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ANIMAL PROTEIN IMPACT ON FUNGICIDAL PROPERTIES OF TREATMENT FORMULATIONS

Bartłomiej Mazela, Maria Bartkowiak
Agricultural University of Poznań, Institute of Chemical Wood Technology, Poland

Izabela Ratajczak
Agricultural University of Poznań, Department of Chemistry, Poland

ABSTRACT

Experiments were carried out with the aim to investigate the impact of animal protein on the fungicidal properties of a treatment formulations. Due to the increased nitrogen concentration, proteins introduced individually into wood, should support fungal development. However, these investigations were undertaken with the aim to find out whether this very component restricts fungicidal properties towards the pure active substance. The objective was to determine the growth dynamics of the mycelium of the *C. puteana* fungus into wood treated with a formulation containing globular protein. The experiments were carried out by measuring the colonization rate of the bait placed inside the sample. Investigations were conducted using boric or propionic acid, fixed in wood with the assistance of protein by means of its denaturation either thermally or chemically. It was concluded that the presence of protein used as a fixing agent in wood preserving formulation does not accelerate the mycelium overgrowing into the treated wood and does not reduce fungicidal properties as well.

KEY WORDS: animal protein, baiting experiment, *Coniophora puteana*, hyphae overgrowing, wood treatment

INTRODUCTION

The idea of the utilisation of various kinds of proteins as components of wood preservatives originates from the need to find a simple and inexpensive way of fixation of the active substance. Such necessity appears in the situation when an effective active substance is employed (fungicide, insecticide, algaecide, fire retardant etc.) to protect wood, but one which does not bind with the wood substance. Comprehensive investigations have been carried out for years aiming at reducing the leaching from wood of boron compounds used as components of wood preservation agents against insects, fungi as well as fire (Peylo and Willeitner 2001). This disadvantageous property of boron compounds frequently prevents it from their application to protect wood used outdoor and in contact with soil. Experiments

on the fixation of boron compounds in wood were carried out, among others, by Kartal and Imamura (2004) as well as by Kartal and Green (2003). Wood treated with boron compounds in the form of boric acid, sodium tetraborate or calcium tetraborate and then treated again with a solution of hydroxylamine naphthalene showed by 30% smaller leaching of boron in comparison with wood treated with pure boron compounds. Thevenon et al. (1998 d) employed a different mechanism of boron fixation in wood using condensed tannins. The idea of this method was a self-condensing polymer network forming under the influence of boric acid (Pizzi and Beacker 1996). Although much less boron was leached out of the treated wood, results concerning long-term wood utilisation were far from satisfactory. Interesting results were obtained following the reaction of boron compounds with proteins (Thevenon et al. 1997, 1998abc, Thevenon and Pizzi 2003). The principal idea behind this method was the exploitation of the ease with which protein forms salts with boric acid following the reaction with amine groups. After the introduction into wood of a small quantity of protein in the form of aqueous solution of boric acid salt, boron becomes fixed in wood in the result of gelation of proteins after treatment with heat or other factor causing protein denaturation.

Bearing in mind the results obtained so far in experiments carried out to investigate possibilities of reducing the leaching of active substances from wood treated with globular protein of animal origin (Mazela and Polus 2003, Mazela and Polus-Ratajczak 2003), experiments were carried out with the aim to investigate the impact of this kind of protein on the fungicidal properties of a treatment mixture. Hypothetically, this natural biopolymer introduced individually into wood, should support fungal development due to the high nitrogen concentration. However, these investigations were undertaken with the aim to find out whether, this very component, made toxic by an appropriate active substance, forming a salt or a physical mixture with it, restricts fungicidal properties towards the pure active substance. The objective of these studies was to determine the growth dynamics of the mycelium of the *C. puteana* fungus into wood treated with a formulation containing globular protein. The experiments were carried out by measuring the colonisation rate of the bait placed inside the sample from the moment it was placed on the mycelium.

Investigations were conducted using two active substances, namely boric acid and propionic acid fixed in wood with the assistance of protein by means of its denaturation either thermally or chemically. The performed experiments comprised the following activities: sample preparation, preparation of treatment solutions, sample treatment, seasoning, conditioning, bait fixing, sample exposure to fungal action, placement of the bait on clean agar substrate, assessment of mycelium overgrowing. Simultaneously, on a separate batch of material, the weight loss of samples treated with the same preparations and subjected to the action of the same test fungus was estimated.

MATERIAL AND METHODS

Sapwood of Scots pine (*Pinus sylvestris* L.) in the form of samples with dimensions: 15 x 22 x 50 mm was used as experimental material. The material used to prepare samples complied with the quality requirements of the EN 113 (1996) standard. All samples were subjected to the action of *Coniophora puteana* (Schum. ex Fr.) BAM Ebw. 15. The sample were divided into two groups. The first group comprised treated samples. Each experimental treatment consisted of six replications. The second group comprised the identical number of control samples which did not undergo any treatment.

Impregnating solutions

The initial raw materials from which treatment formulations were made comprised the following chemical substances: globular protein, boric acid, propionic acid and tannic acid.

Proteins are obtained from the freeze-dried blood plasma through the process of ultrafiltration. They are characterised by very good emulsifying and jellification properties. Denatured proteins are characterised by worse solubility and this determines their application for wood preservation as agents limiting the leaching of the fungicidal substance from wood in the course of its outdoor utilisation as well as in its contacts with soil (Murray et al. 1995).

The remaining constituents of the examined formulations are: boric, propionic and tannic acids. The first two of them act as biologically active substances. The effectiveness of the boric acid in wood preservation has been recognised for years (Lutomski and Neyman 1976, Lloyd et al. 1990, Troya and Navarrete 1991, Drysdale 1994). Propionic acid is not applied commonly in wood treatment as a fungicidal substance. However, there are scientific reports confirming its effectiveness to protect wood against decomposition caused by fungi (Ismail et al. 1989, Eslyn 1973, Mazela et al. 2002). It was employed in the current research, among others, because of the ease with which it forms salts with proteins following binding of the carboxyl and amine groups (Thevenon et al. 1998 a). The third constituent – the tannic acid – despite recognised, albeit limited, fungicidal properties (Dirol and Scalbert 1991, Militz and Homan 1993), fulfils the role of protein denaturing agent (Thevenon et al. 1998 b, Mazela and Polus-Ratajczak 2003).

On the basis of raw materials described earlier, five treatment formulations in the form of aqueous solutions were prepared. The first of them was a 1% protein solution in the form of spray-dried animal blood plasma. The next two formulations were solutions of individual active substances, i.e. boric acid (1%) or propionic acid (4%). Wood samples treated with the above-mentioned three solutions constituted reference material against which samples treated with mixtures of these components were tested. These mixtures constituted the next two test formulations: the mixture of protein (1%) and boric acid (1%) and the mixture of protein (1%) and propionic acid (4%). Solutions containing protein were prepared on a mechanical agitator in order to ensure uniform solution and complete dissolution of protein. In order to enhance protein solubility in the mixture with boric acid, 0.2% solution of sodium hydroxide (NaOH) was added in the amount which allowed obtaining pH above the isoelectric point. The applied low concentrations of active substances were significant. They were determined on the basis of the earlier research results of the fungicidal properties of the tested preparations (Mazela and Polus 2003; Mazela et al. 2005) and, simultaneously, taking into consideration the possibly shortest time of conduction the experiment.

Wood treatment

Wood blocks were dried at 105°C, weighed and treated with the treatment solutions. The samples were treated according to the following cycle: 30 min at 85kPa, 2h at the atmospheric pressure. They were weighed and the retention of the preservatives was evaluated (Tab. 1).

Tab. 1: Mean retention of formulations by wood samples

Preservative	Mean retention [kg/m^3]		Mean retention of 5% tannic acid [kg/m^3]	
Protein 1%	7.2[1)]	(0.5)[2)]	33.7[3)]	(3.1)
Boric acid 1%	7.4	(0.4)	35.5	(3.2)
Propionic acid 4%	30.4	(4.0)	36.0	(4.4)
Protein 1% and boric acid 1%	14.9	(0.8)	32.3	(3.0)
Protein 1% and propionic acid 4%	35.7	(4.4)	30.1	(4.4)

[1)] mean value calculated for the population of 192 samples
[2)] standard deviation
[3)] mean value calculated for the population of 64 samples

Samples were stored in air-conditioned rooms for two weeks until the wood reached air dry moisture content. Because of the applied three different methods of fixation of the biologically active component, the total number of wood blocks was divided into three series. The first series was subjected to a secondary treatment with 5% solution of the tannic acid in order to denature protein. The process of the secondary treatment was performed in a way similar to the primary treatment employing the vacuum method and maintaining identical technological parameters. After the secondary treatment, samples were conditioned for 2 weeks. The second series of samples was heated at the temperature of 100 °C for 4 hours. The third sample series was treated as reference material; therefore, samples were subjected neither to thermal not chemical treatment.

Biological testing

The test with the "bait" was conducted according to the author's own methodology, although partly utilising the experiments carried out by Kleist and co-workers (Kleist et al. 2002) as well as the general methodological assumptions of the EN 113 (1996) standard. These experiments were designed to compare the rate of growth of the mycelium hyphae into the interior of the sample treated with the preservative containing protein against wood treated with the active constituent, without protein. The "bait", in the form of toothpicks, was introduced into all samples by boring in their centres a hole 3 mm in diameter and 40 mm deep along fibres. Toothpicks were 2 mm in diameter and were made of beech wood. They were characterised by high susceptibility to the colonisation with the test fungus as confirmed by initial tests. The hole as well as both cross sections were glued with epoxy resin to prevent mycelium hyphae from overgrowing along fibres. The applied epoxy resin did not affect the growing of the test fungus.

Instead of the traditional sample sterilisation with water steam or gamma irradiation, the samples were subjected only to UV disinfection for 12 h because of the need to avoid undesirable denaturation of protein in wood. Samples prepared in this way were subjected to the action of the fungus for the period of 1 to 9 weeks. Samples were monitored once a week checking the degree of overgrowing by putting the bait of sterile agar substrate in Petri

dishes. After the successive weeks of exposure of samples to the action of the test fungus, the bait was recovered from samples in sterile conditions and then placed on solidified, sterile agar medium. In the cases of bait colonisation, the development of mycelium was observed for three days from the moment it was placed on the medium.

The loss of weight against the test fungus was determined for an independent sample batch which was prepared together with samples to investigate the rate of the mycelium overgrowing. The samples had the same dimensions and were subjected to the same treatments as described earlier. The time of exposure to the action of the fungus was adequate to the time of monitoring of the mycelium growing into the inside of samples. This allowed extrapolating the results of weight losses in the time interval between the 4th and 9th weeks of the mycelium action. The calculation of the weight loss was performed on the basis of methodological assumptions found in the EN 113 standard and are presented in the form of diagram (Fig. 1).

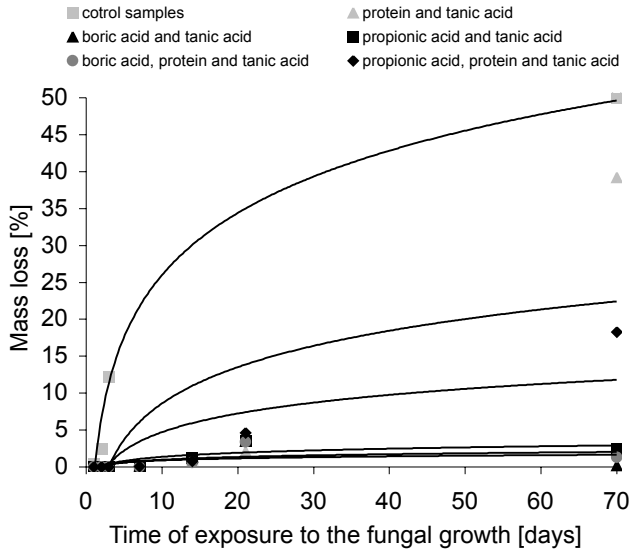

Fig. 1: Wood weight loss during the rate of mycelium growth into samples

RESULTS AND DISCUSSION

The fastest overgrowing of mycelium was recorded in the case of the control samples (untreated). Mycelium hyphae overgrew to the inside of samples after the 6th day of exposure to the action of the tested fungus and colonised 50% of baits (Tab. 2). The mean weight loss of these samples amounted to 0.5% (Fig. 1). During the consecutive three days of sample exposure to the action of the mycelium, the proportion of the colonised baits increased to the level of 85% (Tab. 2) and the weight loss reached the level of 12.2% (Fig. 1).

Tab. 2: The rate of mycelium growth into untreated wood

Samples	Treatment	Time of exposure to the fungal growth [day]							
		2	3	4	5	6	7	8	9
		Colonized baits [%]							
Control	Seasoning	67	100	100	100	100	100	100	100

The lowest resistance, expressed by the shortest time of overgrowing, was observed in the case of samples treated with 1% protein solution. The total of 67% of baits was colonised after the second week of sample exposure to the action of the test fungus (Tab. 3). This refers both to samples air dried after the treatment and those heated in order to denature protein. Overgrowing hyphae were found on all baits already after the third week of exposure of both types of samples to the action of the mycelium. Samples retreated with the tannic acid exhibited a slightly higher resistance to the overgrowing of the mycelium and 50% of them were found overgrown after the 6th week, whereas after week 8 there were 100% of baits found colonised. Different results were obtained for samples treated with 1% solution of the boric acid. In spite of the low concentration of the active constituent in wood, the samples showed resistance to the overgrowing of mycelium hyphae during the entire period of the experiment. None of the samples showed infection symptoms even after the 9th week of exposure to the action of the mycelium. Wood treated with 4% propionic acid showed resistance to the mycelium overgrowing up to the 6th week, when 50% of baits were found colonised (Tab. 3), whereas after the 8th week there were 100% of baits colonised. Wood treated with the same acid but heated, exhibited lower resistance and already in the 5th week 50% of baits were found colonised. The highest resistance was observed in the samples retreated with tannic acid as no bait colonisation was recorded during the entire period of experiment, i.e. 10 weeks. The remaining two variants constitute the examined preservative preparations subjected to verification with regard to the resistance against overgrowing by mycelium hyphae. The first of them is a mixture of boric acid in which the proportion of parts by mass amounted to 1% and protein of the same proportion of part by mass. Wood treated with this mixture exhibited complete resistance to the mycelium overgrowing during the entire experiment. The second of the two tested preparations containing 4% propionic acid and 1% protein was nearly completely effective in its protection of wood against mycelium overgrowing but only in the situation when the wood was retreated with tannic acid (Tab. 3). In this case, 50% of baits were colonised after the 9th week of exposure to the fungus action. Wood treated with this formulation and subjected to heating exhibited resistance up the 7th week, whereas wood which did not undergo secondary thermal or chemical treatment remained resistant up to the 5th week.

Although it is commonly accepted that the rate of the overgrowing of mycelium hyphae deep into the wood structure are not correlated with the wood weight losses which determine the degree of its decomposition (Cartwright and Findlay 1950), nevertheless the performed experiment allows answering the question found in the thesis of this paper.

With reference to the rate of overgrowing of the mycelium hyphae into wood, no differences were observed between the wood treated with boric acid and wood treated with the mixture of this acid and protein. In both cases, the examined wood showed resistance to the overgrowing of mycelium throughout the entire period of the experiment. It is also known from earlier studies that there are no differences in the weight loss of wood treated

with boric acid or the mixture of this acid and protein in the result of action of the same fungus species (Mazela et al. 2005). On this basis, it can be concluded that the presence of protein in the amount of 1% protein failed to change the resistance to the mycelium overgrowing into the treated wood. In additions, the presence of this component did not affect significantly fungicidal properties.

Tab. 3: The rate of mycelium growth into treated wood

Formulation	Treatment	Time of exposure to the fungal growth [week]							
		2	3	4	5	6	7	8	9
		Colonized baits [%]							
Protein 1%	Seasoning	67	100	100	100	100	100	100	100
	100°C; 4 h	67	100	100	100	100	100	100	100
	Tannic acid	0	0	0	0	50	50	100	100
Boric acid 1%	Seasoning	0	0	0	0	0	0	0	0
	100°C; 4 h	0	0	0	0	0	0	0	0
	Tannic acid	0	0	0	0	0	0	0	0
Propionic acid 4%	Seasoning	0	0	0	0	50	50	100	100
	100°C; 4 h	0	0	0	50	100	100	100	100
	Tannic acid	0	0	0	0	0	0	0	0
Protein 1% + boric acid 1%	Seasoning	0	0	0	0	0	0	0	0
	100°C; 4 h	0	0	0	0	0	0	0	0
	Tannic acid	0	0	0	0	0	0	0	0
Protein 1% + propionic acid 4%	Seasoning	0	0	0	50	50	50	100	100
	100°C; 4 h	0	0	0	0	0	100	100	100
	Tannic acid	0	0	0	0	0	0	0	50

A similar regularity was observed when wood samples were treated with the propionic acid and the mixture of this acid with protein. In both cases, the experimental wood maintained its resistance up to the fifth week. It is true that in this case the observed weight losses differed dramatically from the wood treated with boric acid (Mazela et al. 2005). Nevertheless this fact does not mean that the presence of protein supported mycelium growing into the wood but indicates that the treatment mixtures were characterised by poor fungicidal properties due to the low acid concentration.

Samples treated secondarily with tannic acid, in the case of both of the examined preservatives, exhibited higher resistance to the mycelium overgrowing in comparison with samples which were not subjected to the secondary thermal or chemical processing. Apparently, it appears that the tannic acid – due to its well-known fungicidal properties (Dirol and Scalbert 1991, Militz and Homan 1993) - can reduce the fungicidal value of the treatment formulation simultaneously delaying the moment of the mycelium growing into the interior of wood. However, the above-mentioned good fungicidal properties of tannins are limited by the utilisation conditions of the treated wood in hazard class 3 (Dirol and Scalbert 1991). The fungicidal value of wood treated with tannic acid and subjected to leaching is significantly

higher (Mazela et al. 2005). Therefore, there is no doubt that the involvement of the tannic acid in the process of wood treatment with mixtures of the active substance and protein is significant from the point of view of the fixation of a given active substance by means of protein denaturation.

CONCLUSIONS

The presence of 1% (w/w) globular protein in the form of animal blood plasma did not reduce time of the *C. puteana* mycelium hyphae overgrowing through the treated wood. It goes to prove that protein contained in the preservative does not makes wood more attractive to the overgrowing of mycelium hyphae into wood and does not limits the fungicidal properties of the impregnating mixture.

Wood treated with the mixture of boric acid and protein exhibited full resistance to the mycelium overgrowing throughout the period of the experiment. Wood treated with the mixture of propionic acid and protein showed worse resistance to the mycelium overgrowing of the test fungus. This is evident in lower fungicidal properties of this mixture.

The applied chemical protein denaturation by way of the secondary treatment using tannic acid turned out to be the most effective method of fixation of the active substance in wood.

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Bartłomiej Mazela
Agricultural University of Poznań
Institute of Chemical Wood Technology
Wojska Polskiego 38/42
Pl-60637 Poznań
Poland
Tel. +48618487459
Fax: +48618487452
E-mail: bartsimp@au.poznan.pl

Izabela Ratajczak
Agricultural University of Poznań
Department of Chemistry
Wojska Polskiego 75
Pl-60625 Poznań
Poland

Maria Bartkowiak
Agricultural University of Poznań
Institute of Chemical Wood Technology
Wojska Polskiego 38/42
Pl-60637 Poznań
Poland