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PROBING BIOCIDE PENETRATION AND RETENTION IN WOOD PRODUCTS BY IMMUNOLABELING TECHNIQUES

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Abstract

Wood products are commonly degraded by decay fungi, insects (including termites), or other organisms (marine borers). Chemical treatments with biocides are the most effective way to protect wood from these organisms. Surface treatments by immersion, aspersion, spraying, brushing, dipping and injection are extensively used to apply the biocidal products to the wood. The performance of preservative treatments is often evaluated according to their retention and penetration. It is therefore important, for either quality control or research and development purposes, to study the penetration capacity of a biocidal product. The methods commonly used for determining penetration of wood preservatives include observing their migration through their own color, or through color indicators, or extracting samples followed by chemical detection by chromatography analysis. However, these multistep methods are time consuming and do not allow the detection and precise location of active substances within the wood structure. Here, we present the general principle of a novel specific approach for detection, visualization and ultrastructural localization of biocidal active substances in treated wood using immunological techniques together with electron microscopy. In the present work, we raised a polyclonal antibody against cypermethrin and applied it for the detection of the pyrethroid insecticide in Pinus sylvestris samples and its localization within the wood cell walls by transmission electron microscopy. Thus, the distance of penetration of cypermethrin from the sample surface could be evaluated and the results were correlated with chemical analysis. The interest of the immunological detection method is discussed.

Key words: *biocide; insecticide; penetration; immunolabeling; polyclonal antibody; transmission electron microscopy.*

INTRODUCTION

Wood products are often degraded by insects or decay fungi. Chemical treatments by biocidal products are the most effective ways to protect wood from these organisms. In particular in the case of insect attacks, pyrethroids such as permethrin, and cypermethrin, have been widely used for application in agriculture, forestry, horticulture, public health, and households all over the world because of their general high bioefficacy, enhanced stability, and low toxicity to birds and mammals (Wang et al. 2011). It is important to know how wood preservatives are penetrating inside wood. The methods commonly used for determining penetration of wood preservatives include observing their migration through their own color, or through color indicators, or extracting samples followed by chemical detection by chromatography analysis (Aguera et al. 2002). However, these multistep methods are time consuming and poorly sensitive (Goël 2013). Due to their sensitivity, immunochemical techniques have been developed in the last years in veterinary medicine, agriculture, food analysis, to detect active substances and particularly pyrethroids presence (Danks et al. 2001, Lee et al. 1998, 2004, Zhang et al. 2010, Ahn et al. 2011). However, these studies have never been oriented to *in situ* detection and do not allow the detection and precise location of biocides within the wood structure. Thus, a sensitive, selective, and rapid method for monitoring pyrethroids and particularly cypermethrin presence and penetration is presented here. In the present work, we raised a polyclonal antibody against cypermethrin and applied it to its detection in *Pinus sylvestris* samples. The penetration was evaluated to the finest scale using transmission electron microscopy.

OBJECTIVE

The main objective of the present research was to elaborate a specific probe in view of localizing cypermethrin *Pinus sylvestris* wood and following its penetration from the surface. The results will be correlated with chemical analyses of the same wood samples.

MATERIAL, METHOD, EQUIPMENT

Wood material treatment

Pinus sylvestris wooden board were brushed with a 10% dilution of the commercial formulation “AXIL”, a water-based concentrated micro-emulsion product from Sarpap & Cecil Industries from BERKEM Group. The product based on cypermethrin (253F/2), consisted in a mixture of cis/trans isomers in a respective ratio of about 40/60.

The samples were dried in a climatic chamber at 20°C and 65% RH until the day of cutting the wood test specimens as described below.

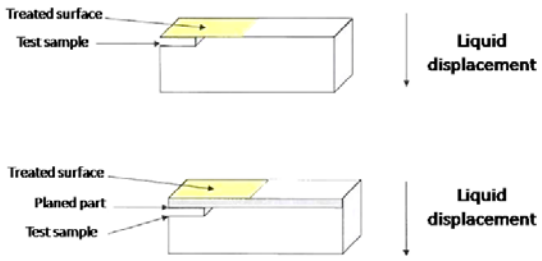

Fig. 1.

Chemical analysis

The protocol comprised two stages: Liquid extraction of the active substance from the wood followed by analysis of the active substance by gas chromatography-electron capture detector: GC-ECD ^{63}Ni. In the first step, the wood specimens were ground and 1g of the ground sample was placed into a 20ml headspace vial in which ethyl acetate (20ml) was added with a pipette. The mixture was subjected to sonication for 2 hrs, cooled to room temperature then filtered on filter paper.

In the second step, a 10ml aliquot of the filtrate was placed into a 20ml headspace vial and 10ml of ethyl acetate containing an internal standard solution (80-100mg) was added. 2µl of the mixture was injected GC-ECD for analysis.

Wood samples preparation for electron microscopy

For TEM studies, small matches (2 x 2mm section, 6 to 7mm height) were cut in test samples and prepared as described (Joseleau and Ruel 1997). Matches were fixed in a mixture of glutaraldehyde / paraformaldehyde 0.2/ 2w/v, and then progressively dehydrated in an ethanol series up to 100%. The samples were then progressively impregnated in solutions of ethanol/LR White acrylic resin, hard grade (Ted Pella, Inc.), before being embedded in fresh LR White pure resin in gelatin capsules and polymerized for 24 h at 50°C.

Ultrathin sections (50nm in thickness) were cut using an ultramicrotome (Leica UC6, Leica Microsystems,Germany) equipped with a diamond knife and collected on plastic rings or carbon coated nickel grids.

Preparation of the polyclonal antibody

Two female New-Zealand white rabbits (sanitary controlled) weighting 3.5 to 4kg were immunized subcutaneously at multiple sites on the back of each rabbit, by injection of 250µg of non-conjugated cypermethrin dissolved in glycol ether and complete Freund' s adjuvant. Animals were boosted at 14-day intervals with nine subsequent injections of the same immunogen suspended in incomplete Freund's adjuvant. Blood was collected from the ear vein of the rabbits and by intracardiac puncture 10 days after the last injection. Blood samples were aliquoted and stored at -20°C in a freezer.

Characterization assays of the antibody

This was performed by dot-blot immunoassays. The immunotests were carried out in 0.5ml semi-micro disposable cuvettes, to reduce the amounts of antiserum. Dots of the antigen and of the substrates to be tested were deposited (1µl) at varying concentrations, on strips of positively charged nylon membranes (Boehringer; 8 x 50mm) as described in Joseleau and Ruel (2007).

Immunogold labeling

All steps were performed at room temperature (18-23°C).

Immuno-gold labelling in TEM was done on ultra-thin transverse sections (50nm) floating downward in plastic rings on 50µl droplets of the reactives as described in Joseleau and Ruel (2007) or collected on carbon-coated nickel grids treated via a *LKB Automate device.* Briefly, after blocking non-specific labelling successively with Tris-HCL(0.01 M Tris-HCl, pH7.6) /glycine then 5% (w/v) non-fat dried milk in TBS500, the sections were incubated on the antiserum diluted in the blocking buffer. After washing on TBS500 and rinsing on Tris-HCl buffer, the sections were floated on the secondary marker coupled to gold particles (protein A or goat anti-rabbit IgGs) diluted in Tris-HCl buffer containing 0.2% fish gelatin. After thorough washes in Tris-HCl buffer and distilled water, sections were then post-fixed in 2.5% glutaraldehyde in H_2O. At this stage, the diameter of the 10 nm gold particles was further enhanced using a silver enhancing kit (SPI-Mark^{TM} silver enhancement kit). Finally, thin sections were post-stained in 2.5% aqueous uranyl acetate. All the observations were carried out on several sections and grids.

Photographs were taken in a JEOL 1200-EX cryo-electron microscope at an accelerating voltage of 80kV.

RESULTS AND DISCUSSION

Cypermethrin is a commonly used pyrethroid insecticide for wood preservation. It is important to determine the presence of the insecticide, its retention in wood and its depth of penetration. A sensitive and specific immunochemical method was developed to be used *in situ* to determine the presence of cypermethrin and the depth of impregnation achieved after application.

Production of a polyclonal antibody against cypermethrin

In the present work, we choose an immunization with the unmodified cypermethrin as the antigen, by directly injecting it to rabbits. This approach, although less efficient than the generally used method consisting in coupling a small antigen molecule to a bulky carrier macromolecule, was successful and produced an antiserum containing a polyclonal antibody against cypermethrin. In spite of the relatively low titer obtained, the interest of directly injecting the antigen is to use the authentic molecule, together with the simplicity of the approach compared to the necessary chemical derivatization and coupling to the carrier protein conventionally performed.

Characterization test of the polyclonal antibody

The injection of rabbits with the non-conjugated technical cypermethrin resulted in the production of an antiserum containing IgGs directed against the insecticide molecule

Since the active substance was not conjugated to a carrier-protein, it could not be satisfactorily fixed in the wells of the ELISA plates for testing the specificity of the antiserum. Thus, an assay was attempted by a mini dot-blot in which the technical cypermethrin was deposited on nylon membrane strips and its fixation confirmed by coloration with toluidine blue. However, during the course of the dot-blot assay, a large proportion of cypermethrin had been washed off from the nylon membrane, rendering the assay inappropriate to this active substance.

To overcome the difficulty in using the conventional assay procedures, we implemented an immuno-TEM assay consisting in applying the antiserum on ultra-thin sections of treated-wood containing the hapten *versus* a control devoid of the hapten, followed by immunogold labeling as described in Joseleau and Ruel, (1997). The best dilutions for the antibody was found between 1/60 and 1/80.The absence of labeling in the controls and the presence of gold labeling in the cypermethrin-treated wood samples, illustrated by the micrographs in Fig. 2, revealed that the antibody had effectively been coupled to its antigen.

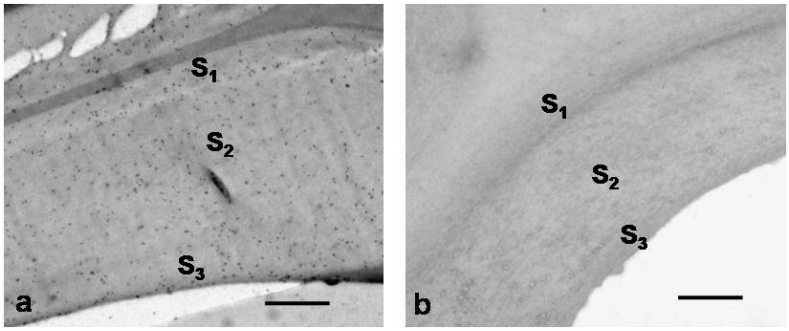

Fig. 2.
a - Cypermethrin labeling of a treated sample of Pine wood; b - Control on untreated wood: absence of gold particles; bar: 1µm

One of the interests of the immunogold-TEM assay is to allow the antigen molecule to be observed in its exact environment in the wood, thereby providing information about the insecticide behavior within the wood structure.

The use of the polyclonal antibody provides a broader recognition potential over monoclonal antibodies and gave us in the past good results with small molecules reported difficult to functionalize (Ruel 2003, Joseleau et al. 2004).

Previous assays in the literature were done using hapten groups chosen and prepared from the insecticide active substance (Lee et al. 2004, Wei et al. 2013). However, a protein-conjugate hapten is no longer the original molecule since it had undergone chemical transformation by binding to the carrier protein. Moreover, if the original molecule does not have a functional group than could be available for conjugating to the carrier protein it has to be transformed in order to gain the required chemical group amenable to conjugation to the protein. As a result, this may impair the specificity of the antibody toward the original molecule by hiding a characteristic functional group essential for the specific immunological recognition of the molecule (Wei et al. 2013). In the case of pyrethroids, the cyano group was shown to be important in cypermethrin immunization process (Zhang et al. 2010).

Localization of Cypermethrin in Wood by Immunolabeling inTransmission Electron Microscopy

The presence of gold particles in the cell walls of the treated wood (see Fig. 2.) showed that cypermethrin had the ability to penetrate in the secondary wall of the wood tracheids.

The gold particles were 10nm in diameter and could be observed directly at high magnification. However further size enhancement of gold grains by silver intensification is sometimes required to underscore the distribution of the tiny gold particles within the wood cell walls and their constitutive sublayers in a more general view letting to see several tracheids. Illustration can be seen in Fig. 3 where both procedures were used. At low magnification (Fig. 3a), the labeling of cypermethrin

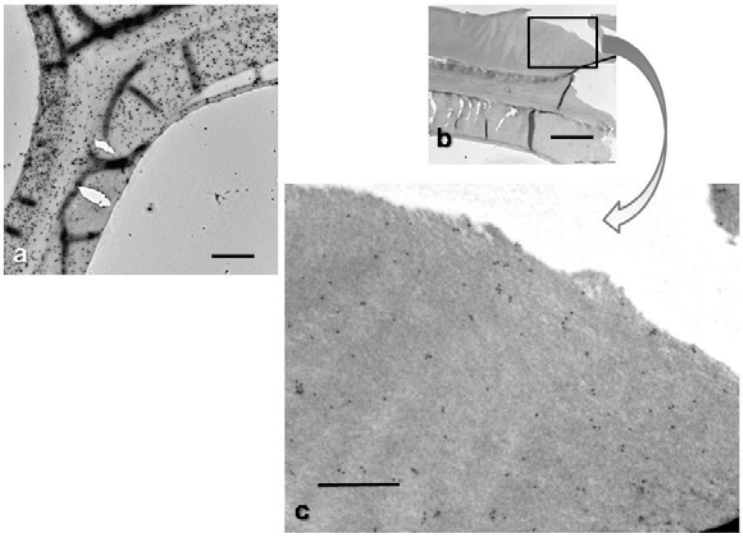

Fig. 3
Comparison of immunogold labeling with and without silver enhancement: a - after enhancement of gold particles, bar = 2µm; b, c - without silver enhancement: b = same magnification as "a", bar = 2µm; c = enlargement of "b", bar = 0.5µm

in the cell walls of two adjacent tracheids and their sublayers is clearly underlined. When gold particles are directly observed (Fig. 3 b and c), it is necessary to strongly enlarge to see the labeling. Both procedures were used in the present work to check possible non specific deposits after enhancing.

The presence of cypermethrin within the thick walls of fibers and tracheids indicates that the insecticide molecules were able to diffuse from the lumen to the internal S_2 layer of the secondary walls. Contrary to Jin and Webster's results (1998) who could not observe any penetration of cypermethrin in the wood tissues, the detection of cypermethrin with the immunological probe associated to the high resolution of electron microscopy brings evidence that the organic active substance actually penetrated the wood and adsorbed in the walls where it is retained. The homogeneous distribution of cypermethrin inside the S_2 layer may be due to its interaction with the wood cell wall ligno-cellulosic macromolecules, where its retention could explain its permanence in wood. Interestingly, the so-called compound middle lamella (primary wall plus middle lamella) was hardly labeled with only very few gold particles. This observation suggests that the fact that cypermethrin could not easily diffuse through the compound middle lamella may be due to the higher lignin concentration and ionized pectic polysaccharides present in this zone that acts as a cytological barrier.

Assessment of Cypermethrin penetration in Wood treated samples

Assessment by chemical analysis

The dried wood test samples were cut into two zones at 0-6mm and 6-8mm from the surface, respectively. The active susbtance was extracted by liquid extraction then evaluated by gas chromatography-electron capture detector: GC-EDC63Ni. The evaluation of the penetration was expressed as the percent of retained cypermethrin (Table 1).

Table 1
Monitoring cypermethrin retention by GC-ECD in board samples of Pinus sylvestris subjected to surface application of the insecticide.

Distance from the surface (mm)	Cypermethrin (% w/w)
0 – 6	0.050 – 0.059
6 - 8	0.004 – 0.008

Assessment by immunogold labeling in TEM

Three consecutive levels of depth relative to the surface of the wood samples delineated and cut at 0-3, 3-6, and 6-9mm, respectively, as in Fig. 1 were immunolabeled with the anti-cypermethrin

antibody. In order to better underscore the localization of cypermethrin, the gold particles (10nm) were silver enhanced. Ultrathin sections of the three levels are shown in Fig. 4.
Between 0 and 3mm from the surface, the gold particles are densely distributed indicating that the active susbstance penetrated easily at this depth (**Fig. 4a**). However, it is noticeable that the concentration in the compound middle lamella remained very low. As the distance from the surface increased, cypermethrin appeared still retained in the cell walls up to about 6 mm from the site of application at the surface (**Fig. 4 b).** Beyond 9 mm, no more significant labeling could be observed (**Fig. 4 c**).

From these results, it appears that, in the present conditions of application of the biocidal product, the retention of cypermethrin is mainly due to its capacity to penetrate the cell wall network in which it remains adsorbed. This is certainly an important factor accounting for its permanence in wood products.

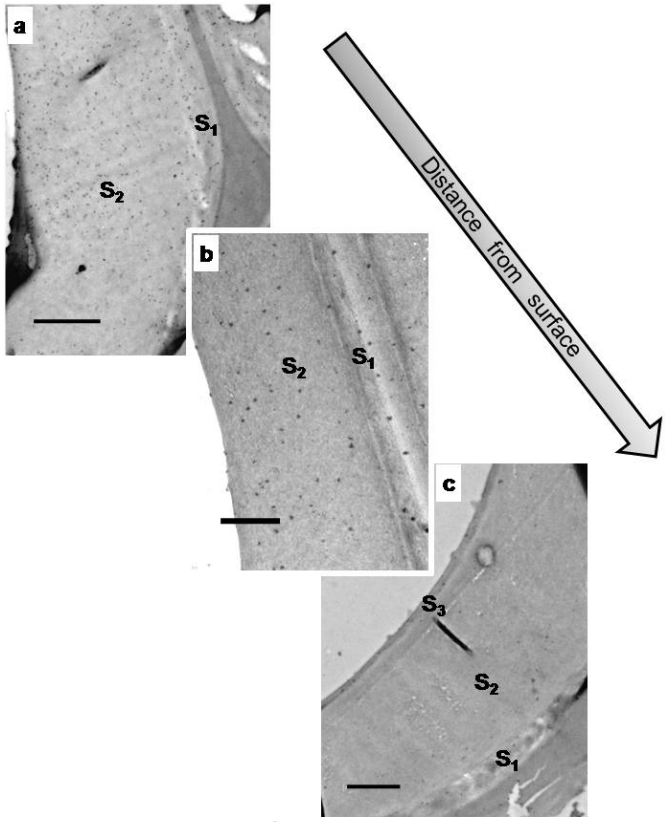

Fig. 4

Cypermethrin penetration in wood impregnated samples after enhancement of gold particles: a - 0-3mm from the wood surface wood; b - between 3 and 6mm in depth; c - between 6 and 9mm from the surface bar = 1.0µm

CONCLUSION

A polyclonal antibody directed against commercial cypermethrin was obtained by a procedure consisting in directly injecting the active substance without previous attachment to a carrier protein. The recognition of cypermethrin in wood by the antibody was validated by adaptation of an immunogold assay in transmission electron microscopy. The polyclonal antibody allowed identifying cypermethrin and monitoring its penetration within wood products at the ultrastructural level. Such a scale of observation revealed that the insecticide active susbstance had the capacity to diffuse within the macromolecular cell wall matrix.

The immunological detection *in situ* of cypermethrin also constituted a useful technique to assess the extent of penetration of the biocidal product in wood products. The depth of penetration corresponded to about 9 mm from the surface of application of the biocidal product. This agrees with the chemical analysis by GC-ECD. By comparison with chemical analysis, it is important to note that the immuno-microscopy approach gives information on the distribution of cypermethrin at the cytological level. Thus, the observation of the capacity of cypermethrin to diffuse and to tightly adsorb on the polymer composite texture of the wood cell wall may explain its permanence and durability.

The present results offer for the first time the visual evidence at the ultrastructural level of the interaction between cypermethrin and the wood cell walls, and provides a new method of visualization of the fate of a biocidal active substance within the wood structure.

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