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SHORT COMMUNICATION

Pine wilt disease: detection of the pinewood nematode (*Bursaphelenchus xylophilus*) as a tool for a pine breeding programme

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Summary

The pinewood nematode (PWN), *Bursaphelenchus xylophilus*, is a serious quarantine pest first detected in Portugal and Europe in 1999. It is the causal agent of pine wilt disease (PWD). A resistance breeding programme has been initiated to contribute to control the evolution of the disease. Five hundred and four adult maritime pine, *Pinus pinaster*, trees were phenotypically selected as candidate trees for this programme from an area affected by PWD. To identify tolerance to the nematode, the selected trees were monitored monthly. Over the course of 1 year, 57 candidate trees died and were tested for the presence/absence of the PWN. As accuracy of detection is of major importance, an ITS-PCR-based method applied directly to wood from adult maritime pine trees was tested and compared with a standard morphological identification method. The results showed that the use of PCR to detect the pathogen provided more rapid and accurate results in comparison with the standard morphological identification. Thus, this method is suitable to be used in the survey of the breeding population for resistance/tolerance to PWD.

1 Introduction

Pine wilt disease (PWD), caused by the pinewood nematode (PWN) *Bursaphelenchus xylophilus*, is one of the most serious biological invasions and damaging diseases that has affected conifer forests worldwide. PWN was introduced in Portugal in 1999 (Mota et al. 1999), has recently spread to Madeira island (Fonseca et al. 2012) and Spain (Robertson et al. 2011) and is listed as a quarantine pest in Europe (Vincent et al. 2008).

Among the different strategies to face this problem is the establishment of a breeding programme for resistance and tolerance of damage to PWD. According to Sniezko (2006), a key factor in determining the fate of the affected species is the frequency and types of genetic resistance naturally present in the host. This author suggests that, even in susceptible host species, there are always rare resistant individuals. Such individuals can form the basis of a resistance breeding programme to develop populations of genetically diverse and resistant trees. Selection is one of the first steps in tree breeding programmes (Zobel and Talbert 1984) and should therefore be implemented in extensively damaged forest areas. In Portugal, a mass selection programme for *Pinus pinaster* has already been initiated. Thus far, 504 apparently healthy trees have been selected as candidate trees in an area with one of the highest incidences of the disease in Portugal.

Monitoring candidate trees as well as rapid and accurate identification of the presence of *B. xylophilus* can provide information about their degree of tolerance and resistance and can be a powerful tool in the selection of trees for the breeding programme. In fact, there are no specific indicators for PWD because other factors, biotic or abiotic, can produce similar symptoms leading to the death of the tree (Sousa et al. 2011). Takeuchi and Futai (2007) refer that both Japanese black and red pine natural stands potentially have asymptomatic trees which strongly suggests that both visual symptoms and resin exudation are not sufficient to detect the PWN. The improvement of detection methods would support the identification of tolerant, symptomatic or even asymptomatic trees (Futai 2003; Takeuchi and Futai 2007; Futai and Takeuchi 2008).

In recent years, a number of DNA-based detection methods emerged and are currently being used to detect the PWN in different pine species, including in maritime pine (Cardoso et al. 2012). Compared with the traditional morphological identification methods, in which nematodes can be previously extracted using the 'tray method' (Whitehead and Hemming 1965), these molecular techniques are more selective and accurate (Cao et al. 2005; Takeuchi et al. 2005; Takeuchi and Futai 2007, 2009; Hu et al. 2010; Wang et al. 2010) and are also able to discriminate between similar pathogen species (Burgermeister et al. 2005; François et al. 2007; Leal et al. 2007; Han et al. 2008; Zhuo et al. 2010), specifically between *B. xylophilus* and the closely related non-pathogenic species *Bursaphelenchus mucronatus* (Akbulut et al. 2008; Li 2008; Kikuchi et al. 2009; Hu et al. 2010). They are also sensitive enough to detect a single nematode in wood tissue, even if the pathogen is already dead, or if it only has eggs or juveniles (Takeuchi et al. 2005). The analysis of specific regions of DNA, such as the ribosomal DNA (rDNA) repeating unit, including the internal transcribed spacers (ITS1 and ITS2), have often been applied for the identification of *Bursaphelenchus* species (Burgermeister et al. 2009).

In this study, we tested the accuracy of an ITS-PCR-based method to detect PWN directly in wood from adult maritime pine trees, by comparing it with the standard morphological identification approach. The implementation of this ITS-PCR-based method would be helpful for the survey of the maritime pine breeding population for PWD resistance.

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2 Materials and methods

From March 2009 to October 2009, 504 candidate *P. pinaster* trees were selected from 'Herdade da Comporta', Alcácer do Sal (38°21′28.52″N; 8°45′49.89″W) and monitored monthly. This area has one of the highest incidences of the disease in Portugal. The criteria for the selection of these candidate trees were dominance (dominant trees), age (adult trees), diameter at breast height (DBH) (higher than 20–25 cm) and absence of external symptoms.

As the trees died, they were felled and sampled. A total of 57 of the candidate trees died during the first monitoring year. Five discs, about eight centimetres thick, were collected per tree at different equidistant stem heights (with the DBH, the base of the crown and the top end of the crown as reference points) (Fig. 1) and incubated at 25°C for 2 weeks, to promote nematode reproduction. For each disc, approximately 60 g of wood shavings were collected from multiple evenly distributed points using an electric drill with a 1.1-cm-diameter bit 14 cm in length. All wood samples were homogenized and divided into two equal parts, one to be used for morphological identification (nematode extraction using the Whitehead and Hemming tray method followed by morphological identification of the nematode) and the other stored at −80°C for the ITS-PCR method.

Based on results obtained using the morphological identification method, 100 mg wood shavings from 51 wood samples, of the total previously stored at −80°C, were evaluated with ITS-PCR method. Total genomic DNA from all samples was isolated using the DNeasy Plant Mini kit (Qiagen, GmbH) following the manufacturer's instructions. Due to the existence of contaminants in DNA extracts of wood that may inhibit PCR, DNA samples were further purified with the Quantum Prep PCR Kleen Spin Columns (Bio-Rad, Hercules, CA, USA).

Total genomic DNA was amplified by PCR using the species-specific primers (from the ITS region) to the PWN: forward primer ITS1 (5′-TACGTGCTGTTGTTGAGTTGG-3′) and reverse primer ITS2 (5′- GCACGGACAAACAGTGCGTAG-3′) (Takeuchi et al. 2005).

All PCRs were carried out in a Biometra TGradient thermocycler in a final reaction volume of 25 μl. The reaction mixture contained 1 μl of template DNA, 0.4 μM of each primer and 1× PCR Master Mix (Fermentas, Germany), which included 0.025 units of Taq DNA Polymerase, 2 mM $MgCl_2$ and 0.2 mM of each dNTP. The thermal cycling programme was as follows: denaturation at 94°C for 1 min, followed by 1 min at 94°C, 1 min at 51°C, 2 min. at 72°C for 35 cycles and a final extension of 72°C for 5 min. After amplification, 10 μl of the amplified product was loaded onto a 2% agarose gel containing 0.5 μg/ml ethidium bromide and 0.5× TBE running buffer and electrophoresed at 5 V/cm. Data analysis was visualized using the VersaDoc Gel Imaging System (Bio-Rad). Amplification products from BxPt46GO *B. xylophilus* isolate (Laboratory of Nematology, ICAAM, Universidade de Évora) and BmPt0 *B. mucronatus* isolate (Laboratory of Nematology, ICAAM, Universidade de Évora) DNA extracts were used as positive and negative controls, respectively. DNA extracts of nematode-free wood tissues taken from 3-year-old *P. pinaster* seedlings were used as an additional control.

To statistically compare the results (presence/absence of *B. xylophilus*) assessed by the morphological identification and ITS-PCR methods, the McNemar's test (nonparametric statistical test) was used (Siegel 1975).

3 Results and Discussion

The results for morphological identification allowed us to detect 47 trees infected by *B. xylophilus* from a total of 57 dead ones (82.5%). Ten trees remained as negative or not conclusive, as indicated in Table 1.

Table 1 also presents the comparison between morphological identification and ITS-PCR methods. This table shows that there were 29 discordant pairs (56.9%). According to McNemar's test, chi-squared test equalled 27.03 with 1 degree of freedom. The two-tailed p value is <0.0001, which means that the two methods used to identify *B. xylophilus* display a highly significant difference. All morphologically analysed positive cases were confirmed by ITS-PCR. Figure 2 shows PCR amplification of the ITS region of rDNA yielded only one fragment with the length of 470 bp. All these results demonstrate the increased accuracy of the molecular method and its suitability to be used to test candidate trees. In fact, several authors consider that molecular detection can provide more accurate results (Cao et al. 2005; Takeuchi et al. 2005; Takeuchi and Futai 2007, 2009; Hu et al. 2010; Wang et al. 2010).

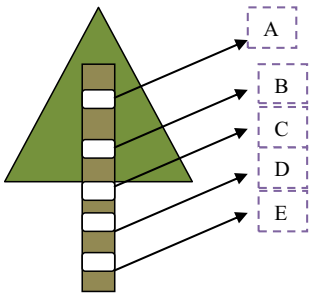

Fig. 1. Wood disc sampling, at different stem heights in adult *Pinus pinaster* trees, to test the presence/absence of *Bursaphelenchus xylophilus*; A, top end of the crown; C, base of the crown; E, diameter at breast height (DBH).

Table 1. Tree infection status according to the morphological analysis, and comparison between morphological identification and ITS-PCR method for the detection of *Bursaphelenchus xylophilus* in discs collected from dead *Pinus pinaster* adult selected trees at 'Herdade da Comporta', Alcácer do Sal.

			Pinewood nematode detection methods	
Tree infection status	Tree	Wood discs	Morphological identification	ITS-PCR
Infected	122	E	0	1
	299	A	0	1
		B	1	1
		C	0	1
		D	1	1
		E	1	1
	304	A	1	1
		B	1	1
		C	Nc	1
	308	A	1	1
		B	1	1
		C	0	1
	452	A	Nc	1
Not infected/not conclusive	20	A	0	0
		B	0	0
		C	0	0
	54	A	0	0
		B	0	0
		C	0	0
		D	0	0
		E	0	0
	195	A	0	0
		B	0	0
		C	0	0
		D	0	0
		E	0	0
	243	A	0	1
		B	0	1
		C	0	1
	244	A	Nc	1
		B	Nc	1
		C	0	1
	352	A	Nc	1
		B	0	1
		C	0	1
		D	0	1
		E	Nc	1
	362	C	Nc	1
		E	Nc	1
	401	A	0	1
		B	0	1
		C	0	0
		D	0	0
		E	0	1
	427	C	0	1
		E	0	1
	523	A	0	1
		B	0	1
		C	0	1
		D	0	1
		E	0	1

0, *Bursaphelenchus xylophilus* not detected; 1, *B. xylophilus* detected; Nc, not conclusive as nematode life stages required for the morphological identification were not found.

As the detection of the PWN in one sample within a dead tree was enough to consider the tree as infected, ITS-PCR method results allowed the screening of the remaining 10 trees from which PWN had not been detected (negative or not conclusive) using the morphological identification method. In fact, seven from those 10 trees were identified as positive for *B. xylophilus*. Trees number 243, 401, 427 and 523, initially considered to be negative, were demonstrated to be positive for PWN using ITS-PCR. The other three trees, number 244 (discs A and B), 352 (discs A and E) and 362 (discs C and E) initially considered not conclusive (nematodes were extracted but the life stages required for morphological identification were not found), were demonstrated to have PWN using the molecular technique (Table 1, Fig. 2). The assemblage of the

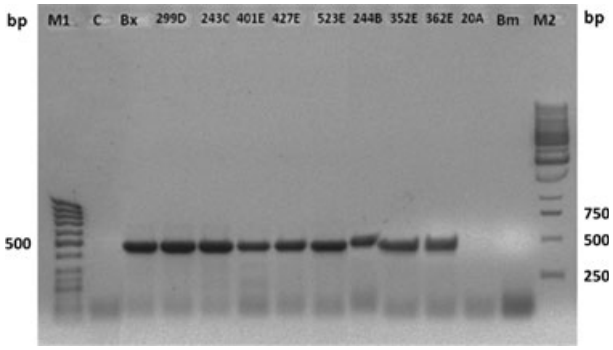

Fig. 2. Efficacy of the detection of *Bursaphelenchus xylophilus* by ITS-PCR method in wood samples, taken from adult *Pinus pinaster* trees, previously assessed as positive, negative and not conclusive using morphological identification method. M1: DNA marker (GeneRuler 100-bp DNA Ladder Fermentas); C: PCR control; Bx: *B. xylophilus* control; sample 299D: *B. xylophilus* previously detected; samples 244B, 352E, 362E: not conclusive as nematode life stages required for the morphological identification were not found; samples 243C, 401E, 427E, 523E, 20A: *B. xylophilus* not previously detected; Bm: *Bursaphelenchus mucronatus* control; M2: DNA marker (GeneRuler 1-kb DNA Ladder Fermentas).

two approaches used, morphological and molecular, helped us to strengthen the screening of PWN dead trees. Overall, 54 of the dead trees (94.7%) were identified as positive for *B. xylophilus*. However, the efficacy of the detection of PWN differed between the two methods. We realize that the molecular method is more suitable to be used as a detection technique directly from adult maritime pine wood tissue. In accordance with Hu et al. (2010), with a single set of species-specific primers and only one-step PCR, it represents a gain in time and cost and allows *B. xylophilus* early accurate detection in candidate trees.

The usage of five discs cut from different trunk heights increased the PWN detection efficiency in each dead tree. As seen in Table 1, the ITS-PCR method provided a more accurate PWN detection in the lowest part of tree (Disc E), the easiest to be sampled in the field without felling the tree. As Schröder et al. (2009) refers, the quality of survey/monitoring, as well as the results of diagnosis, is strongly dependent on the sampling procedure. According to these authors, sampling should be done at multiple heights along the stem to maximize the probability of detecting the PWN. Zhao et al. (2009) indicated that the PWN is difficult to detect using traditional extraction methods followed by morphological identification in wood discs taken from breast height.

Selecting apparently healthy trees from PWD-infested stands keeping them under observation, cloning, testing them and analysing progenies for tolerance and resistance to this disease constitute our methodology to obtain the core of the breeding activity, which is the breeding population. As our selection was carried out in highly infested stands, trees that become symptomatic and finally die must be analysed for the presence of PWN. The positive results represent a valuable source of genetic diversity for breeding purposes related to nematode infection.

More detailed studies on the possibility of early detection of infected candidate trees and detection of asymptomatic trees using the ITS-PCR method should be investigated. These trees may be a gateway to find tolerance/resistance for breeding purposes.

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