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COMPARATIVE EVALUATION OF FIVE LABORATORY SCREENING TESTS FOR ASSESSING THE ANTIFUNGAL POTENTIAL OF PRODUCTS FOR WOOD PRESERVATION

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Abstract: *This paper refers to a number of laboratory tests designed to allow a quick and reliable evaluation of the potential biocidal effect of chemicals intended to be used as wood preservatives with antifungal properties. A total of five tests were run in parallel employing two biocides with recognised efficiency as reference products in comparison with distilled water as control. The relevance of these tests in highlighting the antifungal properties of the tested reference products and the practical aspects of the actual testing procedures were considered in the comparative evaluation of the five tests. Two tests were selected for further experiments.*

Key words: *mycological tests, antifungal potential, reference products, qualitative evaluation.*

1. Introduction

Wood is a natural material, excellent in terms of functional, environmental, and aesthetic aspects. Its composition and structure make it susceptible to degradation by destructive biotic and abiotic factors (physical and chemical), leading to the need of wood protection. Known and effective classic biocidal substances have unacceptable side-effects and ecological impact, thus appearing to increase the trend of replacement with natural biocides from plant resources [9].

Nowadays, there is now, more and more research on wood preservation employing different extracts of vegetal origin: juices and chili extracts against mould fungus attacking wood [9], essential oils used against mould fungus [16] and rot fungus [15], various extracts of sustainable wood species [13], natural biocides from citrus waste [8].

This paper presents a preliminary research phase, within the topic approached in a PhD thesis, which deals with wood preservation in the polarity efficiency versus ecological impact, focussing on testing and implementing novel eco-products from natural resources.

Testing the efficiency of wood preservation products using the classic EN

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113 method, based as principle on the quantitative determination of mass losses of treated wood compared to control samples, following fungal attack by exposure to inoculated culture media, is laborious and time consuming (16+1 weeks). In this respect, screening tests for a first selection of natural products with potential biocide antifungal properties are necessary.

This research resulted from the need to develop a relatively quick and simple testing methodology to highlight and compare the biocidal potential of possible novel wood preservation products, especially vegetable extracts with low ecological impact.

Various screening tests are presented in the literature, but they are either general or with specific applicability in fields other than wood preservation: the protection of documentary patrimony [1], [2], [3], [4], agriculture [11], nutrition [3], [5], [7], [12], [14], medicine [10].

The presented research stage aimed to establish a methodology for assessing the biocidal potential of different products, by rapid and reliable tests, appropriate to the objectives of the thesis. For this purpose, experiments employing tests from literature and original methods were run in parallel. Two biocides with recognised efficiency were used as reference products, in comparison with distilled water as control. On the other hand, it was intended to correlate the screening test method with the standard test method EN 113.

2. Objectives

The research objectives in this preliminary phase were as follows:

- Selecting and adapting tests from the literature (4 tests) and developing one original test (1);
- Performing the selected tests in the laboratory, employing reference biocides;
- The comparative evaluation of results by qualitative methods.

3. Methodology (materials and methods)

Five tests were chosen and run comparatively, as summarized in Table 1 and illustrated as principles in Fig. 1.

The biocide reference products with recognized efficacy (BRE) used were: Copper Sulphate and Romalit N (55% $CuSO_4.5H_20$, 45% $K_2Cr_2O_7$), as 5% aqueous solutions. Distilled water was used as control.

For all tests, standard sterilized Petri dishes for mycology (Φ 100 mm) and agar - malt extract culture medium were employed. The fungus was *Coniophora puteana* (brown rot).

The culture medium was prepared from malt extract (standard from ROTH) and agar (purified and free from inhibitors, microbiology grade, MERCK) using 40g malt and 20g agar for 1 litter of distilled water. Dissolution was carried out at room temperature under stirring, the medium being then sterilized at 121°C for 20 minutes in the autoclave (REYPA AES 75). After sterilization, the medium was distributed in Petri dishes and allowed to cool for 24 hours in the bacteriological cabinet (APRIL, HVF 1200 with vertical laminar flow) to prevent infestation.

The inoculation was done after 24 hours in the central position. The 5 tests differed from one another in the manner of bringing in contact the biocidal substance with the culture medium (see Table 1 and Figure 1). For the HP, HPI, R, CT assays, chromatographic filter paper (Whatman No. 1), previously sterilized, was used as support for the biocidal substance. In the DH test, the biocidal substance was applied directly on the surface of the medium. Each Petri dish was sealed with para-film and properly inscribed.

Types of screening tests performed experimentally Table 1

No.	Test name	Code	Details	Reference
1.	Humar and Pohleven method	HP	Biocide/Whatman paper, Disks ϕ 13 mm; Application: dripping 100 µl	Vek et al. (2013)
2.	Humar and Pohleven method	HPI	Biocide/ Whatman paper Disks ϕ 13 mm Application: immersion	Vek et al. (2013)
3.	R type test	R	Biocide/ Whatman paper Disks ϕ 80 mm Application: immersion	
4.	CT test (Test adapted after the R type test)	CT	Biocide/ Whatman paper Application: immersion	
5.	ICWSE test	DH	Biocide/culture medium Application: On the surface of the culture medium	Delenk et al. (2015)

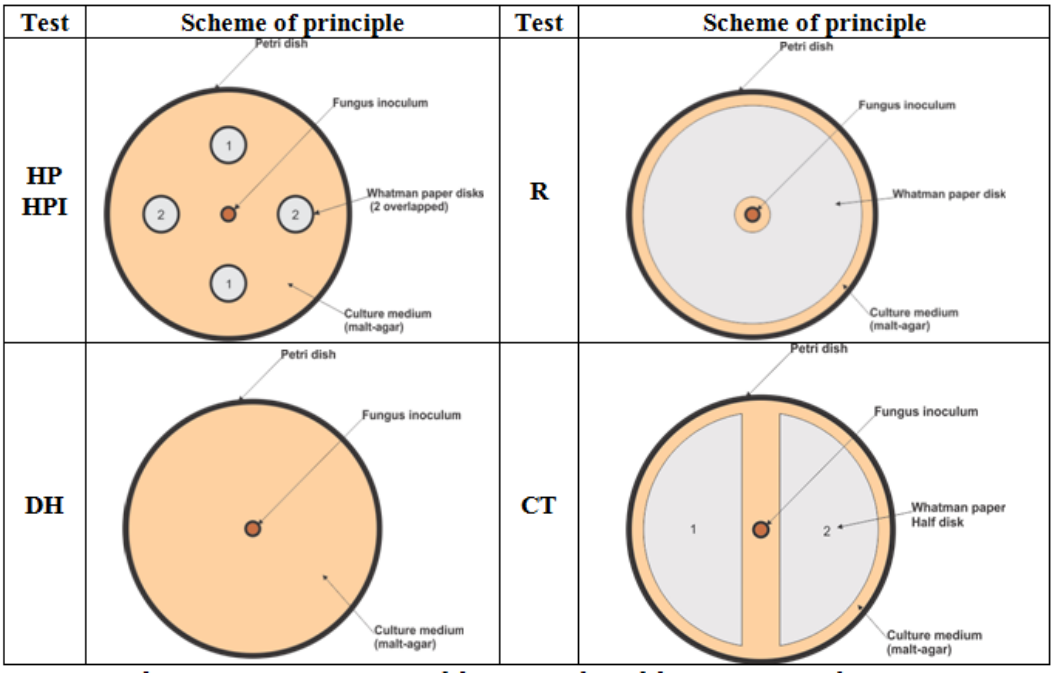

Fig.1. *Schematic representation of the principles of the experimental screening tests*

The dishes were placed in the culture chamber (CLIMACEL 404 by Comfort BMT, Czech Republic) at a temperature of 23 ± 2° C and a humidity of 75 ± 5%.

The evaluation was performed qualitatively by visual analysis and

photographic documentation of the fungal development from the original inoculum, after the following time intervals: 7, 9, 11, and 15 days. The degree of development of the fungal mycelium and the possible preferential orientation (water versus biocide) were monitored. This was necessary both to observe the development of the fungus in relation to the test conditions, and to determine the relevant examination periods and maximum testing times.

In the HP test, the paper disks played the role of "reservoirs" for the biocidal substance, and for the distilled water as control. The biocides were dripped on the paper disks, which had already been placed on the culture medium. The test principle was the diffusion of the substances from the paper disks into the culture medium, which would result in stopping or restraining the development of the fungus in the case of a product with fungicidal properties.

In the HPI, R, and CT assays, the reference biocides were applied on the filter paper disks by dipping, prior to their placement on the culture medium. This approach was considered to be closer to the EN 113 test procedure in which control wood and wood impregnated with the biocidal substance are tested. For a substance with antifungal properties the expectation is either that the treated paper will not be colonised, or that the development of the fungus will be delayed.

In the DH test, the biocidal agent was uniformly applied on the surface prior to inoculation, and it was therefore expected that the development of the fungus would be delayed or totally inhibited, if the substance applied was fungicidal.

HP, HPI, and CT tests had the advantage of cumulating the control sample and the potential biocidal solution in the same dish. For R and DH tests, the biocidal substance and control were tested separately in different Petri dishes.

4. Results and Discussions

Figure 2 shows gradual development of the fungus for the control samples (distilled water) and the copper sulphate (in the same dish or different dishes, according to the test). By periodic analysis of the samples, as the images show, the required and relevant time periods for each test could be established. This aspect had to be associated with a visible, measurable development of the fungus in the control samples. It was noted that the maximum duration of testing would be 11 days. This period of time was associated with the necessary time for the fungus to grow and completely cover the surface of the culture medium. After this period, the development of the fungus could no longer provide information relevant to the test purpose. For all tests, the relevant time durations were 7-9 days. This period of time was enough for observing progressive and measurable stages of the fungus. A previous analysis, 3-5 days following inoculation, might have provided additional information on the initial stages of fungus development, an idea that was retained for further experiments.

At the same time, the analysis of these images highlights the specific way in which the fungicidal potential can be assessed in these tests. In HP, HPI, and CT tests, which aggregated the control and test samples, the biocidal effect was clearly and measurably observed by the preferential development trend towards the control area. Among these tests, this selective trend of development appeared clearer in the CT test, which has been selected for future experiments.

The DH and R tests, the biocidal effect was observed by the total inhibition of the fungal growth in the Petri dishes containing copper sulphate and Romalit N.

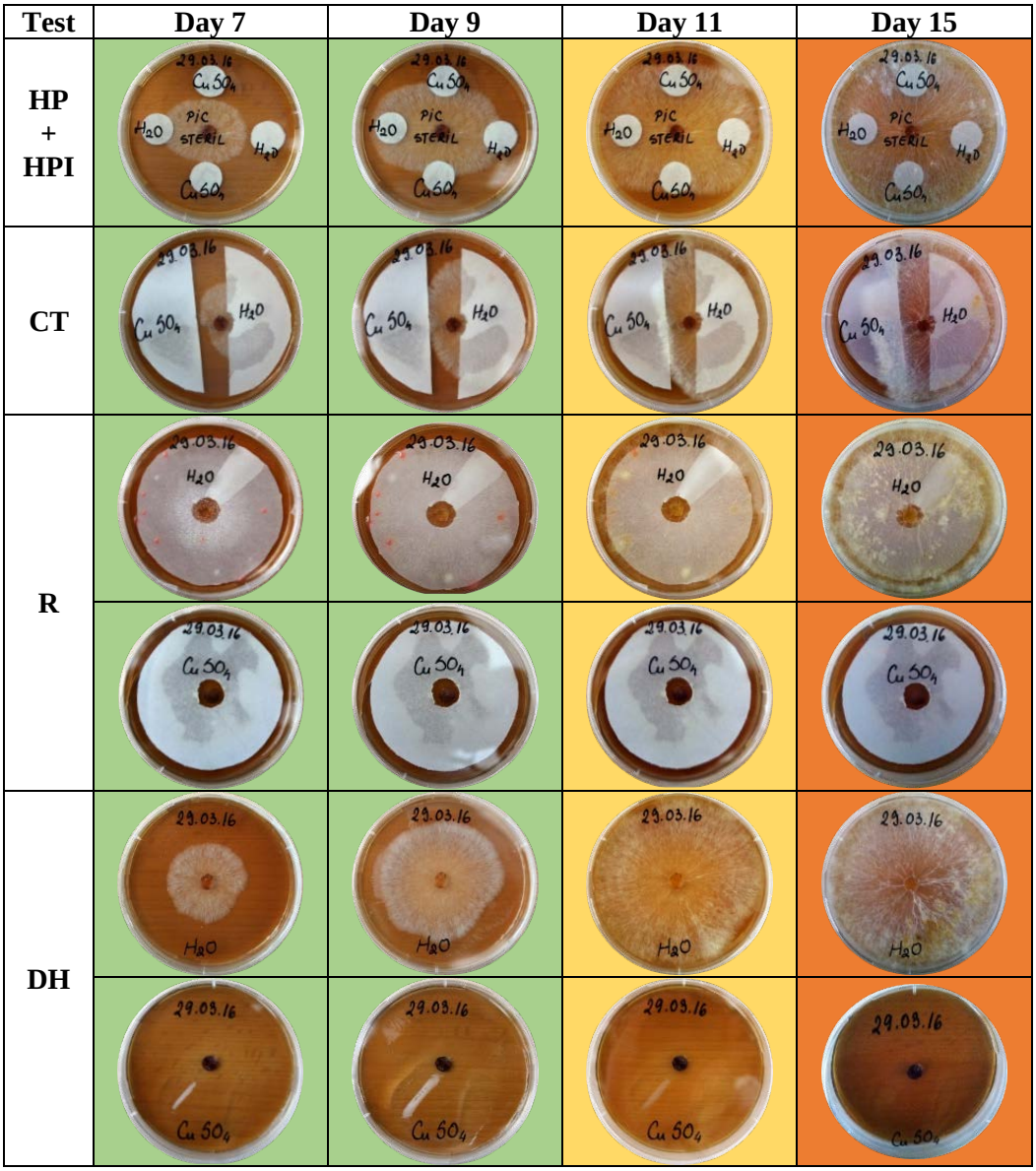

Fig.2. *Evolution of fungal development on control samples and copper sulphates samples in the five experimental tests; color codes: green - periods relevant for evaluation, yellow - maximum test duration, orange - prolonged test period, not relevant*

Considering the above observations, Figures 3 and 4 summarize the results of the biocidal potential effect of the reference substances, from all 5 experimental tests carried out. The pictures in these figures present comparatively, for the control and both BREs, the degree of fungal mycelia development 9 days after inoculation, a time evaluation interval previously established as being relevant for the assessment of all tests.

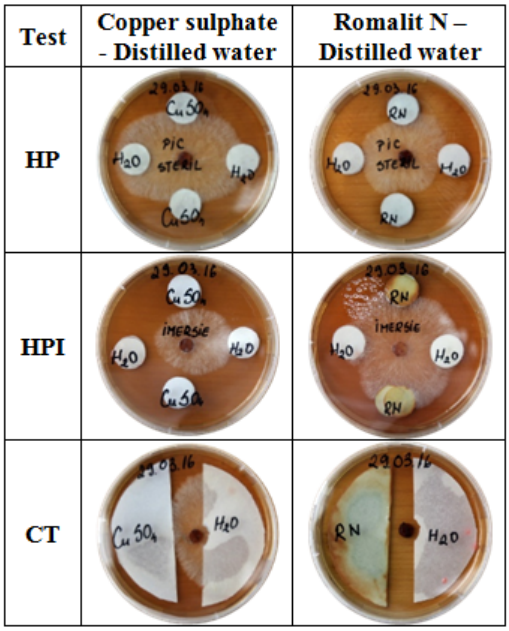

Fig.3. *The degree of fungal development 9 days after inoculation for the tests: HP (top), HPI (middle), and CT (bottom)*

The HP test (Fig. 3 top) clearly indicated the biocidal effect of copper sulphate (the fungus development was preferential towards the distilled water control area), but was inconclusive for Romalit N, although its biocidal efficiency was clearly proven in practice.

The HPI test (Fig. 3 middle) showed results similar to the HP test, meaning the biocidal effect of copper sulphate was evident by the preference of the fungus development on the distilled water area. For Romalit N the results were also inconclusive in this test.

The CT test (Fig. 3 bottom) provided an obvious confirmation of the biocidal effect of copper sulphate by the preferential development of the fungus towards the control zone (distilled water). For the sample containing Romalit N there was no growth of the fungus, which confirmed its biocidal effect.

Both tests, R (fig.4 top) and DH (Fig. 4 bottom), confirmed the biocidal effect for both copper sulphate and Romalit N. In the Petri dishes containing BRE, the fungus did not develop at all, while in the control with distilled water, it developed very well, showing a uniform growth.

Based on the experimental results referring to the reliability of the five tests to reveal the biocidal effect of the two reference products, the decision was to select two out of the five tests for further experiments, namely the tests coded CT and DH.

Also, it was decided that in the future tests, the monitoring should begin from day 3 after inoculation, at intervals of 2 days until the maximum testing interval of 11 days.

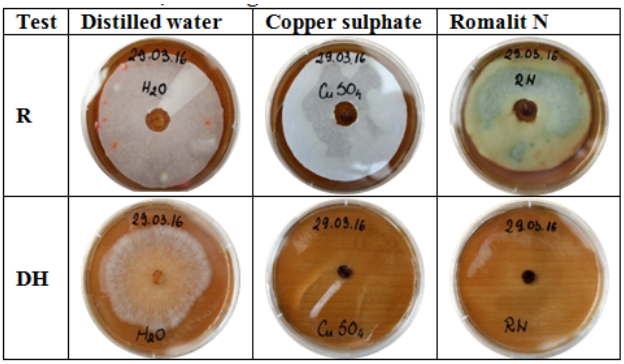

Fig.4. *The degree of fungal mycelial development 9 days after inoculation for R (top) and DH (bottom)*

5. Conclusions

Based on the recent literature in the field of screening tests to assess the potential biocidal fungicide effect of different products, four tests were chosen and a fifth one was developed.

All five tests were performed experimentally in the laboratory, using two reference biocides (BRE): copper sulphate and Romalit N in comparison with distilled water as control.

Based on the analysis of the experimental results, two tests, coded CT and DH, seemed to be relevant and appropriate for use in future experiments. These conclusions should be confirmed by additional experiments on several parallel samples employing more biocidal products and more fungi.

The period of 9 days after inoculation, was established as being relevant for assessment for all tests.

In addition, it was considered necessary to complete the qualitative evaluation with a quantitative assessment of the results of these tests. The system considered and the results obtained will be presented in a future paper.

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