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STUDY OF POPULATION DYNAMICS OF BACTERIA ASSOCIATED WITH PINE WOOD NEMATODE AFTER INOCULATION WITH DIFFERENT STRAINS OF *BURSAPHELENCHUS XYLOPHILUS* IN MARITIME PINE (*PINUS PINASTER)*

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

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under the supervision of

Dr. Marta Wilton Pereira Leite de Vasconcelos

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RESUMO

Durante algum tempo pensou-se que o *Bursaphelenchus xylophilus* era o único agente etiológico da doença do nemátode da madeira do pinheiro. Recentemente, descobriu-se que existem bactérias associadas ao nemátode que contribuem para a patogénese desta doença, sobretudo através da libertação de toxinas que promovem a morte dos pinheiros. De entre as espécies mais comummente encontradas estão bactérias pertencentes aos géneros *Bacillus, Pantoea, Pseudomonas* e *Xanthomonas*.

Este trabalho teve como principal objectivo o estudo do efeito da inoculação de pinheiro bravo (*Pinus pinaster*) com quatro isolados diferentes de nemátodes, na população bacteriana dos nemátodes e das árvores, em diferentes fases da progressão da doença. A monitorização da progressão dos sintomas da doença foi igualmente registada. Pretendeu-se também identificar as bactérias isoladas do xilema das árvores e da superfície dos nemátodes através de métodos de identificação clássicos, do sistema de identificação API20E e da sequenciação de ADN bacteriano.

Os resultados obtidos demonstraram que, relativamente à progressão dos sintomas da doença, a diferença mais marcante foi verificada para os pinheiros inoculados com o isolado não virulento C14-5, onde se visualizou um agravamento de sintomas mais lento e menos severo do que nos pinheiros inoculados com os isolados virulentos. Verificou-se que numa fase inicial da doença, no geral, a população bacteriana dos ramos inoculados foi menor do que a que foi verificada passados 7 e passados 14 dias de inoculação. Num dos métodos de quantificação foi recuperado um maior número de bactérias nos pinheiros inoculados do que nos controlos aos 7º e ao 14º dias de inoculação. Os isolados que levaram à obtenção de maior quantidade bacteriana foram o HF e o 20, sendo que, comparativamente, o isolado não virulento levou à obtenção de menor quantidade bacteriana. Relativamente à identificação bacteriana, concluiu-se que o sistema API20E não foi suficiente na identificação das espécies bacterianas isoladas, mostrando-se pouco discriminatório, tendo sido identificada a espécie *Enterobacter cloacae* em 79% dos isolados e não sendo possível a identificação de sete colónias bacterianas. Assim, a adopção de métodos moleculares de identificação, através da sequenciação do ADN bacteriano, permitiu uma identificação mais fiável, tendo sido identificadas onze espécies bacterianas diferentes dentro dos géneros *Klebsiella*, *Bacillus*, *Enterobacter, Paenibacillus, Terribacillus, Citrobacter, Pantoea* e *Escherichia.* No geral, a diversidade bacteriana aumentou ao longo da progressão da doença. A espécie *Bacillus* spp. predominou na fase mais precoce da doença e a espécie *Klebsiella oxytoca* nas fases mais tardias. As espécies bacterianas isoladas da superfície dos nemátodes não diferiram muito das isoladas do xilema dos pinheiros.

Neste trabalho foram identificadas espécies bacterianas nunca antes reportadas neste tipo de estudo, podendo estas estar associadas a Portugal. A espécie de pinheiro utilizada neste estudo foi diferente das que são usualmente utilizadas no Japão e na China. Foi a primeira vez que foram isoladas e identificadas bactérias de um isolado não virulento do nemátode da madeira do pinheiro.

ABSTRACT

For a long time it was thought that *Bursaphelenchus xylophilus* was the only agent of the pine wilt disease. Recently, it was discovered that there are bacteria associated with the nematodes that contribute to the pathogenesis of this disease, mainly through the release of toxins that promote the death of the pines. Among the species most commonly found, are bacteria belonging to the *Bacillus*, *Pantoea, Pseudomonas* and *Xanthomonas* genera.

The main objective of this work was to study the effect of inoculation of maritime pine (*Pinus pinaster)* with four different nematode isolates, in the bacterial population of nematodes and trees, at different stages of disease progression. The monitoring of progression of disease symptoms was also recorded. Also, the identification of bacteria isolated from the xylem of trees and the surface of nematodes was performed by classical identification methods, by identification system API20E and by sequencing of bacterial DNA.

The results showed that for the symptoms progression, the most striking difference was observed for the pines inoculated with the avirulent isolate, C14-5, which led to a slower and less severe aggravation of symptoms than in pines inoculated with the virulent isolates. It was found that at an earlier stage of the disease, in general, bacterial population of inoculated twigs was lower than what was observed 7 and 14 days after inoculation. In one of the quantification methods more bacteria were recovered from the inoculated pines than from the control pines on the 7th and 14th days after inoculation. A bigger bacterial quantity was isolated from pines inoculated with the nematode isolates HF and 20, and, comparatively, few bacteria were isolated from pines inoculated with the avirulent isolate. The identification system API20E proved to be insufficient and poorly discriminatory in the identification of bacterial species; *Enterobacter cloacae* species was identified in 79% of the isolated bacterial colonies and seven of these colonies couldn't be identified by this method. Thus, the adoption of identification molecular methods, through bacterial DNA sequencing, allowed a more reliable identification: eleven different bacterial species within the *Bacillus, Citrobacter, Enterobacter, Escherichia, Klebsiella, Paenibacillus, Pantoea* and *Terribacillus* genera were identified. General bacterial diversity increased with the progression of the disease. *Bacillus* spp. species were predominant at the earlier stage of disease progression and *Klebsiella oxytoca* species at the later stages. Bacterial species isolated from the surface of nematodes were similar to those isolated from the xylem of pines.

In the present work new bacterial species were identified which have never been reported before in this type of study and may be associated with Portugal. *P. pinaster*, the pine species used in this study, was different from those commonly grown in Japan and China. Furthermore, it was the first time that bacteria were isolated and identified from an avirulent pine wood nematode isolate.

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LIST OF ABBREVIATIONS AND SYMBOLS

ANOVA	Analysis of variance
ATB	Automatic Testing Bacteriology
blastN	Nucleotide Basic Local Alignment Search Tool
bp	Base pair(s)
CFU	Colony-Forming Unit(s)
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate(s)
EDTA	Ethylenediaminetetraacetic Acid
ETI	Effector-Triggered Immunity
H_2O_2	Hydrogen Peroxide
IN	Isolated from the surface of nematodes
MAMP	Microbe-Associated Molecular Pattern(s)
NA	Nutrient Agar
NaCl	Sodium Chloride
NB-LRR	Nucleotide-Binding Leucine-Rich Repeat(s)
O_2	Oxygen molecule
PAMP	Pathogen-Associated Molecular Pattern(s)
PCR	Polymerase Chain Reaction
PROLUNP	Programa Nacional de Luta Contra o Nemátodo da Madeira do Pinheiro
PRR	Pattern Recognition Receptor(s)
PTI	PAMP-Triggered Immunity
PWD	Pine Wilt Disease
PWN	Pine Wood Nematode(s)
rRNA	Ribosomal Ribonucleic Acid
sd	Standard deviation
TAE	Tris-Acetate-EDTA
(W × D × H)	(Width × Depth × Height)
Ø	Control pine(s) (non-treatment)
#	Number
3hai	Three hours after inoculation
7dai	Seven days after inoculation
14dai	Fourteen days after inoculation

INTRODUCTION

Pine Wilt Disease, *Bursaphelenchus xylophilus* and *Monochamus galloprovincialis*

The pine wilt disease (PWD), as its name implies, is a disease found in pine species (*Pinus* spp.) whose main and best known etiologic agent is *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle, the pine wood nematode (PWN) (Please see Figure 5 on page 13 of the Materials and Methods section).

The endoparasitic nematodes, unlike other nematodes, are able to overcome plant defenses, survive and feed on the plant (Jones *et al.,* 2008). Particularly, the migratory endoparasitic nematodes, which include *B. xylophilus*, move within the plant, causing serious damage as they move and feed (Duncan and Moens, 2006; Jones *et al.,* 2008). These nematodes feed mainly on fungi, appearing also in dead or dying trees (Jones *et al.,* 2008). *B. xylophilus* has the particularity to parasitize aboveground parts of trees, not entering the soil and migrating through the tissues of the plant (Kikuchi, 2008).

One of the stages of *Bursaphelenchus*'s life cycle occurs at the same time as the formation of the late instar larvae or pupae of Cerambycidae beetles, that are the vectors for this nematode, being transported in the body of the insect (Aikawa, 2008; Jones *et al.,* 2008). These longhorn beetles belong to the genus *Monochamus* spp. and exist in the same place of the tree occupied by the nematodes (Jones *et al.,* 2008). In Portugal, the vector of PWN is *Monochamus galloprovincialis* Olivier 1795 (Sousa *et al.,* 2001, 2002) (Figure 1). The PWN is transmitted to a diseased tree by the beetle during its oviposition, starting a new life cycle, and the infection period occurs between May and September (Jones *et al.,* 2008). Once infected, trees can die in less than a year, if environmental conditions are favorable (Yoshimura *et al*., 1999; Jones *et al.,* 2008). However, the main and foremost responsible agent for the rapid spread of the disease to other countries is the human (Jones *et al.,* 2008), and there is no treatment for a susceptible tree infected with PWN (Zhao *et al.,* 2007).

Figure 1: Portuguese PWN vector beetle, *Monochamus galloprovincialis* (Vitali, 2009).

After infection, PWN feeds on parenchymal cells (Jones *et al.,* 2008) and moves through the resin canals of the xylem and cortex, where he nourishes and reproduces itself, and also through the cambium cells (Ichihara *et al.,* 2000). This process blocks the tree vascular system due to the appearance of secondary resin as a result of damage to the radial parenchyma cells by the nematodes; moreover, a cavitation phenomenon occurs, which affects water transportation (Jones *et al.,* 2008) to the shoots and leads to rapid needle discoloration.

The severity of the symptoms depends on host species and the season of the year in which infection occurs, mainly due to temperature values (Jones *et al.,* 2008). In the summer there is a rapid death of trees, and in the winter symptoms may not even manifest (Kiyohara and Tokushige, 1971). So, environmental factors (high air temperature and water stress) influence PWD incidence and disease development (Kiyohara, 1973; Suzuki and Kiyohara, 1978). The much talked about climate change adds concerns about this problem worldwide (Mota and Vieira, 2008a). It was also reported that PWN invasion depends on the age of the tree, mainly due to the absence of resin ducts in the stems of young trees (Mamiya 1975, 1980; Ichihara *et al.,* 2000); however there are some authors that defend that PWD appears apart from the age of the host tree (Toda, 1997).

As mentioned above, one of the earliest symptoms of infection is the reduction or cessation of resin's exudation on tree trunks, followed by the discoloration of pine needles (Figure 2) and death of the tree (Jones *et al.,* 2008). PWN can survive in the host tree for long periods without causing any symptoms (Takeuchi and Futai, 2007).

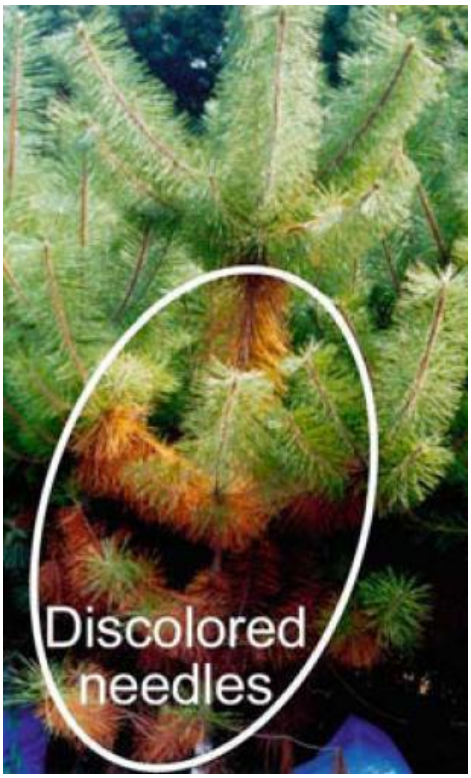

Figure 2: *Pinus thunbergii* old needles discoloration, 3 weeks after inoculation (adapted from Kuroda, 2008).

Table 1 lists the appearance of external symptoms of trees infected with PWN, over time.

Table 1: External symptoms of trees infected with PWN (adapted from Zhao *et al*., 2008)

		Early phase		Developing phase	
	Stage	**1**	**2**	**3**	**4**
External	Symptom	None →		Discoloration of old needles	Discoloration of young needles → Death
	Oleo-resinosis	Normal →	Decreasing →	None	
Pine wood nematode		Low population →		Propagation	Extensive propagation
Time (weeks) Example of *Pinus thunbergii*		1	2	3	4 and beyond

The PWN is native from North America; here, the autochthonous conifers are naturally resistant or tolerant to this agent, whereas the exotic tree species (non-native ones) are affected (Mamiya and Tamura, 1983; Wang *et al*., 2010). The disease firstly emerged in Canada, USA and Mexico, then spread to Japan, Korea and China, where it affected native species such as *P. thunbergii* and *Pinus densiflora.* When the disease arrived in Portugal, it was the first report of this disease in Europe (Yano, 1913; Cheng, 1983; Mamiya and Tamura, 1983; Tzean and Jan, 1985; Guiran and Bruguir, 1989; Yi *et al.,* 1989; Dwinell, 1993; Mota *et al.,* 1999). It is thought that Portuguese *B. xylophilus*'s populations derive from those from North America (Webster, 2004; Takemoto, 2008).

Thus, the PWD is known as the most severe threat to pine forests worldwide, representing a major economic problem for all affected countries (Mota and Vieira, 2008a; Wang *et al.,* 2010).

In Portugal, after the discovery of the nematode in 1999, a national program, named "PROLUNP", for the control of PWN, was created, and a national survey to determine the affected area was undertaken, creating a zone with about 30 km of radius in south-east Lisbon, where all the symptomatic trees were razed, creating later a called “buffer zone” with about 5 km wide around the affected area, where all *Pinus pinaster* trees were killed, even if they weren’t symptomatic (Jones *et al.,* 2008; Mota and Vieira, 2008b).

Unfortunately, Portugal assembles all the necessary conditions to the spread of this disease, in particular the fact that the most important pine species are susceptible to the pest (Braasch, 1997), nematodes are present and there is a compatible vector. The pine species *Pinus sylvestris* and *Pinus halepensis* are also favorable hosts for the PWN, but their distribution and abundance is limited in Portugal (Mota and Vieira, 2008b). The maritime pine, *P. pinaster,* is the most popular host as the insect vector feeds from it during its maturation (Mota and Vieira, 2008b). Stone pine, *Pinus pinea*, is one of the species considered "resistant" or less susceptible to the disease, not being consumed or colonized by the vector *M. galloprovincialis*; however, it is known that PWN is able to infect and kill this species, but more slowly than for *P. pinaster* (Mota and Vieira, 2008b). The reasons behind this phenomenon are still unclear.

P. pinaster trees play an important role in pine production, in the wood and resin industry, as well as coastal protection, being distributed throughout most of the country (Figure 3) and so the appearance of a problem like this is very serious for the Portuguese economy and environment (Mota and Vieira, 2008a).

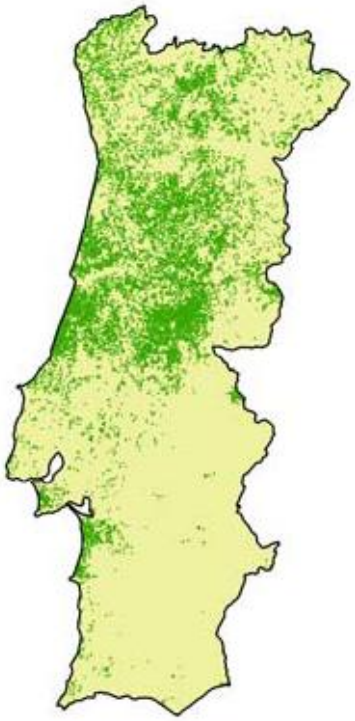

Figure 3: *P. pinaster* distribution in Portugal (DFG, 2001).

Control/prevention of PWD

Various approaches to control or prevent the disease have been studied in Japan and China: eradication, preventive sprays, host tree resistance to PWN, forest sanitation, trapping of the insect vector, biocontrol methods, breeding of natural parasites of the pine sawyer, cultivation of resistance pine species, among others.

- *Eradication*

This method is effective because it kills vector beetles when they are in the larval galleries and pupal chambers and when the PWN transmission has not yet begun (Kamata, 2008).

One example of this approach is the physical controls. These include three techniques: cut and crush, cut and burn and cut and burry. The cut and crush technique consists of cutting and crushing the infected wood without any other treatment. The cut and burn technique is based on carbonizing the wood into charcoal; the complete mortality of vector beetles can be achieved. In the cut and burry technique the logs are buried in the ground or soaking in the water. The first one is efficient for coastal pine stands; the second one is not too useful because it takes too long to kill vector beetles (Kamata, 2008).

A second approach, the chemical control, is useful for diseased trees present in accessible and inaccessible zones. The cut and chemical application encompasses spray and fumigation techniques. After being cut into sections a chemical insecticide is applied to affected trees in the spray technique. These chemicals are composed of one or more chlorpyriphos-methyl, pyridaphenthion, prothiophos, or BPMC (2-sec-butylphenyl methylcarbamate). If this approach is applied at the end of October (fall treatment) about total mortality of pine sawyer can be achieved because that's when larvae live beneath the host bark or are constructing pupal chambers. This treatment is not to efficient if applied from November to March (winter treatment) or after April (spring treatment) because it's impossible for the chemical to reach the pupal chambers, unless an oil solution of chemicals is used. When the diseased trees are in inaccessible areas chemical insecticide can be sprayed from a helicopter; however this approach raises issues such as the adverse impact upon ecosystems. In the fumigation technique the concerns of the application seasons are overcome. The main components used are metam-ammonium carbam NCS or carbam sodium. Also, about 100% of pine sawyer mortality can be achieved and in addition to killing the vector beetle it also kills the nematodes. However, this technique is more expensive than the spray one. The procedure involves treating diseased stacked branches and logs with the chemicals which are then sealed with soil over a PVC (polyvinyl chloride) sheet (Figure 4) (Kamata, 2008).

Figure 4: Fumigation technique for the treatment of wood infected with PWN (picture taken in a pine affected area in Tsukuba, Japan, kindly provided by Dr. Marta Vasconcelos).

- *Treatment of dead pine trees*

The clearance of dead pine trees from forests is one of the main strategies to be adopted for PWD control. Even one infected pine tree left can spread the disease to other pines that are near. China adopted five treatments to wood to stop PWN reproduction and insect vector propagation: clear-cutting of pine trees to chipping and processing chipboard, heat treatment, heating and hydraulic pressing treatment, hot-water treatment and bag-fumigation and microwave treatment (Xu, 2008).

- *Traps for the insect vector*

The choice of the host by the Japanese and Chinese vector beetle, *Monochamus alternatus*, is related to the chemical composition of pines. The terpene (+)-α-pinene is the main attractive compound to the beetle (Xu, 2008). Ethanol acts as a synergistic compound increasing the activity of attraction (Yang *et al*., 2003). An experiment conducted by Siegfried (1987) proved that traps with α-pinene, a β-phellandrene-limonene mixture, limonene and β-pinene are effective to catch weevils. There is still no available, effective trap of this type for *M. galloprovincialis*.

- *Biocontrol strategies*

Pine sawyer can also be controlled by biological methods: bird and insect predators, entomophatogenic fungi and entomophilic nematodes (Kamata, 2008). There are several natural enemies for *M. alternatus*: insect parasites (*Scleroderma guani* and *Dastarcus helophoroides*), parasitic fungi (*Beauveria bassiana, B. brongniartii, Metarhizium anisopliae*, among others), parasitic bacteria (*Serratia marcescens*) and parasitic nematodes (*Steinernema feltiae*). *C. massonianus* is used to release *B. bassiana* in the larva canal of the beetle (Xu *et al.,* 2000); this approach can achieve 95% of pine mortality. *B. bassiana* can also be transferred by *S. guani* however an undesirable effect arises because *B. bassiana* also infects *S. guani* (Xu *et al.*, 2000). A good solution is to propagate *S. guani* alone on the the larvae of *M. alternatus*, not harming the environment (Xu, 2008). Again, no data is available regarding possible biocontrol strategies for *M. galloprovincialis.*

- *Growth of Resistant Pine Species*

Cleared areas previously affected by PWD need to be cultivated again. In these areas resistant pine species, previously examined for their resistance, should be preferred for cultivation (Xu, 2008). It is thought that this resistance has a genetic nature (Nose and Shiraishi, 2008). Thus the study of gene expression (for example genes of the peroxidase and ethylene biosynthesis pathway that are important in the disease response (Miller *et al*., 2005; Shin *et al.,* 2009)) of susceptible pine species is crucial and will help in the understanding of molecular mechanisms of tree resistance and susceptibility. It is required that this resistance is long lasting; however this is not always possible. The PWN isolate and virulence in each region should be taken into account to make this possible.

- *Tree vaccination*

The injection of a nematicide or a chemical solution into a tree, which stops PWN reproduction or potentiates its death, is known as preventive vaccination. This control approach is very efficient because it's long lasting (reinoculation once every 3-4 years) and does not depend on environmental conditions; however it cannot be applied in large scale due to costs and can lead to problems due to the inoculation (Kamata, 2008).

The content of the vaccine formulations can also be an avirulent nematode; it was found that pre-inoculation of trees with avirulent nematode isolates induces resistance on *P. thunbergii* to a

subsequent post-inoculation with virulent nematodes (Kosaka *et al*., 2001). Also in the experiment of Takeuchi *et al.* (2006) they concluded that pre-inoculated seedlings with C14-5 showed lower mortality than seedlings without pre-inoculation. In these cases the cambial zone is determinant for survival or death of pines (Fukuda, 1997). Resistance degree of pre-inoculated trees increases with avirulent nematode concentration and multiple pre-inoculations with C14-5, at different times, induces the resistance more effectively, than a single pre-inoculation, to post-inoculation with a virulent nematode. It has also been demonstrated that climate conditions influences the effect of inoculation with avirulent nematodes (Kosaka *et al.*, 2001).

- *Plant innate immunity system*

Plants possess a response to pathogen/microbe-associated molecular patterns (PAMPs or MAMPs), called PAMP-triggered immunity (PTI) (Boller and Felix, 2009; Boller and He, 2009). However it's difficult to understand the role of this PTI because pathogen virulence effectors inhibit it (Boller and He, 2009). This innate immunity system present in plants detects and moves pathogens away (Chisholm *et al.,* 2006; Jones and Dangl, 2006; Boller and Felix, 2009). The first step of this system is the recognition, in the plant's cell surface, of MAMPs by pattern recognition receptors (PRRs); these are extremely sensitive and specific, able to detect all classes of microbes and with a nonself recognition characteristic (Boller and Felix, 2009; Boller and He, 2009). Although pathogen effectors try to suppress PTI, as stated earlier, plants possess other receptors (nucleotide-binding leucine-rich repeat (NB-LRR) proteins) which constitute an effector-triggered immunity (ETI) or R-gene-based or vertical resistance, as a second line of defense (Boller and Felix, 2009; Boller and He, 2009).

Thus, as plant pathogenic bacteria, fungi and nematodes excrete a lot of virulence effectors into plant cells, the recognition of the plant targets of these effectors will bring a new vision of plant immunity, pathogenesis and plant biology and will help us to better control plant diseases, such as PWD.

The role of bacteria in the infection mechanism

The pathogenic mechanism of PWD has not been well elucidated. For several years it was thought that the PWN was the only etiologic agent of the disease (Mamiya, 1975; Nickle *et al.,* 1981; Mamiya and Tamura, 1983;; Nobuchi *et al.,* 1984; Myers 1988; Fukuda *et al.*, 1992; Yang, 2002). In fact, it was found that this agent produces phytotoxins and cellulases and that its invasion stimulates the production of ethylene and terpenoids by pine trees which could lead to their death (Wang *et al.,* 2010). Inoculation studies with PWN leading to the death of pine also supported this viewpoint, noting that nematodes of different pine trees had different pathogenicity (Kiyohara and Bolla, 1990; Fukuda *et al.,* 1992; Kojima *et al.,* 1994; Hu *et al.,* 1995). However, the rapid increase in the number of nematodes was posterior to the histological and physiological changes in an infected tree, raising the suspicion that there may be other microorganisms involved in the pathological process (Xie and Zhao,

2008). In addition, sterilization of the surface of the nematode leads to loss of pathogenicity (Cao, 1997; Kawazu and Kaneko, 1997).

More recent approaches report the existence of bacteria in symbiosis with nematodes that somehow have a crucial role in the pathogenesis of the disease; however, this hypothesis is still controversial (Oku *et al*. 1979; Kawazu *et al.,* 1996b, 1998; Cao*,* 1997; Kawazu and Kaneko, 1997; Han *et al.,* 2003; Zhao *et al.,* 2003, 2005; Xie and Zhao*,* 2008). It is thought that the ability of the nematode to carry bacteria is nothing more than a natural phenomenon (Zhao *et al.,* 2003, 2007, Guo *et al.,* 2007). However, some authors think that these bacteria are not accidental contaminants but exist as symbionts of nematodes, and co-evoluted with them for a long period (Zhao *et al.,* 2005, 2006). Others think they were obtained by the nematodes from the environment, possibly through the inoculation wounds, by mechanical injury, or via the scars left by the insects when they feed on trees (Zhao and Li, 2008). In fact bacteria are present in many environments, including soil and water, and are associated with some plant diseases. Species of *Acidovorax, Afrobacterium, Burkholderia, Clavibacter, Erwinia, Pantoea, Pectobacterium, Phytoplasma, Pseudomonas, Ralstonia, Spiroplasma, Streptomyces, Xanthomonas* and *Xylella* genera are involved in different kinds of symptoms: galls, overgrowths, wilts, leaf spots, specks and blights, soft rots, scrabs and cankers (Ellis *et al*., 2008). Some of them produce toxins, inject proteins to kill cells or synthesize enzymes that break down structural components of cells and walls (Ellis *et al*., 2008). An example is the bacterial wetwood or slime flux disease, affecting many shade and ornamental trees, including pines, caused by species of *Bacillus megaterium, Enterobacter agglomerans* (also called *Pantoea agglomerans*), *Enterobacter cloacae*, *Klebsiella oxytoca* and *Pseudomonas fluorescens* (Anonymous, 1999). This chronic disease usually does not manifest in trees with less than 10-years-old and is affected by environmental factors (Anonymous, 1999).

Several studies report the existence of a symbiosis between bacteria and entomopathogenic nematodes and promotion of development and reproduction of the nematode by the bacteria (Marainede *et al.,* 1993; Forst and Nealson, 1996; Han and Ehlers, 2000). In the studies of Zhao *et al.* (2000) and Guo *et al*. (2002) it was observed under electronic microscopy, the presence of bacteria on the surface of PWN in an amount of 2.9×10^2 bacteria per nematode. No bacteria were found within the body of the nematode (Zhao *et al.*, 2000). Kusunoki (1987) also found the presence of bacterial cells in resin ducts and parenchymal tissue of pine trees. However, several studies indicate that no bacteria were detected in healthy trees (Zhao *et al.*, 2003; Zhang *et al.,* 2004). Bacteria were also found in the insect vector, *M. alternatus*, and were similar to bacteria from PWN (Xie *et al.*, 2005).

Among the main species of bacteria that are associated with PWN are the genus *Pantoea, Pseudomonas* and *Xanthomonas* (Higgins *et al*. 1999; Han *et al.,* 2003). It was also found that bacteria in different geographic zones may differ (Han *et al.,* 2003; Zhao *et al.,* 2003; Wang *et al.,* 2010). The main species found in China belong to *Pseudomonas* genus, in Japan to *Bacillus* genus and in Korea both are present (Zhao, 2008). Such variations cause differences in the susceptibility of plant species (for example *Cedrus deodara*); this plant dies after infection with PWN in Japan, while in

China it remains healthy (Jiao *et al*., 1996). It is thought that new bacteria from the local flora are acquired by PWN when a new local region is achieved (Zhao, 2008). Zhao *et al*. (2006, 2007) concluded that bacteria such as *Pantoea* sp. and *Peptostreptococcus asaccharalyticus* are not beneficial for the nematodes and even have an inhibitory role on their growth and development. The species *Escherichia coli* also exerts an inhibitory effect on the PWN and vice-versa (Zhao *et al.,* 2005). *Pseudomonas* and *Pantoea* were found in trees infected with *B. xylophilus* but not in uninfected trees (Han *et al.,* 2003). Xie and Zhao (2008) concluded that at later stages of the disease, when the number of nematodes increases rapidly, the bacteria population increases in volume and variety of species, indicating that the environment created due to wilt was good for the nematode and the bacteria.

It is also known that trees infected with bacteria alone or only with aseptic nematodes did not develop the disease, but the combination of nematodes and bacteria leads to the manifestation of the disease symptoms (Oku *et al.,* 1980; Zhao *et al.,* 2000; Han *et al.,* 2003; Zhao *et al.,* 2003). Thus, axenic PWN is not pathogenic compared to the wild PWN (Kawazu and Kaneko, 1997; Cao *et al.,* 2001), and they cannot cause disease even if they survive in the xylem of the pine tree (Chi *et al.,* 2006).

Several studies report the existence of toxins that play an important role in the process of pine wilt (Mamiya, 1980; Oku, 1988, 1990; Zhang *et al.,* 1997), which cannot be produced by the nematode alone (Cao and Shen, 1996; Cao *et al.,* 2001). Experiments in callus showed that the wilting observed after inoculation of the liquid where the bacteria grew, was due to the existence of toxins produced by bacteria (Han *et al.,* 2003; Zhao *et al.,* 2003, 2005). Kawazu *et al*. (1996a) and Kawazu (1998) confirmed the presence of three toxin producing species of *Bacillus* spp. that cause PWD and identified the toxic substance as phenylacetic acid. Oku *et al*. (1979, 1980) and Zhao *et al.* (2003) reported that the wilt toxin is associated with bacteria, namely to a bacteria belonging to the *Pseudomonas* genus associated with the nematode.

As in any symbiotic relationship both species benefit from each other; in this particular case alive or dead nematodes promote reproduction and pathogenicity of bacteria by the supply of essential metabolites and nutrients and in some way protect bacteria within the host tree, and bacteria also increase the reproductive rates of *B. xylophilus* in trees, serving as a power source and/or providing essential nutrients (Zhao *et al.,* 2003, 2005, 2006, 2007; Wang, 2004; Guo *et al.,* 2006). Living nematodes have a stronger stimulatory effect than the dead ones (Zhao, 2008). It was also found that phytotoxin-producing bacteria associated with the nematodes also increase egg production and accelerate the growth and development of *B. xylophilus* in cultured callus (Zao *et al.,* 2007). This symbiotic relationship was classified as an ectosymbiosis (Zhao *et al.,* 2007). The species *Escherichia coli* is reported as inhibitory of PWN reproduction (Zhao and Lin, 2005).

Thus, the PWD is a complex process that involves the PWN and the phytotoxin-producing bacteria associated with it (Zhao *et al.,* 2003; Xie and Zhao, 2008; Kwon *et al.,* 2010; Wang *et al.,* 2010) whereas bacteria alone are not capable of causing disease (Zhao *et al.,* 2003; Jones *et al.,*

2008). Zhao *et al*. (2003) reported that bacteria cannot invade a healthy tree alone; Xie (2003) and Lindberg *et al. (*2004) attribute this fact to the presence of certain metabolites in pine tree extracts that have antibacterial activity. Bacteria must be transported by nematodes to overcome and survive the defenses of the host tree, gaining protection from nematodes (Zhao *et al.,* 2005). These and PWN then collaboratively invade and kill the host tree (Zhao *et al.,* 2007).

Controversial opinions do not support this approach, since in trees inoculated with *B. xylophilus* the blocking of water in the xylem was found before the presence of fungi and bacteria were detected (Kuroda and Ito, 1992) and some think that because bacteria exist inside and outside of the tree, they are contaminants and are not pathogenic (Yang, 2002). All the different experimental data indicate that there is no consensus about the actual role of bacteria in disease progression, which supports the use of sterilization procedures in all experiments during the inoculation process to guarantee that the bacteria found are not mere contaminants (Zhao *et al.,* 2003).

Methods for identifying bacteria

The identification and phenotypic characterization of microorganisms began in 1677 with Antony van Leeuwenhoek. Later, the need for isolation in pure culture lead to the advent of solid culture media by Robert Koch and analysis of physiological characteristics, metabolic and biochemical abnormalities (Ferreira and Sousa, 2000). However, the simplicity of this analysis did not make it suitable for a for proper microorganism identification (Ferreira and Sousa, 2000).

A disadvantage of traditional methods of microbial identification is the fact that they only analyze the phenotypic characteristics of organisms, such as cultural and morphological characteristics, biochemical and metabolic utilization of different substrates, etc (Ferreira and Sousa, 2000). These features often do not allow the differentiation of microbial species (Ferreira and Sousa, 2000).

An example is the Gram stain that allows separating the bacteria according to their staining properties, being the most widely used staining in bacteriology. It is a differential staining because it separates bacteria into two groups: gram-positive and gram-negative ones. The second dye used, the iodine solution, acts as a mordant promoting a stronger stain. Alcohol or acetone is used to decolorize the cells: gram-positive bacteria retain the first stain (crystal violet) and gram-negative bacteria lose the stain, becoming colorless. At the final step of the Gram staining procedure, safranin allows gram-negative bacteria to stain pink or red while the gram-positive bacteria remain with the first dye, appearing dark purple (Prescott, 2002).

The known oxidase test allows detecting the presence of the enzyme cytochrome c oxidase, which is able to reduce O_2 and artificial electron acceptors. The catalase test serves to detect the presence of the enzyme catalase, which converts hydrogen peroxide to water and O_2. Another test is the starch hydrolysis test that detects the presence of the enzyme α-amylase, which hydrolyzes starch, produced by certain bacteria (Prescott, 2002).

Some bacteria have structures of locomotion, the flagella, with 15-20 µm long and 14-20 nm thick, which can only be viewed directly under an electronic microscope or under an optical microscope with special stains (Ferreira and Sousa, 2000). The use of a mordant like tannic acid or potassium alum allows increasing the thickness of flagella being stained with pararosaniline (Leifson method) or basic fuchsin (Gray method) (Prescott, 2002).

The advent of molecular biology techniques allowed the fast, easy, inexpensive and accurate identification of microorganisms, allowing to overcome some limitations of classical methods. One example is the sequencing of nucleic acids that involves determining the sequence of genes with phylogenetic and taxonomic information, such as the 23S and 16S rRNA genes. This technique thus provides information about the identity and/or relationship of the new sequences obtained with those existing in a database (Ferreira and Sousa, 2000).

Main objectives

As it has already been described, bacteria play an important role in the pathogenicity of PWN. However, little is known regarding the bacterial population in Portuguese nematode isolates. Also, nothing is known regarding the bacteria associated with the Japanese avirulent isolate C14-5, and how it compares to virulent isolates. This work is therefore intended to study the effect of inoculation of healthy *P. pinaster* trees, with different nematode isolates, in the bacterial population of either the nematode or the tree itself, at different stages of disease progression (3 hours, 7 days and 14 days after inoculation). It is also proposed to know the bacterial species isolated from the xylem of the trees and from the surface of the nematodes.

- Specific objectives

Thus, one year old *P. pinaster* trees will be inoculated with four different nematode isolates: three virulent isolates (HF and 20, isolated from *Setúbal* region and 8A isolated from Portuguese central region) and an avirulent one (C14-5 isolated from Japan). At the three experimental points several samples will be monitored for symptoms of disease progression such as resin exudation and discoloration of needles. The same samples will be subjected to nematode extraction by the Baermann funnel technique to confirm the efficiency of the inoculation process.

After incubation of twigs from inoculated samples collected at the three experimental points in Nutrient Agar (NA) medium, the quantity of inoculated twigs with bacterial colonies will be registered. Also, colony-forming units (CFU) will be studied after dilution of solutions of bacteria extracted from inoculated twigs and incorporation on NA medium. Bacteria will also be isolated from the four nematode isolates.

Bacterial colonies grown on NA medium from inoculated twigs and from the four nematodes will be successively purified and subjected to a series of identification tests such as Gram and flagella stain, cytochrome c oxidase and catalase tests, starch hydrolysis, growth at 41ºC and API20E identification system.

Finally total genomic bacterial DNA will be extracted from all isolated colonies and a 1500 bp fragment of 16S rRNA gene will be PCR amplified with bacterial universal primers. PCR products will be purified and sequenced at Macrogen Ltd (Seoul, Korea).

Source and culture of nematodes

Three virulent isolates of *B. xylophilus* (HF (Figure 5) and 20, geographical isolates from *Setúbal* region and 8A from Portuguese central region), and an avirulent isolate (C14-5, from Japan), were used in the experiments.

Figure 5: HF virulent pine wood nematode isolate.

All *B. xylophilus* cultures were grown (Figure 6 B) on barley seeds with *Botrytis cinerea* Pers. mycelium (Figure 6 A) at 26ºC, in the dark, for 7 days. Juveniles and adult nematodes were extracted using the Baermann funnel technique (van Bezooijen, 2006) (Figure 7) during 24h at 25ºC.

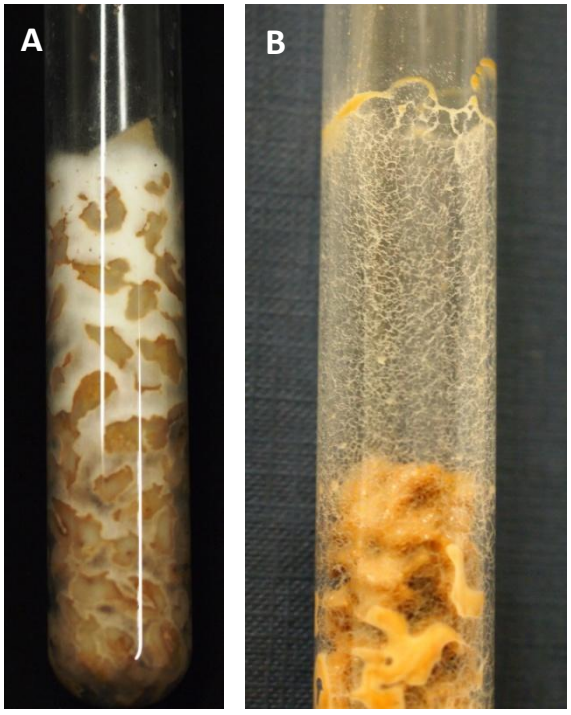

Figure 6 (A-B): A - Barley seeds with *Botrytis cinerea*; B - Nematodes growing on the walls of the tube and feeding on *B. cinerea* on barley seeds.

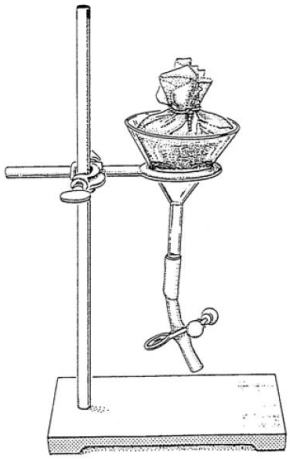

Figure 7: Baermann funnel technique (van Bezooijen, 2006).

The total number of nematodes was determined as follows: 20 µl of the nematode suspension obtained from the Baermann funnel were placed in a nematode counting dish (Figure 8) and living nematodes were counted and estimated for the initial solution. The solution was then adjusted to a final concentration of approximately 2000 nematodes/ml sterile deionized water and used in the inoculation experiments.

Figure 8: Nematode counting dish.

Source, culture and inoculation of pines

The experiments were carried out on one year old Portuguese Maritime pine specie *P. pinaster* at *Escola Superior de Biotecnologia*. Pines were provided by *Sociedade Agrícola Pecuária Melo & Cancela Lda*. and kept in a plant growth chamber (*Fitoclima* S600, Aralab Portugal) scheduled for 80% humidity, photoperiod of 8 hours light and 16 hours darkness and temperatures of 26ºC and 24ºC for periods of dark and light, respectively.

Nematodes were inoculated in pines according to Futai (1980) method (Figure 9): at about 3 cm from the top of the tree the pine needles were removed and three vertical cuts were made in the stem, with the help of a blade; after inoculation with the nematode suspension, the cutting area was sealed with absorbent paper and parafilm. 144 pines were inoculated with the different PWN isolates and 27 others without treatment (non-inoculated) were used as controls (Ø). Thus, a total of 171 pines were used in the experiments.

Figure 9: Inoculation of *P. pinaster* trees.

Three experimental points were considered: an early response of three hours, a mid response of seven days and a later response of fourteen days after inoculation.

Development of symptoms by the diseased pines

One of the earliest symptoms of pine wilt disease is the cessation of resin exudation. For this reason, in order to confirm disease progression, resin exudation was monitored. After every experimental point, a 5 mm hole in the trunk was made using a sterile blade for each of the inoculated and control pines to monitor the resin flow. Also, a visual scale of symptoms that consists of four levels of disease progression was used, ranging from healthy to dead plant (Please see Table 1 on page 3 of the Introduction section).

Determination of the presence of nematodes at different experimental time points

In all experimental time points, the whole pine stem was cut into small pieces and PWN were extracted with the Baermann funnel technique to check if the inoculation process was effective and if the nematodes had survived.

Calculating the quantity of inoculated chips with bacterial colonies

The quantity of inoculated chips with bacterial colonies was determined according to Xie and Zao (2008) (Figure 10): a 20 cm long, 1 year old inoculated stem was sterilized with 75% ethanol and both ends were cut; the central wood was then cut into 2 mm × 2 mm × 5 mm pieces (W × D × H). Eight pieces of the stem of a sample from inoculated and control pines were placed into a Petri dish containing nutrient agar (NA) medium (Frilabo, Portugal) and incubated at 26ºC for 3 days. Bacteria appeared in the site were the chip was placed in NA and in the marks left by the nematodes. These bacteria were also selected for identification (as described later).

Five stem samples were collected from the different experimental points and five replicate dishes, for each sample, were used to calculate the amount of bacterial colonies on the inoculated chips over all the chips from a treatment.

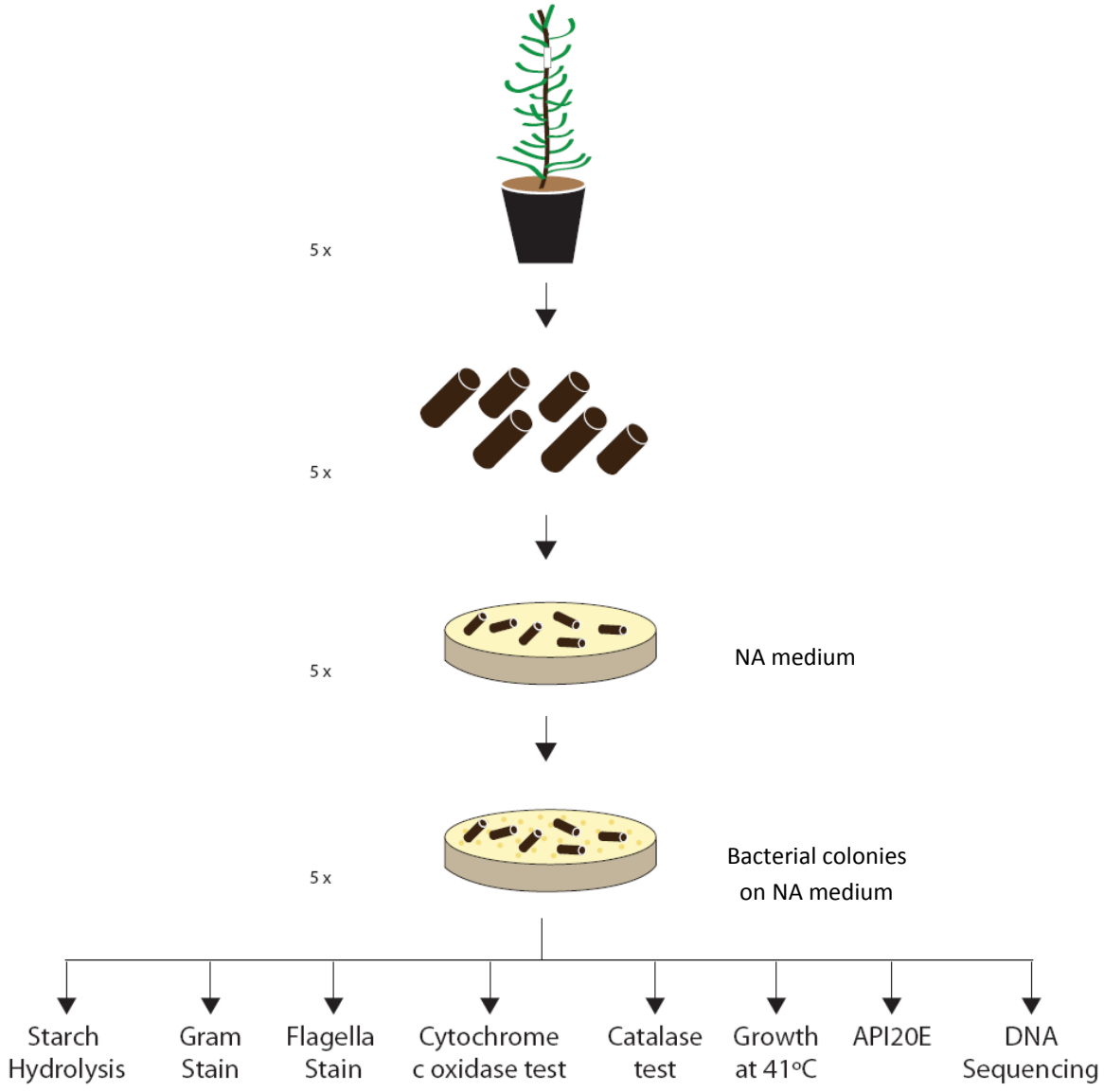

Figure 10: Schematic illustration of the method used for the calculation of inoculated chips with bacterial colonies performed at each experimental time point, with each treatment.

Calculating the colony-forming units (CFU) on inoculated chips

Colony-forming units (CFU) were also calculated in the stem wood using the dish count method, based on dishes with colonies between 25 and 250, according to Xie and Zao (2008) (Figure 11): 1 gram of the stems described above were placed in a glass tube with 4.5 ml of a 0.85% NaCl solution. The tubes were then shaken vigorously for 5 minutes and left standing for 10 minutes. 0.5 ml of the previous solution was diluted in a series of concentrations (10^0, 10^{-1}, 10^{-2}, 10^{-3} e 10^{-4}) and 0.5 ml of each concentration was placed on a sterile Petri dish. Ten milliliter of NA medium at 45°C was then added to the plate, which was incubated at 26°C for 72 hours. Three replicates from each treatment were used to calculate the CFU.

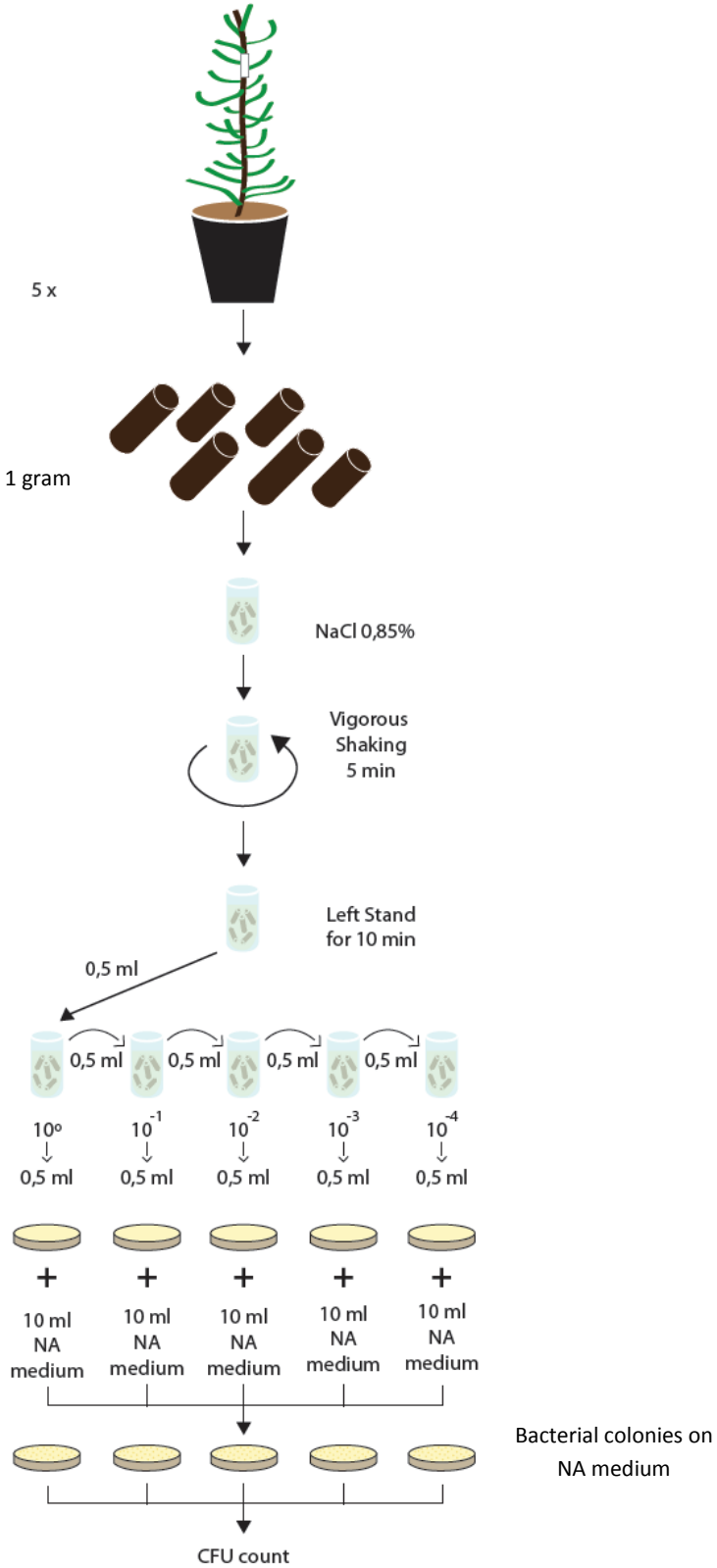

Figure 11: Schematic illustration of the method used for the calculation of CFU on inoculated chips performed at each experimental point, with each treatment.

Isolation of bacteria from the surface of nematodes

Bacteria were also isolated from the surface of the four nematode isolates as described by Han *et al*. (2003): the solution of PWN obtained from the Baermann funnel was centrifuged at 17 *g* for 6 minutes and the supernatant was discarded; the remaining PWN were disinfected with 3% H_2O_2 for 5 minutes; finally, a single nematode was removed with a thin metal needle and placed on a plate with NA media which was incubated at 26ºC. Bacterial colonies which appeared in the track left by the nematodes were successively transferred to NA for colony purification.

Identification of bacteria by classical methods

Both isolated bacterial colonies from xylem and the surface of nematodes were selected for identification based on macroscopic differences. Morphologic characteristics (size, color, shape, transparency, prominence, edge and viscosity) of the purified isolated bacteria from the trees and nematodes were registered. Each isolate was tested for Gram stain: the smear was first stained with crystal violet for 30 seconds, water rinsed for 2 seconds, stained with Gram's iodine for 1 minute, water rinsed, washed with 95% ethanol for 10-30 seconds, stained with safranin for 30-60 seconds, water rinsed and finally dried (Prescott, 2002).

Flagella stain was also performed as described by BD Flagella Stain Droppers manufacturer's instructions (Difco, BBL).

Cytochrome c oxidase and catalase tests were carry out: for the oxidase test - fresh growth from the culture plate was scraped with an inoculation loop, rubbed on filter paper and examined for blue color (positive result) within 10 seconds (NHS, Oxidase Test; Oxidase Test Sticks – Frilabo, Portugal); for the catalase test - a drop of 3% hydrogen peroxide was placed on a glass slide and a colony from the culture plate was placed on the drop. The formation of bubbles indicated a positive result (Murray *et al*., 1998).

Starch hydrolysis was also studied according to manufacturer's instructions: the surface of a 48 hour culture, grown in Difco™ Starch agar was flooded with Gram's Iodine; a positive result is indicated by the presence of a colorless zone surrounding the colonies.

The study of bacterial growth at 41ºC was also performed.

All identification tests described above were performed five times to confirm results.

The bacterial species were finally subjected to the identification systems API20E (bioMérieux Company, Craponne, France). After obtaining the numerical profile, isolated bacterial colonies were analyzed using the analytical catalog of the API20E (API20E Analytical Catalog, 1999).

Identification of bacteria by molecular methods

Total genomic bacterial DNA was successfully extracted for all 38 bacterial colonies (except colony 10) according to Wiedmann-Al-Ahmad *et al*. (1994): one bacterial colony was resuspended in 70 µl pure water, heated 5 min at 95ºC and sedimented at 16,000 x g for 5 min in a microcentrifuge (Thermo Scientific Heraeus Pico 17). The extracted DNA was quantified spectrophotometrically using a NanoPhotometer^{TM} UV/Vis spectrophotometer (Implen GmbH, Germany). 16S rRNA genes were then PCR amplified: the mixture contained 25 mM $MgCl_2$ (Fermentas, USA), 10 × Taq Buffer with KCl (500 mM KCl, 100 mM Tris-HCl (pH 8.8), 0.8% (v/v) Nonidet P40) (Fermentas, USA), 25 mM of each primer 27F (5'-GAGTTTGATCCTGGCTCA-3´) and 1492R (5'-TACCTTGTTACGACTT-3´), 500 U Taq DNA polymerase (Fermentas, USA) and 10 mM dNTPs (Bioron, Frilabo, Portugal). The amplification was performed in a thermocycler DOPPIO (VWR, USA) with the following parameters: an initial denaturation step at 95°C for 5 minutes, followed by 25 cycles at 95°C for 30 seconds, 54°C for 30 seconds and 72°C for 1 minute and a final extension at 72°C for 5 minutes. The amplified products were analyzed by electrophoresis in a 1.5% agarose gel in Tris-acetate-EDTA (TAE) buffer, with SYBR® Safe DNA gel stain (Invitrogen, UK) for 45 minutes at 120 V.

PCR products from 34 of the total 38 bacterial colonies were sent for purification and sequencing by Macrogen Korea. The obtained sequences were finally subjected to a blastN.

Statistical analysis

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

RESULTS AND DISCUSSION

The important role of bacteria in the pathogenicity of pine wilt disease (PWD) has already been demonstrated in several works.

Han *et al.* (2003), in order to examine the role of bacteria in the pathogenicity of the pine wood nematode (PWN), first isolated *Bursaphelenchus xylophilus* from naturally infected black pine wood and *Bursaphelenchus mucronatus* from naturally wilted *Pinus massoniana*; bacteria were then isolated from xylem and from the surface of nematodes and identified. Two species of *Pseudomonas* genus and one of *Pantoea* genus were isolated. Inoculations on aseptic black pine seedlings and *callus* were performed, as well as the study of toxic effects of bacteria on *callus* cultures. They concluded that the mixture of aseptic nematodes with bacteria led to the disease and that the two isolated species of *Pseudomonas fluorescens* produced toxic substances. Also in 2003, a geographical survey of host distribution of bacteria carried by PWN in China and the role of bacteria to pines were studied in the work of Zhao *et al*. (2003); bacteria were isolated from naturally infected black pine and Masson pine and identified. The production of phytotoxins by bacteria was also studied (17 of the 24 identified species produced phytotoxins and 11 of the 17 phytotoxin-producers belonged to *Pseudomonas* genus). Zhao and Lin (2005) studied the interactions between PWN and its associated bacteria by cultivating axenic nematodes and bacteria using callus of *Pinus thunbergii*. The effect of bacteria on reproduction of PWN and vice-versa were studied: 10 of the 29 bacterial species tested (all belonging to *Pseudomonas* genus) increased nematode reproduction rate and also the presence of the nematodes improved bacterial growth. One year later, Zhao *et al.* (2006) studied the interactions between PWN and three bacterial species (*P. fluorescens*, *P. putida* and *Pantoea* sp.) isolated from *B. xylophilus*. The effect of bacteria on fecundity, reproduction rate and body volume of PWN and the effect of PWN on reproduction of bacteria were studied: the two first isolated bacteria increased the three components studied and nematodes also promoted reproduction of these two bacteria; *Pantoea* sp., on the other hand, completely inhibited reproduction of nematodes. The same authors, in the following year, conducted a study on the effects of bacteria carried on the surface of the PWN on its egg hatch, development rate and egg production. *Pseudomonas* spp. species were strong phytotoxin producers and promoted egg production, developmental rate, body length and diameter growth in nematodes, while *Pantoea* sp. and *Peptostreptococcus asaccharalyticus* did not produce phytotoxins and completely inhibited egg production. Xie and Zhao (2008) observed the population dynamics of the PWN and its accompanying bacteria after inoculation of *P. thunbergii*. At the early stages of the disease only a few bacterial species were detected on non-inoculated twigs and only when a few needles became yellow; as the disease progressed the populations of both nematodes and bacteria started to increase rapidly (also bacterial species variety increased). The dominant species found were *Pseudomonas* spp., *Pantoea* sp. and *Sphingomonas paucimobilis*.

The nematode C14-5 is an avirulent isolate used in programs of tree “vaccination” (Kosaka *et al.*, 2001; Takeuchi *et al.,* 2006), whose bacterial population has never been investigated.

This work has compiled some of the techniques used in previous experiments, in order to study the effect of inoculation of four different nematode isolates (three virulent and the avirulent one, C14-5) in the bacterial population of the xylem of *Pinus pinaster*, in three different experimental time points. First, the appearance of disease symptoms was studied; also the presence of nematodes on inoculated trees was investigated. Bacteria were then isolated from the xylem of the trees by to different methods and the number of chips with bacterial colonies as well as CFU values, were registered. Bacteria were also isolated from the surface of the nematodes. Finally, all isolated bacterial colonies were identified.

Stage symptoms and oleoresin flow

The two most common symptoms of PWD are the reduction or cessation of oleoresin's exudation (the earliest one) and discoloration of pine needles (Jones *et al.,* 2008). In the present work, these two symptoms were studied in order to confirm if the inoculation procedure was effective and to compare these results with those obtained in the study of bacterial populations isolated from xylem and surface of *B. xylophilus* (discussed ahead), as it is thought that as the disease progresses, bacterial quantity increases in number and species (Xie and Zhao, 2008).

After inoculation of pines with the four different nematode isolates, two pines for each treatment were studied for the presence/absence of oleoresin flow and the general appearance of the plant was also registered according to Table 1 (Please see page 3 of the Introduction section). The results are shown in Table 2.

Table 2: General appearance and oleoresin flow study of pines after inoculation with the four nematode isolates at three experimental time points: 3 hours after inoculation (3hai), 7 days after inoculation (7dai) and 14 days after inoculation (14dai)

Experimental time point	Treatment	Symptom	Oleoresin flow	Stage
3hai	Ø	None	Normal	1
	8A	None	Normal	1
	HF	None	Normal	1
	20	None	Normal	1
	C14-5	None	Normal	1
7dai	Ø	None	Normal	1
	8A	None	Normal	3
	HF	Discoloration of old needles	Decreasing	3
	20	Discoloration of old needles	Decreasing	3
	C14-5	Discoloration of old needles	Decreasing	3
14dai	Ø	None	Normal	1
	8A	Discoloration of young needles	None	4
	HF	Discoloration of young needles	None	4
	20	Discoloration of young needles	None	4
	C14-5	Discoloration of old needles	Decreasing	3

Three hours after inoculation (3hai), the pines showed a normal oleoresin flow and had no external symptoms, as expected, and so were included in stage 1 (Please see Table 1 on page 3 of the Introduction section); control pines (Ø – non-inoculated) were also healthy.

Seven days after inoculation (7day), pines inoculated with the nematode isolate 8A showed a normal oleoresin flow and had no symptoms; those inoculated with the other nematode isolates (HF, C14-5 and 20) had decreasing oleoresin flow and a yellowing of the older needles, and so these last ones were included in stage 3 of symptoms; controls showed a normal oleoresin flow, as expected.

Finally, 14 days after inoculation (14dai), oleoresin flow in the control pines was normal and pines had no external symptoms; in the pines inoculated with the virulent isolates HF, 20 and 8A, oleoresin secretion totally ceased and almost all needles were brown (Stage 4); pines inoculated with the avirulent isolate C14-5 remained in the Stage 3 of the symptoms.

The progression of symptoms was as expected – healthy pines in the early infection (3hai) and severely diseased pines at 14dai. For pines inoculated with the avirulent isolate C14-5 a slower aggravation of symptoms was observed, consistent with what would be expected; this avirulent isolate, although causing disease symptoms, they were not as fast and severe as with pines inoculated with the virulent isolates. Other groups have inoculated *P. thunbergii* with this avirulent isolate, in order to study if this inoculation led to the emergence of resistance with post-inoculation with a virulent nematode isolate (Kosaka *et al.*, 2001). They concluded that the mortality level of trees inoculated with the avirulent isolate was less than 10%, compared to the 90% obtained with inoculation with virulent nematodes. Takeuchi *et al.* (2006) found in a preliminary experiment, that the nematode C14-5 survived for a long time in the host tree without causing any symptoms. Twenty percent of 4-year-old seedlings of *P. thunbergii* inoculated with the avirulent nematode isolate died within 5 weeks of inoculation; also, 7 months after inoculation, C14-5 nematodes were present in the plants and these still exuded oleoresin (Takeuchi and Futai, 2007). In the present work, pines inoculated with C14-5 started to show some discoloration of old seedlings and decreased oleoresin exsudation, 7dai (1 week after inoculation) differing with the previous experiments. However, 1-year-old *P. pinaster* trees were used, which may justify this discrepancy, as younger trees are more sensitive to the infection (Kuroda, 2008).

Comparing the time to symptom development (Table 2) with those obtained in previous studies with *P. thunbergii* (Zhao *et al*., 2008), at 14dai all the plants from these authors reached stage 2 of symptom progression, whereas in our experiments, at the same time point, a subset of trees reached stage 4 of disease progression. As explained before, this can be explained by the fact that the pines used in the current experiment were young (1-year-old). In previous experiments authors reported data on 5- to 10-year-old samples of *P. thunbergii* and *Pinus densiflora*, and observed a stop in xylem sap ascent in the stem 4-6 weeks after inoculation (Kuroda, 2008). This suggests that tree's age and size are factors that affect the duration of symptom appearance. It is possible that pine specie is also a variation factor.

Determination of the presence/absence of nematodes at different stages of disease progression

In order to verify the effectiveness of the inoculation process, five pines for each treatment were chosen to check whether the nematodes were present in the inoculated trees. The stems were cut into small pieces (5 mm$\times$5 mm$\times$2 cm – W$\times$D$\times$H) and nematodes were extracted using the Baermann funnel technique. The results of the extraction process showed that the nematodes had survived in the inoculated pines for all treatments, 3hai and 7dai. At 14dai, nematodes were recovered from pines inoculated with nematode isolates 20 and HF but were not recovered from those inoculated with nematodes 8A and C14-5. This indicates that either the extraction process wasn't efficient or nematodes may have migrated to other plant parts (needles or roots, for example) or even to the soil. In addition, no nematodes were detected in the control pines, as expected, which was also observed in the experiments of Han *et al*. (2003) and Xie and Zhao (2008).

Quantification of bacterial colonies on inoculated chips

The first approach on the understanding the effect of PWN inoculation on the bacterial population dynamics in the xylem of *P. pinaster* consisted in quantifying the number of chips with bacterial colonies, after placing chips of the inoculated tree in nutrient agar medium. Figures 1 to 9 show an example of the obtained results. In Tables 3-5 and in Figure 15, the average number of chips with bacterial colonies and the standard deviation for the various treatments in the different experimental time points are visible.

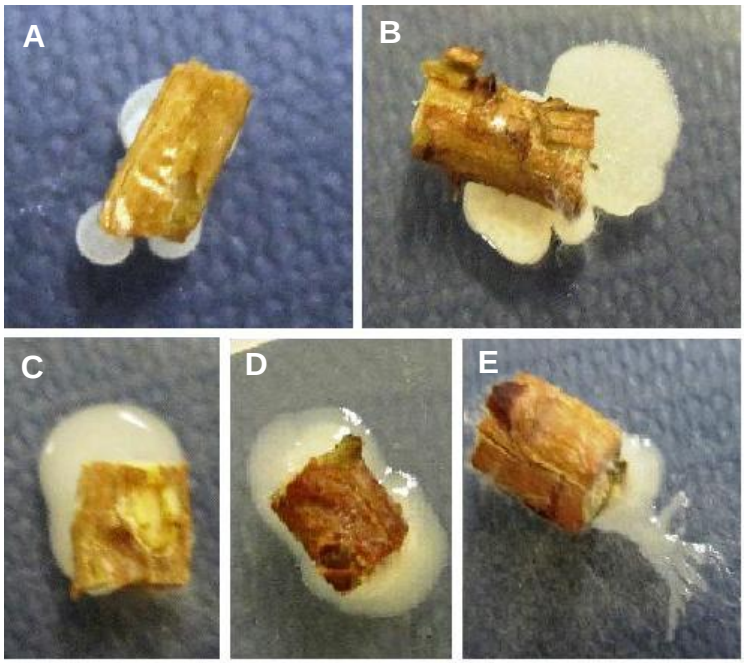

Figure 12 (A-E): Bacterial colonies in the chips inoculated with virulent nematode isolate 8A (A and B), with virulent nematode isolate 20 (C and D), and bacterial colonies along the trails of avirulent nematode isolate C14-5 (E), 3hai.

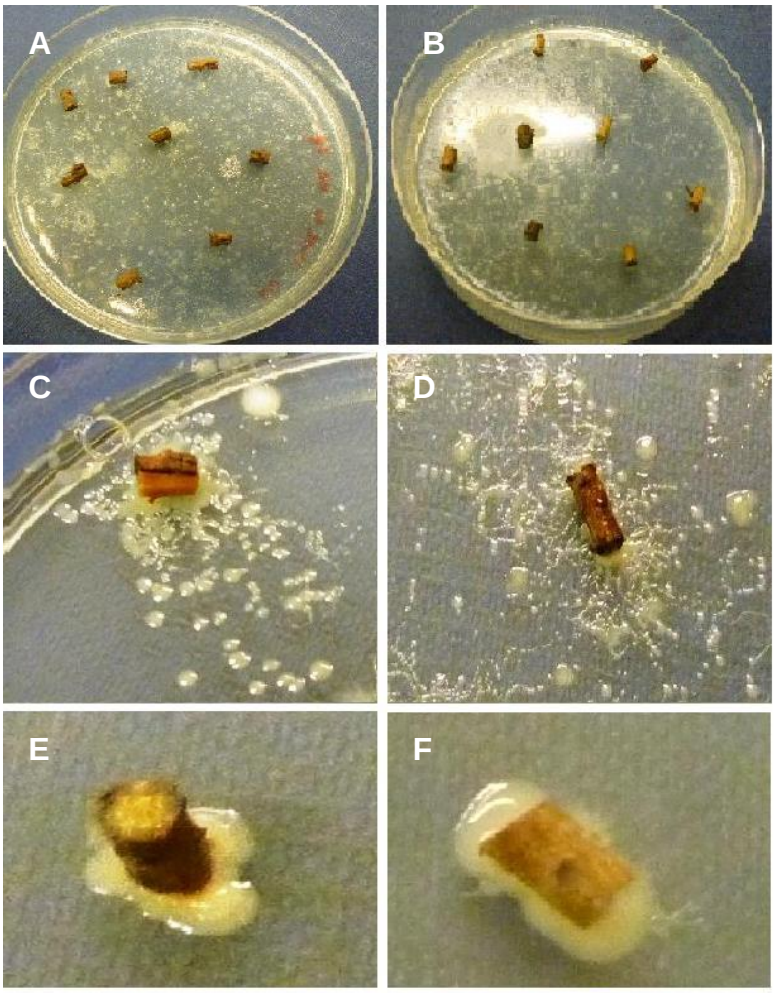

Figure 13 (A-F): Bacterial colonies along the trails of virulent nematode isolate 20 (A and D), HF (B), 8A(C) and bacterial colonies in the chips inoculated with virulent nematode isolate 20 (E) and with avirulent isolate C14-5(F), 7dai.

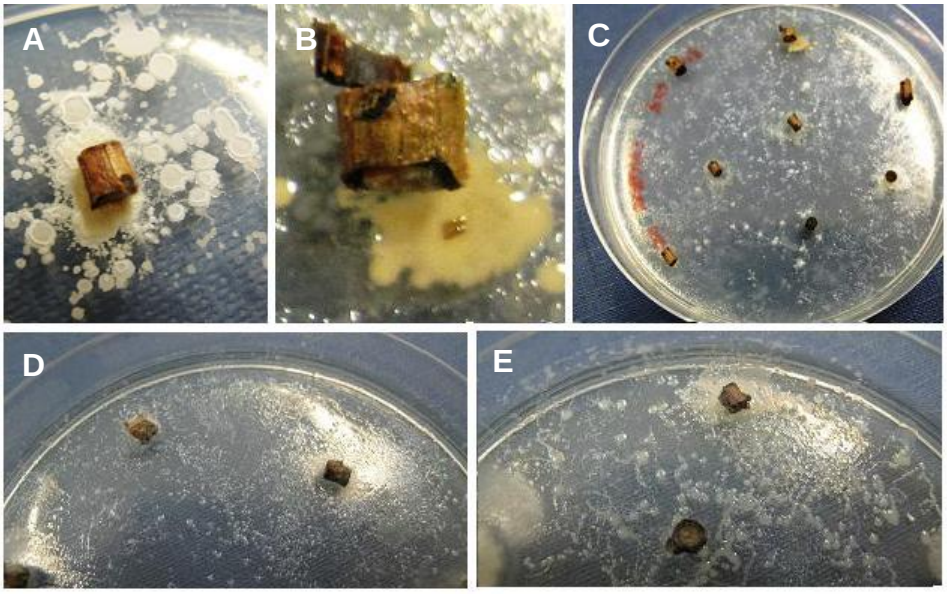

Figure 14 (A-E): Bacterial colonies along the trails of virulent nematode isolate 8A (A-C) and HF (D and E), 14dai.

The quantity of chips with bacterial colonies varied between and within the different experimental time points, as can be observed in Tables 3-5.

Table 3: Number of inoculated chips with bacterial colonies, 3hai, with different treatments (values represent an average of eight samples ± standard deviation)

Experimental point	**3hai**				
Treatments	*Ø*	*C14-5*	*HF*	*20*	*8A*
# of chips with bacterial colonies mean (total # of samples)	$0^{b,c,d,e}$ (8)	$1^{a,c,d,e} \pm 0.4$ (8)	$0.2^{a,b,d,e} \pm 0.7$ (8)	$0.2^{a,b,c,e} \pm 0.4$ (8)	$1.4^{a,b,c,d} \pm 1.5$ (8)

Legend: [a] represents no significant difference between control (Ø); [b] represents no significant difference between isolate C14-5; [c] represents no significant difference between isolate HF; [d] represents no significant difference between isolate 20; [e] represents no significant difference between isolate 8A. ($p > 0.05$)

Xie and Zhao (2008) reported that no bacteria were found in the diseased pines at the first two stages of disease and in healthy trees (our control) at all stages. In this experiment, 3hai, the quantity of chips with bacteria between treatments was not significantly different and was generally low compared to disease progression (7dai and 14dai); as in the experiment of Xie and Zhao (2008) no bacteria were found in the control (Table 3). Han *et al.* (2003) and Zhao *et al*. (2003) also showed that there were no bacteria in healthy pine trees and enhanced the importance of sterilization measures during the experiments to prevent contamination. At 3hai bacteria along the trails of nematodes were only visible in isolate C14-5 (Figure 12 E); in the other isolates (8A, 20 and HF) bacteria were visible only in the site were the twig was placed (Figure 12 A-D). The highest quantity of chips with bacterial colonies was found in 8A and C14-5 nematode isolates (Table 3).

Table 4: Number of inoculated chips with bacterial colonies, 7dai, with different treatments (values represent an average of eight samples ± standard deviation)

Experimental point	**7dai**				
Treatments	*Ø*	*C14-5*	*HF*	*20*	*8A*
# of chips with bacterial colonies mean (total # of samples)	$0.6^{b,C,D,e}\pm$ 0.5 (8)	$2^{a,C,D,e}\pm$ 1.6 (8)	$5.4^{A,B,d,E}\pm$ 2.2 (8)	$4.8^{A,B,c,E}\pm$ 1.3 (8)	$1.4^{a,b,C,D}\pm$ 0.5(8)

Legend: [A] represents significantly different between control (Ø); [B] represents significantly different between isolate C14-5; [C] represents significantly different between isolate HF; [D] represents significantly different between isolate 20; [E] represents significantly different between isolate 8A. ($p < 0.05$)

Seven days after inoculation, the amount of bacteria between treatments was significantly different. In general, bacterial quantity increased, compared to 3hai experiment, with exception of isolate 8A which remained the same (Table 4). This increase indicates that the presence of the nematode, in some way, raises the bacterial population inside the tree (Jones *et al*., 2008; Xie and Zhao, 2008). This could result from the supply of food or essential nutrients, by the nematodes, which leads to bacterial multiplication (Zhao and Lin, 2005; Zhao *et al.*, 2007; Jones *et al.*, 2010; Wang *et al.*,

2010). Bacteria were found in the control pines but the amount was clearly lower than that obtained in the treatments inoculated with the different nematodes (Table 4), differing from what Han *et al*. (2003), Zhao *et al*. (2003) and Xie and Zhao (2008), obtained in their controls (no bacteria were isolated). This suggests that bacteria found in the controls may be natural contaminants probably from the soil or the plant. This is supported by some authors that defend that microorganisms present in tissue cultures are endophytes (Holland and Polacco, 1994; Holland, 1997). Pirtillä *et al*. (2000) also isolated bacteria from *Pseudomonas* and *Methylobacterium* genera from buds of healthy Scotch pines (*Pinus sylvestris* L.). These bacteria can also be associated with a disease called bacterial wetwood or slime flux that appears in many softwood and hardwood trees, including pines, which may not manifest in trees with younger than 10-years-old; however, the disease-causing bacteria can be present (Anonymous, 1999). Most of these bacteria commonly inhabit soil and water (Gillman, 2005). Comparing the different nematode isolates, it was found that the virulent isolates HF and 20 led to a greater amount of bacteria, compared to 8A and the avirulent isolate C14-5. As bacteria seem to be involved in the pathogenicity of *B. xylophilus*, perhaps the avirulence of C14-5 could be associated to a lower number of bacteria. In fact, when compared to the virulent isolates HF and 20, C14-5 had a lower number of bacteria. One interesting observation was that bacteria were found in the marks left by the nematodes in all plates with chips inoculated with the three virulent nematodes (Figure 13 A-D); but not with the avirulent isolate, where bacterial colonies only appeared on the site of the chip (Figure 13 F); this is another indicator that there are less bacteria in C14-5 than in the virulent isolates.

Table 5: Number of inoculated chips with bacterial colonies, 14dai, with different treatments (values represent an average of eight samples ± standard deviation)

Experimental point	**14dai**				
Treatments	*Ø*	*C14-5*	*HF*	*20*	*8A*
# of chips with bacterial colonies mean (total # of samples)	$0.6^{b,C,d,e}$± 0.5 (8)	$1^{a,C,d,e}$± 0.7 (8)	$5.8^{A,B,D,E}$± 1.1 (8)	$0.4^{a,b,C,e}$± 0.5 (8)	$2.4^{a,b,C,d}$± 2.6 (8)

Legend: [a] represents no significant difference between control (Ø); [A] represents significantly different between control (Ø); [b] represents no significant difference between isolate C14-5; [B] represents significantly different between isolate C14-5; [C] represents significantly different between isolate HF; [d] represents no significant difference between isolate 20; [D] represents significantly different between isolate 20[e] represents no significant difference between isolate 8A; [E] represents significantly different between isolate 8A.

Fourteen days after inoculation (Table 5), the amount of bacteria in the chips between treatments was significantly different. Bacteria were found in the control pines but the quantity obtained was again lower than that obtained with nematode inoculations, with exception of isolate 20 which bacterial amount was relatively lower. These bacteria from control pines can be once again associated with endogenous bacterial population in the tree, as described before. Possibly, in the case of isolate 20, nematodes didn't survive or migrate to other plant parts not used for nematode extraction; bacterial colonies were observed only in one or another chip for this treatment. Another possibility is that the bacterial population inside the tree is very high, whereas the amount of nematode is low (Xie and Zhao, 2008). Again, the presence of nematodes stimulates bacterial growth. For isolate

C14-5 few bacteria were found in the chips at 14dai, compared to those from the 7dai experiment, which indicates that either nematodes didn't survive, or because it is an avirulent isolate, the symbiotic relationship with bacteria is lower or nonexistent compared to that seen with the virulent isolates. Comparing the obtained results with those obtained for nematode extraction it would be expected that, for pines inoculated with nematode isolates C14-5 and 8A, of which no nematodes were recovered, the bacterial amount decreased at this stage; this was only true for isolate C14-5.

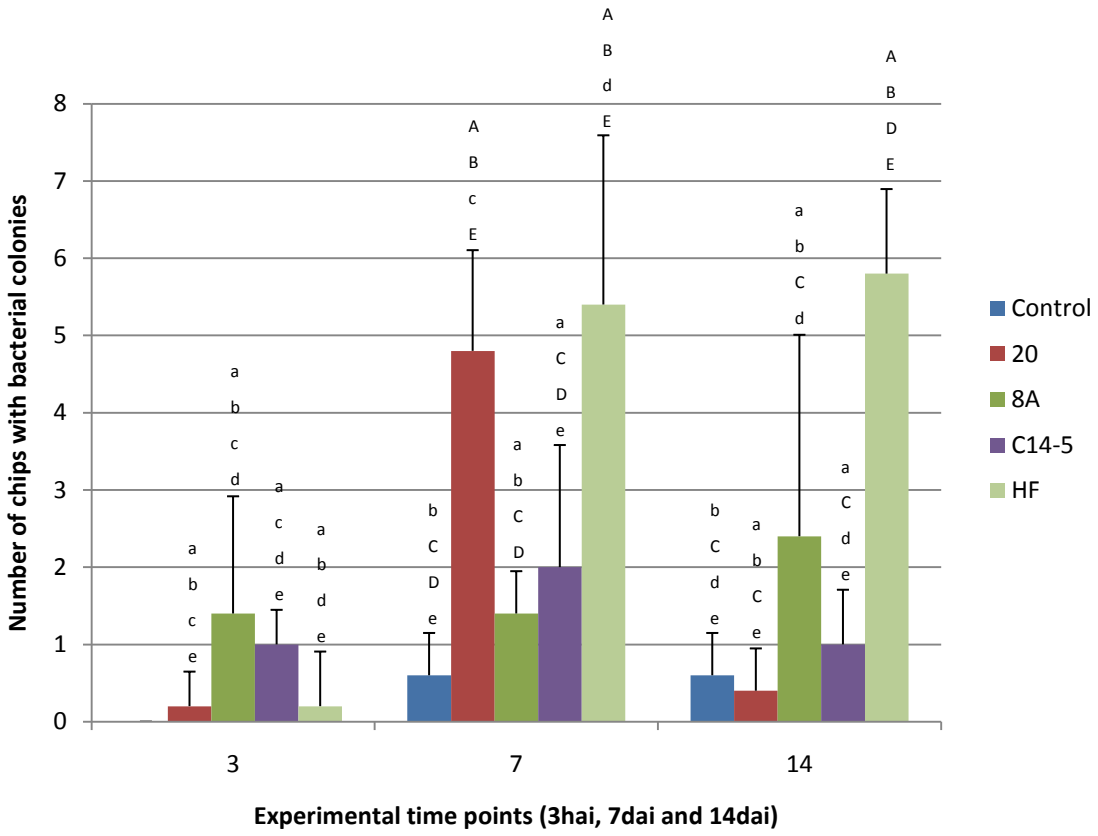

Figure 15: Number of chips with bacterial colonies, non inoculated (control) or inoculated with 4 nematode isolates, at the different experimental time points (values represent an average of eight samples (n=8) ± standard deviation; Legend: [a] represents no significant difference between control (Ø); [A] represents significantly different between control (Ø); [b] represents no significant difference between isolate C14-5; [B] represents significantly different between isolate C14-5; [c] represents no significant difference between isolate HF; [C] represents significantly different between isolate HF; [d] represents no significant difference between isolate 20; [D] represents significantly different between isolate 20; [e] represents no significant difference between isolate 8A; [E] represents significantly different between isolate 8A).

Figure 15 indicates that there is an increase in bacterial quantity, from 3hai until 14dai, for HF isolate, suggesting that this isolate is probably the most virulent one. This is in accordance with the symptom observations that showed that, at the early stage of 3hai, all trees inoculated with the four nematode isolates were healthy whereas for the later experimental time points (7dai and 14dai) pines were included at the stages 3 and 4 and general bacterial population also increased. In the experiment

of Xie and Zhao (2008), they also verified an increase in the percentage of bacteria with the disease stages. Isolate 8A also showed an increase, but not as significant as for HF.

Colony-forming units (CFU) from inoculated chips calculated by dish count

As the previous method did not guarantee that each colony arose from an individual bacterial cell, in this method the results were expressed in terms of CFU, determined for the bacteria present in the inoculated chips.

For the plate count, and to avoid that too many colonies are present and cells are overcrowded, the original inoculum was diluted several times and plates with 25 to 250 colonies were counted (Tortora *et al.*, 2010). This procedure was done for samples collected at the different experimental time points, with different treatments. Tables 6 to 8 show the mean CFU ± the standard deviation.

The CFU between treatments, within and between experimental time points, were not significantly different. No bacterial colonies were found in the control chips in all different experimental time points (Table 6 to 8).

Table 6: Colony-forming units (CFU) average, 3hai, in different treatments (values represent an average of five specimens ± standard deviation)

Experimental point	**3hai**				
Treatments	*Ø*	*C14-5*	*HF*	*20*	*8A*
CFU/ml mean (number of dish count)	$0^{b,c,d,e}$ (5)	$2.7\times10^{2\ a,c,d,e}$ ± 0 (5)	$6.7\times10^{3\ a,b,d,e}$ ± 1.1×10^{4} (5)	$7.4\times10^{2\ a,b,c,e}$ ± 1.1×10^{3} (5)	$7.3\times10^{3\ a,b,c,d}$ ± 1.1×10^{4} (5)

Legend: [a] represents no significant difference between control (Ø); [b] represents no significant difference between isolate C14-5; [c] represents no significant difference between isolate HF; [d] represents no significant difference between isolate 20; [e] represents no significant difference between isolate 8A. ($p>0.05$)

Three hours after inoculation, the number of CFU from the chips inoculated with the avirulent isolate was lower than that inoculated with the virulent nematodes (Table 6), suggesting that if a symbiotic relationship exists between nematodes and bacteria, and if bacteria are important to pine wilt disease, this could be an important reason for the isolate C14-5 being less virulent than the other isolates. This wasn't verified in the previous experiment (calculation of chips with bacterial colonies) (Table 3 and Figure 13). The number of CFU for isolate HF and 8A was relatively higher than that for isolate 20, 3hai; this was seen in the last experiment for isolate 8A (isolate 8A showed a larger quantity of bacteria in inoculated chips when compared with nematode isolates HF, 20 and C14-5 and with the control chips). In the experiment of Xie and Zhao (2008) bacterial values equivalent to ours were only obtained at stages 4 and 5 (1×10^{2} and 8.4×10^{3}, respectively). However, they studied the non-inoculated twigs of 6- to 7-year-old diseased trees, which can explain this difference; as trees

were older and as nematodes have taken longer to achieve the non-inoculated twigs, it's normal that the quantity of bacteria in the early stages was lower than ours.

Table 7: Colony-forming units (CFU) average, 7dai, in different treatments (values represent an average of five specimens ± standard deviation)

Experimental point	**7dai**				
Treatments	*Ø*	*C14-5*	*HF*	*20*	*8A*
CFU/ml mean (number of dish count)	$0^{b,c,d,e}$ (5)	$3.7 \times 10^{3\ a,c,d,e}$ ± 2.8×10^{3} (5)	$5.6 \times 10^{4\ a,b,d,e}$ ± 6.4×10^{4} (5)	$1.1 \times 10^{6\ a,b,c,e}$ ± 1.0×10^{6} (5)	$4.9 \times 10^{3\ a,b,c,d}$ ± 4.2×10^{3} (5)

Legend: [a] represents no significant difference between control (Ø); [b] represents no significant difference between isolate C14-5; [c] represents no significant difference between isolate HF; [d] represents no significant difference between isolate 20; [e] represents no significant difference between isolate 8A. ($p>0.05$)

As expected, seven days after inoculation, the number of CFU increased, compared to the first experimental time point, with exception of isolate 8A (Table 7). For the isolate 20, the number of CFU had a large increase in the order of 10^2 to 10^6; an increase was also verified in the last experiment (mean of chips with bacterial colonies raised from 0.2 to 4.8). This is consistent with what was observed in the NA plates from isolate 20 and HF (Figure 13) where many bacteria were seen in the marks left by the nematodes, indicating that nematodes were alive and in large number and so, more bacterial colonies occurred. Xie and Zhao (2008) reported that when they saw moving nematodes, more bacterial colonies occurred.

Table 8: Colony-forming units (CFU) average, 14dai, in different treatments (values represent an average of five specimens ± standard deviation)

Experimental point	**14dai**				
Treatments	*Ø*	*C14-5*	*HF*	*20*	*8A*
CFU/ml mean (number of dish count)	$0^{b,c,d,e}$ (5)	$5.2 \times 10^{4\ a,c,d,e}$ ± 7.4×10^{4} (5)	$2.0 \times 10^{5\ a,b,d,e}$ ± 2.6×10^{5} (5)	$1.3 \times 10^{6\ a,b,c,e}$ ± 1.8×10^{6} (5)	$6.6 \times 10^{5\ a,b,c,d}$ ± 7.4×10^{5} (5)

Legend: [a] represents no significant difference between control (Ø); [b] represents no significant difference between isolate C14-5; [c] represents no significant difference between isolate HF; [d] represents no significant difference between isolate 20; [e] represents no significant difference between isolate 8A. ($p>0.05$)

Fourteen days after inoculation, the number of CFU increased again for all treatments (Table 8). The increases in CFU and bacterial counts indicate that as the disease progresses, the number of bacteria associated with nematodes increases and it's this complex interaction that induces the disease (Zhao and Lin, 2005).

Identification of bacteria by classical methods

It is important to identify the bacterial species associated with the PWN, since it has been shown that these bacteria differ between geographical regions (Han *et al.*, 2003; Zhao *et al*., 2003; Wang *et al*., 2010). Also, it is thought that these differences make some plant species susceptible to PWD and others not (Zhao, 2008). The understanding of bacteria associated with PWN can also provide valuable information to develop measures to combat the disease, including the development of antibiotics. As an initial exploratory approach, the identification of bacteria was performed using classical methods. Some of these methods were used before for the identification of bacteria associated with Chinese isolates of *B. xylophilus* (Han *et al.,* 2003; Zhao *et al*., 2003; Xie and Zhao, 2008) and *B. mucronatus* (Han *et al*., 2003).

Bacterial colonies that appeared on inoculated stem pieces and those that appeared on the marks left by the four nematode isolates (isolated from the surface of nematodes) were placed in NA medium. They were selected and successively isolated, and morphologic characteristics of the bacterial colony (colony size, color, shape, transparency, prominence, edge and viscosity) were observed. Gram and flagella stain, cytochrome c oxidase and catalase test, starch hydrolysis and growth at 41ºC were also performed. In total, thirty-eight bacterial colonies were isolated and identified from inoculated chips and from nematodes by classical methods (Table 9) and the API20E system (Table 10).

Table 9: Example of results of identification tests performed to bacteria isolated directly from the four nematode isolates (IN), from chips inoculated with different nematode isolates, 3hai, 7dai and 14dai

	Colony	Nematode isolate	Colony appearance	Morphology	Gram stain	Catalase Test	Cytochrome C Oxidase Test	Starch Hydrolysis	Growth at 41ºC	Flagella stain
IN	31	C14-5	Small, beige, transparent edge, mat, bright, smooth	Rod	-	+	-	-	+	-
	32	C14-5	Small, beige, transparent, bright, smooth	Rod	-	+	-	-	+	+
	33	HF	Small, beige, transparent edge, bright, smooth, viscous	Rod	-	+	-	-	+	+
	34	HF	Small, beige, mat, bright, smooth	Rod	-	+	-	-	+	+
	35	20	Small, beige, transparent edge, mat, smooth, viscous	Rod	-	+	-	-	+	-
	36	20	Medium, beige, mat, smooth, viscous	Rod	-	+	-	-	+	+
	37	8A	Small, beige, transparent edge, mat, bright, smooth	Rod	-	+	-	-	+	-
	38	8A	Small, white, transparent, bright, smooth	Rod	-	+	-	-	-	+
3hai	21	8A	Medium, white, mat, rough, jagged edge	Rod	+	+	+	+	-	+
	22	8A	Medium, yellow, mat, smooth, bright	Rod	+	+	+	+	+	+
	23	8A	Small, yellow, transparent, smooth, bright	Rod	+	+	-	-	+	+
	24	8A	Medium, white, mat, rough, jagged edge	Rod	+	+	+	+	+	+
	25	8A	Medium, beige, mat, smooth	Rod	+	+	-	+	+	-
	26	8A	Small, beige, smooth, bright	Rod	-	+	-	-	+	-

Table 9: (Continued)

	Colony	Nematode isolate	Colony appearance	Morphology	Gram stain	Catalase Test	Cytochrome C Oxidase Test	Starch Hydrolysis	Growth at 41ºC	Flagella stain
	27	20	Medium, beige, mat, smooth, bright	Rod	+	+	+	+	+	+
	28	C14-5	Medium, beige, mat, smooth, viscous	Rod	+	+	-	+	+	-
3hai	29	C14-5	Small, beige, transparent, smooth, bright	Rod	-	+	-	-	+	+
	30	HF	Small, beige, transparent, smooth, bright	Rod	-	+	-	-	+	+
	1	20	Small, beige, mat, smooth, transparent edge, bright, viscous	Rod	-	+	-	-	+	-
	2	20	Small, beige, transparent, bright, smooth, viscous	Rod	-	+	-	-	+	+
	3	20	Small, beige, mat, smooth, transparent edge, bright, viscous	Rod	-	+	-	-	+	-
	4	8A	Small, beige, transparent, smooth, viscous, bright	Rod	-	+	-	-	+	+
	5	C14-5	Small, beige, transparent, bright, smooth, viscous	Rod	-	+	-	-	+	+
7dai	6	C14-5	Small, beige, mat, smooth, bright, viscous	Rod	-	+	-	-	+	-
	7	HF	Small, beige, mat, smooth, bright, viscous	Rod	-	+	-	-	+	-
	8	HF	Small, beige, transparent, bright, smooth, viscous	Rod	-	+	-	-	+	+
	9	HF	Small, beige, mat, smooth, transparent edge, bright, viscous	Rod	-	+	-	-	+	-
	10	Ø	Medium, beige, mat, rough	Rod	-	+	-	+	-	-

Table 9: (Continued)

	Colony	Nematode isolate	Colony appearance	Morphology	Gram stain	Catalase Test	Cytochrome C Oxidase Test	Starch Hydrolysis	Growth at 41ºC	Flagella stain
14dai	11	HF	Medium, beige, transparent, bright, smooth, viscous	Rod	+	+	-	-	+	+
	12	HF	Small, beige, transparent, bright, smooth, viscous	Rod	-	+	+	-	+	+
	13	HF	Small, beige, transparent, bright, smooth, viscous	Rod	-	+	-	-	+	+
	14	C14-5	Medium, beige, mat, smooth, bright, viscous	Rod	-	+	-	+	+	-
	15	Ø	Medium, yellow, mat, smooth, bright, viscous	Rod	+	+	+	+	+	+
	16	20	Medium, yellow, mat, smooth, bright, viscous	Rod	-	+	+	-	+	-
	17	20	Small, beige, transparent, bright, smooth, viscous	Rod	-	+	-	-	+	+
	18	8A	Small, beige, transparent, bright, smooth, viscous	Rod	-	+	-	-	+	+
	19	8A	Small, beige, mat, smooth, bright, viscous	Rod	-	+	-	-	-	+
	20	8A	Small, beige, mat, bright, smooth	Rod	-	+	+	+	-	+

IN - Isolated from the surface of nematodes

Two bacterial colonies were isolated from the surface of each nematode isolate (Table 9). The bacterial colonies had no variation in their morphology (rod-shaped bacteria were seen). All of them were gram-negative, positive for the catalase test and negative for the oxidase test. No colonies were able to hydrolyze starch and all of them, with exception of one isolated from 8A isolate, grew at 41°C. The presence of flagella was verified in one of the colonies isolated from isolate C14-5, the two colonies isolated from isolate HF, one isolated from isolate 20 and one from isolate 8A.

Ten individualized colonies from each nematode isolate were isolated from the inoculated pines, at each experimental time point (3hai, 7dai and 14dai) (Table 9). All the 30 bacterial colonies were rod-shaped. One bacterial colony was isolated from control pines (Ø – non-treatment) at each experimental point of 7dai and 14dai; no bacterial colonies were isolated from controls at 3hai. From the three experimental time points, 70% were gram-positive, all were positive for the catalase test, 73% were oxidase negative, 33% didn't hydrolyze starch, 87% grew at 41ºC and 63% showed flagella when viewed by the flagella staining.

In Zhao *et al*. (2003) experiment a total of 24 bacteria were isolated from naturally infected black pine (*P. thunbergii*) and Masson pine (*P. massoniana*) and 22 of them were gram-negative; 79% of their isolated bacteria lack cytochrome c oxidase. Han *et al*. (2003) isolated three predominant bacterial species from naturally infected black pine and *P. massoniana*; two of them presented flagella and were positive from the oxidase test and all were gram-negative, couldn't hydrolyze starch and grew at 41ºC; all were rod-shaped as those from the current experiment. Xie and Zhao (2008) isolated a total of 30 bacterial colonies from 6- to 7-year-old black pine inoculated with *B. xylophilus*; all were rod-shaped, as in the current experiment and 28 of them were gram-negative and all were oxidase negative and catalase positive (bacteria isolated in the current experiment were all also catalase positive).

The most prevalent colony appearances of the isolated bacterial colonies are shown in Figure 17 (A-F) and some of the obtained results in classical test are in Figure 16(A-B) and Figure 18(A-B).

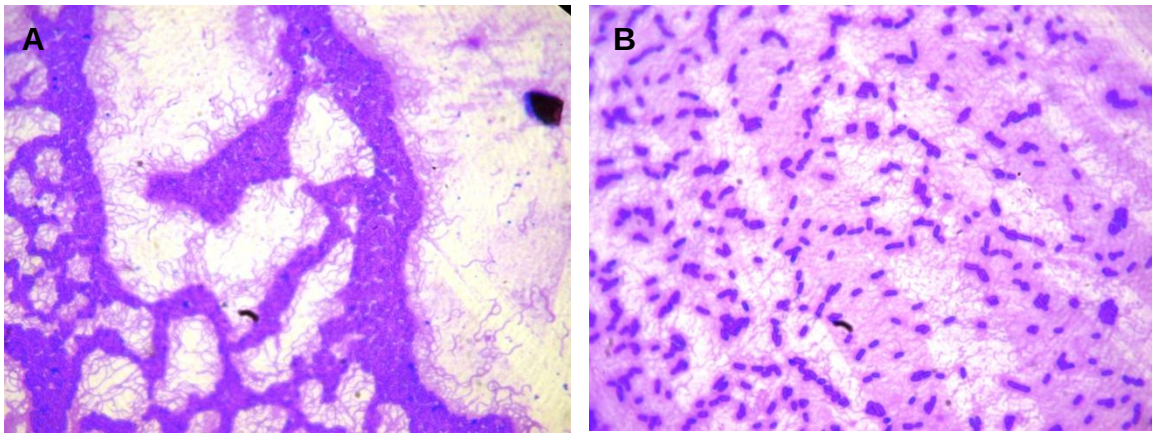

Figure 16 (A-B): Flagella stain for bacterial colonies 5 (A) and 24 (B).

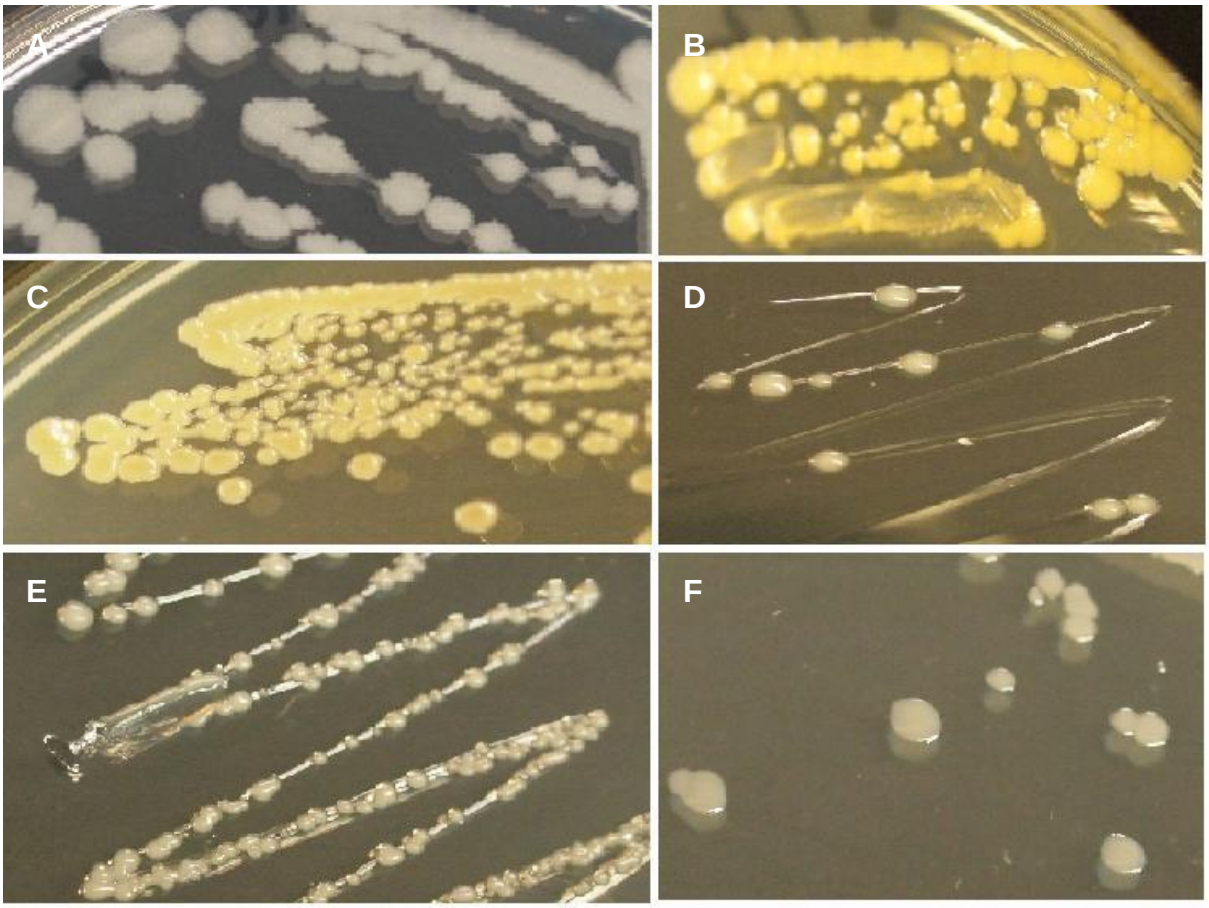

Figure 17 (A-F): Example of bacterial colonies isolated from inoculated twigs and grown on NA medium showing different morphological characteristics. A) Colony 21 - medium, mat, white, rough, with jagged edge; B) Colony 22- medium, yellow, mat, smooth, bright; C) Colony 23 - small, yellow/beige, transparent, smooth, bright; D) Colony 16 - medium, beige, mat, smooth; E) Colony 26 - small, beige, mat, smooth, bright; F) Colony 11 - medium, beige, transparent, bright, smooth, viscous.

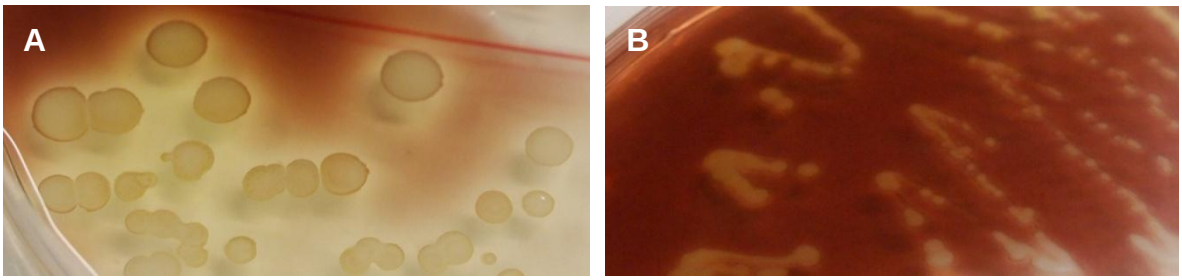

Figure 18 (A-B): Example of a positive (A) and a negative (B) result for starch hydrolysis test (bacterial colony 28 on the left and 29 on the right).

As classical identification methods are based solely on phenotypic bacterial characteristics, and usually only useful in the identification of the bacterial genus, all isolates were selected and tested using the API20E identification system, to try to better identify the bacterial species (Table 10). The API identification system has been used before for the identification of bacteria associated with *B.*

xylophilus by Xie and Zhao (2008). Other systems like automatic testing bacteriology (ATB) (Han *et al*., 2003; Wang, 2003; Zhao *et al*., 2003), ID32GN (Han *et al.*, 2003; Zhao *et al*., 2003), ID32E, 2STAPH, IDAP120A (Zhao *et al.,* 2003), and VITEK GNI+ or VITEK GPI cards (Zhao *et al.*, 2009) have also been used.

Table 10: Summary of the results obtained using the API20E identification system

Experimental time point	Treatment (n)	Possible bacterial species
3 hours	8A (4)*	Unidentifiable
	8A (1)	*Enterobacter cloacae*
	20 (1)*	Unidentifiable
	C14-5 (1)*	Unidentifiable
	C14-5 (1)	*Enterobacter cloacae*
	HF (1)	*Enterobacter cloacae*
7 days	20 (3)	*Enterobacter cloacae*
	8A (1)	*Enterobacter cloacae*
	C14-5 (2)	*Enterobacter cloacae*
	HF (3)	*Enterobacter cloacae*
	Ø (1)	*Enterobacter cloacae*
14 days	HF (1)*	Unidentifiable
	HF (2)	*Enterobacter cloacae*
	C14-5 (1)	*Enterobacter cloacae*
	Ø (1)	*Enterobacter cloacae*
	20 (2)	*Enterobacter cloacae*
	8A (3)	*Enterobacter cloacae*
IN	C14-5 (1)	*Enterobacter cloacae*
	C14-5 (1)	*Citrobacter freundii*
	HF (3)	*Enterobacter cloacae*
	20 (2)	*Enterobacter cloacae*
	8A (2)	*Enterobacter cloacae*

n – Number of bacterial colonies tested

* Positive for Gram stain

IN - Isolated from the surface of nematodes

After analysis of the numerical profile obtained, the main group of bacteria found belonged to the species *Enterobacter cloacae* (30 of the 38 isolated bacterial colonies). This species was also isolated in the work of Xie and Zhao (2008). The bacterial species isolated from control pines 7dai and identified as *E. cloacae*, may be involved in the bacterial wetwood disease, as mentioned above, as it is a species reported to be related to it (Anonymous, 1999). One of the bacterial colonies isolated from the surface of the nematode C14-5 was identified as *Citrobacter freundii*. To the best of our knowledge, this has never been shown before for bacteria associated with *B. xylophilus*.

The API20E gives the possibility to identify and confirm gram-positive bacterial species. Although bacterial colonies identified with an * had a previous positive result for Gram stain, the numerical profile obtained didn't allow the identification of these bacterial colonies.

Bacterial species identification using the API identification system showed an unexpected low bacterial diversity, suggesting that this method had poor discriminatory power. Incorrect reading and interpretation of the results can affect the effectiveness of the identification. Finally, this system can only identify *Enterobacteriacea* and some non-fastidious gram-negative rods; since gram-positive bacteria had been identified, it became necessary to find another identification method for the latter ones. Therefore, DNA sequencing methods were also used to help in the identification process. This method proved effective for Kwon *et al*. (2010).

Identification of bacteria by molecular methods

Total genomic bacterial DNA was extracted from all isolated colonies (except from bacterial colony 10 that failed to grow on NA medium) and an 1500 bp fragment of 16S rRNA gene was PCR amplified with bacterial universal primers. The results of the electrophoresis of PCR products are shown in Figure 19.

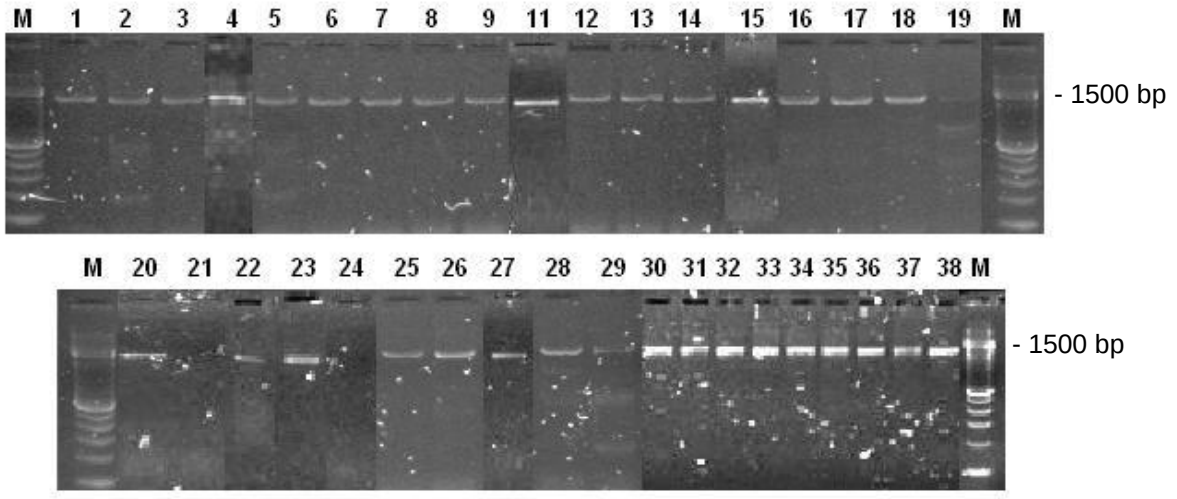

Figure 19: Agarose gel stained with SYBR® Safe DNA gel stain showing the amplification of the 1500 bp fragment corresponding to the 16S rRNA gene (M indicates the marker ladder; columns 1 to 38 represent the isolated colonies).

A total of 34 PCR products were purified and sequenced by Macrogen Ltd (Seoul, Korea) (bacterial colonies 19, 21 and 24, were not sequenced because the obtained DNA concentration was too low). The obtained sequences were finally subjected to a blastN and the results of the bacterial species obtained are shown in Table 11.

Table 11: Bacterial species isolated from trees inoculated with the four nematode isolates in the three experimental time points and bacteria isolated from non-inoculated nematodes identified after blastN of the amplified 16S rRNA gene fragment

Sample	Treatment	Experimental time point	Associated bacterial species
22	8A	3h	*Bacillus megaterium*
23			*Bacillus pumilus*
25			*Bacillus megaterium*
26			*Klebsiella oxytoca*
4		7d	*Klebsiella oxytoca*
18		14d	*Klebsiella oxytoca*
20			*Paenibacillus tundrae*
37		IN	*Klebsiella oxytoca*
38			*Klebsiella oxytoca*
30	HF	3h	*Enterobacter cloacae*
7		7d	*Enterobacter cloacae*
8			*Klebsiella oxytoca*
9			*Klebsiella oxytoca*
11		14d	*Terribacillus shanxiensis*
12			*Klebsiella oxytoca*
13			*Enterobacter oryzae*
33		IN	*Citrobacter freundii*
34			*Klebsiella oxytoca*
27	20	3h	*Bacillus simplex*
1		7d	*Klebsiella oxytoca*
2			*Klebsiella oxytoca*
3			*Enterobacter cloacae*
16		14d	*Klebsiella oxytoca*
17			*Pantoea agglomerans*
35		IN*	*Klebsiella oxytoca*
36			*Escherichia coli*
28	C14-5	3h	*Bacillus megaterium*
29			*Klebsiella oxytoca*
5		7d	*Citrobacter freundii*
6			*Klebsiella oxytoca*
14		14d	*Klebsiella oxytoca*
31		IN	*Klebsiella oxytoca*
32			*Klebsiella oxytoca*
15	Ø	14d	*Bacillus megaterium*

IN - Isolated from the surface of nematodes

Figure 20 shows the preponderance of each isolated bacterial genus in the three experimental time points and those obtained from the four nematode isolates.

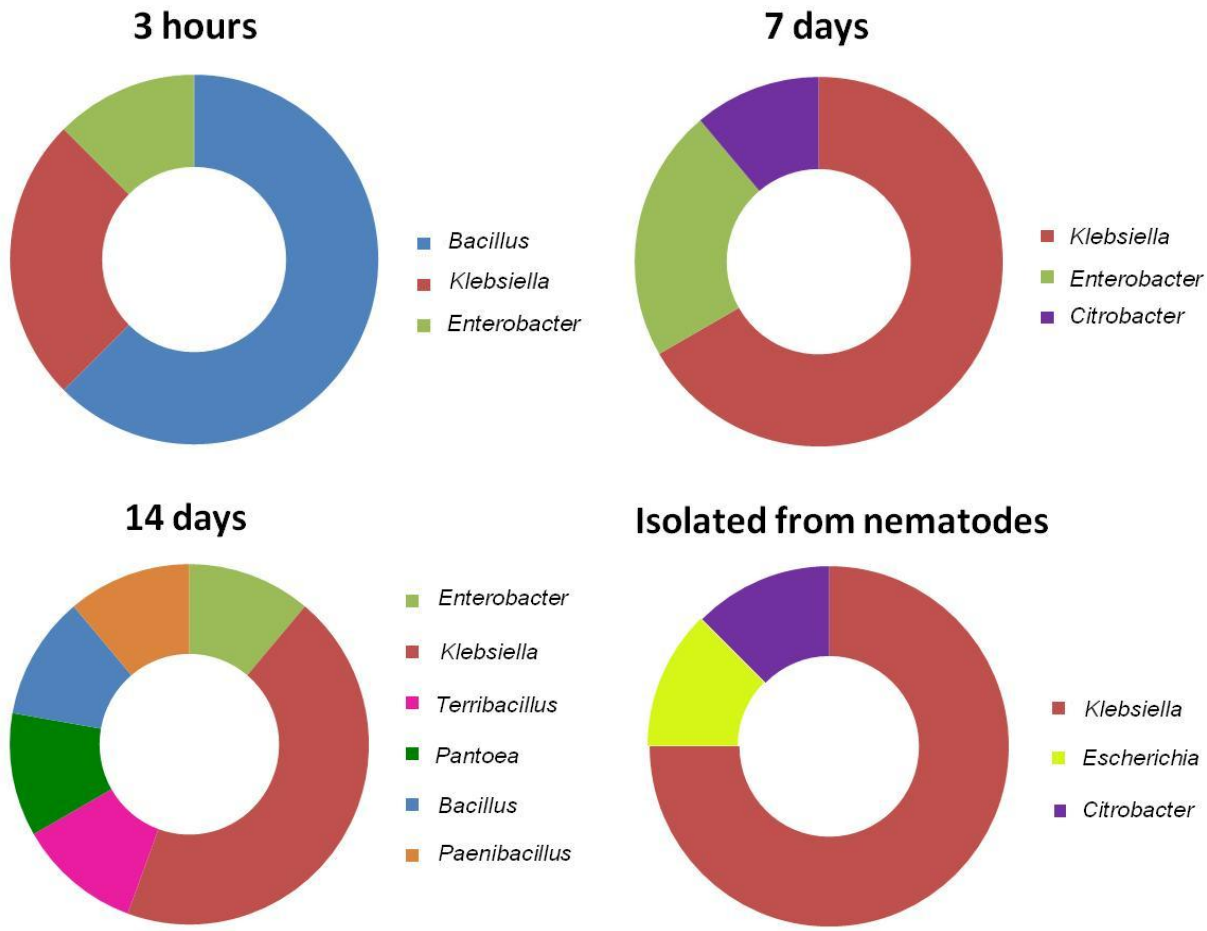

Figure 20: Bacterial genus isolated from trees inoculated with the four nematode isolates in the three experimental time points and isolated from the surface of nematodes.

In this experiment, of the 34 isolated bacterial colonies, 11 different species were identified. The main bacterial species found were *Klebsiella oxytoca* (52.9% of the total), *Bacillus* spp. (17.6% of the total) and *Enterobacter* spp. (11.8% of the total).

When looking at Table 11 and Figure 20, it can be observed that at 3hai, three bacterial genera were identified from the inoculated trees with the four nematode isolates. From nematode isolate 8A, two different species of *Bacillus* spp. were isolated (*B. megaterium* and *B. pumilus*) and one species of *Klebsiella* spp. (*K. oxytoca*). One species of *Enterobacter cloacae* was identified from isolate HF and one species of *B. simplex* from isolate 20. *E. cloacae* species is involved in plant (Roberts *et al*., 1999; Araújo *et al.*, 2002) and insect (Dushay *et al.*, 1996) diseases, being found in terrestrial and aquatic environments (Halda-Alija *et al*., 2001). From isolate C14-5 one species of *Bacillus* sp. and one of *Klebsiella* sp. were identified. The predominant species found at this experimental time point was *Bacillus* spp. Kawazu and Kawazu (1997) and Kaneko (1998); also, three bacteria of *Bacillus* genus (*B. cereus, B. subtilis* and *B. megaterium*), capable of producing toxic substances to callus cells cultivated in a liquid suspension and to black pine seedlings (*Pinus nigra*) were isolated. *Bacillus* spp. constitutes a diverse group of bacteria, widely distributed in soil and aquatic environments (Parvathi *et al.*, 2009). *B. pumilus* is highly resistant to extreme environmental

conditions (Nicholson *et al*., 2000) and produces compounds antagonist to fungal and bacterial pathogens (Aunpad and Na-Bangchang, 2007; Banerjee *et al*., 2007). *B. megaterium* has shown to have a toxic effect *in vitro* against *B. xylophilus* (Hwang *et al.*, 2001; Siddiqui and Shaukat, 2005) being found in diverse environments from soils to seawater (Vary, 1994). Its nematode suppression capacity has been shown in the studies of Biedendieck (2007) and Huang *et al.* (2010). Species of *Enterobacter* spp. have also already been reported from naturally infected black pine with *B. xylophilus*, in China (Zhao *et al*., 2003), from the surface of *B. xylophilus* of naturally infected black pine, in China (Zhao and Lin, 2005) and from inoculated black pine with *B. xylophilus* (Xie and Zhao, 2008). At this initial stage of infection it's not possible to draw conclusions regarding the avirulent isolate, since bacterial species found are both similar, in species and quantity, to those isolated from virulent nematode isolates. Because it's an early stage of the disease, it's possible that part of the bacteria found, namely *Bacillus* spp. species, may result from soil or plant and may not be in association with nematodes. However, this possibility is not very probable, since no bacterial colonies were found in the controls (Ø – non-treatment), which means that if *Bacillus* originated from the trees they should also appear in these samples.

Seven days after inoculation (7dai), which corresponds to a mid-stage of infection, totally three bacterial genera were also isolated from trees inoculated with the four nematode isolates (Figure 20). The predominant species found were *Klebsiella* sp., isolated from all nematode isolates. *E. cloacae* species was also isolated from nematode isolates 20 and HF; species of *Enterobacter* spp. have already been isolated in other studies (Zhao *et al*., 2003; Zhao and Lin, 2005; Xie and Zhao, 2008). From the avirulent isolate, a unique species was identified: *Citrobacter freundii*. This species has been described as a possible control agent for the nematode *Meloidogyne javanica*, decreasing the number of nematode eggs per plant by about 83%, being found in water, sewage, soil and food (Fabry *et al.*, 2007). *Bacillus* spp. species were not found at this disease stage, which can support the hypothesis that this species couldn't be in association with the nematode. As already seen in Table 4, the greatest amount of bacterial colonies was isolated from nematode isolates HF and 20, 7dai; also here, a higher amount of bacteria was isolated from these two nematode isolates, 7dai, with identical bacterial species being found for both. These findings can be an indication that these nematode isolates can probably be the most virulent ones. Again, nothing can be suggested from the avirulent isolate, for the reasons previously mentioned.

Fourteen days after inoculation (14dai), the bacterial colony diversity significantly increased, as expected and according to what Xie and Zhao (2008) obtained in their work. Also in the present work, population and bacterial species number increased as the disease progressed. This increase suggest that as the disease progresses, and assuming that nematode number also increases (as Xie and Zhao (2008) reported), the internal environment is suitable for bacterial development and growing, and more bacteria associates with nematodes, helping and aggravating disease symptoms and leading to host tree death. As in the previous experimental time point, *K. oxytoca* was the predominant species found. Other bacterial species, namely *Terribacillus shanxiensis* and *Enterobacter oryzae* were isolated from trees inoculated with isolate HF and *Paenibacillus tundrae* from isolate 8A. Sequence analysis of the colonies originating from the control pine (Ø) 14dai, showed that this colony

belonged to *B. megaterium* species. This isolate had also been isolated from inoculated pines, suggesting that in the later, the *B. megaterium* may have originated from soil or plant, being a natural contaminant. This species is involved in the bacterial wetwood disease, described above (Anonymous, 1999). A species of *Pantoea agglomerans* was isolated from isolate 20. In the experiment of Han *et al*. (2003) no *Pantoea* sp. species were recovered from uninfected trees (control pines) as in the current work. This isolate was also isolated in previous studies, having an inhibitory effect on *B. xylophilus* (Han *et al*., 2003); it inhibits nematode reproduction and diminishes male adult body volume (Zhao *et al*., 2006).

At this late stage of infection, isolate HF produced the largest amount of bacterial colonies (Table 5), followed by isolate 8A and 20. From the avirulent isolate only one colony of *K. oxytoca* was isolated, so the amount and bacterial diversity decreased, compared with the virulent nematode isolates. This decrease and differentiation in relation to the virulent isolates, is consistent with the fact that we are in the presence of an avirulent isolate. The main bacterial species found at this late stage was *K. oxytoca* (44.4% of the total isolated colonies) indicating that this species is a potential bacteria related to the disease and probably the one truly involved in it, in this geographic zone. *K. oxytoca* species make part of an endophytic population of bacteria that play an important role in plant growth and development (Hallmann *et al*., 1997); this nitrogen-fixing bacterium has been isolated from rice roots (Nguyen *et al*., 1989), and is associated with bacterial wetwood of elms (Anonymous, 1999).

Bacterial colonies were also isolated from the surface of the four nematodes isolates. *K. oxytoca* was isolated from all nematode isolates, indicating that this species is associated with the nematode and establishes a symbiosis with it. No previous work had identified *K. oxytoca*, suggesting that this species is specific to this geographic area. It was also isolated from isolate HF a *C. freundii* species, also found in trees inoculated with isolate C14-5. A species of *Escherichia coli* was isolated from isolate 20, not being isolated from any tree inoculated with the four nematode isolates. This species has already been found in previous work, being isolated from wild PWN (Zhao *et al*., 2003), from *P. massoniana* naturally infected with *B. xylophilus* (Zhao and Lin, 2005) and from inoculated black pine with *B. xylophilus* (Xie and Zhao, 2008). Zhao and Lin (2005) reported that this species inhibited PWN reproduction. As described for *C. freundii*, *E. coli* also proved to be efficient for the control of the nematode *Meloidogyne javanica*, reducing number of galls in 80% (Fabry *et al.*, 2007). This species can be found in soil associated with plant roots (Ritchie *et al.*, 2003). Species diversity from nematodes was not very large compared to those isolated from trees, which indicates that nematodes, apart from owning bacteria in their body, establish symbiotic relationships with bacteria from inside the tree during the infection process, and so all these dynamics can contribute to symptom disease development.

Bacterial species of *Bacillus* spp., *Enterobacter* spp., *Terribacillus* sp., *Pantoea* sp. and *Paenibacillus* sp. were only isolated after tree inoculation, suggesting that these species may have been from trees or soil. However *Bacillus*, *Pantoea* and *Enterobacter* genus have already been reported as being associated with PWN in the experiments of Kawazu (1997, 1998), Han *et al*. (2003),

Zhao *et al.* (2003), Zhao and Lin (2005), Zhao *et al.* (2006), Zhao *et al.* (2007) and Xie and Zhao (2008).

In the current work, at any experimental time point and with any treatment, *Pseudomonas* spp. species was not found. This is an interesting finding, since this species was isolated in several previous works (Han *et al*., 2003; Zhao *et al*., 2003; Zhao and Lin, 2005; Xie and Zhao, 2008) which supports the fact that bacteria differ between geographic zones (Han *et al*., 2003; Wang *et al*., 2010); in fact when PWN occupies a new region, new bacteria are taken from the local flora (Zhao, 2008). In China the dominant bacterial species belong to *Pseudomonas* genus (Zhao *et al.*, 2003; Tan and Feng, 2004), in Japan *Bacillus* spp. are dominant (Kawazu *et al.*, 1996a; Kawazu *et al*., 1996b) and in Korea both genera exists (Zhao, 2008). *P. tundrae*, *T. shanxiensis* and *C. freundii* have never been isolated in any previous experiment, suggesting that along with *K. oxytoca*, these may be specific to the Portuguese geographic zone, as the plant material originated from *Anadia*, Portugal, and the bacteria were isolated from *Setúbal* region and Portuguese central region. Furthermore, this is the first report on the bacterial population of an avirulent nematode isolate and contrary to previous studies, the experiments were carried out on *P. pinaster* and not in *P. thunbergii*, *P. densiflora*, or *P. massoniana*.

Classical vs. molecular identification methods

As classical and molecular methods of bacterial identification differ in their analysis, a comparison between these two methods will be described below to check if the results obtained in the two methods are in concordance.

Bacterial colonies 15, 22, 23, 25, 27 and 28 were identified by sequencing as belonging to *Bacillus* genus. Identification tests revealed that all these bacterial colonies were gram-positive rods, which is correct for this genus (Holt *et al*., 1994; Ferreira and Sousa, 2000; Bottone and Peluso, 2003). Also true for this genus, all bacterial colonies were positive for catalase test (Holt *et al*., 1994; Bottone and Peluso, 2003; Madigan *et al.,* 2004). *Bacillus* spp. species are also positive in oxidase tests, which was not verified in all bacterial colonies (23, 25 and 28 were negative for this test). *B. megaterium* is able to hydrolyze starch, which was confirmed for all bacterial colonies (Figure 18 A for colony 28) (Thomas *et al*., 1980); on the other hand *B. pumilus* can't hydrolyze starch, also confirmed to sample 23 (Lovett and Young, 1969). It has also been described that *Bacillus* spp. species grow at 41ºC, true for all bacterial colonies (Ahmadi *et al*., 2010). This genus is motile by peritrichous flagella (Holt *et al*., 1994), however flagella were not visible for all bacterial colonies (25 and 28 didn't presented flagella).

Bacterial colonies 1, 2, 4, 6, 8, 9, 12, 14, 16, 18, 26, 29, 31, 32, 34, 35, 37, 38 were identified by sequencing as *Klebsiella oxytoca* species. These bacteria are gram-negative rods, positive and negative for catalase and oxidase test, respectively, able to hydrolyze starch, don't grow at 41ºC and are non motile (Holt *et al*., 1994). Not all bacterial colonies had the expected results: isolate 14 grew at

41ºC; isolates 1, 6, 9, 16, 26, 31, 35, 37 were not able to hydrolyze starch and grew at 41ºC; isolates 2, 4, 8, 12, 18, 29 (Figure 18 B), 32, 34 were also unable to hydrolyze starch, grew at 41ºC and has flagella; isolate 38 didn't hydrolyze starch and has flagella. Bacterial colonies 3, 7, 13 and 30 were identified as belonging to *Enterobacter* genus. *Enterobacter* spp. species are gram-negative rods, catalase positive, oxidase negative, motile by peritrichous flagella (Holt *et al*., 1994), unable to hydrolyze starch (Ishii *et al.,* 1994) and not capable of growing at 41ºC (Madigan *et al.,* 2004). Bacterial colonies 3 and 6 didn't produce the expected result for flagella stain and 41ºC growing test; 13 and 30 failed to 41ºC growing test. Bacterial colonies 5 and 33 were identified as *Citrobacter freundii* species. *C. freundii* are gram-negative rods (Reyes and Folwarkin, 1984; Holt *et al*., 1994), catalase positive (Holt *et al*., 1994) and oxidase negative (Holt *et al.*, 1994; Yalçi *et al*., 2006), able to hydrolyze starch (Reisner, 1978) and are motile by peritrichous flagella (Figure 12) (Douka, 1980; Holt *et al.*, 1994). Sample 5 hydrolyzed starch contrary to what one would expect. Sample 11 was identified as *Terribacillus shanxiensis*, a gram and catalase positive species (An *et al.*, 2007), oxidase negative and motile by flagella (Krishnamurthi and Chakrabarti, 2008) as the obtained results. This sample grew at 41ºC and didn't hydrolyze starch. Sample 17 was identified as *Pantoea agglomerans*. *P. agglomerans* is a gram-negative rod, positive for catalase test and negative for oxidase test, motile by peritrichous flagella (Holt *et al.*, 1994) and able to hydrolyze starch (Chung *et al*., 2005). Sample 20 was identified as *Paenibacillus tundrae*. This sample proved to be a gram-negative rod, catalase and oxidase positive, unable to hydrolyze starch, not growing at 41ºC and motile by flagella. Bacterial colony 36 was identified by sequencing as belonging to the species *E. coli;* classical identification tests revealed that this bacterial colony was a gram-negative rod, positive for catalase test, negative for oxidase test, didn't hydrolyzed starch and grew at 41ºC and presented flagella. All results for *E. coli* species are in accordance with the literature (Holt *et al*., 1994; Fotadar *et al.*, 2005)

The data described above indicates that the classical methods, as well as the API methods, were not always concordant with the molecular methods for bacterial identification. Of all the methods used in the current study, DNA sequencing was the most reliable because it analysis the sequence of the 16S rRNA gene and compares this sequence with those existing in a database, a method that is less prone to errors.

GENERAL CONCLUSIONS

PWD is still a serious disease for which there is no effective treatment.

It is certain that other microorganisms, in addition to PWN, are involved in PWD but bacterial role in the disease is still controversial, and further experiments to know the bacterial species associated with the disease are necessary to clarify this hypothesis.

Inoculation of *P. pinaster* trees with three virulent nematode isolates led to a faster and more severe symptom appearance than those verified from trees inoculated with avirulent nematode isolates, more obvious from the 7 to 14 days experiment.

Bacteria were isolated from all inoculated twigs and nematode's surface, indicating that, as in China, Portuguese PWN naturally carries bacteria. Seven and fourteen days after inoculation, bacteria were isolated from control pines (contrary to what was obtained in the previous experiments (Han et al., 2003; Zhao et al., 2003; Xie and Zhao, 2008)), indicating that these came from soil or the plant itself and may be associated with plant disease.

The experiment of the quantification of chips with bacterial colonies showed that the avirulent nematode isolates are associated with the appearance of less bacterial colonies and nematode isolates HF and 20 with the highest number of chips with bacterial colonies, being probably the most virulent isolates. The successive increase in the number of chips with bacterial colonies over time indicates that the presence of nematodes promotes bacterial growth.

The experiment of determination of colony-forming units (CFU) also proved that isolate C14-5 led to the appearance of less bacterial colonies and that CFU increased for all treatments over time.

Identification by classical methods together with the API20E system proved to be insufficient and unhelpful, while bacterial DNA sequencing allowed the identification of a greater diversity of bacterial species. The main bacterial species found was *Klebsiella oxytoca*, followed by *Bacillus* spp. and *Enterobacter* spp., all found in diverse environments from soil to water.

Bacterial species isolated from the surface of nematodes were similar to those isolated from the xylem of *P. pinaster*.

Many new species were isolated in this experiment supporting the fact that bacteria differ between geographic zones; *K. oxytoca* may be associated with the Portuguese region.

Although several species have been isolated in this experiment, it is possible that not all of them are involved in the pathogenicity of PWD and are mere contaminants of PWN.

As 14 days after inoculation no pine actually died, it's possible that with time other differences could be verified, namely in the species diversity and bacterial population dynamic. So, the experiment duration may have been short.

Finally, this work has great importance for Portugal since the disease has serious economic and environmental problems and because the majority of research on this disease focuses on southern Europe and Asian countries.

FUTURE WORK

- As *Pinus pinea* plant species is, along with *Pinus pinaster*, important to Portuguese economy, namely in pinion seed exportation, and as the PWD symptom development, though scarce, is less severe and slow for this species, the same experiments should be conducted in this Pine species. The knowledge of bacterial population dynamics and species will help in the understanding of the reasons that turn this species less susceptible, beyond the fact that is less attractive to the Portuguese vector beetle, *Monochamus galloprovincialis*.

- Several studies reported that inoculation of *B. xylophilus* free of bacteria (aseptic) in callus and aseptic black pine seedlings didn't lead to the browning of this structure or the appearance of symptoms on inoculated seedlings, but the combination of the nematodes with bacteria lead to the browning of the callus and caused strong wilt of seedlings (Oku *et al.,* 1980; Zhao *et al.,* 2000; Han *et al.,* 2003; Zhao *et al.,* 2003). So, to further confirm that bacteria are really involved in PWD, an experiment of this kind should be conducted in *P. pinaster* trees.

- In addition, as some experiments gave inconclusive results, data should be confirmed with the repetition of some tests, namely the DNA sequencing of bacterial species that presented low DNA concentration.

- As 14 days after inoculation no pine actually died, the duration of the experiments may be extended to study bacterial population dynamic and species diversity in later stages of disease progression, including those of pine death.

- Also, as it has already been reported in several works that bacteria associated with PWN produce phytotoxins, particularly produced by *Bacillus* spp. and *Pseudomonas* spp. species (Kawazu *et al.*, 1996a, 1998; Mamiya, 1980; Oku, 1988, 1990; Oku *et al.,* 1979, 1980; Zhang *et al.,* 1997; Han *et al.,* 2003; Zhao *et al*., 2003, 2005), a study of this kind should be conducted.

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