Identification of bacteria associated with different strains of *Bursaphelenchus xylophilus*

Mariana Roriz, Carla Santos, Marta Lima, Marta W. Vasconcelos*

Escola Superior de Biotecnologia, Universidade Católica Portuguesa
*Contact author: : mwvasconcelos@esb.ucp.pt

CATÓLICA
UNIVERSIDADE CATÓLICA PORTUGUESA | PORTO

Plantech

Ministério da Agricultura, do Desenvolvimento Rural e das Pescas

Autoridade Florestal Nacional

INTRODUCTION

The etiology of the Pine wilt disease has not been well understood although the pine wood nematode *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle (**PWN**) was confirmed to be its causative agent [1]. It is suspected that other microbes might be involved in the pathological processes [2]. Evidence has been reported that the bacteria accompanying PWN participate in the pathogenesis and cause death of the diseased pine tree [3]. Using an electron microscope, Kusunoki found quantities of bacteria in tissues (damaged resin ducts and between parenchyma cells) infected with the pine wood nematode [4]. Zhao found there were many bacteria attached to the body surface of the PWN [5]. It is known that independent nematode isolates have varying degrees of virulence. We are interested in understanding the role of bacteria in the virulence mechanism. The main goal of this study was to identify the bacteria associated with three different **virulent** isolates of PWN (HF, 8A e 20) and with one Japanese **avirulent** strain, C14-5.

MATERIALS AND METHODS

- Nematode isolates (HF, 8A, 20 and C14-5) were grown on PDA (potato dextrose agar) with sterilized barley seeds and *Botrytis cinerea*, at 26ºC, in the dark;
- The nematodes were then extracted with a Baermann funnel;
- The suspension liquid obtained, containing the PWN, was centrifuged at 17 g for 6 min. The supernatant was discarded and 3 % H_2O_2 of an equal volume was added to disinfect for 5 min to partially remove miscellaneous microorganisms. Sterile water was added, centrifuged and washed three times [6];
- A single nematode was sought out under aseptic conditions with a thin metal needle, put on a plate with Nutrient Agar (NA) media and incubated at 26ºC for 24h;
- Finally, the bacterial colonies obtained were identified, trough preliminary tests: Gram staining, Cytochrome C oxidase test, Catalase test and growing at 42ºC. We also performed biochemical tests, namely API20E.
- Nematode suspensions were observed under scanning electronic microscope (SEM) (Jeol, Oxford)

RESULTS AND DISCUSSION

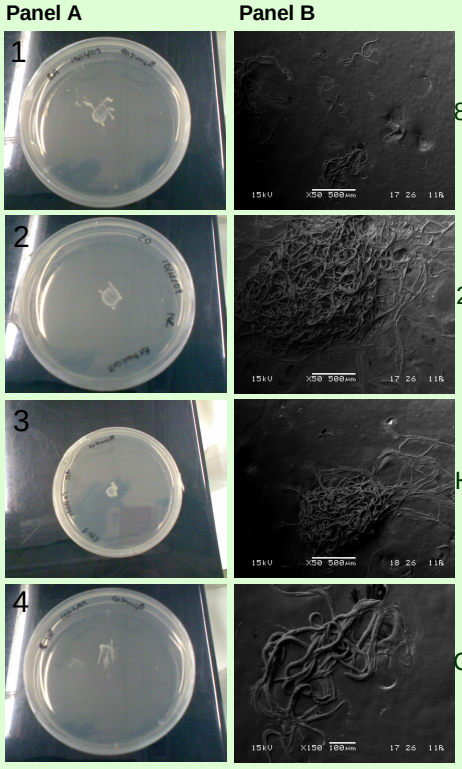

Figure 1: **Panel A:** Growing pattern of the bacteria associated with the different nematode isolates after 24h in Nutrient Agar (NA) media. **Panel B:** The nematodes under Scanning Electron Microscopy. 1) Virulent strain 8A; 2) Virulent strain 20; 3) Virulent strain HF; 4) Avirulent strain C14-5

Table 1: Results obtained in the identification tests of the bacteria associated with the different nematode strains

Nematode strain	Colony appearance	Morphology	Gram staining	Catalase test	Cytochrome C oxidase	Growing at 42ºC
HF	Transparent, bright, small	Small rod-shaped	-	-	+	+
20	Pale brown color, with prominence, bright, mat, big	Rod-shaped	-	-	+	-
8A	Transparent, bright, small	Cocci	+	-	+	+
C14-5	Pale brown color, with prominence, bright, mat, big	Small rod-shaped	-	+	+	+

HF: possibly *Pseudomonas* supported by the preliminary tests; though API20E did not support the suspect and it was not possible to identify other possible bacterial strain.

C14-5: all results (including the API20E system) point to a bacteria belonging to the *Pseudomonas* genus, more specifically to the *Pseudomonas fluorescens/putida* specie.

20: possibly *Pseudomonas* supported by the preliminary tests and API20E whose analysis of the Analytical Profile Index, provided in the kit, supports this hypothesis, with the exception of Trisodium citrate (CIT) and Gelatin (GEL) tests that should have been positive.

8A: impossibility to perform the API20E system that only applies to Gram negative bacteria. It will be therefore necessary to perform other tests to check the identity of the bacterial specie (possible belonging to *Staphylococcus* genus or *Peptostreptococcus asaccharolyticus* specie due to the results of previous studies [7]).

All tests should be repeated to confirm the results, namely the catalase test, which proved inconclusive, and so, not considered for the determination of strains. Other complementary tests will also be made, such as the test of starch hydrolysis, staining of flagella, testing of L-tryptophan and sodium pyruvate.

FUTURE WORK

- ❑ Future experiments will be carried out in *Pinus pinaster* and *Pinus pinea* to study the effect of the bacterial population in the pathogenicity of *B. xylophilus.*
- ❑SEM and light microscopy protocols will be optimized for bacterial visualization
- ❑ DGGE technique will also be applied to the bacterial DNA in order to help on the identififfivation the different bacteria associated to PWN.

References
[1] Mamiya & Enda (1972)J Nematol ***18:159-162***;
[2] Oku *et al.* (1980) *J For Res* ***67:198-199***;
[3] Xie & Zhao (2008) *J Phytopathology* ***156:*** *385-389*;
[4]Kusunoki (1987) Ann Phytopathol Soc Jpn 5:622–629;
{5] Zhao *et al.* (2000) J Nanjing For Univ **24:**72–74;
[6] Han *et al.* (2003) *J Phytopathology* ***151:683–689;***
[7] Zhao & Lin (2005) For Path **35:**339-345.