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THE PROTECTION OF BEECH WOOD (*FAGUS SYLVATICA*) AGAINST THE BROWN ROT *POSTIA PLACENTA* USING CLOVE (*EUGENIA CARYOPHYLLATA*) ESSENTIAL OIL IN A LINSEED OIL MEDIUM

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Abstract: *The present research investigates the antifungal efficiency of clove (Eugenia caryophyllata) essential oil (C-EO) combined with linseed oil (LO) at different concentrations (1%, 5%, 10%) using two types of mycological tests: a qualitative screening test by agar diffusion method and a quantitative mini-block test on treated beech (Fagus sylvatica) wood.The agar diffusion test indicated improved protection of wood should be possible with a mixture of C-EO and LO from a concentration of 5%. In contrast, the mini-block test indicated that wood is partially protect by LO alone and that adding increasing quantities of C-EO gradually reduces this protection.One possible explanation of this unexpected result could be the antioxidant effect of C-EO which could negatively interfere in the oxidative curing process of LO. ESEM investigation revealed the penetration of LO and C-EO/LO mixtures into the wood structure and non-uniform fungal colonization of all the samples exposed to Postia placenta, as well as some characteristic features of consequent wood structure degradation, which was found more advanced for the untreated beech wood samples.*

Key words: *clove essential oil, brown rot, Postia placenta, mycological test, screening, mini-block, ESEM.*

1. Introduction

The protection of wood against fungal attack is of great practical importance for different applications in all situations when the moisture content of wood exceeds the threshold of 20% [7], [14], [46]. EN 335 [2] indicates that this

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threshold can be exceeded in all use classes except Use Class 1. Also, the conservation of wood cultural heritage is a very special field where fungal degradation risk might lead to the loss of an irreplaceable object [3], [16], [24], [37] and often requires both preventive and curative treatments [26].

From the large variety of fungi that can be involved in wood bio-deterioration, the decay fungi are the most dangerous as they are capable of degrading selectively the structural wood polymers via characteristic enzymatic and non-enzymatic processes, resulting in specific chemical and anatomical deterioration patterns, changes of physical properties and ultimately important strength loss of the wood material [8], [14], [15], [17], [46]. These fungi belong to three major groups with generic names related to the main macroscopic features of the decayed wood: brown-rots (*Basidiomycetes*), white-rots (*Basidiomycetes*) and soft-rots (*Ascomycetes* - fungi imperfecti). *Postia placenta* (currently *Rhodonia placenta*) is a common brown-rot fungus in forest ecosystems and is often responsible for destructive decay of wooden structures. *P. placenta* is capable of degrading both softwood and hardwood species, causing rapid depolymerisation of cellulose and hemicelluloses by enzymatic and oxidative processes and is one of the fungi employed in standardized decay tests (EN 113) [1].

Due to environmental concerns and for safety reasons, there is an increasing interest in testing and employment of natural products with antifungal protection effect, low toxicity for humans and minimal ecological impact. Sustainability is another important point to consider and therefore products from renewable resources, including various plants and crops, are preferred [8], [21], [39]. Essential oils (EOs) and drying oils, such as linseed oil (LO) are among these products.

Due to their natural origin, LO and EOs are generally accepted *ab initio* as human and environmental safe, although these properties must be carefully analysed for each EO in relation to its chemical composition, concentration and mode of employment [10], [27].

Essential oils (EOs) are natural organic liquid volatile products that have complex and diverse chemical composition, often with a characteristic odour. They originate from medicinal and aromatic plants as secondary metabolites and can be extracted by various procedures. EOs are biological active products with antifungal, antibacterial, antimycotoxigenic and antioxidant properties [23], [35] with useful applications in diverse fields from medicine and pharmacology to food industry, presenting also potential for wood protection [4], [30], [47]. The antifungal properties of EOs depend on their chemical composition, especially their main components [6], [11], [45]. A loss of fungal membrane integrity, inhibition in cell wall formation, inhibition of some genes involved in hyphae adhesion, growth and sporulation are included in the complex mechanism of EOs action against fungi, though this is not yet fully understood [11].

Linseed oil, also known as flax seed oil, is obtained by pressing from the seeds of the herbaceous plant *Linum usitatissimum.* There are four varieties of linseed oil sold in the market: raw, boiled, stand and refined linseed oils. Boiled linseed oil is processed by heating up to 150°C with the addition of metallic dryers

(e.g. zinc oxide, cobalt or manganese naphtenate) to speed up drying, and diluted with hydrocarbon solvents [25], [29]. Boiled LO has been used to protect wood for many years. The protection is thought to arise mainly to the oxidative polymerization process of LO forming a protective cross-linked hydrophobic outer (on the surface of wood) and inner layer (in the lumens of wooden cells) which reduces water uptake, with direct effect on the colonization of wood by decay fungi and its biological degradation [18], [40], [41].

Nevertheless, some fungicidal activity of raw LO against mould fungi (*Aspergillium, Penicillium, Trichoderma*) was observed *in vitro* tests (diffusion method on culture medium) and was attributed to its high content in unsaturated linoleic and α-linolenic fatty acids [28], [31] though it is well known that wood surfaces treated with raw LO are susceptible to colonization by staining fungi (*Aureobasidium*) producing a black biofilm in outdoor conditions [22], [42].

A study on the antifungal properties of three drying oils (soybean, linseed and Tung) applied and cured on wood, by an *in vitro* agar diffusion test with a white rot decay fungus, was recently reported [38]. The different and limited capacity of the studied oils to inhibit / delay fungal growth on wood was related to their chemical composition, respectively the number and type of unsaturated double bonds which are active in the oxidative curing process leading to a cross-linked structure, the best results being obtained for Tung oil, known as the most fast-drying oil due to its high content (82%) in α-eleostearic acid containing 18C and three conjugated double bonds in trans configuration. In comparison, linseed oil contains about 53% α-linolenic acid with 18C and three unconjugated double bonds in cis configuration, alongside linoleic acid (2 double bonds) and oleic acid (1 double bond).

As the antifungal protective effect of LO as a wood treatment is not usually sufficient, several improvement methods have been proposed: chemical modification by epoxidation to reduce the long drying times and the sensitivity of the oil films to microbial colonization [9], [22], combination with biocides or other natural active ingredients [18], [21] or technologies combining impregnation with oils with a thermal treatment [13], [43].

Clove essential oil (C-EO) extracted from *Eugenia caryophyllata* is one of the EOs with antifungal activity against moulds and decay fungi due to its high content (67-78%) in eugenol [6], [45]. The antifungal propertiesof C-EO have been demonstrated mostly by screening tests on culture medium [6], [30], [45], [47] and less frequently by mini-block tests on wood samples [30].

Previous research has demonstrated the antifungal activity of clove essential oil (CE-O) in ethyl alcohol solutions against the brown rot (*Postia placenta, Serpulla lacrymans*) and the white rot (*Trametes versicolor*) [33], [34] using diffusion tests on culture medium. It would seem that CE-O also provides opportunities for curative treatments of historic wood objects [32].

The present research investigates the antifungal efficiency of C-EO combined with LO at different concentrations using two types of mycological tests: a qualitative screening test by agar diffusion method and a quantitative mini-block test on treated wood. Furthermore, ESEM investigation was employed to highlight internal wood colonization by fungi and

any subsequent structural changes for a better assessment of the proposed treatment.

2. Materials and Methods

2.1. Treating Products

Clove (*Eugenia caryophyllata)* essential oil (C-EO), as a commercial product (purity 100 %, eugenol content 82.63%), available on the Romanian market under the label of "Steaua Divina", and drying boiled linseed oil (LO) as technical product from SC Fabryo Corporation SRL (solids content 49.8%, curing time 24 h), were employed in this research. C-EO/LO mixtures were prepared at three volumetric ratios (C-EO: LO) of 1:100, 5:100 and 10:100, resulting three treating oily solutions, referred to as 1% C-EO/LO, 5% C-EO/LO and 10% C-EO/LO in this paper.

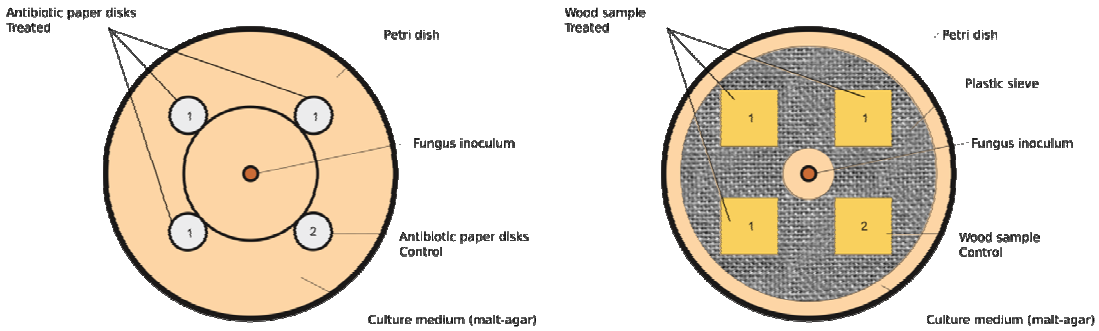
2.2. Paper and Wood Samples; Treating Procedures

Antibiotic test paper disks with a diameter of 12.7 mm, from FiltresFioroni (www.filtres-fioroni.com), were used in the screening mycological test on agar medium, after sterilization by steaming at 121°C for 20 minutes (Raypa autoclave). The sterile paper disks were impregnated with the oily solutions described above by fully immersing them for 30 s at room temperature, then squeezing them on blotting paper to remove excess oil and then air drying for 2 min under sterile conditions in a laminar flow bench, before being placed on petri dishes as described under mycological tests.

Beech (*Fagus sylvatica*) was chosen as a reference non-durable hardwood species for the mini-block test. Test specimens of (20x20x5) mm on the (LxRxT) directions were prepared from healthy, defect-free material. The wood samples were sterilized by steaming at 121°C for 20 minutes and then conditioned in sterile climatic chamber at 20°C and 65% RH until constant weight. The samples were further dried at 103±2°C for 24 h, cooled down in a desiccator and weighed before treating by impregnation under slight vacuum (100 kPa) for 24 h at 20°C followed by another 2 h under atmospheric pressure. The impregnated samples were allowed to cure by oxidative polymerisation in sterile conditions for 24 h, surface sterilized by exposure to UV for 30 min on each face and then reconditioned at 20°C and 65% until constant weight (approximately 96 h). The mini-block samples were again surface sterilized by exposure to UV radiation (30 minutes on each face) in a laminar flow bench immediately before placing on agar plates. The uptake of treating solution (C-EO/LO mixtures or LO) in kg/m^3 and the weight percent gain (WPG, %) after the oil curing were calculated on the basis of the conditioned weights in 65% air relative humidity at 20°C.

2.3. Mycological Tests

The brown-rot fungus *Postia placenta* was employed to evaluate comparatively the antifungal efficacy of LO and C-EO/LO mixtures by: (A) a screening test on culture medium based on the diffusion method [30], [34] and (B) a mini-block test adapted from EN 113 [1] on beech samples, schematically presented in Figure 1.



A B

Fig. 1. *Mycological tests employed in research: A. screening test by diffusion –Reinprecht method; B. mini-block test - adapted from EN 113 and similar setup as the screening test*

2.3.1. Screening test on malt extract agar-agar medium (MEAA)

Petri dishes (diameter 100 mm) containing a 3-4 mm thick layer of solidified sterile malt extract/ agar-agar culture medium (MEAA: 40g malt extracts ROTH and 20g agar-agar ROTH for 1 litre distilled water) were prepared. These were centrally inoculated with a 6 mm diameter block of fungus mycelium. Then four antibiotic test paper disks (three treated with a C-EO/LO mixture and one treated with LO as a control (LO-Control)), were placed directly on the medium at a distance of 20 mm from the border of the fungus inoculum (Figure 1a). Similarly LO-control Petri dishes containing four papers treated with LO and controls of fungus virulence containing just the inoculum were prepared. All tests were performed as duplicates for each treating solution. All the dishes were sealed with parafilm and placed in a culture chamber (Climacell BMT 400) at 23±2°C and 75±5% RH. Fungal development was evaluated and documented by photographs after 3, 5, 7, 9 and 11 days from inoculation. As a principle, the antifungal effect of a certain product is revealed qualitatively by the trend of emerging fungal mycelium to grow preferentially towards the control paper avoiding the area in the proximity of the treated samples where the medium can be poisoned by the antifungal active products diffusing from the treated (poisoned) paper into the medium.

2.3.2. Mini-block test

Petri dishes of 90 mm diameter containing MEAA sterile medium were similarly prepared and inoculated. The fungus was allowed to grow and cover the whole surface of the plate (2 weeks) and afterwards four beech wood samples (three treated with a C-EO/LO mixture and one treated with LO as a control) were placed on top of a sterile plastic mesh on each Petri dish (Figure 1b). Two Petri dishes were prepared for each concentration of the C-EO/LO mixtures. In addition, 2 dishes containing four samples treated with unmodified LO (Control LO) and 2 dishes containing untreated beech wood (Untreated control M) were prepared. The sealed dishes were incubated for 8 weeks in a culture chamber (Climacell BMT 400) at 23±2°C and 75% RH. At the end of the test the qualitative aspects of fungal growth were documented by photographs, before

removing and carefully cleaning each sample. The cleaned samples were disinfected by heating (minimum 2h/103°C) and reconditioned until constant weight at 20°C and 65%. The weight loss (WL,%) following fungal degradation was calculated on the before and after conditioned weights. Moisture control samples and correction samples were included in the test as defined by EN 113 [1].

2.3.3. Microscopic evaluation

A transverse face of block examined was cut with a new razor blade so as to facilitate the observation of individual cells. An Environmental Scanning Electron Microscope (FEI, Quanta 250) was used in LoVac mode to examine the cut surface. Images were collected using an excitation voltage of 5-7 kV, a partial pressure of 70-90 Pa and spot size 3-4.The observed areas were located at about 1-2 mm from the surface closest to the agar and pictures were taken at 300x, 600x and 1200x magnification.

3. Results and Discussions

3.1. Screening Test on MEAA Medium

Some images taken during the screening test by diffusion method on MEAA medium are presented in Figure 2. For the fungal control and LO control dishes a relatively uniform radial growth of mycelium can be observed, which completely covered the dish in 11 days. A slightly delayed but still uniform (no preferential) fungal growth was observed for the dishes testing 1%°C-EO/LO mixture (not included in Figure 2). In contrast, the antifungal properties of C-EO/LO solutions of 5% and 10% concentrations were evident from the 7th day after inoculation by the preferential development of the mycelium towards the control paper treated with unmodified LO.

One reason why this effect of preferential growth was not evident after 3 and 5 days might be the slow diffusion of C-EO from the oily hydrophobic solution (LO medium) into the hydrophilic MEAA medium. Similar screening tests with five EOs (including C-EO) employed as solutions in ethyl alcohol showed a preferential growth towards controls from days 3-5 [34]. Limitations of this type of diffusion test for assessing the antifungal efficiency of chemicals with low water solubility, fixation on wood substrate or those with high volatility have to be acknowledged [46]. Also, the growth of fungi on artificial media is sometimes markedly different from growth in wood [46], so that different effectiveness results might occur when testing treated wood (e.g. mini-block test).

3.2. Mini-Block Test on Beech Wood Samples

Experimental data referring to the treatment of test beech samples and their comparative resistance to the fungal attack of *Postia placenta* expressed by the weight loss after the mini-block test are summarized in Table 1.

Treatment with LO improved decay resistanceof beech wood as shown by the reduced weight loss compared to the untreated control (Table 1). This is in good accordance with literature [41].

The weight loss values did not reveal the same expected effect of increased anti-decay protection by addition of C-EO in LO. On the contrary, all the WL values for the samples treated with LO modified with

C-EO were higher (statistically significant) than those for the control samples treated with LO alone, though inferior to those for the untreated wood M. Moreover, the WL increased as the percent of C-EO in LO increased from 1 to 10%, though the difference between the variants 1%C-EO/LO and 5% C-EO/LO was found not statistically significant. Entrapping of C-EO into the cured film of LO and thus preventing the active antifungal components of C-EO (mainly eugenol) to get in contact and act against the fungus could have explained similar WL values as for the LO controls, but not higher values of WL with increased concentrations of C-EO.

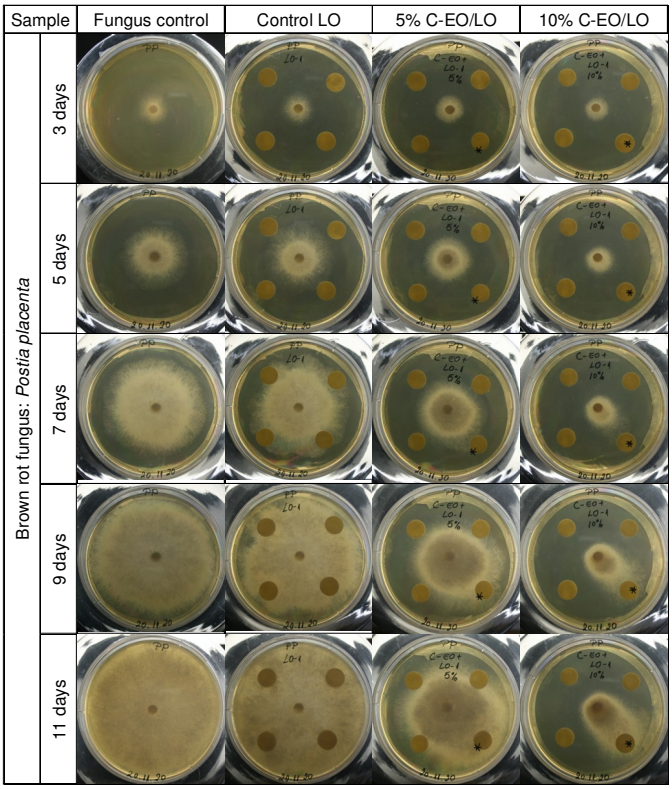


Fig. 2. *Screening the antifungal properties of clove (Eugenia caryophyllata) essential oil (C-EO) in linseed oil (LO) medium by agar diffusion method: fungal growth after 3, 5, 7, 9 and 11 days from inoculation*

The anti-decay protective properties of LO are thought to be linked to its capability to form a cross-linked hydrophobic film which reduces water uptake [40], [41] and, more recently, it was demonstrated that the antifungal protection effect of drying oils can be directly related to their curing properties and might be improved by formulations ensuring a faster and better curing causing a more advanced cross-linking in the resulting film [38]. Accordingly, the above presented elements should be considered in an attempt of understanding and possibly explaining the unexpected results reported in this paper.

Table 1

Summary of quantitative results of wood treatment and mini-block test (adapted from EN 113 [1]) – Beech (Fagus sylvatica) / brown rot fungus Postia placenta – exposure time 8 weeks

Type of sample	Treatment and conditioning (65%TH/20°C)			Mini-block test	
	Solution uptake [kg/m^3]	WPG [%]	MC_i [%]	WL [%]	MC_f [%]
Untreated control M	-	-	10.1 (*0.2*)	20.7 (*2.0*) a	72.6 (*4.3*)
Treated LO (Control LO)	237.3 (*33.7*)	20.3 (*3.0*)	11.7 (*0.1*)	7.3 (*3.0*) b	35.9 (*3.2*)
Treated 1% C-EO/LO	214.3 (*31.7*)	17.2 (*2.9*)	10.3 (*0.1*)	12.1 (*2.7*) c	41.0 (*4.9*)
Treated 5% C-EO/LO	212.2 (*12.4*)	17.3 (*1.1*)	9.9 (*0.2*)	13.7 (*3.1*) c	48.3 (*6.2*)
Treated 10% C-EO/LO	206.7 (*15.6*)	16.1 (*1.4*)	9.9 (*0.2*)	16.6 (*1.4*) d	52.1 (*3.4*)

Notes:
Data are average values of 6-8 samples /treatment; *values in brackets* represent standard deviations; corrections for weight losses independent of fungal attack were applied.
Statistical analysis of WL data by Anova single factor method was applied for each two groups of samples in order to determine if the differences are significant or not. Different indicatives in letters (a-d) in WL column indicate values statistically different at a confidence level of 95% (α critical = 0.05)

All the samples were conditioned at 20°C and 65% RH before being placed in the mini-block test, so that their initial moisture content (MC_i) varied between 9.9% and 11.7%. However, under the test conditions the samples reached different levels of the moisture content so that at the end of the test the average moisture content (MC_f) of the untreated controls (M) was twice that of the LO treated controls (see Table 1), which indicates a hydrophobic and physical barrier effect of the cured LO film. For the samples treated with LO modified with C-EO the MC_f values increased as a function of the concentration of C-EO in the treating solution, a higher amount of C-EO leading to higher MC_f (lower hydrophobicity). This could be an explanation of the WL values increasing with the increase of C-EO content, actually an increased moisture content of the samples.

According to literature data based on laboratory decay tests, the optimum wood moisture levels for decay fungi lie between 40-80% (Scheffer 1973 cited by [46]. Benitez and co-workers [5] studied the influence of the initial moisture content on the decay process by two brown–rot fungi and determined an increase of the wood moisture content during the test due to production of water as a consequence of the degradation of cell wall polymers (carbohydrates) by fungi. It was also found that an increased initial moisture content influenced the rate of deterioration and that wet samples showed higher weight losses than dry samples.

A reasonable explanation for a reduced hydrophobicity of the samples treated with C-EO/LO mixtures compared to LO should be related to their curing and cross-linking process, namely a chemical interaction with a negative effect on the oxidative polymerization of the unsaturated fatty acids (oleic, linoleic, linolenic) responsible for the curing of LO by a chain radical mechanism with oxygen

absorption. Metals, light, heat and enzymes accelerate the oxidation of unsaturated acids in the oil, while antioxidants inhibit oxidation [25]. LO is very sensitive to oxidation and various phenolic compounds are effective for anti-oxidative stabilization of raw LO for dietary and medical uses [20]. Eugenol, the major constituent of clove essential oil, was found to have an inhibitory activity against lipid peroxidation by interfering with chain reactions of free radicals and would be transformed into dehydrodieugenol following this process [19].

Clove essential oil was placed on the first place on a series of essential oils with good radical-scavenging activity [23], [35], and its capacity of inhibiting or delaying lipid peroxidation was experimentally demonstrated [12]. Accordingly, we suppose that the C-EO/LO mixtures could not cure properly by oxidative polymerisation to provide an effective, highly cross-linked protective layer due to the antioxidant effect of C-EO and this effect was more pronounced for the mixture with the highest C-EO concentration. This could also explain the higher MC_f and WL values determined for the samples treated with C-EO/LO mixtures.

3.3. ESEM investigations

ESEM investigation of LO treated samples highlighted that the oil entered into the wood matrix and penetrated some of the cells. A film covering the cell walls in the lumens of the vessels or even filling part of the fiber lumens is visible for the Control LO samples, presented in Figure 3 (right) comparatively with untreated wood M (left). This treatment could not stop some non-uniform fungal colonization via the vessels (Figure 4 - top-right).

Characteristic features of brown-rot are the selective degradation of carbohydrates, a preferential degradation of the S2 layer, leaving the S3 layer and middle lamella largely intact. The lignin in cell walls is modified but not removed, so that the microscopic structure (anatomical elements, cell walls) might remain relatively unchanged even at higher weight losses (40-50%) [15], [36]. The blocks lost less than 20% of their mass during the tests conducted here and so it is not surprising that there are few differences between the treated and none treated blocks. However, untreated wood (M) is cracked in the areas adjacent to the colonized vessels, which indicates a weakened cell wall that breaks rather being cut during surface preparation.

ESEM investigation of the mini-block test samples treated with C-EO/LO after fungal exposure revealed non-uniform fungal colonization, with hyphae visible especially in some vessel elements, as illustrated in Figure 4 (bottom) for the treatment with C-EO/LO of higher concentrations (5%, 10%).

When comparing the ESEM images of the samples treated with C-EO/LO solutions at different concentrations, it would be difficult to objectively assess and compare the level of the fungal attack (extent of colonization by the presence of hyphae), but it seems again that the integrity of their anatomical structure was less affected than for the control unmodified wood. Conclusions should not be drawn, however, from investigations on small areas. Moreover, it was demonstrated that apparent slow and asynchronous growth of hyphae in lignocellulosic substrates leads to uneven decay caused by brown-rot and by white-rot fungi even in adjacent wood cells [17].

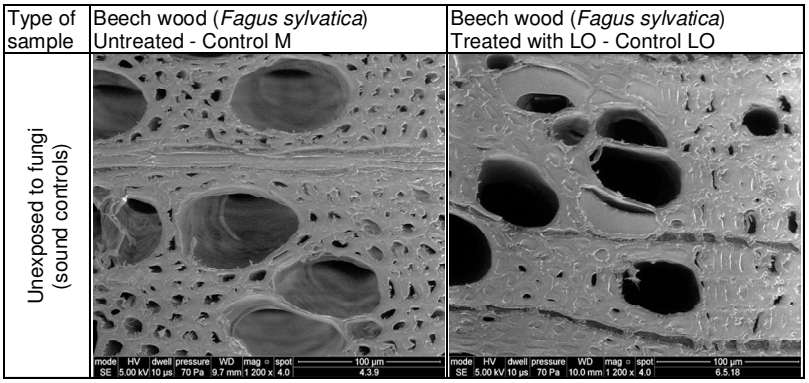


Fig. 3. *ESEM images (1200x magnification) of beech wood (Fagus sylvatica) samples highlighting comparative structural features for the untreated control M (left) and the control LO treated with drying linseed oil (right)*

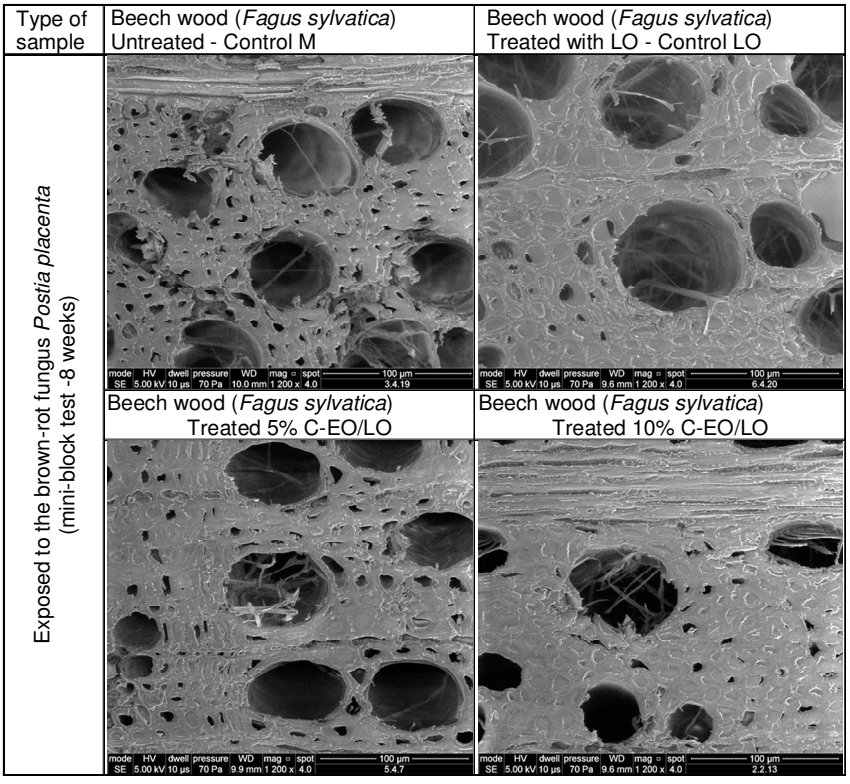


Fig. 4. *ESEM images of beech wood (Fagus sylvatica) samples: untreated M (top-left) and treated with LO (Control LO –top-right) or clove essential oil in linseed oil medium C-EO-LO 5% (bottom-left) and C-EO-LO 10% (bottom right), highlighting comparative aspects of their fungal colonization and structural damages after 8 weeks exposure to the brown-rot fungus Postia placenta (magnification1200x)*

4. Conclusions

A screening test of the efficacy of fungal control using an agar diffusion test indicated improved protection of wood should be possible with a mixture of C-EO and LO. Mini-block tests did not provide the same conclusions. The mini-block test indicates that wood is partially protect by LO alone and that adding increasing quantities of C-EO gradually reduces this protection.

ESEM investigation revealed the penetration of LO and C-EO/LO mixtures into the wood structure and non-uniform fungal colonization of the samples exposed to *Postia placenta,* as well as some characteristic features of consequent wood structure degradation.

Mixing LO with C-EO seems impractical as the expected benefit of increasing LO efficacy in protecting wood against fungal attack due to the contribution of the active antifungal ingredients of C-EO could not be demonstrated by this research. Further research should consider compatibility aspects and investigate the chemical interactions between C-EO and LO to better understand the current results and identify novel approaches for employment of clove essential oil as an alternative antifungal for wood protection.

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