Forest Pathology WILEY-BLACKWELL

For. Path. doi: 10.1111/efp.12136

SHORT COMMUNICATION

Chitosan as a biocontrol agent against the pinewood nematode (*Bursaphelenchus xylophilus*)

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Summary

The pine wilt disease (PWD) is caused by *Bursaphelenchus xylophilus* and poses great environmental and economic challenges. Thus, the development of sustainable techniques for the control of this epidemic disease is of major importance. This work aimed at evaluating if the application of different molecular weight (MW) chitosans as a soil amendment could be used to control the PWD in maritime pine (*Pinus pinaster*, very susceptible to the disease) and stone pine (*Pinus pinea*, less susceptible). At the end of the experimental period (24 days after inoculation), *P. pinaster* and *P. pinea* untreated plants presented ca. 3825 ± 100 and 70 ± 47 nematodes, respectively. In *P. pinaster*, the high-MW chitosan prompted the most drastic results, inducing a 21.9-fold reduction in nematodes numbers, whereas in *P. pinea,* the most effective was the low MW chitosan, which reduced nematodes numbers up to 7-fold, compared with untreated plants. *P. pinea* seems to be highly resistant to the disease, presenting nematode numbers up to 54.6-fold lower than *P. pinaster* and less severe chlorophyll loss (ca. 2-fold).

1 Introduction

The pinewood nematode (PWN) *Bursaphelenchus xylophilus* is a microscopic worm native to North America, where it is not associated with pathogenic disease. However, it was introduced in Japan, China, Taiwan and Korea, becoming a threat to the Far East forests. The PWN was detected in Portugal in 1999 and, since then, reached some northern Spanish regions. The PWD affects mainly *Pinus pinaster* (maritime pine) trees, leading to the cut down of thousands of symptomatic and asymptomatic trees every year, thus posing great challenges to the wood industry and increased environmental impacts.

The nematode is transported within the trachea of *Monochamus galloprovincialis* beetles, and it infects the trees' stems through the beetles' feeding wounds. It feeds and reproduces within the resiniferous ducts inducing the formation of secondary resin by radial parenchyma cells and the dysfunction of water transport, which leads to water deficiency, photosynthetic deficit and, subsequently, plant death (Kuroda 2008). Current control strategies include the extermination of diseased trees, use of traps and insecticides against the insect vector, tree vaccination with nematicides or selective breeding of resistant trees. However, these methods have very low efficiency and are extremely labour intensive and expensive. Due to the rapid spread of the disease and the severity of its consequences, the development of sustainable, cost-effective and environmentally friendly methodologies is urgently needed.

Chitosan is a deacetylated polysaccharide derived from chitin, which can be obtained from the outer shell of crustaceans and cell walls of certain fungi. It is known to induce plants' defence mechanisms, such as increased biosynthesis of phytoalexins, callose, lignin and other phenolic compounds. In fact, it was reported that chitosan displayed elicitor activity by inducing local and systemic resistance mechanisms of tomato plants against the root-knot nematode *Meloidogyne incognita* (Radwan et al. 2012). Moreover, Khalil and Badawy (2012) evaluated the nematicidal activity of different molecular weight (MW) chitosans against *M. incognita* infecting tomato seedlings and reported that its low MWs had the highest efficiency. Nevertheless, no data regarding the nematicidal activity of chitosans against stem infesting nematodes, such as the pinewood nematode, is available. In the present study, soil amendment with different MW chitosans was evaluated as a strategy to improve *P. pinaster* defences against the PWD and *Pinus pinea* was evaluated for its suggested resistance against this pathogen.

2 Materials and methods

Three acid-soluble chitosans were acquired from Sigma–Aldrich (St Louis, MI, USA), each one with a specific MW and deacetylation degree (DD): high MW (HMW, 310–375 kDa; DD > 75%), medium MW (MMW, 190–310 kDa; DD = 75–85%) and low MW (LMW, 50–190 kDa; DD = 75–85%). Chitosan solutions of 0.35% (w/v) were prepared in 0.5% (v/v) acetic acid (Sigma–Aldrich) and stirred in a horizontal shaker for 24 h.

Cultures of *B. xylophilus* strain 65 GO were grown on barley grains and *Botrytis cinerea* mycelium at 25°C in the dark for 7 days. The inoculum was prepared according to (Nunes da Silva et al. 2013).

During the experimental period, 1-year-old *P. pinaster* and *P. pinea* plants were kept in a growth chamber (Fitoclima 10 000 EHF; Aralab, Albarraque, Portugal) under a 16-h light/8-h darkness photoperiod at 25°C/18°C, respectively, and 80% RH. Photon flux density during the day was 380 μmol m^{-2} s^{-1}. One month after acclimation, 40 random plants of each species were transferred to substrate (COMPO SANA Universal substrate; COMPO GmbH & Co KG, Münster, Germany),

Received: 15.5.2014; accepted: 16.7.2014; editor: S. Woodward

supplemented with 40 ml of each chitosan solution (to achieve a total amount of 2% (w/w) chitosan in the subtract), and 40 additional plants of each species were treated with 40 ml of 1% (v/v) acetic acid as control. The substrate approximate composition, as described by the supplier, was (mg l^{-1}): 200 –450 N; 200–500 P_2O_5; 300–550 K_2O, pH 5.0–6.5. To ensure a uniform distribution of chitosan in the soil, the solutions were applied to the substrate as 4 × 10 ml applications with a pipette in different areas of the substrate of each individual pine tree. Ten days after soil enrichment with chitosan, 80 randomly selected *P. pinaster* and *P. pinea* plants (20 for each chitosan solution and 20 controls) were inoculated with ca. 1000 PWNs using the technique described by Nunes da Silva et al. (2013). In addition, 80 plants of each species (20 for each chitosan solution and 20 controls) were inoculated with deionized water.

Six, 12, 18 and 24 days after inoculation (dai), the stems of 40 *P. pinaster* and *P. pinea* plants (five for each inoculation condition and chitosan treatment) were cut into small segments, weighted and placed in centrifuge tubes with 5 ml of deionized water for 24 h at 25°C for nematode extraction. After 24 h, the nematodes were counted under a transmitted light stereo microscope (Motic K Series; Motic Deutschland GmbH, Wetzlar, Germany). Additionally, at the end of the experiment (24 dai), leaves of each plant species inoculated with PWN or water under all chitosan treatments were also collected for total chlorophylls determination. Chlorophyll loss in inoculated plants is represented by the difference ($\Delta_{\text{total chlorophyll}}$) between the average of total chlorophylls in PWN- and water-inoculated plants.

Significant differences were determined by Student's *t*-test corrected for multiple comparisons using the Holm-Sidak method in GraphPad Prism v. 6.01 software (GraphPad Software, La Jolla, CA, USA). Statistical significance was considered at $p < 0.05$.

3 Results

In *P. pinaster* control plants, the nematode population did not suffer significant changes up to 12 dai, with nematode numbers showing a slight increase from the initial inoculum nematode numbers (ca. 1000 nematodes). However, from 12 to 24 dai, there was an abrupt increase in nematode numbers, ca. 2.8-fold, reaching a total of 3825 ± 100 nematodes in the stem at the end of the trial. *P. pinaster* plants supplemented with LMW chitosan did not show significant differences in nematode numbers throughout the experimental period; nonetheless, there was an evident decrease in the amount of nematodes from 18 to 24 dai (ca. 2.6-fold). In addition, in chitosan-treated plants, nematode numbers were always ca. 1.7- to 9.6-fold lower than in control plants. In *P. pinaster* plants treated with the MMW chitosan, nematode numbers showed a slight increase (ca. 1.9-fold) from 6 to 18 dai; 18 dai the number of nematodes was significantly lower (ca. 1.3-fold), compared with control plants. More importantly, the nematode population significantly decreased from 18 to 24 dai, attaining a total of 662 ± 138 nematodes at the end of the experimental period, which was ca. 5.8-fold less than control plants and ca. 2.5-fold lower than the initial inoculum nematode numbers. Treatment with HMW chitosan did not induce significant changes in nematode numbers up to 18 dai; however, nematode numbers were 1.6- to 3-fold lower than control plants and 1.2- to 1.6-fold lower than the initial inoculum nematode numbers. Additionally, from 18 to 24 dai, the nematode population showed a significant decreased, achieving numbers ca. 21.9-fold lower than control plants (175 ± 25 nematodes).

In *P. pinea* plants (Fig. 1), no significant differences in the nematode population were observed up to 12 dai; however, from this time point until the end of the experimental period, nematode number significantly decreased, even in control plants (in which final nematode numbers was ca. 70 ± 47). This decrease was more pronounced in chitosan-treated plants, particularly in plants treated with LMW chitosan, in which nematodes decreased 28.5-fold from 6 to 24 dai until a minimum of 10 ± 6 nematodes. In fact, 24 dai, comparing with *P. pinea* untreated plants, LMW chitosan produced the most

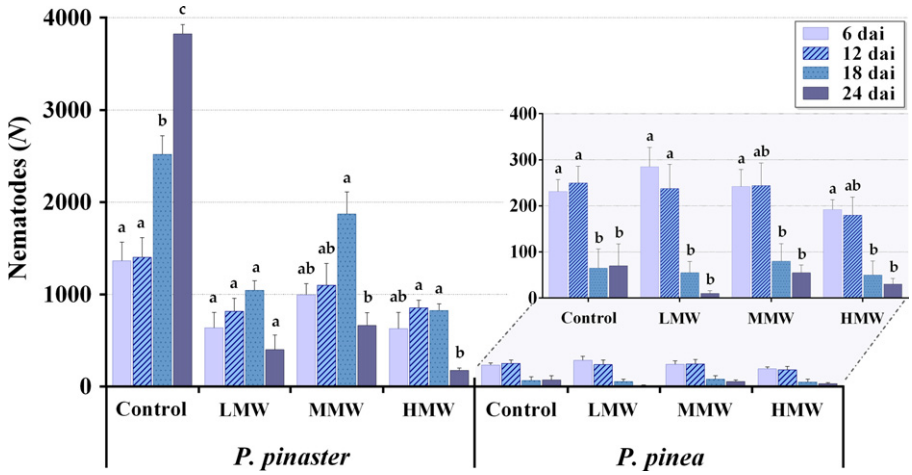

Fig. 1. Number of nematodes in the stem of *Pinus pinaster* and *Pinus pinea* plants inoculated in the absence (Control) or in the presence of low (LMW), medium (MMW) or high molecular (HMW) weight chitosans, 6, 12, 18 and 24 days after inoculation (dai). Each value is the mean of 5 replicates, and vertical lines represent the standard error. Within each species and treatment, bars with the same letter are not significantly different ($p < 0.05$, Students' *t*-test).

drastic result, inducing a ca. 7-fold decrease in nematode numbers; nevertheless, this difference was not statistically significant ($p = 0.141$). Regardless of the treatment, the nematode population in *P. pinea* plants was always 2.2- to 54.6-fold lower than in *P. pinaster* plants and 3.5- to 100-fold lower than the initial inoculum number.

Figure 2 shows the difference between total chlorophylls of PWN- and water-inoculated plants, with larger difference representing higher total chlorophyll loss. Twenty-four dai both *P. pinaster* and *P. pinea* control plants presented a decrease in chlorophylls when inoculated with PWN, compared with water-inoculated plants (Fig. 2). Additionally, this decrease was ca. 2-fold greater in *P. pinaster* than in *P. pinea*. LMW treatment reduced the loss of total chlorophylls in *P. pinaster* PWN-inoculated plants in ca. 2.5-fold, compared with control plants. With MMW and HMW chitosans, chlorophyll content was identical in both PWN- and water-inoculated plants. In *P. pinea* plants, all chitosan treatments prevented total chlorophyll loss in PWN-inoculated plants, comparing with water-inoculated plants. Both PWN- and water-inoculated plants presented similar chlorophyll contents (Fig. 2).

4 Discussion

As the number of nematodes markedly increased from 12 to 18 dai (Fig. 1), it seems that in 1-year-old *P. pinaster* plants, the PWD develops from the early to the advanced stage within this time period. Contrastingly, in *P. pinaster* trees treated with chitosan, this pattern did not occur. Although nematodes still slightly augmented from 6 to 18 dai in plants treated with MMW chitosan, this increase was evidently less abrupt than in control plants. Moreover, the nematode population significantly decreased from 18 to 24 dai, indicating that not only nematodes were not able to successfully reproduce, but also nematode mortality was induced. In plants treated with LMW chitosan, nematode numbers were markedly lower than in control plants in all time points and there was a slight decrease in nematode number along time. This tendency was also observed in *P. pinaster* plants treated with HMW chitosan; however, in this case, the decrease in nematode numbers was significant from 6 to 24 dai.

For all MWs, the application of chitosan seemed therefore to decrease the severity of the disease, probably due to the impairment of nematode reproduction and/or to the induction of physiological alterations in both nematodes and plants (Khalil and Badawy 2012), which did not allowed the successful reproduction of the nematodes. In fact, Khalil and Badawy (2012) reported that chitosan treatments as soil amendments against *M. incognita* reduced the extent of nematode invasion in tomato and eggplant and affected the morpho-physiological and populational parameters of *M. incognita* itself. Thus, treatment of PWN-inoculated *P. pinaster* plants with chitosan could have impaired the ability of the nematode population to migrate within the plant and to reproduce, resulting in the reduction of nematode numbers, comparing with untreated plants. Moreover, studies have shown that chitosan can induce plant resistance to several pathogens by restricting pathogen growth and by eliciting several defence mechanisms, suggesting that chitosan induces a direct antagonistic effect in the plant and, additionally, enhances plant resistance mechanisms. In fact, it has been reported that LMW chitosan, in particular, displayed elicitor activity by inducing local and systemic resistance mechanisms of tomato plants against the root-knot nematode *M. incognita*. This could be the reason why LMW chitosan produced the more radical nematicidal activity from an early stage of the disease, compared with MMW and HMW chitosans.

It has been suggested that the PWN may be associated with ectosymbiotic bacteria living in tight associations in their body surface, especially Gram-negative bacteria belonging to the Genus *Serratia*, *Enterobacter*, *Ewingella* and *Pseudomonas*

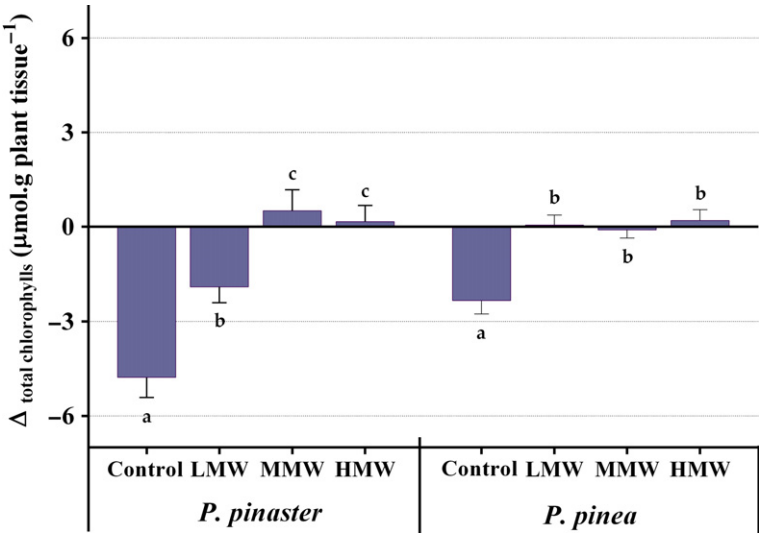

Fig. 2. Difference ($\Delta_{\text{total chlorophylls}}$) between the averages of total chlorophylls in PWN- and water-inoculated *Pinus pinaster* and *Pinus pinea* plants in the absence (Control) or in the presence of low (LMW), medium (MMW) or high molecular (HMW) weight chitosans 24 days after inoculation (dai). Each value is the mean of 5 replicates, and vertical lines represent the standard error. Within each species, bars with the same letter are not significantly different ($p < 0.05$, Students' *t*-test).

(Roriz et al. 2011). It is hypothesized that these bacteria can help the infection process by the PWN through the production of beneficial compounds. In fact, Radwan et al. (2012) and Khalil and Badawy (2012) reported that chitosan treatments as soil amendments against *M. incognita* reduced the extent of nematode invasion in tomato plants and affected the morpho-physiological and populational parameters of *M. incognita* itself. Chitosans have already proved useful for the enhancement of plant defences against several bacteria. Hallmann et al. (1999) reported that soil amendment with chitin caused soil suppressiveness to plant parasitic nematodes and resulted in changes in the bacterial communities of the soil, with Gram-positive bacteria being exclusively recovered from chitin-amended soil. These authors also reported that soil amendments with chitin also induced changes in the bacterial community within the cotton plants tissue. In the current study, the distinct reduction of the nematode population in chitosan-treated *P. pinaster* plants may be a result of the selective pressure of chitosan against Gram-negative bacteria, interfering with their ability to produce ligninolytic enzymes, or inducing a shift in the nematodes' colonizing bacteria, resulting in a predominance of Gram-positive bacteria and therefore in the extent of virulence induced by these nematodes (Hallmann et al. 1999).

The feeding and reproductive activities of the PWNs inside the host's tissues induce several histological and biochemical modifications that eventually lead to leaf discoloration, caused by the impairment of water transport inside the plant and the subsequent photosynthetic deficit (Kuroda 2008). The loss of photosynthetic pigments can occur from an early stage of the PWD and can be used to assess the degree of tissue damage caused by *B. xylophilus* (Nunes da Silva et al. 2013). In the current work, in the absence of chitosan, 24 dai both *P. pinaster* and *P. pinea* PWN-inoculated plants experienced a decrease in total chlorophylls (Fig. 2), most likely due to the activity of the PWN inside the plants' tissues. This decrease was particularly evident in *P. pinaster* at all time points analysed. Contrastingly, when inoculated plants were treated with chitosan, total chlorophyll loss was greatly reduced at the end of the trial (24 dai), which can be attributed to the decrease in the nematode population.

Several studies have shown that chitosan can induce plant resistance to several pathogens by restricting pathogen growth and/or by eliciting several defence mechanisms (Rabea et al. 2003). Thus, in this work, reduced PWN reproduction inside stem tissues could have resulted from the impairment of nematode viability and reproduction ability and/or to the induction of physiological alterations in both nematodes and plants. Alternatively, chitosan may have induced selective pressure against the bacterial communities that live in tight associations with the nematodes, which are thought to increase their virulence (Roriz et al. 2011), with repercussions to the nematode's reproduction and survival ability.

Finally, a reduction of the nematode population throughout the host tissues was related to resistance to PWD. Thus, the fact *P. pinea* plants presented remarkably reduced amount of nematodes (Fig. 1) and decreased total chlorophyll loss (Fig. 2), comparing with *P. pinaster* plants, seems to prove that this species is much more resistant to the PWD, as already hypothesized. As in *P. pinea* nematodes naturally failed to multiply, chitosan application effect was negligible (Fig. 1). Confirmation of *P. pinea*'s resistance to *B. xylophilus* can be of great importance for reforestation policies in affected countries, in which thousands of hectares are lost every year due to the cut down of diseased trees. The current work demonstrated that the application of chitosan as a soil amendment has great potential to be an inexpensive and easy to apply biocontrol agent against the PWN and studies on this matter should be greatly encouraged.

Acknowledgements

The authors would like to thank Fundação para a Ciência e a Tecnologia (FCT) for funding (PTDC/AGR-CFL/120184/2010), to National Funds from FCT through project PEst-OE/EQB/LA0016/2011, and Prof. Manuel Mota (INIAV and Universidade de Évora, Portugal) for providing the nematode strain.

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