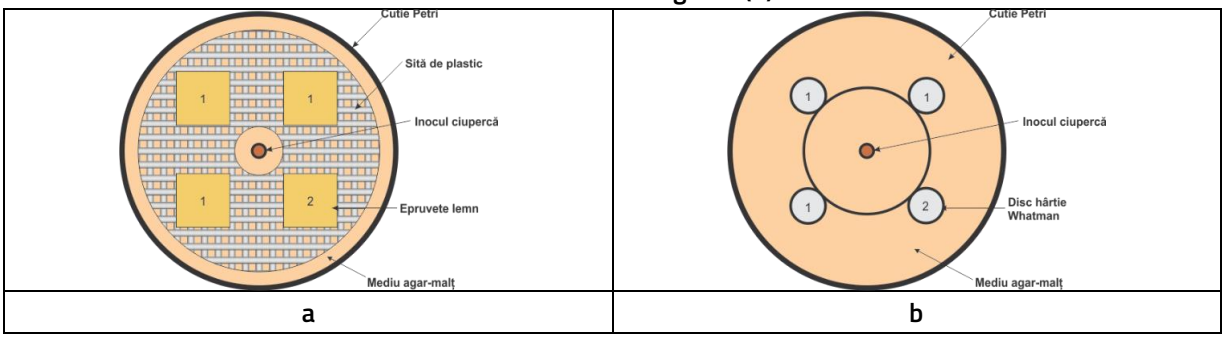


Fig. 4.4 Comparative aspects regarding the arrangement of test samples between the miniblock R test (a) and the RS screening test (b)

These tests were conducted in accordance with the testing principles of EN 113, but using small-sized samples to reduce testing time. The miniblock tests monitor the mass loss of samples due to fungal attack, with the samples placed on a culture medium inoculated with the test fungus. The validation of these tests used two essential oils, selected based on bibliographic research.

Phytotoxicity test

In the context of wood protection, the ecological impact can be both direct (at the time of applying the wood treatment technology) and indirect (through substances that come to influence various environmental components through the use of treated wood).

Following the bibliographic research, a phytotoxicity test on seeds was selected, which analyses both qualitatively - visually and quantitatively how a substance influences germination and sprout growth. (Roccotiello et al., 2011). Similar tests to the chosen one are

available for purchase in kit form as well.[7] In order to accurately test the potential phytotoxic effect of products used in wood treatment (samples from the miniblock R test), it was necessary to conduct a preliminary test before the phytotoxicity test, with the aim of obtaining the solutions used in the test. Thus, a leaching test of the treated wood samples for the miniblock R test was carried out, in accordance with SR EN 84:2000..

Conclusions

Both the RS test and the CT test showed similar results or the same trend. However, due to the RS test measurement method proving more feasible and the existence of literature references for this test (Pánek et al., 2014b), it was decided to use this test further in the present research.

The R miniblock test is validated for continued use, both due to the results obtained in this initial phase of use and its proximity to the requirements of the EN 113 standard.

The environmental impact assessment will be evaluated based on the results obtained through the TF phytotoxicity test, complemented by a prior leaching test.

Chapter 5 - O2 Identification and characterisation of essential oils with potential antifungal biocidal effects

This chapter focuses on the detailed analysis of the chemical composition of certain essential oils, an aspect of particular importance for understanding and explaining how they interact with wood-decay fungi, as well as with solvents/dilution media or the treated wooden substrate.

Based on a thorough bibliographic analysis, various essential oils with antifungal potential were identified in the specialist literature, which could be used against wood decay fungi. Thus, for the research conducted within this doctoral thesis, five essential oils were initially chosen: **B-EO** (***Ocimum basilicum)***, **C-EO** (***Eugenia caryophyllata***), **O-EO** (***Origanum vulgare***), **S-EO** (***Cinnamomum verum***) and **T-EO** (***Satureja hortensis***).

Essential oil profile sheets

By compiling the results obtained from laboratory analyses (FTIR, GC-MS) with information provided by the supplier and other data from the consulted literature, a detailed evaluation of the profile and quality of each essential oil was carried out. This data was originally

[7]https://www.microbiotests.com/toxkit/phytotoxicity-test-with-phytotoxkit-liquid-samples/

synthesised into characterisation sheets for each essential oil, providing a comprehensive overview of these products (BD-02).

Comparative Analysis of the 5 Essential Oils

This section presents a comparative analysis of the main physical properties and chemical composition determined experimentally (GC-MS and FTIR analyses) for the five essential oils: **B-EO** (Basil), **C-EO** (Clove), **O-EO** (Oregano), **S-EO** (Cinnamon) and **T-EO** (Savory) considered in this doctoral thesis. The analysis was conducted based on data and experimental results from individual characterisation sheets of the essential oils.

Physical properties

The comparative data for the 5 essential oils are summarised in the Tab. 5.1

Tab. 5.1 Comparative physico-chemical properties of the 5 essential oils used

No.	Characteristic	B-EO	C-EO	O-EO	S-EO	T-EO
1	Aspect	clear homogeneous liquid				
2	Colour	pale yellow	yellow, yellow-brown	pale yellow – reddish brown	yellow – reddish brown	yellow - yellowish brown
3	Smell	characteristic	characteristic	characteristic. phenolic	characteristic: strong, spicy, warm	characteristic. phenolic
4	Flash point	> 110°C	+100°C	+65°C	115°C	+64°C
5	Density at 20°C, [g/cm³]	0.936 (25°C)	1.042-1.065	0.940-0.980	0.990-1.050	0.875-0.954
6	Refractive index at 20°C	1.5042 (25°C)	1.528-1.538	1.495-1.525	1.545-1.600	1.486-1.505
7	Optical rotation at 20°C	- 6.1°	nespecificat	-3 ° la +1°	-3 ° la +2°	-5° la +4°
8	Solubility	Ethanol, Chloroform	Ethanol	Ethanol Oils	Ethanol Oils	Ethanol

Chemical composition determined by GC-MS

The results regarding the comparative chemical composition, experimentally determined by GC-MS, for the 5 essential oils are summarised in Tab. 5.2 which shows the percentages of areas relative to the total areas of identified components, exclusively for components identified with a percentage ≥ 3% or more.

Tab. 5.2 Comparative chemical composition of the 5 essential oils used, experimentally determined by GC-MS: chemical compounds with relative concentrations ≥3% of total identified compounds

No.	Chemical component identified	Relative concentrations [%] (Percentage of peak area / total area of identified peaks)					Obs.
		B-EO	C-EO	O-EO	S-EO	T-EO	
1	γ –Terpinen			3,49		11,37	MT
2	α –Terpinen					15,07	MT
3	p-Cimen			13,79	3,59		MT ar.
4	Mircen						MT
5	β-Caryophyllene		17,12			7,28	ST
6	α-Linalool	25,63		5,11	3,85	9,42	Alcohol
7	**Carvacrol**			**53,16**		**32,22**	Phenol
8	**Eugenol**		**77,34**				Phenol
9	Eugenyl acetate						Esther
10	**Taragon** (**Estragol**, metil-chavicol)	**60,55**					Ether
11	**Cinam aldehidă**				**47,03**		Aromatic aldehyde
12	Cinamil acetat				7,96		Esther
13	2-Norpinen	3,21					MT
14	α-Pinen			6,91	3,26	7,23	MT
15	Camphene					4,99	MT
16	Eucaliptol (Cineole)			3,36	3,37	4,63	Ether
17	α-Caryophyllene (Humulen)					3,73	ST
18	meta-Eugenol				12,87		Phenol
19	Carryophylene oxide			3,72			Ether
20	para-Isopropil-benzoat de etil				7,75		Esther
21	Cicloundecatriena	4,92					Trienă
	Total	**94,31**	**94,46**	**89,54**	**89,68**	**95,94**	

The experimental data confirm the information regarding the major chemical compounds in the composition of EOs specified in the product technical sheets, while also highlighting differences in concentration and the presence of other compounds at a minimum of 3%. These include monoterpenes (2-Norpinene, α-Pinene, camphene), sesquiterpenes (β-Caryophyllene syn. Humulene), terpene ethers (eucalyptol, caryophyllene oxide), phenols (meta-Eugenol), and esters (ethyl p-isopropylbenzoate), which are found in varying amounts in the composition of the analysed oils. Although the summarised data refer exclusively to components with a concentration of at least 3%, the experimental findings underscore the compositional complexity of essential oils and their individual compositional characteristics.

In this context, it is important to specify that there is generally variability in the composition of essential oils from a particular plant source depending on a multitude of factors including

vegetation conditions, growth period, plant age, harvest time and storage duration. (ex. (Toncer et al., 2017); (Liyanage et al., 2017); (Skubij & Dzida, 2019); (Mohtashami et al., 2018)).

FTIR Analysis

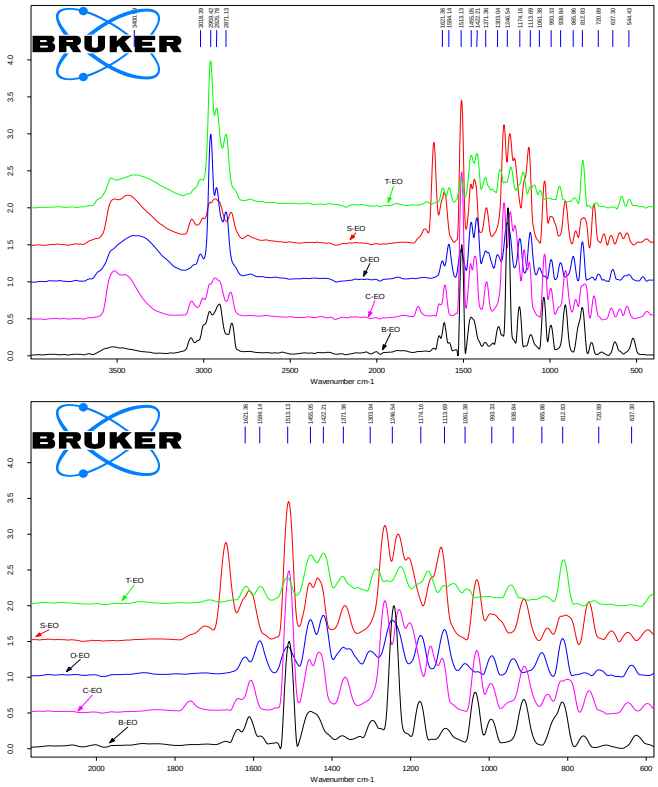


Fig. 5.1 Comparative FTIR-ATR spectra for the 5 essential oils, recorded across the entire range of 4000-6000 cm-1 (up) and the fingerprint region 1800-600 cm-1 (down)

Fig. 5.1 presents the comparative FTIR-ATR spectra of the 5 essential oils analysed. In line with the conclusions of the GC-MS analysis, the FTIR spectra confirm the complexity and compositional individuality of the essential oils examined. Thus, whilst some similarities between spectra are evident (**O-EO** with **T-EO**, **C-EO** with **B-EO**), namely the existence of common absorption bands for various EOs, the spectra do not overlap, being strictly specific, particularly in the fingerprint region.

In conclusion, the results of the investigations in this chapter highlight the physico-chemical and compositional characteristics, similarities and differences between the five essential oils studied (**B-EO**, **C-EO**, **O-EO**, **S-EO**, **T-EO**), providing a fundamental basis for understanding and explaining their biological properties, including fungicidal activity. The active antifungal nature of some major components of these oils, such as eugenol, carvacrol, and cinnamaldehyde, has been demonstrated through specific tests. (ex. (Xie et al., 2017)) and constitutes an argument for a potential biocidal effect of the tested EOs. The difference in composition of the EOs will

likely determine differences in antifungal potential, and efficacy will be influenced by the type of fungi.

Chapter 6 - O3 Comparative evaluation of antifungal biocidal effect - selection of products with potential

This chapter details the testing and evaluation procedure for the fungicidal potential of five selected essential oils (EOs): **B-EO** (***Ocimum basilicum***), **C-EO** (***Eugenia caryophyllata***), **O-EO** (***Origanum vulgare***), **S-EO** (***Cinnamomum verum***) and **T-EO** (***Satureja hortensis***), as well as the results obtained. To determine their antifungal potential against a series of wood-decaying fungi important in wood biodegradation, the essential oils underwent screening tests following the analytical protocol presented in chapter 4.

General considerations on screening tests performed and research stages

To achieve Objective O3 of the thesis, the research conducted and presented in this chapter focused on the following:

- Evaluation of the antifungal potential against representative wood-decaying fungi for each of the 5 essential oils considered;
- Comparative assessment of the results obtained for the 5 essential oils to rank them in terms of antifungal biocidal potential;
- Selection of two essential oils with potential for further research (O4, O5)
- Validation of results by retesting the selected essential oils with potential.

Tab. 6.1 RS screening test: data on essential oil concentrations, solvents and biological material used in testing and validating antifungal potential (stages of O3 implementation)

O3 Stage	Objective	Screening Test	EOs	Solvent	EO concentration in solvent [ml/100 ml]	Biological material
1	Test for antifungal potential (Zvolen) Select 2 EOs	RS	**B-EO** **C-EO** **O-EO** **S-EO** **T-EO**	Ethanol	0,25	Brown rot: ***Serpula lacrymans*** (Wulfen) White rot: ***Trametes versicolor*** (L.)
					1	
					10	
					100	
2	Validate antifungal potential (Brasov) of Selected EOs	RS	**C-EO** **T-EO**	Ethanol	0 / Control	Brown rot: ***Postia placenta*** (Fr.) White rot: ***Trametes versicolor*** (L.)
					1*	
					5	
					10	
		RS	**C-EO** **T-EO**	Linseed oil (LO)	0 / Control LO	Brown rot: ***Postia placenta*** (Fr.) White rot: ***Trametes versicolor*** (L.)
					1*	
					5	
					10	

NOTE: * The 1% concentration of essential oil was used only for Clove essential oil **C-EO**.

The 5 essential oils used were tested at various dilutions in two different solvents: drying linseed oil (code LO) and ethanol (ethyl alcohol pa, 96%), with the testing variants presented in Tab. 6.1.

Results of testing and validation of antifungal potential by screening tests

The test results, comprising photographic documentation of fungal growth progression throughout the trial, calculated specific inhibition indices, and a series of graphical representations, have been synthesised into evaluation sheets assessing the fungicidal potential for each oil and individual experiment..

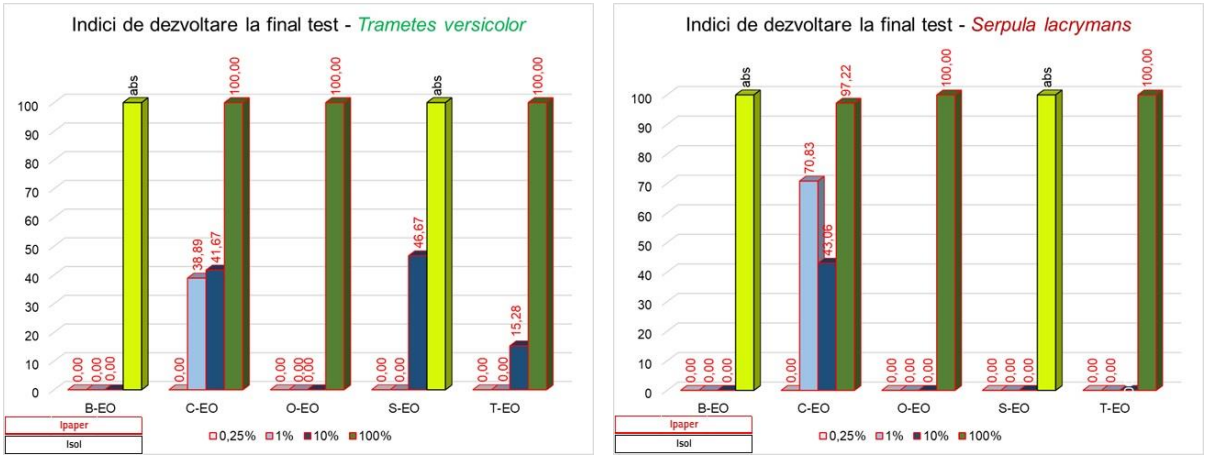
Testing and validation results sheets for antifungal potential

The experimental data resulting from the screening tests were synthesised into 3 types of records:

- Potential antifungal results records (R-O3-1), comprising 5 records created separately for each type of essential oil (BD-O3/1);
- Antifungal potential validation records (R-O3-2), comprising 2 validation records created separately for each type of essential oil selected after the first testing phase (BD-O3/2);
- Comparative antifungal potential results records based on fungal type (R-O3-3), comprising 2 records created for each type of fungus tested, including comparative results for both selected oils (BD-O3/3).

Comparative analysis of the antifungal biocidal potential of tested essential oils

The antifungal potential for each type of oil studied and concentration, depending on the type of fungus used, namely ***Trametes versicolor*** - white rot and ***Serpula lacrymans*** - brown rot, is illustrated in Fig. 6.1(a,b), which graphically presents the experimental results obtained at the end of the test, specifically the inhibition indices on the last day of the test, which may vary depending on the case..



a **b**

Fig. 6.1 The fungal growth inhibition index (Isol or Ipaper) determined at the end of the test (different days for each oil and concentration), comparing the white-rot fungus *Trametes versicolor* (a) and the brown-rot fungus *Serpula lacrymans* (b) for all essential oils tested at the studied concentrations.

TV Conclusion: The data obtained for Clove essential oil (**C-EO**) indicate the highest biocidal potential against white rot (***Trametes versicolor***), as it manifests from a concentration of 1%. Cinnamon (**S-EO**) and Savory (**T-EO**) essential oils also show antifungal potential against **TV**, as can be observed from the comparative values recorded for the fungal growth inhibition index on paper. At a concentration of 10%, the maximum value of Ipaper=46.67% was obtained for **S-EO.**.

SL Conclusion: Clove essential oil (**C-EO**) is the only essential oil that exhibits antifungal biocidal potential, starting from a concentration of 1% up to 100%. Savory oil (10%) shows an initial inhibitory effect on mycelial growth in the medium, but this disappears 7 days after inoculation, when the fungus reaches the paper..

To summarise the results of the comparative analysis of the recorded experimental data regarding the antifungal biocidal potential of the 5 EOs at all concentrations analysed, based on the fungus type, a "chessboard" scheme was created, as presented in Fig. 6.2. In this diagram, essential oils are marked in green at the concentrations where they demonstrated antifungal efficacy.

EO	Conc.	*Trametes versicolor*	*Serpula lacrymans*
B-EO	0.25%		
	1%		
	10%		
	100%	**Z_14 (abs)**	**Z_14 (abs)**
C-EO	0.25%		
	1%	**Z_10 (38.89%)**	**Z_10 (70.83%)**
	10%	**Z_7 (41.67%)**	**Z_7 (43.06%)**
	100%	**Z_14 (100%)**	**Z_14 (97.22%)**
O-EO	0.25%		
	1%		
	10%		**Z_7 (54.39%)**
	100%	**Z_14 (100%)**	**Z_14 (100%)**
S-EO	0.25%		
	1%		
	10%	**Z_10 (46.67%)**	
	100%	**Z_14 (abs)**	**Z_14 (abs)**
T-EO	0.25%		
	1%		
	10%	**Z_7 (15.28%)**	**Z_7 (53.33%)**
	100%	**Z_14 (100%)**	**Z_14 (100%)**
Inhibition index recorded at the end of the test I_{sol} / I_{paper} (Zx – different day)			
Inhibition index throughout the test, up to day Zy			

Fig. 6.2 Schematic presentation, in a "chessboard" style, of essential oils at the studied concentrations, highlighting the concentrations for which antifungal biocidal potential has been demonstrated, compared for the two tested fungi *Trametes versicolor* (white rot) and *Serpula lacrymans* (brown rot)

Conclusion: Following the comparative evaluation of the antifungal biocidal potential of the five tested oils, two essential oils were selected for further research: Clove essential oil **C-EO** (***Eugenia caryophyllata***) and Savory essential oil **T-EO** (***Satureja hortensis***).

Comparative analysis of experimental results obtained in the validation stage of antifungal potential for C-EO and T-EO

To further investigate the biocidal potential of the two selected essential oils: Clove essential oil **C-EO** (***Eugenia caryophyllata***) and Savory essential oil **T-EO** (***Satureja hortensis***), and to validate the results that formed the basis for their selection, a new RS screening test was conducted at ICDT Brasov.

The validation of the antifungal potential was carried out by testing the two selected oils diluted in two different solvents: boiled linseed oil (code LO) and ethanol (ethyl alcohol pa, 96%, code ET). The testing variants were previously presented in Tab. 6.1. The fungi used in the validation test were ***Postia placenta*** - white rot and ***Trametes versicolor*** - white rot.

Validation of the biocidal potential for Clove essential oil C-EO (*Eugenia caryophyllata*)

According to the previous findings, Clove essential oil (**C-EO**) demonstrated biocidal potential starting from a concentration of 1%. The results obtained in the validation test of **C-EO**'s biocidal potential, at various dilutions in the two media considered (LO, ethanol), against the two fungi **TV** and **PP** are graphically represented by the evolution of calculated inhibition indices. The complex graphs show the evolution of growth inhibition indices on medium (Isol) and on paper (Ipaper) throughout the monitoring period, for each concentration of Clove essential oil **C-EO** (***Eugenia caryophyllata***) in the two solvents studied..

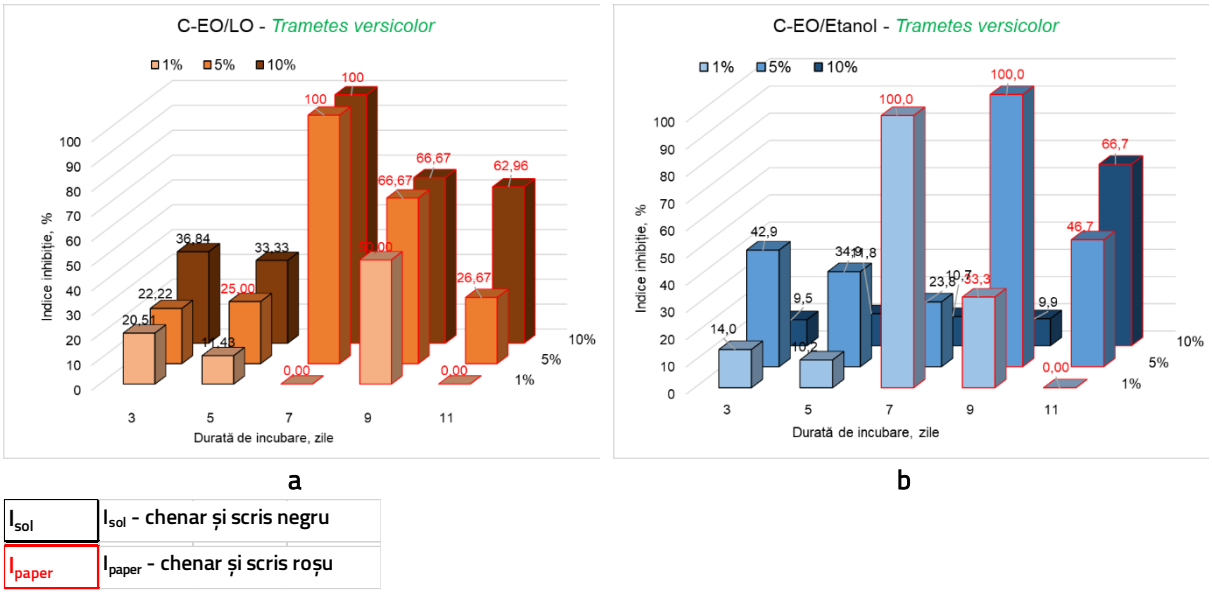


Fig. 6.3 Evolution of growth inhibition indices on medium (Isol) and on paper (Ipaper) for Clove essential oil C-EO (*Eugenia caryophyllata*) at the tested concentrations in the two studied solvents (linseed oil - a; ethanol - b) for the white rot fungus *Trametes versicolor*.

At a concentration of 1% **C-EO** in both solvents used, the fungus appears to develop uniformly (BD-03/2 - R-03-2/**C-EO**) in the first days of monitoring, with slight tendencies towards the control. On day 7, the **TV** fungus reaches only the control paper from the medium in the case of **C-EO**/Ethanol, with the presence of a red border marking, showing a total inhibition of 100% at that point. This diminishes over time, with the inhibition index (Ipaper) decreasing from 100% to 0% by the end of the test (day 11). In the case of **C-EO**/LO, on day 7 the **TV** fungus begins to climb onto the paper, but no preference is observed (BD-03/2 - R-03-2/**C-EO**). Later, on day 9, an inhibitory effect can be seen (Ipaper=50%), which does not persist, however, as by the end of the test all papers are covered with mycelium (Ipaper=0%).

At a concentration of 5% **C-EO**/LO on day 7, the fungus reaches the paper, preferring the control paper, and this inhibitory trend of the **TV** fungus is maintained until the end of the test, with the inhibition index (Ipaper) being 26.67% on day 11. **C-EO**/Ethanol at this concentration (5%) exhibits an inhibitory effect on the development of the **TV** fungus. It is not until day 9 that

the fungus reaches the control paper (Ipaper=100%), but by the end of the test, the index value (Ipaper) reaches 46.7%.

At a concentration of 10% **C-EO**/LO, the **TV** fungus reaches the control paper (Ipaper=100%) on day 7 of monitoring. The inhibitory effect persists until the end of the test, but the index value (Ipaper) decreases from 100% to 62.96%, a value obtained on day 11. Regarding **C-EO**/Ethanol at a concentration of 10%, there is a clear delay in the development of the **TV** fungus, as it only reaches the paper at the end of the test, and the inhibition index (Ipaper) has a final value of 66.7%.

For the brown rot fungus ***Postia placenta***, inhibition of growth was observed in tests conducted with clove essential oil. This effect is presented graphically in Fig. 6.4 where the evolution of growth inhibition indices in medium (Isol) and on paper (Ipaper) can be observed over the 11 days of monitoring, for each concentration of Clove essential oil **C-EO** in the two studied solvents (linseed oil – Fig. 6.4 a; ethanol – Fig. 6.4 b).

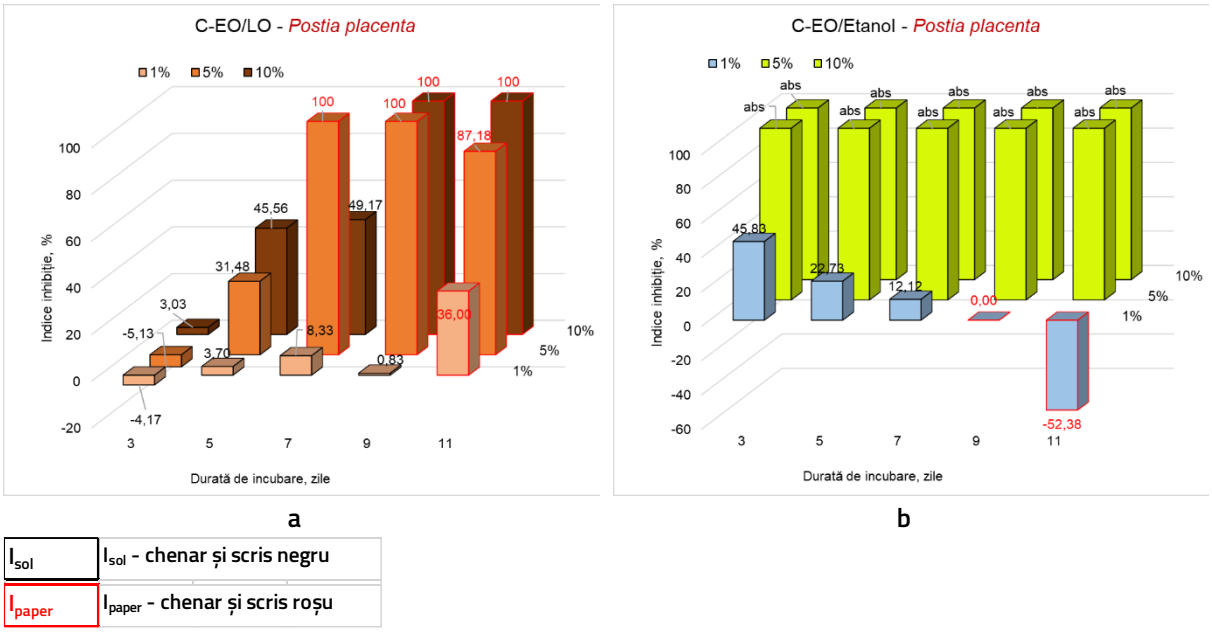


Fig. 6.4 The evolution of growth inhibition indices in medium (Isol) and on paper (Ipaper) throughout the monitoring period, for clove essential oil C-EO (*Eugenia caryophyllata*) at the tested concentrations with the two solvents studied (linseed oil - a; ethanol - b) for the brown rot fungus *Postia placenta*.

At a concentration of 1% **C-EO**/LO, the **PP** fungus experiences delays in development, reaching the paper only on day 11 at the end of the test, when the calculated inhibition index value (Ipaper) is 36%. At this 1% concentration and in the case of **C-EO**/Ethanol, the **PP** fungus behaves differently. The fungus reaches the paper only from day 9, at which point we have absolute inhibition (Ipaper=100%), but at the end of the test (day 11), the inhibition index has a

large negative value (Ipaper = -52.38), which would indicate that the **C-EO**/Ethanol 1% treatment might promote the growth of the **PP** fungus.

At a concentration of 5% **C-EO**/LO on day 7, the **PP** fungus reaches the paper, exhibiting total inhibition (Ipaper=100%). However, by the end of the test on day 11, the index (Ipaper) has a value of 87.18%. At this concentration, **C-EO**/Ethanol demonstrates an absolute inhibitory effect on the growth of the **PP** fungus, which is maintained until the end of the test.

At a concentration of 10% **C-EO**/LO, the **PP** fungus is slowed in its development, only reaching the paper from day 9, and its growth preference is clearly towards the control area. Both on day 9 and at the end of the test, complete inhibition is observed, with the index (Ipaper) having a value of 100%. Regarding **C-EO**/Ethanol at a concentration of 10%, the situation is similar to **C-EO**/Ethanol 5%, namely it exhibits an absolute inhibitory effect on the development of the **PP** fungus and is maintained throughout the duration of the test.

Validation of the biocidal potential of Savory (*Satureja hortensis*) essential oil T-EO

The biocidal potential validation of Savory essential oil (**T-EO**) in the two types of dilution media (LO and Ethanol) is quantified once again by determining the inhibition indices of fungal growth used in testing.

Thus, for the white rot fungus ***Trametes versicolor***, the evolution of growth inhibition indices on medium (Isol) and on paper (Ipaper) throughout the monitoring period, for each concentration of Savory essential oil **T-EO** (***Satureja hortensis***) with the two studied solvents (linseed oil – Fig. 6.5 a; ethanol – Fig. 6.5 b) it is presented in graphical form in Fig. 6.5.

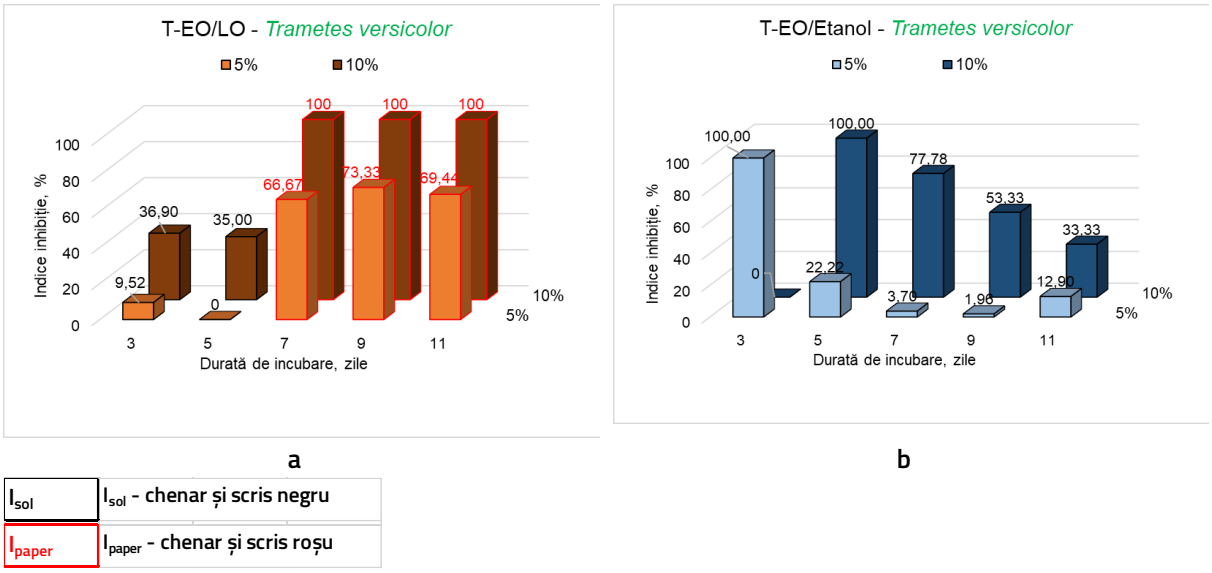


Fig. 6.5 Evolution of growth inhibition indices on medium (Isol) and on paper (Ipaper) for Savory T-EO essential oil (*Satureja hortensis*) at the tested concentrations with the two studied solvents (linseed oil - a; ethanol - b) for white rot fungus *Trametes versicolor*.

At 5% concentration of **T-EO**/LO on day 7, the **TV** fungus reaches the paper (the red marker appears, corresponding to the inhibition index Ipaper), preferring the control paper. This inhibition persists until the end of the test, when the inhibition index (Ipaper) is 69.44%. At this concentration, **T-EO**/Ethanol exhibits an inhibitory effect on the development of the **TV** fungus, with the fungus not yet reaching the paper by the end of the test (day 11).

At the 10% **T-EO**/LO concentration from day 7, total inhibition is observed (Ipaper=100%) and maintained until the end of the test. Compared to the 5% **T-EO**/LO concentration, the **TV** fungus is less developed at the end of the test. Regarding **T-EO**/Ethanol at 10% concentration, we have two different situations in the two Petri dishes. One shows absolute inhibition (the fungus did not develop at all after inoculation), while the other exhibits total inhibition (Isol = 33.33% at the end of the test) (BD-03/2 - R-03-2/**T-EO**).

For the brown rot fungus ***Postia placenta*** in Fig. 6.6 the graphical representation shows the evolution of growth inhibition indices in medium (Isol) and on paper (Ipaper) over the 11-day monitoring period, for each concentration of Savory (***Satureja hortensis***) essential oil with the two studied solvents (linseed oil – Fig. 6.6 a; ethanol – Fig. 6.6 b).

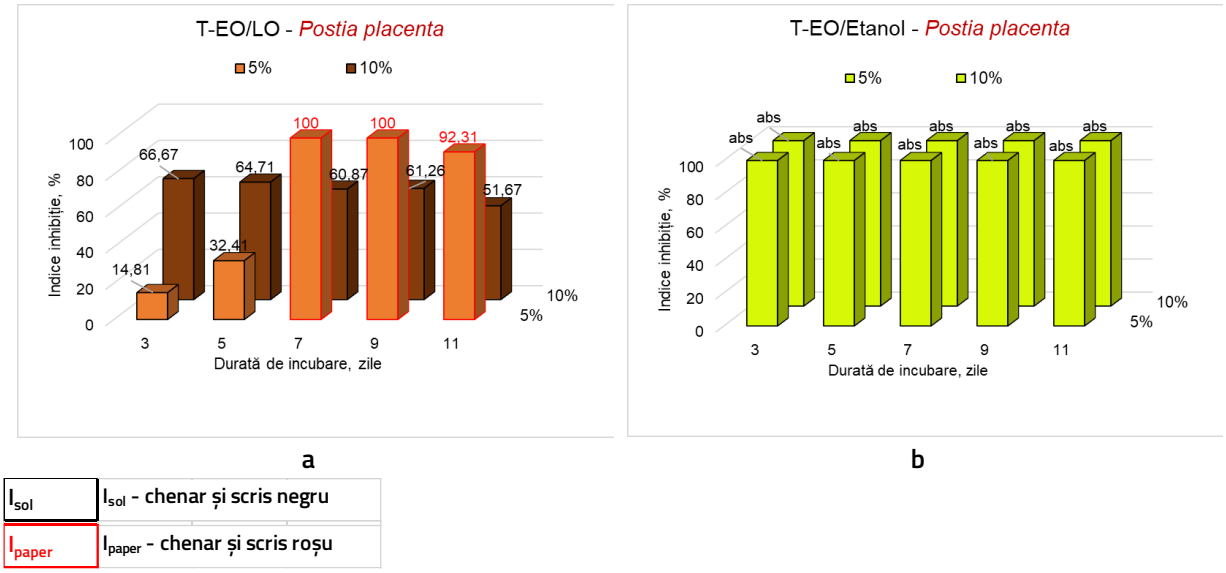


Fig. 6.6 The evolution of growth inhibition indices on medium (Isol) and on paper (Ipaper) throughout the monitoring period, for the essential oil of Savory T-EO (*Satureja hortensis*) at the tested concentrations with the two studied solvents (linseed oil - a; ethanol - b) for the brown rot fungus *Postia placenta*.

At a concentration of 5%, **T-EO**/LO demonstrates antifungal potential by inhibiting the growth of **PP** fungus. On day 11, the inhibition index (Ipaper) is 92.31%, approaching total inhibition. **T-EO**/Ethanol at this concentration exhibits absolute inhibition of **PP** fungus growth, which is maintained until the end of the test.

For the 10% **T-EO**/LO concentration, the **PP** fungus is significantly slowed in development, with a clear preference towards the control. On the last day of testing, the fungus had not yet reached the paper, and the inhibition index (Isol) is 51.67%. Regarding **T-EO**/Ethanol 10%, the absolute inhibition effect on **PP** fungus development is maintained until the end of the test.

Analysis of experimental results obtained based on fungal type

The values of the two indices: growth inhibition on medium (Isol) and growth inhibition on paper (Ipaper) at the end of the test (day 11) for the two essential oils: Clove **C-EO** (***Eugenia caryophyllata***) and Savory **T-EO** (***Satureja hortensis***), using linseed oil LO and ethyl alcohol as solvents at all studied concentrations are presented comparatively in Fig. 6.7, for both types of fungi.

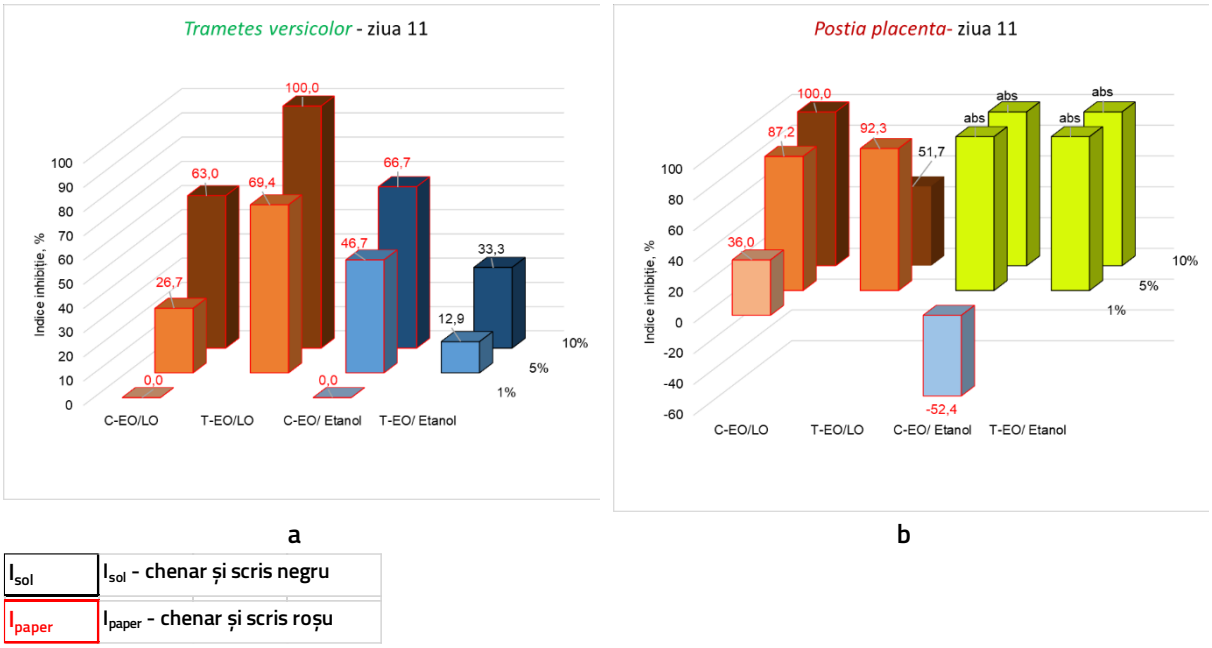


Fig. 6.7 The evolution of growth inhibition indices in medium (Isol) and on paper (Ipaper) at the end of the test, for Clove essential oil (C-EO) and Savory essential oil (T-EO) at the tested concentrations in the two dilution media for white rot - *Trametes versicolor* - a and brown rot *Postia placenta* - b.

In the case of using **C-EO** and **T-EO** in ethanol at concentrations of 5% and 10%, both essential oils produce absolute inhibition of the brown rot fungus ***Postia placenta*** (**PP**), likely due to the volatilisation of EOs and sterilisation of the treatment chamber.

When diluted in ethanol at concentrations of 5% and 10%, Savory essential oil (**T-EO**) has a higher antifungal potential compared to Clove essential oil (**C-EO**) against the white rot fungus ***Trametes versicolor*** (**TV**), in which case the fungus reaches the paper.

When using **C-EO** and **T-EO** in LO, absolute inhibitions were not achieved for either of the two tested fungi at any of the 1-10% concentrations, which could be correlated with the fixation of

EOs in the support paper due to linseed oil, thus preventing sterilisation of the test chamber. However, total inhibition indices (Ipaper =100%) were obtained for **T-EO**/LO 10% for ***Trametes versicolor*** and **C-EO**/LO 10% for ***Postia placenta***..

Conclusions

- All five essential oils studied - **B-EO**, **C-EO**, **O-EO**, **S-EO**, **T-EO** - exhibit antifungal biocidal potential against the tested white rot (***Trametes versicolor***) and brown rot (***Serpula lacrymans***, ***Postia placenta***) fungi when used at full strength, i.e. at the maximum concentration of 100%.
- Based on the fungal growth inhibition indices at the end of the screening tests evaluating the comparative antifungal biocidal potential of the tested EOs, two essential oils were selected for further research: Clove essential oil **C-EO** (***Eugenia caryophyllata***) and Savory essential oil **T-EO** (***Satureja hortensis***).
- Savory essential oil **T-EO** (***Satureja hortensis***) shows a higher inhibition potential compared to Clove essential oil **C-EO** (***Eugenia caryophyllata***) for both **TV** (***Trametes versicolor***) and **PP** (***Postia placenta***) fungi, regardless of the type of dilution medium and concentration.
- Clove essential oil **C-EO** (at 5% and 10%) exhibited a higher antifungal potential against the brown rot fungus ***Postia placenta*** than against the white rot fungus ***Trametes versicolor***, regardless of the dilution medium used.
- Savory essential oil **T-EO** (at 5% and 10%) showed higher antifungal potential against the brown rot fungus ***Postia placenta*** compared to the white rot fungus ***Trametes versicolor***, regardless of the dilution medium used.
- The lower antifungal potential of the essential oils (**C-EO** and **T-EO**) against ***Trametes versicolor***, compared to ***Postia placenta***, correlates with the white rot fungus's (**TV**) ability to degrade phenolic compounds, including eugenol and carvacrol, the main chemical components of **C-EO** and **T-EO**. (Pánek et al., 2014b; Voda et al., 2003).

Chapter 7 - O4: Evaluation of the biocidal efficacy and potential ecological impact of two selected products (Clove essential oil C-EO, Savory essential oil T-EO)

In accordance with the basic concept of the thesis, objective 4 aimed to evaluate two particularly important aspects in the development of potential new products for antifungal

bioprotection of wood, namely: their biocidal efficacy and potential eco-impact. Balancing and evaluating these aspects at the same development stage represents a necessity in an ecological and sustainable approach of wood bioprotection, specifically for the development and testing of new products applicable in the field.

To achieve this objective, two types of tests were conducted in parallel:

- Miniblock test to validate the antifungal effect and evaluate the efficacy on treated wood samples;
- Phytotoxicity test to assess the potential eco-impact due to the leaching of possible toxic compounds from treated wood. (Fig. 7.1).

Methodological aspects

Treatment of wood samples and preparation for the mini-block test

The two essential oils selected based on their previously demonstrated fungicidal potential through screening tests were clove oil (**C-EO**) and thyme oil (**T-EO**). These two EOs were diluted in two different media: linseed oil (LO) and ethanol at various concentrations, with the resulting solutions used to treat the wood material. For **C-EO**, dilutions of 1%, 5%, and 10% were used, while for **T-EO**, the dilutions were 5% and 10%, in both solvents. The wood material used was non-steamed beech wood (***Fagus sylvatica*** L.) and pine sapwood (***Pinus sylvestris*** L.), which constitute non-durable reference species. The results presented in this chapter are exclusively for beech wood. Samples measuring (20x20x5) mm in the L, Tg, and Ra directions were prepared for testing by undergoing several successive processes (Fig. 7.1). The method of cutting samples and the procedures for treatment and preparation for testing are essentially similar to those reported by Reinprecht and colleagues. (Pánek et al., 2014c).

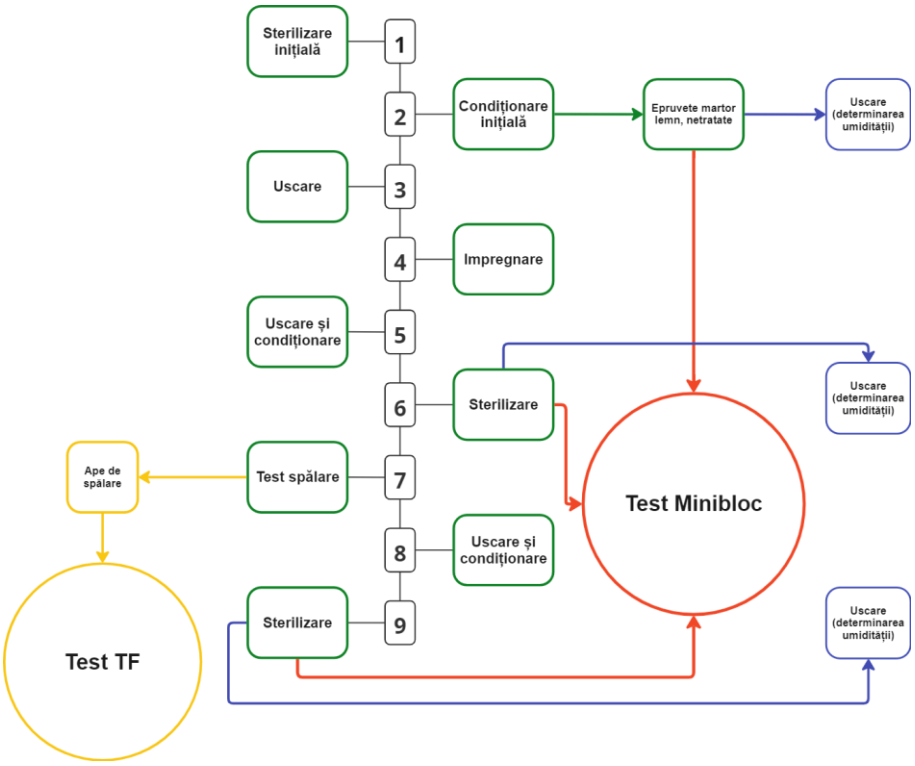


Fig. 7.1 Schema proceselor de pregătire a epruvetelor de lemn pentru testul minibloc și obținerea apelor de spălare pentru testul de fitotoxicitate

Miniblock test

The mini-block R test used is the one presented in detail in Chapter 4. Testing was carried out in parallel with two types of fungi: one brown rot fungus, ***Postia placenta*** (**PP**), and one white rot fungus, ***Trametes versicolor*** (**TV**).

Wood sample washing test and phytotoxicity test

The phytotoxicity test (PT) complemented by the leaching test according to EN 84:2000 applied beforehand are described in detail in chapter 4. In addition to the determined indices presented in chapter 4, the germination percentage and average sprout length, a relative germination index (GI, %) was also calculated, which is a complex indicator that combines the previous indices. (Bae et al., 2014; Di Salvatore et al., 2008).

Experimental Results

Miniblock test

The miniblock tests for diluted essential oils of Clove (**C-EO**) and Savory (**T-EO**) in two solvents, linseed oil (LO) and ethanol, were conducted in two research phases spaced apart in time. Tab. 7.1 presents a summary of the tests conducted in the two stages.

Tab. 7.1 Summary of mini-block tests conducted in the thesis

Tested fungi	Year	EO	Sample type	Ethanol			Linseed oil (LO)		
				1/100	5/100	10/100	1/100	5/100	10/100
PP	2019	C-EO	Unwashed	Yes	Yes	Yes	Yes	Yes	Yes
			Washed	Yes	Yes	Yes	Yes	Yes	Yes
TV		C-EO	Unwashed	Yes	Yes	Yes	Yes	Yes	Yes
			Washed	Yes	Yes	Yes	Yes	Yes	Yes
PP	2020	C-EO	Unwashed	No	Yes	Yes	No	Yes	Yes
		T-EO	Unwashed	No	Yes	Yes	No	Yes	Yes
TV		C-EO	Unwashed	No	Yes	Yes	No	Yes	Yes
		T-EO	Unwashed	No	Yes	Yes	No	Yes	Yes

Within this abstract only a a summary of the results obtained in the second testing stage are reported. An extensive presentation of all the results from both testing stages is available within the thesis.

Wood treated with essential oils in linseed oil

Analysis of C-EO/LO mass losses

The results of the miniblock tests from stage 2 (2020), conducted with **C-EO** and **T-EO** solutions in LO, exclusively on samples not subjected to the washing test, showed the following:

For the **C-EO**/LO test:

Extremely high variability of ML values for untreated control samples: 10.89±23.35 for **PP** and 54.21±15.26 for **TV**, with very large standard deviations, especially for **PP**, correlated with notable differences in mycelium development on similar samples in the same or different boxes. Differences in values between control and independent control samples for both types of fungi indicate an effect of LO treatment on biodegradation by the tested fungi. Notably, for **PP**, the ML value for the independent control (37.95±4.69%) is higher than the control (10.89±23.35%), whilst for **TV** the situation is reversed: (30.98±4.35%) compared to 54.21±15.06%. The differences in ML associated with **PP** and **TV** tests, superior for the latter,

are explained by the preference of white rot fungi (**TV**) for hardwood species, contrary to brown rot fungi (**PP**) which prefer softwoods. As the tests were conducted on beech, the more pronounced attack of **TV** compared to **PP** is explicable for control samples, but the higher ML values for independent control (37.95±4.69%) and total (34.98±9.42%) samples than for the control sample (10.89±23.35) in the case of **PP** are entirely unexpected. These would indicate that the **PP** fungus is not inhibited but rather stimulated in the presence of LO. The very large standard deviations, particularly for control samples, but also for various categories of control and samples treated with **C-EO**/LO 10%, highlight an extreme variability in fungal colonisation and attack.

For PP, the ML values for samples treated with **C-EO**/LO compared to the total control value do NOT show an antifungal effect, but rather a negative effect of increasing sensitivity to biodegradation by approximately 25-40%. This result contradicts the findings of the screening test.

For TV, the ML values for samples treated with **C-EO**/LO compared to the total control value do NOT show an antifungal effect at a concentration of 5%, but rather a ML increase of about 40%, and only a slight protective effect (8% ML decrease) at a **C-EO** concentration of 10%. However, the ML values at both **C-EO** concentrations in LO are lower than for untreated control wood. Thus, LO itself has a positive effect, but this is diminished by the addition of **C-EO** to LO..

For the **T-EO**/LO test:

The existence of value differences between the control and independent control samples for both types of fungi and large standard deviations. The total and independent control ML values indicate that LO treatment has a negative effect on resistance to **PP** attack, which is stimulated by oil treatment. Conversely, the values for total and independent control samples in the **TV** test show a positive effect of increasing resistance to **TV** attack..

For PP, the ML values for samples treated with **T-EO**/LO compared to the total control value show an antifungal effect (ML decrease of approximately 10-45%)).

For TV, The ML values for samples treated with **T-EO**/LO compared to the total control value show a contrary effect (increase in ML by 6-31%).

The variation in average values of mass loss through biodegradation according to sample type/applied treatment is graphically represented in Fig. 7.2, for both types of fungi (**PP**, **TV**), comparing samples treated with **C-EO** and samples treated with **T-EO**, exclusively unwashed samples. These reflect the aspects presented previously.

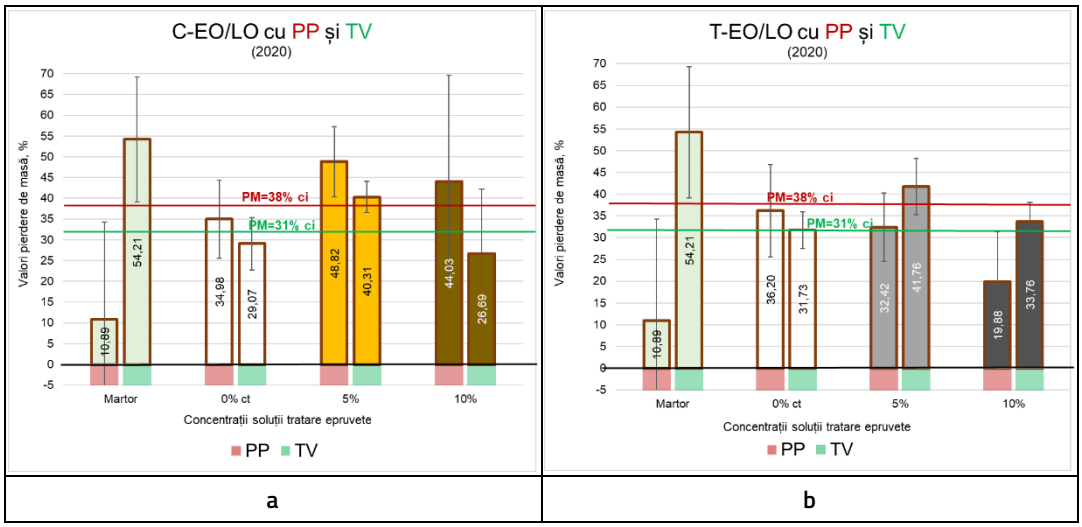
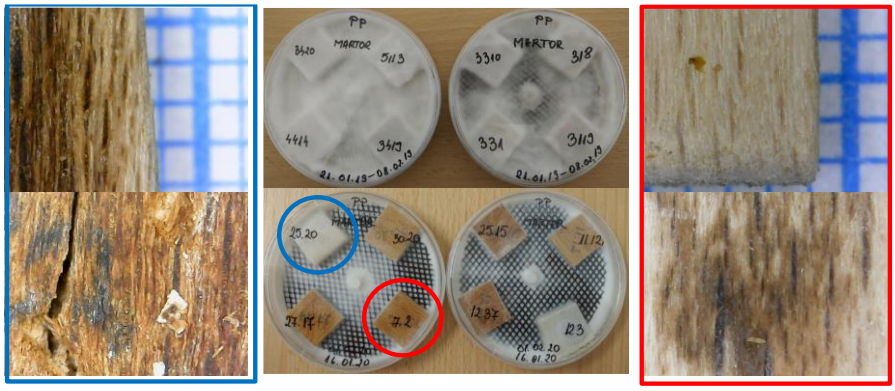


Fig. 7.2 The influence of treating beech wood with EOs/LO on mass losses in a miniblock test with *Postia placenta* (PP) and *Trametes versicolor* (TV), comparing samples with C-EO/LO (a) and samples with T-EO/LO (b)

Macroscopic and microscopic aspects

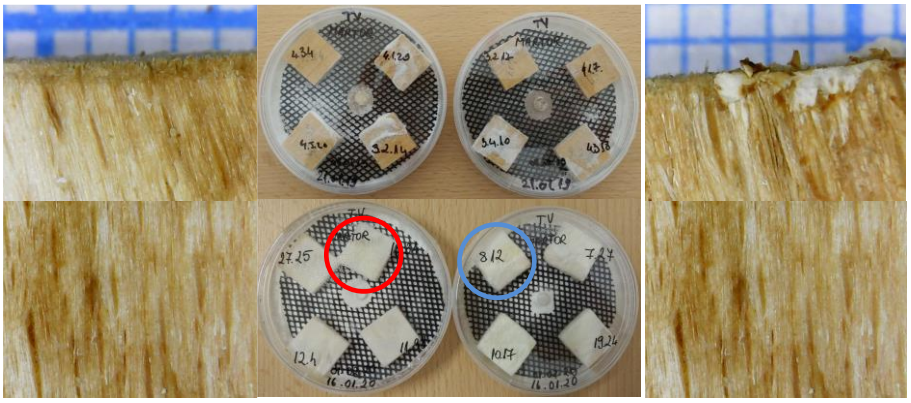
In the previous data analysis, emphasis was placed on the extreme variability of the data in certain situations, which sometimes makes it impossible to draw a conclusion. In this context, it is important to note that this situation does not inherently imply deficiencies in the test's execution but rather internal/specific causes of biological tests, which are difficult to explain and understand.

The microscopic details in the figures (Fig. 7.3, Fig. 7.4, Fig. 7.5) illustrates not only different degrees of degradation on samples with differentiated colonisation, but also the distinctions between types of degradation for ***Postia placenta*** (brown rot, cubic) and ***Trametes versicolor*** (white rot, fibrous)



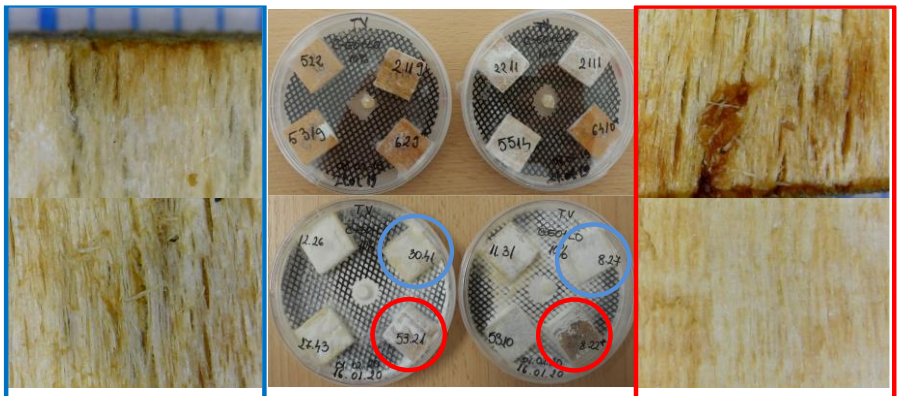
Proba 25.20 PP_Fag_Martor Proba 7.2

Fig. 7.3. Exemplification of fungal growth variability and degree of attack on various similar samples, under identical testing conditions, for untreated beech after miniblock test with PP (2019 and 2020): macro images of Petri dishes and microscopic details of attack



Proba 19.34 **TV_Fag_Martor** **Proba 8.12**

Fig. 7.4 Illustration of fungal growth for two tests under similar conditions and details of the degree of attack on two beech control samples after mini-block test with TV (2019 and 2020): macro images of Petri dishes and microscopic details of attack



Probe tratate
Probe 8.27, 3041

TV_Fag_C-EO/LO 10%

Control LO
Probe 8.22*. 5321

Fig. 7.5 Exemplification of fungal development variability for two tests under similar conditions and attack severity details on control beech samples and those treated with C-EO/LO after miniblock test with TV (2019 and 2020): macro images of Petri dishes and microscopic attack details, white rot appearance, fibrous texture

EOs/LO Conclusions

In the mini-block tests with **C-EO** and **T-EO** in an LO medium conducted in the two stages, there were some differences in product retention, test duration, fungal virulence and actual ML values determined. However, a series of conclusions emerged:

- The results of the miniblock test are, at least partially, at odds with those of the screening test because they actually tested different aspects and states of LO: compounds with antifungal potential that could migrate into the culture medium, and solidified film inside the wood structure protecting through isolation and hydrophobic character, respectively.

- The concrete effect of reducing mass loss depends on the fungus and its virulence, which can vary and generate high data variability and interpretation difficulties: in the 2019 test, LO protected better against **PP** than **TV**, whilst in the 2020 test the situation was reversed.;
- Modifying LO with **C-EO** has a negative effect in terms of increasing ML through the attack of both **PP** and **TV** fungi, but more pronounced for **PP**;
- Modifying LO with **T-EO** has a positive effect on the resistance of treated wood against **PP**, but reduces resistance to **TV** attack;
- Using LO as a dilution medium for the tested essential oils is not recommended, as their protective effect is diminished by their interference in the oxidative curing process.
- Delaying or preventing the formation of a film reduces or nullifies the protective effect of LO. This hypothesis was developed in a previous publication in which the thesis author is a co-author. (Timar et al., 2021)

Wood treated with essential oils diluted in ethanol

From the analysis of the miniblock test results from stage 2 (2020), conducted with **C-EO** and **T-EO** solutions in ethanol exclusively on samples not subjected to the washing test, the following can be concluded:

For the **C-EO**/Ethanol test:

The existence of value differences between control and independent control samples for both types of fungi, with the observation that for **PP** the ML value for the independent control (10.89±23.35%) is higher than the control (37.89±20.15%), whilst for **TV** the situation is reversed: 54.21±15.06% compared to 24.91±23.24%. The ML differences associated with **PP** and **TV** tests, superior for the latter, are explained by white rot fungi's (**TV**) preference for hardwood species, contrary to brown rot fungi (**PP**) which prefer softwoods. As the tests were conducted on beech, **TV**'s more pronounced attack compared to **PP** is understandable. Very large standard deviations, especially for control samples (Fig. 7.3, Fig. 7.4) and control of various categories, but also for the samples treated with 5% **C-EO** in the **TV** test, highlights an extreme variability in fungal colonisation and attack.

For PP, the ML values for samples treated with **C-EO**/Ethanol compared to the total control value show an antifungal effect (ML decrease of about 90%), although the small calculated ML values (about 2%) are slightly higher than the values for the control in the sample boxes. This confirms the fungal inhibition effect due to the volatility of certain compounds toxic to fungi..

For TV, the ML values for samples treated with **C-EO**/Ethanol compared to the total control value do NOT show an antifungal effect (contrary to a ML increase of up to 20%), although the

calculated ML values (approximately 32-39%) are lower than the values for the untreated control (54%).

For the **T-EO**/Ethanol test:

The existence of value differences between control and independent control samples for both types of fungi, and large standard deviations:

For PP, the ML values for samples treated with **T-EO**/Ethanol compared to the total control value show an antifungal effect (ML decrease of approximately 10-45%)..

For TV, the ML values for samples treated with **T-EO**/Ethanol compared to the total control value show an antifungal effect (ML decrease of approximately 81-94%), demonstrating greater efficacy against white rot fungi (**TV**) compared to brown rot fungi (**PP**).

The variations in average mass loss values due to biodegradation, depending on the sample type or applied treatment, are graphically represented in Fig. 7.6, for both types of fungi (**PP**, **TV**), comparing samples treated with **C-EO** and samples treated with **T-EO**, exclusively for unwashed samples. These reflect the aspects presented previously.

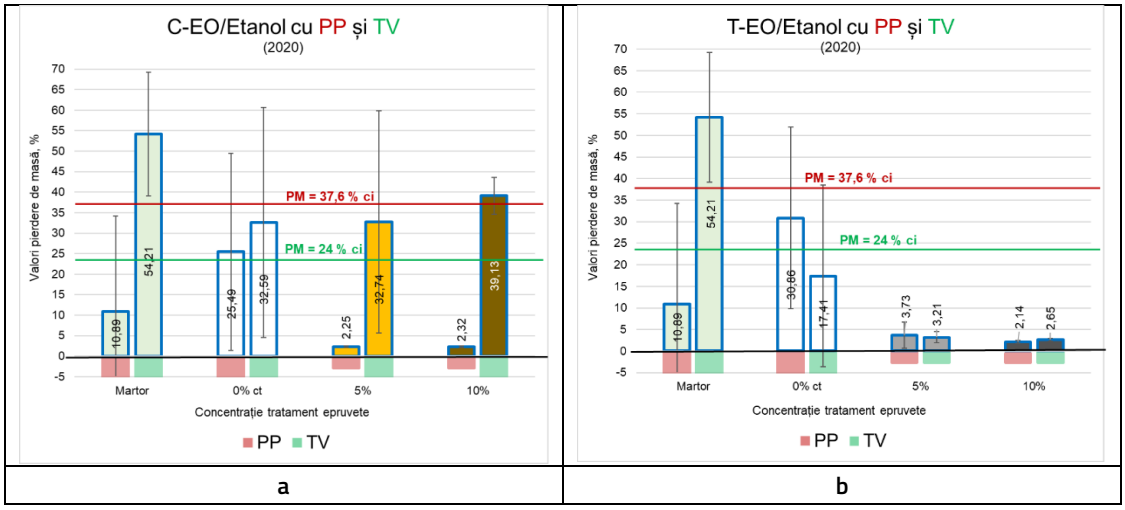


Fig. 7.6 The influence of treating beech wood with C-EO/Ethanol (a) and T-EO/Ethanol (b) on mass loss in mini-block tests with *Postia placenta* (PP) and *Trametes versicolor* (TV)

Conclusions C-EO and T-EO/Ethanol

In the miniblock tests with **C-EO** and **T-EO** in ethanol conducted in two stages, there were some differences in product retention, testing duration, fungal virulence and actual ML values determined. However, a series of common conclusions emerged:

- The miniblock tests with **C-EO** and **T-EO** in alcohol confirm the screening test results, demonstrating antifungal effects on treated wood from concentrations of 5%;

- **C-EO** and **T-EO** solutions with 5-10% concentrations in ethanol show potential as alternative products for antifungal wood protection, but exhibit differentiated specific efficiency correlated with their chemical composition;
- **C-EO** is rich in eugenol and has antifungal activity against the brown rot fungus **PP** (which degrades cellulose and hemicelluloses in wood but cannot cleave lignin), reducing mass losses by 90% compared to the control;
- **C-EO** does not have antifungal efficacy against the white rot fungus **TV**, which can degrade complex polyphenolic compounds such as wood lignin; eugenol is a phenol with a phenyl-propane structure similar to the basic units in lignin structure.
- **T-EO** is rich in carvacrol and terpinenes and shows antifungal activity against both **PP** (45% mass reduction) and **TV** (80-90% mass reduction).

These conclusions are in agreement with similar research in the specialist literature. (Cheng et al., 2008; Medeiros et al., 2016; Pánek et al., 2014c; Voda et al., 2003; Xie et al., 2017; Yingprasert et al., 2015; Zhang et al., 2016; Zyani et al., 2011).

The phytotoxicity test

The potential eco-impact of new alternative wood bioprotection antifungal products was assessed using a phytotoxicity test with lettuce seeds (*Lactuca sativa*), following the procedure outlined in the analytical protocol (chapter 4). The germination index (GI, %) is recommended in specialist literature as a complex indicator that combines two possible toxicity effects of leachable toxic compounds on seed germination and seedling development, namely the number of germinated seeds and seedling length. Moreover, this index is a relative percentage index compared to germination parameters obtained using (distilled) water as a control sample.

The analysed samples were the aqueous extracts (E1, E5, E10) obtained through the water leaching test according to EN 84/2000. Reporting against a common control sample, specifically distilled water from the same batch used in the leaching tests, allows for unambiguous comparison of all analysed samples. Biotin and Diffusit are two classic wood bioprotection products, included in the research as comparison products.

Experimental Results

Fig. 7.7 illustrates through images the end of the test and the differentiation of various extracts (E5) from treated wood (5%) compared to the control sample with distilled water.

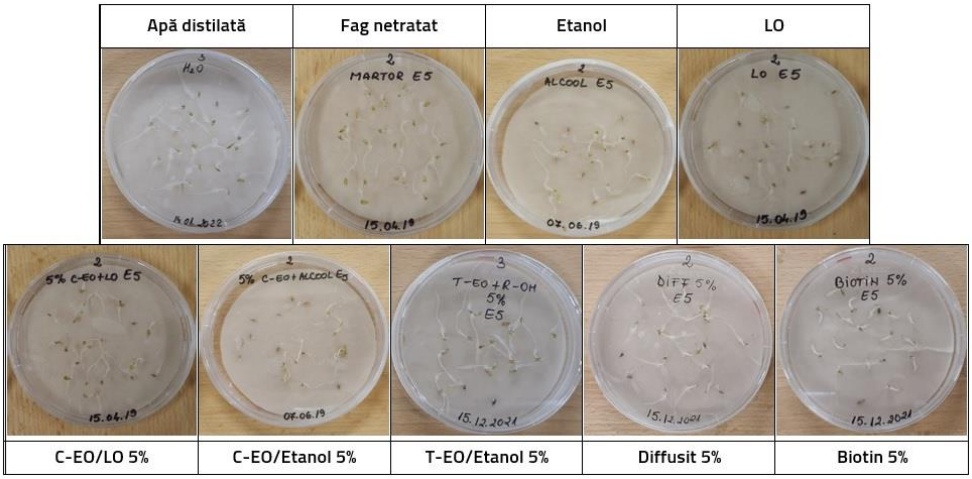


Fig. 7.7 End of test for aqueous extracts (E5) from untreated beech wood and beech wood treated with various 5% concentration bioprotection solutions: C-EO and T-EO in ethanol (ET) and linseed oil (LO), two classic wood bioprotection products Biotin and Diffusit, and distilled water

The following can be observed from the analysis of the experimental data:

- The germination index varied decreasingly from E1 to E10, with an average value of 105.75%, compared to the distilled water control at 100%. These data indicate that for untreated beech wood, no potential negative eco-impact is observed in terms of phytotoxicity of water-soluble compounds.

- For beech wood treated with ethanol (ethanol control), the germination index GI was 88.28% as an average of 3 extracts, indicating a low potential eco-impact.
- For beech wood treated with linseed oil (LO control), the germination index GI = 75.59% as an average of 3 extracts, indicating a higher potential negative eco-impact compared to the control wood and ethanol control.
- For beech wood treated with **C-EO** solutions in ethanol (concentrations 1%, 5%, 10%), the overall average GI for the three extracts E1, E5, E10 were 90.41%, 70.56% and 50.64%. Thus, the phytotoxicity effect manifests at concentrations starting from 5%, which must be considered in practical application, requiring additional water isolation treatments to preserve treatment effectiveness and avoid negative eco-impact.
- For beech wood treated with **T-EO** solutions in ethanol (concentrations 1%, 5%, 10%), the overall GI values (average for E1, E5, E10) decreased from 108.6% to 101.5% and 78.5% as the **T-EO** concentration increased. The phytotoxicity effect is lower than for **C-EO**.
- For beech wood treated with **C-EO** solutions in LO, the variation of phytotoxicity indices based on extract and **C-EO** concentration does not show a clear trend, but the overall GI values (average of 3 extracts) are in the range of 88.6-92%, higher than the LO control (GI = 75.6). This means that linseed oil, through its film-forming qualities, better fixes the toxic compounds

from **C-EO** on wood, even if its hardening is partially disturbed by the strong antioxidant effect of **C-EO**..

- For beech wood treated with 5% and 10% Biotin solutions, GI values of 44.2% and 41.4% were obtained for the 5% and 10% concentrations, respectively. These were the lowest values in the entire series of experiments and indicate a potentially significant negative ecological impact through phytotoxicity if measures are not taken to reduce leachability by fixing the solution to the wood, for example through film-forming or water-repellent treatment. This is also supported by the experimental observation of maximum phytotoxicity for the first extract E1.

- For beech wood treated with Diffusit S solutions (5%, 10%), the overall GI values were 91.4% and 83.6%. Therefore, this product also presents a risk of impact through phytotoxicity in the absence of wood fixation measures.

- An overview of the results, considering the global germination index GI values (%) as an average of the independent values for the three extracts (E1, E5, E10), shows that only untreated beech wood and that treated with **T-EO**/ethanol solutions at 1% and 5% concentrations would not present a negative eco-impact through phytotoxicity. A reduced eco-impact, associated with GI values ≥90% (value proposed by the author), would be possible for beech wood treated with **C-EO**/ethanol 1%, **C-EO**/LO 5% and Diffusit 5%. For all other variants, GI values below 90% were obtained, with minimum values of 44-41% for 5-10% Biotin solutions..

All these aspects are clearly highlighted by the graphs in the figures Fig. 7.8, Fig. 7.9, which also reflect the differences in phytotoxicity between successive extracts.

The effect of the treatment solution concentration is evidently important and covered in the previous discussion, but a comparison of different products/treatments at a constant concentration (e.g. 5%, 10%) is better illustrated by the graphs in Fig. 7.10, Fig. 7.11.

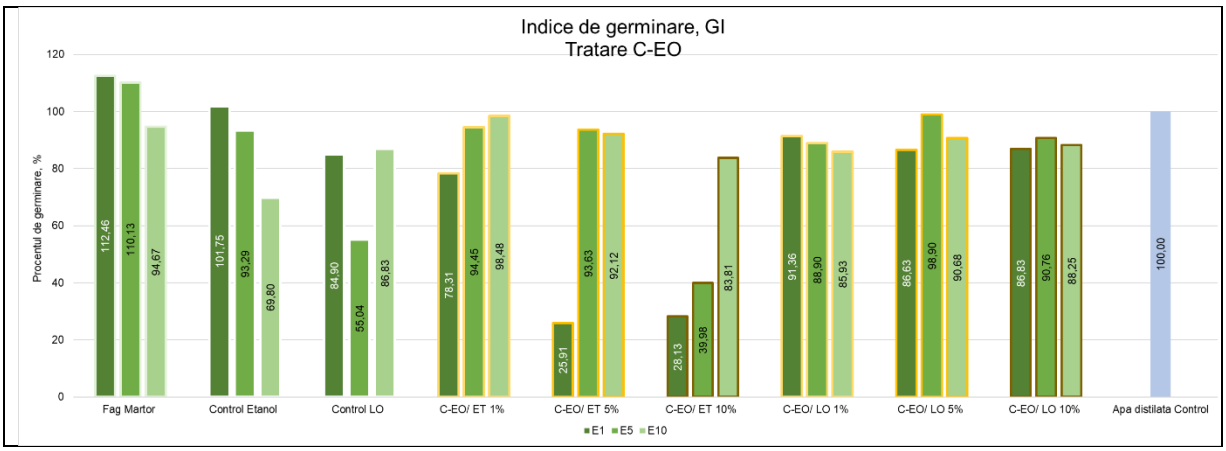


Fig. 7.8 Germination index for aqueous extracts from untreated beech wood and beech wood treated with C-EO solutions in ethanol (ET) and linseed oil (LO), compared to distilled water control.

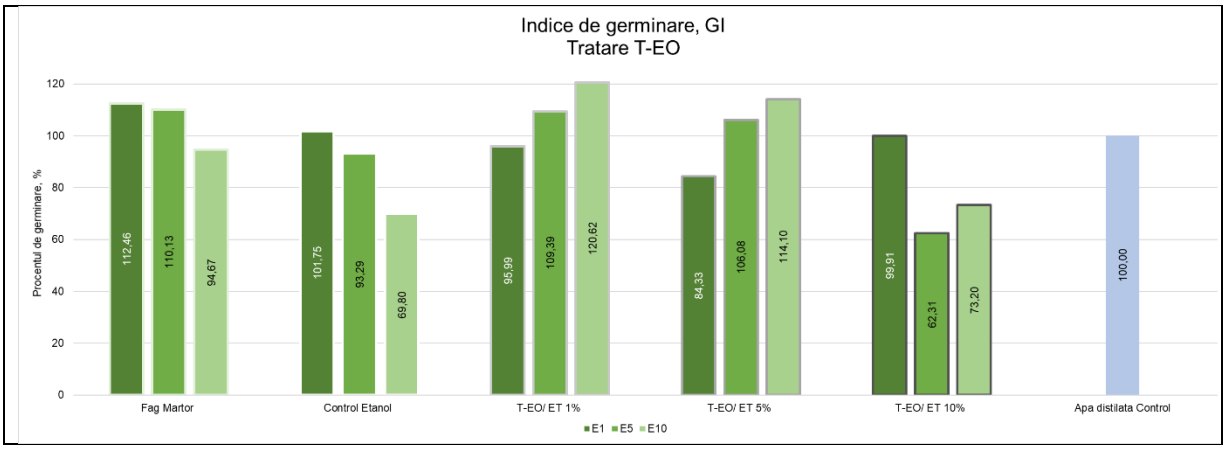


Fig. 7.9 . The germination index for aqueous extracts from control and T-EO ethanol solution-treated beech wood, compared to distilled water control

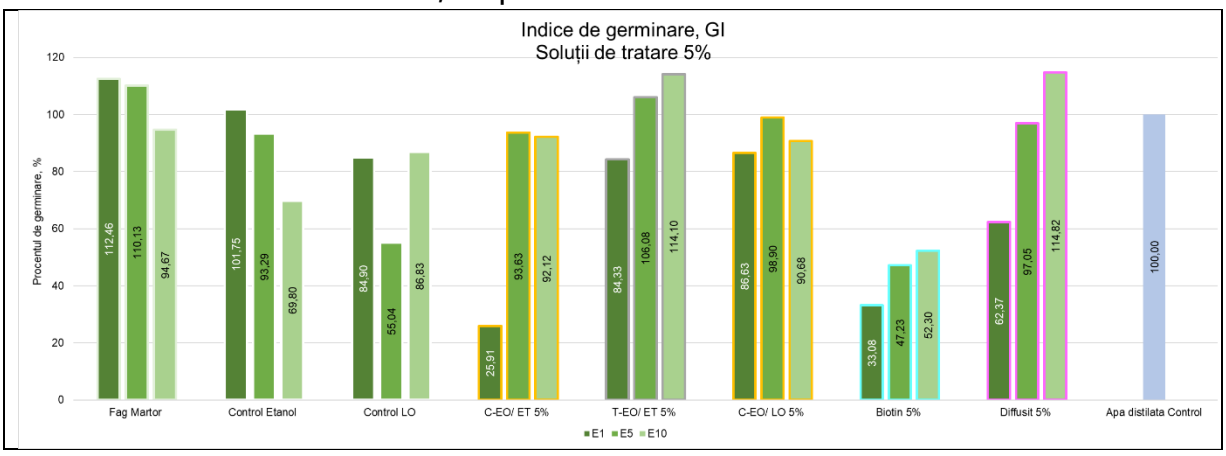


Fig. 7.10 Germination index for aqueous extracts from untreated beech wood and beech wood treated with various 5% concentration bioprotection solutions: C-EO and T-EO in ethanol (ET) and linseed oil (LO), compared to two classic wood bioprotection products, Biotin and Diffusit, and distilled water

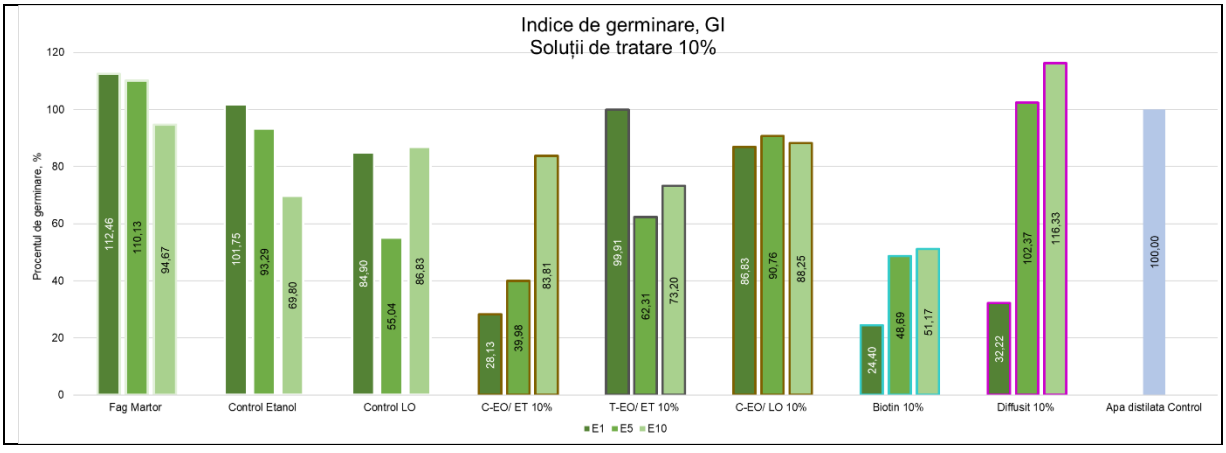


Fig. 7.11 Germination index for aqueous extracts from untreated beech wood and beech wood treated with various 10% concentration bioprotection solutions: C-EO and T-EO in ethanol (ET) and linseed oil (LO), compared to two classic wood bioprotection products, Biotin and Diffusit, and distilled water

Phytotoxicity test conclusions

- Performing this test in correlation with the mini-block test to verify the antifungal protection activity of the two essential oils on treated wood samples represents the implementation of the fundamental idea of the doctoral thesis in the research approach: efficiency versus eco-impact.
- The use of washing water obtained in accordance with EN 84 in testing anchors the assessment of potential eco-impact through phytotoxicity within the testing methodology for biocidal products for implementation, and constitutes a novel and original element of the thesis.
- Experimental data highlight a greater negative eco-impact potential for **C-EO** compared to **T-EO**. They also provide concrete ecological information for the classic bioprotection products used for comparison (Diffusit, Biotin). Considering the widespread use of these classic products, the information in the thesis can contribute to more environmentally friendly usage, in terms of the need for measures/treatments to reduce washability.
- The thesis data reveal important information regarding the potential eco-impact of treatments with linseed oil/drying oils, which have a long history of use and wide applicability, but have been less studied from this perspective..
- Among all the studied variants of beech wood treatment, the minimum negative eco-impact through phytotoxicity was obtained for treatments with **T-EO** solutions in ethanol at concentrations of 1% and 5%, whilst the maximum potential negative eco-impact was observed for Biotin solutions (at the tested concentrations of 5% and 10%).

Chapter 8 - O5: Preliminary tests for implementation in the field of conservation of wooden heritage assets

Arguments and objectives

Cultural heritage is "*an expression of cultural identity, a collection of material and spiritual values inherited to be preserved, valued and passed on to future generations*", and is therefore protected by law.

The research objectives were directly correlated with the practice of conserving and restoring wooden artefacts (necessary operations, materials) and a series of specific requirements imposed on conservation-restoration (C-R) materials, such as compatibility with original natural materials and other materials used in the C-R process, and limiting colour changes to surfaces.

Thus, the research focused on the following concrete objectives:

- O5.1: Evaluating the effectiveness of EOs (C-EO, T-EO) in preventive treatments against fungal attack activated in humid conditions for new, apparently healthy wood that has been naturally infested under normal storage conditions;
- O5.2: Assessing the efficacy of EOs in treatments to combat active fungal attack (xylophagous fungi isolated from heritage objects);
- O5.3: Implementation tests in specific cases of wood/museum artefact conservation, with evaluation of treatment effectiveness through an original laboratory mycological test developed for this purpose;
- O5.4: Assessing the influence of EO treatments on the colour of wood surfaces and their behaviour when exposed to UV-VIS light (accelerated test);
- O5.5: Evaluating the compatibility of bioprotection treatments using EOs with subsequent finishing using beeswax and shellac, and their influence on the colour and light behaviour of finished surfaces.

Achieving objectives O5.1-O5.3 involved developing original mycological laboratory tests specific to each objective. Achieving objectives O5.4-O5.5 involved conducting an accelerated UV-VIS light exposure test, simulating the effect of natural indoor light filtered through glass, as well as colour measurements and FTIR investigations. All tests involved in carrying out the experiments to achieve the stated objectives were performed comparatively for the considered EOs and two classic biocidal products, used as comparison products: Diffusit S and Biotin T (similar with phytotoxicity test).

O5.1: Evaluation of EO efficacy in treatments to prevent fungal attack activated under humid conditions (Test Pr)

This study aimed to demonstrate the importance of applying preventive treatment to new, apparently sound wood used in conservation-restoration interventions to replace missing elements. The experiments were carried out on beech (***Fagus sylvatica***) wood and pine sapwood (***Pinus sylvestris***). is. New, apparently healthy wood, stored under normal conditions is ultimately infected with fungal spores and other microorganisms ubiquitous in the environment and transported by air currents. The risk is increased when new wood is placed in contact with old, already infected/biodegraded wood, even if active conservation measures are taken through curative bioprotection treatments. Under favourable, namely a biodegradable organic substrate, such as wood, and atmospheric humidity above 70%, or wood moisture content above 20%, the conditions for fungal incubation and growth are created, with obvious negative consequences for wooden cultural artefacts or other

biodegradable organic materials. Besides demonstrating this real risk, the main objective was to evaluate the effectiveness of EO solutions compared to classic biocides in preventing wood degradation due to this inevitable natural infestation. Clove essential oil **C-EO** diluted in ethanol (at 5% and 10% concentrations) and Diffusit S (Diff) diluted in distilled water (also at 5% and 10%) were used for comparison. The evolution of the colonisation process was documented through observations and high-resolution photographs. A (semi)quantitative evaluation was conducted using an ImageJ software-based method. The calculated FCA size, %, represents the percentage of the Petri dish surface covered by mycelium.

Results and Discussion

The test results confirmed the initial hypothesis, demonstrating that seemingly healthy wood stored under normal conditions can be infested, and that this infestation may manifest under favourable temperature and humidity conditions.

In Fig. 8.1 there is an exemplification of the qualitative and quantitative results of this test (at its conclusion) for beech wood and all treatments. Observations showed that both analysed wood species exhibited signs of fungal infestation, predominantly including mould fungi but also decay fungi, after just one week of incubation. The fungal attack evolved and diversified over time during the testing period. Regarding the efficacy of the applied treatments, it was found that **C-EO** solutions, both at 5% and 10% concentrations, proved effective in combating infestation. Moreover, compared to the recognised biocidal product Diffusit S, the **C-EO** treatments demonstrated markedly superior efficiency.

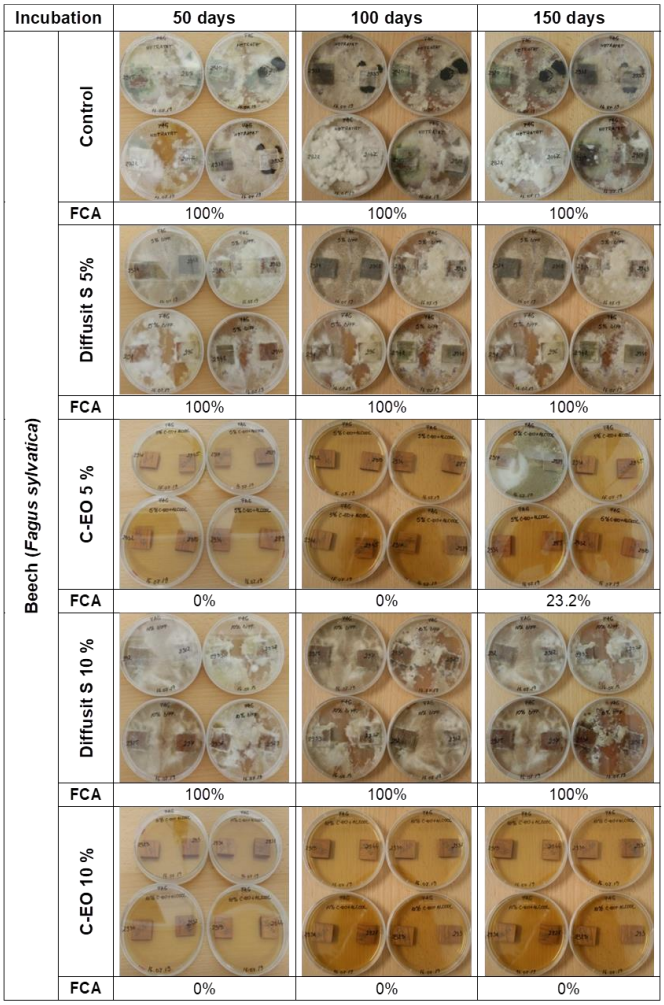


Fig. 8.1 Beech wood: influence of Diffusit S and C-EO solutions (5, 10%) on fungal growth after 50, 100 and 150 days of incubation, as a result of natural infestation of new wood under laboratory conditions[8]

O5.2: Evaluation of EO efficacy in treatments to combat an active fungal attack (wood-decay fungi isolated from heritage objects)

This part of the research aimed to evaluate the effectiveness of two essential oils - Clove (**C-EO**) and Savory (**T-EO**) - diluted in ethanol, compared to two recognised biocides used in wood preservation: Diffusit S and Biotin T, as part of a curative treatment. This test used beech (***Fagus sylvatica***) and Scots pine sapwood (***Pinus sylvestris***) samples, which were infected in a controlled manner in the laboratory with decay fungi isolated from wooden elements of heritage objects. The infected samples were incubated to allow an active fungal attack to develop and establish.

[8] (D.-M. Pop et al., 2021) https://www.scielo.cl/scielo.php?script=sci_arttext&pid=S0718-221X2022000100411

The study focused on three fungal strains isolated from wood sourced from a historic building in Brașov and a piece of furniture with wood weakened by fungal attack. These fungi were isolated through successive transfers onto sterile medium and were coded in correlation with their origin (FC1, S) and macroscopic appearance, specifically the mycelium colour of the mature fungus: brown (B) or white (W). The three isolated fungi had the following codes: FC1-B, FC1-W and SW.

To this end, a laboratory mycological test was conducted to evaluate the effectiveness of a curative treatment for wood with active rot fungus attack, coded as the TCu test (chapter 3).

Results and Discussion

This research was conducted in two stages:

- The first stage aimed to test both the effectiveness of Clove essential oil in ethanol with Diffusit S, and more importantly, to develop this TCu test and establish the methodology.
- The second stage comprised comparative testing of the effectiveness of the two essential oils (**C-EO** and **T-EO**) (10%) diluted in ethanol compared to the two recognised biocidal products (Diffusit S and Biotin T) at the same concentration.

Thus, following stage 1, it was found that overall the treatment with 10% **C-EO** in ethanol is better than the Diffusit treatment, but the effectiveness varied depending on the type of fungus: from absolute inhibition of fungal growth in the case of FC1-B through treatment with 10% **C-EO**, to (reduced) growth in both parallel samples for the SW attack. (D.-M. Pop et al., 2021)

In the second stage, both essential oils (**C-EO** and **T-EO**) diluted in ethanol were tested in parallel on beech and pine wood at a concentration of 10%, compared to Diffusit and Biotin at the same concentration.

Fig. 8.2 shows an example of the evolution of fungal growth (FC1-B) for beech wood samples, including control samples and samples treated with EOs: **C-EO** and **T-EO** solutions in ethanol (c=10%), as well as classic biocidal products: Diffusit S and Biotin in aqueous solutions of the same concentration.

Tab. 8.1 presents a summary of the FCA values (%) determined by processing with ImageJ software. The values represent the percentage area covered by mycelium in Petri dishes, 90 days after curative treatment. For **C-EO**, FCA values ranged from 0% (FC1-B), 11.57% (SW) to 37.57% (FC1-W) for beech wood, and from 0% (FC1-B), 0% (SW) to 90.19% (FC1-W) for pine sapwood. For **T-EO**, FCA values ranged from 0% (FC1-B), 0% (FC1-W) to 5.06% (SW) for beech wood, and from 0% (FC1-B), 0% (SW) to 34.23% (FC1-W) for pine sapwood. The comparison product Diffusit was markedly inferior in terms of curative treatment efficiency, with FCA

values of 100% except for beech wood attacked by SW (FCA=88.78%). The comparison product Biotin completely counteracted the active attack (FCA=0%) for all tested fungi, both for beech and pine, demonstrating maximum efficiency under the test conditions. We specify that this product is recommended for use at concentrations of up to 3%. In practice, there are situations where it is used at a 5% concentration. The 10% concentration used in experiments was motivated by a comparison of products at the same concentration. It is likely that even at a 5% concentration, the efficiency would be satisfactory.

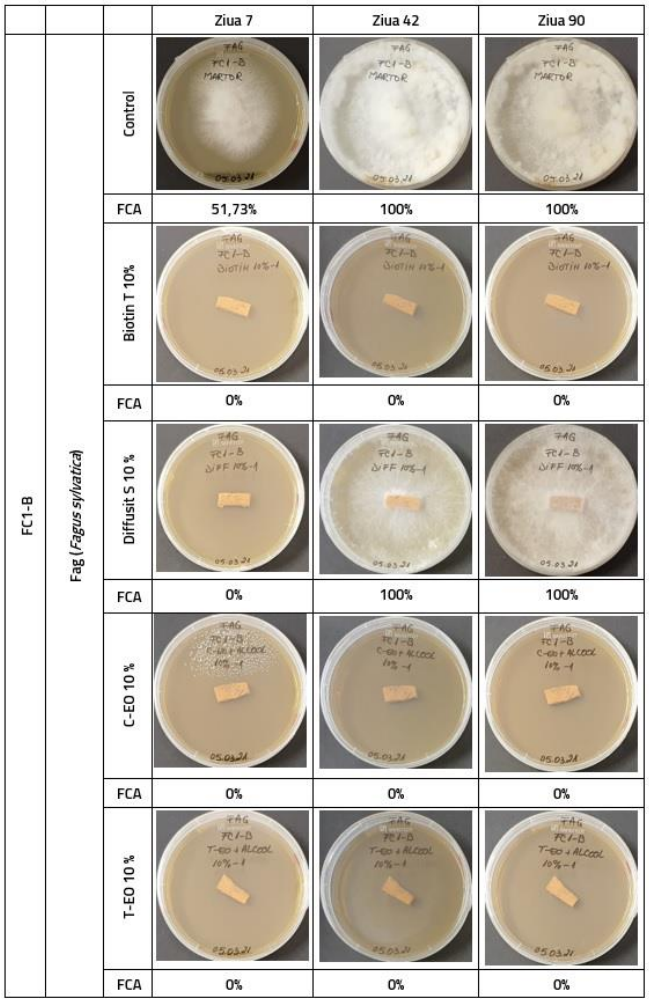


Fig. 8.2 Comparative efficacy of Biotin, Diffusit, C-EO and T-EO (10%) solutions in the curative antifungal treatment of beech wood, degraded following controlled infestation with fungus 1 FC1-B: development progression after 7, 42 and 91 days of incubation

Tab. 8.1 Centralisation of results regarding the effectiveness of curative treatments with classic biocidal products Diffusit S, Biotin T and alcoholic solutions of C-EO and T-EO, at a constant concentration of 10%, depending on the type of wood-decaying fungus and wood species: FCA (Fungal Coverage Area) values, %, after 90 days of incubation following treatment

Treatment product (c=10%)	FC1-B		FC1-W		S-W	
	Beech	Pine	Beech	Pine	Beech	Pine
Untreated control	100%	100%	86,79%	86,67%	97,48%	73,34%
Biotin 10%	0%	0%	0%	0%	0%	0%
Diffusit 10%	100%	100%	100%	100%	88,75%	100%
C-EO/ET	0%	0%	37,57%	90,19%	11,57%	0%
T-EO/ET	0%	0%	0%	34,23%	5,06%	0%

O5.3: Implementation test in concrete cases of wood/museum artefact conservation

This subchapter presents a detailed and comparative analysis of the antifungal biocidal efficacy of Clove essential oil (**C-EO**) (5,10%) in ethanol in relation to two recognised biocidal products (Biotin T (5%) and Diffusit S (10%)), commonly used in the field of wood preservation.

The TcuP test developed and encoded encompassed both in situ and laboratory actions. Two old wooden objects with severe degradation, belonging to the Astra Museum in Sibiu, were chosen as case studies for this research, as part of a restoration camp organised in 2019. The first object subjected to analysis was a sawhorse (Fig. 8.3), made of beech wood, a traditional element of rural households, representative of the country's ethnographic heritage. The second object was a fruit crusher (Fig. 8.4), made from poplar wood, a woody species frequently found in traditional artefacts due to its availability and processing properties. During the restoration camp, the chosen objects underwent restoration and conservation processes, which included treatment with various antifungal products. Both initially and after treatment and conditioning in natural conditions, wood samples were taken from each treated element/part (for each object). At the L5 ICDT Brasov Biological Testing Laboratory, all these wood samples collected during the restoration camp were placed on sterile culture medium (MEA).

The documentation of the colonisation process evolution was carried out through high-resolution observations and photographs, and a quantitative assessment was performed using ImageJ software, which enabled advanced processing and analysis of the obtained images.

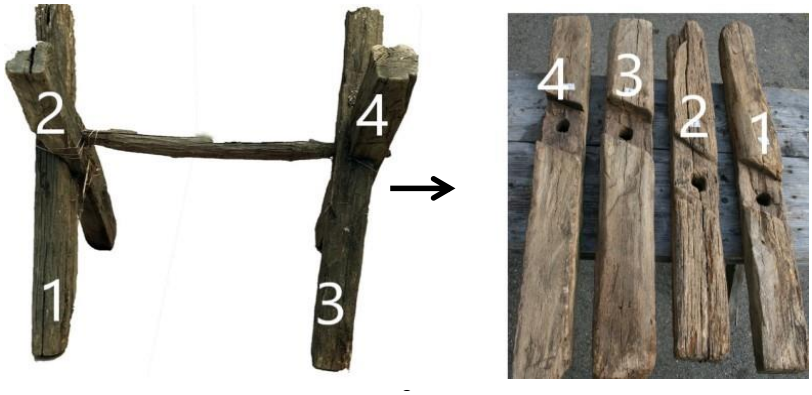


Fig. 8.3 Case study: Beech wood goat belonging to the heritage of the Astra Museum in Sibiu: image of the assembled object - left and component elements - right [9]

Fig. 8.4 Case study: Wooden fruit crusher from poplar wood belonging to the heritage of the Astra Museum in Sibiu: image of the assembled object on the left and parts on the right

Results and Discussion

The test results obtained in the first case study, Capra, have been published and can be accessed. The conclusion was that treatments with **C-EO**/Ethanol at both 5% and 10% concentrations showed higher efficacy compared to treatments with Biotin 5% and Diffusit 10%, even though they did not completely halt fungal growth.

For the second case study, the Fruit Crusher, after 7 days it can be observed (Fig. 8.5) that already shows a complex fungal attack, both in the case of the control sample and in the case of treatment with Paraloid B72, indicating severe wood contamination. On day 7, no signs of fungal growth appeared with the 10% Diff treatment, but for 10% **C-EO**, incipient development is observed, with an FCA value of 1.4%, compared to 0% FCA for Diff. (Fig. 8.5)

[9] **Extras din (D. M. Pop et al., 2020)** https://bioresources.cnr.ncsu.edu/resources/combined-testing-approach-to-evaluate-the-antifungal-efficiency-of-clove-eugenia-caryophyllata-essential-oil-for-potential-application-in-wood-conservation/

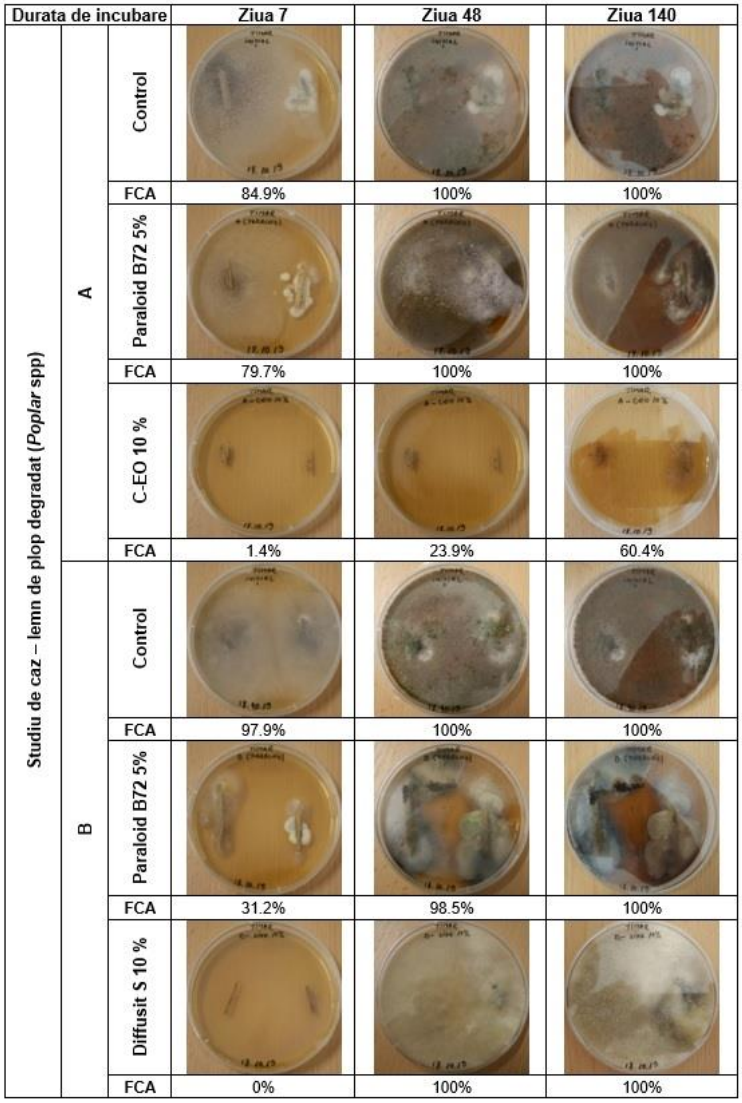


Fig. 8.5 The comparative efficiency of C-EO and Diffusit (10%) solutions in the curative antifungal treatment of poplar wood, compared to untreated wood (control) and wood consolidated with Paraloid B72 (5%), sourced from a heritage object: Fruit crusher. Monitoring conducted at 7, 48 and 140 days.

On day 48, it was observed that in the case of untreated wood samples (control), wood from part A consolidated with Paraloid B72, and also in the case of samples treated with Diffusit, the fungal mix completely covered the surface of the Petri dish (FCA=100%). The wood sample with Paraloid B72 from part B of the object shows a slight delay in fungal development with an FCA value of 98.5%. The treatment with 10% **C-EO** proves to have a good inhibitory effect on the development of the fungal mix, with an FCA value of 23.9%. At the end of the test, day 140, in all Petri dishes with control wood samples, consolidated with Paraloid B72 and treated with 10% Diffusit, FCA=100%, meaning the fungi completely covered the surface of the Petri dish. For the sample treated with 10% **C-EO**, at the end of the test, the FCA value was 60.4%, indicating a pronounced inhibitory effect.

In conclusion, following the TcuP test performed on object 2: Fruit crusher, the Clove essential oil (**C-EO**) showed significantly superior effectiveness compared to the Diffusit S treatment. Although it did not kill the existing fungal mix in the wood, a clear effect of strong delay in fungal development can be observed. Analysing the macroscopic appearance of the fungi developed in the Petri dishes, a stark difference can be seen between the fungi in the dish with 10% **C-EO** treatment and all other dishes containing treated or untreated wood. Thus, it can be understood that from the entire mix of fungi present on the control wood, **C-EO** had a biocidal effect on some of them, but there were also some resistant fungal species.

O5.4: Evaluation of the influence of EO treatments on the colour of wooden surfaces and their behaviour when exposed to UV-VIS light

The materials and treatments used in conservation-restoration (C-R) must meet several conditions specific to the field, such as compatibility with original traditional materials, reversibility, resistance to ageing, and preservation of properties over time. Additionally, C-R materials should not (significantly) alter the colour of the treated substrate. In the case of wood, we are dealing with very different species that naturally vary in colour and behave differently over time in terms of colour stability. (Liu, 2017)(Timar et al 2016). The colour of wooden surfaces can be altered through treatments with coloured materials, as well as by finishing with various film-forming materials, depending on their nature and colour. Additionally, the ageing process will affect finished wooden surfaces, with the effect depending on the wood species and finishing material, as well as ageing factors (UV-Vis light, temperature), ageing time (natural or accelerated artificial), environmental conditions and their variations.

The research in this thesis highlighted the antifungal potential of **C-EO** and **T-EO** as ethanol solutions (chapters 6, 7), but also a certain phytotoxic effect (chapter 7) if there were a risk of toxic compounds being washed off the wood. Moreover, the tests presented earlier in this chapter revealed the effectiveness of preventive and curative treatments with these EOs and their potential application in C-R. In this context, it was deemed necessary to evaluate the colour changes associated with **C-EO** and **T-EO** treatments and their influence on colour changes due to light exposure in indoor conditions. These investigations have been previously published.[10] (Beldean et al., 2024) and is presented only in summary.

For testing, sycamore wood (*Acer pseudoplatanus*) was chosen, as it is a light-coloured wood that is also highly sensitive to light. (Timar & Beldean, 2022). These characteristics were considered important for achieving the proposed objectives. To evaluate colour changes,

[10] https://doi.org/10.35511/978-963-334-518-4

colour measurements were taken using the CIELab system before and after pre-treatment, and colour differences were calculated. The procedure is described in the published paper. (Beldean et al., 2024).

whilst pre-treatment with **C-EO** slightly modifies the colour, resulting in a barely perceptible darkening and shift towards red. From this perspective, the treatments may be acceptable in C-R.

O5.5: Evaluation of the compatibility of bioprotection treatments with EOs with subsequent finishing and their influence on the colour and light behaviour of finished surfaces

In C-R practice, preventive bioprotection treatments are followed by finishing with traditional film-forming materials identified on the artefact (e.g. wax, linseed oil, shellac). For surfaces that were not originally finished, a layer of beeswax is applied to insulate the wood against moisture variations. Consequently, objective O5.5 was established, focusing on the compatibility of surfaces pre-treated with EOs with shellac and beeswax, traditional finishing materials. Additionally, colour changes of the finished surfaces were evaluated following accelerated light exposure tests to highlight the potential influence of pre-treatments with **C-EO** and **T-EO**.

The light exposure was conducted in a climate-controlled chamber equipped with a UV-VIS lamp and H2 filter to simulate natural light passing through window glass, as described in (Timar & Beldean, 2022).

Experimental Results and Conclusions

Some of the experimental results are concentrated in Fig. 8.6 cumulative for objectives O5.4 and O5.5, with a detailed presentation included in the previously published work (Beldean et al., 2024).

From the images of unfinished test samples, not exposed to light (the left column in Fig. 8.6 a) It can be observed that pre-treatment with **T-EO** does not noticeably change the colour of maple wood,

Pre-treatments with **C-EO** and **T-EO** were compatible with subsequent finishing using shellac and beeswax, but extended drying periods were required between coats.

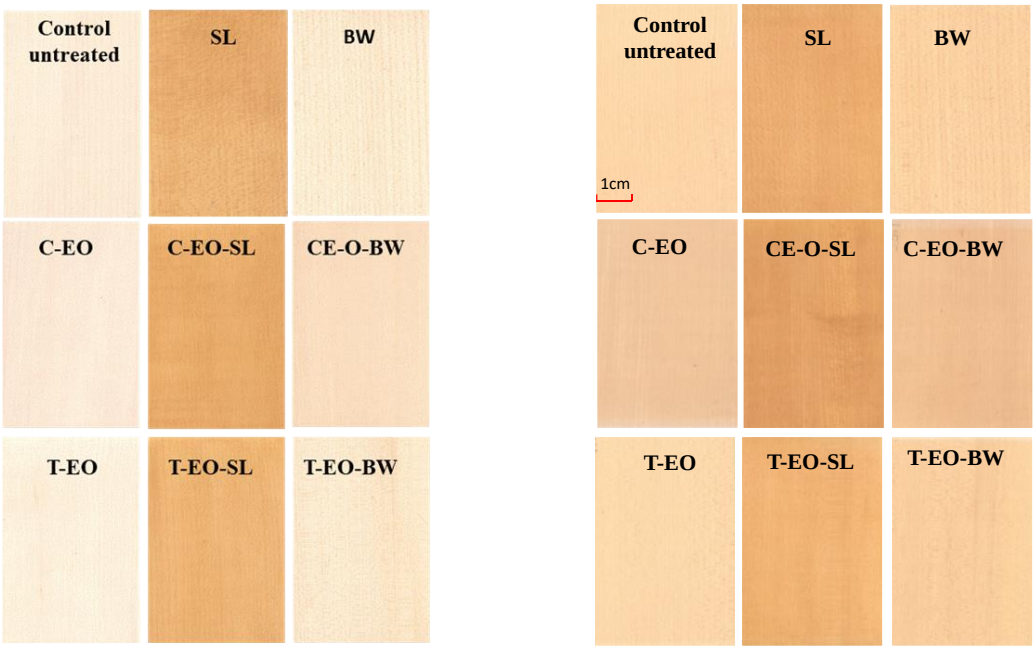


Fig. 8.6 General appearance of sycamore maple (*Acer pseudoplatanus*) samples, control and pre-treated with C-EO and T-EO, unfinished and finished with shellac (SL) and beeswax (BW): a. before light exposure; b. after 96-hour accelerated UV-VIS light exposure test[11]

Conclusions:

- Pre-treatment of maple wood with **C-EO** and **T-EO** barely altered the colour of unfinished maple wood and did not significantly influence colour changes upon light exposure.
- The colour of finished surfaces is primarily determined by the type and colour of the film-forming material (**SL** or BW) and slightly influenced by pre-treatment with **C-EO** and **T-EO**; maximum influence is observed with **C-EO** pre-treatment followed by wax finishing.
- Pre-treatment of maple wood with **C-EO** followed by finishing with **SL** and BW resulted in greater colour changes after exposure to UV-VIS light.
- Pre-treatment of maple wood with **T-EO** followed by finishing with **SL** and BW did not negatively affect the UV-VIS light resistance of the finished surfaces; on the contrary, a slight improvement is observed in the case of **T-EO**.

Based on all these results, the use of Savory (***Satureja hortensis***) essential oil **T-EO** (5-10% solutions in ethanol) can be recommended as an alternative product for preventive and curative bioprotection in the conservation and restoration of wooden heritage items. The use of **C-EO** cannot be completely contraindicated, but further research is recommended.

[11] (preluat din Beldean et al 2024).

Chapter 9 - Final conclusions, original contributions, future research directions

Final conclusions

To achieve the proposed aim, five major research objectives (O1-O5) were formulated and pursued in the thesis, interlinked within an original and rational methodological concept. The formulation of research objectives, identification of possible implementation methods, and verification and validation of results were based on an in-depth study of the specialist literature.

Through the thesis design, the research conducted, and the laborious and rational centralisation and interpretation of results, the proposed objectives were fully resolved. In correlation with these, the following conclusions can be drawn:

- A **complex analytical laboratory protocol** was established and validated, initially comprising three categories of testing methods, to meet the research needs associated with the thesis objectives. The established analytical protocol served to fulfil all thesis objectives (O1).
- Based on an extensive and in-depth study of the specialist literature, synthesis of information and correlations between the composition and properties of essential oils, five essential oils with potential antifungal biocidal properties were identified and selected for testing within the doctoral thesis: Basil essential oil (***Ocimum basilicum***) **B-EO**, Clove essential oil (***Eugenia caryophyllata***) **C-EO**, Oregano essential oil (***Origanum vulgare***) **O-EO**, Cinnamon essential oil (***Cinnamomum verum***) **S-EO** and Savory essential oil (***Satureja hortensis***) **T-EO**.
- A **comprehensive characterisation of the 5 essential oils** was achieved by combining and corroborating information from the technical/safety data sheets of the products with the results of our own experimental investigations on their chemical composition, determined by gas chromatography coupled with mass spectrometry (GC-MS) and FTIR, as well as comparative data from other worldwide research. All this valuable information was compiled into original characterisation sheets for the essential oils, which represent results of the thesis and also serve as a useful scientific resource, in database form, for future research (**BD-O2**).
- The **antifungal biocidal potential of the 5 essential oils** against representative wood-decay fungi causing brown rot (***Serpula lacrymans***, ***Postia placenta***) and white rot (***Trametes versicolor***) was evaluated and compared through screening tests. The result sheets containing details on testing methods, image monitoring of fungal growth, quantitative evaluation using specific inhibition indices, graphical representations of their evolution, with interpretation and

conclusions for each of the 5 essential oils, represent valuable original results, forming a useful database for future research (**BD-O3**).

- Following the screening tests of the antifungal biocidal potential of the 5 essential oils, two essential oils were selected: Clove oil (**C-EO**) and Savory oil (**T-EO**) (O3). These were subsequently subjected to mini-block tests to evaluate their antifungal efficacy on treated wood and phytotoxicity tests, applying the established and validated methods included in the analytical protocol.
- The **miniblock tests confirmed** the antifungal potential of **C-EO** and **T-EO alcoholic solutions** at 5-10% concentrations and revealed different and specific efficacy against brown or white rot fungi, correlating with their chemical composition and the specific attack mode of these fungi; **C-EO** is more active against brown rot, while **T-EO** is more active against white rot (O4).
- The miniblock tests with **C-EO** and **T-EO in linseed oil** demonstrated that this **variant is not viable**, especially for **C-EO**, as it negatively interferes, due to its antioxidant properties, with the oxidative polymerisation hardening process of LO (O4).
- **Phytotoxicity tests** revealed a potential negative eco-impact through phytotoxicity for classical bioprotection products, linseed oil and the essential oils studied, more pronounced for **C-EO** compared to **T-EO** (O4).
- Among all the beech wood treatment variants studied, **minimal negative eco-impact** through phytotoxicity was obtained for treatments with **T-EO** solutions in ethanol at concentrations of 1 and 5%, while **maximum potential negative eco-impact** was observed for Biotin T solutions (at the tested concentrations of 5 and 10%) (O4).
- **Awareness** of these aspects is a necessary first step for coupling bioprotection treatments with water-repellent or film-forming treatments in applications where there is a risk of treatment products being washed off wood and consequently affecting the environment.
- Preliminary implementation tests of **C-EO** and **T-EO** (5-10% solutions in ethanol) as alternative products for **preventive and curative bioprotection in the field of conservation-restoration** of wooden cultural assets have revealed an antifungal efficacy at least equal to or superior to the classic bioprotection products considered for comparison (O5).
- Bioprotection treatments with **C-EO** and **T-EO** are **compatible with subsequent finishing with shellac and beeswax**. Considering aspects related to maintaining unchanged colour and

light resistance, combined with phytotoxicity data, we currently believe that **T-EO** solutions have **potential for application in the conservation of wooden heritage items** and recommend testing in case studies on real objects. For **C-EO**, a series of additional tests are required (O5).

- Implementation **tests in C-R** have generated two **new specific mycological laboratory methods** for preventive and curative protection and an **original combined test**, which enrich the initially developed analytical protocol (O5, O1).

Original contributions

The doctoral thesis brings a series of original contributions to the investigated field (wood bioprotection), starting from the topic itself, namely the potential of essential oils in antifungal bioprotection of wood, and continuing with the fundamental idea of developing the entire scientific approach, specifically the phrase efficiency versus eco-impact, as a sustainable and responsible approach.

Addressing this bold topic required in-depth literature study, creation of specific research tools, use of diverse research methods, recording, processing and analysis of an immense volume of experimental data, and synthesis capacity. All these considerable efforts led to a series of original elements, of which the following stand out:

1. The methodological concept of the doctoral thesis;

2. The complex analytical protocol developed, including original mycological tests for evaluating the applicability and efficiency of certain products in the field of wooden heritage conservation;

3. Synthesis of literature data on essential oils with antifungal biocidal potential, with useful correlations between chemical composition and biocidal efficiency; (DB1)

4. Database containing the results of screening tests performed (DB-O1/1-DB-O1/5)

5. Database of complex characterisation for 5 essential oils (DB-O2)

6. Databases on antifungal biocidal potential against wood-decay fungi representative of brown rot (***Serpula lacrymans***, ***Postia placenta***) and white rot (***Trametes versicolor***), determined through screening tests, for 5 essential oils (Basil essential oil (***Ocimum basilicum***) **B-EO**, Clove essential oil (***Eugenia caryophyllata***) **C-EO**, Oregano essential oil (***Origanum vulgare***) **O-EO**, Cinnamon essential oil (***Cinnamomum verum***) **S-EO** and Savory essential oil (***Satureja hortensis***) **T-EO**) (DB-O3/1), validation sheets for antifungal potential based on dilution medium (DB-O3/2) and type of wood-decay fungus (DB-O3/3)

7. Testing and experimental data on the antifungal biocidal effect against ***Postia placenta*** (brown rot) and ***Trametes versicolor*** (white rot) determined by mini-block test for **C-EO** and **T-EO** in two dilution media (ethanol, linseed oil)

8. Testing and experimental data on potential eco-impact through phytotoxicity for **C-EO** and **T-EO** based on dilution medium and concentration, compared to two classic biocidal products and distilled water.

9. Development of testing concept and specific mycological tests for evaluating the implementation potential of essential oils as alternative antifungal products for preventive and curative treatments in heritage conservation.

Future research directions

We believe that based on the results and conclusions of the thesis, which stem from considerable work and effort, further in-depth and diversified research in this field is justified. As future research directions, the following (non-exhaustively) are envisaged:

1. Testing mixtures of essential oils (from the 5 selected) to enhance the effectiveness of antifungal protection.

2. Mini-block and phytotoxicity tests for essential oil blends with increased antifungal efficacy, comparing standalone treatments and treatments followed by waterproofing with waxes.

3. Implementation tests of thyme **T-EO** essential oil in the conservation-restoration practice of historic furniture/old wooden artefacts (case studies combined with mycological tests).

4. Collaboration with microbiology/mycology specialists to identify fungi isolated from heritage items for a better understanding of the obtained results and extension of research with other individual and mixed essential oils.

5. Development of effective eco-products based on essential oils encapsulated in nanoparticles.

The analytical protocol developed in the thesis, as well as the experimental results in the form of essential oil characterisation sheets, evaluation and validation of the antifungal potential of essential oils constituted in original databases, can provide useful methodological and informational support for future research, without being limited to the directions identified in this thesis.

Selective Bibliography

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Abstract

The doctoral thesis addresses the topic of wood bioprotection in terms of efficiency *versus* eco-impact, exploring in this context the opportunities and limits of some essential oils as potential alternative solutions for antifungal bioprotection of wood. The main concerned applicability area is that of the cultural heritage conservation, considering the importance of wooden cultural assets (artefacts) in terms of percentage representation, diversity, significance and value. However, the limited resistance to biodegradation of wood represents an element of vulnerability, while the fungal attack of wood, occurring under inappropriate conservation conditions, represents an aggressive degradation factor and a significant risk, requesting preventive and curative treatments as counteractions. The doctoral thesis proposes, validates and implements a methodological concept and an analytical protocol that allow the testing and responsible promotion of effective antifungal products with low eco-impact. Five essential oils (EOs) were identified and tested for their antifungal potential against xylophagous brown and white rot fungi: basil essential oil (B-EO), clove (C-EO), oregano (O-EO), cinnamon (S-EO) and thyme (T-EO). C-EO and T-EO were selected for further testing their antifungal effectiveness on wood and evaluation of the potential ecological impact of these treatments, by the phytotoxicity of the water leachates from treated wood. The C-EO and T-EO solutions at 5% concentration demonstrated adequate efficacy and low ecological impact. Preliminary implementation tests in the active conservation of wooden heritage goods have demonstrated an antifungal efficacy at least equal to that of some classical biocidal products considered for comparison (Diffusit S, Biotin T), as well as the compatibility of these treatments with subsequent finishing with beeswax and shellac. The experimental research data and results from this thesis have generated useful databases for future research.

Rezumat

Teza de doctorat abordează tema bioprotecției lemnului în sintagma eficiență *versus* eco-impact, explorând în acest context oportunitățile și limitele unor uleiuri esențiale ca potențiale soluții alternative pentru bioprotecția antifungică a lemnului. Domeniul de aplicabilitate vizat cu precădere este cel al conservării bunurilor de patrimoniu cultural, în care artefactele din lemn/pe suport lemn ocupă un loc important ca pondere, diversitate, semnificație, valoare. Rezistența limitată la biodegradare a lemnului reprezintă însă un element de vulnerabilitate, iar atacul fungic al lemnului, în condiții improprii de conservare, reprezintă un factor de degradare agresiv și un risc semnificativ, pentru a cărui contracarare sunt necesare tratamente preventive și curative, Teza de doctorat propune, validează și implementează un concept metodologic și un protocol analitic care permit testarea și promovarea responsabilă a unor produse antifungice eficiente și cu eco-impact redus. Au fost identificate și testate din punct de vedere al potențialului biocid antifungic, față de ciuperci xilofage de putregai brun și alb, cinci uleiuri esențiale (EOs): ulei esențial de busuioc (B-EO), cuișoare (C-EO), oregano (O-EO), scorțișoară (S-EO) și cimbru (T-EO). C-EO și T-EO au fost selectate pentru testarea eficacității pe lemn și evaluarea potențialului impact ecologic al tratamentelor, prin fitotoxicitatea apelor de spălare a lemnului tratat. Soluțiile de C-EO și T-EO cu concentrații de 5% prezintă eficacitate și impact ecologic acceptabile. Testele preliminare de implementare în conservarea bunurilor de patrimoniu din lemn au demonstrat o eficacitate antifungică cel puțin egală cu a unor produse biocide clasice considerate pentru comparație (Diffusit S, Biotin T), precum și compatibilitatea tratamentelor cu finisare ulterioară cu ceară de albine și șelac. Rezultatele experimentale obținute în teză au generat baze de date utile pentru cercetări viitoare.