Sampling design for efficient detection of pine wood nematode, *Bursaphelenchus xylophilus*, in diseased trees using a DNA detection kit: variation across branch, trunk and tree

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Summary – Detection of pine wood nematode, *Bursaphelenchus xylophilus*, is fundamental for effective control of pine wilt disease. Recent molecular techniques, such as DNA detection, have enhanced detectability of the nematodes whereas appropriate field sampling has received less attention. In order to elucidate a sampling design that most efficiently detects *B. xylophilus* using a commercially-distributed DNA detection kit, we compared detection levels of *B. xylophilus* using wood chips taken from various positions on dead trees. Results showed that the DNA kit had a higher detection level than the conventional method, and that trunk samples had higher levels than branch samples. Statistical model revealed that among-tree variation influenced the detectability more strongly than within-tree variation. Our results suggest that, in practice, with limited resources for control, it is more efficient to take samples from many trees with a minimum number from each tree, rather than taking many samples from a small number of trees.

Keywords – foliage colour, nematode control, pine wilt disease, sampling efficiency.

Pine wilt disease is an epidemic disease of pine trees caused by the pine wood nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970 (Kiyohara & Tokushige, 1971), and vectored mainly by the insect *Monochamus alternatus* (Mamiya & Enda, 1972; Morimoto & Iwasaki, 1972). This disease has caused severe damage to pine forests in eastern Asia, including Japan, Korea and China, and recently has invaded Portugal and Spain (Kishi, 1995; Mota *et al.*, 1999; Abelleira *et al.*, 2011; Robertson *et al.*, 2011). The degradation of pine stands caused by heavy damage from this forest pest has led to a variety of concerns ranging from domestic forestry productivity through wood quality and the timber trade to cascading effects on species interactions and ecosystems (Mota & Vieira, 2008; Rodrigues *et al.*, 2010; Fukasawa, 2016).

In controlling the disease, detection of the infection source is fundamental for adopting various control methods. Classically, the detection of *B. xylophilus* has been based on the Baermann funnel technique (Southey, 1986), which extracts nematodes in water using wood chips taken

from the trunks of dead trees. However, this conventional method has some difficulties. First, when the nematode density is quite low nematode extraction is likely to be unsuccessful (Mamiya, 1975; Zhao *et al.*, 2009), leading to a ‘false negative’ result. Second, in dead pine trees a number of nematode species co-occur (Kiyohara, 1989), and species identification is thus necessary for any extracted nematodes. For the precise identification of species microscopic observations are required, which is only feasible using trained personnel.

Recently, several molecular-based detection methods for *B. xylophilus* have been developed (Matsunaga & Togashi, 2004; Takeuchi *et al.*, 2005; Leal *et al.*, 2007; Kikuchi *et al.*, 2009). These new methods can detect *B. xylophilus* faster and with a greater sensitivity than the Baermann funnel method and thus provide opportunities to mitigate the false negative results. Among those, the *Bursaphelenchus xylophilus* Detection Kit (Nippon Gene Co. Ltd, Tokyo, Japan), based on loop-mediated isothermal amplification (LAMP; Notomi *et al.*, 2000), amplifies only *B. xylophilus* DNA (Kikuchi *et al.*, 2009),

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and provides users with an easy and powerful tool for inspecting for *B. xylophilus* (Aikawa *et al.*, 2010). This DNA kit does not require particular training in molecular techniques, such as PCR, or species identification of nematodes, and is advantageous for users in terms of cost and necessary equipment compared with other molecular-based detection methods that require costly equipment (*e.g.*, real-time PCR).

However, in terms of practice, less attention has been paid to appropriate field sampling efforts for inspection *via* molecular diagnosis methods, in contrast to the Baermann funnel technique for which useful recommendations have been provided (Schröder *et al.*, 2009). In the case of the *Bursaphelenchus xylophilus* Detection Kit, several authors have found substantial horizontal (*i.e.*, among-tree) variation (Kanetani *et al.*, 2011; Hoshizaki *et al.*, 2016). It remains to be determined if inspection along a vertical direction helps to reduce false negatives. In addition, diagnosis *via* the DNA detection kit is based on only a few wood chips. The developers of the kit recommend testing each sample repeatedly (Aikawa *et al.*, 2010), but repetitions should preferably be minimised for practical reasons (*i.e.*, the detection kit is costly). Examination could thus be significantly improved by using an efficient sampling method that minimises the cost but provides a robust diagnosis.

In the present study, we first checked if the DNA detection kit really showed a higher diagnosis performance, *i.e.*, better nematode detection, than the conventional Baermann funnel method. Then we compared the detectability of *B. xylophilus* *via* the DNA detection kit, based on samples taken from various positions on diseased trees. Specifically, we performed the following comparisons: *i*) branch *vs* trunk; *ii*) green foliage *vs* brown foliage on branches; and *iii*) lower *vs* middle *vs* higher position of the trunk. Based on these, we evaluated what is the most efficient sampling design for users to detect pine wood nematodes using the DNA kit, thereby minimising false negatives.

Materials and methods

TEST TREES

The present study was undertaken in a coastal pine forest in Akita, northern Honshu, Japan (39°48'N, 140°02'E; 15–25 m a.s.l.; Hoshizaki *et al.*, 2005, 2016). The annual mean temperature in Akita City is 11.8°C, with a maximum of 25.0°C in August. Precipitation is 1708 mm year⁻¹ and falls as snow during December–March. Dam-

age in this forest has remained mild. We have continued to monitor tree damage in the study area on a monthly basis and the annual incidence of damaged trees has ranged from 0.1 to 2.4% (Ohta *et al.*, 2012). The flight season of the nematode vector *M. alternatus* is from late June to late September (infection period of the disease; Hoshizaki *et al.*, 2005). In this region, pine wilt disease invaded at least 20 years ago (Ohta *et al.*, 2012), and in recent years we have no record of *B. mucronatus*, which does not cause pine wilt.

In August 2012 we identified three test trees (Trees 1–3) and added an additional test tree (Tree 4) in September 2012. At the selection of the test trees, all were showing symptoms of the early stages of disease development; branches with green foliage and those with brown foliage were mixed within the tree crown, and oleoresin exudation was lost. We recorded the position of branches with green and brown foliage within the crown for each tree. The foliage colour in three out of the four test trees turned brown before all the test trees were felled in November (Table 1).

SAMPLING OF WOOD CHIPS

Within 2 weeks after the selection of each test tree, wood chip samples were taken from the trunk and branches (Fig. 1). For trunks, the wood chips were collected at 1.3, 4.0 and 7.0 m high by drilling into the trunk (*i.e.*, cardinal directions) from the surface (four samples per height per tree; *i.e.*, $n = 48$ for trunk samples). Each wood chip sample was sealed in a plastic bag. In this procedure, we rinsed the drill bit in 99% ethanol before each sample was collected to avoid cross-contamination of samples (Aikawa *et al.*, 2010). For branches, we cut off four primary branches (30 mm diam. at the base, two with green foliage and two with brown foliage) from each test tree, and cut off two twigs from each of the branches (eight twigs from each tree; in total, $n = 80$ samples). Cross-contamination of samples was avoided as described. The collected trunk and twig samples were incubated at 25°C for 4 weeks to facilitate nematode propagation. After incubation, each twig was sliced and sealed in a plastic bag (hereafter, 'branch sample').

For the tree that showed a low detection level (Tree 2, see Results section), the twigs and trunk were resampled 3 months after the first sampling to test if the first inspection suffered from a false negative result. In this second sampling, branch and trunk samples were collected in the same way as above ($n = 20$). The samples were incubated for nematode propagation in the same manner.

Table 1. Basic description of the four test trees (*Pinus* spp.) and the progress of pine wilt disease.

Observation parameter:	Tree 1	Tree 2	Tree 3	Tree 4
Species:	<i>P. densiflora</i>	<i>P. thunbergii</i>	<i>P. thunbergii</i>	<i>P. thunbergii</i>
Tree height (m)	12.5	18.4	16.7	20.1
Tree diam. (cm)	23.6	23.2	30.9	33.4
August 25				
Foliage colour ¹	B, G	B, Y, G	B, G	Not detected ³
% brown foliage ²	50	50	60	–
September 25				
Foliage colour	B	B, Y	B, G	B, G
% brown foliage	100	90	60	60
October 29				
Foliage colour	B	B	B, G	B
% brown foliage	100	100	80	100
November 21				
Foliage colour	B	B	B, G	B
% brown foliage	100	100	80	100

¹ B, brown; Y, yellow; G, green.² Percentage of branches with brown foliage.³ This tree was not recorded in August in our monthly monitoring of damaged trees (Ohta *et al.*, 2012). **Fig. 1.** The sampling scheme in each test tree.

NEMATODE DETECTION

To quantify *B. xylophilus* densities in the test tree, we adopted the Baermann funnel method, using approximately 5 g for each branch and trunk sample at 25°C for 48 h, and *B. xylophilus* individuals were counted after microscopic identification of species. The amount of wood chips per sample was determined based on findings of Mamiya (1975), in which 4 g of wood chips was sufficient. The total amount of wood chips per tree (100 g tree⁻¹) was much larger than the minimum level recommended for the diagnosis for a tree (>60 g tree⁻¹; Schröder *et al.*, 2009). The detection trial was conducted once for each sample (n = 80 for first diagnosis for all trees; n = 20 for the second diagnosis for Tree 2).

For molecular detection of *B. xylophilus* we used the *Bursaphelenchus xylophilus* Detection Kit. This detection kit amplifies *B. xylophilus*-specific DNA sequences through LAMP (Notomi *et al.*, 2000), and then a fluorescence reaction is used for confirmation of the amplified DNA (Kikuchi *et al.*, 2009; Aikawa *et al.*, 2010). Compared with PCR-based nematode detection (*e.g.*, Takeuchi *et al.*, 2005), this LAMP-based detection kit enables detection of *B. xylophilus* more quickly (*ca* 90 min. including DNA extraction) (Kikuchi *et al.*, 2009). The amplification is highly sensitive (Kikuchi *et al.*, 2009), and only two wood chips (*ca* 0.12 g) are used for a single diagnosis trial. This high sensitivity, in turn, suggests a risk of false negatives due to the small amount of wood chips. Therefore, we carried out four trials for each sample.

As described in the instructions for the kit, nematode DNA was extracted by soaking wood chips in 800 µl of extraction buffer (provided in the kit) and incubating them at 55°C with shaking (65 rpm) for 30 min. The extraction was stopped by heat shock at 100°C for 10 min. Then, to perform the LAMP reaction, the extracted DNA solution (2 µl) and a kit-provided reaction buffer (18 µl) was incubated at 63°C for 90 min. The LAMP reaction was stopped by heat shock at 80°C for 2 min. After the reaction, we checked if *B. xylophilus* DNA was present ('positive'), based on fluorescence. For the test Tree 2, which showed the lowest level of *B. xylophilus* detection in the first sampling, additional tests were conducted in the same way using wood chips taken in the second sampling. Again, in order to avoid contamination by *B. xylophilus* DNA, we kept the laboratory clean, *e.g.*, by wiping the laboratory bench with sodium hypochlorite before every detection trial.

To assure that DNA of *B. xylophilus* was precisely detected in our laboratory protocol, the detection kit was

also applied to solutions with suspended microscope-identified nematodes, and we confirmed that our diagnosis resulted in 'positive' for the solution with *B. xylophilus* but 'negative' for the solution with other species of *Bursaphelenchus* nematodes.

DATA ANALYSES

Analysis 1

First, we compared the two detection methods (Baermann funnel method *vs* DNA detection kit) to test the sensitivity of nematode detection. Because our sampling was nested within each test tree, we adopted generalised linear mixed models (GLMM). The mixed model, in which random effect(s) are incorporated, is powerful in analysing data with nested/skewed structures avoiding pseudo-replication (Grafen & Hails, 2002; Bolker *et al.*, 2009). We examined both detection levels and nematode densities for each sample.

In Analysis 1, the response variable for detection level was the frequency of positive samples, the explanatory variable (fixed effect) was the detection method, and the random effect was tree identity. For nematode density, the response variable was the number of nematodes detected, specifying dry weight of sample wood chips as an offset term, and the same for others for the detection level. For all model calculations, R ver. 2.15.3 (R Core Team, 2013) and package 'lme4' were used.

Analysis 2

In this analysis, we compared the detection level and nematode density between sampling positions in the trees. The detection level was based only on the DNA detection kit, because the results of Analysis 1 showed a significantly higher detection with the DNA detection kit than the Baermann funnel method. We adopted GLMMs with similar structures and parameters and examined the effects of 'branches *vs* trunk', 'foliage colour (green foliage *vs* brown foliage) of the branch and 'sampling height' (1.3 m *vs* 4 m *vs* 7 m) of the trunk (see Table 2 for complete model descriptions). For Tree 2, for which the result of the above model might have suffered from false negatives, we ran additional GLMMs, using pooled data for first and second sampling, in order to test whether the timing of tissue sampling (at early *vs* later stage) affected the detection.

Finally, to compare the extent of variation at the individual tree level (*i.e.*, difference in means) and that of variation among positions within the tree, variance component analysis was performed using Analysis 2. We

Table 2. Structures, sample sizes, and estimated variance components of generalised linear mixed models in Analysis 2.

Response variable	Fixed effect	Random effects ¹	Parameter distribution	Link function	n	Variance component	
						Among parts within tree	Among trees
Stem <i>vs</i> branch							
Detection level	Branch <i>vs</i> stem	Tree/part	Binomial	Logit	80	1.64	4.20
Nematode density ²	Branch <i>vs</i> stem	Tree/part	Poisson	Log	80	10.62	7.18
Foliage colour (branch)							
Detection level	Brown <i>vs</i> green	Tree/branch	Binomial	Logit	32	1.22	5.59
Nematode density	Brown <i>vs</i> green	Tree/branch	Poisson	Log	32	17.28	5.22
Sampling height (stem)							
Detection level	1.3 m/4 m/7 m ³	Tree/stem direction	Binomial	Logit	48	1.49×10^{-15}	7.34
Nematode density	1.3 m/4 m/7 m	Tree/stem direction	Poisson	Log	48	2.78	8.37

¹ A random effect 'part' ('branch' or 'stem direction' (N, E, S or W at each stem height)) was nested within 'tree'.

² Sample dry weight (DW) was specified as an offset term.

³ 1.3 m was set as the baseline.

calculated variance components based on the deviance values in each model, and compared the degree of different sources of variation.

Results

ADVANTAGE OF THE DNA DETECTION KIT

The densities of *B. xylophilus* varied greatly among the test trees (Fig. 2). The density level for Tree 3 was highest (mean = 365 nematodes (g DW)⁻¹; range = 0-2760), whereas that for Tree 2 was low (mean = 15.9; range = 0-295).

The overall level of *B. xylophilus* detection using the Baermann funnel method and the DNA detection kit was 66.3% (53/80 samples) and 81.3% (65/80 samples), respectively. The difference was statistically significant (Analysis 1; $z = 2.92$, $P = 0.004$). The detection level was highest for Tree 1, followed by Tree 4 (Table 3). Tree 2 showed lowest level of detection in both methods (Table 3). For Tree 2, there was no significant difference in the detection levels between the two sampling timings ($z = 1.23$, $P = 0.22$). For complete results on detection levels and densities of *B. xylophilus*, see Tables S1-S3 in the Supplementary material.

The detection kit revealed *B. xylophilus* infection in 14 (51.9%) out of 27 samples for which *B. xylophilus* was not found using the Baermann funnel method. In the reverse comparison, only two samples were found to be positive for *B. xylophilus* infection via the Baermann funnel

method out of the 53 samples for which the detection kit result was negative for *B. xylophilus* infection.

EFFECTS OF SAMPLING POSITION ON DETECTABILITY

Based on the DNA kit, the mean detection for trunk samples ranged from 88 to 100% for Trees 1, 3 and 4, whereas it was 27% for Tree 2 (Fig. 3A). On the other hand, branch samples showed a variety of detection levels: low (Tree 2), moderate (Tree 3) and high (Trees 1, 4) (Fig. 3A). The detection levels were on average 2.2 times higher for trunk samples than for branch samples from the same tree (Analysis 2; $z = 3.47$, $P < 0.001$). There was no significant difference in the density of *B. xylophilus* between branch and trunk samples (Analysis 2; $z = 0.70$, $P = 0.48$) (Fig. 2A).

The variance component analysis revealed that variation in the detection of *B. xylophilus* originated largely from among-tree variation ($\text{Var}_{\text{among trees}} = 1.6 < \text{Var}_{\text{among branches}} = 4.2$; Table 2), whereas the source of variation in *B. xylophilus* densities was primarily from among-branch variation ($\text{Var}_{\text{among branches}} = 10.6 > \text{Var}_{\text{among trees}} = 7.2$; Table 2).

Branch samples differing in foliage colours showed no significant trend in the detection level ($z = -1.0$, $P = 0.30$; Fig. 3B). This was almost the same for the densities of *B. xylophilus* ($z = -0.06$, $P = 0.96$), although branches with green foliage from Tree 3 had higher *B. xylophilus* densities (Fig. 2B). The variance component analysis showed a similar trend for both nematode detection ($\text{Var}_{\text{among twigs on the same-colour branch}} =$

Fig. 2. Comparison of densities of *Bursaphelenchus xylophilus*. A: Branches vs trunk; B: Green vs brown foliage branches; C: 1 m vs 4 m vs 7 m (trunk). Bars represent the mean density of nematodes, and vertical lines represent standard error.

$1.2 < \text{Var. among trees} = 5.6$) and nematode density ($\text{Var. among twigs on the same-colour branch} = 17.3 > \text{Var. among trees} = 5.2$) (Table 2).

Detection levels for samples taken from differing heights were generally high (>80% detection; Fig. 3C), except for Tree 2 (6.2–37.5%). The effect of sampling height (4 m and 7 m relative to 1.3 m as the baseline) was marginally significant ($z = 1.80$, $P = 0.07$ for both). As for *B. xylophilus* densities, samples taken from 1.3 m high had greater densities than samples taken from 4 m high ($z = -26.5$, $P < 0.001$) and had

Fig. 3. Comparisons of percentages of samples showing a positive reaction for *Bursaphelenchus xylophilus* using the DNA detection kit. A: Branches vs trunk; B: Green vs brown; C: 1 m vs 4 m vs 7 m. Vertical lines represent standard error.

lower density than those taken from 7 m ($z = 66.1$, $P < 0.001$) (Fig. 2C). Analysis of variance components revealed that variation in both the detection and density of *B. xylophilus* originated mostly from among-tree variation ($\text{Var. among replicates within each height} = 1.5 \times 10^{-15} \ll \text{Var. among trees} = 7.3$ for detection level; $\text{Var. among replicates within each height} = 2.8 < \text{Var. among trees} = 8.4$ for nematode density) (Table 2).

Table 3. Detection levels of *Bursaphelenchus xylophilus* using the Baermann funnel technique and the DNA detection kit. Detection level is based on 20 samples for each test-tree.

Tree ID	Baermann funnel technique		DNA detection kit	
	No. of 'positive' samples	Detection level (%)	No. of 'positive' samples	Detection level (%)
1	19	95.0	20	100.0
2	3	15.0	7	35.0
3	14	70.0	18	90.0
4	17	85.0	20	100.0
Total	53	66.3	65	81.3

Discussion

In the present study, we examined the effectiveness of a DNA-based *B. xylophilus* detection kit in conditions in which the density of *B. xylophilus* varied greatly among host trees. Of the four trees examined, Tree 2 exhibited much lower *B. xylophilus* densities than other trees. For all the trees, higher detection levels were observed when the DNA-based kit was applied than when the Baermann funnel method was applied. For Tree 2, both detection and nematode density were lower than for the three other trees, and the difference between the two detection means was remarkable.

Before interpreting these results, we briefly examined whether our results suffered from false positive results, because even when the Baermann funnel method failed to detect *B. xylophilus*, the diagnosis with the detection kit showed positive reactions in >50% of those samples and because this might occur due to the high sensitivity of the kit (Kikuchi *et al.*, 2009). However, this possibility is very unlikely; in our previous paper, the same sampling protocol was adopted in both the field and laboratory, and we had positive results in only 1.5% of >800 samples with the same DNA detection kit (Hoshizaki *et al.*, 2016). This percentage may not be obtained if we suffer from cross-contamination of DNA. Because we paid the same level of care to avoid cross-contamination in the laboratory in both the previous and present studies, false positives can be excluded in the present study.

In Analysis 1, although higher detectability was found for the detection kit, the detection level for Tree 2 was less than 40%. This was consistent with the notion that false negatives may be inevitable when detecting *B. xylophilus* from trees with low nematode density (Mamiya, 1975) and suggested, in turn, the importance of efficient design of wood chip sampling in minimising false negatives.

In Analysis 2, trunk samples showed significantly higher detection levels than branch samples. This may reflect the migration habits of *B. xylophilus* after infection in a host tree. Because the nematodes usually move from infected branches to the stem (Kiyohara & Suzuki, 1978; Zhao *et al.*, 2009), so it may be assumed that variance in *B. xylophilus* density (and presence, too) may be smaller for samples taken from the stem than branch samples.

Foliage colour had no significant effect on the detection level. Intuitively, we might expect that the detection level would be higher for brown foliage branches than for green foliage branches because the population of infected *B. xylophilus* is likely to increase drastically after wilting of the host tree becomes apparent (Kiyohara & Suzuki, 1978). However, based on our results, the reverse was true at the early stage of disease symptoms. This paradoxical phenomenon could be explained based on discordance between the positions at which *B. xylophilus* infection occurs and those in which wilting is triggered. In the first phase of disease symptom development, *B. xylophilus* is likely to infect pine trees in the branches and then move down to the stem. Settlement of *B. xylophilus* individuals in the stem often leads to xylem cavitation at the second phase, resulting eventually in wilting of the tree (Fukuda, 1999). Importantly, the branch experiencing wilting, *i.e.*, having discoloured foliage, may be determined by the position at which the cavitation occurs in the stem, not by the position at which infection occurs. Therefore, in the present study the branches with discoloured foliage were independent of the branch with *B. xylophilus* infection. Although *B. xylophilus* was frequently detected from trunk samples in our results, xylem cavitation was inferred to have occurred partially in the stem of the test trees.

The effect of sampling height was marginally significant. This result has several non-decisive implications in practice. Sampling at 1.3 m high would be advantageous

in terms of labour cost, but ‘negative’ results might be due to false negatives. Resampling at a higher position would be better when a negative result is obtained at a lower height. On the other hand, access to 4 m or higher on the trunk may be costly. If access to higher positions can be obtained easily, samples taken from higher positions could be better. In other words, the decision on sampling height may depend upon the forest manager(s).

Our results suggest that sampling position could be better replicated, but when we have a closer look at our data (Tables S1–S3), diagnosis for a single sample is mostly 4/0 or 3/1 (denoted as ‘positive’/‘negative’ trials), implying a marginal effect of repetition for *B. xylophilus* detectability. Within-tree variation in *B. xylophilus* densities in pine trees has been investigated in detail, where the densities varied both within a tree along the vertical direction and among branches (Kiyohara, 1989; Zhao *et al.*, 2009; Hoshizaki *et al.*, 2016). In a case study using the same DNA detection kit as in the present study, detection levels varied among positions in the wood samples used (Kanetani *et al.*, 2011). These findings suggest the importance of replication of wood chip samples for accurate diagnosis for a given tree. However, in our GLMMs for detection level, among-tree variation was consistently larger than within-tree variation (Table 2). This indicates that tree identity is more influential for the results than within-tree positions. In other words, prioritising efforts to increase the number of trees to be examined could be more effective compared with devoting efforts to increasing the number of samples per tree. This is in accordance with the general recommendation of Schröder *et al.* (2009) (preferably five trees per site), although it should be noted that the number of trees to be taken for diagnosis may depend upon the disease status of the forest (Schröder *et al.*, 2009) and the climate of the site (Zinno *et al.*, 1987; Ohta *et al.* 2012).

In practice, resources for control, *e.g.*, funding or labour costs, are usually constrained. Based on our results, and in accord with the general recommendations (Schröder *et al.*, 2009), we recommend the following for efficient sampling for the detection of pine wilt disease; first, taking wood chips (at least 60 g tree⁻¹) from the trunks of as many trees as possible, and the sampling is better from higher positions (4 m or higher). A higher sampling position is not mandatory, keeping on the ‘safe side’, but false negatives cannot be discounted when nematode density is quite low. Second, diagnosis should preferably be based on the DNA detection kit with repetition (sampling with four repetitions is acceptable).

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Table S1. Densities of nematodes (*Bursaphelenchus xylophilus* and other species) based on the Baermann funnel technique and diagnosis results of *B. xylophilus* infection based on the DNA detection kit (denoted as 'positive'/'negative' trials) for branch samples.

Tree ID	Foliage colour	No. of branches	No. of twigs	<i>B. xylophilus</i> (nematodes (g DW) ⁻¹)	Level of other species ¹	DNA detection kit
1	Green	1	1	113	—	4/0
			2	2	—	4/0
		2	1	769	—	4/0
			2	668	—	4/0
	Brown	1	1	218	—	4/0
			2	734	—	4/0
		2	1	10	—	2/2
			2	18	—	4/0
2	Green	1	1	0	++	0/4
			2	0	+	0/4
		2	1	0	+	0/4
			2	0	+	0/4
	Brown	1	1	295	+	2/2
			2	9	++	0/4
		2	1	0	+	0/4
			2	0	++	0/4
3	Green	1	1	2005	+	2/2
			2	665	+	4/0
		2	1	0	—	1/3
			2	0	—	2/2
	Brown	1	1	0	—	0/4
			2	0	—	2/2
		2	1	1	—	0/4
			2	0	—	1/3
4	Green	1	1	22	+	4/0
			2	406	+	4/0
		2	1	12	+	3/1
			2	29	+	3/1
	Brown	1	1	49	—	3/1
			2	284	—	2/2
		2	1	2	—	4/0
			2	7	+	4/0

¹ +++, >500 nematodes; ++, 50-500 nematodes; +, <50 nematodes; —, Not detected.

Table S2. Densities of nematodes (*Bursaphelenchus xylophilus* and other species) based on the Baermann funnel technique and diagnosis results of *B. xylophilus* infection based on the DNA detection kit for trunk samples.

Tree ID	Sampling height (m)	Side	<i>B. xylophilus</i> (nematodes (g DW) ⁻¹)	Level of other species ¹	DNA detection kit
1	7.0	N	4	+	3/1
		E	43	+	4/0
		W	4	+	3/1
		S	18	+	4/0
	4.0	N	89	++	4/0
		E	18	+	4/0
		W	17	–	4/0
		S	15	+	3/1
	1.3	N	4	–	2/2
		E	10	+	4/0
		W	0	–	3/1
		S	5	–	4/0
2	7.0	N	0	+	0/4
		E	13	+	4/0
		W	0	–	0/4
		S	0	+	2/2
	4.0	N	0	–	2/2
		E	0	+	0/4
		W	0	+	3/1
		S	0	+	0/4
	1.3	N	0	–	0/4
		E	0	–	0/4
		W	0	–	0/4
		S	0	–	0/4
3	7.0	N	214	+	4/0
		E	513	+++	4/0
		W	2760	+	4/0
		S	166	++	4/0
	4.0	N	233	++	4/0
		E	29	–	4/0
		W	269	+	4/0
		S	229	+	4/0
	1.3	N	528	+	4/0
		E	100	++	4/0
		W	303	+++	4/0
		S	96	+	4/0
4	7.0	N	1033	+	4/0
		E	90	+	4/0
		W	3	+++	4/0
		S	0	+++	4/0
	4.0	N	0	+	4/0
		E	3	+++	4/0
		W	7	+	4/0
		S	0	++	3/1
	1.3	N	26	+	4/0
		E	34	+	4/0
		W	73	+	4/0
		S	726	+	4/0

+++ , >500 nematodes; ++ , 50-500 nematodes; + , <50 nematodes; – , Not detected.

Table S3. Nematode densities based on the Baermann funnel technique and diagnosis results of *Bursaphelenchus xylophilus* infection based on the DNA detection kit for Tree 2, along with differing timings of sampling.

Foliage colour ¹ or sampling height (m)	No. of branches	No. of twigs	Side ²	<i>B. xylophilus</i> (g DW) ⁻¹		Level of other species		DNA detection kit	
				Early symptom	After 3 months	Early symptom	After 3 months	Early symptom	After 3 months
A. Branch									
Green	1	1	NA	0	0	++	+	0/4	0/4
		2	NA	0	0	+	+	0/4	0/4
	2	1	NA	0	0	+	+	0/4	0/4
		2	NA	0	0	+	+	0/4	0/4
Brown	1	1	NA	295	0	+	—	2/2	0/4
		2	NA	9	0	++	++	0/4	0/4
	2	1	NA	0	0	+	++	0/4	0/4
		2	NA	0	0	++	+	0/4	0/4
B. Trunk									
7.0	NA	NA	N	0	0	+	—	0/4	0/4
	NA	NA	E	13	0	+	+	4/0	4/0
	NA	NA	W	0	24	—	—	0/4	1/3
	NA	NA	S	0	35	+	—	2/2	4/0
4.0	NA	NA	N	0	5	—	—	2/2	3/1
	NA	NA	E	0	0	+	—	0/4	0/4
	NA	NA	W	0	33	+	—	3/1	4/0
	NA	NA	S	0	0	+	—	0/4	0/4
1.3	NA	NA	N	0	0	—	—	0/4	4/0
	NA	NA	E	0	2	—	—	0/4	1/3
	NA	NA	W	0	0	—	+	0/4	0/4
	NA	NA	S	0	0	—	—	0/4	0/4

¹ Colour in early symptoms.² Injection side (see Fig. 1).