Population dynamics of bacteria associated with different strains of the pine wood nematode *Bursaphelenchus xylophilus* after inoculation in maritime pine (*Pinus pinaster*)

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A B S T R A C T

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 the API20E identification system 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. In general, it was found that bacterial population, inside the tree, increased with disease progression. A superior 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 was insufficient in the identification of bacterial species; *Enterobacter cloacae* species was identified in 79% of the isolated bacterial colonies and seven of these colonies could not be identified by this method. Molecular identification methods, through bacterial DNA sequencing, allowed a more reliable identification: eleven different bacterial species within the *Bacillus, Citrobacter, Enterobacter, Escherichia, Klebsiella, Paenibacillus,* Bacillus, *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Paenibacillus*, *Pantoea* and *Terribacillus* genera were identified. General bacterial diversity increased with the progression of the disease. *Bacillus* spp. were predominant at the earlier stage of disease progression and *Klebsiella oxytoca* at the later stages. Furthermore, 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 their geographical origin (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.

1. Introduction

1.1. Pine wilt disease, Bursaphelenchus xylophilus, Monochamus galloprovincialis and Pinus pinaster

The pine wilt disease (PWD), as its name implies, is a disease found in pine species (*Pinus* spp.) whose main and best known

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etiologic agent is *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle, the pine wood nematode (PWN).

In Portugal, the vector of PWN is *Monochamus galloprovincialis* Olivier 1795 (Sousa et al., 2001, 2002), as the nematode is transported in the body of the insect (Aikawa, 2008; 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). 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 and death of the tree (Jones et al., 2008).

The maritime pine, *P. pinaster*, is the most popular host as the insect vector feeds from it during its maturation that causes the transmission of PWN to host trees (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*. *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 (Mota and Vieira, 2008a).

1.2. 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, 1983; Nickle et al., 1981; Nobuchi et al., 1984; Myers, 1988; Fukuda et al., 1992; Yang, 2002). 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; Tamura, 1983; Kawazu et al., 1996b; Cao, 1997; Kawazu and Kaneko, 1997; Kawazu, 1998; Han et al., 2003; Zhao et al., 2003, 2005; Xie and Zhao, 2008).

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 the genus *Pseudomonas*, in Japan to the genus *Bacillus* and in Korea both are present (Zhao, 2008). 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.

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, 2003; Han et al., 2003).

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). However, 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.

As little is known regarding the bacterial population in Portuguese nematode isolates and the bacteria associated with the Japanese avirulent isolate C14-5, and how it compares to virulent isolates, this work is intended to study the effect of inoculation of healthy *P. pinaster* trees, with four different nematode isolates (HF, 20, 8A and C14-5), in the bacterial population of either the nematode or the tree itself, at different stages of disease progression (3 h, 7 days and 14 days after inoculation). It also aims at identifying the bacterial species isolated from the xylem of the trees and from the surface of the nematodes.

2. Materials and methods

2.1. Source and culture of nematodes

Three virulent isolates of *B. xylophilus* (HF 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.

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

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 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.

2.2. Source, culture and inoculation of pines

The experiments were carried out on 1-year-old Portuguese maritime pine specie *P. pinaster* at CBQF – *Escola Superior de Biotecnologia da Universidade Católica Portuguesa*. 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 h light and 16 h darkness and temperatures of 26 and 24 °C for periods of dark and light, respectively.

Nematodes were inoculated in pines according to the Futai (1980) method. One hundred and forty-four pines were inoculated with the different PWN isolates and 27 others without treatment (non-inoculated) were used as controls (Ø).

Three experimental points were considered: an early response of 3 h (3hai), a mid response of 7 days (7dai) and a later response of 14 days after inoculation (14dai).

2.3. 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 according to Zhao et al. (2008).

2.4. 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.

2.5. Calculating the quantity of inoculated chips with bacterial colonies

The quantity of inoculated chips with bacterial colonies was determined according to Xie and Zhao (2008): 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 $2 \times 2 \times 5$ mm pieces ($W \times D \times H$). 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 26 °C for 3 days. Bacteria that appeared in the site around the chips and by the nematode movement were placed in NA. 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.

2.6. 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 min and the supernatant was discarded; the remaining PWN were disinfected with 3% H_2O_2 for 5 min; finally, a single nematode was removed with a thin metal needle and placed on a plate with NA media which was incubated at 26 °C. Bacterial colonies which appeared in the track left by the nematodes were successively transferred to NA for colony purification.

2.7. 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 s, water rinsed for 2 s, stained with Gram's iodine for 1 min, water rinsed, washed with 95% ethanol for 10–30 s, stained with safranin for 30–60 s, 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 s (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 h 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 41 °C 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).

2.8. 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 95 °C and sedimented at 16,000*g* for 5 min in a microcentrifuge (Thermo Scientific Heraeus Pico 17). The extracted DNA was quantified spectrophotometrically using a NanoPhotometer™ 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 min, followed by 25 cycles at 95 °C for 30 s, 54 °C for 30 s and 72 °C for 1 min and a final extension at 72 °C for 5 min. 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 min 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.

2.9. Nucleotide sequence accession numbers

The 16S rRNA gene sequences of the bacterial colonies isolated reported in this study have been deposited in EMBL database under the accession numbers from FR821638 to FR821671.

2.10. 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 ($p < 0.05$).

3. Results and discussion

3.1. Stage symptoms and oleoresin flow

After inoculation of pines with the four different nematode isolates, pines for each treatment were studied for the presence/absence of oleoresin flow and the general appearance of the plant

Table 1
General appearance and oleoresin flow of inoculated plants at the three experimental time points.

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

was also registered according to Zhao et al. (2008). The results are shown in Table 1.

The progression of symptoms was as expected – healthy pines in the early infection (3hai) and severely diseased pines at 14dai. It was observed that pines inoculated with the avirulent isolate C14-5 led to a slower aggravation of symptoms, consistent with what was observed in other experiments (Kosaka et al., 2001).

3.2. 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 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 was not efficient or nematodes may have migrated to other plant parts (needles or roots, for example). 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).

3.3. Quantification of bacterial colonies on inoculated chips

In Tables 2–4 the average number of chips with bacterial colonies and the standard deviation for the various treatments in the different experimental time points are visible.

Three hours after inoculation, 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 Han et al. (2003), Zhao et al. (2003) and Xie and Zhao (2008) no bacteria were found in the control (Table 2). The highest quantity of chips with bacterial colonies was found in 8A and C14-5 nematode isolates (Table 2).

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 3). 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; Wang et al., 2010). At this experimental point, and contrary to what Han et al. (2003), Zhao et al. (2003) and Xie and Zhao (2008) reported for their experiments, 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 3). These bacteria may be natural contaminants from the soil or the plant. In fact bacteria have been isolated from buds of healthy Scotch pines (*Pinus sylvestris* L.) (Pirtillä et al., 2000); they can also be associated with some plant diseases as the bacterial wetwood disease (Anonymous, 1999). Seven days after inoculation, 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.

Fourteen days after inoculation (Table 4), the amount of bacteria in the chips between treatments was significantly different. Again, bacteria were found in the control pines and generally in a lower amount than that obtained with nematode inoculations. The presence of nematodes stimulated, once again, bacterial growth. For the isolate C14-5 few bacteria were found in the chips at 14dai, compared to those from the 7dai experiment, which indicates that either nematodes did not 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.

Over the three experimental time points, the bacterial quantity for the isolate HF always increased, suggesting that this is probably the most virulent isolate.

3.4. Identification of bacteria by classical methods and API20E system

As an initial exploratory approach, the identification of bacteria was performed using classical methods. In total, 38 bacterial colonies were isolated and identified from inoculated chips and

Table 2
Number of inoculated chips with bacterial colonies, 3hai.

Experimental point	3hai				
Treatments	Ø	C14-5	HF	20	8A
# Of chips with bacterial colonies mean ± SD (total # of samples)	0(8)	1 ± 0.4 (8)	0.2 ± 0.7 (8)	0.2 ± 0.4 (8)	1.4 ± 1.5 (8)

Table 3
Number of inoculated chips with bacterial colonies, 7dai.

Experimental point	7dai				
Treatments	Ø	C14-5	HF	20	8A
# Of chips with bacterial colonies mean ± SD (total # of samples)	$0.6^{C,D}$ ± 0.5 (8)	$2^{C,D}$ ± 1.6 (8)	$5.4^{A,B,E}$ ± 2.2 (8)	$4.8^{A,B,E}$ ± 1.3 (8)	$1.4^{C,D}$ ± 0.5(8)

Means marked with different letters are significantly different ($p < 0.05$; Tukey): [A]from control (Ø); [B]from isolate C14-5; [C]from isolate HF; [D]from isolate 20; [E]from isolate 8A.

Table 4
Number of inoculated chips with bacterial colonies, 14dai.

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

Means marked with different letters are significantly different ($p < 0.05$; Tukey): [A]from control (Ø); [B]from isolate C14-5; [C]from isolate HF; [D]from isolate 20; [E]from isolate 8A.

from individual nematodes by classical methods and the API20E system.

First, two bacterial colonies were isolated from the surface of each nematode isolate. 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 recovered from the inoculated pines, at each experimental time point (3hai, 7dai and 14dai). All the 30 bacterial colonies were rod-shaped, as in the experiments of Han et al. (2003) and Xie and Zhao (2008). 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 (93% of Xie and Zhao (2008) isolates were catalase positive), 73% were oxidase negative, 33% did not hydrolyze starch, 87% grew at 41 °C and 63% showed flagella when viewed by the flagella staining. In the experiment of Zhao et al. (2003), 79% of their isolates were oxidase negative (similar to our results) and 92% were gram-negative.

The API20E identification system was performed to help in the identification of the bacterial species. 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*. As the API20E system only identifies gram-negative bacterial species, and we have isolated seven gram-positive bacteria, these last ones could not be identified by this method; so, 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) and Proença et al. (2010).

3.5. 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. 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 5.

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).

The predominant species found 3hai was *Bacillus* spp. Kawazu and Kaneko (1997) and Kawazu (1998) also isolated three bacteria of the genus *Bacillus* (*B. cereus, B. subtilis* and *B. megaterium*); these constitute 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).

Seven days after inoculation, the predominant species found was *Klebsiella oxytoca*, isolated from all nematode isolates. 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*, being found in water, sewage, soil and food (Fabry et al., 2007).

Fourteen days after inoculation, the bacterial colony diversity significantly increased, as expected and according to what Xie and Zhao (2008) obtained in their work (Fig. 1). Also in the present work, population and bacterial species number increased as the disease progressed. As in the previous experimental time point, *K. oxytoca* was the predominant species found, 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

Table 5
Bacterial species isolated from trees inoculated with the four nematode isolates in the three experimental time points and bacteria isolated from non-inoculated nematodes.

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

[a] Bacteria were identified after blastN of the amplified 16S rRNA gene fragment; IN, isolated from the surface of nematodes.

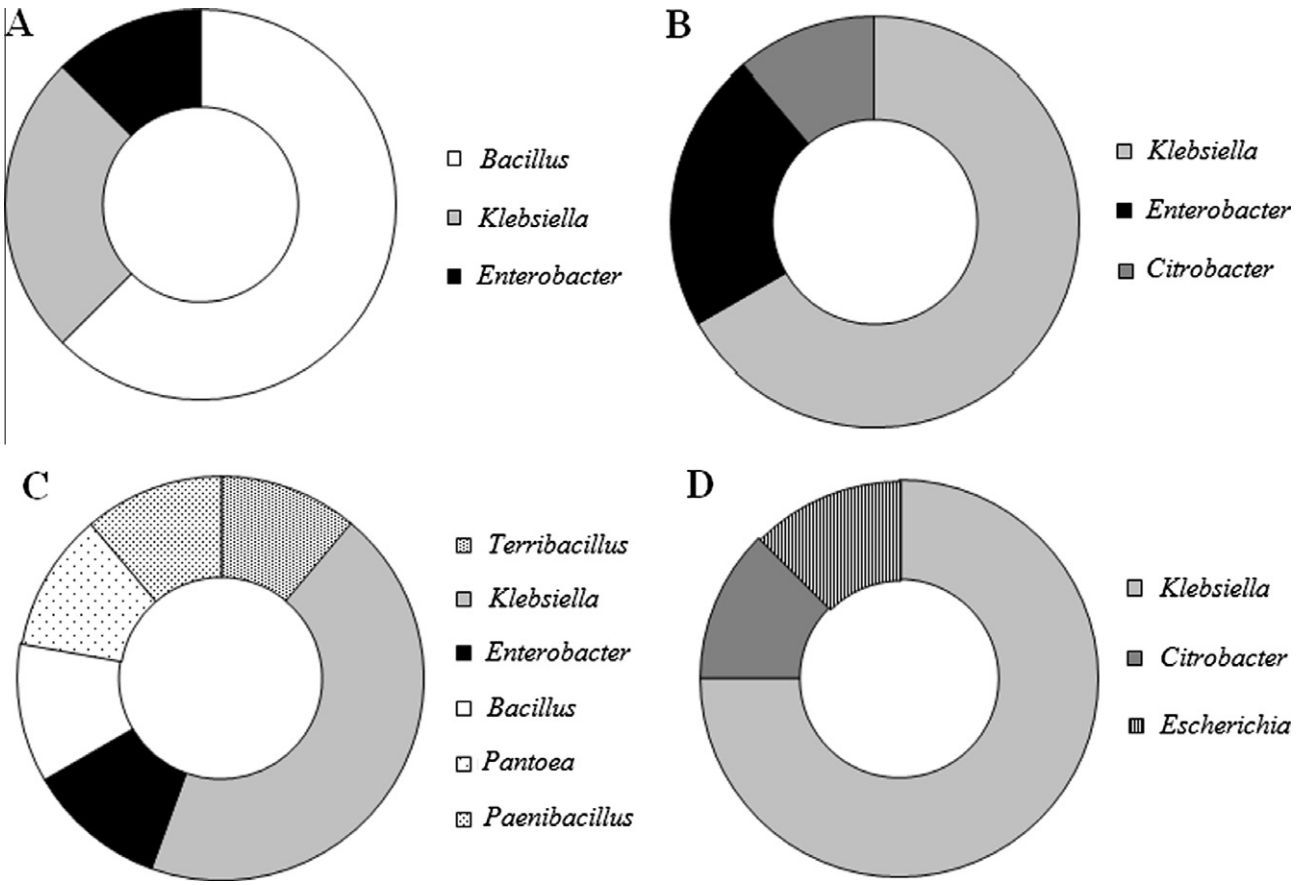

Fig. 1. Population dynamics of main bacterial genus isolated from trees inoculated with the four nematode strains in the three experimental time points and isolated from the surface of nematodes. (A) 3hai. (B) 7dai. (C) 14dai. (D) Bacteria isolated directly from the nematodes.

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 again that this species is specific to this geographic area. A *C. freundii* species, also found in trees inoculated with isolate C14-5, was also recovered from isolate HF. 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 (*Pinus thunbergii*) 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). 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.

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 the genus *Pseudomonas* (Zhao et al., 2003; Tan and Feng, 2004), in Japan *Bacillus* spp. are dominant (Kawazu et al., 1996a,b) 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 Po rtuguese 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*.

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