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Relationship between resistance to pine wilt disease and the migration or proliferation of pine wood nematodes

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Abstract To study the relationship between resistance to pine wilt disease and the migration or proliferation of pine wood nematodes (PWN) (*Bursaphelenchus xylophilus*), we conducted experiments using clonally-propagated Japanese black pines (*Pinus thunbergii*) with pre-evaluated individual resistance levels. Bark including the cortical resin canals—one of the main migration pathways of PWN—was removed by girdling, but neither the migration of PWN nor symptom development of pine wilt disease were inhibited by this treatment in non-resistant clones. Histological observations showed no significant differences in the lumen area or the number of cortical- and xylem- axial resin canals between resistant and susceptible clone groups from a half-sib family. A bioassay using methanol extracts from resistant and susceptible clones showed that extracts from both clones showed similar attractant effects to PWN, but neither had repellent effects. The resistant clones were multi-inoculated with PWN into three split points to mimic migration in the stem. The proportion of damaged plants was not significantly different from that in single-inoculated plants (control). In this experiment, the number of PWN detected from partially-damaged plants was much higher than that from non-damaged plants. An inoculation test using stem cuttings showed that the population of PWN increased in susceptible cuttings at 1–20 days after inoculation (dai), while it remained unchanged or gradually decreased in resistant cuttings. These findings suggested that the factors contributing to resistance were associated with inhibiting the proliferation of PWN, rather than inhibiting their migration.

Keywords *Bursaphelenchus xylophilus* · Clonal propagation · Pine wilt disease · *Pinus thunbergii* · Resistance

Abbreviations

Dai	days after inoculation
LSD	least significant difference
PWN	pine wood nematodes
SDW	sterilized distilled water

Introduction

Japanese black pine (*Pinus thunbergii*) forests have been seriously damaged by pine wilt disease, which is caused by pine wood nematodes (PWN), *Bursaphelenchus xylophilus*. The area of damaged pine forests

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has already spread to East Asia, including Japan, China, Taiwan, and Korea (Sun 1982; Kishi 1995; La et al. 1998). In 1999, PWN were detected for the first time in Europe, from damaged *P. pinaster* trees in Portugal (Mota et al. 1999). Pine wilt disease has thus become a threat to Asian and European pine forests.

In Japan, a breeding project to obtain Japanese black pine resistant to PWN began in 1978 (Fujimoto et al. 1989). To date, resistant seedlings have been produced commercially from the seed orchard composed of grafting clones selected in the project. Recently, resistant plants have been efficiently produced by clonally propagating the resistant seedlings through cuttings (Sasaki et al. 2004; Mori et al. 2004, 2006a, b). We showed that clonally-propagated plants retained high resistance against two virulent PWN isolates (Mori et al. 2004, 2006a, b). However, the mechanisms of defence or resistance against PWN are poorly understood. It is, therefore, difficult to predict whether these plants will retain resistance against various PWN isolates during their entire growth and development period. It seems important for the regeneration of damaged pine forests to identify the factors involved in resistance, and to elucidate the resistance mechanisms.

According to previous reports, the PWN population increased dramatically in susceptible pine species (e.g. *P. sylvestris*, *P. thunbergii*, *P. densiflora*) after inoculation; however PWN were detected in smaller numbers in resistant pine species (e.g. *P. resinosa*, *P. rigida*, *P. taeda*) (Bedker et al. 1987; Oku et al. 1989). Kuroda (2004) reported that the PWN population remained lower in the stem of selected PWN-resistant Japanese red pine (*P. densiflora*) seedlings than in the non-selected seedlings of the same species. These reports suggest that proliferation of PWN is inhibited in resistant pine trees. On the other hand, Oku et al. (1989) examined migration of PWN in resistant or susceptible pine stem cuttings (5 cm-long). They observed that none of the 200 inoculated PWN migrated through the cuttings of a resistant species (*P. rigitaeda*), while approximately 30 PWN migrated through those of a susceptible species (*P. thunbergii*). Kuroda et al. (1991) reported that PWN were distributed around the base of inoculated branches of resistant species (*P. taeda*) at early stages, and took three weeks to migrate throughout the trunk. In contrast, PWN were distributed throughout the trunk only one week after inoculation in susceptible species (*P. thunbergii*) (Kuroda et al. 1991). These reports suggest that PWN can migrate rapidly or readily in susceptible pine trees, but cannot do so in resistant pine trees.

Thus, it is likely that factors inhibiting proliferation or migration of PWN are related to resistance in pine trees. Suga et al. (1993) screened methanolic fractions with nematicide activity from branches of resistant species (*P. massoniana*, *P. strobus*, *P. palustris*) and susceptible species (*P. thunbergii*, *P. sylvestris*), and found that pinosylvin monomethyl ether was commonly detected in heartwood of the three resistant species. Hanawa et al. (2001) found that 3-*O*-methyldihydropinosylvin isolated from inoculated branches of a resistant species (*P. strobus*) had activity to immobilize PWN. Accordingly, stilbenoids are considered to be one of the metabolites related to resistance in pine trees. On the other hand, Kawaguchi (2006) stated that the lumen area of cortical resin canals was closely related to the migration of PWN and resistance in *P. thunbergii*, and was significantly larger in non-selected seedlings than in resistant family seedlings. Moreover, there was a positive correlation between the lumen area of cortical resin canals and the number of PWN that migrated through the 10 cm-long cutting (Kawaguchi 2006). Kuroda (2004) speculated that the multibranched, shoot-rich features of resistant families of *P. densiflora* might be one of the factors related to resistance, because disorientation of resin canals at the joints between branches and the main stem might disrupt migration of PWN.

Seedlings from resistant and susceptible pine species (or families) have been used as test plants to identify the characteristics that differ between resistant and susceptible pine trees. These seedlings have been used for experiments without evaluation of their individual resistance level, yet the resistance level of seedlings greatly varies even among individuals within the same family (Goto et al. 2002; Mori et al. 2004, 2006a, b). Therefore, it is possible that some of the different characteristics observed between resistant and susceptible seedlings might be attributed to species- or family-specific factors but not to resistance factors.

To determine resistance factors, we focused on evaluating the individual resistance levels of test plants using clonally-propagated *P. thunbergii* plants (Mori et al. 2004, 2006a, b). Because they are genetically uniform and reproducible, we can pre-evaluate each plant's individual resistance level by determining the

damaged proportion of distinct clonal ramets prior to examination. In this study, we used these test plants to examine whether inhibiting the migration or proliferation of PWN affected resistance.

Materials and methods

Pine wood nematodes

A virulent PWN isolate, Ka-4, (Aikawa et al. 2003) and a moderately virulent PWN isolate, Shimabara, (Fujimoto et al. 1989) were gifts from the Forestry and Forest Products Research Institute. Both strains were cultured on *Botrytis cinerea* grown on autoclaved barley grains in Petri dishes for 7–10 days at 25°C in the dark, and were isolated using the Baermann funnel technique (Thorne 1961).

Evaluation of clonally-propagated plant resistance levels

At the Fukuoka Prefecture Forest Research and Extension Centre, *P. thunbergii* seedlings from the seed orchard were grown and clonally propagated by cutting. To evaluate the individual resistance level of test plants, 2 year-old clonal plants (ramets) propagated from each seedling were inoculated with 5,000 PWN (Shimabara). At six months after inoculation, each clone was examined to assess the damaged proportion (total amount of discoloured foliage, wilted or dead ramets/number of inoculated ramets). If the damaged proportion of a clone was <20%, the clone was evaluated as resistant; if it was >90%, the clone was evaluated as susceptible.

Inoculation experiment with or without girdling

Fifteen 2 year-old ramets propagated from five non-resistant clones (damaged proportion: 57.4–100%) were divided into three groups to obtain uniform genetic composition among the groups. In the first group, a 2 cm-wide strip of bark in the middle of a current-shoot was removed (girdling), then 50 μl of 5,000 PWN (Ka-4) suspension was inoculated into the apical side point 3 cm away from the debarked area (girdling + PWN). In the second group, 50 μl of 5,000 PWN (Ka-4) suspension was inoculated into the middle portion of a current-year shoot without girdling (non-girdling + PWN). In the third group, 50 μl of sterilized distilled water (SDW) was inoculated into the apical side point 3 cm away from the debarked area by girdling (girdling + SDW). The debarked areas were covered with petroleum jelly to prevent passage of PWN across them (Ishida et al. 1993). Symptom development was visually inspected six months after inoculation. To examine whether PWN can migrate over the debarked area, 3 cm-long segments of each ramet (girdling + PWN) were harvested from both apical side (including the inoculation point) and basal side points 3–6 cm from the debarked area at 55–68 days after inoculation (dai). Bark or xylem from the segments was cut into small pieces separately. PWN were isolated from the pieces by the Baermann funnel technique (Thorne 1961), and the number of living PWN per segment was counted under a stereomicroscope.

Histological observation of axial resin canals

Samples were harvested from the middle of a current-year shoot of seven resistant (damaged proportion: 0–15.4%) or susceptible (damaged proportion: 92.9–100%) 13 year-old clones in a half-sib family. The samples were fixed in FAA (37% formalin, 99.7% acetic acid, 50% ethanol; 5:5:90 *v*/*v*/*v*) for 48 h at 4°C, dehydrated through an alcohol series (60%, 70%, 80%, 90%, 95%, 99.5%), cleared in chloroform, and then embedded in paraffin. A sliding microtome was used to cut 20 μm-thick cross-sections, which were stained with safranine and mounted on glass slides with Canada balsam. Slides were observed under a light microscope (×40), and three parameters of axial resin canals in the cortex and xylem were measured; the number of resin canals mm^{-2}, total lumen area of resin canals mm^{-2}, and average lumen area of a resin canal. Images were analyzed with WinROOF 5.03 software (Mitsutani Shoji Co. Ltd.).

Bioassay of methanol extracts from *P. thunbergii* with PWN

Stem cuttings (21 cm-long) were harvested from current-year shoots of a resistant clone (damaged proportion: 0/9=0%) and a susceptible clone (damaged proportion: 26/28=92.9%). Each cutting was cultured in water-absorbable polyurethane foam (11×4×4.75 cm; Toyo Quality One Co. Ltd) in a plastic

container (40×32×15 cm) filled with distilled water. They were placed in a growth chamber (KG-50HLA, Koito Industries Co. Ltd) at 28°C and 70% relative humidity, under a regime of 14 h light: 10 h dark. After pre-incubation for three days, they were inoculated with 5,000 PWN (Ka-4). At 6 dai, bark and xylem from each cutting was recovered separately and homogenized (Milser, IFM-600D, Iwatani International Corporation). The homogenates were extracted with methanol for 24 h at room temperature, and the extracts were concentrated with a rotary evaporator. The bioassay was carried out using methods described previously (Futai 1979) with some modifications. Methanol extracts from each sample (10 μl of 10 mg ml^{-1}) were spotted onto filter paper (No.707, 8 mm diam). An equal volume of methanol was spotted on the filter paper as a solvent-only control. Filter paper spotted with neither methanol nor extracts was used as a blank. The papers were placed on 1.5% agar plates, as shown in Fig. 1. A piece of absorbent cotton (1×1 cm) containing 5,000 PWN (Ka-4) was placed on the centre of each plate. After incubation for 24 h at 25°C in the dark, an agar disk beneath each filter paper was punched out using a cork borer (12 mm diam). PWN were isolated from both agar disks and filter paper, and counted as described above. The arrangement of each filter paper containing extracts in three different assays is shown in Fig. 1.

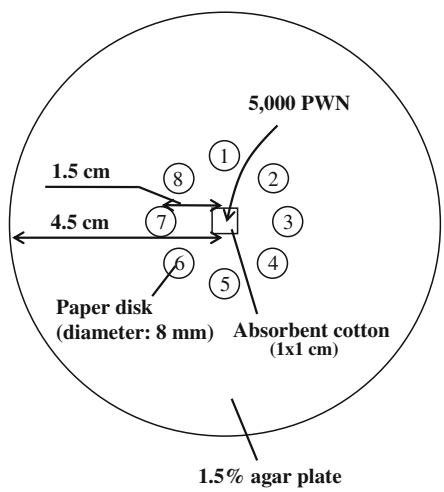

Fig. 1 Arrangement of paper disks spotted with methanol extracts from resistant or susceptible *Pinus thunbergii* on a 1.5% agar plate. Assay I: 1=extract from bark of resistant clone, 5=extract from xylem of resistant clone, 3,7=solvent-only control, 2,4,6,8=blank. Assay II: 1=extract from bark of susceptible clone, 5=extract from xylem of susceptible clone, 3,7=solvent-only control, 2,4,6,8=blank. Assay III: 1=extract from bark of resistant clone, 3=extract from xylem of resistant clone, 5=extract from bark of susceptible clone, 7=extract from xylem of susceptible clone, 2,4,6,8=solvent-only control

Effect of multiple inoculations on symptom development

Fifty ramets (2 to 4 year-old) propagated from eight resistant clones (damaged proportion: 0–15.4%) were divided into two groups to obtain uniform genetic composition between the groups. The three inoculation points were 3 cm (upper), 8 cm (middle), or 13 cm (lower) away from the apical bud of a current-shoot in both groups. In the first group, 50 μl of 5,000 PWN (Ka-4) suspension, 50 μl of SDW, and 50 μl of SDW were inoculated into upper, middle and lower points, respectively (control). In the second group, 50 μl of 1,667 PWN suspension was equally inoculated into each point (5,000 PWN in total). Splitting of the inoculation among three points, i.e. 'split-inoculation', was designed to mimic the migration of 5,000 PWN within the stem. Symptom development was visually inspected six months after inoculation. Three ramets were selected from each of the partially-damaged or non-damaged ramets in each group for determining the distribution of PWN around the inoculation points. PWN were isolated from 5 cm-long segments (including each inoculation point) of the selected ramets, and counted as described above.

Inoculation experiment using stem cuttings

Stem cuttings (21 cm length) were harvested from current-year shoots of two resistant clones (damaged proportion: 0–3.3%) or two susceptible clones (damaged proportion: 100%) in a half-sib family. Six cuttings per clone were inserted into the water-soaked polyurethane foam, and cultured in the growth chamber under the conditions described above. After pre-incubation for 4 days, 50 μl of 5,000 PWN (Ka-4) suspension was inoculated into a point 4 cm from the apical bud. To determine distribution of PWN, two cuttings per clone were harvested at 24 h, 6 days, and 20 dai. PWN were isolated from three segments per cutting, each 3 cm long, taken from the proximal point (0–3 cm away from the inoculation point towards the apical bud), the middle point (5–8 cm away from the inoculation point towards the basal end), and the distal point (13–16 cm away from the inoculation point towards the basal end).

Results

Effects of girdling on inhibition of pine wilt disease in non-resistant clones

To examine the role of cortical resin canals in pine wilt disease development, the symptoms in girdled ramets were compared with those in non-girdled ramets. All ramets inoculated with PWN were damaged, irrespective of girdling (Table 1), while girdled ramets were not damaged by inoculation with SDW. The distribution of PWN in girdled ramets was observed. Our results showed that more PWN were detected from the basal side segment over the debarked area than from the apical side segment including the inoculation point, except for the clone '12-03'.

Relationship between histological characteristics of axial resin canals and resistance

To examine whether histological characteristics of axial resin canals were related to resistance, we compared cortical- and xylem- resin canals between resistant and susceptible clone groups from a half-sib family. Three parameters were measured; the number of resin canals mm^{-2}, total lumen area of resin canals mm^{-2}, and average lumen area of a resin canal. There were no significant differences between the two groups in sectional areas of cortex or xylem (*t*-test, $P>0.33$) (Table 2). In fact, no significant differences between the two groups were observed among all parameters of axial resin canals (*t*-test, $P>0.46$) (Table 2).

Response of PWN to methanol extracts from resistant or susceptible clones

To examine the response of PWN to methanol extracts from resistant or susceptible clones, three different bioassays were conducted. Significantly larger numbers of PWN were detected from the disks spotted with bark or xylem extracts from both clones than from the solvent-only control or blank (LSD (least significant difference) multiple comparison, $P<0.05$) (Fig. 2A and B). More PWN were attracted to the disks spotted with xylem extracts than to disks spotted with bark extracts from both clones (LSD multiple comparison, $P<0.05$) (Fig. 2A and B). When the attractant effects of bark and xylem extracts from both clones were directly compared on the same plate, no significant differences were observed between the clones (LSD multiple comparison, $P>0.39$) (Fig. 2C). Xylem extracts were better attractants than bark extracts in both clones (LSD multiple comparison, $P<0.01$) (Fig. 2C) as shown in assay I (Fig. 2A) and II (Fig. 2B).

Effects of split-inoculation on symptom development in resistant clones

To further examine the relationship between the migration of PWN and resistance, a split-inoculation test was carried out on resistant clones to mimic the

Table 1 Symptom development of non-resistant clonal ramets with or without girdling, and the number of PWN detected from the girdled ramets

Clone no.	Damaged proportion of ramets[a]		Symptom at 6 months after inoculation			No. of PWN[b] (Girdling + PWN)			
			Girdling + PWN	Girdling + SDW	Non-girdling + PWN	Apical side (Inoculation point)		Basal side	
	(%)					Bark	Xylem	Bark	Xylem
14-02	57.9	(11/19)	Partially-damaged[c]	Non-damaged	Dead	26	0	116	9
14-04	87.5	(7/8)	Dead	Non-damaged	Dead	2	0	49	36
14-27	63.6	(14/22)	Dead	Non-damaged	Partially-damaged[d]	0	0	32	26
12-01	66.7	(2/3)	Dead	Non-damaged	Partially-damaged[c]	217	25	3,770	1,047
12-03	100.0	(3/3)	Dead	Non-damaged	Partially-damaged[c]	4	22	4	19

[a] Values indicate resistance level to PWN

[b] PWN were detected at 55–68 dai

[c] Inoculated and non-inoculated shoots were damaged

[d] Inoculated shoot was damaged, but non-inoculated shoots were not damaged

Table 2 Number or cross-sectional lumen area of cortical- or xylem- resin canals in resistant and susceptible *Pinus thunbergii* clones from a half-sib family

	Clone no.	Damaged proportion of ramets[a]		Observed cross sectional area		Cortical resin canal			Xylem resin canal		
		(%)		Cortex (mm^2)	Xylem (mm^2)	No. of resin canals mm^{-2}	Total lumen area mm^{-2} ($\times 10^{-3}$ mm^2)	Average lumen area of resin canal ($\times 10^{-3}$ mm^2)	No. of resin canals mm^{-2}	Total lumen area mm^{-2} ($\times 10^{-3}$ mm^2)	Average lumen area of a resin canal ($\times 10^{-3}$ mm^2)
Resistant	14-22	15.4	(2/13)	13.07	10.42	2.07	42.5	20.6	5.56	5.63	1.01
	14-23	0.0	(0/7)	9.12	7.12	1.97	41.8	21.2	6.47	5.03	0.78
	14-26	3.3	(1/30)	6.41	9.21	3.43	33.4	9.7	5.97	6.21	1.04
	14-31	14.3	(1/7)	11.48	12.28	2.09	33.1	15.8	6.02	7.58	1.26
	14-33	9.1	(2/22)	14.57	8.66	2.75	67.7	24.7	3.58	3.81	1.06
	14-35	0.0	(0/12)	12.55	13.71	2.95	34.7	11.8	5.62	6.52	1.16
	14-49	11.1	(1/9)	8.29	7.05	4.10	58.2	14.2	7.38	7.84	1.06
	Mean[b]	7.6		10.78	9.78	2.77	44.5	16.8	5.8	6.09	1.05
	SE	2.5		1.10	0.95	0.30	5.1	2.1	0.4	0.5	0.1
Susceptible	14-03	93.8	(15/16)	10.40	8.76	2.88	31.3	10.8	6.05	6.19	1.02
	14-07	92.9	(26/28)	12.09	8.63	1.82	34.5	19.0	5.56	6.74	1.21
	14-16	100.0	(16/16)	11.59	8.76	2.85	39.7	13.9	5.59	5.00	0.89
	14-18	100.0	(15/15)	13.80	11.62	1.81	48.4	26.7	5.25	4.52	0.86
	14-29	100.0	(9/9)	10.85	5.96	3.32	70.7	21.3	4.70	5.48	1.16
	14-34	100.0	(17/17)	12.51	12.38	2.48	34.0	13.7	8.16	8.38	1.03
	14-50	100.0	(16/16)	12.53	8.19	2.23	42.7	19.1	6.47	6.79	1.05
	Mean[b]	98.1		11.97	9.19	2.48	43.0	17.8	6.0	6.16	1.03
	SE	1.2		0.43	0.82	0.22	5.1	2.0	0.4	0.5	0.0

[a] Values indicate resistance level to PWN

[b] Means of all histological parameters are not significantly different between resistant and susceptible clones (*t*-test).

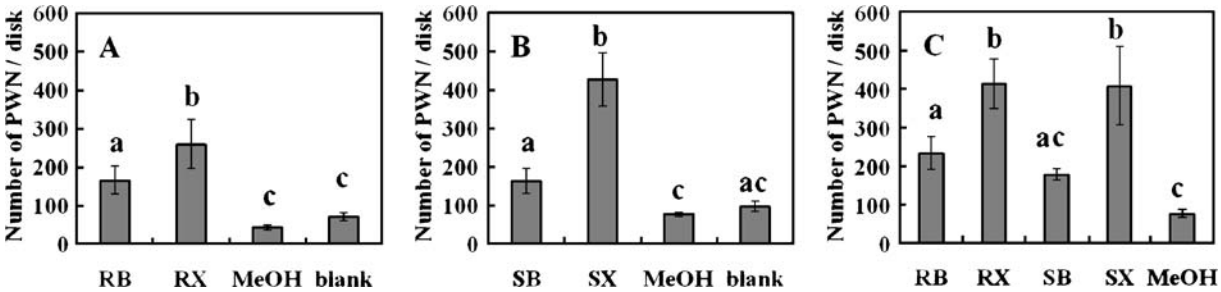

Fig. 2 Number of PWN detected from each disk (both paper and media) at 24 h after inoculation in assay I(**A**), II(**B**), or III (**C**). Values are means ± SE of three replicates. Significantly different means are indicated by different letters (LSD multiple comparison, $P<0.05$). *RB* extract from bark of resistant clone, *RX* extract from xylem of resistant clone, *SB* extract from bark of susceptible clone, *SX* extract from xylem of susceptible clone, *MeOH* solvent-only control, *blank* no spotting

migration of PWN. The proportion of non-damaged, partially-damaged and dead ramets among the total inoculated ramets was not significantly different between the split-inoculated group and the control group (chi-square test, $P=0.60$) (Table 3). At 50 dai, numerous PWN were detected from the segments of partially-damaged ramets, while a small number of PWN was detected from non-damaged ramets (Table 4).

Distribution of PWN in stem cuttings of resistant and susceptible clones

To examine the distribution of PWN between resistant and susceptible clones, an inoculation test using stem cuttings was conducted under controlled conditions to exclude various experimental biases. At 24 h after inoculation, the number of PWN was not significantly different between resistant and susceptible groups at every point (*t*-test, $P>0.28$) (Fig. 3). Most of the invaded PWN were detected around the inoculation points in both groups. At 6 dai this trend was weaker. The number of PWN detected from the distal point was larger than that detected from the middle point in both groups. At the proximal point, the number of PWN did not differ significantly between the two groups throughout the entire incubation time. On the other hand, the number of PWN in the resistant group was smaller than that in the susceptible group at the middle

Table 3 Symptom development of resistant clonal ramets at six months after inoculation with 5,000 PWN into one point (control) or split among three points (split-inoculation) on a current-year shoot

Clone no.	Age	Damaged proportion of ramets[a]		Control			Split-inoculation		
		(%)		Non-damaged	Partially-damaged	Dead	Non-damaged	Partially-damaged	Dead
14-22	4	15.4	(2/13)	1[b]	1		2		
14-33	4	9.1	(2/22)			2			2
14-49	4	11.1	(1/9)	1	1			1	1
14-26	3	3.3	(1/30)	1				1	
14-33	3	9.1	(2/22)	1	2			2	1
01-01	2	0.0	(0/3)	1			1		
08-48	2	0.0	(0/5)	1			1		
11-49	2	0.0	(0/5)	2			2		
12-10	2	0.0	(0/4)	2			1	1	
14-22	2	15.4	(2/13)	2			2		
14-26	2	3.3	(1/30)	7			7		
Total[c]				19	4	2	16	5	4

[a] Values indicate resistance level to PWN

[b] No. of ramets

[c] Total proportion of non-damaged, partially-damaged or dead ramets between control and split-inoculation is not significantly different (chi-square test, $P=0.60$)

Table 4 Number of PWN detected from each segment of non-damaged or partially damaged clonal ramets at 50 dai with 5,000 PWN into one point (control) or split among three points (split-inoculation)

Symptom	Clone no.	Age	Control			Split-inoculation		
			Upper[a]	Middle[b]	Lower[b]	Upper[c]	Middle[c]	Lower[c]
Non-damaged	14-22	4	403	231	38	3	11	4
	14-22	2	2	3	3	0	0	0
	14-26	2	1	7	6	4	3	0
Partially damaged	14-33	4	2,192	983	2,464	4,536	2,736	8,339
	14-33	3	2,298	2,588	11,866	2,681	2,387	5,704
	14-49	3	254	543	4,678	333	268	211

[a] Segment including inoculation point with 5,000 PWN

[b] Segment including inoculation point with SDW

[c] Segment including inoculation point with 1,667 PWN

(*t*-test, $P<0.01$) and distal points (*t*-test, $P=0.09$). At 20 dai, the PWN population increased dramatically at middle and distal points in the susceptible group. The total number of PWN detected from the three segments at 20 dai was 19.3-fold more than that detected 24 h after inoculation. In contrast, the PWN population remained unchanged or gradually decreased throughout the experimental period at every point in the resistant group.

Discussion

In this study we used clonally-propagated plants with pre-evaluated individual resistance levels to examine whether inhibiting migration or proliferation of PWN was related to resistance in *P. thunbergii* trees. By using these plants, we were able to characterize resistant and susceptible plants without ambiguity: for example, some seedlings are actually susceptible even though they are propagated from resistant species (or families), and *vice versa*.

According to previous reports (Mamiya 1980; Ichihara et al. 2000), more PWN were often detected from resin canals in inoculated *P. thunbergii* or *P. densiflora* plants. Hence, resin canals were considered to be one of the migration pathways of PWN. In particular, it was suggested that cortical resin canals may be main pathways (Ishida et al. 1993; Kawaguchi 2006). Kawaguchi (2006) suggested that narrow cortical resin canals functioned as bottlenecks for migration of PWN and the lumen area of cortical resin canals was one of the indicators for resistance level. Based on this report, migration of PWN and symptom development might be inhibited by removing the bark that contains cortical resin canals. However, neither could be inhibited by girdling in non-resistant clones (Table 1). In addition, there was no significant difference in the lumen areas or the number of cortical resin canals between resistant and susceptible

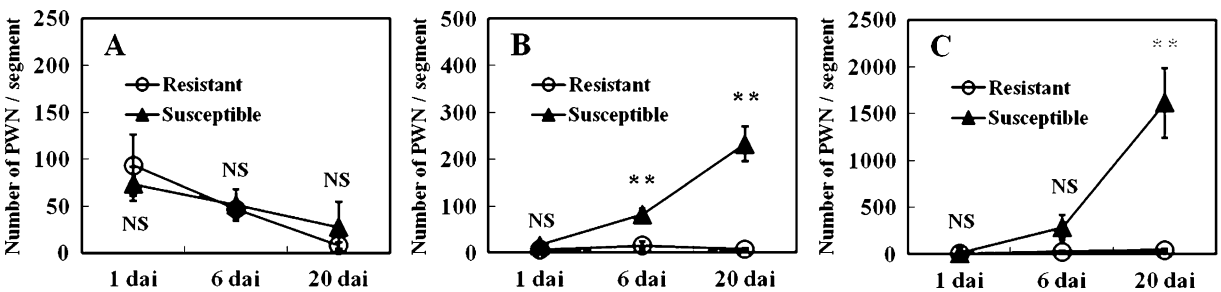

Fig. 3 Time-course of number of PWN detected from segments at the proximal point (**A**), the middle point (**B**) and the distal point (**C**) in resistant and susceptible cuttings. Values are means ± SE of four cuttings from two resistant or susceptible clones. Significantly different means between resistant and susceptible clones are indicated by *double asterisk* ($P<0.01$) with *t*-test (*NS* no significant difference). *dai* days after inoculation

groups from a half-sib family (Table 2). Similar results were obtained with respect to xylem resin canals. Our observations agreed with the previous observations made by the same authors, Kawaguchi et al. (1998), in which no significant difference was observed in the lumen areas or the number of cortical resin canals between damaged and non-damaged seedlings within the same half-sib family. These findings suggest that the lumen areas of cortical resin canals are dependent on familial (or genetic) background rather than the individual's resistance level. Thus, cortical resin canals may be one of the migration pathways of PWN, but the resistance level would be not dependent on the lumen areas or the number of cortical resin canals.

The migration of PWN was likely to be affected by chemical components from *P. thunbergii*. Methanol extracts from bark and xylem of both resistant and susceptible clones showed attractant effects to PWN, but neither showed repellent effects (Fig. 2). In particular, xylem extracts were greater attractants for PWN than bark extracts in both clones. However, there was no significant difference between the two clones in attractant effects of bark or xylem extracts. Thus, it seems that migration of PWN was affected by certain extractable compounds in bark or xylem but these compounds were not involved in the expression of resistance.

The relationship between the resistance and the migration of PWN was examined by a split-inoculation test, which was designed to mimic the migration of PWN. The damaged proportion of the split-inoculated group was not significantly different from that of the genetically identical control group (Table 3). This result suggests that the nature of resistance does not change in resistant plants, even though PWN are migrated artificially by split-inoculation. In this experiment, the number of PWN detected from segments including each inoculation point was smaller in non-damaged ramets. In contrast, very large numbers were detected in damaged ramets, irrespective of split-inoculation (Table 4). Based on these data, it appears that inhibiting proliferation of PWN is essential for the expression of resistance, but inhibiting migration of PWN is not closely related to resistance.

An inoculation test using stem cuttings showed that distribution of PWN was not significantly different between resistant and susceptible groups at 24 h after inoculation (Fig. 3). Most of the PWN that invaded remained around the inoculation points, and the number of PWN decreased with distance from inoculation points in both groups. During 6–20 dai, these trends weakened in both groups. Finally, more PWN were detected from the distal point rather than the middle or proximal points. Even in the resistant group, PWN did not remain around the inoculation point. Thus, we did not observe a close relationship between resistance and inhibiting migration of PWN. The PWN population dramatically increased in the susceptible group (except at the proximal point) from 6 to 20 dai; however populations were unchanged or gradually decreasing in the resistant group from 24 h to 20 dai. These results strongly suggest that inhibiting proliferation of PWN is more crucial for expression of resistance than inhibiting migration of PWN.

To explain the mechanism of resistance, it is necessary to identify the factors for inhibiting proliferation of PWN. In previous studies, compounds possessing nematicide activity such as stilbenoids (e.g. pinosylvin monomethyl ether and 3-*O*-methyldihydropinosylvin) were detected from resistant pine species (e.g. *P. massoniana*, *P. strobus* and *P. palustris*) (Suga et al. 1993; Hanawa et al. 2001). However, these acute nematicidal compounds may not associate with inhibiting PWN proliferation because the number of PWN did not abruptly decrease in resistant cuttings throughout the entire incubation time (Fig. 3). Wang et al. (2005) showed that virulent PWN isolates have higher proliferation ability than avirulent isolates or *Bursaphelenchus mucronatus* (an avirulent species), due to a shorter life-cycle and higher fecundity. It is likely that other, as yet unknown compounds specific to resistant plants inhibit the normal life-cycle, reproduction and/or sexual behaviour of PWN.

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