

EFFECT OF A CHITOSAN-ENRICHED BIOFERTILIZER ON INDUCED RESISTANCE AGAINST THE PINWOOD NEMATODE, *BURSAPHELENCHUS XYLOPHILUS*

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INTRODUCTION

The pine wood nematode (PWN, *Bursaphelenchus xylophilus*), is a microscopic worm native from North America, where it is not associated with pathogenic disease. However, in Portugal the PWN is responsible for the pine wilt disease (PWD), which affects mainly *P. pinaster* (maritime pine) trees, leading to the cut down of thousands of trees every year. *P. pinea* (umbrella pine) trees, on the other hand, present a high degree of resistance to the pathogen.

This pathogen was detected for the first time in Portugal in 1999 [1], probably due to the importation of timber from North America. Despite the measures taken at the time, the nematode has spread through the entire country [2], inclusively reaching some Northern Spanish regions [3].

The PWN is transported within the trachea of *Monochamus galloprovincialis* beetles and infects the trees through the beetles' feeding wounds during their feeding period, which occurs between May and September [4,5]. After invasion, the nematodes move rapidly from the point of entry into the tissues and penetrate through the resiniferous ducts, feeding on their epithelial cells [6]. At this stage, the population of nematodes remains low and no visible external symptoms appear for some time; this stage is called "primary stage". Within two days after the first contact, the majority of the nematodes has developed to the adult stage [7] and has begun to migrate, feed and reproduce within the resiniferous ducts (advanced stage; [8]). Once symptoms evolve to the advanced stage, the tree inevitably dies within 3/4 months.

As a result of nematode infection, the plants' vascular system can be blocked by the formation of secondary resin produced by radial parenchyma cells damaged by the nematode migration [9]. In addition, due to cavitation and embolism processes, there is also dysfunction of the water transport within the plant, which leads to water deficiency and other alterations in leaves and subsequent photosynthetic deficit, which was characterized as a symptom of advanced stage [10-12].

The exportation of timber and wood-manufactured products from countries affected by the PWD is greatly restricted and regulated, involving the need to inspect the timber and

submit it to high temperature treatments or even to the prohibition to export timber and wood-manufactured products [14]. However, these measures are very costly for the companies involved in the wood industry (about 16 million euros per year), turning them less competitive in relation to other countries. Thus, efficient and sustainable alternative methods for the control of the PWD are urgently needed.

Chitosan, a deacetylated derivative of chitin, is mainly obtained from the outer shell of crustaceans, such as crabs and shrimps [15,16], and has been used to enhance plant defenses against several microorganisms, including bacteria [17-19], fungi [17, 19, 20-22] and nematodes [23].

This compound induces plants' defense mechanisms, including biosynthesis of phytoalexins [24-27], callose [26, 28-30], lignin [31, 32] and other phenolic compounds [33-36]. Thus, exogenous application of chitosan seems to be extremely promising in controlling several plant diseases. Nevertheless, this potential was never evaluated for the PWN. The aim of this work was, therefore, to evaluate if the application of chitosan as a soil amendment to PWN infected plants could improve the plants' defenses against the disease.

MATERIALS and METHODS

PWN maintenance and inoculum preparation

The virulent *B. xylophilus* strain 65 GO was grown on barley grains with *Botrytis cinerea* mycelium at 25°C in the dark for 7 days. Nematodes were extracted overnight at room temperature using the Baermann funnel technique [37]. The nematode suspension was prepared according to Roriz *et al.* [38] in order to obtain a final concentration of approximately 1000 nematodes mL⁻¹.

Preparation of the biofertilizer enriched with chitosan

The enriched biofertilizer was produced by mixing biofertilizer from phosphate and potash rocks (PKB) and organic matter (OM) using beverage residue (AMBEV Industry) enriched in N by inoculation with selected diazotrophic bacteria and *Cunninghamella elegans* fungi, which contain chitin and chitosan in their cellular walls.

Plant maintenance and transplantation

During the experimental period, plants (approximately 1 year-old) were kept in an environmental growth chamber (Fitoclima 10 000 EHF, Aralab) under a 16-h light/8-h darkness photoperiod at 25°C/18°C, respectively, and 80% relative humidity. One month after acclimation, 36 plants of each species were transplanted to the enriched biofertilizer. For that, the soil was carefully removed from each plant and 12 plants of each species were transplanted into a commercial substrate (COMPO SANA ® Universal) mixed with 0 %, 7.5 % or 15 % of the enriched biofertilizer (v/v).

Inoculation

Five days after transplantation, 18 *P. pinaster* and *P. pinea* seedlings (12 for each biofertilizer concentration) were inoculated with 1000 PWN and other 18 plants with autoclaved deionized water (control) using the technique described by Asai & Futai [39].

Sample collection and processing

Seven and 24 days after inoculation (dai), 18 *P. pinaster* and *P. pinea* plants (3 for each inoculation and biofertilizer condition) were sacrificed for subsequent analysis: stems were

used for nematode quantification and leaves were collected and stored at -80°C for total chlorophyll, carotenoids, water content and total soluble phenolics analysis.

Nematode extraction and quantification

For assessment of nematode survival and reproduction, the stems of PWN-inoculated plants, collected as previously described, were cut into seven segments corresponding to the inoculation site, the segments up to 2 and 4 cm above and up to 2, 4, 6 and 8 cm below the inoculation site and nematodes were extracted and quantified following a protocol described by Nunes da Silva *et al.* [40]. The amount of nematodes present in each stem segment was determined taking into account the weight of each sampled segment (nematodes.g⁻¹) and the total amount of nematodes was calculated using the sum of the number of nematodes counted in each stem segment and the total stem biomass (sum of the biomasses of each individual stem segment).

Determination of total chlorophylls and water content

About 0.5 g of leaves (fresh weight), obtained as described above, were used for the quantification of total chlorophylls according to Sims and Gamon [41].

In addition, approximately 0.5 g of leaves (fresh weight) was lyophilized for 72 h and the water content was calculated as demonstrated by Tan *et al.* [42].

Statistical analysis

Means and standard errors were calculated using Microsoft Excel 2010 software. Significant differences were determined by Student's T-test in GraphPad Prism software (version 6.01, San Diego, CA). Statistical significance was determined at $p < 0.05$.

RESULTS and DISCUSSION

Nematode Population

The PWN is a microscopic worm that infects primarily pine trees, such as *P. pinaster*, causing the PWD. After reaching the inner tissues of the plant, the nematode population remains low for some time (the primary stage of the disease), beginning to feed on the epithelial cells of the resin ducts and reproduce (advanced stage of disease) afterwards [13]. This leads to the interruption of water flow and, subsequently, to the death of the plant. The dispersion pattern of the nematode population in *P. pinaster* and *P. pinea* trees inoculated with PWN, 7 and 24 dai is shown in Tables 1 and 2.

Seven dai, both *P. pinaster* and *P. pinea* trees, for all biofertilizer concentrations tested, had higher concentration of nematodes at the site of inoculation. However, in *P. pinaster* inoculated trees the nematodes were dispersed throughout all stem segments, whereas in *P. pinea* the nematode population was restricted to the inoculation site. This trend for the restriction of the nematode population to the inoculation site has already been described by Nunes da Silva *et al.* [40] in other plant species apparently resistant to nematode, thus confirming the resistant character of *P. pinea*. Twenty-four dai, there was a substantial increase in the density of nematodes in *P. pinaster* stems in the absence of chitosan, mainly in stem segments below the site of inoculation. This seems to indicate that nematodes successfully reproduced throughout the experimental period and migrated preferentially downwards, probably to search for fresh tissue from which they could feed. However, in *P. pinaster* plants grown in the presence of chitosan, there was no significant increase in the population of nematodes (Table 1 and 2). Nevertheless, the dispersion pattern of the nematodes throughout the stems remained identical to plants grown in the absence of

chitosan. These results seem to indicate that the presence of chitosan in the soil had a negative impact on nematode reproduction.

Table 1 – Nematode concentration (n° nematodes.g⁻¹ plant tissue) in each stem segment analyzed and total n° of nematodes in stems of inoculated *P. pinaster* plants . Each value is the mean of three replicates (mean ± standard error). For the total number of nematodes, values with the same letter are not significantly different.

Stem segment	7 dai			24 dai		
	0 %	7.5 %	15 %	0 %	7.5 %	15 %
[-8cm; -6cm]	57 ± 16	0	5 ± 4	4947 ± 289	149 ± 15	55 ± 8
[-6cm; -4cm]	160 ± 37	16 ± 11	10 ± 10	5639 ± 1159	88 ± 71	14 ± 2
[-4cm; -2cm]	142 ± 68	16 ± 9	16 ± 13	3241 ± 252	343 ± 56	46 ± 6
[-2cm; inoc]	77 ± 18	8 ± 4	25 ± 11	2235 ± 1082	180 ± 44	66 ± 8
inoc	686 ± 239	103 ± 74	53 ± 3	1145 ± 403	128 ± 11	0
[inoc; +2cm]	115 ± 53	45 ± 31	8 ± 8	1064 ± 528	160 ± 93	199 ± 39
[+2cm; +4cm]	96 ± 23	0	12 ± 12	112 ± 29	0	76 ± 62
TOTAL	199 ± 8 ^a	29 ± 17 ^b	20 ± 6 ^b	2433 ± 154 ^c	166 ± 11 ^{a, d}	68 ± 12 ^d

Table 2 - Nematode concentration (n° nematodes.g⁻¹ plant tissue) in each stem segment analyzed and total n° of nematodes in stems of inoculated *P. pinea* plants . Each value is the mean of three replicates (mean ± standard error). For the total number of nematodes, values with the same letter are not significantly different.

Stem segment	7 dai			24 dai		
	0 %	7.5 %	15 %	0 %	7.5 %	15 %
[-8cm; -6cm]	0	0	0	0	17 ± 14	24 ± 24
[-6cm; -4cm]	0	0	0	0	0	0
[-4cm; -2cm]	0	0	0	0	0	0
[-2cm; inoc]	8 ± 8	0	0	0	0	0
inoc	383 ± 383	95 ± 13	143 ± 127	15 ± 15	79 ± 21	94 ± 63
[inoc; +2cm]	0	20 ± 16	0	0	0	0
[+2cm; +4cm]	11 ± 11	0	0	0	0	0
TOTAL	38 ± 21 ^{a, b}	14 ± 2 ^{a, b}	23 ± 18 ^{a, b}	2 ± 2 ^a	16 ± 4 ^{a, b}	19 ± 5 ^b

In *P. pinea* plants, 24 dai, no significant differences were observed in the number of nematodes in all stem segments analyzed (Table 2). When compared with the data from 7 dai, it seems to indicate that the nematodes were not able to reproduce. However, 24 dai there was a significant increase in the total number of nematodes in plants treated with chitosan, comparing with untreated ones. Although this result seems to indicate that the ability of the nematode to reproduce appears to be promoted by the presence of chitosan, this result may be simply due to variations in the inoculation protocol between the 3 replicates for this experimental condition. In fact, only one of the replicates showed nematode numbers different from 0, which may have resulted from deficient inoculation, thus resulting in the present results.

Total chlorophylls and water content

Chlorophylls are photosynthetic pigments found inside chloroplasts and enable plants to convert solar energy into chemical energy. The decrease in the photosynthetic activity and chlorophyll content has been described during the development of the disease in plants infected with PWN [40, 44] and it is considered a symptom of the advanced stage of the disease induced by water stress in leaves [45]. Furthermore, it is suspected that the decrease in photosynthesis also decreases the resistance mechanisms against the PWN [12, 46].

In this study, there were no significant differences in the concentration of total chlorophylls in *P. pinea* plants, in both control and inoculated plants for all chitosan treatments tested [Table 3]. Thus, inoculation with PWN did not appear to interfere with the density of chlorophylls in inoculated *P. pinea* plants, probably due to their resistant nature.

Table 3 - Total chlorophylls ($\mu\text{mol.g}^{-1}$ plant tissue) in control and inoculated *P. pinea* plants 7 and 24 days after inoculation (dai). Each value is the mean of three replicates (mean $\pm$ standard error) and values with the same letter are not significantly different.

	<i>Control</i>			<i>Inoculated</i>		
	0 %	7.5 %	15 %	0 %	7.5 %	15 %
7 dai	4,24 $\pm$ 0,01 ^a	4,73 $\pm$ 0,48 ^a	4,61 $\pm$ 0,21 ^a	4,59 $\pm$ 0,31 ^a	4,97 $\pm$ 0,22 ^a	4,63 $\pm$ 0,36 ^a
24 dai	4,17 $\pm$ 0,48 ^a	4,58 $\pm$ 0,22 ^a	4,12 $\pm$ 0,61 ^a	4,06 $\pm$ 0,39 ^a	4,09 $\pm$ 0,66 ^a	4,39 $\pm$ 0,41 ^a

However, in *P. pinaster* plants inoculated with PWN in the absence of chitosan, a significant decrease in total chlorophylls throughout the experimental period was observed, which probably led to a decrease in the photosynthetic rate (Table 4). However, in plants inoculated in the presence of chitosan, no significant changes in total chlorophylls were observed, even in plants inoculated with PWN. These results seem to demonstrate that the presence of chitosan in the soil prevented the destruction of chlorophylls, thus ensuring the photosynthetic capacity of infected *P. pinaster* plants and, therefore, maintaining its resistance against the PWN.

Table 4 – Total chlorophylls ($\mu\text{mol.g}^{-1}$ plant tissue) in control and inoculated *P. pinaster* plants 7 and 24 days after inoculation (dai). Each value is the mean of three replicates (mean $\pm$ standard error) and values with the same letter are not significantly different.

	<i>Control</i>			<i>Inoculated</i>		
	0 %	7.5 %	15 %	0 %	7.5 %	15 %
7 dai	4.17 $\pm$ 0.51 ^{a, b}	4.54 $\pm$ 0.18 ^a	4.35 $\pm$ 0.51 ^{a, b}	4.16 $\pm$ 0.13 ^a	4.38 $\pm$ 0.17 ^a	4.26 $\pm$ 0.14 ^a
24 dai	4.51 $\pm$ 0.28 ^a	4.19 $\pm$ 0.17 ^a	4.42 $\pm$ 0.24 ^a	3.07 $\pm$ 0.22 ^b	4.61 $\pm$ 0.34 ^a	4.06 $\pm$ 0.08 ^a

As previously mentioned, after infection with the PWN, nematodes feed on host's resin ducts epithelial cells, causing tracheid embolism and a decrease in the water potential within the plant, which cause the water flow to stop and, eventually, the death of the tree [13].

Concerning water content, no significant differences were observed in control and inoculated *P. pinea* plants, regardless of the amount of biofertilizer tested (Table 5). In *P.*

pinaster plants inoculated with PWN, water content significantly decreased over time, compared to control plants, in the absence of chitosan (Table 6). However, this difference was not observed when plants were treated with both concentrations of biofertilizer, probably due to the reduction of nematode population (Table 1). Since the key phenomenon responsible for the death of the plant is the interruption of the water flow in their tissues, the application of chitosan to the soil of *P. pinaster* plants may be useful in retarding the death of infected plants or even in the reduction of mortality.

Table 5 – Water content (%) in control and inoculated *P. pinea* plants 7 and 24 days after inoculation (dai). Each value is the mean of three replicates (mean $\pm$ standard error) and values with the same letter are not significantly different.

	<i>Control</i>			<i>Inoculated</i>		
	0 %	7.5 %	15 %	0 %	7.5 %	15 %
7 dai	63.74 $\pm$ 0.80 ^a	60.92 $\pm$ 1.28 ^a	54.62 $\pm$ 2.10 ^a	62.57 $\pm$ 1.27 ^a	59.37 $\pm$ 1.33 ^a	65.54 $\pm$ 1.53 ^a
24 dai	57.55 $\pm$ 1.68 ^a	63.42 $\pm$ 1.27 ^a	62.00 $\pm$ 1.90 ^a	58.74 $\pm$ 0.20 ^a	60.95 $\pm$ 0.49 ^a	56.22 $\pm$ 2.02 ^a

Table 6 - Water content (%) in control and inoculated *P. pinaster* plants 7 and 24 days after inoculation (dai). Each value is the mean of three replicates (mean $\pm$ standard error) and values with the same letter are not significantly different.

	<i>Control</i>			<i>Inoculated</i>		
	0 %	7.5 %	15 %	0 %	7.5 %	15 %
7 dai	65.11 $\pm$ 2.74 ^a	61.30 $\pm$ 0.97 ^a	62.05 $\pm$ 2.54 ^a	60.05 $\pm$ 2.54 ^a	58.50 $\pm$ 1.67 ^a	59.00 $\pm$ 2.02 ^a
24 dai	58.52 $\pm$ 2.76 ^a	60.82 $\pm$ 2.02 ^a	62.03 $\pm$ 2.04 ^a	45.26 $\pm$ 1.55 ^b	58.75 $\pm$ 2.65 ^a	60.33 $\pm$ 2.68 ^a

In conclusion, the addition of chitosan as a soil amendment to areas colonized by *P. pinaster* trees seems to be a promising strategy to control PWD. Further studies are underway to optimize this process and to analyze at a molecular level the genetic variations induced by chitosan that may be responsible for the apparent resistance acquired by chitosan-treated plants.

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