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ARTICLE
SHORT COMMUNICATION

Durable resistance to pine wilt disease in *Pinus pinaster*: evidence of nematode elimination in asymptomatic genotypes

Resistencia persistente a la enfermedad del marchitamiento del pino en *Pinus pinaster*: evidencia de la eliminación de nematodos en genotipos asintomáticos

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Abstract: *Aim of study*: To determine if specific *Pinus pinaster* trees exhibit resistance or tolerance to pine wilt disease, and to clarify whether artificially inoculated nematodes survive or are eliminated in asymptomatic plants. *Area of study*: Experiments were conducted in the greenhouse facilities of the Lourizán Forest Research Center (42° 24′ 33″ N, 8° 39′ 46″ W, Pontevedra, Spain). *Material and methods*: This study involved two sets of assays. In the first set, seedlings from 32 families of the Galician *P. pinaster* breeding program were artificially inoculated with *Bursaphelenchus xylophilus* three times over a year. Only 23 plants remained asymptomatic by the assay´s end, and four of these continued to be asymptomatic fifteen months after the final inoculation. At that point, these four 'mother plants' were clonally propagated via cuttings. Both the mother plants and their cuttings were subsequently checked for nematode presence at various time points. The second set of experiments comprised three inoculation experiments, evaluating clones derived from nematode-resistant *P. pinaster* parents of families. At the conclusion of these assays (109-141 days after inoculation), each plant was assigned a symptom stage, and nematode density was assessed. From each assay, 15 asymptomatic plants were sampled for nematode counting at 8 and 18 months post-inoculation. *Main results*: In the nematode counting performed in mother plants fifteen months following the last inoculation, nematodes were extracted solely from a section below the inoculation point of a single mother plant. Years later, none of the mother plants or their cuttings contained nematodes. In the inoculations experiments with resistant clones, only 16.5% of the plants that remained asymptomatic at the end of the assays contained nematodes. *Research highlights*: The possibility of producing pine wood nematode-free resistant plants by grafting or cutting is crucial to control pine wilt disease progression.

Keywords: cutting; maritime pine; mother plants; pine wilt disease; pine wood nematode.

Resumen: *Objetivo del estudio*: concluir si determinados árboles de *Pinus pinaster* presentan resistencia o tolerancia a la enfermedad del marchitamiento del pino, y esclarecer si los nematodos inoculados artificialmente sobreviven en plantas asintomáticas o son eliminados. *Área del estudio*: Los experimentos se llevaron a cabo en los invernaderos del Centro de Investigación Forestal de Lourizán (42° 24′ 33″ N, 8° 39′ 46″ W, Pontevedra, España). *Material y métodos*: Este estudio comprendió dos conjuntos de ensayos. En el primer grupo, se realizaron tres inoculaciones artificiales a lo largo de un año con *Bursaphelenchus xylophilus* en plantas de 32 familias del programa de mejora genética de *P. pinaster.* Solo 23 plantas permanecieron asintomáticas al final del ensayo, y cuatro de ellas continuaron asintomáticas quince meses después de la última inoculación. En ese momento, estas cuatro "plantas madre" se propagaron clonalmente mediante estaquillado. A continuación, la presencia del nematodo se examinó tanto en las plantas madre como en sus estaquillas en diferentes momentos. El segundo grupo de experimentos, consistió en tres ensayos de inoculación en los que se evaluaron clones de progenitores de familia de *P. pinaster* resistentes al nematodo. Al final de estos ensayos (109-141 días tras la inoculación), a cada planta se le asignó un nivel de daño, y se determinó la densidad de nematodos. De cada uno de los ensayos, 15 plantas asintomáticas se muestrearon para el conteo de nematodos a los 8 y 18 meses tras la inoculación. *Resultados principales*: En el conteo de nematodos llevado a cabo en plantas madre tras quince meses desde la última inoculación, se extrajeron nematodos únicamente en una sección por debajo del punto de inoculación de una sola planta madre. Años después, ninguna de las plantas madre ni sus estaquillas contenían nematodos. En los ensayos de inoculación con clones resistentes, solamente el 16.5% de las plantas que permanecieron asintomáticas al finalizar el ensayo contenían nematodos. *Aspectos destacados de la investigación*: La posibilidad de producir plantas resistentes libres de nematodo mediante injertado o estaquillado es fundamental para el control de la propagación del nematodo de la madera del pino.

Palabras clave: enfermedad del marchitamiento del pino; estaquilla; nematodo de la madera del pino; pino marítimo; plantas madre.

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Supplementary information ↓

1. INTRODUCTION

In Galicia, northwestern Spain, *Pinus pinaster* Ait. is a crucial tree species for afforestation. *Bursaphelenchus xylophilus* (pine wood nematode, PWN), the pathogen causing pine wilt disease (PWD), constitutes one of the main threats that this species faces.

Despite its susceptibility to PWD, *P. pinaster* is a species with broad genetic diversity (González-Martínez et al., 2004). It has exhibited significant variation in PWN susceptibility in inoculation assays under controlled conditions, both at provenance (Menéndez-Gutiérrez et al., 2017, Torres-Sánchez, 2023) and family levels (Menéndez-Gutiérrez et al., 2017). Currently, clones within the most resistant families of the Galician *P. pinaster* breeding program are being evaluated to identify genotypes with higher resistance.

Preventive planting of resistant *Pinus* spp. seedlings in still unaffected or slightly PWD-affected areas can effectively control the spread of PWN in Spain (Díaz-Vázquez et al., 2020). Such planting can also be of great significance in severely damaged areas. Japan successfully adopted this strategy by first selecting surviving trees in forests widely affected by PWD. Subsequently, grafts of these trees or their progenies were tested for resistance to PWN, and the surviving individuals were then used to create seed orchards. Since then, resistant seedlings from these orchards are planted in areas with varying degrees of PWD-affectation (Fujimoto et al., 1989).

Vegetative propagation via cutting or grafting are appropriate methodologies for multiplying PWN-resistant *P. pinaster* clones or establishing seed orchards, as they preserve the resistance trait. However, unlike in Japan, the disease has not yet spread throughout the entirety of Spain. Therefore, when propagating resistant clones evaluated by artificial nematode inoculation, it is crucial to ensure that the shoots used for cutting or the scions for grafting are free of nematodes.

Irreversible symptoms are a common characteristic of pine wilt disease. Once needle discoloration begins, the tree usually dies within a short period (Torres-Sánchez et al., 2023). However, some infected trees never show any symptoms; these are considered resistant or tolerant trees. Even so, these asymptomatic trees or their cuttings might still harbour nematodes (Uchiyama & Futai, 2025).

This study aimed to clarify whether certain *P. pinaster* trees could display resistance or tolerance to PWN. These are controversial terms, but commonly in nematology, resistance refers to the host's ability to prevent or impede pathogen invasion, development, and multiplication, while tolerance denotes the host's capacity to endure infection with minimal damage (Trudgill, 1985). It is of great importance to elucidate whether nematodes remain alive in asymptomatic plants or eventually disappear, to enable cuttings or grafts to be made without the risk of spreading nematodes. We investigated individuals that showed no symptoms despite multiple PWN inoculations, examining them for PWN presence at several time points after inoculation and from different tree positions.

2. MATERIAL AND METHODS

2.1. Cuttings of asymptomatic seedlings

A *Bursaphelenchus xylophilus* inoculation assay was conducted on two-year-old *Pinus pinaster* seedlings in a greenhouse at the Lourizán Forest Research Centre. Seedlings originated from 32 families of the Galician breeding program. All seedlings were inoculated three times: in April 2016, September 2016, and April 2017. For all inoculations, we used a highly virulent *B. xylophilus* strain (SpPO1; Menéndez-Gutiérrez et al., 2021) at a dose of 600 nematodes per plant, encompassing all developmental stages. The assay was considered concluded six months after the final inoculation, when no seedling exhibited changes in its wilting stage. At this point, the mean mortality of the assay was 59.8%, and only 23 plants out of the 576 inoculated remained completely asymptomatic.

Fifteen months after the last inoculation (June 2018), four *P. pinaster* seedlings, designated "mother plants", were selected among these 23 because they had remained completely asymptomatic since the initial inoculation. At this time, these mother plants were clonally propagated by cutting, yielding at least eight cuttings from each. On the same date that the cuttings were taken, samples were collected from the mother plants to check for nematode presence. Three samples were collected from each mother plant (12 in total): one comprised branches from the area above the inoculation point, another from branches below the inoculation point, and the third consisted of segments immediately below the shoots collected for cutting.

In December 2021, 56 months after the last inoculation of the mother plants and 42 months after cutting, all mother plants and 16 cuttings (four from each mother plant) were processed to detect *B. xylophilus*. Mother plants were sectioned into eight parts (Figure 1), and the cuttings were divided into three sections: root, stem, and branches. Each section served as a distinct sample for nematode analysis.

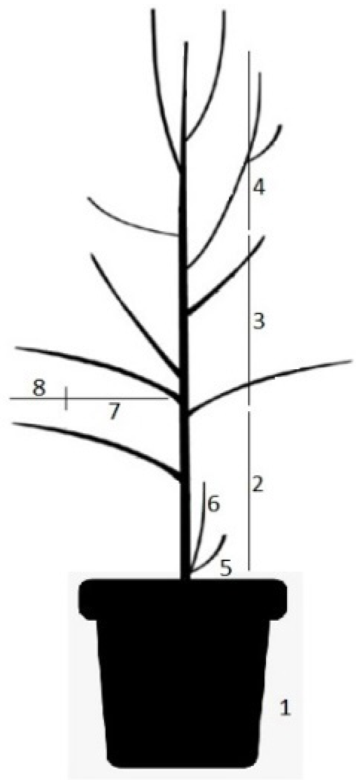

Figure 1. Sections into which the mother plants were divided 56 months following their last inoculation with *Bursaphelenchus xylophilus* to test for their presence. **1**, roots; **2**, **3** and **4**, lower, middle and upper third of the stem; **5** and **6**, lower branches of the previous year's and present year's growth; **7** and **8**, upper branches of the previous year's and present year's growth).

In all cases, samples were kept at 25 ºC for 15 days before extraction. Nematode quantification was carried out by extracting nematodes using a modification of the Baermann funnel technique. Nematodes were then counted under a stereomicroscope (Olympus Co., Ltd., Tokyo, Japan), and density was expressed in nematodes per gram of dry wood.

Spearman's prank correlation coefficients were computed to examine the relationships between the nematode density and the wilting stage.

2.2. Inoculation assays

Three *B. xylophilus* inoculation assays were conducted under controlled greenhouse conditions. These assays evaluated clones derived from three nematode-resistant *P. pinaster* parents of families (BOE 2020). Two assays (Assay 1 and Assay 2) involved two inoculations, separated by 12 months, while the third (Assay 3) received a single inoculation. All assays followed a randomized complete block design with seven blocks, an average of 16 clones per parent of family, three parents of families, and one inoculated plant per experimental unit. A total of 261, 421, and 161 plants were inoculated with *B. xylophilus* in Assays 1, 2 and 3, respectively. Additionally, 18 to 36 control plants (inoculated with distilled water) from three clones, one per parent of family, were included. Each inoculation used a dose of 1,200 nematodes at mixed developmental stages, as described by Menéndez-Gutiérrez et al. (2018).

At the conclusion of the assays (112, 141 and 109 days after inoculation [DAI] for Assay 1, 2 and 3, respectively), the severity of wilting in each plant was assessed using a 7-point scale (1: asymptomatic plant; 7: dead plant; Menéndez-Gutiérrez et al. 2018). Subsequently, nematodes were extracted from the aerial parts of more than 100 plants per assay (136, 153 and 115 inoculated plants in

Assays 1, 2 and 3, respectively). The exact number varied based on the number of clones per parent of family included in each assay.

Plants that remained asymptomatic were maintained in the greenhouse under controlled conditions. Eighteen months (Assays 1 and 2) and eight months (Assays 3) after their respective inoculations, 15 asymptomatic plants per assay were sampled for nematode quantification. In all cases, these plants were divided into three sections (roots, stems, and branches) and incubated at 25 ºC for 15 days before extraction.

Nematode quantification was performed by extracting the nematodes from the different tissues using a modified Baermann funnel technique. Nematodes were counted under a stereomicroscope (Olympus Co., Ltd., Tokyo, Japan), and density was expressed in nematodes per gram of dry wood.

3. RESULTS AND DISCUSSION

At 15 months after the last inoculation, *B. xylophilus* was detected in only one section of one mother plant; specifically, nematodes were found exclusively in the branches located below the inoculation point of that single mother plant. This detection more than a year after the last inoculation, albeit minimal, indicated the presence of nematodes in a host that had been infected a long time ago and remained completely asymptomatic. Upon re-evaluation 56 months after the last inoculation, a notable change occurred: no *B. xylophilus* was detected in any of the eight sections of any mother plant. While other nematode species were found in the roots of all the mother plants, *B. xylophilus* was completely absent. This sustained absence over an extended period strongly suggests that these asymptomatic mother plants possess an active mechanism capable of either preventing *B. xylophilus* establishment or eliminating the pathogen over time, even following artificial inoculation. The consistent asymptomatic status of these plants from 15 to 56 months after inoculation, coupled with the disappearance of the nematode, further supports their robust resistance.

The absence of *B. xylophilus* in any section (roots, stems or branches) of the 16 processed cuttings from the mother plants 41 months after cutting is a significant finding for disease management. Although *B. xylophilus* was present in one mother plant when the cuttings were originally taken, the nematode was not detected near the shoot sections collected for the cutting process. This indicates that the vegetative material used for propagation was completely *B. xylophilus*-free. Our results align with the findings of Nakajima et al. (2019), who reported that newly formed shoots of previously artificially inoculated *Pinus thunbergii Parl.*-resistant clones could serve as PWN-free material for multiplication. These findings collectively provide crucial evidence supporting the viability of propagating PWN-resistant *P. pinaster* cuttings for plantation initiatives, offering a pathway for expanding resistant material without inadvertently spreading the nematode.

Results from the three inoculation assays showed a distinct distribution of wilting symptoms among the randomly selected plants used for nematode counting at the end of the assays (112, 141, and 109 DAI for Assays 1, 2, and 3, respectively). A substantial proportion of plants remained asymptomatic or displayed only very mild symptoms (Stages 1-2: 68.4, 79.1 and 69.2% for assay 1, 2 and 3, respectively). In contrast, a smaller proportion of plants died (Stages 6-7: 16.9, 5.9 and 28.7%, respectively). A minority of plants exhibited few symptoms (Stage 3: 7.4, 13.7 and 0.7%, respectively), and an even smaller percentage remained alive but with significant decay symptoms (Stages 4-5: 7.4, 1.3 and 1.4%, respectively) (Table 1). Overall, the number of nematodes found in the plants correlated with the wilting stage, increasing with higher levels of damage (data not shown).

Table 1. Prevalence of *Bursaphelenchus xylophilus* in plants at assays conclusion (112, 141 and 109 days after inoculation, DAI, in Assays 1, 2 and 3, respectively) categorized by severity of wilting symptoms

Wilting severity	No. plants	NP_NemR	NP_NemS	NP_NemT	Nematode density (range)
Assay 1					
1	30	4	2	6	0.31-1.30
2	63	13	10	19	0.24-7.50
3	10	4	7	7	0.41-19.71
4	8	3	7	7	0.19-27.20
5	2	0	1	1	32.24
7	23	23	23	23	106.31-2128.29
All (1-7)	**136**	**47**	**50**	**63**	
Assay 2					
1	12	0	0	0	0
2	109	4	13	14	0.11-1.76
3	21	2	6	6	0.14-4.33
4	2	0	1	1	0.26
7	9	9	9	9	6.63-1348.18
All (1-7)	**153**	**15**	**29**	**30**	
Assay 3					
1	79		7		1.11-1.54
2	20		4		0.61-8.60
3	1		1		3.05
4	1		1		42.61
5	1		1		88.30
7	41		41		6.71-2,166.00
All (1-7)	**143**		**55**		

Wilting severity, symptom stages (1-7); No. Plants, total number of plants; NP_NemR, NP_NemS, NP_NemT, number of plants with *B. xylophilus* presence in the roots, stems and whole plant, respectively; Nematode density (range), minimum and maximum values of the total number of nematodes per gram of dried weight; estimated for the whole plant in assays 1 and 2, and for stem in assay 3.

Furthermore, the percentage of plants with presence of *B. xylophilus* was low when they were classified as Stage 1 and 2 at the end of each assay (Stage 1: 20.0, 0.0 and 8.9%, and Stage 2: 30.2, 12.8 and 20.0% for in assays 1, 2 and 3, respectively) (Table 1). Moreover, they had a low number of nematodes, since only one plant harbored more than two *B. xylophilus* per gram of dried weight (data not shown). Eight (Assay 3) and 18 months (Assays 1 and 2) after inoculation, none of the 15 processed asymptomatic plants per assay contained any *B. xylophilus* in their roots, stems, or branches.

These findings from the inoculation assays strongly corroborate the presence of resistance in the tested *P. pinaster* clones. The consistent observation of a high proportion of inoculated plants remaining asymptomatic or with only mild symptoms (Stages 1-2) across all assays is particularly significant. This outcome contrasts sharply with the high mean mortality (59.8%) observed in the broader initial inoculation assay from which these breeding program clones were derived, clearly demonstrating the effectiveness of the selection for resistance. The observed correlation between increasing nematode numbers and higher wilting stages further indicates that susceptibility leads to disease progression, while the very low nematode presence in asymptomatic or mildly symptomatic plants points towards an effective host resistance mechanism.

The notable absence of *B. xylophilus* in asymptomatic (Stage 1-2) plants by the end of the assays, and its complete disappearance in all processed asymptomatic plants 8 to 18 months later, reinforces this conclusion. As Wang et al. (1998) defined, in nematology, resistance refers to the host's capacity to avoid pathogen invasion and/or multiplication, whereas tolerance indicates host´s ability to withstand infection without experiencing excessive harm. Our results suggest that these *P. pinaster* clones exhibit true resistance, inhibiting nematode multiplication from the initial stages or actively preventing their establishment and proliferation within plant tissues even after initial proliferation.

Supplementary information ↑

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Supplementary material

Not applicable.

Data availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Authorship contribution statement

Raquel Díaz: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Lucía Villar: Data curation, Investigation, Methodology, Writing – review & editing.

María Menéndez-Gutiérrez: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing.

Competing interests

The authors have declared that no competing interests exist.

Statement on the use of Artificial Intelligence

Not applicable.

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