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NEW APPROACHES FOR THE CONTROL OF THE PINE WOOD NEMATODE (*BURSAPHELENCHUS XYLOPHILUS*)

by

Francisca de Sá Nogueira Santarém Mergulhão

[December 2015]

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NEW APPROACHES FOR THE CONTROL OF THE PINE WOOD NEMATODE
(BURSAPHELENCHUS XYLOPHILUS)

Novas abordagens para o controlo do nemátode da madeira do pinheiro (*Bursaphelenchus xylophilus*)

Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in Applied Microbiology

by
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Resumo

O nematode da madeira do pinheiro (NMP) *Bursaphelenchus xylophilus* é o agente etiológico da doença do nemátode da madeira do pinheiro em *Pinus pinaster*. Não existe ainda um método eficiente para controlar e/ou eliminar este agente patogénico. O quitosano é um produto extraído da parede celular de alguns fungos ou da desacetilação da quitina, um produto amigo do ambiente e amplamente usado como agente antimicrobiano.

O objetivo deste trabalho foi o de testar a influência do quitosano no aumento da defesa da planta contra o NMP e também compreender as bases fisiológicas de ação deste composto a múltiplos níveis: efeito na população de nemátodes na planta, na produção de compostos de defesa e na modulação da carga microbiana no nemátode. Também se testou o quitosano como agente de esterilização da madeira comparando também este composto com outros métodos como o tratamento térmico em estufa e por microondas.

Para este trabalho de investigação infetaram-se pinheiros com NMP onde 75 pinheiros foram mantidos em solo sem quitosano e 75 em solo com quitosano. Verificou-se que até aos 28 dias após a infeção o quitosano evitou a morte dos pinheiros e o número total de nemátodes foi inferior ao número de nemátodes dos pinheiros mantidos em solo sem quitosano e idêntico ao longo de todo o tempo experimental. Este composto aumentou a produção de carotenoides, (53.8 ± 4.6 mol/g^{-1} aos 21 dai para 111.5 ± 11.9 mol/g^{-1} aos 28 dai) antocianinas (63.4 ± 5.5 mol.g^{-1} para 142.3 ± 20.4 mol.g^{-1} aos 28 dai), polifenóis totais (2.70 ± 0.1 µg.g^{-1} aos 7 dai para 3.2 ± 0.7 µg.g^{-1} aos 14 dai) e lenhina (0.7 ± 0.1mg.g^{-1} aos 14 dai para 1.5± 0.1 mg.g^{-1} aos 28 dai) demonstrando a sua capacidade para aumentar os mecanismos de defesa da planta. Foi estudada também a influência do quitosano nas bactérias associadas ao nemátode na planta e *Enterobacter* sp. foi o género predominante encontrado ao longo do tempo, o que sugere que as bactérias deste género estejam associadas à doença. De forma a se compreender se as bactérias obtidas no estudo anterior provinham realmente do nemátode ou se teriam sido transmitidas a este pela planta realizou-se um estudo "*in vitro*" do efeito do quitosano no NMP. Verificou-se que *Stenotrophomonas* sp., *Bacillus* sp., *Kluyvera* sp., *Buttiauxella* sp., *Klebsiella* sp. e *Paenibacillus* sp. não foram encontrados associados com o nematode mantido em cultura no laboratório, o que pode indicar que estas bactérias são transmitidas pela planta. Para a comparação do quitosano com outros métodos de controlo, como o tratamento térmico através de uma estufa e de energia microondas, utilizou-se serrim de madeira de pinheiro infetado com NMP. Este estudo demonstrou que o tratamento térmico com estufa e com micro-ondas tiveram uma maior capacidade nematicida quando comparado com soluções de quitosano a diferentes concentrações.

Este estudo permitiu concluir que o quitosano apresenta-se com um potencial método de prevenção da doença do NMP aumentando a defesa da planta contra este agente patogénico e evitando a evolução da doença e consequente morte do pinheiro. O quitosano influencia a carga microbiana do nematode, mas mais testes têm que ser realizados para perceber qual o efeito na evolução da doença. Este composto apresenta um efeito limitado nematicida em serrim quando comparado com outros métodos de tratamento.

Abstract

The pine wood nematode (PWN) *Bursaphelenchus xylophilus* is the causal agent of the pine wood disease (PWD) in *Pinus pinaster*. There isn't an effective method for control and/or elimination of this pathogen. Chitosan is a product extracted from the cell wall of some fungi or deacetylation of chitin, an environment friendly product, widely used as an antimicrobial agent.

The objective of this study was to test the influence of chitosan in the increase of plant defence against the PWN and also understand the physiological bases of action of this compound at multiple levels: effect on the population of nematodes in the plant, production of defence compounds and modulation the microbial load in the nematode. Chitosan was also tested as a wood sterilizing agent and compared with other methods such as heat-treatment by kiln-drying and microwave radiation.

For this research project trees were infected with PWN and 75 were kept in soil without chitosan and 75 trees were kept in soil with chitosan. It was found that up to 28 days after infection chitosan prevented the death of pine trees and the total number of nematodes was below the number of nematodes of pine trees kept in soil without chitosan. This compound increased the production of carotenoids, (from 53.8 ± 4.6 mol / g-1 at 21 dai to 111.5 ± 11. 9 mol / g-1 at 28 dai) anthocyanins (from 63.4 ± 5.5 mol g-1 to 142.3 ± 20.4 mol g-1 at 28 dai), total polyphenols (from 2.7 ± 0.1 µg.g-1 at 7 dai to 3.2 ± 0.7 µg.g-1 at 14 dai) and lignin (rising from 0.7 ± 0.1 mg. g-1 at 14 dai to 1.5 ± 0.1 mg.g-1 at 28 dai) demonstrating its ability to increase the defence mechanisms of the plant. The influence of chitosan on the bacteria associated with the nematodes was also studied in the plant, and Enterobacter sp. was the predominant genus found over time, suggesting that bacteria of this type are associated with the disease. In order to understand if the bacteria obtained actually came from the nematode or was transmitted to the nematode by the plant, an "*in vitro*" study was performed, were the effect of chitosan on the PWN kept in laboratory culture conditions was studied. It was found that *Stenotrophomonas* sp., *Bacillus* sp., *Kluyvera* sp., *Buttiauxella* sp., *Klebsiella* sp. and *Paenibacillus* sp. were not found associated with the nematode, which may indicate that these bacteria were transmitted by the plant. To compare chitosan with other control methods, such as heat- treatment through kiln-drying and microwave energy, wood chips infected with PWN were used. The heat-treatment by kiln-drying and microwave energy had a higher nematicide capacity when compared to chitosan solutions with different concentrations.

This study found that chitosan is a potential prevention method for the PWD increasing plant defence against this pathogen and preventing disease progression and consequent death of the tree. Chitosan influences the microbial load of the nematode but more tests have to be performed to understand the effect on the progression of the disease. This compound has a limited nematicide effect on wood chips when compared with other treatment methods.

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List of Abbreviations and Symbols

AC agar – All Culture Agar

CFU – Colony Forming Units

Cu – Copper

Dai – Days After Infection

DNA - Deoxyribonucleic Acid

dATP - Deoxyadenosine triphosphate

dCTP - Deoxycytidine triphosphate

dGTP - Deoxyguanosine triphosphate

dTTP - Deoxythymidine triphosphate

EDTA – Ethylenediaminetetraacetic Acid

H_2O_2 - Hydrogen Peroxide

ISPM – International Phytosanitary Measures

LB agar – Luria Bertani Agar

$MgCl_2$ – Magnesium Chloride

MW - Molecular Weight

NaCl – Sodium Chloride

NMP – nematode da madeira do pinheiro

PCR - Polymerase Chain Reaction

PWD – Pine Wood Disease

PWN – Pine Wood Nematode

RH – Relative humidity

rRNA - Ribosomal Ribonucleic Acid

Tris-HCL - *Tris hydrochloride*

TSA – Tryptic Soy Agar

TAE - Tris-Acetate-EDTA

27F – 27 Forward

1492R – 1492 Reverse

1. Introduction

1.1 Pine wood disease history, geographic expansion and economic impact

First report of Pine Wood Disease (PWD)

The nematode *Bursaphelenchus xylophilus* (Steiner & Bührer, 1934) Nickle, known as pine the wood nematode (PWN) is considered the causal agent of the pine wilt disease (PWD). The PWN is one of the few plant parasitic nematodes that can kill an adult tree in a relative short period of time (Vicente *et al*., 2012). The genus *Bursaphelenchus spp*. belongs to the family *Aphelenchoididae* and 70 species have been described (Ryss *et al*., 2005; Ye *et al*., 2007). Most nematodes of the *Bursaphelenchus spp*. are fungal feeding and are mostly found on dead or dying trees, eating the fungus that has colonized the tree (Jones *et al*., 2008).

The first report of PWD was made in Japan and dates back to 1905 (Nascimento *et al*., 2015). The PWN was also reported in Louisiana, North America, where it as identified as *Aphelenchoides xylophilus* (Steiner and Buhrer, 1934). The nematode *Bursaphelenchus lignicolus* was shown to be the causal agent of PWD in 1968 by a forest pathologist that noticed something moving on a petri dish in which he incubated pieces of wood from dead pine trees (Futai, 2013). The name was later on changed from *B. lignicolus* to *B. xylophilus* (Nickle *et al*., 1981).

PWD in Portugal

Since being reported, the disease has spread to many Asian countries such as: Japan, China, Korea and Taiwan (Yano, 1913; Cheng *et a*l., 1983; Tzean and Jan, 1985; Yi *et al*., 1989) (Figure 1.1.1). More recently, was detected in Europe (Portugal) (Mota *et al*., 1999). In Portugal, the PWD was first reported in 1999 and at that time it was distributed in a relatively small, but highly affected area in the Setubal Peninsula. The nematode isolates obtained from those infected trees presented little genetic diversity (Mota *et al*., 1999; Vieira *et al.,* 2007). In 2006, a strategy was implemented by the forestry and plant quarantine authorities (DGRF and DGPC) in which a three kilometer wide phytosanitary strip surrounding the infected area was cleared of *Pinus pinaster* trees*,* in an attempt to control and eradicate the nematode (Zhao *et al*., 2008). However, in 2009, the PWD had spread and was detected in other areas of Portugal like the Madeira Islands (Fonseca *et al.,* 2012), and as such the whole country is now classified as an “affected area” (Rodrigues, 2008). Recently, the PWN has also been reported in Spain, indicating the danger of *B. xylophilus* spread into new areas (Robertson *et al.,* 2011; Abelheira *et al.,* 2014).

This geographic expansion is mostly due to human activity. The nematodes are transported in the timber that is being traded and that is frequently used in the production of packaging materials. Nematodes can be transported inside their vectors (a pine sawyer beetle, to be described further on in this report) or on their own in the wood (Jones *et a*l., 2008; Futai, 2013). Different factors such as climatic conditions (temperature and precipitation), edaphic, topographic and biological features also influence the PWN spread (Jones *et a*l., 2008).

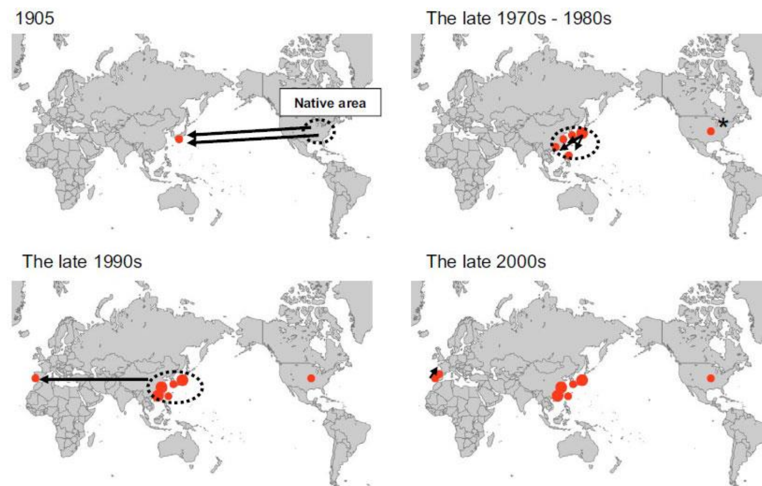

Figure 1.1.1 –History of the invasion of *B. xylophilus* and PWD through the world (adapted from Shinya *et al*. 2013).

Economic impact

The PWD embodies a great economic and environmental impact with very large annual losses of timber. This disease also increases the cost of management procedures for disease control (Mamiya, 2004; Yang, 2004; Shimazu, 2006). Native forest ecosystems have also suffered a major loss in biodiversity, wildlife habitat destruction, soil, water and tree species conservation (Kiyohara and Bolla, 1990; Suzuki, 2002).

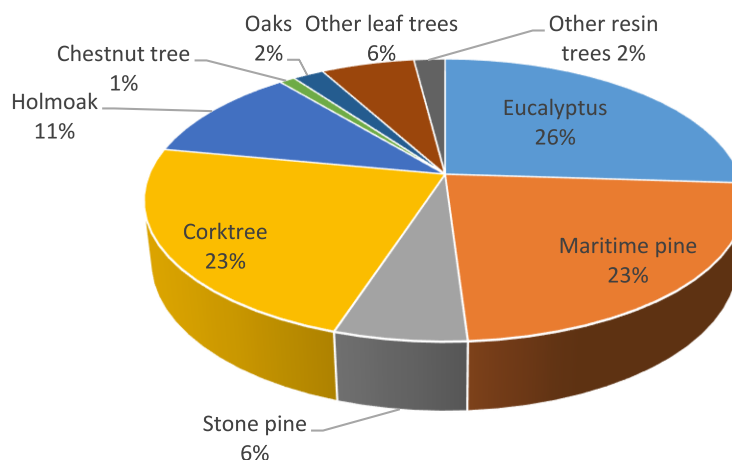

Figure 1.1.2 – Distribution of the total areas by species/group of species (adapted from IFN6, 2013).

In Portugal, the forestry sector plays a major role accounting for 12% industrial gross domestic product, 10% of foreign trade and 5% of national employment. Maritime pine (*Pinus pinaster*) is one of the most important productions, as well as stone pine (*Pinus pinea*) and eucalyptus (*Eucalyptus globulus*) (Figure 1.1.2). Invasive pests such as the PWD have affected tremendously the maritime pine production and forestry industry in Portugal, forcing to make exceptional cuts because of the strict phytosanitary regulations.

This has contributed to a decline in the planted area of *P. pinaster* in last 20 years. The maritime pine has had a decrease in area over time of about 263 000 ha (IFN6, 2013), and national efforts are being made do counteract this tendency, namely through the recently created “Centro de Competências do Pinheiro Bravo”, a consortium of national experts aiming to protect and increase maritime pine research, production and commercialization.

1.2 Pine wilt disease agent, host, infection and symptoms

The PWN is vectored by the wood-boring beetles of the genus *Monochamus*, but the most important species are *Monochamus alternatus* and *M. galloprovincalis* (Sousa *et al.,* 2001). Studies have shown that M. *galloprovincialis* is the only insect vector of PWN in Portugal (Sousa *et al*., 2001, 2002). In healthy trees the nematodes enter through the wound made by the maturation feeding of the *Monochamus* beetles and the nematodes multiply where the *Monochamus* lay their eggs (Tóth, 2011). Still this beetle favours the attack of dying trees due to other biotic or abiotic agents over healthy trees (Iwasaki, *et al.,* 1971).

The transmission of the disease can occur by two different methods, primary and secondary. In the primary transmission method the nematode enters the tree by wounds caused by the beetle feeding. In the secondary transmission method the nematode enters the tree through ovoposition wounds caused by the insect vectors (Dwinell, 1997) (Figure 1.2.1). After infection, the PWN feeds off the fungi that colonize the dying tree and feeds and reproduces in the epithelial cells that line the resin canals (parenchymal cells) (Futai, 2013). The PWN moves fast and enters the wood tissues through the resin canals of the xylem (Ichihara *et al*., 2000; Nunes da Silva *et al*., 2015).

The severity of the symptoms and incidence of PWD are related to: host species, bacteria, temperature and time of year. Infection of trees by the PWN in the summer results in a rapid death of the trees, in the spring the symptoms take longer to develop, whereas in the autumn and winter there can be no development of symptoms (Kiyohara and Tokushige, 1971). In Portugal, the pine species susceptible to the nematode are *P. pinaster* (Webster and Mota, 2008) and *P. nigra* (Inácio *et al*., 2015).

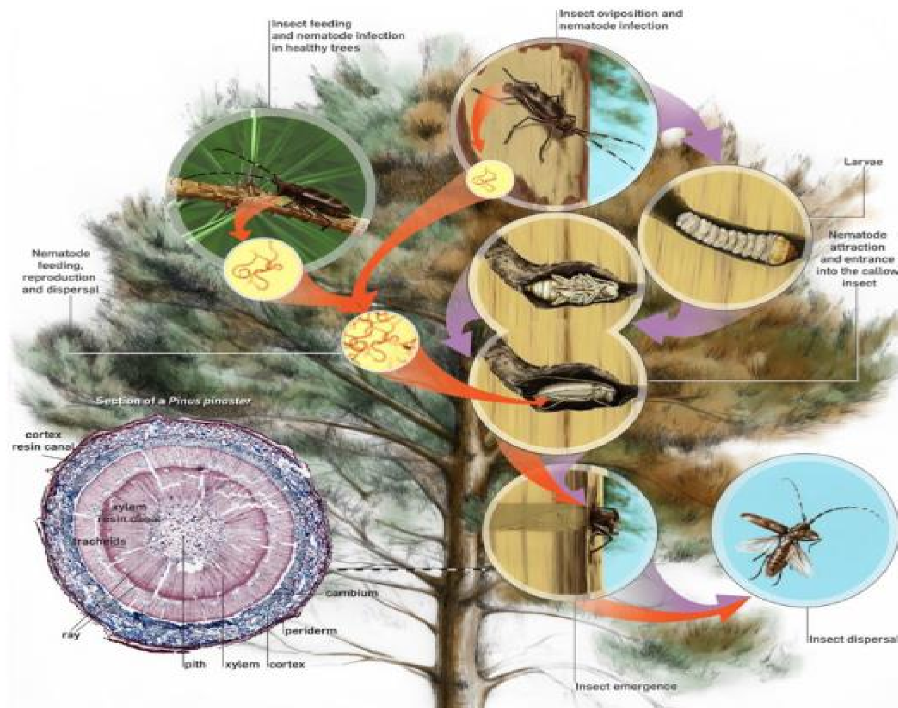

Figure 1.2.1 – PWN life cycle (adapted from Nascimento *et al.,* 2015).

The appearance of the first wilting symptoms suggest a successful nematode infection (Nascimento *et al.,* 2015). The destruction of the epithelial cells leads to a significant decrease of resin flow and resin plays an important role in tree defence by presenting antimicrobial properties and toxic effects on insects and other organisms (Langenheim, 2003; Sipponen and Laitinen, 2011; Futai, 2013). Induced cavitation and embolism in tracheids occur due to the terpenoids synthesised in the xylem ray cells which promotes dysfunction of water flow (Nascimento *et al.,* 2015).

The modifications in the xylem, induced by the nematode, such as the decrease in water potential and transpiration and photosynthesis lead to the manifestation of the external symptoms like chlorosis (Fukuda, 1997; Hara and Takeuchi, 2006). A decrease in the photosynthetic rate been reported to be associated with nematode reproduction leading to a drastic advance of the PWD. (Kawaguchi, 2006) and eventually to tree death.

The pine tree will also fight this infection by increasing the concentration of chlorophyll in the needles and by producing secondary metabolites like phenolic compounds and lignin (Lattanzio, *et al*., 2006). Chlorophylls are molecules that absorb light in the needles of the pine trees and use it to drive photosynthesis. Some studies suggest that the browning of the plant tissues during PWD, might be a result of the accumulation of secondary metabolites like polyphenols (Futai, 2003; Nunes da Silva *et al.,* 2013). Phenolic compounds are the plants most common and widespread secondary metabolites formed from the shikimate-phenylpropanoids-flavonoids pathways, producing monomeric and polymeric phenols and polyphenols. Contradictory to this information, Kuroda *et al*. (2011) suggested that higher production of phenolic metabolites could explain the resistance of *Pinus densiflora* against the PWN.

Lignin is a complex phenolic polymer that when polymerized forms cell walls more resistant to mechanical and enzymatic disturbance, acting as a major line of defence against pathogens (Lewis and Yamamoto, 1990). Lignin is also found to be produced in higher amounts during the advanced stages

of PWD, which suggests that it is associated with PWD-resistance. Constitutive lignin was also found to be related to defence mechanisms against nematodes (Kawaguchi, 2006; Nunes da Silva *et al.,* 2013).

1.3 Bacteria associated with PWN and PWD

In nature there is a mutualistic and beneficial relationship between nematodes and bacteria, and this relationship is mostly due to nutritional dependence and pathogen protection. This is intrinsically related with the environment, ecological conditions and nematode life stages. It has been suggested that bacteria associated with the PWN play an important role in the disease by producing toxins that can damage the plant cells (Roriz et al., 2011; Nascimento *et al*., 2015). The relationship between the PWN and bacteria in pathogenicity is a controversial topic (Zhao *et al*., 2008). Some studies found that trees infected with only the bacteria or axenic nematodes did not develop the disease while nematodes with their associated bacteria lead to the manifestation of the symptoms (Oku et al., 1980; Zhao et al., 2000, 2003; Han et al., 2003). However, some studies suggest that the bacteria that exist in the tree are mere contaminants and not pathogenic (Yang, 2002).

The tree resin canals and tracheids in which nematodes move and reproduce are naturally colonized by bacteria (Nascimento *et al.,* 2015). When the nematode invades and moves in the host plant the bacteria associated with the nematodes tend to increase in number and diversity (Xie and Zhao, 2008; Roriz *et al*., 2011). Thus, there are complex interactions between nematodes, their associated bacteria, and the colonized tree that influence disease severity. These interactions can be explored to reduce the nematodes ability to cause disease symptoms.

PWN-associated bacteria tend to differ geographically but some bacterial species are more predominant in association with PWN in different *Pinus spp.* independently of the geographical location. Gram-negative bacteria are the most predominant, distributed among the *Proteobacteria* (mostly *Burkholderiaceae*, *Enterobacteriaceae, Pseudomonadaceae* families), whereas Gram-positive are mostly *Firmicutes* and *Actinobacteria* (Nascimento *et al.,* 2015). Nevertheless, not every bacteria associated with the PWN are phytopathogenic in the same extent (Han *et al.,* 2003; Zhao *et al.,* 2003; 2009; Vicente *et al.,* 2012b) and some might not even be phytopathogenic (Proença *et al.,* 2012a,b). *Burkholderia*, *Pseudomonas* and other bacteria from the Enterobacteriaceae are the most predominant in Portugal has been associated with PWN from different geographic regions (Proença *et al.,* 2010; Roriz *et al.,* 2011) (Figure 1.3.1).

Symbiotic species	Japan	China	Korea	Europe
Associated bacteria	Serratia Bacillus Pseudomonas Burkholderia Enterobacter Erwinia	Serratia sp. M24T3 Pseudomonas Pantoea Stenotrophomonas	Serratia Brevibacterium Burkholderia Enterobacter Ewingella	Serratia Pseudomonas Burkholderia Enterobacter Ewingella

Figure 1.3.1 – Bacteria associated with PWN by region (adapted from Zhao *et al.* 2014).

Studies suggest that PWN-associated bacteria have a putative adjuvant effect on PWN pathogenicity (Roriz *et al*., 2011; Cheng *et al.,* 2013; Vicente *et al.,* 2013b). Bacteria associated with the nematode could improve its survival under strong and prolonged stress conditions. Also, enzymes like cellulases and pectinases, produced by the nematodes, help its dispersion through the plant cells (Shinya et al. 2013) and cellulose enzyme genes are thought to have been acquired from bacteria by horizontally gene transfer to *B. xylophilus* (Kikuchi et al., 2011; Martins, 2012). Also supporting this theory, the PWN-associated bacteria seem to be cellulose producers (Niu et al., 2012; Vicente et al., 2012b). The PWN doesn't have a fully functional pathway that is necessary for the terpanoid degradation, however, a lot of the enzymes involved in the metabolism of xenobiotics were found in PWN-associated bacteria. This means that PWN and bacteria have developed a mechanism to degrade xenobiotics by a functional complementary mutualism, allowing them to overcome toxic compounds produced by the secondary metabolism of the pine host (Kikuchi *et al*., 2011; Cheng *et al.,* 2013).

1.4 Pine Wilt Disease prevention and treatment

Prevention and control methods

Prevention, alongside treatment, is an important approach to reduce the PWD and control the *Bursaphelenchus* and its vector (Dwinell, 1997). Different strategies have been suggested either to prevent or treat the disease, either targeting the nematode itself, the vector, the host, or a combination of thereof.

Variable degrees of intra and interspecific resistance (Figure 1.4.1) to the nematode have been reported, and this information can be used in reforestation efforts (Nunes da Silva *et al*., 2015). It is also important to study the genes that may induce tree resistance to the PWN and use breeding or genetic transformation techniques for generating resistant trees that can be cultivated where the susceptible ones have been taken down (Santos *et al*., 2012). In 1978, the first resistant species to the PWN were established in Japan (Kamata, 2008). In Portugal, efforts are being made to identify naturally resistant *P. pinaster* genotypes, by looking at surviving trees from the heavily infested area in the Setubal peninsula, with promising results.

The application of nematicides (Figure 1.4.1) (a solution that can stop the reproduction or kill the nematode), has been used since 1994 with limited degree of success. This method can be very effective, can last for several years and is not highly influenced by external variables. Also it does not have a human impact and can be used on all trees, although it could develop some phytotoxicity. Some disadvantages of this method are the fact that it is very expensive and labour-intensive when applied in large pine forests, phytotoxicity can be a problem and discoloration of the bark and needles can occur (Kamata, 2008). Thus, nematicides are more suitable for botanical gardens or when trying to preserve a limited number of value added trees.

The use of birds and other insect predators (Figure 1.4.1), entomopathogenic fungi and entomopholic nematodes have been suggested as a biological control tools for the pine wilt disease. For the vector *M. alternatus* there are several natural predators, such as insect parasites, parasitic fungi and parasitic nematodes. (Kamata, 2008). Many studies were performed in order to verify the efficiency of different trapping methods for the monitoring of *Monochamus*, but the results are still controversial.

The most common traps used are the cross-vane and multi-funnel and the lures used are based on host monoterpenes, usually α-pinene, and ethanol (Ikeda *et al*., 1980) but these are not specific to M. *galloprovincialis*.

If the infected trees are burned (Figure 1.4.1) the hibernating pine sawyer larvae can be killed. This method has a complete mortality of the vector beetle and nematode. It is highly effective, but implies destroying the trees, and if used without strict supervision may increase the likelihood of forest fires (Kamata, 2008). In Portugal this method is not routinely utilized.

Fumigation (Figure 1.4.1) consists of application of metam-ammonium carbam NCS, carban sodium and methyl bromide. With this method almost 100% mortality is obtained by killing the nematode and its insect vector. Fumigation is costly and methyl bromide is considered an ozone-depleting substance and its production and use has been banned from the United States. Thus, fumigation is not a viable long-term option (Dwinell, 1997; Kamata, 2008).

The heat-treatment (Figure 1.4.1) of wood is described in the ISPM Nº 15 (FAO, 2009) which says that wood packaging materials must be heated so that a minimum temperature of 56ºC for a minimum time of 30 continuous minutes must be achieved in the wood core. Different methods can be used to achieve these parameters like, kiln-drying, heat-enabled chemical pressure impregnation, microwave, etc, as long as they meet the heat treatment parameters specified in the standard. This method has been proven to be effective but it is energy intensive, time consuming and sometimes not feasible (Uzunovic *et al.,* 2013). It also increases the costs for the wood packaging business.

Method	Type	Advantages	Disadvantages	References
Resistant Trees	Prevention	High efficiency.	Very few naturally resistant pine trees. Production is very expensive.	Zhao *et al*., 2008
Nematicides	Control	Can be very effective and last for a long time.	Very expensive and labour-intensive Some phytotoxicity.	Kamata, 2008.
Burning of infected trees	Control	Low cost and high efficiency	Use is restricted to periods when forest risk is low. Heat from the fires can damage other pine trees.	Zhao *et al*., 2008
Biological control	Control	There are several predators of the vector.	Not specific to M. galloprovincialis.	Zha *et al*., 2008
Fumigation	Control	Easy to apply, 100% mortality of the nematodes.	Toxic, harms the environment, high costs.	Zhao *et al*., 2008
Heat – Treatment	Control	High mortality of the nematodes.	Energy intensive, time consuming and sometimes not feasible.	Uzunovic *et al*., 2013
Biocontrol agents	Prevention/control	Simple application method. High efficiency. Non-toxic and environme friendly.	More research needed.	Vasconcelos, 2014 Nunes da Silva *et al*., 2014

Figure 1.4.1 – Summary of the advantages and disadvantages of different prevention/control methods.

1.5 Chitosan as a novel prevention method

The current control and treatment strategies have low efficiency and can be very labour intensive and expensive. Biocontrol agents (Figure 1.4.1) are a cost-effective and environmentally friendly methodology. Chitosan is an aminopolysaccharide that has been shown to have a nematicidal activity against the PWN and improve plant defences (Nunes da Silva *et al*., 2014; Khalil and Badawy, 2012). This aminopolysaccharide also presents a variety of advantages such as being environmentally friendly and non-toxic (Vasconcelos, 2014) which makes it a possible alternative for other control and treatment methods.

Chitin is a structural polysaccharide that is extracted from the exoskeleton of crustaceans (shrimps, crabs, etc.), nematodes eggs and the cell wall of some fungi (No *et al.,* 1997; Bueter *et al.,* 2013). Chitosan, a linear aminopolysaccharide of glucosamine and *N*-acetylglucosamine units, is obtained by the alkaline deacetylation of chitin. It is much less prevalent in nature then chitin and it was only found to be a part of the cell wall of some fungi (Muzzarelli *et al.,* 1986). It is composed of a long-chain homopolymer of *N*-acetyl-D-glucosamine (GlcNAc), (1-4)-linked 2-acetamido-2-deoxy-β-glucan (Park and Kim, 2010).

Chitosan presents various advantages like the fact that it's physically and biologically functional and it's biodegradable and biocompatible with a lot of organs, tissues and cells. For this reason chitosan has a lot of applications in different areas of study (Hirano, 1996; Shigemasa e*t al.,* 1996; Shahidi *et al.,* 1999; Rabea *et al.,* 2003). It is widely used as an antimicrobial agent, by itself or with other natural polymers, due to the fact that it has antimicrobial properties (Badaway *et al.,* 2011). It presents as a disadvantage the fact that its insoluble in water, has a high viscosity and a tendency to coagulate with proteins at high pH. It is soluble in dilute organic acids such as acetic acid, formic acid, succinic acid, lactic acid and malic acid.

Chitosan antimicrobial effects depend on several variables, including molecular weight, degree of deacetylation, solubility, positive charge density, chemical modification, pH, concentration, hydrophilic/hydrophobic characteristic, chelating capacity and type of microorganism (Badaway *et al.*, 2011; Vasconcelos, 2014). It shows the biggest activity against yeasts and moulds, followed by Gram-positive bacteria and lastly Gram-negative bacteria (Kendra and Hadwiger, 1984). It has been shown that chitosan also presents antiviral activity inhibiting the productive infection by bacteriophages (Kochkina *et al.,* 1995). The mechanism of chitosan's antimicrobial activity is still unknown but it is thought to be influenced by various factors that act sequentially and independently (Rabea *et al.,* 2003).

Chitosan has mostly been used in the human biomedical industry (Park and Kim, 2010). The application of chitosan on agriculture is less described and is mostly linked to its antimicrobial properties. Studies suggest that chitosan can be used to enhance the antimicrobial activity in plant-defence against bacteria (Tikhonov *et al*.,, 2006; Rabea and Steurbaut, 2010), fungi (Park *et al*., 2002; Trotel-Aziz *et al*., 2006) and nematodes (Khalil and Badawy, 2012; Nunes da Silva *et al.,* 2014). It induces plant defence mechanisms by producing compounds such as phytoalexins (Vasyukova *et al*., 2001), callose (Ren *et al*., 2001), lignin (Barber *et al*., 1989) and other phenolic compounds. When applied at optimal concentration it can induce a delay in the disease leading to a reduced plant wilting (Benhamou *et al*.,

1994). Chitosan has already been used in soil amendment and as seed and foliar treatment to control the fungus *F. oxysporum* (Rabea *et al.,* 2003).

It is also thought that chitosan enhances plant-defence responses, linked to the production of chitinase that will make chitin and chitosan into more soluble compounds by breaking down the chain and by altering the pathways involving the jasmonic acid (Walker-Simmons and Ryan, 1984; Farmer and Ryan, 1990; Doares *et al*., 1995). Chitosan induces the expression of different genes involved in plant defence (Doares *et al*., 1995; Bell *et al*., 1998; Eikemo *et al.*, 2003). Chitosan can also modulate the soil microbiome by stimulating the activity of beneficial microorganisms in the soil like *Bacillus*, fluorescent, *Pseudomonas*, Actinomycetes, Mycorrhiza and Rhizobacteria. These beneficial microorganisms will alter the equilibrium in the rhizosphere and the pathogen will become disadvantaged (Bell *et al*., 1998; Murphy *et al*., 2000; Daayf *et al*., 2003; Hassni *et al*., 2004; Pal and Gardener, 2006; Uppal *et al*., 2008). It also has the ability to create a physical barrier, preventing the pathogen from spreading. As stated before, it is also a known chelating agent of nutrients and minerals like Fe and Cu, not allowing pathogens to access those nutrients (Hadrami *et al*., 2010).

The nematicidal activity was described by Khalil and Badawy (2012) by studying the effect of chitosan of different molecular weights (MW) against *M. incognita* in tomato seedlings, reporting that low MWs had the highest efficiency. Nunes da Silva *et al.* (2014) studied the effect of chitosan as a biocontrol agent against the pinewood nematode (*Bursaphelenchus xylophilus*) and found that low MW chitosan showed the highest nematicidal effect, displaying a decrease in nematode numbers from 18 to 24 days after infection. The high MW chitosan did not induce significant changes in the nematode population. In the current study we hypothesise that this decrease in the nematode population maybe was due to a possible modulation of chitosan over Gram-negative bacteria, interfering with the ability of the nematodes to produce ligninolytic enzymes and/or inducing a shift in the nematode's colonizing bacteria reducing the virulence of this nematodes.

2. Main Objectives

As already stated, previous studies suggested that chitosan presents a nematicidal against the PWN, opening the possibility of using chitosan as a biocontrol agent. But little is known about the mechanisms underlying these effects. Also, chitosan could be used as an alternative method for nematode inactivation in infected wood, representing an alternative to the conventional wood heat treatment method. Therefore this study aims to study the influence of chitosan amended soil in *P. pinaster* trees infected with PWN, looking at the expression of defence mechanisms by the plant and at the modulation of bacterial population. It also compares chitosan treatment of wood chips to the conventional heat treatment methods in their ability to eliminate nematode infested wood. The specific objectives of this thesis are to:

- ✓ Conduct a time course study and evaluate the symptoms of infected *P. pinaster* trees kept in chitosan enriched soil when compared to plants without chitosan treatment.
- ✓ Evaluate the synthesis of photosynthetic pigments and defence compounds such as polyphenols and lignin.
- ✓ Monitor, at each time point, the evolution of the nematode population in the plants.
- ✓ Monitor, bacterial population in nematodes extracted from infected trees which were treated or not with chitosan.
- ✓ Compare this bacterial profile with bacteria isolated from nematodes kept in laboratorial conditions also subjected to chitosan treatment and how the bacterial communities compare to the ones that the nematode acquires within the plant.
- ✓ Evaluate chitosan as an alternative nematode sterilization method of wood chips comparing it to existing treatment methods such as heat treatment with kiln-drying and microwave irradiation.

3. Materials and Methods

3.1 Plant material and treatments

Throughout the entire experimental period, one year old *P. pinaster* trees were maintained in a growth chamber (Fitoclima 10 000 EHF; Aralab, Albarraque, Portugal) under a 16 h light/8 h darkness photoperiod at 25/18 ºC, respectively, and 80% RH. The photon flux density during the day was 380 μmol m^{-2} s^{-1}. The trees were kept in commercial substrate (COMPO SANA Universal substrate; Compo GmbH & Co KG, Munster, Germany) composed of (mg l^{-1}): 200-450 N; 200-500 P_2O_5; 300-550 K_2O, pH 5.0-6.5). The soil/chitosan mixture was prepared by adding 80 ml of a 4.38 % chitosan solution prepared in 0.5% acetic acid. For the preparation of the chitosan solution, 43.8 g of low molecular weight (LMW) chitosan (Sigma Aldrich) was added to 1 L of 0.5 % acetic acid solution. The chitosan in the soil had a final concentration of 2.5 % (w/w).

3.2 Nematode culture and inoculations

A virulent strain of the PWN (65 GO) was used in this experimental work. Nematodes were maintained in agar plates with *Botrytis cinerea* and then transferred to mycoboxes with barley seeds at 25 ºC for 14 days. Different stages nematodes were extracted using the Baermann funnel technic for 24 h at 25 ºC. The total number of nematodes extracted was determined by pipetting 500 μl of the extracted nematode solution and placed in a nematode counting dish. Adjustments were made so that a solution with 2000 nematodes in 750 μl of sterilized water was obtained and used in the inoculations of the *P. pinaster* trees.

A total of 150 trees were used in this experiment: the chitosan mixture was applied to a group of 75 pine trees, in which 50 were inoculated with nematode and 25 with water; and another group of 75 pine trees served as control, where a solution of 0.5 % acetic acid was added to the soil, and in which 50 were inoculated with nematode and 25 with water (Figure 3.2.1).

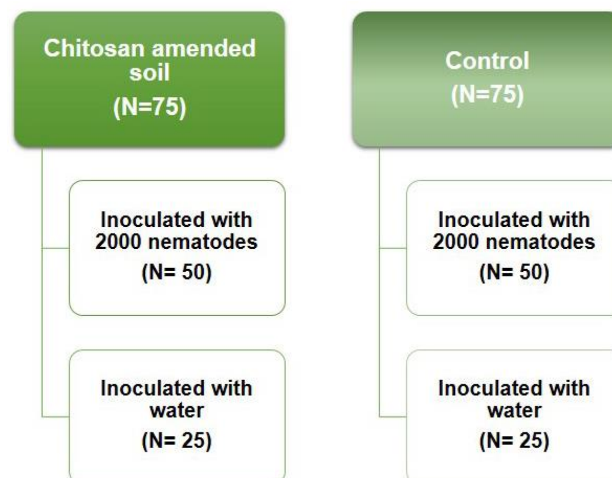

Figure 3.2.1 – Schematic representation of the treatments applied to the soil and of the inoculations performed on the P. *pinaster* trees.

The nematode inoculation methods were performed as described by Futai (1980). Briefly, at about 3 cm from the top the needles of each pine tree needles were removed and three transversal cuts were made on the stem using the help of a blade. A piece of absorbent paper was placed on the wound and parafilm was used to seal it. A nematode suspension of 2000 nematodes/750 μl, of the strain 65 GO, was pippeted into the wound (Figure 3.2.2).

Figure 3.2.2 – Inoculation of the Pine trees with the nematode suspension.

Five biological replicates of each treatment were harvested at five different time-points: one, seven, 14, 21 and 28 days after inoculation (dai). Needles were separated from the stems and both were immediately frozen in liquid nitrogen and a piece of the stems was stored at room temperature for the microbiological analysis. Five additional plants from the nematode-inoculated plants were harvested at the same time-points for the nematode counting.

3.3 Symptom development

The development of chlorosis was registered at each time-point, following a colour and disease scale adapted by Nunes da Silva *et al*. (2013), where 0 corresponds to plants completely green; 1 – <10% needles with chlorosis; 2 - 10-25 % needles with chlorosis; 3 - 25-50 % needles with chlorosis; 4 - >50 % needles with chlorosis and 5 – Plant death (Figure 3.2.3).

Figure 3.2.3 – Pines trees presenting chlorosis symptoms (left, stage 3 and right, healthy).

3.4 Nematode quantification

The whole pine stem was cut into small pieces and the nematodes were extracted using the Baermann funnel technique (Baermann, 1917). The total number of nematodes were determined using a nematode counting dish under a transmitted light stereo microscope.

3.5 Photosynthetic pigments quantification

To quantify the chlorophylls and carotenoids the Sims and Gamon (2002) method was used. To this end, 0.1 g of needles were grinded in a mortar with liquid nitrogen, and 10 ml of cold acetone/Tris buffer solution at 1 M was added (80:20 vol:vol, pH= 7.8). The samples were incubated at 4 ºC for 24-72 hours leaving the tubes laying down. The tubes were centrifuged (U-320 R, Boeco, Germany) at 13 000 rpm for 5 min, and absorbances were recorded at 470, 537, 647 and 663 nm. The amount of pigments was calculated as follows:

$$\textbf{Anthocyanin} = 0.08173A_{537} – 0.00697A_{647} – 0.002228A_{663}$$

$$\textbf{Chl}_{\textbf{a}} = 0.01373A_{663} – 0.000897A_{537} – 0.003046A_{647}$$

$$\textbf{Chl}_{\textbf{b}} = 0.02405A_{647} – 0.004305A_{537} – 0.005507A_{663}$$

$$\textbf{Carotenoids} = (A_{470} – (17.1 \times (Chl_a + Chl_b) – 9.479 \times \text{Anthocyanin}) / 119.2$$

3.6 Total soluble phenolics determination

To quantify the total soluble phenolics the Azevedo and Herlânder (2005) method was used. Needles were frozen in liquid nitrogen and stored at -20 ºC. The samples were then lyophilized for 72 hours. The lyophilized plant material was grounded to powder and 0.1-0.2 g of the lyophilized powder was transferred to 15 ml falcon tubes. Five ml of methanol was added and the samples were let to extract for 24 h in the dark at room temperature. The extract was recovered after centrifugation at 5000 g for five minutes.

For the quantification of soluble phenolics five ml of ultrapure water and 0.5 ml of Folin-Deni’s reagent were added to 0.1 ml of methanolic extract. The mixture was stirred vigorously and the reaction allowed to occur for five minutes. After, 1.5 ml of sodium carbonate at 35 % (w/v) was added and let to react in the dark for two hours. Finally, 2.9 ml of ultrapure water was added. The absorbances were read at 760 nm using a NanoPhotometer^{TM} UV/VIS spectrometer (Implen GmbH, Germany) and the quantity determined using a quercetin calibration curve.

3.7 Lignin quantification

The concentration of lignin was determined by the acetyl bromide method (Hatfield *et al*., 1999; Fukushima and Hatfield 2001). Briefly, the extraction was performed as previously described for the total soluble phenolic determination. The extract was discarded and the extraction made until the supernatant was clear. The samples were dried in a kiln at 60 ºC for 24-48 hours. The quantification was performed by adding 10 mg to 500 µl of glacial acetic acid and 500 µl of acetyl bromide 25 %. The mixture was digested at 50 ºC for two hours with vigorous stirring. The samples were sedimented by centrifuging for ten minutes at maximum speed. One hundred µl were recovered to a new micro tube that contained 200 µl of acetic acid and 150 µl of glacial acetic acid. Finally, 50 µl of hydroxylamine hydrochloride and 500 µl of glacial acetic acid were added. The quantification was determined in a NanoPhotometer^{TM} UV/VIS spectrometer (GmbH, Implen, Germany) at 280 nm (quartz cuvettes).

3.8 Analysis of the impact of chitosan in the nematode bacterial population

The analysis of the impact of chitosan on the bacterial population was performed on: a) nematodes extracted from the control versus chitosan treated plants and from b) nematodes kept in laboratory conditions in culture vessels.

3.8.1 From the inoculated trees

The pine tree segments of six plants per treatment and time-point were first sterilized by submerging a 3 cm piece of the stem in 75 % ethanol for 15 seconds and removing the excess ethanol by washing in two passages of deionized water. Next, the ends of the stem where removed and the stem was cut horizontally so that the middle portion was exposed and the nematodes are able to leave the stem to the medium. After being cut horizontally both pieces were cut in half vertically and those four pieces cut again in three, so that in the end 12 pieces were obtained and plated.

One tree segment was then inoculated in All Culture Agar medium (AC), Luria Bertani Agar medium (LB) and Tryptic Soy Agar medium (TSA) by placing it in the middle of the petri dish with the interior of the stem faced down and incubated at 26 ºC for 3 days. For each treatment 3 stems where used and for each stem two replicas in each medium where made. The isolates obtained were further preserved at -80 ºC in a modified Luria Bertani broth, supplemented with 15 % of glycerol as described in Calheiros *et al.* (2010).

CFUs were calculated by utilizing the method of Xie and Zhao (2008) with a slight modification. In summary, the dish count method was used, in which a piece of a wood chip obtained as previously described was placed in a falcon tube with 5 ml of 0.85 % NaCl solution. The tubes were shaken vigorously using a vortex for five minutes and left standing for ten minutes. With 0.5 ml of the previously prepared solutions dilutions were performed in a series of concentrations (10^{0}, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}). 0.5 ml of those dilutions were pipetted into the middle of the petri dishes containing TSA, LB and AC agar and the pipetted solution was spread evenly trough the plate and incubated at 25ºC for 72 h. For each dilution of wood chip (three wood chips per treatment), three replicas were made on each medium.

3.8.2 From cultured nematodes

For the *in vitro* analysis of the effect of chitosan on the nematode bacterial population kept in laboratorial conditions with barley grains and *Botrytis cinerea* (standard protocol for nematode culture and maintenance) a solution of 0.15 % chitosan was prepared by adding 0.15 g of LMW chitosan to 100 ml of acetic acid at 0.5 % and left to mix overnight. The pH was adjusted to 6.0 with NaOH 5 M. One hundred and fifteen µl of a nematode suspension with about 100 nematodes were transferred to 1.5 ml eppendorfs and 850 µl of the previously prepared 0.15 % chitosan solution was added. As controls the nematodes were also resuspended in 0.5 % acetic acid and in water. Eight hundred and fifty µl of acetic acid at 0.5 % was added to 150 µl of a nematode suspension and to another three 1.5 ml eppendorfs. The same was prepared with sterile H_2O. The samples and the controls were incubated for 24 h at 25 ºC. After the incubation period, individual nematodes were recovered with the help of a pipette, isolated on AC, LB and TSA medium and the petri dishes were incubated for 24 h at 25 ºC. Six treatment replicates and three biological samples for each control treatment were applied.

Bacterial identification

The total genomic DNA of pure bacteria isolates from the previous tasks was extracted using the heat-shock method as performed by Calheiros *et al.* (2010). Colonies of pure bacterial isolates were added to 200 µl of sterile ultra-pure water, vortexed to homogenize and heated at 95 ºC for 10 min. Samples were then put into ice for 5 min and vortexed. Finally, samples were centrifuged at maximum velocity (17.000 x g) for 5 min (Heraeus Pico 17, Thermo Scientific, Germany) and the DNA was stored at -20 ºC. The extracted DNA was quantified spectrophotometrically using a NanoPhotometer^{TM} UV/VIS spectrometer (GmbH, Implen, Germany).

16S rRNA genes were amplified by PCR. The mixture (NZYTaq) contained 50 mM Tris-HCl, pH 9.0, 50 mM NaCl, 2.5 mM $MgCl_2$, 200 µM each of dATP, dCTP, dGTP and dTTP, 5 µg of activated salmon sperm DNA, 0.2 U/µl of Taq DNA polymerase and 0.5µM each of 27F (5'-GAGTTTGATCCTGGCTCA-3) and 1493R (5'-TACCTTGTTACGACTT-3´) primers (Eurofins MWG), in a final volume of 25 µl. The PCR reactions were performed on a thermocycler (DOPPIO, VWR, USA) using the following parameters: one cycle of initial denaturation at 95 ºC for 120 s, 25 cycles of denaturation at 95 ºC for 30 s, annealing at 54 ºC for 30 s and extension at 72 ºC for 1 min and finally one cycle of a final extension at 72 ºC for five min. The final product were analysed by electrophoreses in a 1 % agarose gel in Tris-EDTA (TAE) buffer with DNA stain Gel Red^{TM} (Biotium) for 45 min at 120 V and 400mA. PCR products of all 129 bacteria isolates were sent to sequencing by STAB vida, Portugal.

3.9 Chitosan versus heat treatment methods on wood sterilization

Samples of wood chips were obtained by drilling wood blocks using a DeWalt DCD732D2 drill making a hole with 12.0 mm of diameter and 15 cm of depth. A nematode suspension of five ml H_2O with about 2000 nematodes was made and pipetted into a plastic bag were three g of wood chips where later added and mixed with the nematode suspension. The wood chips with the nematode were then incubated for 48 h at 25 ºC.

For the kiln treatment (WTB Binder 7200 tuttlingen), three different temperatures (25, 55 and 65 ºC) and four different exposure times (15, 30, 45 and 60 min) for each temperature were performed. For the microwave treatment (Candy CMG 2392 DW) two different exposure times (30 s and 1 min) of 1000 watts were applied. For each condition three samples of wood chips with 2000 nematodes, obtained as previously described, were considered. As control, three samples of wood chips were inoculated with water.

To test if chitosan could be used as an alternative wood treatment solution, a solution was made by adding 21.9 g of chitosan with LMW (Sigma Aldrich) to 500 ml of a 5 % acetic acid solution to a final concentration of 43.8 % of low MW chitosan. For this experiment nine samples of wood chips where submitted for 24 h to the chitosan treatments which consisted of adding chitosan solutions with different concentrations (10.95 %, 21.9 % and 43.8 %). Three replicates per treatment were considered (N=9). The samples were inoculated with 2000 nematodes and finally incubated at 25 ºC for 48 h. The nematodes were extracted using the Baermann funnel technique and determined using a nematode counting dish under a transmitted light stereo microscope.

3.10 Statistical analysis

The data obtained was analyzed using GraphPad InStat for Windows (Version 3.05, 16 bit, GraphPad Sotware, Inc.). Treatment differences were tested by one-way ANOVA – Tukey comparison ($p < 0.05$).

4. Results and Discussion

4.1 Evaluation of the effect of soil amendment with a chitosan solution in *P. pinaster* trees infected with *B. xylophilus*

Symptom development

Successful nematode infection in the pine trees is firstly visualized by the presence of needle discoloration (Nascimento *et al*., 2015). In the current work these visual symptoms were registered in order to understand and compare the effect of chitosan on the tree survival. Also it will allow us to conclude if the nematode infection was successful. After the inoculation of the pine trees with the nematode suspension the presence of discoloration in the needles was observed and registered at each time-point (*dai*, days after inoculation). The external symptoms classification was adapted from Nunes da Silva *et al* (2013), with the difference that a new classification was added with the number 0 (completely green) and 5 (tree death). The results are shown in Figure 4.1.1.

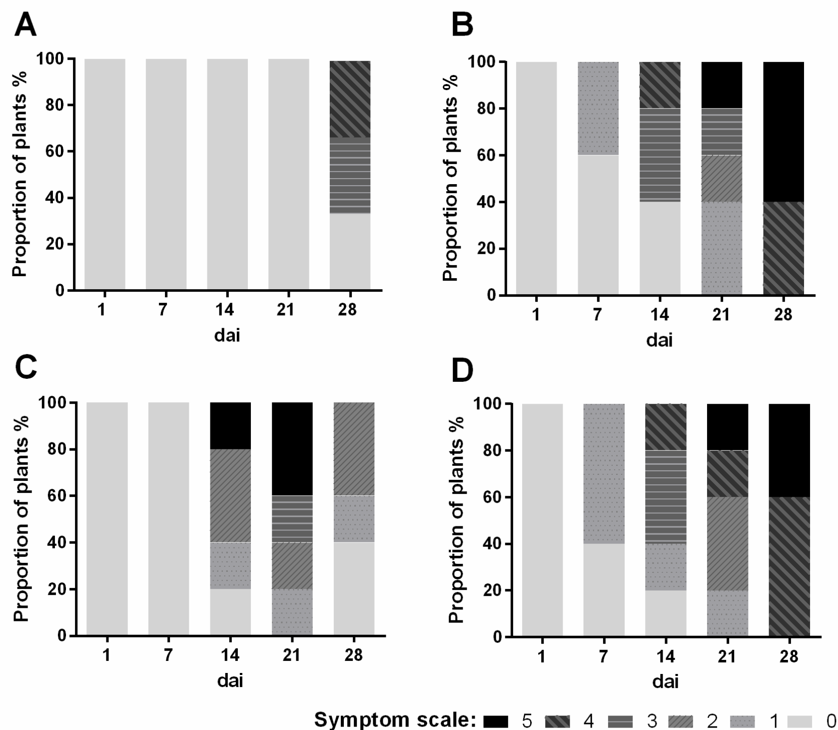

Figure 4.1.1 – Progression of the visual symptoms in the pine trees treated: (A) without chitosan, without nematodes; (B) without chitosan, with nematodes; (C) with chitosan, without nematodes; (D) with chitosan, with nematodes. Symptoms were classified as: 0 – plant completely green; 1 – <10% of brown needles; 2 – 10-25% of brown needles; 3 – 25-50% brown needles; 4 - >50% brown needles; 5 – tree death.

Until 7 dai most plants did not present visual symptoms of infection. The control plants, kept in soil without chitosan (Figure 4.1.1), did not present symptoms of chlorosis through the experimental time period, recording symptoms only after 28 dai (the emergence of less than 10 % of yellow leaves). This low percentage of chlorosis could come from the fact that the soil was supplemented with a solution of

acetic acid that could have led to acidification of the soil and consequently yellowing of the leaves. In the plants kept in soil with chitosan and without nematodes (Figure 4.1.1), a yellowing of the leaves from 14 to 21 dai followed by a recovery of the normal coloration of the leaves at 28 dai was registered. This transient visual toxicity symptoms could also be due the activation of the plant defence mechanisms by chitosan (Agrawal *et al*. 2002). In the plants inoculated with nematode, kept in soil without chitosan (Figure 4.1.1), there was a progression of the symptoms until the death of the trees at 28 dai with 60 % of plant death. In the plants inoculated with nematodes and treated with chitosan (Figure 4.1.1), even though there was a trigger of symptoms at 14 dai, the symptoms evolved slower, reaching 40 % of plant death which is lower than the 60 % of plant death obtained in the treatment without chitosan and with nematode.

This result is in conformity with a previous study that showed that chitosan reduces the loss of chlorophylls and reproduction of nematodes (Nunes da Silva *et al*., 2014) when compared to control plants. Previous studies show that the reddish discolouration of old needles is one of the first symptoms observed in *P. thunbergii* and *P. densiflora* infected with PWN (Zhao *et al*., 2008). The time that it takes for visual symptoms to develop depends on tree age, weather and soil mixture. In some studies this period was found to be three weeks after inoculation, after which discoloration of the younger needles and subsequent host death occurs in about 1-2 months after inoculation (Kuroda *et al*., 1988).

Nematode quantification

In order to monitor nematode multiplication in plants with or without chitosan five samples of each treatment were harvest at different time-points and nematodes were counted. The results obtained are described in figure 4.1.2.

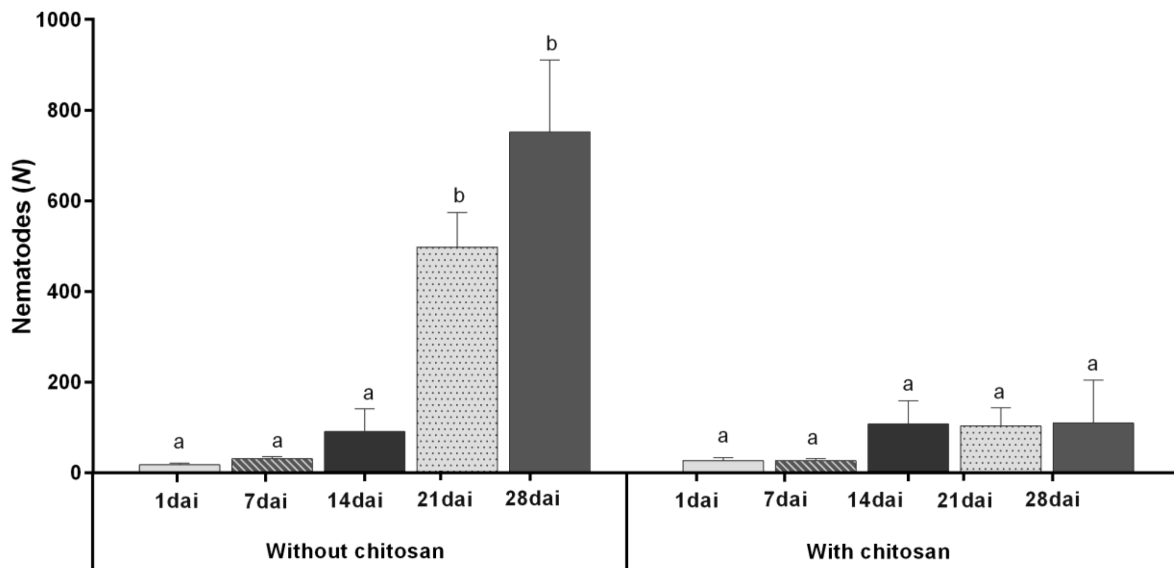

Figure 4.1.2 – Total number of nematodes (N) extracted from the stems at each time-point (1, 7, 14, 21 and 28 dai). Each value is the mean of 5 seedlings. Error bars represent standard deviation. Bars showing the same letter are not significantly different ($P < 0.05$).

Figure 4.1.2 shows that without chitosan there was a significant increase ($P < 0.05$) in the total number of nematodes (17 ± 4 nematodes at 1 dai to 751 ± 158 nematodes at 28 dai), indicating the aggravation

of the disease over time. In fact, as the visual symptoms progressed from 14 days until death of the plant at 28 days, it was found that the number of nematodes also increased significantly since this experimental point until the end of the analysis period, reaching about 751 ± 158 nematodes per plant. In contrast, with chitosan the number of nematodes was low and statistically identical over the entire experimental time-period. In fact, only 110 ± 93 nematodes were recovered from plants inoculated in the presence of chitosan, which is about one-seventh the number found in plants not treated with this compound. This last result indicates that this compound prevented, or at least, slowed the beginning and the progression of the pine wood disease. Since the PWD is divided into two stages, where in the advanced stage there is a drastic increase in the number of nematodes in the plant tissues, we can conclude that in the plants inoculated with nematode in the absence of chitosan the disease progressed to the advanced stage. These results are concordant with Nunes da Silva *et al* (2014), where low MW chitosan prevented nematode multiplication over time.

Photosynthetic pigments quantification

For the quantification of the photosynthetic pigments the Sims and Gamon (2002) method was used. The results are described in Figure 4.1.3 and 4.1.4.

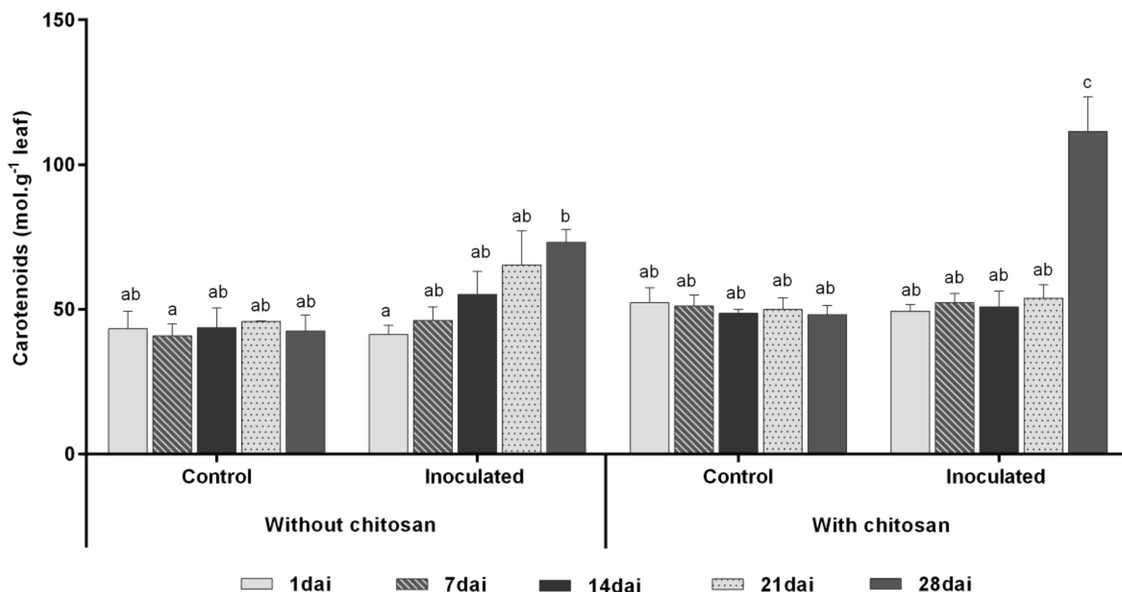

Figure 4.1.3 – Carotenoids (mol.g^{-1} leaf) extracted from the needles at each time-point (1, 7, 14, 21 and 28 dai). Each value is the mean of 5 seedlings. Error bars represent standard deviation. Bars showing the same letter are not significantly different ($P < 0.05$).

From the analysis of Figure 4.1.3 we can observe that the control plants (not inoculated with nematodes) did not show significant variances in the content of carotenoids throughout the trial period; however, the inoculated plants, either without or with chitosan, showed a significant increase in the concentration of carotenoids from day 1 to 28 dai. This increase in the synthesis of carotenoids at 28 dai was greater in plants treated with chitosan (going from 53 ± 4.6 mol/g^{-1} of leaf at 21 dai to 111 ± 11.9 mol/g^{-1} of leaf at 28 dai) compared with plants inoculated without chitosan. The carotenoids have the capacity to capture electrons and reduce and capture free radicals (Griffiths et al., 1955; Sistrom et al., 1956). This means

that the treatment with chitosan also induced the synthesis of carotenoids so that it enhances the plant defence mechanisms as described before by Nunes da Silva *et al* 2014. Furthermore, the carotenoids are accessory pigments that participle in the harvest of light, have photo protective functions, and stabilize the complexes protein-pigment of the photosynthetic apparatus. The increase of this metabolite in the plants inoculated with nematode can be explained as an attempt of the plants to minimize the adverse effects that the nematode induces in the plant tissues, trough the maintenance of photosynthetic capacity (Nunes da Silva *et al*., 2014).

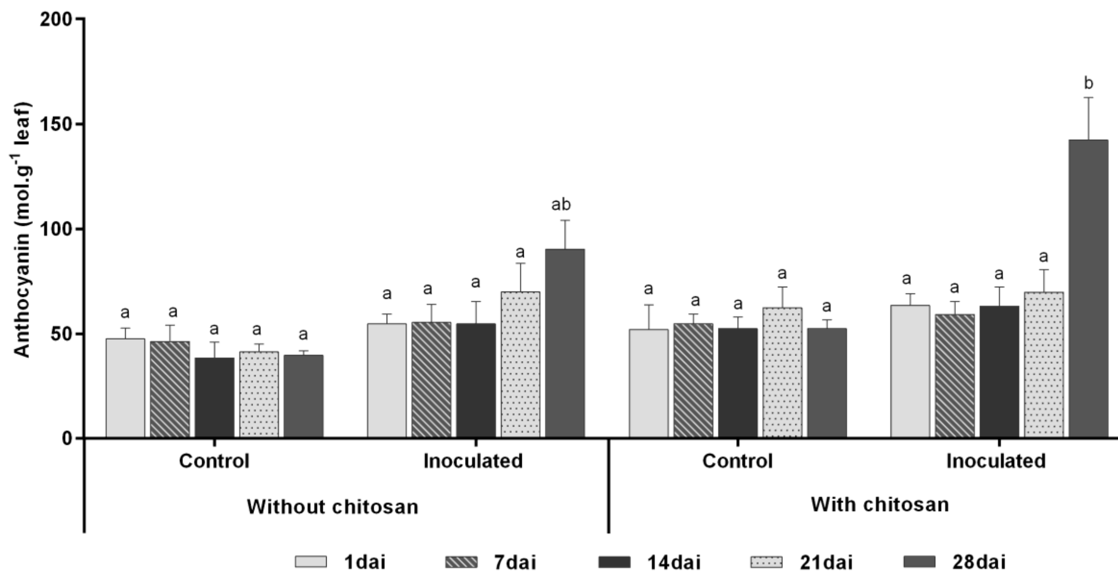

Figure 4.1.4 – Anthocyanin (mol.g^{-1} leaf) extracted from the needles at each time-point (1, 7, 14, 21 and 28 dai). Each value is the mean of 5 seedlings. Error bars represent standard deviation. Bars showing the same letter are not significantly different ($P < 0.05$).

Anthocyanins are pigments that belong to the flavonoids and their function is the protection of the plants against the ultraviolet (UV) light and the oxidative stress (Quina, 2009). In this work the anthocyanin concentration (Figure 4.1.4) increased (going from 63 ± 5.5 mol.g^{-1} of leaf to 142 ± 20.4 mol.g^{-1}) significantly in the plants inoculated and treated with chitosan at 28 dai, but not in the other treatments. This shows, once again, the ability of chitosan to improve the defence mechanisms of the plants against the biotic stress as previously reported (Nunes da Silva *et al*., 2014).

Total soluble phenolics determination

Total phenolic determination was realized using the Azevedo and Herlânder (2005) method. The results are described in Figure 4.1.5.

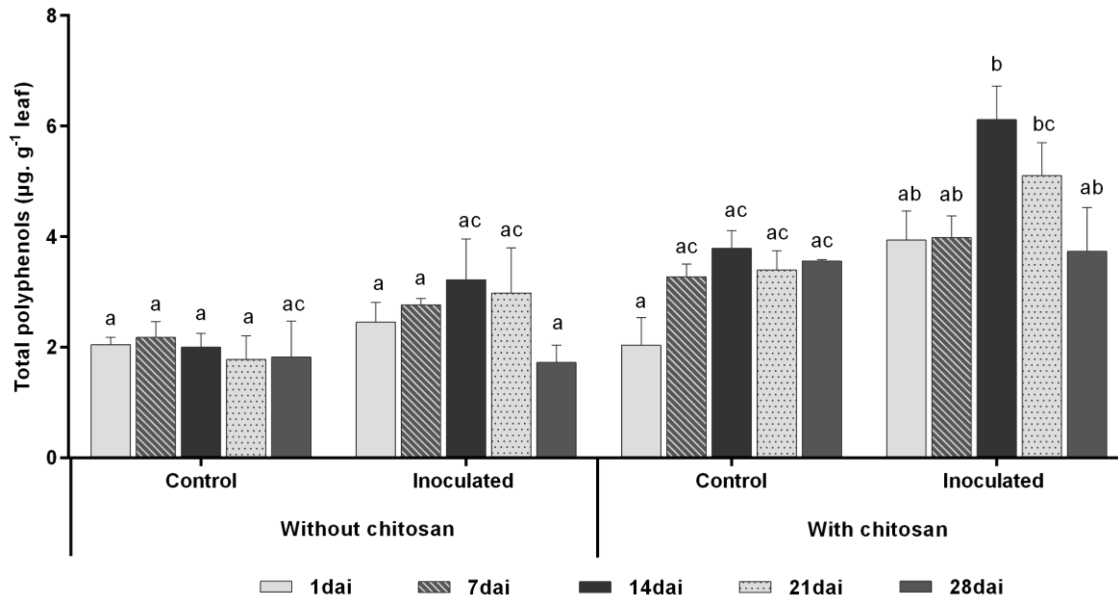

Figure 4.1.5 – Total polyphenols (µg.g^{-1} leaf) extracted from the needles at each time-point (1, 7, 14, 21 and 28 dai). Each value is the mean of 5 seedlings. Error bars represent standard deviation. Bars showing the same letter are not significantly different (P < 0.05).

From the analysis of Figure 4.1.5 we can observe that the synthesis of total polyphenols was about two times higher in the treatments with chitosan (6.1 ± 0.6 µg.g^{-1} leaf) than in those without chitosan (3.2 ± 0.7 µg.g^{-1} leaf). In the experiment with chitosan and inoculated with nematodes, nematode infection increased the production of total polyphenols in the plant (going from 2.7 ± 0.1 µg.g^{-1} at 7 dai to 3.2 ± 0.7 µg.g^{-1} at 14 dai) as expected. Treatment with nematodes and chitosan presented the highest levels of total polyphenols through time with a peak at 14 dai (6.1 ± 0.6 µg.g^{-1}). The production and accumulation of polyphenols is suggested to be the reason for the browning in the pine tissues. Studies show that polyphenols accumulate in the plant as a response to PWD when compared to controls (Futai, 2013) and that some phenolic compounds have nematicidal activity (Suga *et al.*, 1993). Recently, a study by Kuroda *et al* (2011) showed that there was an increase in the production of total polyphenols in PWD-resistant variety of *Pinus densiflora*, suggesting that it could lead to the tree resistance. This increase in total polyphenols in the presence of chitosan might be one of the reasons why the *P. pinaster* trees treated with this compound prevented, or at least, slowed the beginning and the severity of the pine wood disease.

Lignin quantification

Lignin quantification was determined using the acetyl bromide method (Hatfield et al. 1999; Fukushima and Hatfield 2001), and the results are described in Figure 4.1.6.

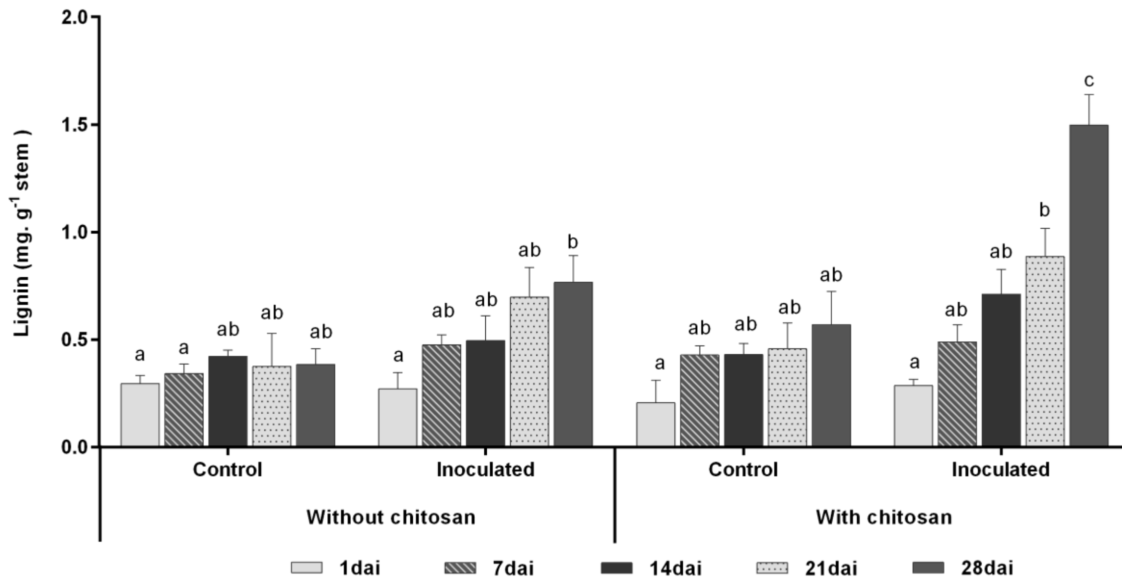

Figure 4.1.6 – Lignin (mg.g^{-1} leaf) extracted from the needles at each time-point (1, 7, 14, 21 and 28 dai). Each value is the mean of 5 seedlings. Error bars represent standard deviation. Bars showing the same letter are not significantly different (P < 0.05).

The analysis of Figure 4.1.6 shows that without chitosan nematodes infections increases the levels of lignin from 0.5 ± 0.1 mg.g^{-1} at 14 dai to 0.7 ± 0.1 mg.g^{-1} at 28 dai. This might be due to the fact that the plant tries to minimize the adverse effects that the nematode induces in the plant tissues, since constitutive lignin was already associated with defence mechanisms against nematodes (Kawaguchi, 2006). Lignin biosynthesis usually occurs in the advance stage of the infection as a defence mechanism to the PWN and is thought to be a resistance factor to nematode inoculation because harder tissues might restrict the movements of the nematode inside the plant (Franco et al. 2011). With chitosan at 28 dai, the inoculated pine trees showed significantly higher concentrations of lignin than the pine trees inoculated with nematode and without chitosan (0.7 ± 0.1 mg.g^{-1} at 28 dai and 1.5 ± 0.1 mg.g^{-1} at 28 dai, respectively). This result confirms the finding that chitosan induces the biosynthesis of several antimicrobial compounds such as lignin (Barber *et al.*, 1989).

4.2 Analysis of the impact of chitosan on nematode bacterial population

4.2.1 From the inoculated trees

Chitosan molecules are positively charged and when they encounter the negatively charged microbial cell walls, they lead to leakage of proteinaceous and other intracellular constituents (Seo *et al.,* 1992; Fang *et al.,* 1994; Chen *et al.,* 1998; Jung *et al.,* 1999). It is also a chelating agent that selectively binds trace metals and inhibit the production of toxins and microbial growth (Cuero *et al.,* 1991). It also acts as a water-binding agent, inhibiting various enzymes and activates different defence processes in the

host tissue (Ghaouth *et al*., 1992). Another possible mechanism is by the interaction between positively charged chitosan with the cellular DNA of some fungi and bacteria that will inhibit RNA and protein synthesis (Hadwiger *et al.,* 1986; Sudarshan *et al.,* 1992). It is only capable of its antimicrobial activities when in acidic medium due to its poor solubility in pH above 6.5 (Rabea *et al.,* 2003). In order to understand if the protective role of chitosan observed above was also linked to a modulation of the bacterial population in infected plants, segments of the previously analysed trees were analysed for their bacterial community.

The inoculation of pine tree chips was made by using a modified method of Zie and Xhao (2008). The bacteria present in the trails left by the nematode in the mediums (AC agar, LB agar and TSA) were isolated and identified. Figure 4.2.1.1 show examples of the results.

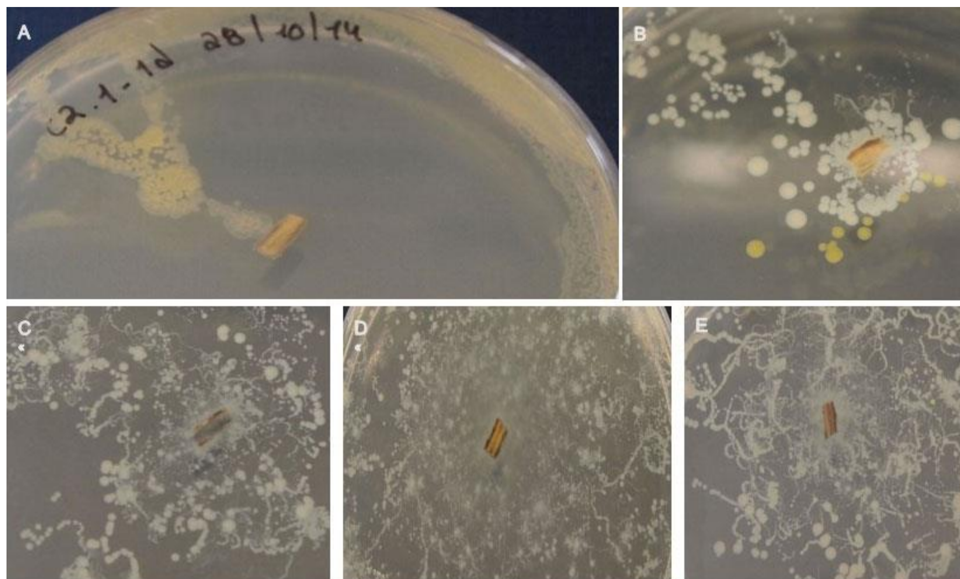

Figure 4.2.1.1 – Bacterial colonies left by the trail of the nematodes in the agar mediums (AC, LB and TSA). A – Bacteria present in 1 dai; B - Bacteria present in 7 dai; C - Bacteria present in 14 dai; D - Bacteria present in 21 dai; E - Bacteria present in 28 dai.

The colonies obtained from the bacteria present in the trail of the nematode did not present major differences in terms of appearance (size, colour, shape, transparency, proeminence, edge and viscosity), being mostly medium sized, white with bege center, round, convexed, transparent and smooth.

The number of bacteria was determined in the stems at each time-point and treatment, and the results expressed in terms of CFU. A number of dilutions were performed from the original inoculum in order to be able to count the CFU. The results varied from 0 to 300 CFU, if more then 300 CFU were counted then the >300 identification was given. Figure 4.2.1.2 shows the results obtained.

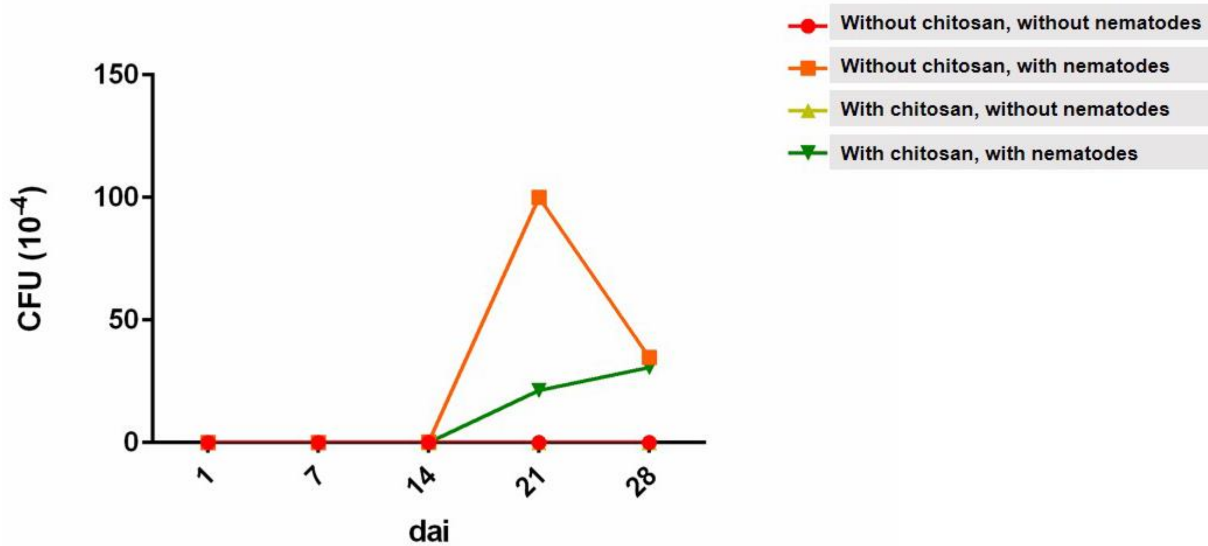

Figure 4.2.1.2– Colony-forming units (CFU) average formed in the medium Luria-Bertani (LB) agar.

The control treatments (with or without chitosan and without nematode) did not present any CFU as in the experiment of Xie and Zao (2008) and Roriz *et al* (2011). Fourteen dai the plants with and without chitosan and inoculated with nematode showed an increase in the number of CFU. In the plant inoculated with nematode and without chitosan the number of CFU increased until the 21 dai after which it showed a decrease in number. This decrease could be explained by the death of the tree and subsequent death of the nematodes and nematode associated bacteria. In the treatment inoculated with nematode and with chitosan there was a slight increase until the 28 dai followed by a tendency to a stabilization of the CFU number.

Total genomic DNA of the bacteria was extracted and the 1500 bp fragment of the 16S rRNA gene was amplified by PCR. An example of the results obtained on electrophoreses of the PCR samples is shown in Figure 4.2.1.3.

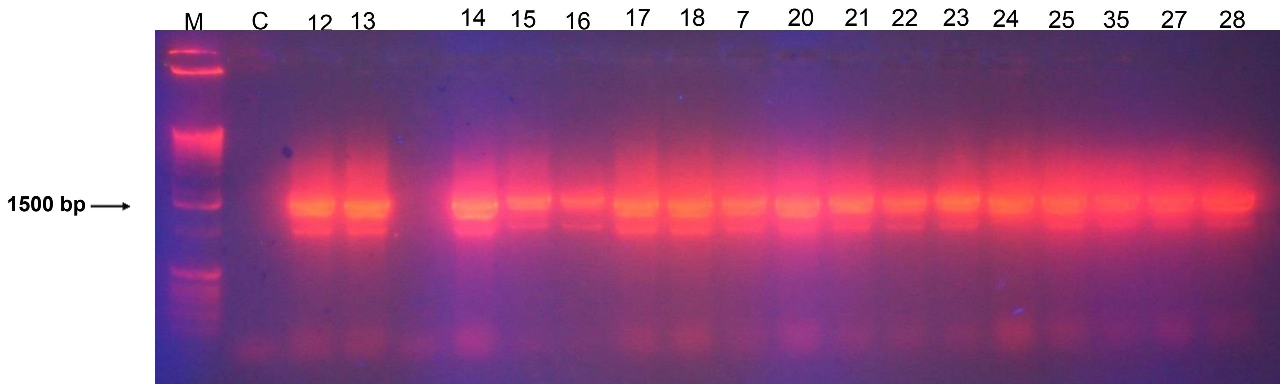

Figure 4.2.1.3 – Agarose gel stained with Gel Red™ (Biotium) showing the results of the amplification of the 16s rRNA gene corresponding to a 1500bp fragment (M corresponds to the marker ladder; C corresponds to the negative control and columns 12 to 28 represent the isolated colonies).

The 129 PCR products obtained from the colonies isolated from the tree segments were sent for sequencing to a commercial providor (STAB VIDA, Portugal), the sequences were submitted to a blastN for strain identification, and the results of the identifications are presented in the appendix (Tables 6.1; 6.2; 6.3; 6.4 and 6.5)

Of the 129 isolates obtained, the main bacteria species found was *Enterobacter ludwigii* and *cloacae* (81.40%), followed by *Paenibacillus* sp. (5.43%), *Buttiauxella noackiae* and *warmboldiae* (4.65%), *Bacillus cereus* (2.33%), *Stenotrophomonas maltophilia* (2.33%), *Pseudomonas* sp. (1.55%), *Pantoea agglomerans* (0.78%), *Klebsiella oxytoca* (0.78%) and finally *Klyuvera ascorbata* (0,78%).

At 1 dai only a *Pseudomonas koreensis* was obtained from the treatment with the plants inoculated with PWN and without chitosan (Figure 4.2.1.4). *Pseudomonas koreensis* was found associated with the PWN (Morais, *et al*., 2013) and with its insect vector (Vicente *et al*., 2013) in Portugal. *Pseudomonas* sp. has also been extensively described as being associated with the PWN in Portugal (Proença *et al*., 2010; Roriz *et al*., 2011; Vicente *et al*., 2011; Vicente *et al.,* 2012) and in other countries (Zhao *et al*., 2003; Zhao and Guo, 2004; Zhao and Lin 2005; Zhao *et al*., 2009; Tian *et al*., 2011; Zhu *et al*., 2012; Wu *et al*., 2013) being the most isolations occuring in China (Han *et al*., 2003; Zhao *et a*l., 2003; Zhao *et al*., 2005; Tian *et al*., 2010) and one of the most isolated in Japan (Oku *et al*., 1980).

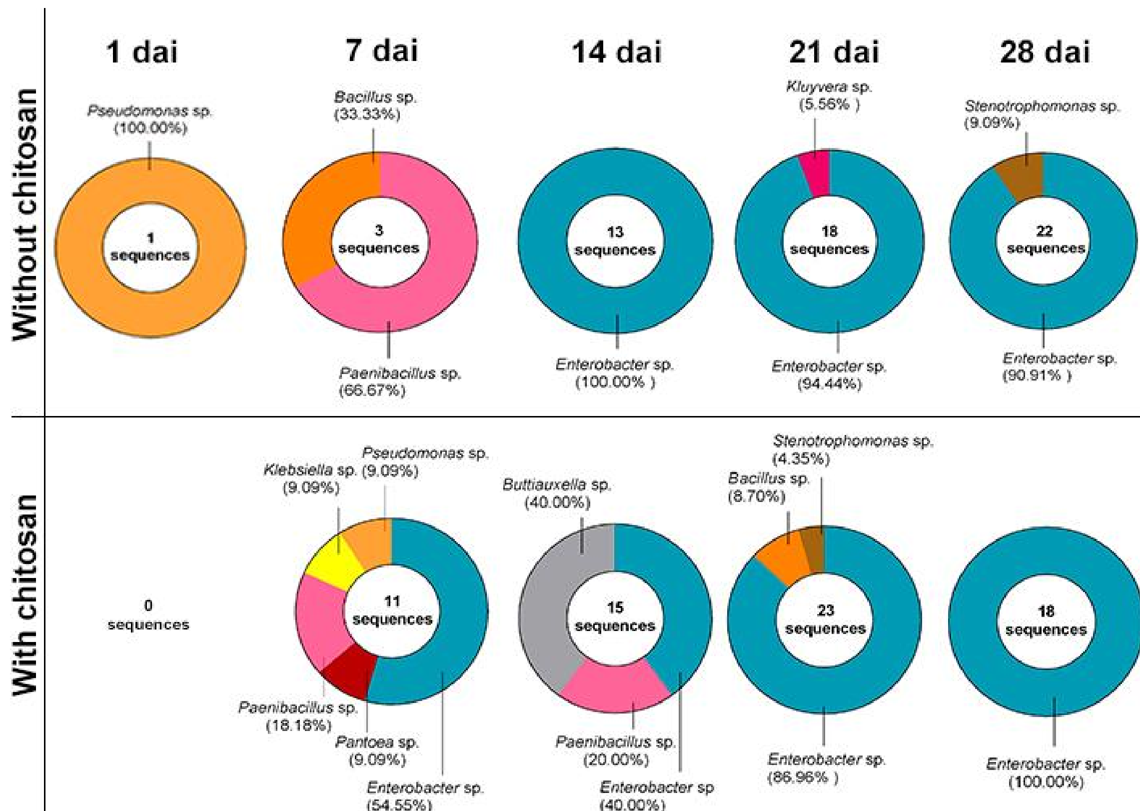

Figure 4.2.1.4 – Results of the percentage of each strain isolated from the nematodes not treated and treated with chitosan.

Seven dai three bacteria isolates were obtained and identified as being, two *Paenibacillus* sp. and one *Bacillus* sp. Previous studies associate the *Bacillus* sp. with the PWN (Tamura and Mamiya, 1973; Kawuzu *et al*., 1996C; Tan and Feng, 2004; Ma *et al*., 2009; Roriz *et al.*, 2011) and *Monochamus galloprovincialis* (Vicente *et al*., 2013a). *Bacillus* sp. is the predominant genus in Japan (Kawazu, *et al.*, 1996a). Strains of this genus are able to suppress some plants pathogens, including nematodes, and promote plant growth (Gokta and Swarup, 1988; Li *et al*., 2005). The *Bacillus* sp. is usually found in water and soil (Parvathi et al., 2009), this could tell that this genus was originated from the soil, but since no bacteria was isolated in the controls, this suggests that it was associated with the nematode. *Paenibacillus* sp. was reported in a few studies as a bacteria associated with the PWN (Tian et al., 2011; Roriz et al., 2011) and it’s insect vector *Monochamus galloprovincialis* (Vicente et al., 2013a). Strains of this genus have been documented to be present in a variety of places, like soil (Berge et al., 2002), water, rizhosphere (Silveira, 2003) vegetable matter, foods, tree roots, forage and insect larvae (Daane et al., 2002).

At 14 dai, 13 isolates were obtained in treatment with nematodes and without chitosan. All of this isolates were identified as being *Enterobacter cloacae* or *Enterobacter ludwigii*. Treatment with nematodes and chitosan presented 15 isolates, three of which were not sequenced due to low DNA purity, six colonies were identified as *Enterobacter* sp., three *Paenibacillus* sp. and six *Buttiauxella* sp. (one *Buttiauxella warmboldiae* and five *Buttiauxella noackiae*). *Buttiauxella* sp. was described as being associated with the PWN (Zhao *et al*., 2003).

At 21 dai, 17 isolates were obtained in the treatment with nematode and without chitosan, sixteen were identified as *Enterobacter* sp*.* and one as *Klyuvera ascorbata*. This genus and strain was never described as being associated with the PWN which supports the theory that this bacteria differ

between geographic regions and suggests that this strain might be endemic of the geographic region of Portugal from where the strain 65 GO of the nematode was obtained. *Kluyvera* sp. is usually found in soil, water, sewage, hospitals sinks and food products of animal origin (Farmer *et al*., 1981). Treatment with nematodes and chitosan presented 23 isolates, were one of them was not sequenced due to low DNA purity, twenty were identified as *Enterobacter ludwigii* or *cloacae,* two as a *Bacillus cereus* and one as a *Stenotrophomonas maltophilia*. *Stenotrophomonas* sp. was already described in many studies as being associated with the PWN (Zhao et al., 2003; Yuan et al., 2011; Zhao et al., 2011; Tian et al., 2011; Vicente et al., 2011) and it's insect vector, *Monochamus galloprovincialis* (Vicente et al., 2013a). *Stenotrophomonas* is ubiquitous in the environment, soil and plants. *S. maltophilia* is the strain predominant in plants with a worldwide distribution and can be beneficial for them (Denton and Kerr, 1998; Berg et al., 2010). A study by Zhao et al (2011) shows that *S. maltophilia* was the most toxic to pine seedlings.

28 dai, treatment with nematodes and without chitosan presented 22 isolates, twenty-one were identified as *Enterobacter ludwigii* and *cloacae* and one as *Stenotrophomonas maltophilia*.

As infection progressed, the isolates of the genus Enterobacter became more abundant. At 28 dai, 18 isolates were obtained and all of them were identified as being *Enterobacter* sp. In the treatment with nematode and chitosan, after seven dai, 11 bacteria isolates were obtained. Five of those isolates were identified as being *Enterobacter* sp., specifically *Enterobacter ludwigii* and *Enterobacter cloacae*, two were *Paenibacillus* sp., one *Pseudomonas* sp., one *Klebsiella oxytoca* and one *Pantoea agglomerans*. Strains of the *Enterobacter* genus have been report in association with the PWN (Zhao *et al*., 2003; Zhao and Lin 2005; Xie and Zhao, 2008; Zhao *et al*., 2009; Know *et al*., 2010; Tian *et al*., 2011; Vicente *et al*., 2011; Yuan *et al*., 2011; Roriz *et al*., 2011; Vicente *et al*., 2012b). A study by Vicente *et al* (2011) describes Enterobacter sp. as one of the most commonly isolated species in pinewood nematode cultures. Another study by Vicente *et al* (2012) also reported that bacteria associated with PWN, namely *Serratia* sp., *Enterobacter* sp. and *Pantoea* sp., induce similar symptoms to the ones made by the PWN in *P. pinaster*, 45 dai.

Klebsiella sp. was already described as being associated with the PWN (Zhao *et al*, 2005; Proença *et al*., 2010; Vicente *et al.*, 2011; Roriz *et al*., 2011). *Klebsiella oxytoca* was referred by Roriz *et al*. (2011) as the main bacterial strain associated with the nematode and it established a symbiosis with it. Previous studies associate *Pantoea* sp. with the PWN (Hong *et al.,* 2002; Han *et al*., 2003; Zhao and Guo, 2004; Zhao and Lin, 2005; Zhao *et al*., 2009; Proença *et al*., 2010; Roriz *et al.,* 2011). *Pantoea agglomerans* can be found in soil and plants (Andersson *et al*., 1999).

Enterobacter sp. was the predominant genus found in this study through time and in both treatments which indicates that this genus is associated with the disease and the nematode and geographic region. A previous study by Vicente *et a*l (2011) isolated and identified the bacteria associated with the PWN in laboratory pine wood nematode cultures and PWN from a symptomatic tree. This study showed that in the laboratory cultures, the most associated bacteria was *Enterobacter* sp. and concluded that these bacteria showed a strong specific bacteria nematode relationship, since it's still present after successive generations of PWN. Still, we observed that chitosan increases the defence response of the plant to the PWN triggered at 14 dai. And at this time-point, the bacteria found in the

nematodes treated with chitosan were more abundant and diverse than the ones found in the nematodes without chitosan at the same time-point, which were all *Enterobacter* sp. It is possible that the protective effect of chitosan at 14 dai may be linked to this effect on the bacterial population.

4.2.2 From cultured nematodes

In order to isolate the effect of chitosan on the bacterial population of the nematode without the effect of the tree inoculation process, an "*in vitro*" study was performed on nematodes obtained in the laboratory cultures and subjected to a 24 h chitosan treatment.

Forty seven isolates were obtained from nematodes**.** Of these, the main bacteria found were *Pseudomonas japonica* and *P. putida* (50%), *Enterobacter cloacae* and *ludwigii* (41.3%) and *Pantoea agglomerans* (8.7%).

The nematodes treated with H_2O were used as control since the bacteria isolated from these samples would be natural PWN associated bacteria that did not suffer influence from the plant host, from chitosan or from the acetic acid in which the chitosan is diluted. From these, nine isolates were *Enterobacter cloacae* and *ludwigii*, seven isolates were *Pseudomonas japonica* and *P. putida* and finally three isolate were *Pantoea agglomerans*. *Pseudomonas putida* has been found to be associated with the PWN insect vector (*Monochamus galloprovincialis*) (Proença *et a*l., 2010) and already been described as associated to the PWN in China (Zhao *et al*., 2003). This is the first report of *Pseudomonas japonica* in association with the PWN.

Previous studies associate *Pantoea* sp. with the PWN (Hong *et al.,* 2002; Han *et al*., 2003; Zhao and Guo, 2004; Zhao and Lin, 2005; Zhao *et al*., 2009; Proença *et al*., 2010; Roriz *et al.,* 2011). *Pantoea agglomerans* can be found in soil and plants (Andersson *et al*., 1999).

The bacteria of the nematodes maintained in 0.5% acetic acid were studied to verify if the acetic acid in which the chitosan is dissolved does influence the bacteria population. In these samples five isolates were *Pseudomonas japonica*, one isolate was *Pantoea agglomerans* and eleven isolated were *Enterobacter ludwigii* and *cloacae*. The results are shown in figure 4.2.2.1.

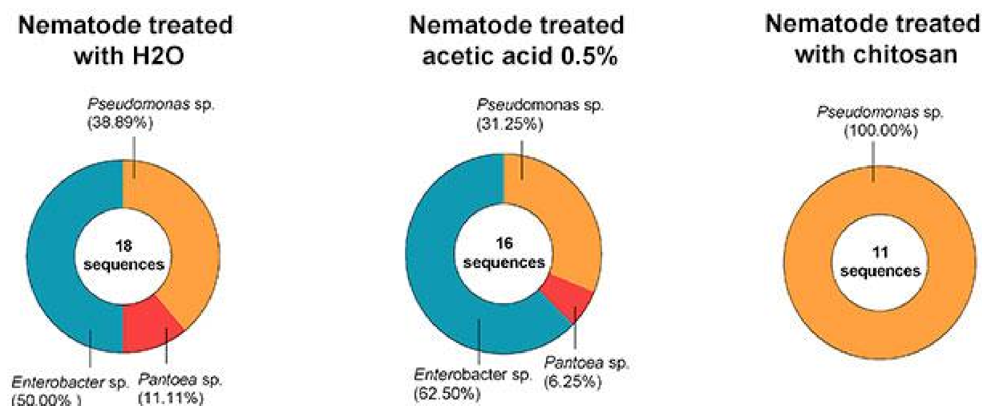

Figure 4.2.2.1 – Results of the percentage of each strain isolated from the nematodes treated with water, acetic acid 0.5% and chitosan.

The bacteria isolated from the nematodes maintained in 0.15% low MW chitosan were studied to understand the effect of chitosan on the population of the PWN associated bacteria. From these samples all eleven isolated were identified as *Pseudomonas putida* and *japonica*.

In this study the PWN associated bacteria found on both nematodes isolated from H_2O samples and 0.5% acetic acid samples don't seem to differ and *Pseudomonas* sp., *Enterobacter* sp., and *Pantoea* sp. were found. The PWN associated bacteria found on the nematodes isolated from the chitosan solution changed drastically and only *Pseudomonas japonica* and *putida* were found. This may indicate that chitosan does influence the population of PWN nematode associated bacteria and the severity and progression of the PWD and that *Pseudomonas* sp. might be more resistant to this concentration of chitosan than *Enterobacter* sp. and *Pantoea* sp. In this analysis of the nematode bacterial population *Stenotrophomonas* sp., *Bacillus* sp., *Kluyvera* sp., *Buttiauxella* sp. *Klebsiella* sp. and *Paenibacillus* sp. weren't found associated with the PWN which might indicate that this bacteria are transmitted from the plant to the PWN when it migrated through it.

4.3 Chitosan versus conventional treatment methods on wood sterilization

Given the promising results of chitosan on reducing nematode population in inoculated trees, and given the high costs of the mandatory heat-treatment of wood, we decided to test the efficacy of chitosan in eliminating nematodes on infected wood. We compared chitosan's efficiency to the conventional heat-treatment methods, such as kiln-drying and microwave energy

Heat-Treatment

Heat-treatment is a method applied to wood packing materials to significantly reduce the spread of pests and their negative impacts. In this method, a specific time-temperature schedule is used to achieve a minimum temperature of 56 ºC for 30 continuous minutes throughout the entire profile of the wood (ISPM-15). However, the efficacy of this treatment has been questioned. For the performance of the heat-treatment various sources of energy might be used as long as they achieve the previously described parameters. Such as kiln-drying, heat-enabled chemical pressure impregnation, microwave or others treatments. Heat-treatment using microwave energy was showed to being an effective method in previous studies (Kim and Suh 2014, Payette et al., 2015). In this study, the heat-treatment by kiln-drying and microwave energy was evaluated.

Three different temperatures were used for this evaluation (25 ºC, 56 ºC and 65 ºC). The ideal temperature for nematode reproduction is 25 ºC and so control samples were submitted to this temperature. The temperature used in the heat treatment of wood, as described in ISPM-15, is 56ºC and 65 ºC was used to evaluate if nematodes survive at higher temperatures then 56 ºC. For each temperature four different times were applied (15, 30, 45 and 60 minutes) to the wood chip samples. In this study about 4 g of *P. pinaster* wood chips infected with about 2000 PWN as previously described were used. The results are shown in Figure 4.3.1.

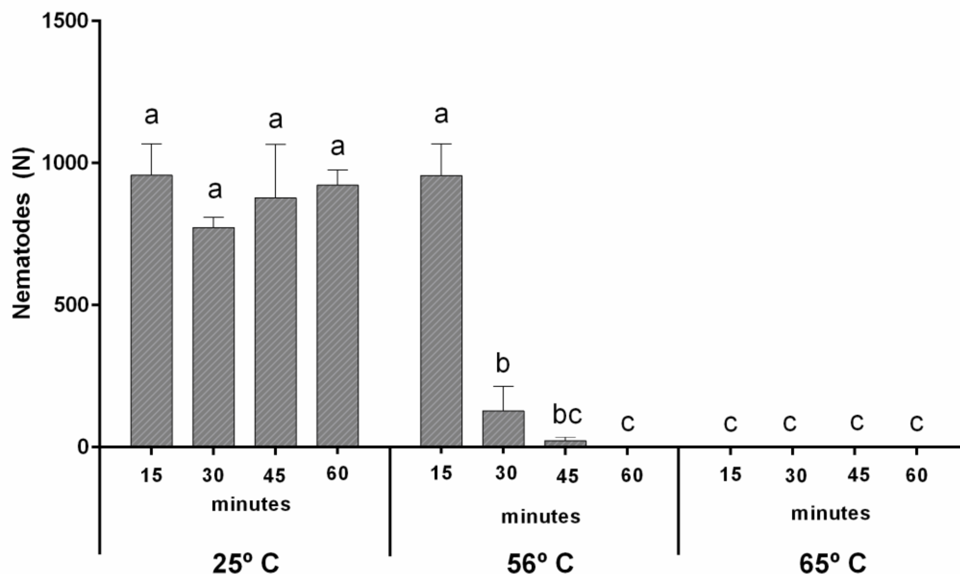

Figure 4.3.1 – Results of the quantification of total nematodes number in wood chips after heat treatment with different temperatures (25, 56 and 65ºC) applied for different times (15,30,45 and 60 minutes).). Each value is the mean of 3 wood chip samples. Error bars represent standard deviation. Bars showing the same letter are not significantly different (P < 0.05).

At 25 ºC the number of nematodes was the same for every heating time. At 56 ºC the number of nematodes started to decrease (from 956 ± 111 nematodes to 1 ± 1 nematode) after 15 min of heating and at 60 min there were no nematodes in the wood chips which shows that after 45 min of heating there were still viable nematodes in the wood chips (23 ±12 nematodes). This result was different then what expected since one of the phytosanitary treatments proposed in the ISPM-15 is the heat treatment in which 56 ºC are applied to the wood for 30 continuous minutes and in previous studies this heat treatment was shown to be efficient at eliminating the nematode population (Tomminen et al., 1992). At 65 ºC there were no viable nematodes visualized in the wood chips at every temperature. These results show that the 65 ºC for 15 min seems to be a viable and more efficient alternative for the still implemented heat-treatment.

For the evaluation of the effectiveness of microwave energy as a heat-treatment method against the PWN, wood chips were infected with 2000 nematodes and samples were submitted to of 1000 watts microwave energy for 30 s and 60 s. The results are showed in figure 4.3.2.

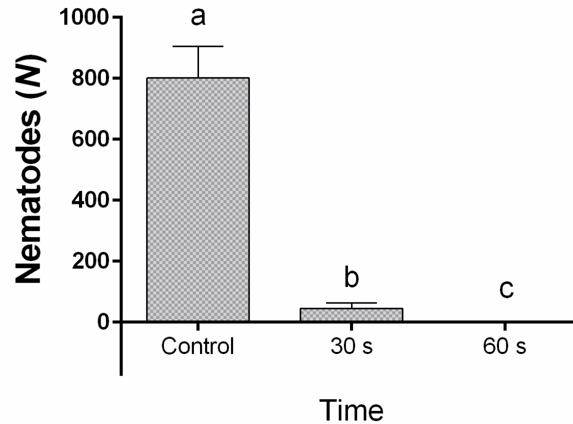

Figure 4.3.2 – Results of the quantification of total nematodes number in wood chips after heat treatment with microwave irradiation applied for different times (0, 30 seconds and 1 minute). Each value is the mean of 3 wood chip samples. Error bars represent standard deviation. Bars showing the same letter are not significantly different (P < 0.05).

Figure 4.3.2 shows that with the applications microwave irradiation for 30 s only 45 ± 17 nematodes survived. But with the application of microwave irradiation for 1 minute 100 % of the total nematode population was killed. This result was also achieved in previous studies (Hoover *et al*., 2010, Kim and Suh 2014, Payette *et al*., 2015) which shows that heat-treatment with microwave irradiation is a viable alternative to kiln-drying.

Chitosan

Chitosan is a aminopolysaccharide obtained from the deacetilation of chitin or from the cell walls of some fungus. Recent studies suggest that chitosan has a nematicidal activity (Khalil and Badawy 2012) and most specifically agaisnt the PWN (Nunes da Silva *et al*., 2014). However, the reasons behind this protection are still unknown. In this experiment the effect of different concentrations of chitosan (10.95, 21.9 and 43.8 g/L) on the total nematode population of infected wood chips was studied and compared to more convencional methods. A suspension of 2000 nematodes was used to infect wood chips. The results are presented in figure 3.1.3.

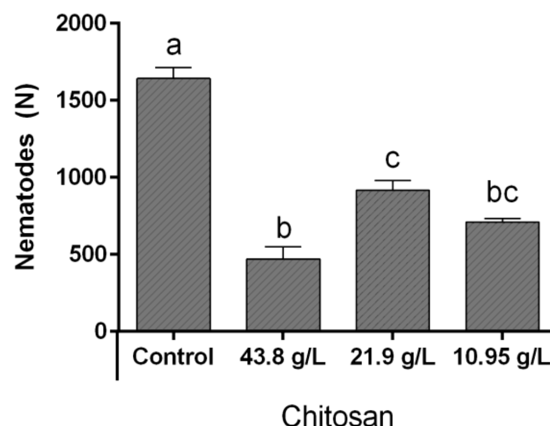

Figure 4.3.3 – Results of the quantification of the total nematode number in wood chips after treated with three chitosan solutions with different concentrations (43.8 g/L, 21.9 g/L and 10.95 g/L). Each value is the mean of 3 wood chip samples. Error bars represent standard deviation. Bars showing the same letter are not significantly different (P < 0.05).

In the wood chips treated with chitosan, all solutions presented a lower number of nematodes (709 ±24 nematodes with 10.95 g/L , 916 ± 64 nematodes with 21.9 g/L, 471 ± 80 nematodes with 43.8 g/L of chitosan) than the control (1643 ± 72 nematodes) 48 h after infection and the most efficient concentration was 43.8 g/L. This study suggests that chitosan presents a limited nematicidal effect on wood chips when compared to heat-treatment of wood. Also, further testing is necessary to validate its upscale utilization.

5. Conclusions

The PWD has a big economic impact, leading to major losses in the volume of *Pinus pinaster* species and other *Pinus* species susceptible to this disease. *Pinus pinaster* is one of the most import raw materials in the wood industry and this industry is one of the most important in Portugal. There are still no effective control methods for this disease which may lead to its spreading throughout Europe and others countries. Chitosan is an environmentally friendly compound that has demonstrated in previous works to have a nematicidal effect (Nunes da Silva et al., 2014). It also has been shown to enhance the antimicrobial activity in plant-defence against bacteria (Tikhonov *et al*., 2006; Rabea and Steurbaut, 2010), fungi (Park *et al*., 2002; Trotel-Aziz *et al*., 2006) and nematodes (Khalil and Badawy, 2012; Nunes da Silva *et al.* 2014).

Heat-treatment is a method used to sterilize wood from the PWN, were wood is submitted to 56 ºC for 30 minutes throughout the whole surface (ISPM-15). In wood chips infected with PWN submitted to different temperatures for different times, viable PWN were found at 56 ºC for 30. No viable PWN were found at 65 ºC for 15 min. In the wood chips infected with PWN submitted to heat-treatment through 1000 W microwave irradiation for one min was sufficient for the elimination of all the PWN population. Chitosan reduced the number of nematodes in infected wood chips and 43.8 g/L was the most effective concentration.

In the plants infected with PWN and kept in soil treated with chitosan there was still a trigger of the symptoms at 14 dai but the symptoms evolved slower and did not reach the plant death. With chitosan, the number of nematodes in the plant was statistically identic over the entire experimental time-period, which indicates that this compound prevented, or at least slowed, the beginning and the severity of the pine wood disease. Plants maintained in soil amended with chitosan produced higher amounts of photosynthetic pigments (carotenoids and anthocyanins) at 28 dai when compared with the plants maintained in soil without chitosan. This shows that the treatment with this compound enhances the plant defence mechanisms by minimizing the adverse effects that the nematode induces in the plant tissues. Total polyphenols in the plants treated with chitosan was higher than the ones without the treatment, reaching a peak at 14 dai. Previous studies (Kuroda *et al*., 2011) show that *Pinus densiflora* species, resistant to the PWN, accumulate polyphenols in their needles suggesting that it could lead to the tree resistance. This increase in total polyphenol levels with chitosan might be one of the reasons why it prevented, or at least, slowed the beginning and severity of the pine wood disease. In the plants treated with chitosan the lignin levels were higher than in the ones not treated with chitosan, reaching a peak at 28 dai. This results show, once again, the ability of chitosan to improve the plant defence mechanisms against nematodes (Kawaguchi, 2006) and induce the biosynthesis of several antimicrobial compounds (Vasconcelos, 2014) such as lignin (Barber *et al.*, 1989).

In this study, *Enterobacter sp* was the most predominant genus in nematodes treated and not treated with chitosan. The previous results show that chitosan triggers the plant defence mechanisms at 14 dai and at this time-point the bacteria present in the nematode treated with chitosan were more abundant and diverse than the ones found in the nematodes without chitosan which were all

Enterobacter sp. In cultured nematodes analysis of the effect of chitosan on the nematode bacterial population, *Stenotrophomonas* sp., *Bacillus* sp., *Kluyvera* sp., *Buttiauxella* sp. *Klebsiella* sp. and *Paenibacillus* sp. weren't found associated with the PWN which might indicate that this bacteria are transmitted from the plant to the PWN when it migrated through it.

6. Future Work

This study was performed until 28 days after the infection with the nematode, a similar study but with an extended duration of the experiment could be performed so more data could be collected and analysed since an increase of some metabolites was achieved at 28 dai and the plants treated with chitosan did not die throughout the experiment.

This study was performed under controlled conditions in laboratory and with young pine trees, a field research could be conducted were chitosan would be applied on the soil of adult pine trees.

Several PWN associated bacteria are known to produce key enzymes like cellulases and pectinases, toxins and other substances that help the nematode infect the plant. A study of the production of this compounds by the bacteria isolated from the PWN could be performed.

To understand if chitosan does influence PWN associated bacteria, a study could be designed were the PWN were first treated with chitosan and then used to infect *Pinus pinaster* trees.

7. Annex

Table 6.1 – Results of the sequencing and blastN of the bacteria associated with the PWN obtained from the treatments inoculated with and without chitosan amended soil at 1 dai.

Isolate	Treatment	Time-point	Bacteria	Gram	Acc. No. of similar sequence	Similarity
1	B	1dai	*Pseudomonas koreensis*	Rod, negative	HM367599.1	0.0 (99%, 1,200 bp)

Table 6.2 - Results of the sequencing and blastN of the bacteria associated with the PWN obtained from the treatments inoculated with and without chitosan amended soil at 7 dai.

Isolate	Treatment	Time-point	Bacteria	Gram	Acc. No. of similar sequence	Similarity
2	B	7dai	*Paenibacillus* sp.	Rod, positive	KJ781901.1	0.0 (99%, 307 bp)
3		7dai	*Bacillus* sp.	Rod, positive	JX311857.1	0.0 (99%, 1,609 bp)
4		7dai	*Paenibacillus* spp.	Rod, positive	KJ944134.1	0.0 (99%, 1,200 bp)
6	D	7dai	*Enterobacter ludwigii*	Rod, negative	JQ038222.1	0.0 (99%, 1,172 bp)
7		7dai	*Enterobacter ludwigii*	Rod, negative	KC139450.1	0.0 (99%, 1,200 bp)
8		7dai	*Enterobacter ludwigii*	Rod, negative	JQ038222.1	0.0 (99%, 1,200 bp)
9		7dai	*Pantoea agglomerans*	Rod, negative	KJ781904.1	0.0 (99%, 1,200 bp)
10		7dai	*Paenibacillu*s spp.	Rod, positive	KF925453.1	0.0 (98%, 469 bp)
11		7dai	*Klebsiella pneumoniae*	Rod, negative	JQ039993.1	1,00E-146 (99%, 328 bp)
12		7dai	*Paenibacillus* sp.	Rod negative	KJ781901.1	6,00E-180 (100%, 361 bp)
13		7dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (99%, 1,088 bp)
14		7dai	*Pseudomonas* sp.	Rod, negative	JF772541.1	0.0. (82%, 1,192 bp)
15		7dai	*Enterobacter ludwigii*	Rod, negative	KC355280.1	0.0. (98%, 1,200 bp)
16		7dai	*Enterobacter cloacae*	Rod, negative	EF446900.1	0.0 (79%, 1,200 bp)

Table 6.3 - Results of the sequencing and blastN of the bacteria associated with the PWN obtained from the treatments inoculated with and without chitosan amended soil at 14 dai.

Isolate	Treatment	Time-point	Bacteria	Gram	Acc. No. of similar sequence	Similarity
17	B	14 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (99%, 1,259 bp)
18		14 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (96%, 1,200 bp)
19		14 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (97%, 1,200 bp)
20		14 dai	*Enterobacter ludwigii*	Rod, negative	JQ308602.1	0.0 (99%, 1,198 bp)
21		14 dai	*Enterobacter spp.*	Rod, negative	KF788246.1	0.0 (100%, 453 bp)
22		14 dai	*Enterobacter ludiwigii*	Rod, negative	JN700133.1	0.0 (99%, 1,200 bp)
23		14 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (99%, 1,200 bp)
24		14 dai	*Enterobacter cloacae*	Rod, negative	JQ038222.1	0.0 (97%, 1,200 bp)
25		14 dai	*Enterobacter cloacae*	Rod, negative	JQ038222.1	0.0 (99%, 1,200 bp)
26		14 dai	*Enterobacter ludwigii*	Rod, negative	JQ308602.1	0.0 (98% 1,199 bp)
27		14 dai	*Enterobacter cloacae*	Rod, negative	KC835100.1	0.0 (97%, 670 bp)
28		14 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (99%, 1,200 bp)
29		14 dai	*Enterobacter ludwigii*	Rod, negative	LC015547.1	0.0 (99%, 1,200 bp)
30	D	14 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (99%, 1200 bp)
31		14 dai	*Paenibacillus sp.*	Rod, negative	KF011678.1	0.0 (91%, 1,200 bp)
32		14 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (99%, 1,200 bp)
33		14 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (98%, 1,200 bp)
34		14 dai	*Enterobacter cloacae*	Rod, negative	EU733519.1	0.0 (99%, 1,200 bp)
35		14 dai	*Enterobacter ludwigii*	Rod, negative	KC355281.1	0.0 (94%, 1,095 bp)
36		14 dai	*Paenibacillus sp.*	Rod, negative	KM253131.1	0.0 (86%, 1,200 bp)
37		14 dai	*Paenibacillus sp.*	Rod, negative	KJ733988.1	0.0 (99%, 1,197 bp)
38		14 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (99%, 1,200 bp)
39		14 dai	*Buttiauxella warmboldiae*	Rod, negative	NR_028893.1	0.0 (97%, 1,200 bp)
40		14 dai	*Buttiauxella noackiae*	Rod, negative	NR_036919.1	0.0 (98%, 1,200 bp)
41		14 dai	*Buttiauxella noackiae*	Rod, negative	NR_036919.1	0.0 (99%, 1,200 bp)
42		14 dai	*Buttiauxella noackiae*	Rod, negative	NR_036919.1	0.0 (98%, 1,200 bp)
43		14 dai	*Buttiauxella noackiae*	Rod, negative	NR_036919.1	0.0 (99%, 1,274 bp)
44		14 dai	*Buttiauxella noackiae*	Rod, negative	NR_036919.1	0.0 (99%, 1,193 bp)
45		14 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (99%, 1200 bp)
46		14 dai	*Paenibacillus sp.*	Rod, negative	KF011678.1	0.0 (91%, 1,200 bp)
47		14 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (99%, 1,200 bp)

Table 6.4 – Results of the sequencing and blastN of the bacteria associated with the PWN obtained from the treatments inoculated with and without chitosan amended soil at 21 dai.

Isolate	Treatment	Time-point	Bacteria	Gram	Acc. No. of similar sequence	Similarity
48	B	21 dai	*Enterobacter ludwigii*	Rod, negative	LC015546.1	0.0 (97%, 1,200 bp)
49		21 dai	*Enterobacter ludwigii*	Rod, negative	LC015546.1	0.0 (97%, 1,200 bp)
50		21 dai	*Enterobacter ludwigii*	Rod, negative	JQ308602.1	0.0 (98%, 1,200 bp)
51		21 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (97%, 1,200 bp)
52		21 dai	*Kluyvera ascorbata*	Rod, negative	FJ823021.1	1,00E-08 (96%, 1,197 bp)
53		21 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (99%, 1,138 bp)
54		21 dai	*Enterobacter clocae*	Rod, negative	EU733519.1	0.0 (98%, 1,200 bp)
55		21 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (99%, 1200 bp)
56		21 dai	*Enterobacter ludwigii*	Rod, negative	LC015546.1	0.0 (97%, 1,200 bp)
57		21 dai	*Enterobacter cloacae*	Rod, negative	JQ038222.1	0.0 (99%, 1,200 bp)
58		21 dai	*Enterobacte ludwigii*	Rod, negative	KC139450.1	0.0 (98%, 1,199 bp)
59		21 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (99%, 1,200 bp)
60		21 dai	*Enterobacter cloacae*	Rod, negative	JQ038222.1	0.0 (98%, 1,200 bp)
61		21 dai	*Enterobacter sp.*	Rod, negative	LN829595.1	0.0 (100%, 704 bp)
62		21 dai	*Enterobacter cloacae*	Rod, negative	JQ038222.1	0.0 (99%, 1,198 bp)
63		21 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (99%, 1,200 bp)
64		21 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (99% 1,200 bp)
66	D	21 dai	*Enterobacter cloacae*	Rod, negative	JQ038222.1	0.0 (98%, 1,200 bp)
67		21 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (99%, 1,200 bp)
68		21 dai	*Bacillus cereus*	Rod, positive	KR063190.1	1,00E-17 (100%, 131 bp)
69		21 dai	*Enterobacter cloacae*	Rod, negative	JQ038222.1	0.0 (99%, 1,200 bp)
70		21 dai	*Enterobacter ludwigii*	Rod, negative	JQ308602.1	0.0 (99%, 1200 bp)
71		21 dai	*Bacillus cereus*	Rod, positive	DQ870690.1	0.0 (98%, 1,157 bp)
72		21 dai	*Enterobacter cloacae*	Rod, negative	KP257787.1	0.0 (99%, 696 bp)
73		21 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (99%, 1,125 bp)
74		21 dai	*Enterobacter cloacae*	Rod, negative	JQ038222.1	0.0 (99%, 1,200 bp)
75		21 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (98%, 1,200)
76		21 dai	*Stenotrophomonas maltophilia*	Rod, negative	KF177140.1	0.0 (99%, 1,200 bp)
78		21 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (98%, 1,200 bp)
79		21 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (96%, 1,200 bp)
80		21 dai	*Enterobacter ludwigii*	Rod, negative	JQ308602.1	0.0 (98%, 1,200 bp)
81		21 dai	*Enterobacter ludwigi*	Rod, negative	JQ308602.1	0.0 (99%, 1,199 bp)
82		21 dai	*Enterobacter cloacae*	Rod, negative	JQ038222.1	0.0 (99%, 975 bp)
83		21 dai	*Enterobacter cloacae*	Rod, negative	JQ038222.1	0.0 (99%, 1,200 bp)
84		21 dai	*Enterobacter cloacae*	Rod, negative	JQ038222.1	0.0 (99%, 1,200 bp)

85		21 dai	*Enterobacter ludiwigii*	Rod, negative	KC139450.1	0.0 (99%, 1,258 bp)
86		21 dai	*Enterobacter ludiwigii*	Rod, negative	KC139450.1	0.0 (98%, 1,196 bp)
87		21 dai	*Enterobacter cloacae*	Rod, negative	JQ038222.1	0.0 (99%, 1,200 bp)
88		21 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (96%, 1,200 bp)

Table 6.5 – Results of the sequencing and blastN of the bacteria associated with the PWN obtained from the treatments inoculated with and without chitosan amended soil at 28 dai.

Isolate	Treatment	Time-point	Bacteria	Gram	Acc. No. of similar sequence	Similarity
89	B	28 dai	*Enterobacter cloacae*	Rod, negative	JQ038222.1	0.0 (97%, 1,200bp)
90		28 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (99%, 1,200bp)
91		28 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (98%, 1,200bp)
92		28 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (99%, 1,200bp)
93		28 dai	*Enterobacter cloacae*	Rod, negative	JQ038222.1	0.0 (99%, 1,200bp)
94		28 dai	*Enterobacter cloacae*	Rod, negative	JQ038222.1	0.0 (99%, 1,200bp)
95		28 dai	*Enterobacter cloacae*	Rod, negative	JQ038222.1	0.0 (98%, 1,200bp)
96		28 dai	*Enterobacter ludwigii*	Rod, negative	LC015541.1	0.0 (99%, 1,046bp)
97		28 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (99%, 1,200bp)
98		28 dai	*Enterobacter cloacae*	Rod, negative	JQ038222.1	0.0 (97%, 1,200bp)
99		28 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (98% 1,200bp)
100		28 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (99%, 1,200bp)
101		28 dai	*Enterobacter cloacae*	Rod, negative	JQ038222.1	0.0 (99%, 1,200bp)
102		28 dai	*Enterobacter cloacae*	Rod, negative	JQ038222.1	0.0 (97%, 1,200bp)
103		28 dai	*Enterobacter clocae*	Rod, negative	JQ038222.1	0.0 (99%, 1,200bp)
104		28 dai	*Enterobacter sp.*	Rod, negative	JF783987.1	0.0 (96%, 1,200bp)
105		28 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (99%, 1,200bp)
106		28 dai	*Stenotrophomonas sp.*	Rod, negative	FJ404810.1	0.0 (95%, 1,200bp)
107		28 dai	*Stenotrophomonas maltophilia*	Rod, negative	KF177140.1	0.0 (97%, 1,200bp)
108		28 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (99%, 1,200bp)
109		28 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (99%, 1,200bp)
110		28 dai	*Enterobacter cloacae*	Rod, negative	JQ038222.1	0.0 (98%, 1,200bp)
111		28 dai	*Enterobacter cloacae*	Rod, negative	JQ038222.1	0.0 (99%, 1,200bp)
112		28 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (99%, 1,199bp)
113		28 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (99%, 1,200bp)
114		28 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (99%, 1,200bp)
115		28 dai	*Enterobacter cloacae*	Rod, negative	JQ038222.1	0.0 (99%, 1,200bp)
116		28 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (99%, 1,200bp)
117		28 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (98%, 1,200bp)
118		28 dai	*Enterobacter cloacae*	Rod, negative	JQ038222.1	0.0 (98%, 1,200bp)

119	D	28 dai	*Enterobacter ludwigii*	Rod, negative	LC015546.1	0.0 (96%, 1,200bp)
120		28 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (99%, 1,200bp)
121		28 dai	*Enterobacter ludwigii*	Rod, negative	LC015547.1	0.0 (99%, 1200bp)
122		28 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (99%, 1,200bp)
123		28 dai	*Enterobacter cloacae*	Rod, negative	JQ038222.1	0.0 (99%, 1,200bp)
124		28 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (96%, 1,143bp)
125		28 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (99%, 1,200bp)
126		28 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (100%, 1,200bp)
127		28 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (98%, 1,200bp)
128		28 dai	*Enterobacter ludwigii*	Rod, negative	JN700133.1	0.0 (98%, 1,200bp)

Table 6.6 – Results of the sequencing and blastN of the bacteria associated with culture nematodes treated with H_2O.

Isolate	Treatment	Bacteria	Gram	Acc. No. of similar sequence	Similarity
3	H_2O	*Enterobacter* sp.	Rod, negative	KT201614.1	0.0 (91%, 1,286 bp)
8	H_2O	*Pseudomonas* Sp	Rod, negative	FJ950615.1	0.0 (95%, 889 bp)
9	H_2O	*Pantoea agglomerans*	Rod, negative	KF756696.1	0.0 (92%, 1,298 bp)
10	H_2O	*Enterobacter cloacae*	Rod, negative	KF756696.1	0.0 (90%, 1,272 bp)
12	H_2O	*Pseudomonas putida*	Rod, negative	EF641266.1	0.0 (94%, 1,269 bp)
13	H_2O	*Pantoea* sp.	Rod, negative	EU302840.1	0.0 (95%, 1,275 bp)
14	H_2O	*Enterobacter* sp.	Rod, negative	KF788246.1	1.00E-103(90%, 1,254 bp)
15	H_2O	*Enterobacter cloacae*	Rod, negative	FJ608256.1	3.00E-89(90%, 312 bp)
16	H_2O	*Pseudomonas japonica*	Rod, negative	KT825519.1	0.0 (98%, 1,256 bp)
17	H_2O	*Pseudomonas putida*	Rod, negative	EF641266.1	0.0 (95%, 1,337 bp)
18	H_2O	*Pseudomonas* sp	Rod, negative	KR063209.1	0.0 (94%, 1,226 bp)
20	H_2O	*Enterobacter ludwigii*	Rod, negative	KJ396900.1	0.0 (96%, 1,175 bp)
21	H_2O	*Enterobacter ludwigii*	Rod, negative	KC139450.1	0.0 (98%, 1,322 bp)
22	H_2O	*Pseudomonas japonica*	Rod, negative	KT825519.1	0.0 (95%, 995 bp)
35	H_2O	*Enterobacter ludwigii*	Rod, negative	KJ396900.1	0.0 (96%, 1,251 bp)
53	H_2O	*Enterobacter ludwigii*	Rod, negative	KJ396900.1	0.0 (95%, 1,268 bp)
55	H_2O	*Pantoea* sp	Rod, negative	EU302841.1	0.0 (96%, 1,131 bp)
56	H_2O	*Pseudomonas* sp	Rod, negative	KR054996.1	0.0 (99%, 1,112 bp)
66	H_2O	*Enterobacter* sp.	Rod, negative	KT201614.1	0.0 (99%, 1,307 bp)

Table 6.7 – Results of the sequencing and blastN of the bacteria associated with culture nematodes treated with acetic acid 0.5%.

Isolate	Treatment	Bacteria	Gram	Acc. No. of similar sequence	Similarity
1	Acetic acid 0.5%	*Pseudomonas* sp.	Rod, negative	KR063209.1	0.0 (93%, 1,273 bp)
4	Acetic acid 0.5%	*Pantoea* sp.	Rod, negative	EU302841.1	0.0 (96%, 1,342 bp)
5	Acetic acid 0.5%	*Enterobacter cloacae*	Rod, negative	KC009688.1	0.0 (96%, 1,325 bp)
6	Acetic acid 0.5%	*Enterobacter* sp.	Rod, negative	KT201614.1	0.0 (96%, 1,151 bp)

23	Acetic acid 0.5%	*Enterobacter ludwigii*	Rod, negative	KJ396900.1	0.0 (94%, 1,308 bp)
24	Acetic acid 0.5%	*Enterobacter ludwigii*	Rod, negative	JQ308602.1	0.0 (96%, 1,354 bp)
26	Acetic acid 0.5%	*Pseudomonas japonica*	Rod, negative	KT825519.1	0.0 (95%, 1,340 bp)
28	Acetic acid 0.5%	*Enterobacter ludwigii*	Rod, negative	EU557027.1	0.0 (96%, 1,477 bp)
30	Acetic acid 0.5%	*Enterobacter sp.*	Rod, negative	KR189861.1	0.0 (97%, 1,210 bp)
31	Acetic acid 0.5%	*Pseudomonas japonica*	Rod, negative	KT825519.1	0.0 (99%, 1,108 bp)
33	Acetic acid 0.5%	*Enterobacter cloacae*	Rod, negative	CP011798.1	0.0 (95%, 1,389 bp)
34	Acetic acid 0.5%	*Pseudomonas japonica*	Rod, negative	KT027724.1	0.0 (97%, 1,320 bp)
42	Acetic acid 0.5%	*Enterobacter ludwigii*	Rod, negative	KJ396900.1	0.0 (95%, 1,306)
45	Acetic acid 0.5%	*Enterobacter ludwigii*	Rod, negative	KJ396900.1	0.0 (95%, 1,222 bp)
47	Acetic acid 0.5%	*Enterobacter ludwigii*	Rod, negative	KC139450.1	0.0 (98%, 1,111 bp)
65	Acetic acid 0.5%	*Pseudomonas japonica*	Rod, negative	KT825519.1	0.0 (97%, 1,368 bp)
69	Acetic acid 0.5%	*Enterobacter* sp	Rod, negative	KR067593.1	0.0 (94%, 1,221 bp)

Table 6.8 – Results of the sequencing and blastN of the bacteria associated with culture nematodes treated with chitosan 0.15%.

Isolate	Treatment	Bacteria	Gram	Acc. No. of similar sequence	Similarity
39	Chitosan 0.15%	*Pseudomonas putida*	Rod, negative	EF034390.1	0.0 (93%, 1,252 bp)
44	Chitosan 0.15%	*Pseudomonas putida*	Rod, negative	KF070875.1	8.00E-165 (95%, 1,266 bp)
50	Chitosan 0.15%	*Pseudomonas japonica*	Rod, negative	KT825519.1	0.0 (91%, 1,275 bp)
51	Chitosan 0.15%	*Pseudomonas japonica*	Rod, negative	KT825519.1	0.0 (95%, 919 bp)
52	Chitosan 0.15%	*Pseudomonas sp*	Rod, negative	KR054996.1	0.0 (98%, 1,411 bp)
57	Chitosan 0.15%	*Pseudomonas japonica*	Rod, negative	KT825519.1	0.0 (98%, 1,150 bp)
58	Chitosan 0.15%	*Pseudomonas sp*	Rod, negative	FJ950639.1	0.0 (94%, 1,297 bp)
59	Chitosan 0.15%	*Pseudomonas japonica*	Rod, negative	KT825519.1	0.0 (99%, 1,081 bp)
60	Chitosan 0.15%	*Pseudomonas putida*	Rod, negative	KP731360.1	0.0 (98%, 940 bp)
63	Chitosan 0.15%	*Pseudomonas putida*	Rod, negative	KP739807.1	0.0 (93%, 1,244 bp)
68	Chitosan 0.15%	*Pseudomonas putida*	Rod, negative	EF641266.1	0.0 (96%, 1,315 bp)

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