全国高校素质教育教材研究编审委员会审定

松萎蔫病致病新机理论文选编

赵博光 编著

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图书在版编目（CIP）数据

松萎蔫病致病新机理论文选编/赵博光　编著. —中国：中国科学文化出版社，2008.1

ISBN 978-988-17372-3-6

Ⅰ.松…　Ⅱ.赵…　Ⅲ.森林树种—病虫防治—论文集

Ⅳ.S763.7

中国版本图书馆CIP数据核字（2008）

松萎蔫病致病新机理论文选编

赵博光　编著

责任编辑：高爱云

封面设计：张骐年

出版发行：中国科学文化出版社

地　　址：香港干诺道中168-200号信德中心西翼3703室

电　　话：00852-34210868　　**传真**：00852-34262347

排　　版：科士洁文印中心

印　　刷：新颖印务有限公司

开　　本：787mm×1092mm　1/16

印　　张：19.5

字　　数：462千字

版　　次：2008年1月第1版

印　　次：2008年1月北京第1次印刷

书　　号：ISBN 978-988-17372-3-6

定　　价：39.80元

序

赵博光，南京林业大学森林资源与环境学院教授、博士生导师。赵博光教授对我国荒漠植物的活性化学成分及其开发新产品和生产工艺进行了 20 余年的研究和开发，在国际上首次提出“利用高新技术，大面积种植和综合利用荒漠有毒灌草防治荒漠化”的新途径。他首次提出从荒漠植物提取研制杀松材线虫的植物源天然化合物-苦豆碱制剂，并于 1998 年通过国家林业局科技司的鉴定，获得国家发明专利。此项研究成果于 1999 年被国家自然科学基金委员会评为庆祝中华人民共和国建立五十周年优秀应用推广成果项目，同年又被国家林业局列为林业科技成果推广项目。赵博光教授以其科研成果与内蒙金驼药业集团合作申请并承担国家发改委的 2000 年国家重点高科技产业化示范工程项目“内蒙古金驼药业集团总公司有毒灌草资源开发利用产业化示范工程”。项目国家投资 1.18 亿元（见国家计委计司高计函【2000】082 号文件）。2002 年国家计委决定向赵博光教授与内蒙金驼药业集团合作的“有毒灌草资源综合开发利用产业化示范工程”项目等全国 100 项高技术产业化项目授牌（见国家计委文件：计高技【2002】1690 号）。目前该工程基建已经完工、投产。

赵博光教授于 2001 年承担国家自然科学基金重点项目“松材线虫病的致病分子机理及防治新途径”，通过研究和实验发现松材虫病的真正病原是其携带的产毒细菌，并在国际上首次提出松材线虫病是由松材线虫与其携带的致病细菌引起的复合侵染病害的松材线虫病致病机理新假说。并首次发现松材线虫与其携带的致病细菌的互惠共生现象。2004 年该项研究以“松材线虫与其携带的细菌共生致病机理”为题连续获国家自然科学基金重点项目资助。该成果已经引起了国内外有关专家的关注，赵博光教授被国际线虫学家学会邀请在该学会 2003 年在美国康乃尔大学举行的年会上做有关的学术报告。2004～2005 年先后被日本京都大学、俄罗斯科学院莫斯科寄生虫研究所、莫斯科全俄检疫研究所、俄罗斯科学院圣·彼得堡动物研究所邀请做有关松材线虫病致病机理的学术报告。2006 年 7 月在葡萄牙，里斯本举行的“松萎蔫病：对世界森林生态的威胁”国际研讨会。在会上赵教授做了关于松萎蔫病的复合侵染及线虫与细菌互惠共生的学术报告。大会授予赵博光教授杰出的贡献奖，以表彰他对松萎蔫病致病机理研究的创新性成果。2007 年赵教授与国际林联松萎蔫病组协调人日本学者 Dr.Futai 共同主持了在日本京都大学召开的“松萎蔫病在亚洲”为题的国际研讨会。

本论文集收集了赵博光教授及在国内外学术期刊上发表了关于松萎蔫病致病机理及防治方面的论文为 50 篇。赵教授毕业于北京林业大学林学系森保专业，大学毕业后，先后在黑龙江巴林林业局做技术员、宁夏盐池制药厂工程师。文革后考入南京林业大学读研究生。于上世纪 80～90 年代赴美国和加拿大留学继而以高级访问学者的身份合作研究。主要从事森林昆虫化学生态和松萎蔫病方面的研究。

1992 年以来已主持 15 项国家自然科学基金项目，其中包括国家自然科学基金重点项目 2 项、农业倾斜项目 2 项；国家 948 项目 2 项；及国家发改委项目 1 项；国家公益项目 1 项；江苏省科技厅及外专局项目 4 项；国家林业局项目 3 项及多项横向科研项目等。先后在国内外发表学术论文 160 余篇。2000 年起被国家自然科学基金委先后特聘为生命科学部动物学科组和林学学科组二审评委。现为国际化学生态学会会员、国际林联会员、中国治沙暨沙业学会理事、中国昆虫学会理事、国家林业局林业生物基因工程安全委员会委员。

赵博光教授是我在北京林业大学读书的同学，我为赵博光教授在学术上取得的成果感到由衷的高兴，特此作序以记。

中国工程院　院士
北京林业大学　校长　　尹伟伦（签字）
2007 年 11 月 20 日

目　录

第一章　松萎蔫病致病新机理的提出和试验证据

Nematology, 2003, Vol. 5(6), 899～906

Distribution and pathogenicity of bacteria species carried by *Bursphelenchus xylophilus* in China

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Abstract: Bacteria carried by the pine wood nematode (PWN), *Bursaphelenchus xylophilus*, from both healthy and diseased *Pinus thunbergii* and *P. massoniana* were studied in five main pine wilt disease epidemic provinces in P. R. China. No bacteria were found in healthy pines but were found on PWN from all samples collected from diseased trees. 24 bacteria strains were isolated from the nematodes and were identified by a combination of classical and automatic testing bacteriology (ATB) expression methods. Bioassay showed that 17 of the 24 identified strains produced phytotoxins. 11 of these 17 phytotoxin producers belonged to the genus *Pseudomonas*. Glasshouse and field inoculation tests using sterile techniques showed that both PWN and the toxin-producing bacteria carried were necessary to induce disease. We hypothesise that pine wilt disease is a complex, induced by both PWN and the bacteria it carries.

Key words: bioassay; field test; *Pinus massoniana*; *P. thunbergii*; *Pseudomonas*; sterilised nematode

Pine wood nematode (PWN), *Bursaphelenchus xylophilus*, is thought to be native to North America, since it is less pathogenic to some of the native pine trees. The nematode was first identified as the pathogen of pine wilt disease in Japan after pine forests were damaged seriously in the last century (Mamiya & Kiyohara, 1972). Since it was found in 1982 in Nanjing, Jiangsu Province, P. R. China (Cheng *et al.*, 1986), the disease has spread to Anhui Province, Hubei Province, Zhejiang Province, Guangdong Province, Hongkong and Taiwan. The nematode has also been found in Portugal in Europe (Mota *et al.*, 1999). The disease

Received: 21 April 2003; revised: 13 August 2003
Accepted for publication: 28 August 2003
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associated with PWN has become a worldwide threat to forests, tourism and the environment.

Bursaphelenchus xylophilus has been though to be the only pathogen of pine wilt disease (Mamiya, 1975, 1983; Myers, 1988; Yang, 2002), though mechanism of the disease has not been clearly elucidated. However, surface-sterilised PWN was reported to lose its pathogenesis (Cao, 1997; Kawazu & Kaneko, 1997). It was also reported that bacteria were associated with PWN (Oku *et al.*, 1980; Higgins *et al.*, 1999). Bacteria on the body wall of PWN were observed by electron microscopy (Zhao *et al.*, 2000). The average number of bacteria carried by one nematode isolated from the diseased tree was 2.9×10^2 (Guo *et al.*, 2002). A nationwide sampling of healthy black pine, *Pinus thunbergii* and Masson pine, *P. massoniana*, in P. R. China, and culturing of wood chips under sterilised conditions, showed that neither bacteria nor fungi existed in healthy pines. All the above findings led us to hypothesise that bacteria might play a role in pine wilt disease. Therefore, we undertook a geographic survey of host distribution of bacteria carried by PWN in China. In addition, we investigated the possible pathogenic role of the bacteria to pines.

Materials and methods

Bacteria carried by pwn

Sampling

Pine branches from dead trees naturally infected by pine wilt disease were collected in September and October, 2001 at 7 locations in 5 main pine wilt epidemic provinces, Enshi, Hubei; Nanjing, Jiangsu; Chaohu and Hanshan, Anhui; Fuyang and Pinghu, Zhejiang; and Conghua, Guangdong. Samples were collected from black pine and Masson pine. Branches of healthy black pine and Masson pine were also collected as control at the same time. In each location, 3 diseased trees and 3 healthy trees of each host species were selected and 3 sample branches per tree collected from 3 levels of the crown, up, middle and low.

Isolation and identification of bacteria

The bark of the sample branches was removed and the wood cut into 5 cm lengths. Under sterile conditions, the surface of the wood was sterilised with 70% ethanol and the sapwood was removed with a sterilised knife. 4 chips (1× 0.5 cm × 0.5 cm) of the central part of the wood were put directly on a Petri dish with nutrient broth media and incubated at 28℃. Three replicate plants were made for each sample from each tree. Bacteria colonies were selected from the trails made by nematodes on the medium, 2～3 days after establishing the culture. To avoid losing any bacteria species, colonies with different characters from those already selected were intentionally chosen and coded for further purification. The selected and purified colonies were stored at −30℃.

To ensure all the colonies came from *B. xylophilus*, the nematodes were collected from the dishes in which bacteria colonies had been isolated and identified morphologically under a

microscope. Only those colonies from a dish in which all the nematodes were identified as *B. xylophilus* were selected for further identification. Colour, transparency, prominence, edge, viscosity, etc. of the isolated and purified colonies were observed and Gram and oxidase reactions were performed (Krieg & Holt, 1984). Then identification was carried out by an automatic bacteria identification system, ABT Expression, with a fully automated drug susceptibility identifier (bioMerieux Inc., Marcy—Etoile, France). The ID 32GN system was adopted for Gram negative bacteria; ID32E for Enterobacteriaceae and other Gram negative bacteria; 2STAPH for *Staphylococcus* and IDAP120A for anaerobes. 32 biochemical parameters were included in the analysis system, containing D-ribose, maltose, acetates, *etc.* Final results were taken from anslysis with an ATB Plus computer and the related software (ATBLAB Plus, Marcy—Etoile, France).

Bioassay of toxin production by bacteria

All identified strains of bacteria were separately inoculated into nutrient broth loquid medium (3 g $\cdot L^{-1}$ nutrient broth, 10 g $\cdot L^{-1}$ peptone, 5 g $\cdot L^{-1}$ NaCl, pH 7.2～7.4). The medium was then agitated at 110 rotations min^{-1} and 28℃ for 48 h. The original liquid was adjusted to a final concentration of 1×10^6 bacteria mL^{-1} and was centrifuged at 800 g for 15 min. Then the supernatant liquid without bacterial cells was used in biossays.

All glassware and tools were sterilised and the bioassay was conducted in a 300 mL glass bottle with a 4.5 cm diam. Mouth in which there were three 1.5 mL ampoules. Under aseptic conditions, a 60-day-old aseptic seedling with the root cut off was put into an ampoule containing the supernatant liquid from the identified bacteria. Enough water was added into the bottle to maintain humidity. 6 replidats were set up for each strain of identified bacteria. The blank nutrient broth medium treated identically to the inoculated media was used as the control. The bottle with 3 seedlings was sealed and kept at 27℃ and 14 h light : 10 h dark. The seedling were observed and their appearance recorded daily for 15 days. The time taken before a seedling wilted was recorded and the mean for the six replicates was used to indicate the phytotoxicity of each bacterium. If the average period was 12 days or less, the strain was considered to produce phytotoxin(s).

Inoculations of balck pine seedlings and trees

Aseptic culture of black pine seedlings

Seeds of black pine were washed in running water for 4 h, dipped in 0.1% potassium permanganate for 2 h and then washed three times in sterile water. The seeds were afterwards treated with 70% ethanol for 30 s, then with 0.1% mercuric chloride for 1 min and washed five times in sterile water. Under aseptic conditions, the episperm was peeled off and the seeds washed three times in sterile water, and treated with 5% hydrogenperoxide solution for 3 min, then washed five times in sterile water. The treated seeds without episperm were soaked for 24 h and then treated with 3% hydrogen perocxide solution for 3 min and washed five times in sterile water. They were then put on a potato dextrose agar (PDA) plate for germination at

25～27℃. If a plate was contaminated, the seeds or seedlings were discarded. When new seedlings sprouted, they were transplanted into tubes, diam. 20 mm and cultured in MS media at 25～27℃ under a regime of 14 h light：10 h dark. The seedlings were grown for periods of time required by the experiments.

Culture of PWN

Pine wood nematodes were isolated by Baermann funnel from naturally diseased black pine trees from Nanjing, Jiangsu. The nematodes were cultured on *Botytis cinerea* on PDA medium in a Petri dish. Nematodes were washed off the agar and dish with sterile water and single adult nematodes were picked under a microscope. After nematodes were confirmed to be *B. xylophilus*, one female and one male were cultured on a mat of *B. cinerea* in a test tube kept at 25℃ for a week. Species of bacteria carried by the strain of PWN were identified by the procedures previously described.

Culturing black pine callus

Following the procedure of Gao (2001) and Zhang (2002), calli were successfully induced on the tissue culture medium, 1/2 MS + 2, 4-D 10.0 mg • L^{-1} + KT 4.0 mg • L^{-1} + 6-BA 4.0 mg • L^{-1}, from mature embryos of *P. thunbergii* at 27 ± 1℃ in the dark. The callus was subcultured on the tissue culture medium, 1/2 MS + NAA 0.5 mg • L^{-1} + IBA 0.1 mg • L^{-1} + GA_3 1.0 mg • L^{-1} + LH 100 mg • L^{-1} + 2, 4-D 1.0 mg • L^{-1}, in the same conditions as above.

Production of bacteria-free nematodes

Nematodes with culture medium were washed with sterile physiological salt solution (0.9% NaCl) onto filter paper in a funnel and left for 24 h when the living nematodes in 10 ml of liquid were centrifuged at 800 g for 6 min. The supernatant was discarded and the nematodes were washed six times with sterile physiological salt solution to which penicillin-G 1 600 units/mL, streptomycin 10 mg/mL and fungicidin 5 mg/mL were added. Each time, 10 mL of the liquid was added to the precipitate and centrifuged. Finally after washing and centrifuging three times with sterile physiological salt solution, the nematodes were cultured on the black pine callus. To examine whether the nematodes were bacteria-free, several nematodes were put on nutrient broth media in a plate and cultured at 27℃ to observe if bacterial colonies grew.

Inoculation of 4-month-old black pine seedlings

Bacteria-free PWN cultured on black pine callus were washed with sterile water and the suspension adjusted to 2 000 nematodes ml^{-1}. Bacteria strains, GcM5-1A (*Pseudomonas fluorescens*), GcM1-3A (*P. cepacia*), ZpB1-2A (*P. putida*), ZpB1-1A (*Pantoea* sp.) and AcB1C (*Peptostreptococcusasa ccharolyticus*) (Table 1), were selected for use in the inoculation tests. A bacterial lawn was removed from the slant nutrient broth agar media after incubation for 48 h and transferred into nutrient broth liquid medium and rocked for 48 h at 28℃. The bacterial culture was then centrifuged at 40 000 g for 15 min, the supernatant discarded and the precipitate adjusted to 1×10^7 bacteria mL^{-1} by adding sterile physiological salt solution. For inocula with bacteria and nematodes, the suspension was left for 1 h to allow the bacteria

to combine with the nematodes. Untreated nematodes were obtained using Baermann funnels after culturing wild PWN on a mat of *B. cinerea* and washing three times with sterile physiological salt solution. The nematode suspension was adjusted to a concentration of 5 000 nematodes ml^{-1}. To ensure that axenic nematodes did not carry any bacteria, before use they were checked again as described previously. Under aseptic conditions, the upper stem of a 4-month-old axenic black pine seedling in a test tube was lightly punctured with a needle, then a piece of sterilised cotton was introduced onto the wound and 500 nemafodes and/or 1×10^6 bacteria added onto it. Each treatment consisted of 6 replicates. Inoculation with sterile physiological salt solution was used as a control.

The seedling were observed, symptoms recorded daily for 5 days and incidence of disease symptoms calculated for each treatment. Symptoms were classified as: 0: no symptoms; Ⅰ: brightness of needles lost; Ⅱ: needles turned yellowish or wilting; Ⅲ: seedlings wilted and brown. 5 days after inoculation the pine seedling was uprooted, treated with 0.1% mercuric chloride for 4 min, washed three times with sterile water and cut into 3~5 mm lengths. Bacteria were isolated by culturing 2~3 pieces of seeding segments on nutrient broth media. The pieces of the seedlings were put into a dish to which 5 mL water was added. After soaking for 12 h at room temperature, the pieces were examined under a microscope for presences of nematodes.

Greenhouse inoculation of 3-year-old black pine trees

Tests using potted 3-year-old black pine trees were conducted in a greenhouse at Nanjing Forestry University; maintaining temperature and relative humidity were kept at 28~29℃ and 50%~60%, respectively. Soil used to grow the black pines was steam-sterilised before use. Lysol and ultraviolet rays were used to sterilize the compartment before the potted trees were moved in. GcM5-1A (*Pseudomonas fluorescens*) and PWN isolated as previously described were used in the experiment. Inoculations were made with 5 000 wild or axenic PWN and/or 2×10^6 bacteria in 0.5 mL of sterile physiological salt solution and a control treatment with 0.5 mL of sterile physiological salt solution. 20 trees were used for each treatment. All tools and materials used in the experiment were sterilised. A cut about 5 mm deep was made on the tree trunk after surface-sterilising the bark with 70% alcohol, and inoculum was introcuced onto a piece of sterilised cotton inserted into the cut. Then the cotton was covered with a piece of gauze and a second piece treated with streptomycin paste was added and the whole bound tightly on the trunk with a bandage. Symptoms on the trees were observed and recorded 3 months after inoculation. Symptoms were classified as 5 levels: - : healthy looking, resin flow normal; +: resin flow reduced; + +: resin flow ceased and needles yellowish; + + +: most needles yellowish or yellow; + + + +: all needles brown, tree dead. 3 branch samples were cut from each tree for laboratory recovery examination. The nematodes and bacteria in the samples were checked in the laboratory with the same procedure as described for seedling. Mean nematode number g^{-1} dry wood was calculated for each treatment.

Filed inoculation experiments with 7-year-old black pine trees

Field tests were conducted in 7-year-old black pines in a forest in Nanjing, Jiangsu from 30 September 2002 to 15 July 2003. Inocula used in the experiment were the same as those used in the glasshouse experiment. To keep conditions in the field as aseptic as possible, tools and materials used in the experiment were sterilised. To keep dust away from the crown, plastic sheeting was wrapped around the tree. A 5 mm diam. Hole was drilled in the trunk after the bark was surface-sterilised with 70% alcohol. Inoculum was introduced into the hole, and then the hole was covered with cotton, gauzes and bandage as previously described.

A wooden framework covered with plastic mesh was set up for each tree to animal damage, especially from beetles and other pests. Symptoms of the treated trees were checked and recorded on 15 July 2003. Meanwhile, the amount of resin flow from drill holes was recorded in the following days. Three branch samples were cut from each tree for laboratory recovery examination. The nematodes and bacteria in the samples were checked in the laboratory with the procedure described above and symptoms were classified as before.

Table 1 Identification of 24 bacteria strains carried by *Bursaphelenchus xylophilus* in China and the phytotoxicity (means of 6 replicates) of their cell-free culture fitrates to black pine seedlings in vitro

Isolate[1]	Gram reaction	Oxidase reaction	Species	Similarity (%)	Phytotoxicity (Days to wilting)
GcM5-1A	–	+	*Pseudomonas fluorescens*	88.0	4.2
ZpB2-1A	–	+	*P. fluorescens*	70.8	4.5
GcM6-1A	–	–	*P. putida*	83.1	4.2
ZpB1-2A	–	+	*P. putida*	97.4	4.5
HeN-139A	–	–	*Pseudomonas* sp.	99.9	9.0
ZpB1-2B	–	–	*Pseudomonas* sp.	93.7	4.5
HeM-127B	–	–	*Pseudomonas* sp.	99.9	7.3
HeM-2A	–	–	*Pseudomonas* sp.	99.8	9.0
HeM142B	–	–	*Pseudomonas* sp.	77.8	9.3
AhM2D	–	+	*P. cepacia*	60.0	9.2
JnB619	–	+	*P. cepacia*	98.7	4.2
HeM3	–	–	*Pantoea* sp.	99.7	12.0
HeMA	–	–	*Pantoea* sp.	99.9	10.3
JnB1B	–	–	*Pantoea* sp.	99.7	12.1
AcM1A	–	–	*Pantoea* sp.	99.9	13.2
ZpB1-1A	–	–	*Pantoea* sp.	91.0	9.8
GcM2-3B	–	–	*Pantoea* sp.	99.9	3.5
JnM1B	+	–	*Peptostreptococcu-sasac charolyticus*	86.5	12.3
AcB1C	+	–	*P. asaccharolyticus*	86.5	12.8
ZpB2-3A	–	–	*Enterobacter amnigenus*	88.5	13.2

续表

Isolate[1]	Gram reaction	Oxidase reaction	Species	Similarity (%)	Phytotoxicity (Days to wilting)
AcB2C	–	–	*Buttiauxella agresitis*	99.4	12.5
AhM2A	–	–	*B. agrestis*	99.4	13.0
GcM3-2A	–	–	*Serratia marcescens*	99.9	3.8
GcM6-2B	–	–	*S. marcescens*	99.8	4.2

[1]Sample origin. He: Hubei, Enshi; Jn: Jiangsu, Nanjing; Ac: Anhui, Chaohu; Ah: Anhui, Hanshan; Zf: Zhejiang, Fuyang; Zp: Zhejiang, Pinghu; Gc: Guangdong, Conghua. Host tree sepecies. B: black pine, *Pinus thunbergii*; M: Masson pine, *Pinus massoniana.*

Table 2 Identification of bacteria species carried by wild pine wood nematode, *Bursaphelenchus xylophilus* and their phytotoxicity to black pine seedlings in vitro

Isolate[1]	Gram Reaction	Oxidase reaction	Species	Similarity (%)	Phytotoxicity (Days to wilting)
NJG4	–	+	*Pseudomonas fluorescens*	82.8	4.2
NJH1	–	+	*P. fluorescens*	84.8	4.5
NJE2	–	–	*P. putida*	71.9	4.0
NJK2	–	–	*p. putida*	80.1	4.5
NJG2	–	–	*Pseudomonas* sp.	68.4	4.5
NJG3	–	–	*Pseudomonas* sp.	68.4	7.3
NJI1	–	+	*Escherichia coli*	98.5	13.2
JnB619	–	–	*P. cepacia*	98.7	4.2
NJPn7	–	–	*Sphingomonus paucimobilis*	99.4	4.5
NJP4	–	–	*Enterobacter amnigenus*	85.0	12.8
JnB1B	–	–	*Pantoea* sp.	99.7	12.1

[1]Sample origin: NJ: Nanjing; Jn: Jiangsu, Nanjing.

Results

Bacteria colonies appeared in cultures on the tracks left by nematodes from all the samples collected from the 5 main epidemic provinces in China. The isolation experiments demonstrated that it was a universal phenomenon for PWN to carry bacteria in their natural environment. There were no bacteria in all samples collected from healthy trees.

In the inoculation experiments using 4-month-old seedlings, 3-year-old black pine trees in the glasshouse and 7-year-old trees in the forest showed that none of the bacteria tested invaded healthy pines by themselves (Tables 3, 4, 5).

24 strains of bacteria were isolated and identified from the collected samples by combination of the classical and the ATB system. In addition, their phytotoxicity was also assayed (Table 1).

Table 3 Effects of combinations of pine wood nematode (PWN: bacteria-free and wild isolates) and strains of bacteria isolated from PWN on symptoms (0: none; Ⅰ: brightness of needles lost; Ⅱ: needles turned yellowish or wilting; Ⅲ:seedlings wilted and brown) at 3 and 5 days after inoculation of 4-month-old axenic black pine seedlings and on recovery (+) or not (–) of nematodes and bacteria from stem segments at 5 days (6 seedlings per treatment)

Inoculum			Days from inoculation										Recovery	
			3d					5d						
Nematode	Baterium	Strain	Symptom level				Incidence	Symptom level				Incidence	Bacteria	Nematodes
			0	Ⅰ	Ⅱ	Ⅲ	%	0	Ⅰ	Ⅱ	Ⅲ	%		
Control			6	0	0	0	0	6	0	0	0	0	–	–
None	*Pseudomonas fluorescens*	GcM5-1A	6	0	0	0	0	6	0	0	0	0	–	–
None	*P. cepacia*	JnB619	6	0	0	0	0	6	0	0	0	0	–	–
None	*P. putida*	ZpB1-2A	6	0	0	0	0	6	0	0	0	0	–	–
None	*Pantoea* sp.	ZpB1-1A	6	0	0	0	0	6	6	6	6	6	–	–
Axenic PWN	*Peptostreptococcus Asaccharolyticus*	AcB1C	6	0	0	0	0	6	0	0	0	0	–	–
Axenic PWN	None	None	5	1	0	0	17	4	2	0	0	33	–	+
Axenic PWN	*Pseudomonas Fluorescens*	GcM5-1A	0	5	1	0	100	0	0	3	3	100	+	+
Axenic PWN	*P. cepcia*	JnB619	2	4	0	0	67	0	3	1	2	100	+	+
Axenic PWN	*P. putida*	ZpB1-2A	0	5	1	0	100	0	2	2	2	100	+	+
Axenic PWN	*Pantoea* sp.	ZpB1-1A	0	5	1	0	100	0	0	4	2	100	+	+
Axenic PWN	*Peptostreptococcus Asaccharolyticus*	AcB1C	4	1	0	0	17	4	2	0	0	33	+	+
Wild PWN	–	–	1	5	0	0	83	0	1	2	3	100	+	+

Table 4 Pathogenicity (symptoms: −: none; + + + +: all needles brown, tree dead) and recovery of bacteria (− or +), nematodes (number per gram dry weight wood) or fungus (− or +) from 3-year-old black pine trees grown in a glasshouse after inoculation with axeinic pine wood nematode(PWN) and a phytotoxin-producing bacterium carried by nematode

Inoculum			N	Symptom	Recovery		
Nematode	Bacterium	Strain			Bacteria	PWN/g	Fungus
Axenic PWN	None	None	16/20	−	−	1.2	−
			4/20	+ + + +	+	264.1	−
Axenic PWN	*Pseudomonas fluorescens*	GcM5-1A	20	+ + + +	+	201.6	−
None	*P. fluorescens*	GcM5-1A	20	−	−	0	−
Wild PWN	−	−	20	+ + + +	+	220.4	−
	Control		20	−	−	0	−

Table 5 Pathogenicity (symptoms: −: healthy, resin flow normal; +: resin flow reduced; + +: resin flow ceased, needles yellowish; + + +: most needles yellowish or yellow; + + + +: all needles brown, tree dead) and recovery of bacteria (− or +), nematodes (number per gram dry weight of wood) or fungus (− or +) from 3-year-old black pine trees grown in a forest after inoculation with axenic pine wood nematode (PWN) and a phytotoxin-producing bacterium carried by the nematode

Inoculum			n	Symptoms	Recovery		
Nematode	Bacterium	Strain			Bacteria	PWN/g	Fungus
Axenic PWN	None	None	3	−	−	<20	−
			1	−	−	−	−
			2	+ + +	+	20~200	−
			1	+ + +	+	>200	−
			1	+ + + +	+	>200	+
Axenic PWN	*Pseudomonas fluorescens*	GcM5-1A	1	−	−	−	−
			1	+	−	−	−
			6	+ + + +	+	20-200	−
None	*P. fluorescens*	GcM5-1A	8	−	−	−	−
	−		1	+	−	−	−
Wild PWN		−	5	+ + + +	+	>200	−
			2	+ + + +	+	>200	+
	Control		8	−	−	−	−

The main group of bacteria (11 of the 24 strains) carried by PWN belonged to the genus *Pseudomonas*. This genus was isolated from samples collected in every province and host species, *i. e.*, Hubei, Jiangsu, Anhui, Zhejiang and Guangdong Provinces, in both black pine and masson pine. All bacteria strains of the same genus showed a certain level of phytotoxicity (Table 1). This indicates that the PWN carry certain bacteria and that *Pseudomonas* spp. might play a key role in pine pathogenicity. This finding is also true for bacteria species carried by wild PWN used in inoculation tests (Table 2).

Six bacteria strains (*Pantoea* spp.) were also isolated from samples from the five

provinces in both black and Masson pine. However, some strains of this genus showed no phytotoxicity. Other bacteria strains of low frequency were isolated from particular locations.

To examine the role of the isolated bacteria strains in the disease, GcM5-1A (*Pseudomonas fluorescens*), JnB619 (*P. cepacia*), ZpB1-2A (*P. putida*), ZpB1-1A (*Pantoea* sp.), AcB1C (*Peptostreptococcu sasacc harolyticus*), *B. xylophilus* and axenic *B. xylophilus* were used to inoculate 4-month-old black pine seedlings (Table 3). None of the seedlings inoculated with a single bacterium stain showed any symptom of disease and no bacteria were recovered. There were nematodes found in the seedling inoculated with axenic PWN (see Table 3). This indicates that these nematodes could invade seedlings but not induce the disease, because only 2 of the 6 seedlings showed minor symptoms at the 5th day (Table 3). Such symptoms could result from nematode feeding, since the seedlings showed only shrinkage at the inoculated site but no wilting or browning. In the tests with axenic PWN and a bacterium, all seedlings showed disease symptoms. In those treatments, two or three of the six seedlings in each were dead at day (Table 3). Both the bacterium and nematodes were recovered from these seedlings (Table 3). There were no significant differences between these tests and those with wild PWN in incidence and symptom rating at day 5 (Table 3). The only exception was AcB1C + axenic PWN, where symptoms were the same as those for axenic PWN, indicating that strain AcB1C is not pathogenic. The result was consisitent with that in Table 1 in which AcB1C showed no phytotoxin production.

In the glasshouse tests with 3-year-old black pine, all trees treated with wild PWN were dead at the end of the tests (Table 4). All 20 trees treated with axenic PWN + GcM5-1A were also dead. However, trees treated with GcM5-1A alone showed no disease symptoms. 16 of the 20 trees treated with axenic PWN showed no symptoms and only nematodes were recovered from these trees; the other four trees were dead and both nematode and bacteria were recovered (Table 4).

In the field experiment, 7-year-old trees were inoculated with GcM5-1A (*Pseudomonas fluorescens*) and/or *B. xylophilus* or axenic *B. xylophilus*. No trees treated with GcM5-1A had any disease symptoms and neither bacteria nor nematodes were found in the recovery tests. Three of the eight trees inoculated with axenic PWN showed no disease symptoms and PWN but no bacteria were recovered (Table 5). Failure of the inoculation was thought to be the case for one tree in which no bacteria or nematodes were found (Table 5). The other 4 trees of this treatment had serious disease symptoms and both bacteria and many nematodes were recovered (Table 5). The mortality and recoveries were not significantly different between wild PWN alone and axenic PWN with GcM5-1A with respectively, seven and six dead trees of the eight inoculated. It was noteworthy that only those trees from which both nematodes and bacteria were recovered had symptoms greater than class "+".

Discussion

The isolation experiments showed that, in China, it is a general and natural phenomenon