PINE OIL POTENTIAL AS AGENT TO PRESERVE THE *Pinus pinaster* WOOD

Ofélia ANJOS[(1,3)*], Lúcia GARISO[(1)], Helena MACHADO[(2)], Miguel PESTANA[(2)]
[1]*Superior Agrarian School of Castelo Branco, Quinta da Senhora de Mércules, Apartado 119 – 6001 Castelo Branco – Portugal (ofelia@esa.ipcb.pt)*
[2] *INRB-National Institute of Biological Resources, Quinta do Marquês, 2780-159 Oeiras (helena.machado@efn.com.pt; miguel.pestana@efn.com.pt)*
[3] *CERNAS – Centro de Estudos de Recursos Naturais, Ambiente e Sociedade, Bencanta, 3040-316, Coimbra, Portugal*

Abstract:
Pine oil can be obtained from α-pinene and this is one of the constituents from gun-turpentine. Is a natural product and is known for its insecticide, bactericide and fungicide proprieties. In this study dry pinewood samples, cut in radial section, were impregnated with pine oil and stabilised at room temperature. Two degradative fungi were tested: *Trametes versicolor* and *Trichoderma citrinoviride*. Sterilized polypropylene vessels filled with glass beads and distilled water were prepared. Wood samples, deposed over the glass beads surface, were inoculated with fungal plugs collected from actively growing colonies and maintained at 25ºC in the dark during 29 days. The fungi development was assessed with an image analysis system using a digital camera. Analysis of variance (ANOVA) was use to study the effect of impregnation treatments, sterilization and fungal treatments.
The results showed that the pine oil impregnation could be a good wood preserver product with an additional advantage of its non toxicity for the human health.

Key words: Pine oil, fungal biodegradation, *Trametes versicolor*, *Trichoderma citrinoviride*, wood preservation.

1. INTRODUCTION

Pinus pinaster wood is very susceptible to fungal degradation and that could be a great problem for its commercialization causing important economic lost. Usually industrial products used for wood preservation treatments are made by synthetic chemical compounds with boron and chloride added with some antibiotics.

Decay in standing trees is of major concern in relation to human safety, since it often weakens stems, branches or roots enough to increase the chance of a mechanical failure. In trees decay commonly occur in small extension without causing any mechanical weakness and it is therefore inappropriate to regard a tree hazardous merely because decay has been detected (Schwarze, 2007).

Wood is composed by cellulose (40-50%), hemicelluloses (25-40%), lignin (20-35%) and other structural components as terpene, phenol, alkaloids and tannins (Sjostrom, 1993). The ability of fungi to degrade lignocellulosic materials is due to their highly efficient enzymatic system. Depending on wood components attacked during decomposition process wood decay fungi can be split up in several groups: blue-staining, soft-rots, white-rots and brown-rots (Knapp, 1985). Many fungi digest only one of the components of wood leaving the others behind. A lignin-digesting fungus leaves the white cellulose behind; this is called a white-rot. *Trametes versicolor* (L.) Lloyd is a frequent white-rot fungus especially efficient in lignin degradation (Cabral *et al.*, 1985-1987). Brown-rot fungi are the most destructive ones primarily causing a rapid despolymerization of the cellulose and hemicellulose components of wood but shortly removing the lignin (Chang *et al.*, 1981). Although genera *Trichoderma* can include species with different behaviours, from endophytic to plant pathogenic fungi (Samuels, 1996), the species *Trichoderma citrinoviride* Bissett can be considered a brown-rot fungus as it shows more capability to degrade cellulose and hemicellulose than lignin (Henriques, 2007).

The copper and zinc preservative products were been substitute by the boron products cased by its favourable environmental characteristics. Borates are better preservatives because offer adequate protection to lumber in non-ground contact situations and building applications (Obanda *et al.*, 2008). Moreover with the borates systems the industry need use other products like anti foams and insecticides that are toxic for the environmental. So it is important continue the research in this subject in order to found new products that could be a better solution in environmental terms.

Limonene is a natural hydrocarbon, cyclical, unsaturated compound and belongs to the terpene group. It occurs naturally in certain trees and shrubs, being the largest constituent in several essential oils, as pine trees and citrus fruit. It's used as a solvent, in rubber industries, fragrance and food (Derfer and Traynor, 1989). Its antiseptic proprieties made it a good choice as a natural wood preserver. In other to improving the knowledge in environmental friendly products the research team started a preliminary study with limonene and conclude that wood impregnation with this product can increase its durability reducing the development of wood decay fungi (Francês *et al.*, 2008).

Pine oil can be obtained by extraction of aged pine wood (natural pine oil) or by hydration of α-pinene with aqueous mineral acids, followed by distillation to separate de pine oil from de excess monocyclic hydrocarbons (synthetic pine oil). Pine oil is consumed in industrial and household cleaning products, disinfectants, solvents, fragrances, medicine and aromatherapy (Kelkar *et al.* 2006).

In the present work we evaluated the potential of pine oil to prevent the development of *T. versicolor* and *T. citrinoviride* on pinewood. The capability of pine oil as disinfectant product was also tested. Further research (scale-up) is needed in order to evaluate eventual effects of pine oil impregnation on wood resistance.

2. MATERIAL AND METHODS

Trametes versicolor TR489 obtained from fructification on eucalyptus wood and *Trichoderma citrinoviride* B2 isolated from *Platypus cylindrus* Fab. (Coleoptera: Platypodidae) galleries on cork-oak, both cultures from INRB-Oeiras collection were tested. Cultures were maintained on PDA medium (potato dextrose agar) at 25ºC in the dark.

Dry pinewood samples (2x2x1 cm) cut in radial section, were impregnated with pine oil or distilled water used as control for impregnation. All samples were stabilised during 24 hours at room temperature.

Polypropylene MAGENTA® vessels (GA7, Sigma-Aldrich 77×77× 97 mm) filled with 10 ml glass beads (5 mm diameter) and 10 ml of distilled water were prepared. All the materials were sterilized in autoclave during 20 minutes at 121ºC and 1 bar pressure. Wood samples were deposed over the glass beads surface and inoculated with fungal plugs (l6 mm diameter) collected from actively growing colonies. Boxes were sealed with PARAFILM® and maintained at 25ºC in the dark during 29 days. Moisture level was verified regularly.

The fungi development was assessed with an image analysis system using a digital camera with 6Mega Pixel and software COGNEX VISION PRO4 CR(2).

Experimental design was factorial (2x2x3x5) with two impregnation treatments (pine oil/distilled water), two wood sterilization treatments (autoclave sterilized wood/unsterilized wood), three fungal treatments (*T. versicolor* TR 489/*T. citrinoviride* B2/control without fungi) and 5 repetitions.

Data were subjected to variance analysis (ANOVA), multiple comparing test and least significant difference with programme STATISTICA® version 6.0.

For the study of the capability of pine oil as disinfectant product different sterilization methods were tested. Pine oil impregnated wood and untreated samples were sterilized in autoclave during 20 minutes at 121ºC and 1 bar pressure autoclaved or superficial treated with alcohol and flame burned. After sterilization wood samples were transferred into Petri dishes with PDA medium and incubated at 25ºC in the dark during 15 days or until fungi development. Isolation and observation under microscope permitted to identify some contaminant fungi to the genera (Barnett and Hunter, 1988; Halin, 1997).

3. RESULTS AND DISCUSSION

Fungi development after 29 days of incubation for both species is showed in Figure 1. Both fungi failed to develop on wood impregnated with pine oil independent of the application of sterilization treatment. In control (distilled water impregnation) *T. versicolor* showed a good development in both autoclave sterilized (Figure 2) and unsterilized treatments (Figure 3). For this species the development is superior on autoclave sterilized wood indicating that *T. versicolor* benefits from environment lacking contaminant species.

T. citrinoviride showed an unusual behaviour with weak growth on control (distilled water impregnation) making data interpretation meaningless.

During the experiment, contaminant fungi were only reported on unsterilized non impregnated treatments showing clearly the preserving effect of pine oil impregnation on wood.

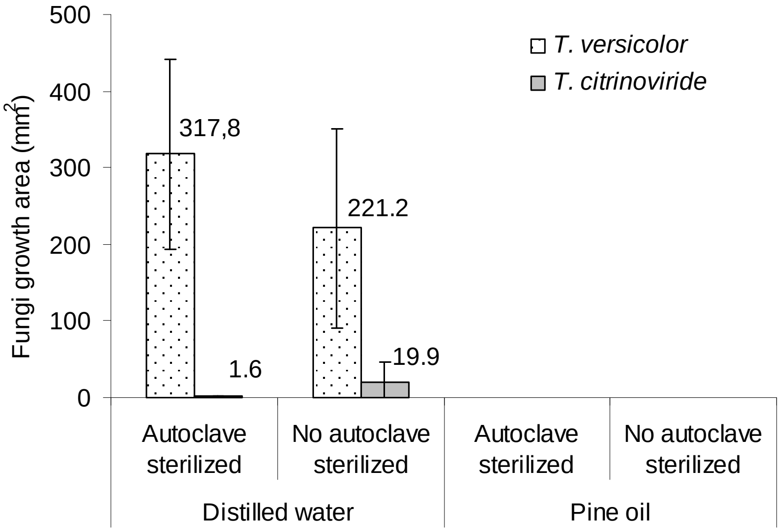

Figure 1 – Growth area of *T. versicolor* and *T. citrinoviride* in wood with two impregnation treatments and two sterilisation treatments (mean of 5 replicates, bars represent the standard deviation of the mean).

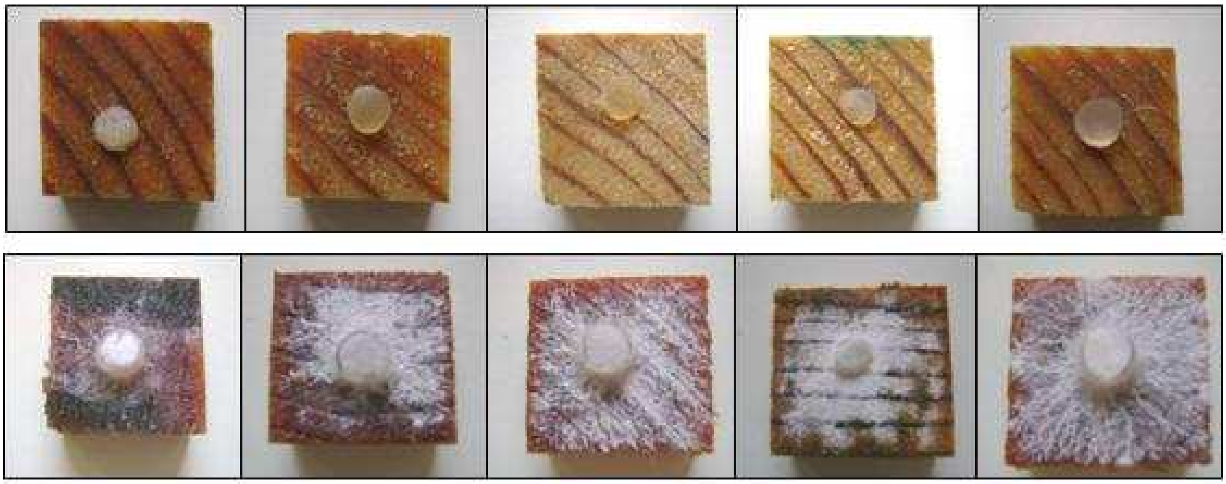

Figure 2 – Growth of *T. versicolor* in 5 wood samples with pine oil impregnation (first line) and distilled water impregnation (second line) treatments. All samples were autoclave sterilized.

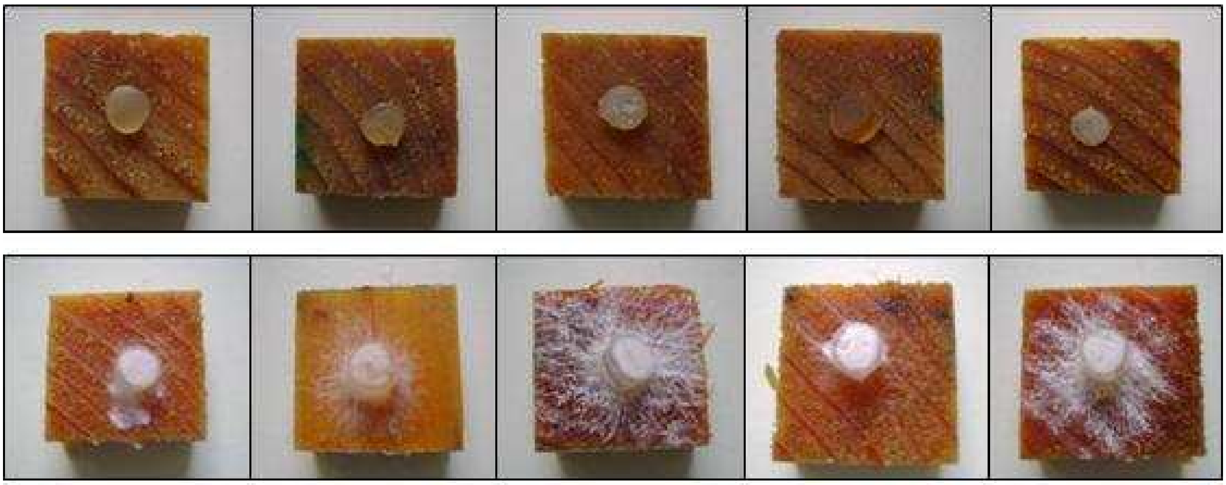

Figure 3 – Growth of *T. versicolor* in 5 wood samples with pine oil impregnation (first line) and distilled water impregnation (second line) treatments. All samples were unsterilized.

The study of the capability of pine oil as disinfectant product (Table 1) showed an excellent effect on preventing the development of all contaminant fungi in treatments with pine oil impregnated wood. All unsterilized wood treatments (control) were contaminated. The contaminants fungi included species from *Trichoderma*, *Aspergillus* and *Penicillium* genera (Figure 4). Autoclave sterilization was a good control treatment with no contaminations. Treatments with alcohol for superficial sterilization presented one repetition contaminated with *Aspergillus* sp. indicating the presence of endophytic species in wood samples. These contaminations were also prevented with pine oil impregnation.

In order to compare the results of this test with a previous tests using limonene as wood preserver (Francês *et al.*, 2008), antifungal activity based on area of *T. versicolor* development (mm^2) using the formula [AFA = (Treatments/Control) x 100] and expressed in percentage, is presented in Table 2.

Table 1 – Results of contaminant fungi in pine oil impregnated wood and untreated wood after application of different methods of sterilization (n=5).

Wood Treatment	Sterilization Method	Contaminations (Nb.)	Contaminant Genera
Pine oil impregnation	Unsterilized	0	
	Alcohol	0	
	Autoclave	0	
Untreated wood	Unsterilized	5	*Trichoderma* *Aspergillus* *Penicillium*
	Alcohol	1	*Aspergillus*
	Autoclave	0	

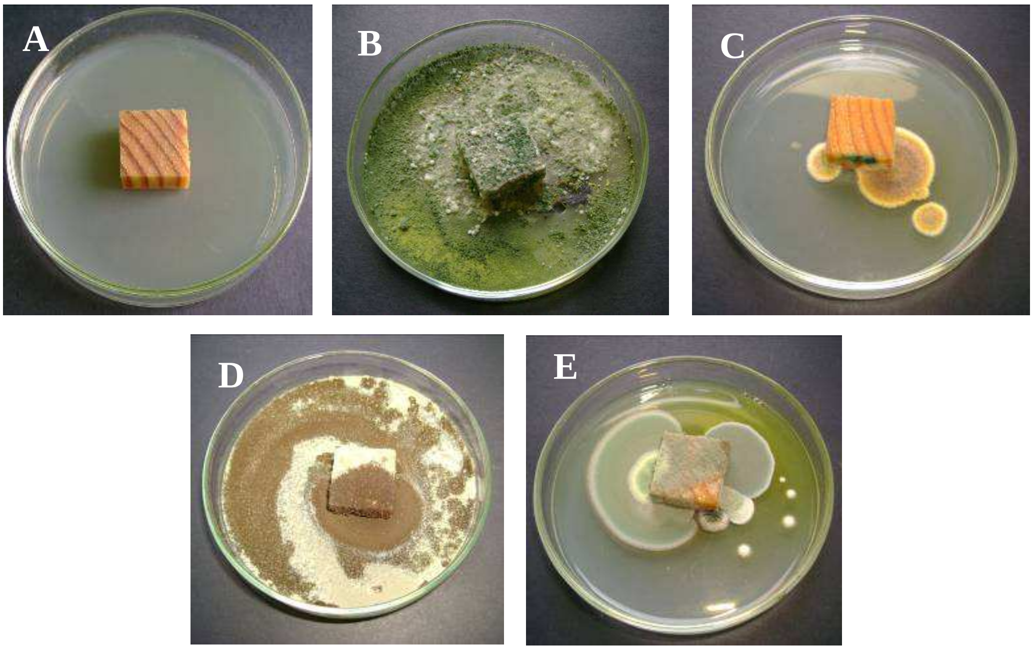

Figure 4 – Contaminant fungi observed in pine wood samples with oil impregnation (A) *versus* non impregnated wood. Examples of detected contaminant fungi: *Trichoderma* (B), *Aspergillus* (C and D), *Penicillium* (E).

Table 2 – Antifungal activity against *T. versicolor* of limonene and pine oil expressed in percentage.

Wood Treatment	Sterilization Method	Antifungal activity (%)
Pine oil impregnation	Unsterilized wood	100.0
	Sterilized wood	100.0
Limonene impregnation	Unsterilized wood	43.8
	Sterilized wood	43.5

More studies are in progress to evaluate the preserve action of both products with the fungi referred in the EN113 norm and its durability. Moreover the present results show clearly that the pine oil could be a good wood preserver product, better than the limonene product previously studied.

4. CONCLUSIONS

Wood impregnation with pine oil prevents the development of the wood decay fungi tested, *T. versicolor* and *T. citrinoviride*. Pine oil impregnation also prevents the incidence of contaminant fungi, namely *Trichoderma* sp., *Aspergillus* sp. and *Penicillium* sp..
Although some *Trichoderma* species are also known for their antagonistic abilities in soil (Cox *et al*. 2001) the species tested in this study (*T. citrinoviride*) showed an unusual weak development in wood. Further tests should use species recommended in European Standard EN113 as *T. versicolor*.
The results show that the pine oil could be a good wood preserver product with an additional advantage of its non toxicity for the human health. It showed an antifungal activity of 100% against *T. versicolor*. Limonene can also be an attractive product for wood preservation with an antifungal activity of 43%. Based on results from both tests ongoing research was oriented to find a mixture of both products joining a good antifungal activity and an attractive price. Different dilutions were also considered.

BIBLIOGRAPHY

BARNETT H.L.; HUNTER, B.B., 1988. Illustrated Genera of Imperfect Fungi. 4ª ed, APS Press, Minnesota, USA.

CABRAL T., SARDINHA R.A., PIMENTEL MG., 1985-1987. Actuação de três basidiomicetas sobre a madeira de *Eucalyptus globulus* Labill. An. Inst. Sup. Agron. 42:55-77.

CHANG M.M., CHOU T.Y.C., TSAO G.T., 1981. Structure, pre-treatment and hydrolysis of cellulose. In: ZIECHTER A (ed.), Advances in Biochemical Engineering. Berlin: 15-42.

COX P.; WILKINSON S.P.; ANDERSON, J.M., 2001. Effects of fungal inocula on the decomposition of lignin and structural polysaccharides in Pinus sylvestris litter. Biol. Fertil. Soils 33:246-251.

DERFER J.M., TRAYNOR S.G. 1989. Chemistry of turpentine in Naval Stores: Production, Chemistry and Utilization. DF Zinkel and J Russell Eds., New York.

EN113 (1996). Wood Preservatives - Test Method for Determining the Protective Effectiveness Against Wood Destroying basidiomycetes - Determination of Toxic Values. European Committee for Standardization (CEN), Brussels, Belgium.

FRANCÊS, S., ANJOS, O., PESTANA, M., MACHADO, H. 2008. Knowledge of the potential of limonene as agent to preserve the pinus pinaster wood. Ecowood – 2008, 3rd International Conference on environmentally–compatible forest products. Fernando Pessoa University, Porto-Portugal: 263 – 266.

HALIN, R.T., 1997. Illustrated Genera of Ascomycetes. Vol I., APS Press, Minnesota.

HENRIQUES J.M., 2007. Fungos associados a *Platypus cylindrus* Fab. (Coleóptera: Platypodidae) e sua relação com o declínio do sobreiro em Portugal. Mestrado em Biologia de Pragas e doenças de plantas. Universidade de Évora.

KELKAR V.M., GEILS B.W., BECKER D.R., OVERBY S.T., NEARY D.G., 2006. How to recover more value from small pine trees: Essential oils and resins. Biomass and Bioenergy *30*:316–320

KNAPP J.S., 1985. Biodegradation of celluloses and lignins. Compreensive Biotechnology. MM Young Ed., Vol. 4, Pergamon Press, Oxford, pp.835-846.

OBANDA D.N., SHUPE T.F., BARNES H.M., 2008. Reducing leaching of boron-based wood preservatives - A review of research. Bioresource Technology *99*: 7312–7322

SAMUELS, G. J. (1996). *Trichoderma*: A Review of Biology and Systematics of the Genus. Mycol. Res. *100 (8)*: 923-935.
SCHWARZE F.W.M.R. 2007. Review - Wood decay under the microscope. Fungal biology reviews *21*:133 – 170
SJOSTROM E., 1993. Wood chemistry, second edition: fundamentals and applications. Academic Press.