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A Study on Pathogenicity of Bacteria Carried by Pine Wood Nematodes

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Abstract

Three bacterial strains Njh, Njt and Njw, have been isolated with a high frequency from the xylem of the wilted black pine (*Pinus thunbergii*) and from the surface of the pine wood nematodes (*Bursaphelenchus xylophilus*). These bacteria have been identified as *Pseudomonas fluorescens* biotype I, *P. fluorescens* biotype II and a species of the genus *Pantoea* respectively. To determine pathogenicity, callus and aseptic black pine seedlings were inoculated as follows: aseptic nematodes only (each of *B. xylophilus* and *B. mucronatus*); bacterium only (each of bacterial strain Njh, Njt, Njw) and aseptic nematodes plus bacterium (each of *B. xylophilus* and *B. mucronatus* in combination with each of Njh, Njt, Njw). The results showed that inoculation with either aseptic *B. xylophilus* or *B. mucronatus* did not lead to browning of the callus or wilt of aseptic black pine seedlings. But those inoculated with aseptic nematodes plus any one of Njh, Njt, Njw showed some wilting or browning symptoms. The combination of nematodes plus either Njh or Njt caused severe symptoms but the combination of nematodes plus Njw had fewer symptoms. The bacteria carried by pine wood nematodes played an important role in pathogenicity. In addition, bacteria were cultured in liquid media through shaking. The filtered liquid was directly applied to the callus and the ability of each kind of bacterium to induce browning was determined. The results showed that Njh and Njt caused severe browning but Njw did not. It is suspected that this is due to wilt related toxins in the bacterial culture fluid. Therefore, it's suggested that the disease was caused by co-infection of both pine wood nematodes and bacteria and possible toxic effect of bacteria.

Introduction

Pine wilt disease which leads to wilt and subsequent death of infected trees is one of main diseases found in conifer forests (Nickle et al., 1981; Mota et al., 1999). It is distributed mainly in Japan and China, and is also

found in Canada, Korea, Nigeria, United States and recently Portugal etc (Mamiya, 1973, 1975; Dropkin et al., 1981; Khan, 1991; Mota et al., 1999). In China, it mainly damages black pine (*Pinus thunbergii* Parl.) and Masson's pine (*P. massoniana* Lamb.) (Cheng et al., 1986). Although it was found in Japan in 1905 and researched for more than half a century (Yono, 1913), its pathogenicity is still not clear. For a long time it was thought that *Bursaphelenchus xylophilus* (Steriner and Buhner 1934) Nickle 1970, was the only pathogenic agent causing the disease (Mamiya, 1975, 1983; Nickle et al., 1981; Nobuchi et al., 1984). This viewpoint was supported by many phenomena such as inoculation with the pine wood nematode led to wilt of pine trees and that nematodes from different pine trees differ in their pathogenic abilities (Kiyohara and Bolla, 1990; Fukuda et al., 1992; Kojima et al., 1994; Hu et al., 1995). Further studies found that toxins play an important role in the process of wilting (Mamiya, 1980; Oku, 1988, 1990; Zhang et al., 1997) and that the pine wood nematode itself does not produce these toxins (Cao et al., 2001). Oku et al. (1979) suggested that the production of toxins which led to wilt were associated with bacteria. Using an electron microscope, Kusunoki found quantities of bacteria in tissues (damaged resin ducts and between parenchyma cells) infected with the pine wood nematode (Kusunoki, 1987). Zhao found there were many bacteria attached to the body surface of the pine wood nematode (Zhao et al., 2000). In order to examine bacteria's role in pathogenicity of the pine wood nematode, this study first isolated bacteria and treated nematodes aseptically, then inoculated black pine seedlings and callus with them and finally determined bacteria's pathogenicity.

Materials and Methods

Source, purification and culture of nematodes

Bursaphelenchus xylophilus was isolated from naturally diseased and wilted black pine wood in the Zijin Mountain of Nanjing (Jiangsu, China) and *B. mucronatus*

Mamiya and Enda 1979, from naturally wilted *Pinus massoniana* wood in Yixing of Jiangsu. The diseased wood was cut into chips of $2 \times 10 \times 10$ mm. Twenty gram of samples (fresh weight) were used to isolate the nematodes with Baermann funnels. The nematodes from the two different sources were multiplied on *Botrytis cinerea* Pers. cultures on potato-dextrose-agar (PDA) medium at 25°C. The nematodes were washed off the plate with sterile water and a single adult nematode was sought out under a dissecting microscope. After the nematode was identified as either *B. xylophilus* or *B. mucronatus*, it was cultured on a slant medium covered by *Botrytis cinerea*. There were one female and one male adult in each tube and the tube was put in a 25°C incubator for further use. Thus both *B. xylophilus* and *B. mucronatus* were grown separately in pure culture.

Acquisition of bacterium-free nematode

Nematodes together with the agar medium were washed onto a piece of filter paper in a Baermann funnel with sterile physiological salt solution containing 0.9% NaCl and left there for 24 h. Dead nematodes and the medium were removed and 10 ml of nematode containing liquid at the bottom of the funnel was collected and centrifuged at 17 g for 6 min. The supernatant was discarded and the nematode-containing precipitate was washed and disinfected six times with a liquid containing 0.9% aseptic NaCl solution to which 1600 units/ml penicillin-G, 10 mg/ml streptomycin and 5 mg/ml fungicidin were added. Each time 10 ml of the liquid was added to the precipitate and centrifuged. Finally after washing and centrifuging three times with the sterilized physiological salt solution, the bacterium-free nematodes were cultured and reproduced on the callus of black pine. To determine whether the nematodes carried bacteria, they were cultured on a nutrient broth agar media (nutrient broth 3 g, peptone 7 g, NaCl 5 g, agar 17 g and water 1000 ml, NB medium) at 28°C for 48 h. There was no colony appearance on the agar medium so it was assumed that bacterium-free nematodes had been obtained.

Isolation and purification of the bacteria

Bacteria were isolated in two ways: (1) Isolation of bacteria from the xylem: samples of diseased pine wood infected by pine wood nematode were taken, and their bark was removed and the wood was cut into 5 cm lengths with a saw. The surface of the wood was sterilized with 70% ethanol and the sapwood was removed under aseptic conditions with a sterilized knife. The central part of the wood was directly put on the plates with NB media and incubated at a temperature between 25–28°C. The plates were examined daily for the appearance of bacterial colonies. (2) Isolation of bacteria from pine wood nematodes: the suspension liquid obtained from the Baermann funnel containing the pine wood nematode was centrifuged at 17 g for 6 min. The supernatant was discarded and 3% H₂O₂ of an equal volume was added to disinfect

for 5 min to partially remove miscellaneous microorganisms. Then sterile water was added to centrifuge and washed three times. Finally a single nematode was sought out under aseptic conditions with a thin metal needle, put on a plate with NB media and incubated at a temperature between 25–28°C. The bacterial colonies appeared on the marks left by nematodes that had passed.

Bacterial colonies isolated were purified with the help of dilution

Some purified strains were transferred to a slant NB medium and cultured between 25–28°C for 2–3 days to be used as an inoculum. The rest of the strains were kept at –30°C in a 1/2 NB liquid medium to which 25% glycerol was added.

Identification of bacteria

Bacteria were identified on the basis of colony culture, staining tests and both physiological and biochemical characteristics together with ATB expression with a fully automated drug susceptibility identifier (bio-Merieux China Limited Company, River Enterprise Co. Ltd, Guangzhou, China). For observation of colony culture, three purified strains were cultured on NB media and their single colony shape, colour, transparency, prominence, edge and viscosity, were observed. Staining tests included Gram reaction, and flagellum staining, both physiological and biochemical characteristics assayed included oxidase, gelatin liquefaction, starch hydrolysis, nitrate reduction, growth at 41°C. An ID32GN system was adopted in the analysis with the fully automated drug susceptibility identifier. The 32 biochemical parameters included in the analysis contained D-ribose, maltose, acetates, rhamnose, inositol, saccharose, itaconic acid, suberate, malonate, lactate, L-alanine, mannitol, D-glucose, salicin, D-melibiose, L-fucose, D-sorbierite, arabinose, propionate, caprate, valerate, citrate, histidine, glycogen, L-serine, ornithine, lysine, arginine, phenylalanine, L-proline, lactose, and urea.

Culture of aseptic black pine seedlings

Plump black pine seeds were washed in running water for 5 min and then dipped in water for 24 h with frequent water changes. The floating seeds on the surface of water were discarded when the seeds had imbibed water. The rest were dipped in 70% ethanol for about 30 s, treated with 0.1% mercuric chloride for 10–15 min, and washed with sterile water three to five times. Washed seeds were then put on a PDA plate for accelerated germination, with each seed separated from the other. Frequent examinations were made during the process of accelerated germination and contaminated seeds were removed, while uncontaminated seeds were selected and cultured on fresh PDA plates. Finally, uncontaminated germinated seeds were transferred to a container with MS media (Yinglong, 1985) and cultured there for 3–4 months under controlled conditions (temperature 25°C, light for 12–14 h daily of 1300 lx).

Callus culture of black pine

Induction of callus Explanted tissues were from aseptic black pine seedlings with newly opened leaves. Young leaves were cut into lengths of 1–2 cm each with sterilized scissors, washed with sterile water once and cultured on a medium for induction.

Using MS as an essential medium, 10.0 mg/kg 2,4-dichlorophenoxyacetic (2,4-D), 3.5 mg/kg kinetin (KT), 200 mg/kg hydrolysed casein, 100 mg/ml ascorbic acid, 0.6–0.7% agar and 3% sucrose were added. The media were distributed in conical flasks and then sterilized at 121°C for 20 min. The tissue mass from sterilized black pine seedlings was put on inducing media, with each flask containing 3–4 tissue masses, and cultured at 24–26°C under a light for 12–14 h daily at 1300 lx.

Successive culture of callus A 1/2 MS (with major elements reduced by half) was used, to which 4.0 mg/kg 2,4 - D, 200 mg/kg hydrolysed casein, 100 mg/ml ascorbic acid, 0.6–0.7% agar and 3% sucrose were added. Undifferentiated explants and browning tissue were cut off the newly induced callus mass and the callus, in good condition, was transferred to the medium for successive culture. Each flask had two callus masses, which were cultured at 24–26°C in the dark.

Inoculation

Preparation of inoculum Unsterilized nematodes cultured on fungi and aseptic nematodes cultured on the callus of black pine were washed with sterile water and adjusted to a concentration of 2000 nematodes/ml. Thus a suspension liquid with nematodes in it was obtained. A suspension liquid with bacteria in it was prepared as follows: bacterial lawn was sought out from the bacteria cultured on slant NB media for 48 h and put in NB liquid media containing bacteria was shaken for 4 days at 28°C and then adjusted to a concentration of 2×10^6 bacteria/ml. If inoculation was conducted with either of the two suspension liquids individually, the liquid was diluted to half of the original concentration. If inoculation was conducted with a mixture of the two suspension liquids, the mixture was left there in a static state for 1 h so that the bacteria could completely adhere to the nematodes. Each suspension liquid was adjusted as follows: 10 000 nematodes/ml and 1×10^6 bacteria/ml. Before inoculation, aseptic nematodes were cultured on NB agar media at 25°C for 48 h to examine whether they carried bacteria.

Inoculation of callus A 0.5 ml of the inoculum suspension nematode containing liquid was added to the surface of the callus (Callus had been successively cultured for 25 days). The treated calluses were placed at 24–26°C in the dark. The different treatments of the callus are shown in Table 1. Each treatment was duplicated three times, with aseptic water as a control. Examinations were made 24 h after inoculation to observe changes in callus colour. The criteria for classifying the symptoms of the tested callus were as follows:

No change in callus colour, was expressed as –;
The callus colour showed yellowish, was expressed as +;
The callus colour showed dark yellow, was expressed as ++ and
The callus colour showed dark brown, was expressed as +++.

Inoculation of aseptic seedlings The upper stem of an aseptic black pine seedling cultured in a test tube for 2 months was lightly punctured with a needle, and then a piece of aseptic absorbent cotton was placed on the wound. A 0.25 ml of the inoculum suspension liquid with a concentration of 250 nematodes/ml and 2.5×10^6 bacteria/ml was added to the cotton. The treated seedlings were placed under controlled conditions (temperature 25°C, light for 12–14 h daily of 1300 lx). The different treatments are shown in Table 2. Each treatment was duplicated five to eight times. Inoculation with the aseptic physiological salt solution was used as a control. Four days after inoculation, the testing pine seedlings were observed.

Recovery of the nematode and the bacterium from the wilted seedlings

Five days after inoculation, the pine seedlings were uprooted, disinfected for 4 min with 0.1% mercuric chloride to remove any surface inoculum and washed three times with sterile water. Then the tested seedling (stem 9–11 cm long) was cut into lengths of 3–5 mm with aseptic scissors and bacteria were isolated from two to three pieces. The isolated bacteria were identified according their morphology and the stain tests. The remaining pieces of the seedling were put into a sterile culture dish, to which 5 ml of sterile water was added. After soaking in water for 24 h, the extracted nematodes were examined and identified according to their morphology.

Culture and bioassay of toxins from bacteria

The three species of bacteria were separately inoculated into NB liquid media, with a final concentration of 1×10^8 bacteria/50 ml. The medium was then shaken at a rotating speed of 110 per min and a temperature of 25°C for 4 days. The cultural medium liquid passed through a bacterial filter and was directly applied to callus. The blank NB liquid medium was used as a control. A callus with a volume of 1.5–1.8 cm³ and successively cultured for 25 days was inoculated with the liquid with a dosage of 0.5 ml/cm³. The treated callus was then cultured in the dark, observed and recorded every day. The criteria for classifying the symptoms of the tested callus corresponded with those mentioned above.

Results

Isolation of bacteria

No microorganisms were isolated from healthy pine trees. To find bacteria in diseased trees the following method was used: To isolate from wood, a number of

Treatment	Replication	Time (day)				
		1	2	3	4	5
Control of aseptic water	1	-	-	-	-	-
	2	-	-	-	-	-
	3	-	-	-	-	-
Aseptic <i>Bursaphelenchus xylophilus</i>	1	-	-	-	-	-
	2	-	-	-	-	-
	3	-	-	-	-	-
Aseptic <i>B. xylophilus</i> + Njw	1	-	-	-	-	+
	2	-	-	-	-	+
	3	-	-	-	+	+
Aseptic <i>B. xylophilus</i> + Njt	1	-	+	++	++	+++
	2	-	+	++	++	+++
	3	-	+	++	++	+++
Aseptic <i>B. xylophilus</i> + Njh	1	-	+	++	++	+++
	2	-	+	++	++	+++
	3	-	+	++	++	+++
Unsterilized <i>B. xylophilus</i>	1	+	++	+++	+++	+++
	2	+	++	+++	+++	+++
	3	+	++	+++	+++	+++
Aseptic <i>B. mucronatus</i>	1	-	-	-	-	-
	2	-	-	-	-	-
	3	-	-	-	-	-
Aseptic <i>B. mucronatus</i> and strain Njw	1	-	-	+	+	+
	2	-	-	-	-	+
	3	-	-	-	-	+
Aseptic <i>B. mucronatus</i> and strain Njt	1	-	+	+	++	+++
	2	-	-	+	++	+++
	3	-	-	+	++	+++
Aseptic <i>B. mucronatus</i> and strain Njh	1	-	-	+	++	+++
	2	-	-	+	++	+++
	3	-	+	+	++	+++
Unsterilized <i>B. mucronatus</i>	1	+	+	++	++	+++
	2	+	+	++	++	+++
	3	-	+	++	++	+++
Njw	1	-	-	-	-	-
	2	-	-	-	-	+
	3	-	-	-	-	-
Njt	1	-	-	-	+	+
	2	-	-	-	+	+
	3	-	-	-	-	+
Njh	1	-	-	-	-	+
	2	-	-	-	+	+
	3	-	-	-	+	+

Table 1
Response of the callus inoculated with nematodes and bacteria

Treatment	The ratio of wilted seedlings in 4 days	Recovery of bacteria		Recovery of nematodes	
		Frequency	Strain	Frequency	Strain
Control of aseptic water	0/8	0/8	/ ¹	0/8	/
Aseptic <i>Bursaphelenchus xylophilus</i>	0/8	0/8	/	8/8	<i>B. xylophilus</i>
Aseptic <i>B. xylophilus</i> + Njw	2/8	2/8	Njw	2/8	<i>B. xylophilus</i>
Aseptic <i>B. xylophilus</i> + Njt	8/8	8/8	Njt	6/8	<i>B. xylophilus</i>
Aseptic <i>B. xylophilus</i> + Njh	8/8	8/8	Njh	7/8	<i>B. xylophilus</i>
Unsterilized <i>B. xylophilus</i>	8/8	8/8	Njh	7/8	<i>B. xylophilus</i>
Aseptic <i>B. mucronatus</i>	0/5	0/5	/	2/5	<i>B. mucronatus</i>
Aseptic <i>B. mucronatus</i> and strain Njw	3/8	3/8	Njw	2/8	<i>B. mucronatus</i>
Aseptic <i>B. mucronatus</i> and strain Njt	8/8	8/8	Njt	7/8	<i>B. mucronatus</i>
Aseptic <i>B. mucronatus</i> and strain Njh	6/8	6/8	Njh	6/8	<i>B. mucronatus</i>
Unsterilized <i>B. mucronatus</i>	8/8	7/8	Unknown ²	7/8	<i>B. mucronatus</i>
Njw (suspension liquid cultured through rocking)	0/8	0/8	/	0/8	/
Njt (suspension liquid cultured through rocking)	4/8	0/8	/	0/8	/
Njh (suspension liquid cultured through rocking)	5/8	1/8	Unknown	0/8	/

Table 2
Percentage of wilted black pine seedlings and recovery of bacteria and nematodes

¹ '/' means that nothing was recovered.

² 'Unknown' means that the bacteria had been recovered but was not identified.

bacterial colonies were found along the trails of nematodes. Colonies from individual trees were compared and identified. It was found that three strains of bacteria occurred more than 97% of the total isolates. These three strains of bacteria were named as strains Njh, Njt and Njw, respectively.

Identification of bacteria

The characteristics of the three strains are as follows:

Strain Njh Single-celled, non-sporing rods, with no pallium, $0.5\text{--}0.6 \times 1.5\text{--}1.8 \mu\text{m}$ in size, Gram negative, and moving with the help of the clumpy flagella at one end. When cultured on nutrient broth agar media, the colony was milky yellow, semi-transparent, round in shape and smooth on the edge. It was positive in oxidase tests, could hydrolyse gelatin, but could not hydrolyse starches, grows at 41°C and is negative for nitrate reduction. Together with the result obtained from the automated analyzer, this strain was identified as *Pseudomonas fluorescens* biotype I (with a confidence of 82% for the identification).

Strain Njt Rod-like, single or paired when present, $0.49\text{--}0.61 \times 1.51\text{--}1.78 \mu\text{m}$ in size, Gram negative, with no spore and palisade, and moving with the help of the clumpy flagella at one end which were short and hooked. When cultured on nutrient broth agar media, the colony was milky white, semi-transparent, round in shape and smooth on the edge. It was oxidase positive, could not hydrolyse starches and grows at 41°C . It worked in the nitrate reduction, produced mucilage on a 2–4% sucrose medium, but could not hydrolyse gelatin. Together with the result obtained from the automated analyzer, this strain was identified as *P. fluorescens* biotype II (with a confidence of 87.4% for the identification).

Strain Njw Rod-like, single or chain-like when present, $0.9\text{--}1.2 \times 4\text{--}3 \mu\text{m}$ in size, Gram negative, with no flagella, and no spore or palisade. When cultured on nutrient broth agar media, the colony was white, opaque, round in shape and prominent. It was oxidase negative, could not hydrolyse starches and gelatin and grows at 41°C . Together with the result obtained from the automated analyzer, this strain was identified as *Pantoea* spp. (with a confidence of 91.1% for the identification).

Inoculation of callus

Callus of black pine was treated with each of *B. xylophilus* or *B. mucronatus* combined with each of the three species of bacteria respectively. Please see Table 1 for results. The table shows that the callus was not browned when inoculated with either aseptic *B. xylophilus* or aseptic *B. mucronatus*. When inoculated only with bacteria, the callus was browned only to a small extent and browning was only confined to the surface of the callus. The combination of the two species of nematode combined with each of the three strains of bacteria respectively could obviously lead to

browning of the callus, with the combination of the two species of nematode with the strain Njw showing the weakest browning. Browning was very conspicuous when inoculation was made with the combination of the two species of nematodes with strains of Njh and Njt. The symptoms were similar to those of inoculation with unsterilized pine wood nematodes.

There was no difference between the two species of nematodes in the extent of browning. But there was an obvious difference between different combinations of nematodes and bacteria in browning of the callus. Browning of the callus was closely related to the species of the bacterium.

Inoculation of aseptic black pine seedlings

The stems of the seedlings were punctured and inoculated with each of aseptic *B. xylophilus*, *B. mucronatus*, each of the three stains of bacterium, and each of *B. xylophilus* and *B. mucronatus* combined with each bacterium. The results are shown in Table 2. Seedlings did not show wilt symptoms on inoculation with either aseptic *B. xylophilus* or aseptic *B. mucronatus*, while the combination of either aseptic *B. xylophilus* or aseptic *B. mucronatus* with one of the three strains of bacteria caused strong wilt. There was no significant difference in wilt between different species of nematodes inoculated onto pine seedlings but this was not the case with different combination of nematodes with different kinds of bacteria. Among the three strains of bacteria tested, strains Njh and Njt were stronger than Njw in ability to cause wilt. In addition, inoculation with unsterilized *B. mucronatus* also led to wilt. These results suggest that wilt of pine seedlings is not in close correlation with species of nematodes, but with the strain of bacteria carried by nematodes.

Recovery of bacteria and nematodes

Results of recovery of the bacteria and nematodes from inoculated seedlings are shown in Table 2. Bacteria and nematodes were recovered from wilted pine seedlings that had been inoculated with either the combination of each bacterium with *B. xylophilus* and the combination of each bacterium with *B. mucronatus*. Pine seedlings did not wilt when inoculated with sterilized *B. xylophilus*, which was recovered from healthy seedlings. This indicated that the seedling could be infected by the nematode, but the nematode was not lethal to the seedling. Inoculation with a suspension liquid containing bacteria strains Njh and Njt led to wilting for a short period of time, but no bacteria were recovered from the wilted seedlings, which reveals that bacteria had not invaded the seedlings. Possibly toxins contained in the suspension liquid caused localized damage to the seedlings.

Possible toxic effects produced by three strains of bacteria

Bacteria were cultured in liquid media through shaking. The filtered liquid was directly applied to the callus of black pine and the ability of each strain of bacterium to induce browning was measured. As

Table 3
Responses of the callus to the cultural liquid medium with bacteria

Bacterium	Replication	Time (days)				
		1	2	3	4	5
Njw	1	-	-	-	-	+
	2	-	-	-	-	+
	3	-	-	-	+	+
Njt	1	-	+	++	++	+++
	2	-	+	++	++	+++
	3	-	-	++	++	+++
Njh	1	-	+	++	++	+++
	2	-	+	++	++	+++
	3	-	+	++	++	+++
CK (NB liquid medium)	1	-	-	-	-	-
	2	-	-	-	-	-
	3	-	-	-	-	-

No change in callus colour = '-';
Callus colour showed slight yellow = '+';
Callus colour showed dark yellow = '++';
Callus colour showed dark brown = '+++'.
'

compared with control (viz. uninoculated liquid media), the culture liquid from three strains of bacteria had an obvious effect on browning. Strains Njh and Njt had stronger effect than Njw in causing brown. Part of the calluses began browning 24 h after the liquid was added. Five days after the liquid from strains Njh and Njt were applied to the callus masses, the masses browned and died. There was no significant change in callus colour for those treated with filtered liquid from the Njw strain culture but it was darker than the control. The result of bioassay is shown in Table 3.

Discussion

The results show that inoculation with aseptically cultured nematodes cause no disease symptoms in callus or aseptically cultured pine seedling. Both callus and the seedling showed only slight symptoms when inoculated with bacteria, but inoculation with a combination of nematodes and bacteria led to serious symptoms. Inoculation with the combination of either *B. xylophilus* or *B. mucronatus* with each of the three strains of bacterium could lead to disease. This was especially true with the combination of nematodes with either strain Njh or Njt. Browning of the callus and wilting of pine seedlings were not associated with species of nematode, but associated with the species of bacteria. This indicates a possible co-action of the bacteria carried by pine wood nematode and pine wood nematode itself on pathogenicity.

Three bacterial strains isolated from the experiment have been identified as two strains of *P. fluorescens* biotype I, *P. fluorescens* biotype II and a species of the genus *Pantoea*, which were not available from previous studies. Kawazu isolated three strains toxic to both the callus and the seedling of black pine, viz. *Bacillus cereus*, *B. subtilis* and *B. megaterium* (Kawazu, 1998) which indicates that the bacteria carried by pine wood nematodes from different regions may be different. This could explain why *Cedrus deodara* in USA and Japan is infect-

able by pine wood nematodes (Dropkin et al., 1981) while in China it is not.

Inoculation with a combination of aseptically cultured *B. mucronatus* or *B. xylophilus* and any one of the bacterial strains led to similar symptoms. Inoculation with unsterilized *B. mucronatus* led to browning of the callus and wilting of the pine seedlings. In natural conditions *B. mucronatus* does not cause disease or is weak in pathogenicity (Mamiya and Enda, 1979; Futai, 1980; Hu et al., 1995) while indoors its pathogenicity increases. Possible explanations for this could be (i) the tissue of the young seedling and callus used in the experiment are more sensitive than mature tissue found in nature, (ii) the nematode's ability to carry bacteria and to spread and reproduce is increased in laboratory conditions and (iii) the wound created artificially when inoculation was made is favourable to invasion and spread of nematodes.

Inoculation with only aseptically cultured nematodes does not lead to wilting of aseptically cultured seedlings, but the inoculated sites of seedlings were diseased and wilted. The wilt caused by aseptically cultured nematodes usually had no browning, but the inoculated site became yellow and subsequently hollow. These symptoms could be caused by mechanical wounds of nematodes feeding and reproducing on a large scale.

Kawazu has isolated three bacteria (*Bacillus* spp.) that could produce substances toxic to black pine seedlings, calluses and the cells cultured in a suspension liquid. He also isolated the toxic substance and identified it as phenylacetic acid (Kawazu et al., 1996; Kawazu, 1998). This study isolated two species of *P. fluorescens* and a species from the genus of *Pantoea*. The two species of *P. fluorescens* were strong in pathogenicity and possibly produced substances toxic to black pine seedlings and its callus. The toxic substances remain to be isolated and identified.

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