RESEARCH ON THE CONTROL OF PINE WILT DISEASE, CAUSED BY THE PINE WOOD NEMATODE *BURSAPHELENCHUS XYLOPHILUS*, IN PORTUGAL

Estudio sobre el control de la enfermedad del marchitamiento del pino causada por el nemátodo de madera de pino *Bursaphelenchus xylophilus*, en Portugal

Edmundo Manuel Rodrigues de Sousa, Luis Bonifácio & Pedro Naves

Instituto Nacional dos Recursos Biológicos. Unidade de Silvicultura e Produtos Florestais. Quinta do Marquês. 2780 - 159-OEIRAS (Portugal). E-mail: edmundo.sousa@inrb.pt

Abstract

Since the detection in 1999 in Portugal of the Pine Wilt Disease caused by *Bursaphelenchus xylophilus* (Pine Wood Nematode, PWN), various research lines improved the strategies for its control. The current control strategies focused on: (i) identification of affected areas, (ii) prevention of the spread of the disease to new locations, (iii) reducing the incidence in affected areas. The identification of affected areas is based on regular survey of pines during autumn and winter and sampling the wood for nematode detection. The spread of the disease occurs through the flight of the insect vector *Monochamus galloprovincialis*, and transportation of infected wood with both the PWN or vector. The spread of the disease can be prevented by insecticide microinjection of healthy trees and the use of a net with insecticide to cover the timber during transportation. The reduction of disease incidence in the affected areas is achieved by removal of symptomatic and dead trees from November to April, when the vector is inside the wood. To control flying vector adults, traps provided with pheromones and cairomones are installed from May to October. The joint strategy of cultural and biotechnical measures achieves a decrease of the mortality caused by PWN within a few years. Additionally, control measures must be applied to bark beetles, currently causing high mortality in the affected areas.

Key-words: *Monochamus galloprovincialis, Pinus pinaster, Pine sawyer, Traps*

Resumen

Desde la detección en 1999 en Portugal de la enfermedad del marchitamiento de pino causada por *Bursaphelenchus xylophilus* (Pine Wood Nematode, PWN), se han abierto varias líneas de investigación encaminadas a mejorar su control. Las estrategias de control han tenido como objetivos: (i) identificar las áreas afectadas, (ii) prevenir la expansión de la enfermedad a nuevas masas, (iii) reducir la incidencia en las áreas afectadas. La identificación de áreas afectadas está basada en el muestreo frecuente de pinos durante otoño e invierno y el examen de la madera para la detección propia del nematodo. La dispersión de la enfermedad se produce mediante el vuelo del insecto vector *Monochamus galloprovincialis*, y mediante el transporte de madera conteniendo PWN y vector conjuntamente. Esta dispersión se puede prevenir mediante la microinyección de insecticida en árboles

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sanos y el empleo de una malla con insecticida que cubra la madera durante el transporte. La reducción de la incidencia de enfermedad se logra retirando árboles sintomáticos y muertos desde noviembre a abril, coincidiendo cuando el vector está dentro de la madera. Para controlar el vuelo del vector, se colocarán de mayo a octubre trampas con feromonas y cairomonas. La estrategia conjunta de medidas culturales y biotécnicas permite reducir, en pocos años, la mortalidad causada por PWN. Además, se han de aplicar medidas adicionales de control ante escolítidos, responsables de una elevad mortalidad de árboles en áreas afectadas por PWN.

Palabras clave: *Monochamus galloprovincialis, Pinus pinaster, Cerambícido, Trampas*

INTRODUCTION

The Pine Wood Nematode (PWN) *Bursaphelenchus xylophilus* (Steiner et Buhrer, 1934) Nickle 1970 (Nematoda: Aphelenchoididae) is endemic to North America, being the causal agent Pine Wilt Disease (PWD), which affects conifers worldwide (MAMIYA & KIYOHARA, 1972). The nematode was introduced in Japan where it caused an abnormal mortality on endemic pine species (MAMIYA, 1984). Through the international trade of infected wood it was afterwards introduced in China, Korea and Taiwan, (DWINELL, 1997), and it was detected for the first time in Europe at Marateca, Setúbal, Portugal, in 1999 (MOTA et *al.*, 1999). Molecular studies (RFLP) carried out on Portuguese populations of the PWN showed genetic similarities with the Asian Far East populations (METGE & BURGERMEISTER, 2005). Meanwhile nematode-infected trees were reported from Spain, in Extremadura (late 2008) and Galicia (late 2010) (ABELLEIRA et *al.*, 2011).

Nowadays, PWD is considered as a complex interaction among the PWN, an insect vector and a tree host. Worldwide, the PWN is the only common element of this tri-parted interaction, while the *Monochamus* vectors and the *Pinus* hosts may vary from one location to another.

PINE WOOD NEMATODE IN PORTUGAL

After the detection of *B. xylophilus* in Portugal, an intensive survey to detect the local insect vectors was performed. The PWN was only found associated with the native pine sawyer *Monochamus galloprovincialis* Olivier. (SOUSA et *al.*, 2001), being its host the maritime pine (*P. pinaster*) (SOUSA et *al.*, 2002).

In Portugal, *M. galloprovincialis* beetles were considered secondary insects from a sanitary point of view, as they did not caused damages to the tree while feeding and do not breed on healthy trees, being attracted to stressed, dying or recently killed trees only (CABRAL, 1995; SOUSA et *al.*, 2001). PWN introduction disrupted the equilibrium between the native *M. galloprovincialis* and its host, and the pine sawyer populations profited from the availability of high number of dying trees to become a primary agent of mortality of adult healthy pine trees (although indirect), with significant economic and ecological impacts (Figure 1).

The transmission of PWN to a healthy host tree occurs by the feeding activity of the insect vector (NAVES et *al.*, 2007a). Eggs are laid in the bark scars of the tree hosts, where the females chew on the bark (EVANS et *al.*, 1996) and during this process secondary transmission of PWN can occur, however in a lesser extent than through the feeding activity (NAVES et *al.*, 2007b).

Nematode and vector coexist in the tree hosts for several months without interaction until late spring, when nematodes aggregate around the insect pupal chambers. As soon as the callow adult is formed, the nematodes transfer to the insect's tracheas or, in lower quantities, to under the elytra, the antennae and the legs of insects (NAVES et *al.*, 2006). Infection rate of *Monochamus* is extremely variable and most of the beetles were found to carry less than 5,000 nematodes when they emerge (SOUSA et *al.*, 2002).

Field trials in Southern Portugal showed that the induction of resin production is an effective qualitative method for early detection of *B. xylophilus* infection. Infected pines stopped producing resin 1.5 months before significant

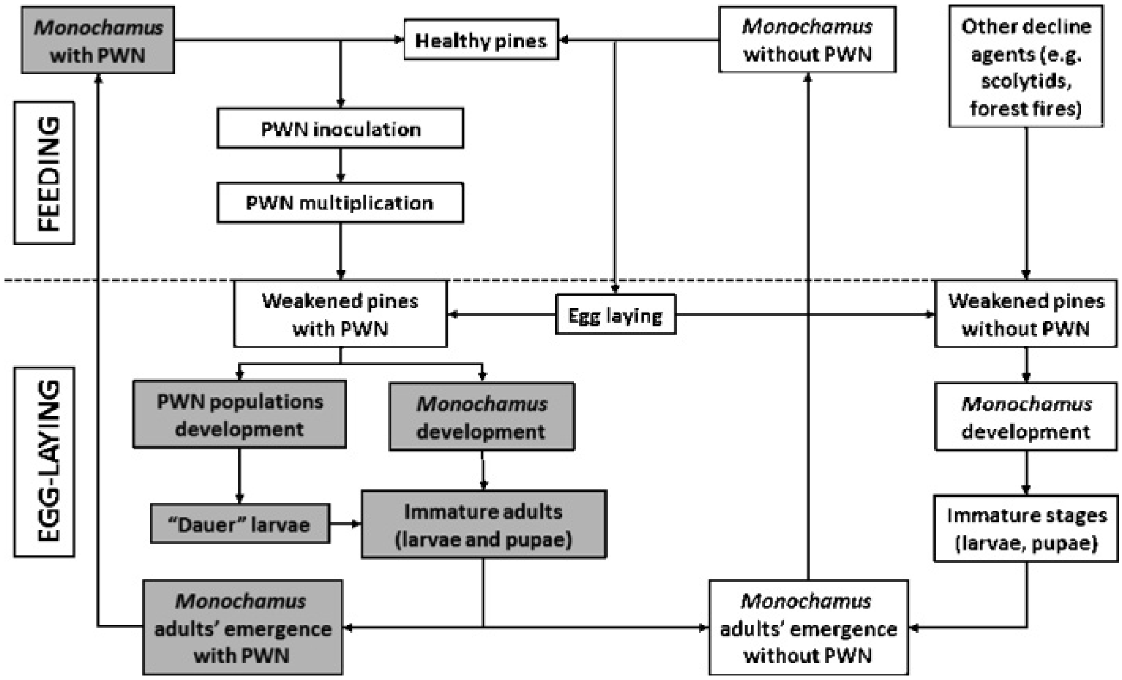

Figure 1. *Interactions established among Monochamus beetles, the host-trees and Bursaphelenchus xylophilus*

yellowing of the needles was visible (>25% crown), usually after July (BONIFÁCIO & SOUSA, 2012) and tree death, due to lack of water, occurs in 2 to 3 months (KURODA, 1991; EVANS, 1992).

The discoloration of the crown is not specific for infection with PWN, since the attack by other biotic agents, such as bark beetles, cause identical symptoms in the trees. The way to confirm the infection by the nematode is either by laboratory analysis of the wood and morphological identification (PENAS et *al*., 2002) or molecular techniques (HU et *al*., 2011).

MANAGEMENT AND CONTROL OF PINE WILT DISEASE

PWD can be controlled aiming either the nematode or the insect vector. However, the nematode is difficult to control because it has a short life cycle, high fecundity and fertility under favourable conditions, alternating phytophagous and micophagous phases and when conditions deteriorate shifts to resistant larval stages. So, the best control strategy against the PWD, has to focus in preventing the spread and dissemination of the nematode by the flight of infected insect vectors. The accumulation of knowledge about the disease during the last 11 years evolved the strategies to manage and control the PWD and allowed the detection of new wilt foci.

Immediately after the detection of the PWN in 1999 and accordingly to Council Directive 2000/29/EC, the Portuguese authorities implemented a phytosanitary strategy to control and eradicate the disease under a legal framework denominated National Eradication Programme for Pinewood Nematode (PROLUNP), based on three main interconnected actions to prevent further dissemination, control of the insect vector and disrupt the interaction between the three organisms:

1) identification and delimitation of nematode-affected areas;
2) prevention of wilt dispersal to new locations;
3) reduction of wilt incidence in affected areas.

Identification of Nematode-Affected Areas

Since the beginning, the identification and delimitation of forests affected by the PWD was essential. To prevent further dissemination of the disease and to control the insect vector an annual countrywide survey of PWN was implemented, mainly in risk areas, such as areas of high concentration of wood parks (surrounding of entry, wood yards and transformation facilities), pine stands with symptomatic trees or borders of forest fires (DGRF, 2003).

PWN survey is based on field sampling during autumn and winter (when pine wilting symptoms are well visible) through wood samples collection from the trees (about 100 g) for laboratory extraction and identification of the nematode (PENAS et *al.*, 2002).

This procedure allowed to establish a Demarcated Area at Setúbal Península, Southern Portugal and, until 2008, the rest of the country was considered a PWN Free Zone (RODRIGUES, 2008). The Demarcated Area included an Affected Zone surrounded by a 20 km radius nematode free Buffer Zone (to limit natural spread of the insect vector) (Figure 2).

Since the first survey in 2000, the Demarcated Area increased from 309,000 ha to over one million ha in 2007. The Affected Zone increased from 48,000 ha to 80,000 ha, and the number of felled pine trees with wilt symptoms (not all infected by PWN) increased from 53,487 to over 200,000 (Table 1).

The expansion of the Demarcated Area forced the Portuguese and European authorities to shift the strategy in 2007, establishing a Clear Cut Belt where nearly five million healthy conifer trees were cut, creating a 3 km-wide corridor free of the vector´s host trees (*Picea orientalis*, *Pinus halepensis*, *Pinus nigra*, *Pinus nigra laricio*, *Pinus pinaster*, *Pinus radiata* and *Pinus sylvestris*) in the periphery of the original Demarcated Area of Setúbal Peninsula (Figure 2).

However, this control strategy in preventing natural dissemination of PWD by the flight of infected insects to new areas was only successful until late 2007, when new foci appeared more than 200 km north of Setúbal Peninsula. They were clearly linked to illegal or accidental transport of wood infected with both the nema-

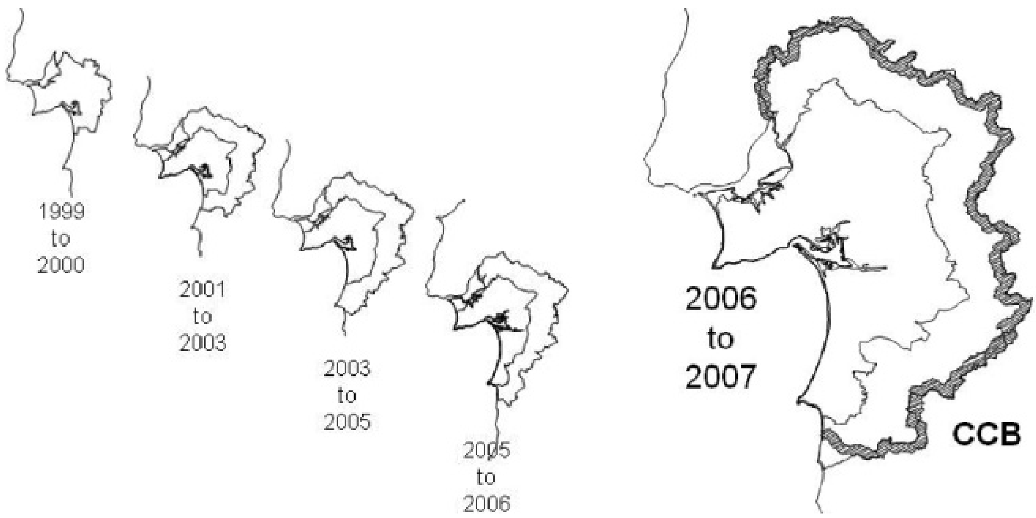

Figure 2*. Evolution of demarcated area in Setúbal peninsula with Affected Zone (inner area) and Buffer Zone (outer area). Clear Cut Belt (CCB) implemented in the limit of Buffer Zone in 2007*

	2000	2001	2002	2003	2004	2005	2006	2007
Total area	309,000	564,000	564,000	564,000	617,000	617,000	641,000	1.010,000
Total area of pine stands	48,000	60,000	60,000	60,000	69,000	69,000	69,500	80,000
Area of stands with symptoms	12,392	16,793	11,623	14,076	16,391	19,519	30,247	28,667
Nº felled pines Affected zone Buffer zone	 53,487 *(1)*	 54,314 4,720	 43,014 7,528	 53,338 8,120	 74,889 10,197	 93,308 9,509	 268,211 20,774	 196,530 17,770

Table 1*. Evolution of area (ha) and number of felled pines during eradication of symptomatic pines against pine wilt disease, in Portugal (DGRF, 2007). (1) – In accordance with European Commission Decision nº 2000/58/EC*

tode or its vector. The intervention that took place in all affected areas, including the new foci, resulted in the felling during 2008 of 279,717 symptomatic pines.

Then a more intensive survey was implemented, based on the National Forest Inventory (NFI) in a 2x2 Km grid (AFN, 2010a). All the symptomatic conifer trees located in the grid points were screened for the PWN presence according to EU established methodology. The initial grid was moved annually, according to one of the four cardinal points (North, South, East and West), so that after 5 years the midpoint and the cardinal points will be prospected (10,850 observations overall), maximizing the detection of the PWN.

As not all points of the grid can be simultaneously sampled, priority is given to forests with higher risk of suffering wilt disease, namely points located at:

- Proximity to PWN-affected forests;
- Proximity to pine forests recently affected by forests fires (which usually have a high abundance of insect vectors);
- Other pine forests with wilt symptoms (in this case is needed field validation of satellite images interpretation).

This survey allowed the delimitation of a new restriction zone and lead to the classification of entire Continental Portugal as an Affected Area, with a 20 km's Buffer Zone along the Portuguese-Spanish border. In spite of this classification there are still large maritime pine forests without *B. xylophilus*. PWN distribution is still concentrated in Portugal Central Region with a South to Southeast extension to original Setubal Demarcated Area, and no additional PWN positive cases were detected in the North region during 2009 and 2010 campaigns. During these interventions the overall number of pine with decline symptoms that were cut increased significantly in 2009 to 935,520 trees and in the following year of 2010 aconsiderable decrease occurred being necessary to cut 603,877 symptomatic trees (not all infected by PWN).

Since the new wilt foci have a scattered distribution, the Intervention Zones (IZ) were created in geographic areas at the parish level (Figure 3). In late 2009, it was also detected in the Portuguese Madeira Island.

Prevention of Wilt Dispersal to New Locations

After 2008, the prevention of PWD dispersal to new locations was focused on the two most important dispersal pathways: the flight of infected insects and the human transport of wood infected with both the PWN or the vector.

Dispersal by the Insect Vector

To avoid the dispersal of infected *M. galloprovincialis* adults out of the affected areas it's necessary to develop strategies based on its flight behaviour and creation of effective barriers. These barriers can be implemented with traps, by the removal of suitable host trees or with preventive chemical treatments.

It's still unknown the maximum flight distance for an infected *M. galloprovincialis* adult and field assays carried out in Portugal suggest the occurrence of an obligatory migratory flight in search of new habitats/hosts just after emergence, possibly genetic driven, similarly to other insect species (e.g., SHIVANKAR et *al.*, 2006), but unknown for this genus.

Mature *M. galloprovincialis* (9-12 days) make short-distance flights for feeding, covering few hundred meters if adequate hosts are available. The average daily flight distances were 4.97 m and 4.30 m for males and females (BONIFÁCIO, 2009), half the distance reported for *Monochamus alternatus* Hope in Japan (10.6 m and 12.3 m, respectively) (TOGASHI, 1990), but both species have identical 14 m maximum flights. The length of the flight depends on the wind direction (*downwind*), and increases with the insect's weight, due to higher physiological capacities. In open pine stands the insects tends to fly more, preferably landing in a pine with large crown (older trees), while in dense pine stands with uniform trees the insects fly less or remains a longer time on the same tree (BONIFÁCIO, 2009).

The efficacy of emamectin benzoate for preventing the spread of wilt disease to new areas was recently tested in Portugal, after being successfully employed in Japan (KAZUYA et *al.*, 1999; TAKAI et *al.*, 2003) and the United States (JAMES et *al.*, 2006). Trunk-injection trials were made in Portugal in 2009 and within 2 years all treated trees remained healthy, while in the control plot 28% of the pines died. Laboratory studies found that the tested dose rate decreased both the longevity and feeding activity of *M.*

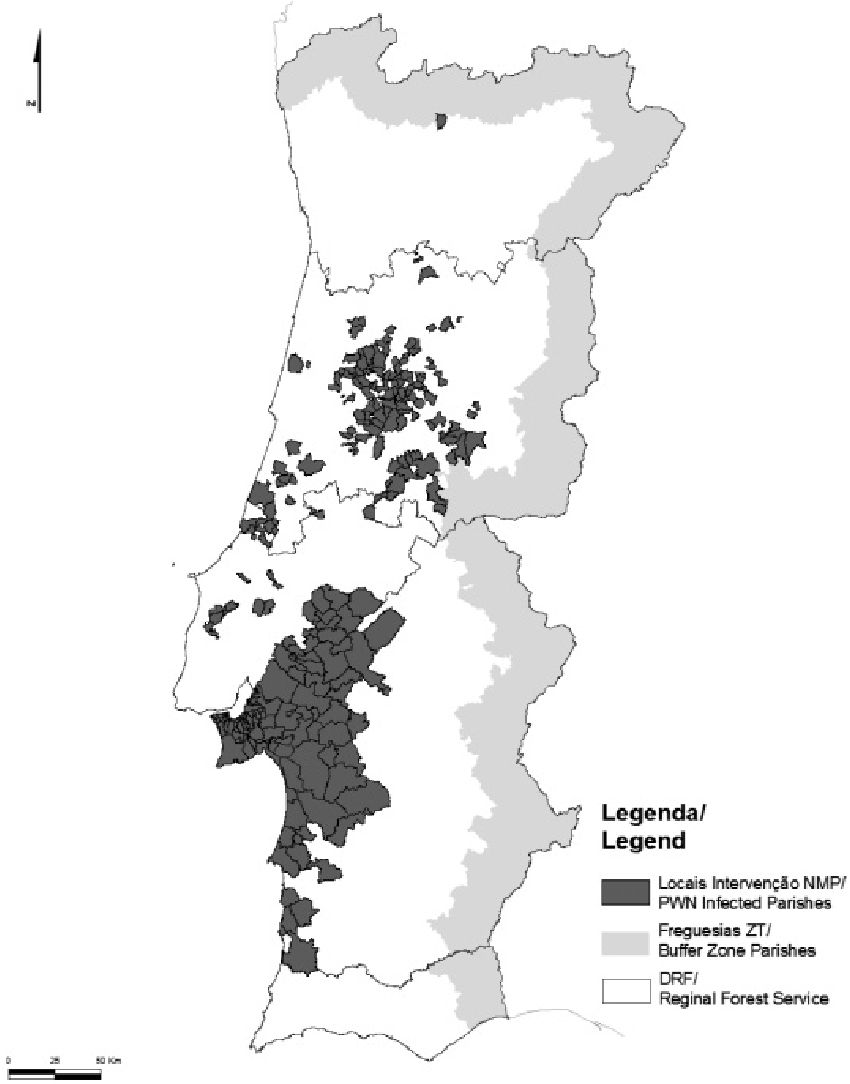

***Figure 3**. Distribution of parishes with Pine Wood Nematode positive detection in 2010*

galloprovincialis exposed to branches collected from treated trees. Overall, the experiments found a preventive effect of emamectin benzoate trunk-injection in adult maritime pines, and the dose rate of 0.064 g a.i.·cm^{-1} of diameter at breast height is currently recommended to protect individual trees and prevent wilt dissemination.

Dispersal by Human Transport of Wood

The human transport of wood infected with both the nematode or the vector is the most important long-distance dispersal pathway for pine wilt disease worldwide (e.g., KAWAI et *al*., 2006). The dispersal of the vector can occur while inside the wood, in immature stages, or as adult insect, on top of wood transport trucks of any other vehicle.

To avoid this, Portuguese and EU laws impose severe phytosanitary requirements to each Infected Zone (IZ), and the management, harvesting, transport and processing of timber (logs, and round wood) resulting from the cut of symptomatic trees, must follow national and international standards (A.F.N., 2010b). Transport of contaminated wood outside the IZ can only be done when the vector insect is not flying (from May to October) (NAVES et *al*., 2008) and to destinations guarantying treatment or processing of the wood before the beginning of the beetles' emergence.

During the spring and summer the transport of infected wood (with diameters above 20 cm) can only be done in closed containers or roofed trucks, after the application of an insecticide and only to destinations assuring immediate processing of the wood. High-risk materials, such as wood and branches with a diameter below 20 cm (where most of the insect population completes its larval development) (BONIFÁCIO, 2009), can only be transported in closed containers or roofed trucks from one affected region to another and only outside the flight season of the beetle, to avoid creating new wilt foci.

Wood destined for export and packaging materials such as pallets are considered high risk for disseminating the PWN, although it can be mitigated by application of International Standards for Phytosanitary Measures regulation 15 treatments (ICPM, 2002) such as heat treatment (HT) applied by Portuguese exporting companies.

Reduction of Wilt Incidence in Affected Areas

In Portugal the most important management and control strategy for PWD control consists in the elimination of symptomatic trees during late autumn, winter and early spring, while the insect vector is inside the host (as late-instar larvae or pupae) and no adults are flying (NAVES et *al.*, 2008). Trees with wilt symptoms are visually identified and marked with ink late in the year, when the vast majority of the nematode-infected pines are already dead or the crown shows conspicuous wilting symptoms. Afterwards, marked pines are felled and since the *M. galloprovincialis* larvae can be found inside very small branches and in the thinner sections of the trunk (SOUSA et *al.*, 2001), this material must be collected and destroyed or transported for processing into materials such as pellets and others. As the pine sawyer is never found in the stumps or roots of Portuguese *P. pinaster*, these parts don't require any action.

Complementary to these practices, the vector's population can also be diminished during the flight season (from late spring to early autumn) using traps baited with attractive lures. During the last decade, several studies have improved the efficiency of trapping against *M. galloprovincialis*, with the comparative testing of different traps, with the transparent cross vane and the 12-element multi-funnel traps having the highest captures (BONIFÁCIO, 2009).

The most effective chemical lures to attract the flying beetles are a combination of pine odours (α-pinene and ethanol) and bark beetles pheromones (Ipsdienol, Ipsenol and Methylbutenol) (PAJARES et *al.*, 2004; BONIFÁCIO et *al.*, 2012). Recently, a specific *M. galloprovincialis* pheromone was described (IBEAS et *al.*, 2008), already commercially available, although its efficiency needs to be further evaluated in PWN affected areas, where high densities of *M. galloprovincialis* and dead pine hosts occur.

The use of specific and efficient natural enemies (bio-control) would also be an interesting and environmental-friendly option to control the insect vector in Portugal, but until now there are no adequate species for such control programs. Field surveys already conducted did not detect any egg parasitoid, and larval parasitism found had low mortality rates and was due to generalist parasitoids (*Coeloides sordidator* Ratzeburg, *Cyanopterus flavator* F. and *Iphiaulax impostor* Scopoli). Similar results have been described elsewhere in Europe (CAMPADELLI & DINDO, 1994), therefore bio-control strategies cannot be based on these species (NAVES et *al.*, 2005).

The systematic wood sampling of symptomatic trees in 2010 revealed that only a small part of those trees were in fact infected by the Pine Wood Nematode (9.7%). Taking advantage of the general decline of pine forests in the nematode-affected areas, bark beetle outbreaks begun to be noticed from 2006 onwards (BONIFÁCIO et *al.*, 2008), with heavy mortalities due to *Orthotomicus erosus* and *Ips Sexdentatus*, as these species have multiple annual generations and were ignored by the control measures focused on the PWN and *M. galloprovincialis*. Considering these developments, an active complementary management approach against these all insect pests is now implemented from May to October, when the bark beetles are most active and are breeding. This consists on the immediate felling of any wilted pine in the field (regardless of being or not infected with the PWN), and the placement of traps baited with specific sexual and aggregation pheromones to capture flying beetles and directly reduce the insect´s populations.

Overall, the management and control strategies against both the PWD and the associated mortality agents has already proved that can be successful when correctly implemented with efficiency and within the correct timing.

CONCLUSION

The experience obtained from more than a decade of managing and controlling the PWN in

Portugal and the scientific knowledge gathered during this period, emphasize the necessity to control the immature stages of the insect vector *M. galloprovincialis* before the emergence of the adults, and the importance of developing efficient methods of capturing/eliminating the recently-emerged beetles before they begin to disperse *B. xylophilus* into new hosts. In fact, the insect's 3-months emergence period, the 2 months mean adult life span and the long nematode transmission period explains why a large number of trees are infected with *B. xylophilus* each year and how wilt disease is an important mortality factor for susceptible pines.

On the other hand, control options are limited, expensive and must be correctly executed to be effective. There is still margin for development and improvement on some techniques, such as increase in the efficiency of traps and lures to capture *M. galloprovincialis* adults, and the development and manipulation of nematode resistant (or less susceptible) varieties or clones of maritime pine.

Overall, and regardless of the joint efforts of various public and private entities, more than 10 years after its detection, the PWN has disseminated to central and northern Portugal, Madeira Island and neighboring Spain. These isolated and scattered foci were caused by human activity, and emphasize the importance of illegal or accidental transport of wood infected with both the nematode or its vector on the long distance dissemination of the disease.

The treatment of wood and wood-based products carried out by industrial units is continuously followed and controlled by trained phytosanitary inspectors of the Ministry of Agriculture, Rural Development and Fisheries.

Despite all the problems, our experience proved that when management and control measures are applied properly and in time, it is possible to maintain a healthy pine forest despite the presence of *B. xylophilus*, and eventually someday this pathogenic nematode will be just one of many organisms associated with the pine forests in Portugal.

Finally, all legal documentation about Pine Wilt Disease in Portugal was gathered in the Law-Decree no. 95/2011 of 8th August.

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