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Multiple factors associated with forest decline in the context of control measures for the pinewood nematode

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Abstract Forest decline is a complex phenomenon; however, there is often a tendency to oversimplify by attributing it to a single cause, neglecting the interaction of multiple factors. Ever since the detection of the pinewood nematode (PWN), *Bursaphelenchus xylophilus*, in Portugal (and in Europe) over two decades ago, pine wilt cases in the country have been primarily attributed to this pathogen, leading authorities to enforce strict containment and control measures. Other contributing factors are generally overlooked, with the system being easily overwhelmed by disturbances such as droughts and fires. In this study, a physiographic approach was used to assess multiple factors potentially affecting tree wilting, including environmental parameters in the landscape, alongside other biological agents, and infestation by the PWN. The study area is a highly managed production forest where control measures have been implemented since the detection of PWN in the 1990´s. Pine decline was found to be aggregated in the landscape, with topographic parameters associated with water availability playing a significant role. Wilting trees were affected by multiple biological agents, even though the density of PWN was positively correlated with the decline. However, *B. xylophilus* was also found to be present in asymptomatic hosts, suggesting an ability to persist for some time in healthy trees within the forest. This research illustrates the multifaceted nature of pine forest decline and the limitations of managing it as originating from a single cause. A critical overview of current PWN management measures is provided, along with proposals for a more heuristic and holistic approach.

Keywords *Bursaphelenchus xylophilus* · Forest management · Landscape analysis · *Pinus pinaster* · Portugal · Water availability

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Introduction

Episodes of massive synchronous tree mortality on extensive forested areas have always intrigued researchers and alarmed stakeholders. This phenomenon can just be part of normal forest succession, since the establishment, maturation, aging and senescence of trees often occur naturally in groups or cohorts (Mueller-Dombois, 1992). However, in most cases, forest decline is considered premature and anomalous, and its causes are unclear and/or hard to pinpoint. For the sake of communication there was the need to name the problem and thus decline concepts have been proposed (Manion & LaChance, 1992; Ostry et al., 2011), resulting in a long list of decline and dieback diseases and syndromes (Ciesla & Donaubauer, 1994; Jurskis, 2005). These concepts were considered useful to label and discuss the sequence and impact of multiple possible causal factors of a complex phenomenon. However, there is always a tendency for researchers, stakeholders and public in general to set a hypothesis about the cause of decline and present it as a fact, oversimplifying the interaction of different biotic and abiotic causal factors, tying the concept of decline to one cause (Ostry et al., 2011; Sinclair & Hudler, 1988). Forest pathologists, when presented with major forest decline, tend to search for a “causal agent” on a pest or pathogen, and although host susceptibility and environmental factors are generally taken into account, as part of the disease triangle, the focus ends up on a biological agent (Ostry et al., 2011). Examples include the efforts to find insect pests and fungal pathogens to explain declines in oaks (e.g. Balci & Halmschlager, 2003), pines (e.g. Jules et al., 2016) and eucalyptus (e.g. Stone & Bacon, 1995). Ecologists and environmental scientists, on the other hand, tend to focus on the abiotic or environmental factor that concern them most. An illustration of this was the increase on reports of forest decline during the 1980s in Europe and North America, which was described as the “Waldsterben syndrome” or “forest death”. The phenomena were mostly attributed to acid rain and air pollution, major environmental concerns at the time, and considered as somewhat overblown by some authors (Kandler & Innes, 1995). In recent decades, concerns over rising temperatures and increasingly severe droughts have translated into this being the preferred explanation for episodes of tree decline, with alarming reports of declining forests across the world and its relationship with climate change (e.g. Hammond et al., 2022; Hartmann et al., 2022).

Pine wilt disease (PWD) was first described in 1905 in forests of native pines located in the island of Kyushu, southern Japan, near the city of Nagasaki. During the next six decades, authorities recorded pine forest decline throughout Kyushu, Shikoku and the southern half of the larger island of Honshu (Mamiya, 1988). The phenomena were popularized by the word "Matsukuimushi", meaning “insects involved in dead pine trees”, and efforts to control pine decline employed strategies aimed at decreasing population levels of bark beetles and wood borers (Futai, 2013; Mamiya, 1988; Suzuki, 2004). In 1969, Japanese plant pathologists reported that an unknown species of *Bursaphelenchus* nematodes was commonly detected in dead pine trees in many localities in Kyushu. Inoculation experiments confirmed it to be pathogenic to Japanese native pines, while American pines proved to be somewhat tolerant (Kiyohara & Tokushige, 1971). The pathogen was described as a new species and named *Bursaphelenchus lignicolous* (Mamiya & Enda, 1972) and in the next decade it was detected in dead pine trees throughout most of Japan (Mamiya, 1988). The frequency and abundance with which this pathogen was found in dead trees led to the entire decline of pine forests recorded in southern Japan since the beginning of the twentieth century being attributed to it a posteriori (Futai, 2013; Mamiya, 1988; Suzuki, 2004).

In 1979, the PWN was found by a visiting Japanese scientist on a wilting Austrian pine, *Pinus nigra*, in Missouri, United States (Dropkin & Foudin, 1979). In the following years, extensive surveys were carried out throughout the country, revealing the nematode to be widespread, apparently only affecting exotic species and debilitated pines (Wingfield et al., 1984). Furthermore, it was found that the species had already been isolated from pinewood samples in Louisiana in 1929 and described as *Aphelenchoides xylophilus* (Steiner & Buhrer, 1934). The taxonomy of the species was revised in 1981, and both Japanese and American nematodes were unified under the name *Bursaphelenchus xylophilus* (Nickle et al., 1981). Since then, *B. xylophylus* was considered as indigenous to North America, where it gained the status of an innocuous saprophytic organism, and invasive to Japan, where it was categorized as a serious pine pest.

Throughout the 1980s, *B. xylophilus* was detected in South Korea, Taiwan and China, associated with forest decline, establishing its status as one of the most important invasive pine pests worldwide (Togashi & Jikumaru, 2007; Yang & Qouli, 1989).

The discovery *B. xylophilus* in North America, and its subsequent detection in traded wood and its derivatives, led to the enactment of regulations and quarantine rules by the European Plant Protection Organization (EPPO) on coniferous chips, sawn wood, and logs imported by the European Union, which became fully effective in 1993 (Dwinell, 1997). Despite all the protective measures, *B. xylophilus* was found in Portugal in 1999, in a few forest sites south of Lisbon (Mota et al., 1999). A quarantine zone of about 300 thousand ha was immediately set around these sites with the purpose of eradicating the invasive pathogen. Within the quarantine zone, all wilting conifers were mandated to be tested for the presence of *B. xylophilus*, promptly removed, and transported to an authorized destination for processing and/or destruction, ensuring the absence of the PWN or its insect vector on woody materials, and its circulation from infected areas tracked through official documentation. This quarantine area expanded progressively in subsequent years, exceeding one million ha in 2007, with approximately 750 thousand trees cumulative felled. Additionally, in that same year, nearly five million healthy conifers were removed within a 3 km-wide corridor surrounding the quarantine zone (Sousa et al., 2011; Fig. 1a). However, these containment efforts proved unsuccessful, and by 2008 *B. xylophilus* was considered established throughout the country, prompting the implementation of nationwide quarantine measures that remain in effect. To avoid dispersal of the nematode to the remainder of Europe, a 20 km buffer zone was established along the Portuguese border, totaling 2.5 million ha (Fig. 1a), where rules dictated that all conifers showing any wilting symptoms should be immediately removed (EU, 2012). Despite of

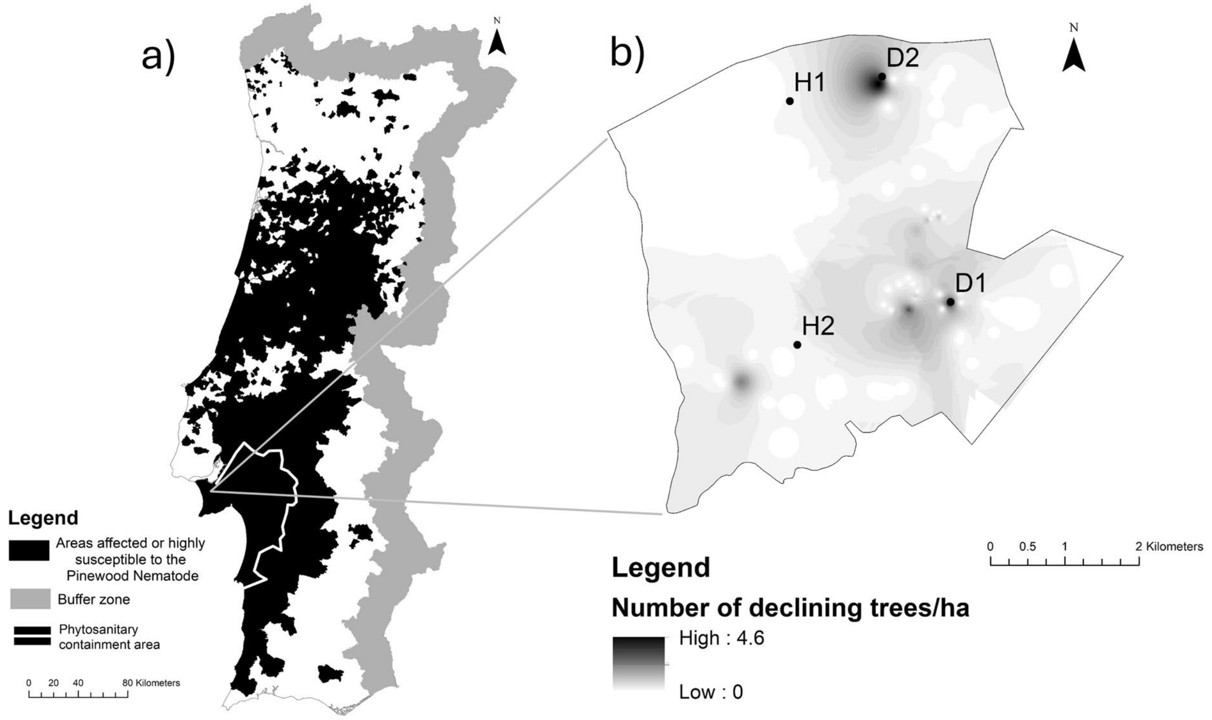

Fig. 1 Map of Portugal (**a**) with the location of Herdade da Apostiça (**b**): a) the map delineates the pinewood nematode quarantine area established in 1999 (depicted by the white line), the current zones affected or highly susceptible to the pests, and the buffer zone designed to curb its spread to the wider European region; b) Prediction map of tree decline across 2700 ha of Herdade da Apostiça, produced using the Inverse Distance Weighting (IDW) method in ArcGIS

these measures, the PWN has since been detected in several localities in Spain (Abelleira et al., 2020; Zamora et al., 2015).

More than 25 years after the initial detection of *B. xylophilus* in Portugal, pine forests remain ecologically dominant, and it has been clear for some time that the pathogen cannot be eradicated. However, the same rules and principles are still being enforced in attempts to contain this invasive species, with successive updates and upholds on the legislation (EU, 2012 and 2018), and when dealing with pine forest health and decline in Portugal, the fear of the PWN is omnipresent. Pine forests still cover around one million ha of the Portuguese territory (https://www.icnf.pt/api/file/doc/0f0165f9df0d0bbe), with the border buffer zone comprising an additional 2.5 million ha (Fig. 1a), often in rugged and remote territory. To make matters even more complicated, these areas are mostly privately owned, divided into small properties, with landowners often absent or simply unknown, making it impossible to establish responsibility for keeping the forest free of symptomatic pines. Consequently, the enforcement of the PWN containment/eradication policy is difficult and costly. Furthermore, the system put in place to deal with this epidemic does not account for other factors affecting pine forests health and decline, becoming easily overwhelmed by natural disturbances in ecosystem dynamics, such as severe drought and forest megafires. Exemplifying this was the exceptional and abrupt increase in the number of declining trees which was recorded in 2006, and again in 2017, following severe droughts, overwhelming the Portuguese authorities (EU, 2018; Sousa et al., 2011). Moreover, bark beetle infestation was recognized as a major factor on pine mortality, but its intercorrelation with damage caused by *B. xylophilus* has not been explored (Sousa et al., 2011).

In this study, our goal was to investigate the factors driving recurrent forest decline, particularly considering the longstanding presence and spread of the PWN, aligning with concepts of decline and dieback diseases and syndromes (Ciesla & Donaubauer, 1994; Jurskis, 2005). Despite rigorous forest management measures aimed at controlling *B. xylophilus*, its persistence alongside recurrent outbreaks of forest decline suggests a complex dynamic of this pest, as well as intricate interactions with other biotic and abiotic factors. To explore this complexity, we employed a physiographic approach to analyze the spatial dynamics of pine forest decline and its association with the PWN, along with other biotic agents and environmental factors, in a production pine forest situated within the PWN quarantine zone established in 1999 (Fig. 1). A recent surge in tree decline, coinciding with the severe drought of 2015–2020, provided an opportune moment for this study.

Material and methods

Studied forest

The area of the present study comprises Herdade da Apostiça (HA), a private forest property of 4000 ha located in the Setúbal Peninsula, in the southwest coast of Portugal (Fig. 1b). It has an altitudinal range of 0 to 90 m above sea level, comprising of Tertiary-age dunes and palaeodunes. Maritime pine, *P. pinaster*, covers about 80% of the forested area, with Stone pine, *P. pinea*, and cork oak, *Quercus suber*, covering the remaining forest. It is located about 40 km west of the localities where *B. xylophilus* was initially discovered in 1999, being under quarantine for over 20 years (Fig. 1). The decline of *P. pinaster* due to *B. xylophilus* has long been a major concern for stakeholders of this forest (Pimentel et al., 2017), with all wilting trees being promptly removed during winter.

This area has a Mediterranean climate, with mild winters and hot dry summers, with an average annual temperature of 16.5ºC and annual precipitation of 700 mm. The characteristic summer dry season enfolds from June to August, with average temperatures rising to 22.3ºC and precipitation dropping to about 25 mm per month (see Supplementary material for more details).

Spatial distribution of pine decline and associated environmental factors

It was assessed how pine forest decline relates to environmental factors known to affect forest health. These factors include topography and its influence on water availability (Binkley & Fisher, 2020; Elias et al., 2019), as well as aspects of vegetation diversity (Fierke et al., 2007; Filloy et al., 2023).

To assess the spatial distribution of decline, we randomly selected 83 sampling points within 2700 ha of HA using ArcMap 10.4 (ESRI). During summer of 2020, we assessed *P. pinaster* decline at each point using an English BAF 10×wedge prism (Mannel et al., 2006), which creates an optical illusion of the tree trunk by refracting light at a specific offset angle. At each point, the surveyor conducted a 360-degree sweep, counting all *P. pinaster* trees where the prism's offset view showed trunk connection. Borderline cases were recorded as 0.5 trees, while completely detached trunks were excluded. We calculated basal area (BA) by multiplying tree counts by the Basal Area Factor (10). Declined trees were considered as the ones presenting the canopy in the stages 1 to 4 of pine wilt disease scale, as described by Malek and Appleby (1984), from 20–50% of the canopy pale green to the entire canopy pale green and/or reddish-brown, a scale commonly used on PWD surveys. Trees with entirely dark green canopies were considered healthy, while standing dead trees (fully reddish-brown with partial or total leaf fall) were excluded. For each sampling point, we calculated decline as the ratio of declining trees' BA to total BA.

Following the completion of the field work, a geospatial analysis was conducted to examine spatial patterns of tree decline. A vector point file (shapefile) was created in ArcMap using the coordinates of all sampling points. The attribute table of this shapefile was then populated with field-collected data on BA and decline. To account for scale-dependent effects, buffers with radii of 50, 100, 150 and 200 m, were delineated around each point in ArcMap. Using tree decline data, several spatial interpolation methods were applied in ArcGIS to generate a continuous surface of decline. Ten percent of the total dataset was set aside for validation. Inverse Distance Weighting (IDW) was found to provide the best results, and this method was used to produce a spatial prediction map of tree decline across the studied area (Fig. 1b).

To determine parameters related with topography and water availability, data was extracted from an altitude raster file (Digital Terrain Model, DTM), with a spatial resolution of approximately 30 m, obtained by the Shuttle Radar Topographic Mission (SRTM) (U.S. Geological Survey 2020; https://www.usgs.gov/centers/eros/science/usgs-eros-archive-digital-elevation-shuttle-radar-topography-mission-srtm, retrieved at 25/04/2023). Several parameters known to be related with local water availability were computed using SAGA version 7.6 (System for Automated Geoscientific Analysis, Olaya, 2004). These include Slope, representing terrain steepness with higher values denoting steeper terrain and lower values indicating flatter terrain; Relative Slope Position (RSP), indicating slope gradient and curvature with higher values signifying positions closer to the top of the slope and lower values indicating positions closer to the bottom; Distance to Channel Network (DCN), where high values denote locations farther from waterlines and low values indicate closer proximity to these lines; and Topographic Wetness Index (TWI), reflecting the relative wetness or moisture availability of different terrain features within the landscape (Sörensen et al., 2006). The mean for each of these parameters was computed individually for every buffer and point. Subsequently, all computed values were incorporated into the attribute table of the shapefile corresponding to each point. In essence, for each sampling point and parameter, there exist four values denoting the variations across different buffers.

To evaluate landscape diversity, we acquired a Pléiades satellite image from 21 February 2020, processed as an orthorectified image using ground control points, with a spatial resolution of 0.5 m. This image was used to generate a land cover map. Using the four spectral bands of the satellite —blue, green, red, and near-infrared bands (NIR)— we applied the ISO Cluster Unsupervised Classification method in ArcMap. This approach is particularly advantageous when training data is unavailable, as it requires no prior knowledge and automatically groups pixels into natural clusters based on spectral similarity, outputting a classified image. Several tests were conducted with this method, varying parameters such as the number of spectral bands, the number of classes, the maximum number of iterations and the minimum number of pixels per class. The test that provided the best discrimination between vegetated areas and bare soil/roads, as well as variations in density and biomass within vegetated regions, was selected as the basis for the land cover map, with additional support of Google Earth imagery. Further validation and refinement were carried out through on-site fieldwork. This raster-format map was then spatially segmented according to shapefiles corresponding to various buffer zones with distinct radii. The resulting segmented rasters were subsequently transformed into polygon files (shapefile format) and employed as input data for V-late—Vector-based Landscape

Analysis Tool, version 2.0, a freely available extension for ArcMap (Lang & Tiede, 2003). This tool facilitated the computation of various diversity indices, namely Richness Diversity Index (RDI), Shannon Diversity Index, Equitability, and Simpson Diversity Index. The values of these diversity indices for each point and buffer zone were incorporated into the attribute table of the shapefile for the study points, following the previously described procedure for topographic parameters.

Assessing pinewood nematode infestation within host trees and its association with decline and co-occurring pests

Sampling asymptomatic hosts during plant epidemics is essential because these individuals may represent either transient or long-term cryptic stages in disease cycles. Understanding these infection patterns is critical for elucidating epidemic dynamics and improving outbreak prediction (Parnell et al., 2017). Accordingly, we sampled both asymptomatic and declining trees as much as possible on each sampling occasion.

In October of 2019, nine *P. pinaster* and two *P. pinea* trees were felled in an area of HA identified by stakeholders as exhibiting generalized tree decline (D1 on Fig. 1b). Among these, three of the *P. pinaster* trees were healthy, while six showed signs of decline; both *P. pinea* trees were healthy. Dendrometric data were collected for all trees, including diameter at breast height (DBH; measured at 1.30 m above ground level), height, and age (determined through the growth rings). The bark was removed, and slices of phloem and xylem tissue were extracted at three points along each tree's stem: at the DBH level (Base), at the middle of the stem (Mid), and at the stem section within the canopy (Top). Additionally, twigs were randomly collected throughout the canopy. The presence or absence of blue-stain fungi (Ophiostomataceae), bark beetles (Scolytinae), and wood-boring insects was also assessed through morphological identification of subcortical galleries and characteristic staining patterns (Pimentel & Calvão, 2023).

In October of 2020, four sites within the study area (HA) were selected based on a spatial prediction map of forest decline, derived using the IDW method and data on *P. pinaster* decline obtained during the previous summer's fieldwork (Fig. 1b). These included two high-decline sites: D1 (a previously b confirmed high-decline area) and D2 (an additional hotspot identified by the map on Fig. 1b). Two healthier sites (H1 and H2) with low decline incidence were also selected (Fig. 1b). On each one of these sites, 10 *P. pinaster* trees within an area of about 2 ha were sampled and, as much as possible, declining and healthy trees were selected. Stone pines were also sampled whenever present. For each one of the selected trees, the DAP and health status of the tree was annotated (healthy or decline as described previously). The presence or absence of blue-stain fungi, bark beetles and wood borers on the stem of each one of these trees was recorded. The evaluation of PWN population densities was carried out in the canopy, since previous work pointed to this being the section of the trees with the highest density of nematodes, even in asymptomatic pine trees. For each sampled tree, one or two large branches were cut from the canopy with telescopic pruning shears (6.5 m max reach). Random twig samples were then taken from these branches. Sampling was repeated on the same sites in May 2021. Additionally in a subset of trees, both twigs from the canopy and phloem/xylem tissues from the stem at a height of about 130 cm, were collected.

In the lab, a subsample of the original homogenized sample collected in the field was cut into 1–2 cm fragments and weighed (10–15 g for phloem tissues and 20–40 g for twigs). Nematodes were then extracted from these samples using the Whitehead and Hemming tray method (Whitehead & Hemming, 1965). To prevent cross-contamination between samples, the sieves were thoroughly immersed in 96% ethanol for at least 5 min. The suspensions obtained from each sample were observed under a stereoscopic microscope (Olympus SZX-12, Olympus corporation, Tokyo, Japan). The number of *B. xylophilus* individuals in each sample was counted. Identification of PWN was confirmed based on the male spicule morphology, female vulval flap, and tail terminus (EPPO Standard, 2023). After the extractions were completed, the samples were oven-dried at 60 ºC for 5 days and weighed again. Dry weight was then subtracted from fresh weight, and the total number of *B. xylophilus* per sample divided by the dry weight. Population density was expressed in gram of dry wood (PWN*g^{-1}dry wood).

Statistical analysis

The spatial variation in pine decline was assessed using a Univariate Moran´s I analysis, while the relationship between decline and the spatial structure of environmental parameters was examined through Bivariate Moran´s I analysis. Both analysis were conducted in GeoDa (version 1.6.7.9; https://geodacenter.asu.edu). We used shapefiles of sampling points containing field data, topographical parameters, and diversity indices for this purpose. To create the spatial weights matrix for selecting neighbouring points, we tested several methods, including Euclidean distances and different exponents of inverse distances. The Euclidean distance method was ultimately selected due to the absence of significant differences between approaches. Significance of Moran's Index values was tested with 999 random permutations, and *P*-values were adjusted using the Holm-Bonferroni method to account for Type I error due to multiple tests across each distance classes. A correlograms was considered statistically significant if at least one autocorrelation value remained significant after correction ($\alpha = 0.05$) (Fortin & Dale, 2005; Legendre & Fortin, 1989; Legendre & Legendre, 1998). We plotted correlograms for both univariate and bivariate analyses, displaying Moran's I against 10 distance classes, ranging from 1000–6000 m. While different numbers of classes were tested, 10 classes produced high intra-class autocorrelation and a clear spatial patterning in the correlogram. Correlograms allow the evaluation of the autocorrelation behaviour across spatial distances, with their characteristic shapes providing insights into underlying spatial processes such as patchiness and gradients. Interpretation of these patterns follows established frameworks linking spatial structure to ecological processes (Fortin & Dale, 2005; Legendre & Fortin, 1989).

Due to zero-inflation and right-skewness in the nematode density data, along with small sample sizes in some comparisons, we employed non-parametric statistical approaches. Differences between groups were assessed using Mann–Whitney Wilcox tests for pairwise comparisons and Kruskal–Wallis followed by post-hoc Dunn tests for multiple tissue type comparisons.

To properly model the zero-inflated and skewed distributions, we implemented zero-inflated gamma regression using the glmm TMB package. This approach simultaneously estimated: (1) a logistic regression submodel for zero-inflation probability and (2) a gamma generalized linear model submodel (with log link) for positive PWN densities. Health status and site identity were included as fixed effects in respective analyses. Model adequacy was verified through residual diagnostics. Effect sizes are reported as fold-changes with corresponding 95% confidence intervals.

For spatial analyses comparing sites (D1, D2, H1, H2) in the 2020 survey, we reported median densities and interquartile ranges (IQRs) to account for distributional skewness. We quantified distributional skewness (γ_1) for all groups and identified outliers using Tukey's method (values exceeding $Q3 + 1.5 \times IQR$). All nematode density measurements are reported in units of PWN·g^{-1} dry wood, presented as either mean ± standard deviation or median [IQR] as appropriate for the distribution. All analyses were performed using R version 4.3.1.

Results

Spatial distribution of pine decline and associated environmental factors

The correlogram for pine decline (Univariate Moran) was significant, indicating positive spatial autocorrelation up to approximately 1300 m, followed by negative autocorrelation at greater distances (Fig. 2;

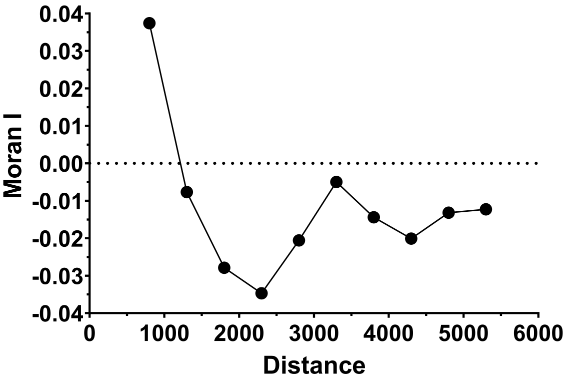

Fig. 2 UnivariateMoran's I spatial correlogram of *Pinus pinaster* decline (percentage of the basal area of dead/dying trees in relation to the total basal area of *P. pinaster*) on Herdade da Apostiça. The correlogram is globally significant at $\alpha = 0.05$ after correction using Holm's procedure

Table S2 Supplementary Material), suggesting clustered distribution of affected trees within the forest. The studied area has an average slope of 8%, with considerable local variation ranging from 0 to 70% (see Fig. S5 Supplementary Material). Bivariate correlograms for topographic variables and *P. pinaster* decline were significant for Slope only at a 150 m distance and for RSP, DCN, and TWI across all distances. These correlograms revealed positive autocorrelation at shorter distances and negative autocorrelation at longer distances for Slop, RSP, and DCN, implying similar values for forest decline and each respective variable in nearby locations. However, TWI exhibited an inverse pattern, indicating dissimilar values at nearby locations (Fig. 3, four upper graphics; Tables S3–S6 Supplementary Material). Correlograms for the four Diversity Indices did not show significance (Fig. 3, four lower graphics; Tables S7–S10 Supplementary Material).

Pinewood nematode infestation within host trees and its association with decline and co-occurring pests

Of the nine *P. pinaster* trees felled and sampled in the fall of 2019 (average ± SD height 14.9 ± 2.1 m, DAP 25.5 ± 7.4 cm, age 22.9 ± 3.2 years), the three asymptomatic pines showed no signs of infection by blue stain fungi, bark beetles, or wood borers, nor any infestation by *B. xylophilus* along the stem. However, *B. xylophilus* was isolated from the twigs of all three trees. In contrast, the six declining trees were infested by blue stain fungi and bark beetles, with two also showing signs of wood borer activity. *B. xylophilus* was detected in the stem tissues of all but one of these trees, while all six had PWN in their twigs. Nematode density in twigs of declining trees was higher than at any of the three sampled stem heights, though the difference was only marginally significant when compared to the base (Kruskal–Wallis $\chi^2 = 7.75$, df = 3, $P = 0.052$; post-hoc Dunn test: twigs vs. base z = -1.5, $P = 0.068$; vs. mid z = -2.3, $P = 0.012$; vs. top z = 2.5, $P = 0.006$; all stem height comparisons were non-significant; Fig. 4). The average density of *B. xylophilus* in twigs of declining trees was significantly higher than in healthy trees (Mann–Whitney $W = 72.5$, $P = 0.012$; Fig. 4). The two *P. pinea* trees felled and sampled at this time were healthy and showed no signs of infestation by any biological agents.

In the subsample of maritime pine trees surveyed in the fall of 2020, where both twigs and stem woody tissues were collected (N = 11, DBH 32,5 ± 24,4 cm), higher densities of PWN were detected in the twigs than in the stem. However this difference was not statistically significant due to high variability (mean ± SD twigs 32.20 ± 62.88; Base 0.49 ± 0.57 PWN·g^{-1} dry wood; Mann–Whitney $W = 65$, $P = 0.790$).

In our 2020 summer survey of maritime pine trees (N = 40; mean DBH 26.8 ± 14.0 cm), most individuals were visually healthy (31 trees) but PWN was detected in the canopy of 58% of these asymptomatic trees (N = 18; 1.34 ± 2.25 PWN·g^{-1} dry wood). The PWN distribution displayed strong zero-inflation and right-skewness in both health categories (healthy trees: $\gamma_1 = 3.1$; declining trees: $\gamma_1 = 1.3$). Notably, extreme PWN densities (> 100 PWN· g^{-1} dry wood) occurred exclusively in declining pines, with a maximum observed value of 195.95 PWN·g^{-1} (216 times the median for this group). Zero-inflated modelling showed comparable probabilities of zero PWN detection between groups (coefficient = -1.64, P = 0.14). However, when PWN was present, declining trees presented a 21.9-fold increase over healthy trees (95% CI: 3.7–128.9; z = 3.42, $P < 0.001$). These differences were confirmed by likelihood ratio testing ($\chi^2 = 9.58$, $P = 0.008$). The density of *B. xylophilus* was more strongly related with the presence of blue stain fungi and bark beetles, than with the activity of wood borers (Table 1).

The zero-inflated gamma model revealed significant spatial heterogeneity in PWN distribution patterns, with notable differences in both zero-inflation probability ($\chi^2 = 25.54$, $P < 0.001$) and positive nematode densities ($\chi^2 = 10.89$, $P = 0.012$) among sites. Site D1 exhibited substantially higher infection levels (median = 0.78 PWN·g^{-1} dry wood, IQR: 0.24–30.60), including two extreme outlier values of 97.18 and 195.95 PWN· g^{-1} dry wood. Site H1 maintained consistently low but detectable infections (median = 0.94 PWN·g^{-1}, IQR: 0.28–1.25) with complete absence of zero values (0% zero-inflation). Site D2 and H2 showed minimal infection, with 78–80% of trees having no detectable PWN (median = 0 PWN· g^{-1} dry wood).

In May, only 5 out of 40 sampled trees (DAP 22.3 ± 8.2 cm), presented symptoms of decline. Notably all trees wilting in October had been

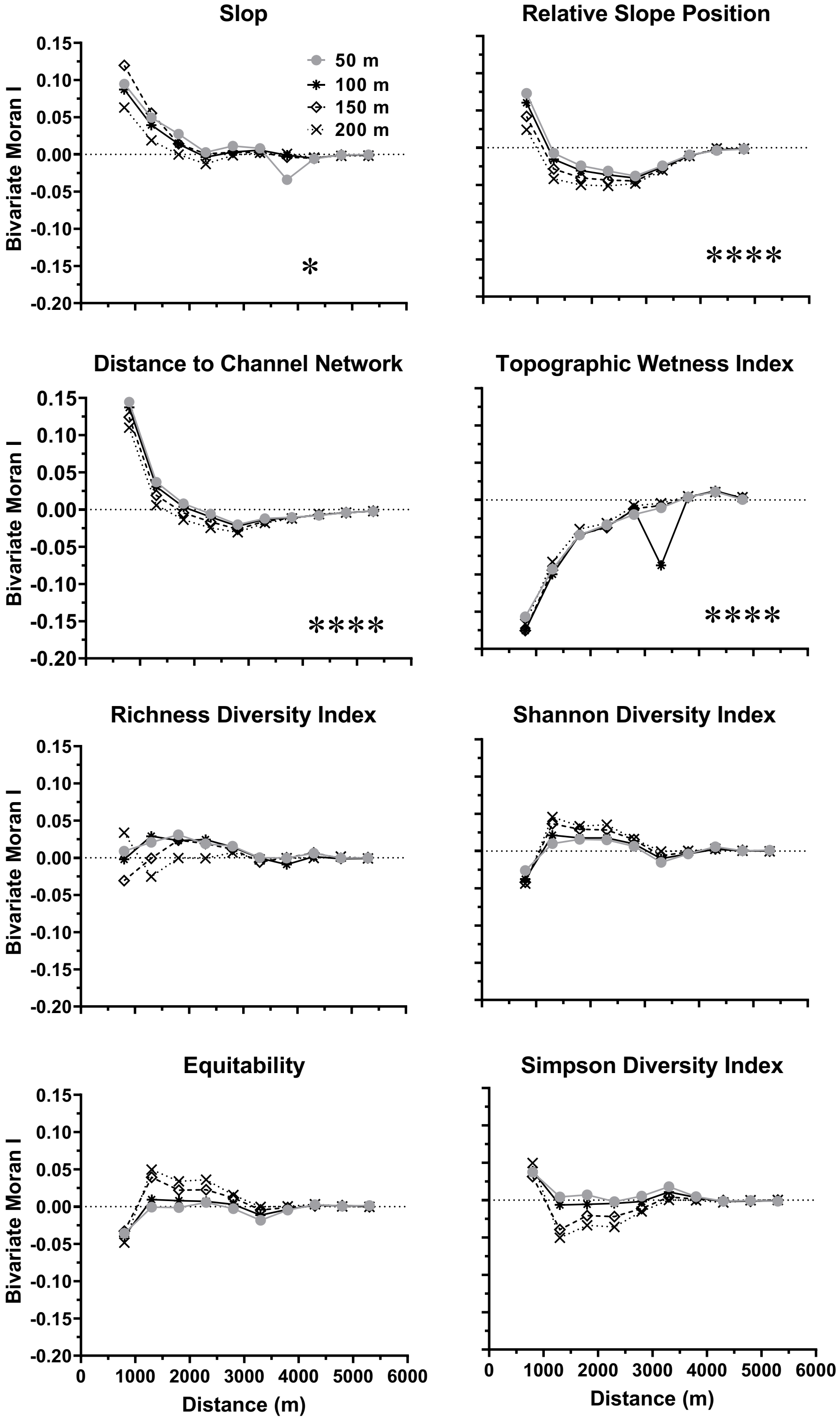

Fig. 3 Bivariate Moran's I correlograms computed for *Pinus pinaster* mortality, calculated as the percentage of the basal area of dead/dying trees in relation to the total basal area of *P. pinaster* in the stand, and various landscape and vegetation diversity metrics, at the distance classes of 50, 100, 150, and 200 m.* correlogram significant at the distance class of 150 m; **** correlograms significant at all distance classes. The level of significance considered was α = 0.05 after correction using Holm's procedure

logged during the winter. No *B. xylophilus* were extracted from the 5 declining trees, with only one asymptomatic tree harboring a detectable population of the pathogen in the canopy.

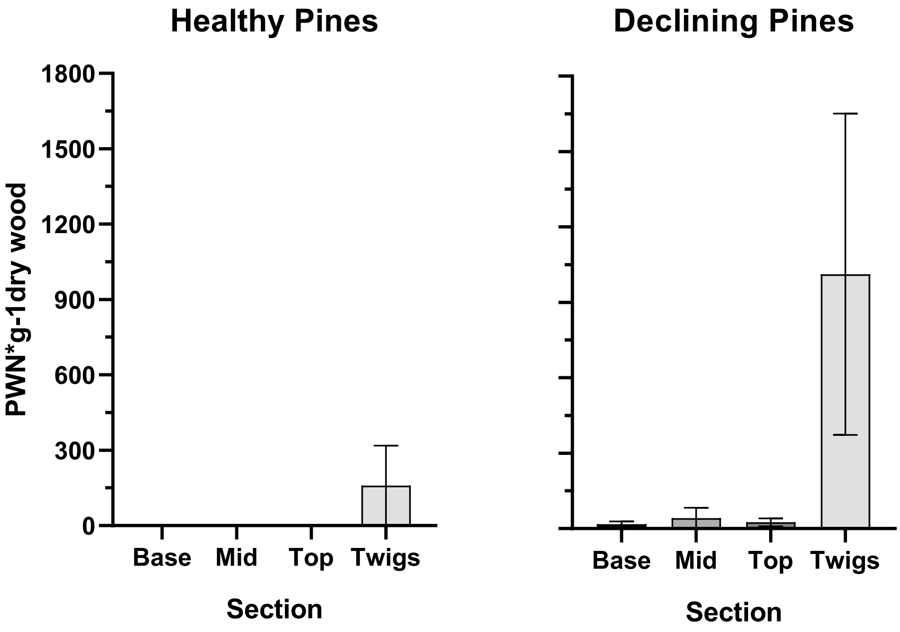

Fig. 4 Mean ± SE of *Bursaphelenchus xylophilus* nematodes per gram of dry xylem and phloem tissue, sampled at three stem levels—Base: ~ 1.30 cm above soil; Mid: stem midpoint; Top: stem portion within the canopy— and from canopy twigs. Data refers to three healthy and six declining *Pinus pinaster*, and samples were collected from felled trees in October 2019 at Herdade da Apostiça, Portugal

Five trees sampled in October of 2020 were resampled in May of 2021, including the sole tree with spring PWN detection. In this tree nematode density increased from 0.03 in October to 15.07 PWN*g-1 dry wood in May. Among the remaining trees: one showed no canopy PWN in either season, while the other three exhibited a decline from an average of 1.95 ± 3.27 PWN*g-1 dry wood in October, to undetectable levels in May.

From the combined number of *P. pinea* trees sampled in October and May ($N = 13$; DAP 28.4 ± 16.1 cm), no PWN was detected and no signs of decline were observed.

Discussion

In this study, a heterogeneous pattern of decline in maritime pine within a production forest was uncovered, due to the spatial aggregation of dead or dying trees within patches spanning approximately 1 km. These patches tended to occur on steeper, elevated terrain, far from watersheds, and on drier soils. Our research highlights the significant impact of water availability on pine decline in the studied forest. Notably, declining trees become more apparent in the fall, following the summer drought. However, during this season, the distribution of *B. xylophilus*-infected pines showed no discernible landscape structure. Most of the sampled pines that carry PWN appeared asymptomatic; however, the density of PWN notably increased on wilting trees.

Table 1 Association analysis between different biological agents in 40 *Pinus pinaster* trees sampled in October 2020 at Herdade da Apostiça, Portugal, trough Spearman's rank correlation coefficients

	Blue stain fungi P/A	Bark beetles P/A	Wood borers P/A	*B. xylophilus* P/A
Bark beetles P/A	0.900***	-		
Wood borers P/A	0.783***	0.630***	-	
B. xylophilus P/A	0.325*	0.293	0.258	-
B. xylophilus density	0.458**	0.501***	0.263	0.862***

P/A refers to presence/absence data; *B. xylophilus* density refers to data on *B. xylophilus**g^{-1} dry wood

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

Topography is a fundamental driver of environmental heterogeneity in forest ecosystems, significantly influencing hydrological processes that regulate soil water content, microclimate variations, and edaphic conditions. These relief-driven moisture gradients promote niche differentiation and landscape-scale forest diversity (Elias et al., 2019). Even modest elevational changes of just a few meters can produce marked declines in both water availability and soil fertility, creating steep transitions from mesic valley bottoms to xeric ridges and steep slopes (Binkley & Fisher, 2020). Our study area, characterized by an ancient dune system with elevation variations of dozens of meters, demonstrates how these topographic moisture gradients directly structure patterns of pine decline. This relationship is evidenced by significant correlations with key topographic variables: Slope, RSP, DCN, all exhibited positive spatial autocorrelation with decline severity, in our study area, while TWI exhibited an inverse relationship. The underlying mechanisms reflect topography's control of hydrological processes through several pathways. Steeper slopes promote thinner, erosion-prone soils, with reduced capacity to retain water, creating progressively xeric conditions as the Slope values increase. RSP is a terrain metric positioning trees along a hillslope, with ridge tops (higher RSP) experiencing greater water stress than depressions. Similarly, DCN values increase with distance from the nearest drainage channel, corresponding to decreasing edaphic humidity. Together, these metrics are inversely related to soil water availability and were found to be positively related with pine decline. In contrast, TWI's positive relationship with soil moisture explains its negative correlation with decline severity. This fluctuation in water availability is of particular importance for a fast growing species like *P. pinaster*, which thrive in nutrient-rich environments but display limited tolerance to drought (Tapias et al., 2004). While pines can develop extensive root systems, this structural adaptation reflects the average moisture conditions at their establishment site (Andivia et al., 2019). During severe drought periods (such as those occurring between 2015–2020 prior to our surveys), these root systems cannot rapidly adjust to compensate for sudden water deficits (Mackay et al., 2020). These hydrological limitations may be particularly pronounced on steep slopes and ridge positions where reduced water retention capacity created especially xeric conditions, ultimately generating a landscape mosaic of differential vulnerability to pine decline.

The association between water availability, *B. xylophilus*, and PWD should not be surprising, given that pine mortality associated with PWN in Portugal can been linked to water scarcity. Outbreaks of *B. xylophilus*-induced pine mortality followed three periods of severe drought in Portugal over the past 30 years. The first was the 1992–95 drought, which preceded the initial detection of *B. xylophilus* in a restricted area in the southern part of the country, coinciding with the decline of maritime pine (Mota et al., 1999). The second was a sharp increase in pine mortality and the spread of the pathogen across the country during the 2004–09 drought (Sousa et al., 2011). The third was a dramatic increase in pine mortality in recent years, coinciding with the most severe drought of the last 50 years from 2015 to 2020 (Silva et al., 2022; see Fig. S3 and S4 Supplementary Material). This surge overwhelmed Portuguese authorities, making it impossible to process all newly declining trees within legal deadlines and resulting in the enactment of a temporary derogation by the EU Commission (EU, 2018). Pine wilt attributed to the recent invasion by the PWN in Central-North Portugal was also found to be spatially aggregated, but at a macrogeographical scale, with warm, dry locations with higher evapotranspiration experiencing the most severe mortality (Calvão et al., 2019). Water stress is known to favor wilting caused by the PWN (Estorninho et al., 2022), but drought stress by itself can directly lead to xylem cavitation and wilting (García de la Serrana et al., 2015). Thus, separating these two effects on tree mortality in a drought-prone Mediterranean forest is a challenging task.

Water stress is also known to promote blue stain fungi and bark beetles, key primary agents of pine forest decline worldwide (Croisé et al., 2001; Erbilgin et al., 2021). These organisms were consistently present in declining *P. pinaster* trees in our studied forest. Infection by the PWN has long been found associated with infestation by bark beetles, starting in the decades after PWD was first described in Japan, at the beginning of the twentieth century (Futai, 2013; Mamiya, 1988; Suzuki, 2004). In south-eastern United States, PWN infections were associated with outbreaks of southern pine beetle *Dendroctonus frontalis*, the most damaging bark beetle affecting pine forests in that region (Kinn & Linit, 1992). In Portugal, bark beetles *Orthotomicus erosus* and *Ips sexdentatus* were recognized as important contributing factors of pine mortality in the southern part of the country, associated with the drought of mid 2000´s and the infestation by *B. xylophilus* (Sousa et al., 2011).

The diversity of vegetation cover within a landscape has also been generally associated with positive effects on forest health, often resulting in reduced incidence of pathogens and pests. While the underlying mechanisms are not always clear, diverse forest communities can serve as natural barriers to pest spread by limiting host availability, disrupting pest life cycles. They also enhance the overall resilience of the community due to the increased probability of including resistant or tolerant species (Ferrenberg, 2016; Fierke et al., 2007; Filloy et al., 2023). However, in our study, we did not observe any significant effect of vegetation diversity on tree mortality. Our analysis focused on a monoculture production with relatively low diversity, which may have influenced the results. Nevertheless, topography and water availability emerged as the predominant landscape factors influencing forest health in our study area, with no interaction with vegetation cover.

In the present study PWN was detected in the canopies of more than half of the asymptomatic trees sampled during autumn across the studied forest, with no apparent spatial distribution pattern. However, *B. xylophilus* densities were notably higher in declining trees, which tended to occur in aggregated spatial clusters. These asymptomatic carriers become rare by the following spring. Previous studies in our region and other affected areas indicate that phoretic *B. xylophilus* peak on early-flying *Monochamus* vectors during spring, suggesting this period as the prime time for pathogen inoculation into host pines (Atkins et al., 2021; Firmino et al., 2017; Togashi et al., 2023). Elevated summer temperatures promote PWN population growth, explaining their higher densities in pine canopies in late summer and fall (Pimentel & Ayres, 2018). In Mediterranean climates like our study area, summer drought stress exacerbates pine susceptibility, enhancing both *B. xylophilus*' pathogenicity and population expansion (Estorninho et al., 2022). The subsequent drop in PWN populations to undetectable levels by the following spring may reflect successful pathogen clearance by previously inoculated asymptomatic trees during winter. Supporting this hypothesis, of the four asymptomatic trees with detectable PWN in autumn that were resampled in the spring, three showed reduced nematode loads to undetectable levels, while only one exhibited increased levels. However, PWN was not detected in the remaining declining trees that persisted in the forest after winter logging operations, whereas most wilting trees in the fall had high nematode densities. This suggests that PWN abundances in the canopy are somewhat dependent on seasonal environmental variation. Thus, it appears that PWN can persist in the canopy of asymptomatic pines for some time, with PWD development heavily reliant on environmental factors. Research in Japan and North America has also shown that PWN can subsist for extended periods in asymptomatic pine hosts, potentially serving as wild reservoirs of the nematode (Atkins et al., 2021; Halik & Bergdahl, 1994; Hoshizaki et al., 2016).

Our study revealed significant prevalence and, at times, high abundance of PWN in tree canopies, highlighting the ecological importance of the phyllosphere for this pathogen. The nematode likely enters the canopy through maturation feeding by its beetle vectors (Naves et al., 2007), and the mycophagous *B. xylophilus* thrives on airborne fungi species inhabiting the pine canopy (Pimentel et al., 2021). These findings suggest this niche plays a critical role in the pathogen's life cycle, highlighting the need for further investigation of the canopy's contribution to PWN population dynamics and PWD development.

Notably, PWN was not detected in any sampled *P. pinea* trees, and although the sample size was small, this finding confirms the species resistance. These results align with previous studies, demonstrating higher resistance due production of higher levels of secondary metabolites than *P. pinaster*, which may inhibit PWN population growth and deter its insect vector (Pimentel et al., 2017; van Halder et al., 2022).

Conclusions and considerations regarding the management of forest decline

The PWN is a confirmed pathogenic agent of pine trees; maritime pine was proved to be very susceptible in inoculation experiments (Pimentel et al., 2017) and declining trees in the forest generally showed an increase in the numbers of the pathogen. However, in the present work, it was not possible to disentangle the direct effects of *B. xylophilus* from other agents of pine decline. PWN containment and eradication measures presently being enforced depend mostly on the detection of wilting trees, and assessment of the dispersion capacity of its insect vector (EU, 2012 and 2018). Criticisms of these measures have also been focused on uncertainties regarding the dispersion and transmission capacity of PWN by *Monochamus* vectors (de la Fuente et al., 2018; Robinet et al., 2010). However, PWN seems to be a highly adaptable pathogen, with a complex life-history, and forest decline is a complex phenomenon. Control measures currently in force do not account for some this complexity, most important: the reasonable probability of *B. xylophilus* subsisting in asymptomatic trees in the forest, at least for short periods of time; the complexity of its life-histories, including variations in virulence and the possibility of an alternative saprophytic lifestyle (Pimentel & Ayres, 2022; Togashi & Jikumaru, 2007); and climate related (e.g. drought) factors. Climate is crucial in this context, as PWD is exacerbated by worsening drought conditions, which are anticipated to get even worse with climate change, resulting in more frequent severe droughts. This factor must be considered in the PWN containment measures to prevent overwhelming the system, as has occurred in the recent past. Furthermore, although the vector is the major focus of control operations, only aspects related to timing and distance of dispersion have generally been considered. Community interactions are also determinant on the abundance and distribution of *Monochamus* within wilting pines. In fact, pine sawyers were found not to be particularly abundant within the wood borers community in southwest Portugal, only occurring in less than one third of declining trees (Pimentel et al., 2023). Thus, strategic salvage of wilting trees in invaded areas could effectively limit PWN spread while requiring removal of only a fraction of affected trees. This targeted approach would reduce operational costs and lower the risk of overwhelming the system. However, implementation would be challenging, as it requires better understanding of both Monochamus' host preferences and the influence of nematode phoresy. Additional research on vector-nematode interactions would be essential to develop such targeted control measures.

Current approaches to managing PWD epidemics tend to focus on a limited set of static variables, offering operational simplicity but potentially insufficient scope to address the complex socioecological dimensions of disturbance management. This challenge reflects broader patterns in forest disturbance management, where more adaptive frameworks have demonstrated value by incorporating stakeholder perspectives, cost–benefit analyses, and iterative evaluation of management outcomes (Ayres & Lombardero, 2018). Incorporating the One Health concept into PWD management could also offer significant benefits by (a) uncovering reservoirs and revising pathogen life histories, (b) elucidating drivers of virulence beyond the context of direct host–pathogen interactions, (c) accounting for the natural highways of long-distance dissemination, and (d) updating disease forecasts in response to changing land use, cultivation practices, and climate (Morris et al., 2022).

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Author's contributions C.S.P. conceptualized the research, acquired funding, defined the methodology, acquired, curated, and analysed the data, and wrote the original draft. D.P. and J.B assisted with conceptualization, methodology, and data acquisition. J.C. assisted with conceptualization, methodology, data acquisition, and analysis. R.M. assisted with data acquisition and analysis. M.M.M. conceptualized the research, acquired funding, provided resources, and supervision. T.C. conceptualized the research and methodology, acquired funding, acquired, curated, and analysed the data, and provided resources and supervision. All authors reviewed and edited the manuscript.

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Declarations

Conflict of interest All authors declare that they have no conflict of interest.

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