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ORIGINAL ARTICLE

Industrial assays to evaluate the efficacy of vacuum pressure impregnation with commercial wood preservatives to eliminate the pinewood nematode, *Bursaphelenchus xylophilus*, and other nematodes from *Pinus pinaster* wood

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Abstract
To demonstrate the efficacy of the vacuum pressure impregnation (VPI) with commercial wood preservative products to eliminate the quarantine organism, pinewood nematode (PWN), *Bursaphelenchus xylophilus*, and other nematodes from maritime pine (*Pinus pinaster*) wood, *in vitro* assays and industrial assays in horizontal industrial autoclave tanks were conducted. *In vitro* nematicidal activity assays through direct exposure of the PWN 3rd-stage dispersal juveniles, the resistance juvenile stage, extracted from naturally infected *P. pinaster* revealed 100% nematode mortality with three commercial wood preservatives. Nematode mortality was also assessed in VPI industrial assays with the three commercial wood preservatives using naturally PWN infected *P. pinaster* experimental units, with various diameters and sizes. After VPI treatment, the nematode mortality ranged from 99.9761 to 100%. After incubation, the mortality of the total number of nematodes increased and, in all sections, the nematode mortality was higher than 99.9981% and in some it was 100% indicating that wood impregnated with preservative products does not constitute an environment favorable to the reproduction and development of nematodes. Overall, our findings demonstrated that the efficiency of the VPI process results from the joint action of the physical effect of pressure and vacuum and of the nematicidal effect of the preservative product. VPI treatment can be considered a valuable approach to eliminate PWN and other nematodes from maritime pine wood avoiding the subsequent application of the heat treatment.

1 Introduction

The introduction of quarantine pathogens into non-native areas has led to the development and application of quarantine procedures worldwide. Research has been conducted to develop phytosanitary methods to guard against the introduction and spread of these organisms. Presently, numerous pathogenic organisms are transported around the world through the international trade of wood and wood by-products. Some of these organisms are responsible for major economic losses and have also caused significant ecological impacts in forested areas. The pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner and Bührer, 1934) Nickle, 1970 (Nickle et al. 1981), the causal agent of the pine wilt disease (PWD), is one of these pathogens considered a A2 quarantine organism by the European and Mediterranean Plant Protection Organization (EPPO) and responsible for great losses in coniferous forests (EPPO 2013, 2022).

Since the international transport of wood and wood packaging material has been recognized as one of the most important means for PWN dispersion to non-affected areas,

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the International Plant Protection Convention (IPPC) has adopted technical requirements for the treatment of wood and wood packaging material (crates, boxes, packing cases, dunnage, pallets, cable drums and spools/reels) following the International Standards for Phytosanitary Measures No. 15 (ISPM 15). This standard describes phytosanitary measures that reduce the risk of introduction and spread of quarantine pests associated with the movement in international trade and establishes the basis for the harmonization of international phytosanitary measures, specifying the treatments for quarantine organisms that are harmful to forests, namely the PWN (FAO 2013).

According to the ISPM 15, all wood packaging material must be subjected to heat treatment (HT), microwave dielectric heating, fumigated with methyl bromide (restricted in many countries) or sulfuryl fluoride. The HT is the most widely used process worldwide and is conducted in industrial heat chambers with the requirement that the wood, including its core, reaches a minimum of 56 °C for 30 continuous minutes (FAO 2013). Nevertheless, the IPPC identified the need to develop and implement alternative treatments. Wood impregnation under vacuum pressure impregnation (VPI) with commercial wood preservative products, used worldwide for wood protection against wood decay organisms (insects, fungi, bacteria), can be effective to eliminate PWN from wood at industrial scale, eliminating the posterior use of HT. This impregnation process permits: (i) durability; (ii) deep penetration of the preservative product; (iii) controlled treatment conditions and (iv) processes adapted to industrial and large-scale production. Most of the wood is impregnated in industrial treatment autoclave tanks by a process called full-cell process or Bethell Method. In this process, the preservative product is applied by vacuum and pressure cycles and the wood cells are filled with the impregnation agent to receive the largest possible amount of preservative product inside the wood (Lebow and Anthony 2012; Kirker and Lebow 2021).

In Europe, chemicals used for wood preservation must be approved and validated by the European Chemicals Agency (ECA) and within this agency there are chemicals approved with different applications and toxicities. Nowadays, most of the commercial brands are composed of a mixture of different substances, including biocides, fungicides, and bactericides.

Data on the effectiveness of the wood impregnation process with a commercial preservative product by vacuum and pressure in the elimination of PWN and other nematodes (100% of mortality) have already been achieved in an industrial assay performed with naturally infected trunks with 15 cm diameter (Fonseca et al. 2017). In addition, *in vitro* laboratory assays conducted with several commercial wood preservatives using PWN inoculated wood blocks also revealed effectiveness in the PWN elimination, concluding that wood impregnated with preservative products does not represent a risk for PWN dissemination (Arcos et al. 2015).

In Portugal, since 1999, when the PWN was reported, in *Pinus pinaster* Aiton, in the Setúbal Peninsula (Mota et al. 1999), all industrial wood impregnation companies have faced a problem. All wood impregnated with preservative products used for sleepers, poles, fences, shingles, decking, cladding, windows, log cabins, and many other applications also need to be subjected to an additional HT, to comply with the requirements of the ISPM 15, increasing costs and production time. In view of this situation, and exploring the scientific results already reported, a multidisciplinary working group was created to evaluate the efficacy of wood impregnation treatment by pressure and vacuum with preservative products in the elimination of PWN and other nematodes from maritime pine wood. This working group, led by the *Associação das Indústrias de Madeira e Mobiliário de Portugal* (AIMMP), is composed of nematologists, wood technicians and industrial wood impregnation companies.

The main objectives of this working group were to evaluate the nematicidal activity of commercial wood preservative products and, to assess the effectiveness of the VPI treatment with different preservative products in the elimination of PWN and other nematodes from *P. pinaster* wood.

2 Materials and methods

2.1 Selection of trunks naturally infected with PWN

Fifty declining *P. pinaster* trees were identified and their trunks debarked and stored. Only the trunks with diameter, at least at one of the ends (base or top) equal or greater than 25 cm, corresponding to the highest diameter of the trunks treated in the companies, were selected for the extraction, identification, and quantification of PWN and other nematodes. Circular sections, 2–3 cm thick, were sawed from each end of each trunk, and 100 g of wood from each section was sampled and nematodes extracted using the tray method (Whitehead and Hemming 1965; EPPO 2013). The identification of PWN was based on female and male morphological diagnostic characters (female tail terminus shape, vulval flap presence and male spicules) (EPPO 2013, 2022). The identification of the other nematodes was based on the main morphological characters (anterior end, stylet, vulva, spicules, posterior end) and followed the nematode classification by Hodda (2022). The quantification of *B. xylophilus* and other nematodes was carried out under an inverted stereoscopic microscope.

The number of *B. xylophilus*/g of wood was calculated to verify whether this number was between 100 and 20 000/g of wood and with more than 60% of 3rd -stage dispersal juveniles (JIII), according to the technical requirements for the development and validation of wood treatments (Magnusson and Schröder 2009). Then, the selected trunks (6) with a high density of PWN (more than 100 PWN/g) were stored at the industrial companies in a covered and dry location to reach the optimum moisture content for impregnation (approximately 25%). The wood moisture content in each trunk was measured in three spots using a digital wood moisture meter (Gann HT65, Germany), and the wood moisture content of each trunk was estimated by using the average of three measurement values. When the trunks reached the optimal moisture content value to impregnate the wood in an autoclave, only three trunks (Trunks 4, 6 and 8) displayed a high population density of PWN and other nematodes. The trunks 4, 6 and 8 were then used in the industrial assays.

2.2 Wood preservatives

Three copper-based preservative products (Table 1) were supplied by the three companies where the industrial wood impregnation assays were performed. The preservative concentrations and retention values used in the industrial assays were the ones applied routinely in the three companies for exterior timbers used in permanent contact with ground or fresh water (Class 4SP) (Table 1).

2.3 Nematicidal activity of preservative products-*in vitro* assays

The nematicidal activity of the three preservative products was assessed in three *in vitro* laboratory assays. through direct exposure of JIII nematodes extracted from naturally infected *P. pinaster* wood. This juvenile stage is the most common developmental stage in naturally infected wood and considered the most resistant stage (EPPO, 2022).

After extracting the nematodes from the wood, using the tray method (EPPO 2013), the aqueous suspension was observed under an inverted stereoscopic microscope and the JIII were separated.

Each treatment consisted of five replicates, with tap water used as a control. In each of the replicates, 1 mL of the preservative product was used with the concentration applied in the impregnation (preservative X – 4.3%; preservative Y – 5%; preservative Z – 5.8%), in each of the companies. The product was transferred to excavated glass blocks and kept in a humid chamber, in the dark, during the assay. Twenty JIII were transferred to five glass blocks/treatment, corresponding to 100 nematodes/treatment, and nematode mortality was monitored. Nematodes that were distended and showed no movement when touched with the eyelash were transferred to tap water and considered dead only when, after one hour, no movement was detected (Esteves et al. 2017; Maleita et al. 2017).

In the 1st assay, the treatments were carried out with the three preservative products, supplied by the companies, and nematode mortality was monitored and quantified at 0, 2, 4, 6, 8, 10, 12 and 14 h. In the 2nd assay, nematode mortality, after exposure to the preservative products X and Y, was monitored and quantified after 30, 60, 75, 90 and 120 min, since that, in the 1st assay, the 1st observation was only performed after 2 h of exposure to the preservatives, where 100% mortality was detected. In the 3rd assay, to confirm the nematicidal effect of the three preservative products, photographic records of PWN (mixed developmental stages) directly extracted from infected wood were obtained after 30 min of exposure to each of the preservative products, using a stereoscopic microscope with a digital camera (Leica M80, IC80 HD, Leica Microsystems, Germany).

2.4 Industrial assays

Trunk 4 was selected to be used with water impregnation and named W4. Trunk 6 that had a high diameter corresponding to a high weight and number of nematodes was divided in half. One of the halves was used in the impregnation assay with the preservative product X and designated as X6. The other half, designated as Y6, was used in the impregnation assay with the preservative product Y. Trunk

Table 1 Wood preservatives composition, concentrations and retention values used in the industrial impregnation assays

Preservative product	Main components	Concentration (%)	Retention (Class 4SP) (Kg/m^3)
X	Basic copper carbonate 17.3% Cyproconazole 0.1%, N-alkyldimethylbenzylammonium chloride 4.75%	4.3	22.5
Y	Basic copper carbonate 18.3% Didecylmethylpoly(oxyethyl)ammonium propionate 10.6%	5.0	23.7
Z	Basic copper carbonate 14.55%, Propiconazole 0.16%Tebuconazole 0.16%, Didecyldimethylammonium chloride, DDAC, 0.5%	5.8	28.6

8 was used in the impregnation assay with the preservative product Z and named as Z8.

Afterwards, the trunks W4, X6, Y6 and Z8 were divided into two sections (W4-T/W4-B, X6-T/X6-B, Y6-T/Y6-B and Z8-T/Z8-B), representative of the top (T) and of the base (B). The two sections of each trunk were divided again to obtain four experimental units/treatment (W4-T1, W4-T2, W4-B1, W4-B2; X6-T1, X6-T2, X6-B1, X6-B2; Y6-T1, Y6-T2, Y6-B1, Y6-B2 and Z8-T1, Z8-T2, Z8-B1, Z8-B2), which were measured, weighed, and used as replicates. To estimate the number of nematodes in each experimental unit, before treatment, a slice approximately 3–4 cm thick, of the corresponding sections, was removed and weighed. The nematodes were extracted, identified, and quantified as described above. The number of nematodes (*B. xylophilus* and other nematodes) was estimated/100 g of wood. Before each assay, the moisture content in each of the experimental units was determined as described before.

Then, the four naturally infected experimental units were introduced into industrial autoclaves for VPI with water and the preservative products X, Y and Z, at room temperature (Fig. 1). In the case of VPI with water, the experimental units were introduced into an empty autoclave (Fig. 1a). In the case of the preservative products, the experimental units were introduced together with wooden posts included in a real treatment (Fig. 1b-d).

2.5 VPI treatment conditions

The VPI treatments with water and with the three preservative products were carried out in horizontal industrial autoclaves at room temperature under vacuum and pressure, being the treatment cycles pre-defined and programmed according to the specifications of the companies where the treatments were conducted (Table 2).

2.6 Nematode extraction and quantification after VPI treatment

After VPI, the four impregnated experimental units were removed from the industrial autoclave, left to drain for 3 days. Each of the four experimental units/treatment was divided into two obtaining eight experimental units/treatment. These units were cut into slices of approximately 3–4 cm, placed in plastic bags, labelled, and transported to the NEMATO-lab for extraction, identification, and quantification of nematodes. Four experimental units were analysed two to five days after the VPI treatment and four after

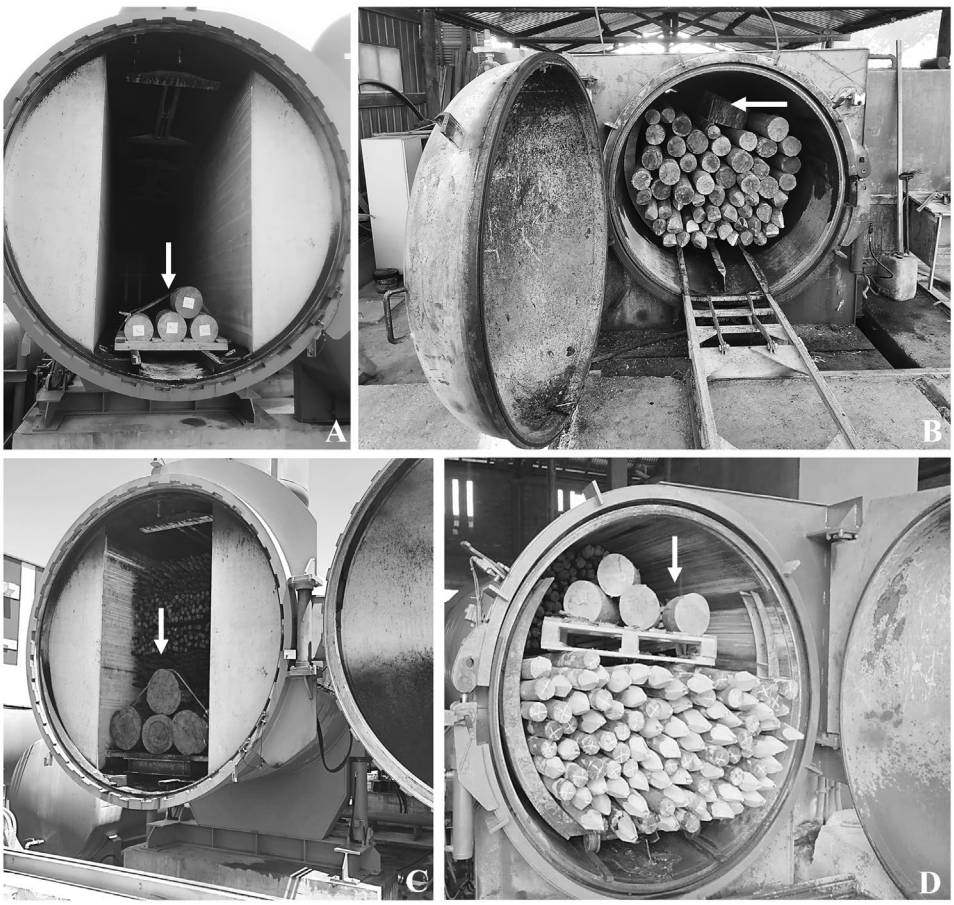

Fig. 1 Wood experimental units in horizontal industrial autoclaves for vacuum pressure impregnation treatment with water (**a**), preservative X (**b**), preservative Y (**c**) and preservative Z (**d**)

Table 2 Treatment cycles (vacuum and pressure) used in the impregnation with water and three preservative products in sections of *Pinus pinaster* wood naturally infected with the pinewood nematode, *Bursaphelenchus xylophilus*

Impregnation agent	Treatment cycle		
	Initial vacuum (minutes/bar)	Impregnation (minutes/bar)	Final vacuum (minutes/bar)
Water	35/0.85	45/12	35/0.85
Preservative X	15/0.85	75/12	15/0.85
Preservative Y	35/0.85	45/12	35/0.85
Preservative Z	15/0.85	75/12	15/0.85

incubation for 21 days at 20–25 ºC to allow for the hatching of the juveniles and/or reproduction of nematodes (EPPO 2013; Bonifácio et al. 2014; Fonseca et al. 2017). In the case of VPI with water, the extraction, identification, and quantification of nematodes after incubation were not conducted as the number of nematodes recovered from the VPI treated wood was high.

The initial number of nematodes in each of the eight experimental units was estimated considering the weight and the total number of nematodes existing in each of the experimental units before treatment. After the VPI treatment and after the incubation period, the respective slices were cut into small pieces of wood (no larger than 1 cm in width, using a grafting scissor), and the nematodes were extracted, identified, and quantified as previously described.

2.7 Data analyses

In the *in vitro* assays, data on nematicidal activity of the three preservative products were converted to corrected mortality percentage, corrected by Schneider Orelli's formula with reference to water, used as experimental control (Schneider-Orelli 1947):

$$\text{Corrected Mortality } \% = \frac{(\%\text{ mortality in treatment} - \%\text{ mortality in control})}{(100 - \%\text{ mortality in control})} X100$$

The effects of the three preservative products on nematode mortality in *in vitro* assays were compared in one-way analysis of variance (ANOVA) followed by post-hoc Fisher's least significant difference (LSD) statistical test.

In the industrial assays, nematode mortality percentage with the water and with the three preservative products was calculated directly using the following formula:

$$\text{Mortality } \% = \frac{(n^{o}\ nematodes\ before\ treatment - n^{o}\ nematodes\ after\ treatment)}{(nematodes\ number\ before\ treatment)} \times 100$$

In this case, the mortality was not corrected with reference to water, because the water was considered as treatment.

The % of mortality of the different VPI treatments was compared statistically based on one-way analysis of variance (ANOVA) followed by post-hoc Fisher's LSD statistical test. Statistical analyses of the data were performed using SPSS Statistics version 26.0 for Windows.

3 Results

3.1 Nematicidal activity of preservative products-*in vitro* assays

In the 1st assay, the results revealed that the three preservative products have nematicidal properties, with 100% mortality being obtained with the three preservatives. However, the effectiveness of nematicidal activity was higher with X and Y, with 100% mortality after 2 h of exposure. For Z, 100% mortality was only achieved after 14 h of exposure (Fig. 2).

In the 2nd assay, it was possible to detect differences in the nematicidal activity of the two preservatives (X and Y). With the preservative X, 100% mortality was achieved after 2 h of exposure, but with Y, 100% mortality was reached after 30 min of exposure (Fig. 3).

In the 3rd assay, 100% mortality was detected in nematodes exposed to preservative Y after 30 min of exposure (Fig. 4c), as in the previous assay, while in preservative X most were dead and some were still alive with the snaking movement, characteristic of nematodes (Fig. 4b). Contrary, in the preservative Z, most of the nematodes were alive and showing movement (Fig. 4d), and in the control all nematodes were alive (Fig. 4a).

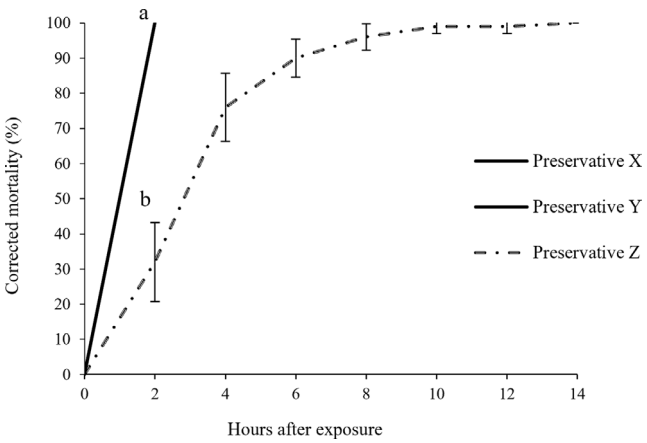

Fig. 2 Corrected mortality of *Bursaphelenchus xylophilus* (3rd-stage dispersal juveniles, JIII) exposed to the preservative products X, Y and Z. Data represent the mean of five replicates and the bars represent standard deviations. Means followed by different lower case letters, at the same exposure time, differ significantly ($p < 0.01$) according to the Fisher's LSD test

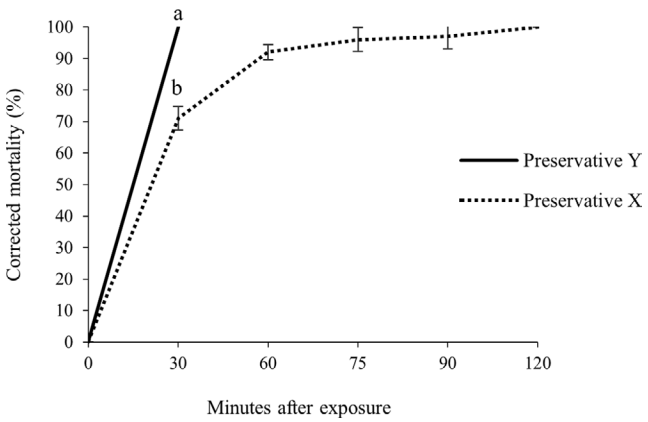

Fig. 3 Corrected mortality of *Bursaphelenchus xylophilus* (3-rd stage dispersal juveniles, JIII) exposed to the preservative products X and Y. Data represent the mean of five replicates and the bars represent standard deviations. Means followed by different lower case letters, at the same exposure time, differ significantly ($p < 0.01$) according to the Fisher's LSD test

3.2 Industrial assays

3.2.1 Water VPI treatment

In the treatment with water, the identification and quantification of nematodes (*B. xylophilus* and other nematodes)/100 g of wood before and after the treatment are shown in Table 3, being the number of *B. xylophilus* before treatment found to be greater than 10 000 nematodes/100 g of wood, corresponding to more than 100 nematodes/g according to the technical requirements for the development and validation of wood treatments and targeting the density of at least 100 000 nematodes/experimental unit (Follett and Neven 2006; Magnusson and Schröder 2009; Schröder et al. 2009; Haack et al. 2011; Schortemeyer et al. 2011; Bonifácio et al. 2014). Other nematodes belonged to the orders Rhabditida and Panagrolaimida, including nematodes from families Rhabditidae, Aphelenchoididae and Aphelenchidae were found (Hodda 2022). The results revealed that the percentage of elimination of *B. xylophilus* ranged from 77.82 to 84.81% and the percentage of elimination of the total number of nematodes ranged from 80.2 to 87.84% (Table 3).

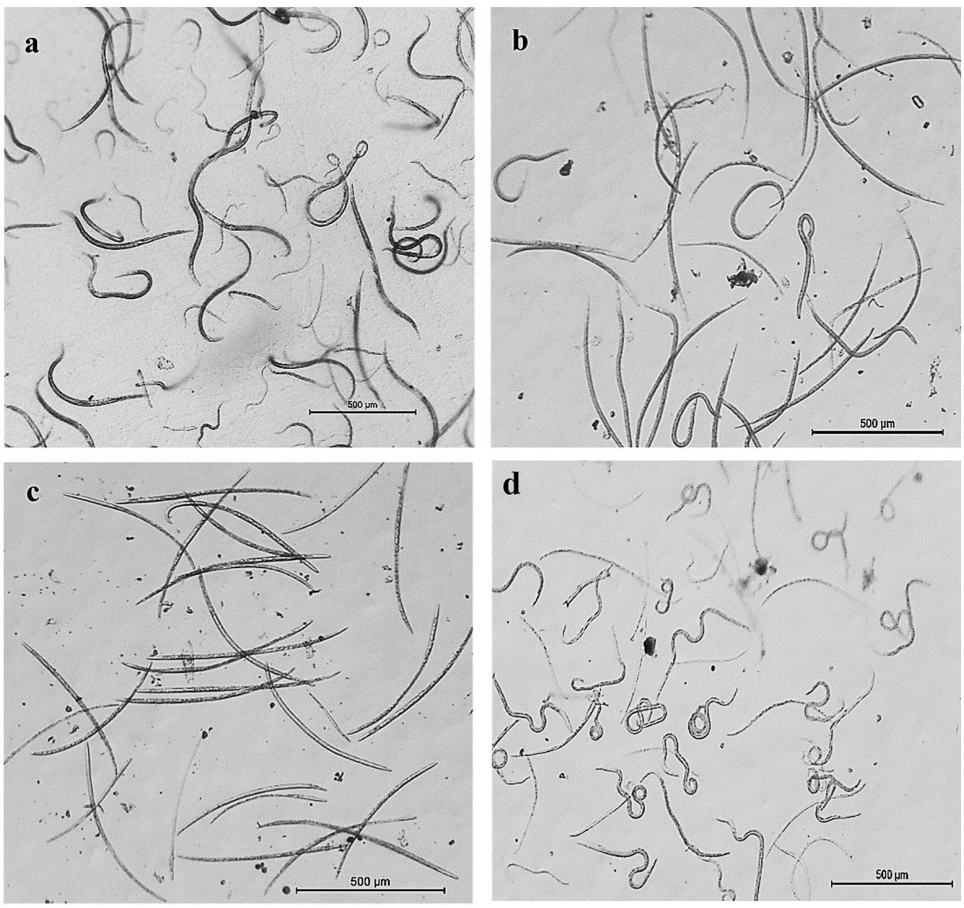

Fig. 4 Stereoscopic microscope photographs of *Bursaphelenchus xylophilus* (mixed developmental stages) after 30 min of exposure to water (**a**) and to the preservative products X (**b**), Y (**c**), Z (**d**)

3.2.2 Preservative X VPI treatment

The number of nematodes (*B. xylophilus* and other nematodes) was estimated/100 g of wood, and it was found that the number of JIII in the four experimental units was higher than 74% (X6-T1–74.3%, X6-T2–75.9%, X6-B1–77.9%, X6-B2–78%). Despite the number of *B. xylophilus* being higher than 10 000 nematodes/100 g of wood, only in one of the experimental units, the total number of nematodes/experimental units exceeded largely the 100 000 nematodes (data not shown). Nematodes (*B. xylophilus* and other nematodes) identification and quantification was carried out as described previously. The results of nematodes identification and quantification in the sections of the experimental units after VPI (X6-T1A, X6-T2A, X6-B1A, X6-B2A), after incubation (X6-T1B, X6-T2B, X6-B1B, X6-B2B) and the respective mortality percentages are shown in Tables 4 and 5, respectively.

After VPI treatment and after an incubation period, 100% mortality was not attained in all experimental units as some live nematodes were still detected.

After treatment, the mortality of the total number of nematodes (*B. xylophilus* and other nematodes) in the four sections (X6-T1A, X6-T2A, X6-B1A, X6-B2A) was higher than 99%, however only two of them (X6-T1A, X6-T2A) exceeded 99.9968% as required for treatment validation according to Probit 9 requirements (Table 4). After incubation, the mortality of the total number of nematodes increased and, in all experimental unit sections (X6-T1B, X6-T2B, X6-B1B, X6-B2B) it was higher than 99.9968%, and in one of them (X6-T2B) it was 100% (Table 5). It should be noted that, in the incubated sections, despite the detection of some live nematodes, there was no growth in the population, with a decrease in the number of nematodes.

3.2.3 Preservative Y VPI treatment

In this treatment, as in the previous one, it was found that the number of JIII in the four experimental units was higher than 74% (Y6-T1–79.3%, Y6-T2–74.9%, Y6-B1–77.8%, Y6-B2–80.7%). As in the previous industrial assay and although the number of *B. xylophilus* higher than 10 000 nematodes/100 g of wood was not detected in any of the experimental units (data not shown), the total number of nematodes in each of the experimental units exceeded largely 100 000 nematodes (Table 6).

Nematodes (*B. xylophilus* and other nematodes) identification and quantification were carried out as described previously. The results of nematodes identification and quantification in the sections of the experimental units after VPI (Y6-T1A, Y6-T2A, Y6-B1A, Y6-B2A) and after incubation (Y6-T1B, Y6-T2B, Y6-B1B, Y6-B2B) and the mortality

Table 3 Diameter, weight, length, moisture content and number of pinewood nematodes (PWN), *Bursaphelenchus xylophilus*, and other nematodes/100 g of wood from each experimental unit, before and after vacuum pressure impregnation (VPI) with water (W)

Experimental units	Diameter (cm)	Weight (Kg)	Length (cm)	Moisture (%)	Nº nematodes before VPI			Nº nematodes after VPI			Total nematodes mortality (%)	PWN mortality (%)
					PWN	Other	Total	PWN	Other	Total		
W4-T1	30	25	60	21.8	14 613	4935	19 548	3321	551	3872	80.20	77.28
W4-T2	30	20	50	20.8	14 798	4997	19 795	3137	490	3627	81.68	78.80
W4-B1	30.5	21	50	22.8	11 859	6709	18 568	1801	456	2257	87.84	84.81
W4-B2	30.5	27	60	23.7	11 844	6701	18 545	2384	263	2647	85.73	79.70

Table 4 Diameter, weight, length, moisture content and number of pinewood nematodes (PWN), *Bursaphelenchus xylophilus*, and other nematodes in four sections of the experimental units before and after vacuum pressure impregnation (VPI) with preservative X

Experimental unit sections	Diameter (cm)	Weight (Kg)	Length (cm)	Moisture (%)	Estimated nematodes number/section before VPI			Nematodes number/section after VPI			Mortality (%) after VPI		
					PWN	Other	Total	PWN	Other	Total	PWN	Other	Total
X6-T1A	42	19.3	14.5	22.6	1 627 376	125 643	1 753 019	11	15	26	99.9993	99.9881	99.9985
X6-T2A	42	16.4	12.5	25	1 696 908	124 148	1 821 056	15	0	15	99.9991	100	99.9992
X6-B1A	47	15.0	11	22.5	988 050	35 250	1 023 300	120	86	206	99.9879	99.7560	99.9799
X6-B2A	47	14.2	11	21.1	1 014 159	37 887	1 052 047	216	35	251	99.9787	99.9076	99.9761

Table 5 Diameter, weight, length, moisture content and number of pinewood nematodes (PWN), *Bursaphelenchus xylophilus*, and other nematodes in four sections of the experimental units before and after vacuum pressure impregnation (VPI) with the preservative X and incubation

Experimental unit sections	Diameter (cm)	Weight (Kg)	Length (cm)	Moisture (%)	Estimated nematodes number/section before VPI			Nematodes number/section after VPI and incubation			Mortality (%) after VPI and incubation		
					PWN	Other	Total	PWN	Other	Total	PWN	Other	Total
X6-T1B	42	19.5	14.5	22.6	1 644 240	126 945	1 771 185	3	0	3	99.9998	100	99.9998
X6-T2B	42	14.4	11	25	1 489 968	109 008	1 598 976	0	0	0	100	100	100
X6-B1B	47	15.1	12	22.5	994 637	35 485	1 030 122	9	0	9	99.9991	100	99.9991
X6-B2B	47	14.4	11.2	21.1	1 029 168	38 448	1 067 616	4	0	4	99.9996	100	99.9996

Table 6 Diameter, weight, length, moisture content and number of pinewood nematodes (PWN), *Bursaphelenchus xylophilus*, and other nematodes in four sections of the experimental units before and after vacuum pressure impregnation (VPI) with the preservative Y

Experimental unit sections	Diameter (cm)	Weight (Kg)	Length (cm)	Moisture (%)	Estimated nematodes number/section before VPI			Nematodes number/section after VPI			Mortality (%) after VPI		
					PWN	Other	Total	PWN	Other	Total	PWN	Other	Total
Y6-T1A	42	18.3	13.8	23.9	1 284 477	83 997	1 368 474	16	7	23	99.9988	99.9917	99.9983
Y6-T2A	42	13.8	10.5	24	1 126 080	58 098	1 184 178	4	0	4	99.9996	100	99.9997
Y6-B1A	47	13.9	11	24	820 656	19 321	839 977	1	0	1	99.9999	100	99.9999
Y6-B2A	47	15.5	12	22.2	939 145	47 895	987 040	0	0	0	100	100	100

percentages are shown in Tables 6 and 7, respectively. After VPI treatment and after the incubation period, 100% mortality was not achieved in all experimental units. After VPI and after the incubation period some live nematodes were still detected. After treatment, the mortality of the total number of nematodes (*B. xylophilus* and other nematodes) in the four experimental unit sections (Y6-T1A, Y6-T2A, Y6-B1A, Y6-B2A) was higher than 99.9968%, and in one of the sections (Y6-B2A) 100% (Table 6). After incubation, the mortality of the total number of nematodes increased in all sections (Y6-T1B, Y6-T2B, Y6-B1B, Y6-B2B) and it was higher than 99.9968%, and in two of them (Y6-T2B and Y6-B1B) it was 100% (Table 7). Similar to the assay with the preservative product X, there was no growth in the nematode population, after incubation.

3.2.4 Preservative Z VPI treatment

The number of nematodes (*B. xylophilus* and other nematodes) was estimated/100 g of wood, and the number of JIII in the four experimental units was higher than 63% (Z8-T1–64%, Z8-T2–68.1%, Z8-B1–63.6%, Z8-B2–67.2%). However, the number of *B. xylophilus* higher than 10 000 nematodes/100 g of wood was only detected in one of the experimental units (data not shown), but the total number of nematodes in each of the experimental units exceeded 100 000 nematodes. Nematodes (*B. xylophilus* and other nematodes) identification and quantification were carried out as described previously. The results of nematodes identification and quantification in the sections of the experimental units after VPI (Z8-T1A, Z8-T2A, Z8-B1A, Z8-B2A) and after incubation (Z8-T1B, Z8-T2B, Z8-B1B, Z8-B2B) and the respective percentages of mortality are indicated in Tables 8 and 9.

The results with the preservative product Z revealed that, after VPI and after the incubation period, 100% mortality was not achieved in all experimental units. After the treatment and after the incubation period some live nematodes were detected (Tables 8 and 9).

After VPI treatment, the % mortality of the total number of nematodes (*B. xylophilus* and other nematodes) in the four sections (Z8-T1A, Z8-T2A, Z8-B1A, Z8-B2A) was higher than 99%, however only two of them (Z8-T2A and Z8-B2A) exceeded 99.9968% (Table 8). After incubation, the mortality of the total number of nematodes increased and, in all sections (Z8-T1B, Z8-T2B, Z8-B1B, Z8-B2B), it was higher than 99.9968%, being in one of them (Z8-T2B) 100% (Table 9). It should be highlighted that in the incubated sections, as in the assays carried out with the preservative products X and Y, despite the detection of some live nematodes, no population growth was observed.

The means of the percentage of mortality of the total number of nematodes (PWN + other nematodes), of the four experimental units, in each VPI assay, after treatment and after the period of incubation (Table 10) revealed that the VPI assay with the preservative Y was the most effective, after treatment and after incubation, with means of percentage of mortality of 99.9995 and 99.9998, respectively. In the other VPI assays, with the preservatives X and Z, the means of percentage of mortality were lower than 99.9968% after the treatment. However, after the incubation, the percentage increased to 99.9996% and 99.9991%. Despite these differences, the differences found among the three assays were not statistically significant ($p > 0.005$) (Table 10).

4 Discussion

The *in vitro* assays, through direct exposure of JIII nematodes, the resistance juvenile stage, extracted from naturally infected wood indicated that the three preservative products have a strong nematicidal activity. However, the most effective preservative product was the product Y. This preservative product induced the mortality of all nematodes in only 30 min of direct exposure to the product. With the product X, 100% mortality was achieved after 2 h of exposure and the product Z was the least effective, with 100% mortality only obtained after 14 h of exposure. Differences in nematicidal activity may be attributed to the chemical composition of each of the preservatives. The preservative product Y has a higher percentage of ammonium in the propionate form of didecylmethylpoly (oxyethyl) ammonium and in dissolved copper in the form of basic copper carbonate. Several studies have already demonstrated the nematicidal activity of compounds rich in ammonium (Oka and Pivonia 2002; Oka et al. 2007; Renco et al. 2011; Wei et al. 2012; Su et al. 2015) and copper (Eloh et al. 2016; Mohamed et al. 2019; Yeon et al. 2019; Akhter et al. 2020) against plant-parasitic nematodes mainly belonging to the genera *Globodera* (potato cyst nematodes) and *Meloidogyne* (root knot nematodes). Regarding the PWN, Shoji (1985) showed the efficient eradication of the PWN in pine logs by application of metam-ammonium. Additionally, the effects of *in vitro* exposure to different concentrations of copper sulphate on the mortality and movement behaviour were evaluated, and the results proved that copper sulphate had strong effects at a low concentration (5 mg/L), revealing that copper sulphate is effective against PWN restricting their harmful effects on plants by increasing mortality and by inhibiting their movement (Tan et al. 2013).

In the industrial assays, conducted in horizontal industrial autoclaves, water (W) and wood preservatives (X, Y and Z) as impregnating agents, PWN naturally infected maritime

Table 7 Diameter, weight, length, moisture content and number of pinewood nematodes (PWN), *Bursaphelenchus xylophilus*, and other nematodes in four sections of the experimental units before and after vacuum pressure impregnation (VPI) with the preservative Y and incubation

Experimental unit sections	Diameter (cm)	Weight (Kg)	Length (cm)	Moisture (%)	Estimated nematodes number/section before VPI			Nematodes number/section after VPI and incubation			Mortality (%) after VPI and incubation		
					PWN	Other	Total	PWN	Other	Total	PWN	Other	Total
Y6-T1B	42	17.3	13	23.9	1 214 287	79 407	1 293 694	6	1	7	99.9995	99.9987	99.9995
Y6-T2B	42	16.4	12.5	24	1 338 240	69 044	1 407 284	0	0	0	100	100	100
Y6-B1B	47	13.2	10.5	24	779 328	18 348	797 676	0	0	0	100	100	100
Y6-B2B	47	12.3	9.5	22.2	745 257	38 007	783 264	0	1	1	100	99.9974	99.9999

Table 8 Diameter, weight, length, moisture content and number of pinewood nematodes (PWN), *Bursaphelenchus xylophilus*, and other nematodes in four sections of the experimental units before and after vacuum pressure impregnation (VPI) with the preservative Z

Experimental unit sections	Diameter (cm)	Weight (Kg)	Length (cm)	Moisture (%)	Estimated nematodes number/section before VPI			Nematodes number/section after VPI			Mortality (%) after VPI		
					PWN	Other	Total	PWN	Other	Total	PWN	Other	Total
Z8-T1A	25	9.5	27	27.5	695 400	86 165	781 565	26	13	39	99.9963	99.9849	99.9950
Z8-T2A	25	6.8	20.5	28.5	746 164	74 324	820 488	13	7	20	99.9983	99.9906	99.9976
Z8-B1A	26	11.8	32	27.5	921 934	151 040	1 072 974	41	15	56	99.9956	99.9901	99.9948
Z8-B2A	26	9.5	28	27.5	614 365	67 165	681 530	16	3	19	99.9974	99.9955	99.9972

Table 9 Diameter, weight, length, moisture content and number of pinewood nematodes (PWN), *Bursaphelenchus xylophilus*, and other nematodes in four sections of the experimental units before and after vacuum pressure impregnation (VPI) with the preservative Z and incubation

Experimental unit sections	Diameter (cm)	Weight (Kg)	Length (cm)	Moisture (%)	Estimated nematodes number/section before VPI			Nematodes number/section after VPI and incubation			Mortality (%) after VPI and incubation		
					PWN	Other	Total	PWN	Other	Total	PWN	Other	Total
Z8-T1B	25	9.3	26.5	27.5	680 760	84 351	765 111	5	9	14	99.9992	99.9893	99.9981
Z8-T2B	25	6.9	21	28.5	757 137	75 417	832 554	0	0	0	100	100	100
Z8-B1B	26	11.7	31.5	27.5	914 121	149 760	1 063 881	6	4	10	99.9993	99.9973	99.9990
Z8-B2B	26	9.0	26.5	27.5	582 030	63 630	645 660	5	0	5	99.9991	100	99.9992

pine trunks were used. In the 1st assay with water, it was verified that the elimination of *B. xylophilus* and other nematodes ranged from 77.82 to 87.84% (Table 3). Although there was not 100% elimination of nematodes, these results permitted to conclude that the physical action of vacuum and pressure has effect on the mortality of nematodes inside the wood. A previous laboratory study performed with naturally PWN infected wood sections (75 mm long/20 mm width) and with wood shavings revealed that high pressures (5 and 15 MPa) had effects on the survival of PWN inside the wood, and a pressure of 30 MPa caused 100% mortality (Fonseca et al. 2014), while in the industrial assays, carried out with the three preservative products, the maximum pressure used was lower (1.2 MPa).

In the VPI, with the preservative product Y, the time for pressure impregnation was 45 min, and the time used in each vacuum step was 35 min, and despite the greater diameter of the experimental units, the mortality of the total number of nematodes was higher than 99.9968% in all the sections (after treatment and after incubation) and with means of percentage of mortality of 99.9995 and 99.9998, respectively. Regarding the two other assays with the preservative products X and Z, the total time for pressure impregnation was much higher (75 min) and the time used for each vacuum step was lower (15 min). In these cases, after treatment, the mortality of the total number of nematodes, in the four sections analysed/treatment was higher than 99%, however two of them (X6-T1A, X6-T2A and Z8-T2A, Z8-B2A) exceeded 99.9968% (Tables 4 and 8). After incubation, with both preservatives, the mortality of the total number of nematodes was higher than 99.9968%, having increased in all sections (Tables 5 and 9). In these two VPI assays, the means of percentage of mortality were lower than 99.9968% after the treatment, but after the incubation the percentage increased to 99.9996% and 99.9991% (Table 10). Overall, these results indicate a greater efficacy of the VPI with the preservative product Y in the nematode mortality in all sections analysed (Tables 6, 7 and 10). In all VPI treatments with the three preservative products, after the incubation period, the percentage of mortality of the total number of nematodes was higher than 99.9968% in all experimental units, requirement for treatment validation, and in some of them it was 100%. Previous assays performed with the preservative Z revealed 100% efficacy in experimental units with 15 cm in diameter after treatment and incubation (Fonseca et al. 2017). The detection of live nematodes in a few experimental units could be due to disparities in the preservative product impregnation through all sapwood in the different experimental units. In order to improve the efficacy of the treatment, conditions (pressure, vacuum, and time) could be adjusted. In the VPI assays, the greater mortality with the preservative Y, corroborates the results obtained in the *in vitro* assays.

5 Conclusion

This study demonstrated the efficiency of the VPI with commercial wood preservative products to eliminate PWN and other nematodes from naturally infected wood. The efficiency of this wood impregnation process is dependent on the diameter of the wood, moisture content, preservative product, product concentration and vacuum/impregnation pressure and time and results from the joint action of the physical effect of pressure and vacuum and the nematicidal effect of the preservative product. Moreover, it should be highlighted that in the incubated experimental wood units with the three preservative products, there was no growth in the nematode population, with a decrease in the number of nematodes, suggesting that impregnated wood does not constitute an environment favourable for the development and reproduction of nematodes. Overall, our findings indicate that the physical action of vacuum and pressure plus the action of the preservative products clearly induce the nematode mortality inside the wood and that this process eliminates PWN and other nematodes from maritime pine wood at industrial scale, eliminating the need of a posterior use of the heat treatment.

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Table 10 Mortality percentage of total number of nematodes (mean ± standard deviation) of the three vacuum pressure impregnation (VPI) treatments with the three preservative products after treatment and after incubation. Statistical analysis based on one-way analysis of variance (ANOVA) followed by post-hoc Fisher's least significant difference (LSD) statistical test (p-value ≤ 0.05). Means followed by the same letters are not significantly different

VPI treatments	Mortality (%) after VPI	Mortality (%) after VPI and incubation
VPI + preservative X	99.9884 ± 0.0121[a]	99.9996 ± 0.0004[a]
VPI + preservative Y	99.9995 ± 0.0008[a]	99.9998 ± 0.0003[a]
VPI + preservative Z	99.9961 ± 0.0014[a]	99.9991 ± 0.0008[a]

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Declarations

Conflict of Interest All authors declared that they have no conflict of interest.

Ethics approval This article does not contain any studies with human participants or vertebrates performed by any of the authors.

Consent to participate Informed consent was obtained from all individual participants included in the study.

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