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A Pathological Study of the Bacterial Tree Diseases Found in Hokkaido, Northern Japan

By

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Summary : Bacterial canker of *Maackia amurensis* var. *buergeri* caused by *Pseudomonas syringae*.

A new disease of *Maackia amurensis* var. *buergeri* was identified in Hokkaido. Affected trees were heavily damaged and had cankers on trunks and branches. The pathogen was isolated and characterized as *Pseudomonas syringae*. Inoculation tests confirmed its pathogenicity to the host. Hence, the disease was designated as a "bacterial canker of *M. amurensis* var. *buergeri* " caused by *P. syringae*. Anatomical observations to elucidate the process of the canker formation were carried out. The initial symptoms included a longitudinal series of swellings and slightly swollen bark. First, abnormalities appeared near the cambial zone. Xylem formation was suppressed, hyperplasia of irregular ray parenchyma cells occurred, then the swellings appeared. The irregular cells increased in number ; after that, the surface of the swollen bark burst open. The ruptured swellings coalesced to form long irregular cankers.

: Watermark disease of willows (*Salix* spp.) caused by *Erwinia salicis* (Day 1924) Chester 1939.

For the first time, the watermark disease of willows was identified in Hokkaido. During spring and summer, the leaves and branches suddenly wilted, and the affected trees sometimes died. When affected branches or trunks were cut, a distinct, watery zone stained reddish brown or brownish black (watermark) was observed in the sapwood. The pathogen was isolated from the watermark and identified as *Erwinia salicis*. Inoculation tests confirmed its pathogenicity to willows. The anatomical characteristics of affected trees and their ability to conduct water were investigated. In the watermark, some of the ray parenchyma cells caused necrosis. Dye-injection tests revealed that water conduction did not take place in the watermark. Therefore, the non-conductive watermark can be considered to be similar to discolored wood, and its formation and expansion in the affected sapwood may be the reason for the wilting symptoms.

Contents

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Preface

Forest pathology is a division of plant pathology that was first systematized by HARTIG (1882).

The first scientific report of forest pathology in Japan was presented by TANAKA in 1888. Although in over 100 years of the history of forest pathology in Japan, research has mostly focused on fungal diseases, except for pine wilt disease caused by the nematode. The study of diseases caused by other microorganisms had been neglected, particularly bacterial diseases. Actually, only a small number of reports about bacterial tree diseases have been presented in Japan (quoted in NISHIYAMA, 1997), and almost all of these were etiological and bacteriological studies. Pathological studies including the physiological aspects, anatomical aspects and mechanisms of disease development are lacking. In Hokkaido, almost no studies on bacterial tree diseases have been carried out.

Recently in Hokkaido, several experimental approaches have been implemented to establish natural forest and watershed management regimes to replace the previous artificial forest management regime (planting conifers, such as *Abies*, *Larix* and *Picea*). In order to establish these two regimes, it is necessary to implement methods to encourage the growth of fine broad-leaved trees, as these trees are very valuable not only for their wood but also for their contribution to maintaining the biodiversity of the natural forest. Therefore, more information about broad-leaved trees, including silviculture, physiology, genetics and protection, is needed. The study of disease is quite important, especially for fatal diseases such as shoot blight, gall, canker and wilt.

Recently, two new diseases of broad-leaved trees, the canker of *Maackia amurensis* var. *buergeri* and the wilt disease of willows (*Salix* spp.), were identified in Hokkaido. After pathological studies of these diseases had been conducted, it was determined that both of them were caused by plant-pathogenic bacteria, and the mechanisms of disease development were outlined (SAKAMOTO *et al.*, 1999 ; SAKAMOTO, 1999 ; SAKAMOTO *et al.*, 2000 ; SAKAMOTO & SANO, 2000). This report describes the results of these studies.

Chapter-I Bacterial canker of *Maackia amurensis* var. *buergeri* caused by *Pseudomonas syringae*.

1. Introduction

Maackia amurensis Rupr. et Maxim. var. *buergeri* Schn. (inu-enju) (hereafter referred to as *M. amurensis*) is a leguminous deciduous tree. In Japan, it is mainly found on the islands of

Hokkaido, Honshu and Shikoku. The wood of *M. amurensis* exhibits a beautiful combination of yellowish white sapwood and dark brown heartwood and is very durable. While there are several small artificial stands and experimental forests in Hokkaido, an adequate forest management regime has not been established yet. Recently, a major outbreak of a new canker disease of *M. amurensis* was reported in two plantations at Kawayu in Teshikaga (eastern Hokkaido) and Higashiyama in Furano (central Hokkaido) (KOIZUMI *et al.*, 1989 ; SASAKI, 1990). Those stands have been heavily damaged due to the disease, which has critically reduced the outlook for wood production. Some trees were also affected in natural forests in Kimobetsu (southern Hokkaido), Chitose and Sapporo (central Hokkaido), in the garden of "Yotei seinen no mori" Forest Park in Makkari (south Hokkaido) and in roadside trees in Sapporo and Tokoro (eastern Hokkaido).

When affected tissues were examined with a phase-contrast light microscope, numerous bacterial cells were observed to stream out from the tissues. The bacterium was isolated from the affected tissues and its pathogenicity to *M. amurensis* was confirmed. The bacterium was characterized tentatively as *P. syringae* by its bacteriological characteristics. This study is the first report of canker disease in *M. amurensis* caused by *P. syringae* in the world.

The initial symptoms of the disease appeared as a longitudinal series of swellings and slightly swollen bark (SSB). Field observations had revealed that these swellings gradually burst and coalesced to form longitudinal cankers with exposed wood tissues. However, to elucidate the process of the canker formation, anatomical studies of symptoms on various stages are needed.

This chapter deals with the description of the symptoms, the isolation of the pathogen and its characterization and pathogenicity tests, then proposes the name of this disease as "bacterial canker of *M. amurensis* var. *buergeri*" caused by *P. syringae*. There is also an anatomical study of the disease development.

2. Symptoms of the disease

Symptoms were observed regularly from 1993 to 97 in the Higashiyama stand. Cankers were observed on trunks and branches alike. On trunks, they grew longitudinally (Fig. 1 a). The initial symptom developed from late spring to early summer as a series of longitudinal swellings (approximately 3×1.5 cm) on the surface of the bark, which were interspersed with SSB (Fig. 1 b). These swellings gradually burst open and coalesced to form longitudinal cracks (Fig. 1 c-1, 2). These long cankers on the trunks sometimes spread horizontally and girdled the trunks. In the Kawayu stand, many cankers were developing from pruning scars (Fig. 1 d). The tissues of the affected inner bark were observed to crack and break off easily. Finally, brownish black, dead inner bark tissues tore and fell off, exposing the xylem tissues.

3. Isolation, characterization and pathogenicity of the pathogen

3-1 Materials and Methods

3-1-1 Isolation

Samples were collected from 24 affected *M. amurensis* in Kawayu and Higashiyama stands from May through October in 1994. Small pieces of inner bark tissue (approximately 5×5×5 mm) were excised from swollen, water-soaked areas in the swellings and from the margins of cankers. Each piece was macerated in 5 m*l* of sterile peptone (Difco, MI, USA) water (1% w/v). The resulting suspensions were then streaked on plates of nutrient agar (NA : Beef extract : 5 g ;

Table 1. Bacterial isolates used in this study

Number	Location	Isolation source	Date isolated	Year
ys-8	Higashiyama	swelling	24 May	1994
ys-9	Higashiyama	swelling	23 June	1994
ys-10	Kawayu	swelling	15 July	1994
ys-11	Kawayu	canker	15 July	1994
ys-25	Higashiyama	canker	6 October	1994
ys-26	Higashiyama	canker	6 October	1994
ys-27	Higashiyama	canker	6 October	1994
ys-28	Higashiyama	canker	6 October	1994
LOB 2-1		*Syringa vulgaris*		
TO 1		*Trema orientalis*		
YAMAMOMO 801		*Myrica rubra*		

peptone : 10 g ; NaCl : 5 g ; agar : 15 g ; distilled water : 1000 m*l*. Eiken E-MC 01) and incubated for 2-3 days at 20°C. The single colonies which regularly grew, were re-streaked on fresh NA plates to establish purity. In total, isolates from 19 different trees (3 isolates from Kawayu and 16 isolates from Higashiyama) were collected. These isolates were either stored at −80 C in liquid skim milk (10% w/v) for working cultures or freeze dried for long-term storage.

3-1-2 Characterization

Eight isolates were tested for bacteriological characteristics (Table 1). Isolates of *P. syringae* pv. *syringae*, *P. s.* pv. *tremae* and *P. s.* pv. *myricae* (stored in Shizuoka Univ.) were selected for comparison tests.

Colony morphology was observed on NA plates. Cell morphology of preparations stained with acid fuchsin were observed by phase contrast microscopy. Gram-reaction was determined by RYU'S (1937) method. Flagella insertion was recorded by light microscopy of slide preparations stained by a modification of YAMANAKA'S silver staining method (SHIRATA & GOTO, 1981). Poly-β-hydroxy butyrate granules were stained with Sudan Black B (SCHAAD, 1988).

Fluorescent pigment production was determined on medium B of KING *et al.* (1954). The oxidation /fermentation (OF) test was carried out in the medium of HUGH and LEIFSON (1953). In the test for indole production, cultures were grown in peptone water and indole was detected using Kovac's reagent (COWAN, 1974). Phosphatase activity was also determined by the method of COWAN (1974). Pectin liquefaction was determined on Paton's medium, by the method of SCHAAD (1988). Urease activity was determined on CHRISTENSEN'S (1946) urea agar plates. Gluconate oxidation was tested by the method of IIZUKA and KOMAGATA (1963). Lecithinase activity and V.P.-M.R. tests were performed as described in the Manual of Microbiological Methods (Society of American Bacteriologists, 1957). Tests for hydrolysis of starch and casein were performed as described in the Manual of Methods for General Bacteriology (GERHARDT *et al.*, 1981). For H_2S production, cultures were grown in nutrient broth, and tested using lead-acetate paper. Growth at 40°C was examined in peptone water (1% w/v). Utilization of organic compounds as sole sources of carbon was determined using the medium of AYERS *et al.* as described in

the Manual of Microbiological Methods (Society of American Bacteriologists, 1957), incorporating the carbohydrates at 1.0% and the other compounds at 0.2%. The tested compounds were glucose, sucrose, D-galactose, D-fructose, D-ribose, inositol, D-xylose, D-raffinose, D-mannitol, L-arabinose, D-arabinose, lactose, maltose, melibiose, L-rhamnose, dulcitol, adonitol, D-salicin, D-mannose, glycerol, D-sorbitol, erythritol, D-cellobiose, trehalose, starch, D-melezitose, α-methyl-D-glucoside, L-malate, gluconate, citrate, succinate, L-arginine, formate, n-butyrate, propionate, mesaconate, levullinate, D-saccharate, glutarate, betaine, malonate, DL-lactate, acetate, L-valine, L-isoleucin, L-serine and L-tyrosine. Positive results were recorded only when visible growth was observed. Reducing substances from sucrose, nitrate reduction and reaction in purple milk were all tested by the methods described by DYE (1968). Finally, the following were tested by the methods described by LELLIOTT *et al.* (1966) ; arginine dihydrolase, oxidase and tyrosinase activities, hydrolysis of aesculin and Tween 80 ; gelatin liquefaction, levan production (mucoid growth), potato soft rot and tobacco hypersensitive reaction.

3-1-3 Pathogenicity

Pathogenicity tests were performed on 7-year-old seedlings of *M. amurensis* growing in the nursery of Hokkaido Research Center, Forestry and Forest Products Research Institute (FFPRI) (Sapporo) in 1994. The inoculation dates, isolates, and numbers of tested seedlings are listed in Table 2. Cultures stored at −80℃ were spread on NA plates and incubated for 3-4 days at 20℃. Holes were bored into the xylem of the trunks of seedlings with a cork borer (5 mm in diam.), then filled with a mass of bacterial cells from NA plates and sealed with vinyl tape. Controls received the same treatment, without bacterial cells. Each plant had three inoculation sites and two control sites. The vinyl tape was removed 2-3 weeks after inoculation. The appearance and development of symptoms was observed up to the end of October 1997.

3-2 Results

3-2-1 Isolation

The same bacterium was regularly isolated from all the affected freshy tissues. The colonies were 1-2 mm in diameter on NA after 3 days at 20℃, with an entire margin, and were circular, convex, smooth and glistening.

3-2-2 Characterization

All the isolates tested were Gram-negative, non-sporing, straight rods, motile with one to three flagella and produced a white-to-cream colored growth on NA. They metabolized glucose oxidatively. All the isolates gave positive reactions in the following tests : reducing substances from sucrose, hydrolysis of Tween 80 and tobacco-hypersensitive reaction. Variable results were obtained for levan production among the isolates (ys-8, 10, 11, 26, 27 : positive ; ys-9, 25, 28 : negative). No visible changes were observed in the test of purple milk. All isolates gave negative reactions in the following tests : fluorescent pigment on King B medium, growth at 40℃, VP-MR tests, H_2S production, gluconate oxidation, hydrolysis of aescrin, casein and starch, indole test, gelatin and pectin liquefaction, arginine dihydrolase, activities of lecithinase, oxidase, phosphatase, tyrosinase and urease, nitrate reduction, potato soft rot. All isolates produced acid from glucose, sucrose, D-galactose, D-fructose, D-ribose, inositol, D-mannose, glycerol, D-sorbitol and erythritol but no isolates produced acid from D-xylose, D-raffinose, D-mannitol, L-arabinose, D-arabinose, lactose, maltose, melibiose, L-rhamnose, dulcitol, adonitol, D-salicin, D-cellobiose, trehalose, starch, D-melezitose and α-methyl-D-glucoside. All isolates utilized L-malate,

Table 2. Schedule of inoculation tests and results [a]

Date	Isolate used	Number of inoculated seedlings	Number of affected inoculation sites
24 May	ys-8	5	7
23 June	ys-9	12	21
15 July	ys-10	3	4
	ys-11	3	8
16 August	ys-9	5	11
22 August [b]	re-isolated bacterium	3	4
7 September	ys-10	3	4

a) All tests were performed with the same procedure in 1994.
b) Re-inoculation test.

Table 3. Characteristics of the present isolates from *M. amurensis* and reference isolates

Characteristics	Present isolates (*)	*P. syringae* pv. *syringae* (*)	*P. syringae* pv. *myricae* (*)	*P. syringae* pv. *tremae* (*)	*P. syringae* pv. *actinidiae* [a]
Fluorescent pigment on King B medium	−	+	−	−	−
Levan (Mucoid growth)	V	+	+	−	+
Growth at 40℃	−	−	−	−	−
Reducing substances from sucrose	+	+	+	+	+
VP-MR tests	−	−	−	−	−
H_2S production	−	−	−	−	−
Gluconate oxidation	−	−	−	−	−
Reaction in purple milk	−(#)	KD	−(#)	−(#)	KW
Aescrin hydrolysis	−	+	−	−	−
Casein hydrolysis	−	+	−	+	+
Starch hydrolysis	−	−	−	−	−
Tween 80 hydrolysis	+	+	+	+	+
Indole test	−	−	−	−	−
Gelatin liquefaction	−	+	−	−	−
Pectin liquefaction	−	−	−	−	
Arginine dihydrolase	−	−	−	−	−
Lecithinase	−	−	−	−	−
Phosphatase	−	−	−	−	
Tyrosinase	−	−	+	+	−
Urease	−	+	−	−	−

* = The author's data.
a = Data from TAKIKAWA *et al.* (1989).
+ = 80-100% isolates positive.
− = 0-20% isolates positive.
K = alkaline reaction
D = digestion
W = weak reaction
# = No visible change was observed.
V = Variable results among the isolates.

Table 4. Acid production from carbohydrates and related carbon sources by the present isolates from *M. amurensis* and reference isolates

Organic compound	Present isolates (*)	*P. syringae* pv. *syringae* (*)	*P. syringae* pv. *myricae* (*)	*P. syringae* pv. *tremae* (*)	*P. syringae* pv. *actinidiae* (a)
Glucose	+	+	+	+	+
Sucrose	+	+	+	+	+
D-Galactose	+	+	+	+	+
D-Fructose	+	+	+	+	+
D-Ribose	+	+	+	+	+
Inositol	+	+	+	+	+
D-Mannose	+	+	+	−	+
Glycerol	+	+	+	−	+
D-Sorbitol	+	+	+	−	+
Erythritol	+	+	−	−	−
D-Xylose	−	+	+	−	−
D-Raffinose	−	+	−	−	+
D-Mannitol	−	+	+	+	+
L-Arabinose	−	+	+	+	+
D-Arabinose	−	−	−	−	−
Lactose	−	−	−	−	−
Maltose	−	−	−	−	−
Melibiose	−	−	−	−	−
L-Ramnose	−	−	−	−	−
Dulcitol	−	−	−	−	−
Adonitol	−	−	−	−	−
D-Salicin	−	−	−	−	−
D-cellobiose	−	+	−	−	−
Trehalose	−	+	+	−	−
Starch	−	+	−	−	−
D-Melezitose	−	−	−	−	−
α-methyl-D-glucosid	−	−	−	−	−

＊ = The author's data.
a = Data from TAKIKAWA *et al.* (1989)
+ = 80-100% isolates positive.
− = 0-20% isolates positive.

gluconate, citrate, succinate, L-arginine, D-saccharate, glutarate, betaine, malonate and L-serine, but no isolates utilized formate, n-butyrate, propionate, mesaconate, levullinate, DL-lactate, acetate, L-valine, L-isoleucin and L-tyrosine.

3-2-3 Pathogenicity

All of the 4 isolates produced pathogenic reactions on *M. amurensis*. In tests started on 24 May, 23 June and 15 July 1994, the inoculated inner bark tissues began to proliferate vigorously in the vicinity of the inoculation points after approximately two weeks. The bark became cracked and the symptoms continued to proliferate until the end of September 1994. In the inoculation test performed on 16 August 1994, the inoculated bark tissues proliferated less rapidly and extensively than the tissues inoculated early in the season. In the inoculation test performed on 7 September 1994, only slight proliferation of inoculated bark tissues was observed. Disease development ceased at the end of September 1994. At the end of April 1995, the symptoms started to develop again. Several swelling (Fig. 2 a -1) and noticeable cankers (Fig. 2 a -2) were observed.

Table 5. Utilization of organic compounds by the present isolates from *M. amurensis* and reference isolates

Organic compound	Present isolates (*)	*P. syringae* pv. *syringae* (*)	*P. syringae* pv. *myricae* (*)	*P. syringae* pv. *tremae* (*)	*P. syringae* pv. *actinidiae* (a)
L-Malate	+	+	+	+	+
Gluconate	+	+	+	+	+
Citrate	+	+	+	+	+
Succinate	+	+	+	+	+
L-Arginine	+	+	+	+	+
D-Saccharate	+	+	−	−	+
Glutarate	+	+	+	−	+
Betaine	+	+	+	−	+
Malonate	+	+	−	+	+
Formate	−	−	−	−	−
n-Butyrate	−	−	−	−	−
Propionate	−	−	−	−	−
Mesaconate	−	−	−	−	−
Levullinate	−	−	−	−	−
DL-Lactate	−	+	−	−	−
Acetate	−	+	−	−	+
L-Serine	+	+	+	−	+
L-Valine	−	−	−	−	−
L-Isoleucin	−	−	−	−	−
L-Tyrosine	−	−	−	−	+

* = The author's data.
a = Data from TAKIKAWA *et al.* (1989).
+ = 80-100% isolates positive.
− = 0-20% isolates positive.

Finally in 1997, 59.1% of the inoculation points had become noticeable cankers. There were no pathogenic reactions on control sites.

Two collections of bacteria were re-isolated on 16 August from the proliferated tissues of a seedling that had been inoculated with the isolate ys-8 on 24 May in 1994. One of them showed pathogenicity when re-inoculated into the host (Table 2).

On the days of high humidity, droplets of bacterial ooze exuded from the affected lesions every year from early May to the end of June (Fig. 2 b). At first, the exudates were transparent-to-white droplets of low viscosity but they gradually turned brown and increased their viscosity as time went by.

3-3 Discussion

The bacterial isolates from *M. amurensis* formed a relatively homogeneous group in their bacteriological properties. They were Gram-negative, non-sporing, straight rods, motile with one to three flagella, produced a white-to-cream colored glistening growth, metabolized glucose oxidatively, did not grow at 40℃, nor did they accumulate poly-β-hydroxy butyrate granules. Hence, they should be included in the genus *Pseudomonas* (PALLERONI, 1984).

Variable results were obtained for levan production among the isolates. All isolates were oxidase negative, did not rot potato, were arginine dihydrolase negative and produced a hypersensitive reaction in tobacco. They, therefore, belonged to LOPAT Group I (LELLIOTT *et al.*, 1966). The bacteriological characteristics of the *M. amurensis* isolates and those of other *P. syringae*

pathovars that were pathogenic to woody plants are compared in Tables 3-5. The results with the present isolates were very similar to the characteristics of *P. syringae* pathovars such as *P. syringae* pv. *syringae* (Tables 3-5, YOUNG, 1991), *P. s.* pv. *myricae* (Tables 3-5, OGIMI & HIGUCHI, 1981), *P. s.* pv. *tremae* (Tables 3-5, OGIMI *et al.*, 1988 b), *P. s.* pv. *actinidiae* (Tables 3-5, TAKIKAWA *et al.*, 1989), *P. s.* pv. *dendropanacis* (OGIMI *et al.*, 1988 a) and *P. s.* pv. *daphniphylli* (OGIMI *et al.*, 1990). Hence, the isolates were tentatively classified as a member of *P. syringae* (SAKAMOTO *et al.*, 2000). The present isolates did not produce fluorescent pigment on King's B medium, but some strains of *P. syringae* are known not to produce the pigment (Tables 3-5, LELLIOTT *et al.*, 1966 ; OGIMI & HIGUCHI, 1981 ; OGIMI *et al.*, 1988 b ; TAKIKAWA *et al.*, 1989).

Bacterial canker of *M. amurensis* has not been previously reported in the world. Hence, the author proposes the common name "bacterial canker of *M. amurensis* var. *buergeri*" caused by *P. syringae* (SAKAMOTO *et al.*, 2000).

4. Anatomical study of the disease development

4-1 Materials and Methods

4-1-1 Sample collections

Samples were collected from 7- 10 year-old branches on 18 affected trees and 5 healthy trees of *M. amurensis*. They were collected in the Kawayu and Higashiyama stands, in the natural forests of Kimobetsu and in the field of Hokkaido Research Center, FFPRI, from 1992-97.

4-1-2 Macroscopic observations

Over 50 transverse wood disks collected from the affected trees representing SSB, series of swellings and cankers, were compared with disks from healthy trees with a dissecting microscope (at×8-40).

4-1-3 Microscopic observations

Samples of the SSB, the swellings, the margins of cankers and the healthy tissues (approximately 1.5×1.5×1.5cm, including phloem and xylem) were fixed with FAA (formalin : acetic acid : 50% ethanol=5 : 5 : 90 v/v). After washing under running water for 3-4 hours, they were dehydrated in ethanol series and embedded in 10% celloidin (Toyokuni Chemical Co.). Transverse, tangential and radial sections of 20-25 μm in thickness were cut on a sliding microtome. They were stained with safranin O (1% solution in 50% ethanol) and fast green (0.1% solution in 95% ethanol), and were then examined under a light microscope.

4-2 Results

4-2-1 Macroscopic observations

On the disks of the SSB, the macroscopic difference with the healthy tissues was the thickening of phloem tissues, but no other characteristic differences were observed.

The most remarkable difference between the swellings and the healthy tissues was the thickening of phloem tissues. The thickened tissues were water-soaked. Dark brown stained spots were frequently observed, and the cambial zone near the spots became fuzzy or disappeared (Fig. 3 a).

On the disks of the cankers, a part of the cambial zone was already dead, the exposed sapwood was also dead, and part of the heartwood was dry. Thickened dark brown, dead phloem tissues were attached to the margins of cankers. The affected phloem tissues could break off easily (Fig. 3 b). Water-soaked areas separated the affected tissues from the healthy tissues at the margins of

cankers.

4-2-2 Microscopic observations

In the tissues of the SSB far from the swelling, the cambial zone was clearly observed. The most remarkable difference between healthy tissues and SSB was a suppressed lignification of the xylem tissues in inner areas of the cambial zone. In these areas, vessel formation was also suppressed (Fig. 3 c, d).

In the SSB near swelling, the cambial zone became fuzzy or disappeared (Fig. 3 e).

In the swellings, hyperplasia of irregular-shaped parenchyma cells (Fig. 3 f-1, 2) was commonly observed. First, the irregular cells were observed near the ray parenchyma cells (Fig. 3 f-1), and they were poor in protoplasm compared with normal ray cells. Masses of bacteria were observed in intercellular spaces of the irregular ray cells (Fig. 3 f-2).

The irregular-shaped parenchyma cells increased in number with the development of the disease until the cambial zone had completely disappeared. In the phloem tissues, the parenchyma cells were degraded by the bacteria, then the bacterial lesions (the areas where the mixture of degraded parenchyma cells and bacteria was observed) became more and more numerous (Fig. 4 a). In many cases, the degradation of the tissues started from the affected ray parenchyma cells (Fig. 4 b). These areas correspond to the stained spots in the macroscopic transverse views (Fig. 3 a). The irregular-shaped parenchyma cells increased in number with degradation, the bacterial lesions got bigger and bigger, then the phloem tissues got thicker and thicker. The causal bacteria were never seen in the living cells even in the tissues of the advanced swellings, but they were always seen in the intercellular spaces of degraded cells as a mixture of the bacteria and degraded parenchyma cells. Masses of bacteria were found in some vessels (Fig. 4 c).

In the tissues of ruptured swellings, wound periderms were formed to protect the living cells in the cortex and phloem. However, bacterial lesions expanded to the cortex and phloem with the development of the disease, and coalesced with the wound periderm (Fig. 4 d). The coalesced tissues were observed to crack and break off easily.

In the margins of cankers, tyloses in the vessels were commonly observed (Fig. 4 e-1). Wound periderms were observed to form repeatedly ; however, they often failed to enclose the bacteria completely (Fig. 4 e-2).

4-3 Discussion

The results of the current research clarified the anatomical characteristics of the development of the disease as follows. The first anatomical abnormalities of the disease appeared near the cambial zone as the suppression of normal xylem formation. Then, the hyperplasia began to form irregular-shaped parenchyma cells, and caused several swellings and SSB on the bark. The irregular cells increased in number with degradation by the bacteria, and the surface of swollen bark burst. The cracks of the swellings coalesced to form long irregular cankers .

Masses of bacteria were observed in the vessels. They may have migrated long distances longitudinally in the vessels. That might be why longitudinal series of swellings and cankers were formed. The route of bacterial migration in radial or tangential directions was not clear in this study. The reason why the swellings were produced at irregular intervals was not elucidated. However, the bacterial lesions first appeared near the ray parenchyma cells in many cases. This fact suggests that the bacteria might have migrated radially along the ray parenchyma cells.

The hyperplasia of irregular ray parenchyma cells suggests some bio-chemical stimulation by the causal bacterium, such as I.A.A. production in the bacterial canker (knot) in *Fraxinus excelsior* (JANSE, 1981 b). The cell degradation in the affected tissues may indicate some kind of enzymatic activity. Although not all the tested isolates produced levan on the artificial medium (levan positive : ys- 8, 10, 11, 26, 27 ; levan negative : ys-9, 25, 28), extra-cellular polysaccharide produced by the causal bacterium may have played an important role in the production of water-soaked tissues as in the case of some other phytopathogenic *Pseudomonas* spp. (EL-BANOBY & RUDOLPH, 1979). Levan negative isolate ys-9 produced water-soaked affected tissues in the inoculation test. This suggests that levan negative isolates can produce levan in host tissues.

5. Conclusion

This study revealed the pathogens and mechanisms of disease development of the disease. It presents many important suggestions to elucidate the mechanisms of the formation of bacterial cankers and galls on other trees. However, the bacterial pathovar of this *P. syringae* needs to be determined.

The infection of bacterial cankers (knots) on *F. excelsior* is thought to occur through bore holes and lesions made by insects, frost cracks, lenticels, leaf scars and possibly bud-scale scars (JANSE, 1981 a). In the case of *M. amurensis*, many cankers, which had pruning scars in the center of the lesion, were found in the Kawayu stands (Fig. 1 d). In spring and summer from 1995 to 1997, several naturally occurring symptoms were observed in some seedlings which had not been used in the 1994 inoculation tests in the nursery (Fig. 2 c). In these cases, infection may have occurred through wounds caused by protection supports used to prevent snow damage in winter. An exudation of bacterial ooze was observed on the branches and trunks of the affected seedlings in the nursery during spring and summer, and the ooze was observed to attract several kinds of insects, such as ants, beetles and moths. This suggests that the bacterium may be spread not only by wind and rain but also by such kinds of insects, subsequently entering healthy trees via wounds and scars during these periods.

After continuous research of symptoms in the nursery and the stands, it was confirmed that the period of symptom development corresponded with the period of active thickening growth of the seedlings of *M. amurensis* (FUJIMURA *et al.*, 1993). This fact suggests a close relationship between the pathogenic reaction and the cambial activity.

Frost damage, freezing stress, and fluctuating temperatures were considered to be necessary to produce pathogenic reactions or to play an important role in the development of symptoms in some bacterial canker diseases (WEAVER, 1978 ; DE KAM, 1982 ; KLEMENT *et al.*, 1984 ; RAMSTEDT *et al.*, 1994 ; VIGOUROUX, 1999). In this study, noticeable pathogenic reactions were observed by inoculation tests performed in the nursery from spring to early summer without cold temperature. However, it is suspected that such climatic factors may promote more severe disease development. Thus, it is critical to examine the relationship between climatic factors and disease development in detail.

The fungi *Fusarium lateritium* and *Phoma riggenbachii* were frequently isolated from the bacterial canker of *F. excelsior*. These two fungi have been considered to increase the severity of the canker symptoms (JANSE, 1981 b). The author isolated *Fusarium* spp. at a high rate from cankers and ruptured swellings on *M. amurensis*. Some of these isolates caused noticeable

necrotic lesions on *M. amurensis* within a year after the inoculation tests. However, in the next year, the necrotic lesions did not develop any further (SAKAMOTO *et al.*, 1994). Nevertheless, the possibility remains that infection or a parasite of *Fusarium* spp. plays an important role in intensifying the canker symptoms.

The masses of bacteria in the affected tissues must migrate in vessels proximally and distally, but the mechanisms of radial and tangential migration have not been explained yet. Moreover, the molecular and cellular function of the causal bacterium, which produces the pathogenic reactions, is uncertain.

Chapter-II Watermark disease of willows (*Salix* spp.) caused by *Erwinia salicis* (Day 1924) Chester 1939.

1. Introduction

Willows (*Salix* spp.) have a high productivity of wood and have played an important role in maintaining biodiversity. Therefore, they are valuable for establishing an adequate watershed management regime in terms of water, soil and landscape conservation.

In August 1993, the author and assistants performed ecological research of wood decay fungi on conifers in natural forests in a mountainous area near Mt. Taisetsu in central Hokkaido. There, the author found a serious disease, which caused shoot blight, wilt and dieback on willows. Currently, this disease is observed on *S. bakko* Kimura (bakko-yanagi), *S. sachalinensis* Fr. Schm. (onoe-yanagi) and *S. kinuyanagi* Kimura (kinu-yanagi) in the mountainous area near Mt. Taisetsu (Kamikawa, Kamisihoro, Rubeshibe and Oketo) and in the natural forests of the upper part of Nissho-Pass (Hidaka). This kind of disease on willows had not previously been reported in Japan.

When affected tissues were examined with a phase-contrast light microscope, numerous bacterial cells were observed to be streaming out from them. The bacterium was isolated from the affected tissues and its pathogenicity to willows was confirmed, then identified as *Erwinia salicis* by its bacteriological characteristics. It is the causal agent of the watermark disease of willows. This causal bacterium was first described by DAY (1924) as *Bacterium salicis*, the name was later changed to *Erwinia salicis* (Day 1924) Chester 1939 (CHESTER, 1939). This disease is one of the most serious diseases of willows. It has been reported in the UK (DAY, 1924 ; DOWSON, 1937), the Netherlands (LINDEIJER, 1931) and Belgium (RIJCKAERT *et al.*, 1984). It is most severe in *S. alba* L. and its many clones, particularly *S. alba* var. *caerulea* (cricket bat willow), which are grown for the production of cricket bats in the UK. In the Netherlands, the causal bacterium causes serious losses to ornamental willows.

Wood discoloration, such as watermarks, is a serious problem for wood quality, in terms of both dirty color and decreased durability. Indeed, the wood of cricket bat willows (*S. alba* var. *caerulea*) affected by watermark disease is brittle and useless for cricket bats (STROUTS & WINTER, 1994). Some anatomical and histological works on affected wood have been carried out (DAY, 1924 ; METCALFE, 1940, 1941 ; WONG and PREECE, 1978 a, b, c ; PREECE *et al.*, 1979). However, the study of the functional characteristics of the watermark in affected trees has been neglected, especially water conductivity. The development of the wilting symptoms in the leaves and shoots of this disease suggests a progressive inhibition in its ability to conduct water. To elucidate the mechanism of wilt, studies should be made about the anatomical and physiological relationships

Table 6. Bacterial isolates used in this study

Host	Number	Location	Date isolated	Year
Salix bakko	ys-4	Kamikawa	7 August	1997
	ys-5	Hidaka	7 August	1997
	ys-7	Kamishihoro	10 September	1997
	ys-30	Kamikawa	15 July	1997
	ys-31	Kamikawa	15 July	1997
	ys-32	Kamikawa	15 July	1997
	ys-33	Kamikawa	15 July	1997
	ys-34	Kamikawa	15 July	1997
	ys-35	Kamikawa	15 July	1997
	ys-43	Kamikawa	18 June	1998
S. sachalinensis	ys-3	Kamishihoro	7 August	1997
	ys-6	Kamikawa	7 August	1997
Mallotus japonicus	AM-1	Shizuoka	June	1976
	ys-38	Shizuoka	1 October	1997
	ys-39	Shizuoka	1 October	1997

between the inhibition of water conduction and watermark in affected trees.

This chapter, which is the first scientific report of this disease outside of Europe, deals with the description of the symptoms, the isolation of the pathogen and its identification and pathogenicity tests. Wood anatomy and water conductivity of affected *S. sachalinensis* are also studied.

2. Symptoms of the disease

Symptoms were observed regularly from 1993 to 1998 in the mountainous area near Mt. Taisetsu. During spring and summer, the leaves on some branches suddenly wilted and turned reddish brown, but remained on the trees for a time (Fig. 5 a). On some trees almost all the branches were affected. Some branches and trunks died and then became leafless. Sometimes recovery shoots developed on the affected branches but they often became affected later. Seriously affected trees died within 4 to 5 years (Fig. 5 b). The internal symptom is called a 'watermark'. It was found in the sapwood of affected branches and trunks as a distinct, watery zone stained reddish brown or brownish black. It forms a circumferential stain (Fig. 5 c), sometimes covering almost the entire transverse section in seriously affected trees (Fig. 5 d). Bacteria tended to ooze from watermark on cutting. On exposure to air, the watermark quickly turned dark brown or black and a dark brownish-black liquid (mixture of bacterial ooze and metabolic substances in watermark) was exuded.

3. Isolation, identification and pathogenicity of the pathogen

3-1 Materials and Methods

3-1-1 Isolation

Plate culture isolations were made from the watermarked tissues from affected willows (*S. bakko*, *S. kinuyanagi* and *S. sachalinensis*) during the summers of 1995-98. Samples were collected

from Kamikawa, Kamishihoro and Hidaka. In total, isolates from 24 different trees (20 isolates from *S. bakko,* 2 isolates from *S. sachalinensis* and 2 isolates from *S. kinuyanagi*) were collected and stored by the same method in the case of *M. amurensis.*

3-1-2 Identification

Twelve isolates were selected for testing of bacteriological characteristics (Table 6). Three isolates of *E. mallotivora* from *Mallotus japonicus* (AM1, made by GOTO in 1976, and field isolates ys-38 and 39 made by TAKIKAWA in 1997) were selected for comparison tests.

Colony morphology was observed on NA plates and autoclaved potato tissues (DOWSON, 1937). Cell morphology of preparations stained with acid fuchsin was observed by phase contrast microscopy. Gram reaction was determined by RYU's (1937) method and flagella insertion was recorded by light microscopy of slide preparations stained by a modification of YAMANAKA's silver staining method (SHIRATA & GOTO, 1981).

The oxidation/fermentation (OF) test was carried out in the medium of HUGH and LEIFSON (1953). In the test for indole production, cultures were grown in peptone water and indole was detected using KOVAC's reagent (COWAN, 1974). Phosphatase activity was determined by the method of COWAN (1974). Pectin liquefaction was determined on Paton's medium, by the method of SCHAAD (1988). Urease activity was determined on CHRISTENSEN's (1946) urea agar plates. Phenylalanine deaminase activity was determined on the medium of EWING *et al.* (1957). Amino acid decarboxylases were determined by the method of MOELLER (1955), with L-lysine hydrochloride, L-ornithine hydrochloride and L-arginine hydrochloride. Gluconate oxidation was tested by the method of IIZUKA and KOMAGATA (1963). Lecithinase activity and V.P.-M.R. tests were performed as described in the Manual of Microbiological Methods (Society of American Bacteriologists, 1957). Tests for hydrolysis of starch and casein, and for catalase and DNAse activity were performed as described in the Manual of Methods for General Bacteriology (GERHARDT *et al.*, 1981). In addition, the following were tested by the methods described by DYE (1968) : H_2S production from cysteine, thiosulphate and peptone, NaCl tolerance and maximum temperature for growth, levan production (mucoid growth), reducing substances from sucrose, nitrate reduction, growth factor requirements, reaction in purple milk, acid production from carbohydrates and related carbon sources (using medium C and peptone water), and utilization of organic acids (sodium salts). The tested compounds were glucose, D-fructose, D-galactose, sucrose, lactose, maltose, D-melezitose, dextrin, adonitol, D-sorbitol, dulcitol, starch, inulin, D-arabinose, rhamnose, D-xylose, D-mannose, D-ribose, trehalose, melibiose, D-cellobiose, D-raffinose, glycerol, D-mannitol, D-salicin, inositol, α-methyl-D-glucoside, acetate, fumarate, gluconate, L-malate, succinate, benzoate, oxalate, propionate, citrate, formate, DL-lactate, tartrate, galacturonate, malonate. Finally, the following were tested by the methods described by LELLIOTT *et al.* (1966) : arginine dihydrolase, oxidase and tyrosinase activities, hydrolysis of aesculin and Tween 80 ; gelatin liquefaction, potato soft rot and tobacco hypersensitive reaction.

3-1-3 Pathogenicity

Pathogenicity tests were performed on 2-year-old seedlings from trees of *S. sachalinensis* and *S. kinuyanagi* growing in the nursery of Hokkaido Research Center, FFPRI in 1998. The inoculation dates, isolates, and number of test seedlings are listed in Table 7. Cultures stored at −80°C were spread on NA plates and incubated for 5-6 days at 20°C. One-year-old branches were pricked to the xylem with a needle through a drop of bacterial suspension (approximately 10^{9} cfu/

Table 7. Schedule of inoculation tests and results [a]

Date	Isolate	Tested seedling	Number of inoculated seedlings	Number of affected seedlings
26 June	ys-4	*S. sachalinensis*	5	3
		S. kinuyanagi	5	4
	ys-43	*S. sachalinensis*	5	4
		S. kinuyanagi	5	4
7 July	ys-4	*S. sachalinensis*	5	5
		S. kinuyanagi	5	4
	ys-43	*S. sachalinensis*	5	4
		S. kinuyanagi	5	3
17 July	ys-4	*S. sachalinensis*	5	3
		S. kinuyanagi	5	4
	ys-43	*S. sachalinensis*	5	3
		S. kinuyanagi	5	4
4 August [b]	re-isolated bacterium	*S. sachalinensis*	5	3

a) All tests were performed with the same procedure in 1998.
b) Re-inoculation test.

mL). In the control inoculations, distilled water was used instead of bacterial suspension. The appearance and development of symptoms was observed up to the end of September 1998.

3-2 Results

3-2-1 Isolation

The same bacterium was regularly isolated from all the freshly affected tissues. The colonies were 1-2 mm in diameter on NA after 4 days at 20℃, with an entire margin, and were circular, convex, smooth and glistening.

3-2-2 Identification

All the isolates tested were Gram-negative, non-sporing, straight rods, motile with peritrichous flagella and produced a transparent-to-white colored growth on NA. They also produced a yellow pigment on autoclaved potato tissues. They metabolized glucose fermentatively. All isolates gave positive reactions in the following tests : levan production, reducing substances from sucrose, VP test, hydrolysis of aesculin, H_2S production from cystein, thiosulphate and peptone, pectin liquefaction and catalase activities. No visible changes were observed in all isolates in the test of purple milk. All isolates gave negative reactions in the following tests : growth factor requirements, MR test, gluconate oxidation, hydrolysis of casein, starch and Tween 80, indole test, nitrate reduction, gelatin liquefaction, amino acid decarboxylases from L-lysine, L-ornithine and L-arginine hydrochloride, arginine dihydrolase, activities of DNAse, lecithinase, phenylalanine deaminase, oxidase, phosphatase, tyrosinase and urease, tobacco-hypersensitive reaction and potato soft rot. The maximum concentration of NaCl for growth was 5% and maximum temperature was 35℃. In medium C and peptone water, all isolates produced acid from glucose, D-fructose, D-galactose, sucrose, D-mannitol, D-mannose, D-ribose, glycerol, salicin, melibiose, raffinose and α-methyl-D- glucoside, but no isolates produced acid from lactose, maltose, D-melezitose, dextrin, adonitol, dulcitol, starch, inulin, D-arabinose, rhamnose, D-xylose, trehalose, D-cellobiose, D-sorbitol and inositol. All isolates utilized acetate, fumarate, gluconate, L-malate, succinate and formate, but no isolates utilized citrate, DL-lactate, tartrate, galacturonate,

Table 8. Characteristics of the present isolates from willows and some species in *Erwinia amylovora* group

	Present Isolates(*)	*E. salicis*(a)	*E. nigliffluens*(a)	*E. quercina*(a)	*E. rubrifaciens*(a)	*E. mallotivora*(*)
Characteristics						
OF test	F	F	F	F	F	F
Growth factor requirements	−	−	−	+	−	+
Levan (Mucoid growth)	+	+	−	+	+	+
Growth at 36℃	−	−	+	+	+	−
Reducing substances from sucrose	+	d	−	+	−	+
VP test (acetoin)	+	+	+	+	−	−
MR test	−	−	+	−	−	−
Gluconate oxidation	−	−	−	−	−	−
Reaction in purple milk	−(#)	−(#)	K(3-7)	K(7-14)	K(7-14)	−(#)
Aesculin hydrolysis	+	+	+	+	−	−
Casein hydrolysis	−	−	−	−	−	d
Starch hydrolysis	−	−	−	−	−	+
Indole test	−	−	−	−	−	−
Nitrate reduction	−	−	−	−	−	−
H_2S from Cystein	+	+	+	+	+	−
Thiosulphate	+	−	+	+	d	+
Peptone	+	−	+	+	−	−
Gelatin liquefaction	−	−	−	−	−	−
Pectin liquefaction	+	+(b)	−(b)	−(b)	−(b)	−
Amino acid decarboxylase						
from L-Arginine	−	−	−	−	−	−
L-Lysine	−	−	−	−	−	−
L-Ornithine	−	−	−	−	−	−
Arginine dyhydrolase	−					−
DNAse	−	−(b)	−(b)	−(b)	−(b)	d
Lecithinase	−					d
Phenylalanine deaminase	−	−	−	−	−	−
Phosphatase	−					d
Tyrosinase	−					−
Urease	−	−	+	−	−	−

*= The author's data.
a = Data from DYE (1968).
b = Data from LELLIOTT & DICKEY (1984).
# = No visible change was observed.
K = alkaline reaction.
+ = 80-100% isolates positive.
d = 21-79% isolates positive.
− = 0-20% isolates positive

malonate, benzoate, oxalate and propionate.

3-2-3 Pathogenicity

Isolates ys-4 and ys-43 both produced pathogenic reactions in more than 70% of the seedlings inoculated (Table 7). The reactions were observed approximately 2-3 weeks after inoculation. Initially a few leaves became light brown and curled, but about 3-4 days later, other leaves also turned brown. Then whole branches wilted (Fig. 5 e), the affected leaves fell off, and the

Table 9. Acid production from some carbohydrates and related carbon sources by the present isolates from willows and some species in *Erwinia amylovora* Group

Organic compound	Present isolates (*)		*E. salicis* (a)		*E. nigliffuens* (a)		*E. quercina* (a)		*E. rubrifaciens* (a)		*E. mallotivora* (*)	
Medium	C	P/W	C	P/W	C	P/W	C	P/W	C	P/W	C	P/W (b)
D-Arabinose	−	−	−	−	+	+	−	−	+	+	−	−
Rhamnose	−	−	−	−	+	+	+	−	+	+	−	−
D-Xylose	−	−	−	−	+	+	−	−	−	−	+	+
D-Mannose	+	+	+	+	+	+	+	+	+	+	+	+
D-Ribose	+	+	+	+	+	+	+	+	+	+	+	+
Trehalose	−	−	−	−	+	+	−	−	−	−	+	+
Melibiose	+	+	+	+	+	+	−	−	−	−	−	−
D-Cellobiose	−	−	−	−	+	−	−	−	−	−	+	(+)
D-Raffinose	+	+	+	+	+	+	−	−	−	−	−	−
Glycerol	+	+	+	d	+	+	+	+	+	d	+	(+)
D-Mannitol	+	+	+	+	+	+	+	+	+	+	+	+
Salicin	+	+	+	+	+	+	+	+	+	−	−	−
Inositol	−	−	+	+	+	+	−	−	−	−	+	−
α-methyl-D-glucoside	+	+	−	−	−	−	+	+	+	+	−	−

* =The author's data.
a = Data from DYE (1968).
b = Data from LELLIOTT & DICKEY (1984).
C = medium C; P/W = peptone water.
+ = 80-100 % isolates positive.
(+) = delayed positive reaction.
d = 21-79 % isolates positive.
− = 0-20 % isolates positive.

branches died within a month. There were no pathogenic reactions in the branches of control seedlings.

The watermark stain was observed in transverse sections of affected branches (Fig. 5 f). A collection of bacterium was re-isolated on 17 July from the stained tissues of a seedling of *S. sachalinensis* that had been inoculated with ys-43 on 26 June. This isolate showed pathogenicity when re-inoculated into *S. sachalinensis* (Table 7).

3-3 Discussion

The bacterial isolates from willows formed a homogeneous group in their bacteriological properties. They were Gram-negative, non-sporing, straight rods, motile with peritrichous flagella, produced a transparent-to-white colored growth, metabolized glucose fermentatively and showed negative reactions in the potato soft rot test and in the oxidase activity and nitrate reduction tests. Hence, they should be included in the *Erwinia amylovora* group (BRADBURY, 1970 b).

The bacteriological characteristics of the willow isolates and those of other species in the *E. amylovora* group that were pathogenic to woody plants, are compared in Tables 8-10. The following characteristics of these isolates corresponded well with those of *E. salicis* (but not the other species) : growth factor requirements, no growth at 36°C, reaction in purple milk, utilization of citrate, lactate, tartrate, galacturonate and malonate, and acid production from sucrose, raffinose and mannitol. Their pathogenicity to *Salix* spp. was also confirmed, indicating that

Table 10. Utilization of organic compounds by the present isolates from willows and some species in *Erwinia amylovora* Group

Organic compounds	Present isolates(*)	*E. salicis*(a)	*E. nigliffluens*(a)	*E. quercina*(a)	*E. rubrifaciens*(a)	*E. mallotivora*(b)
Acetate	+	+	+	+	+	
Fumarate	+	+	+	+	+	+
Gluconate	+	+	+	+	+	
L-Malate	+	+	+	+	+	
Succinate	+	+	+	+	+	
Formate	+	−	+	+	+	−
Citrate	−	−	−	+	+	+
DL-Lactate	−	−	+	+	+	−
Tartrate	−	−	+	−	+	−
Galacturonate	−	−	−	−	−	−
Malonate	−	−	−	−	−	−
Benzoate	−	−	−	−	−	
Oxalate	−	−	−	−	−	
Propionate	−	−	−	−	−	

* = The author's data.
a = Data from DYE (1968).
b = Data from LELLIOTT & DICKEY (1984).
+ = 80-100 % isolates positive.
− = 0-20 % isolates positive.

they were *E. salicis*.

There were some differences in the bacteriological characteristics of these isolates from those reported by DYE (1968), for example, acid production from α-methyl-D-glucoside and inositol, utilization of formate and H_2S production from thiosulphate and peptone. These were weak reactions that tend to be scored differently by some investigators ; therefore it is possible that any differences might reflect differences in interpretation of the tests. To elucidate this point, it will be necessary to make a direct comparison of European and Japanese isolates. A molecular comparison will also be needed.

The external and internal symptoms, and the timing of their appearance, corresponded to the symptoms of watermark disease of willows as previously reported in Europe (DAY, 1924 ; DOWSON, 1937 ; METCALFEE, 1940 ; SMITH *et al.*, 1986 ; STROUTS & WINTER, 1994). This report is the first description confirming the presence of the watermark disease in willows, and its pathogen, *E. salicis*, in Japan. Although *E. salicis* was reported to be present in Japan in one textbook (BRADBURY, 1986), no evidence to support this statement was provided.

4. Anatomy and water conductivity of the affected trees.

4-1 Materials and Methods

4-1-1 Field experiments and sample collections

Field experiments and most samplings were performed on *S. sachalinensis* (6.25 cm in average diameter at breast height) in the natural forests of Kamikawa on 10 July 1998.

Samples for macroscopic and microscopic observations of affected and healthy *S.*

sachalinensis of various ages were taken in Kamikawa from August 1993 to July 1998.

4-1-2 Macroscopic observations

Over 50 transverse wood disks collected from the affected trees were compared with disks from healthy trees with a dissecting microscope (at×8-40).

4-1-3 Microscopic observations

Samples of the watermark and the healthy sapwood (approximately 1.5×1.5×1.5 cm) were collected from 5 affected trees and 2 healthy trees. Samples were also collected from the moderately affected and healthy trees that had been used for the dye injection test. The samples were fixed with FAA (formalin : acetic acid : 50% ethanol=5 : 5 : 90 v/v) for one week, then washed under running water for 3-4 hours. Transverse, tangential and radial sections of 20-25 μm thickness were cut on a sliding microtome. They were stained with safranin O (1% solution in 50% ethanol) and fast green (0.1% solution in 95% ethanol), and were then examined under a light microscope. Some unstained sections were also observed.

4-1-4 Dye-injection test

The dye-injection test was carried out to study the water conductivity of the affected and healthy trees.

One moderately damaged tree (with approximately 30% of branches dead), two seriously damaged trees (with more than 90% of the branches dead) and one healthy tree (for control) were selected. Funnel-shaped plastic collars were attached to the trunks, then the space between the collars and the trunks was sealed with plastic clay and vinyl tape. A safranin O solution (0.2% aqueous solution) was poured in the collars, then four to six holes (8.5 mm in diam., average depth of 59.1 mm) were bored with an electric drill under the surface of the solution in the collars. About four hours later, the trees were cut down and divided into 1m-long sections, which were dissected in the laboratory. The distribution of the dye was examined to detect the zone of xylem that remained conductive.

4-1-5 Soft X-ray photography

Cylindrical specimens were excised from the trunks of one affected tree (with more than 90% of the branches dead) (approximately 5 cm in diameter and 5 cm in length) and one healthy tree (approximately 6 cm in diameter and 5 cm in length). After excision, both cut surfaces of the specimens were coated with petroleum jelly and then wrapped with polyvinyl sheets to prevent dehydration. The specimens were transported to the laboratory and stored in a refrigerator at −80℃ for 5 days. The samples were prepared for soft X-ray photography according to the methods developed by SANO *et al.* (1995). In brief, 2mm-thick transverse disks were sawn off with a disc saw and soaked in liquid nitrogen (LN_2) to prevent thawing. The frozen disks were placed on a film case in which an X-ray film (X-ray FR ; FUJI Photo Co. Ltd.) had been mounted, and irradiated with an X-ray apparatus at 20 kV and 5 mA for 2 minutes from a distance of 1.3 m. Then, the transverse surfaces of the disks were photographed on a commercial black and white film with an ordinary camera. After that, the disks were oven-dried at 45℃ for 24 h, then air-dried at room temperature for one week. Soft X-ray photographs of the dried disks were taken under the same conditions as the green (frozen) samples. The images on each negative were enlarged on ordinary commercial black and white print paper to observe the distribution of water. In the X-ray photographs taken of the green wood, dark zones represent high levels of moisture.

4-1-6 Moisture content

The moisture content was measured in the healthy and affected disks, which were used for the soft X-ray photography. Wood strips containing pith were removed from the cylindrical specimens. The wood strips were serially divided into wood pieces of approximately 5 to 15 mm in radial width from both sides of the outermost sapwood to pith (Fig. 9). Moisture content (MC) as a percentage of the weight of the oven-dried (at 105℃ for 48 hours) wood was calculated from the weight of the wood pieces when green and when dried, respectively.

4-1-7 Cryo-scanning electron microscopy

Cylindrical specimens were excised from the trunks of the affected tree and healthy tree that were used for soft X-ray photography. The samples were collected according to the method developed by UTSUMI *et al.* (1996), and prepared according to the procedures described by SANO *et al.* (1995). In brief, funnel-shaped plastic collars were attached to the trunks following a similar procedure with the dye injection test, then LN_2 was poured into the collars. Ten minutes later, specimens of frozen wood were cut, then stored in LN_2. After the specimens were transported to the laboratory, they were taken out of LN_2, covered with aluminum foil and stored in a refrigerator at −50℃ until used. After the preparation of samples for cryo-scanning electron microscopy, specimens that had been stored at −50℃ were transferred to a low-temperature room kept at −20℃. The specimens were equilibrated at exactly −20℃ to prevent contamination by frost during subsequent treatments. Small cubic blocks (5×5×5 mm) were trimmed from the specimens, and transverse surfaces of the blocks were shaved smoothly with a microtome in the low-temperature room. The transverse surfaces of the samples were examined under the cryo-SEM (JSM 840 A equipped with CRU-40) to observe the distribution of ice (water in natural condition) in the tissues.

4-2 Results

4-2-1 Macroscopic observations

Watermarks were observed in xylem that was one year old or older, but were not usually observed in the xylem of the current year. Even in the case of seriously affected trees, if the whole area of transverse surface was attacked by the watermark, the current year xylem remained almost completely free from it.

4-2-2 Microscopic observations

In the unstained tissues of the watermark, contents of the affected ray parenchyma cells were yellow to brown, and some cells lost their contents.

In the stained tissues of the watermark, some of the vessels were clogged with tyloses and masses of bacteria (Fig. 6 a, b), and some of the ray parenchyma cells caused plasmolysis and necrosis (Fig. 6 c). Masses of bacteria formed in the vessels irrespective of the presence of tyloses. Tyloses were also found in the tissues without the watermark, but the masses of bacteria were only found in the watermark. The ray parenchyma cells adjoining the vessels clogged with bacteria were affected first. Then, the parenchyma cells in the watermark gradually died as the disease progressed. Sometimes, the bacteria were seen in dead ray parenchyma cells in contact with the vessels clogged with the bacteria.

In the healthy tissues, no tyloses, no masses of bacteria in vessels and no necrosis of ray parenchyma cells were observed.

4-2-3 Dye-injection test

S. sachalinensis is a diffuse-porous species. In the healthy tree, all the annual rings of the

sapwood were stained with the dye at 10 cm above the injection holes. The region that was stained with the dye within each annual ring differed between the outer and the middle to inner parts of the sapwood ; the staining was limited to the outer layers of each annual ring of the middle to inner part of the sapwood (Fig. 7 a). In moderately damaged trees, the watermark formed a circumferential stain in sapwood at 10 cm above the injection holes. The outer part of the sapwood and some areas in the middle to inner part of the sapwood had the ability to conduct water. However, no conductivity was observed in the watermark (Fig. 7 b-1, 2). In seriously damaged trees, the entire transverse section was almost completely covered by the watermark at 10 cm above the injection holes. Only part of sapwood in the outer layers was conductive, and no conductivity was observed in the watermark (Fig. 7 c).

4-2-4 Soft X-ray photography

When comparing the normal black and white photograph and the soft X-ray photograph of the affected green wood disks, the watermark was observed to be darker than the surrounding pale sapwood on the soft X-ray photograph taken of the green (frozen) wood (Fig. 8 a, b). This indicated that there was a high accumulation of moisture. There were no black colored areas (a high level of moisture) in the soft X-ray photograph of the dried disk from the affected wood (Fig. 8 c).

The heartwood, both in the affected and healthy trees, was darker than the sapwood on the soft X-ray photographs. This indicates that the heartwood of *S. sachalinensis* contained a high moisture level when green.

4-2-5 Moisture content

Fig. 9 shows that the moisture content in the middle to inner parts of the affected sapwood was higher than in the healthy sapwood. The moisture content of the heartwood was high both in the affected and healthy wood.

4-2-6 Cryo-scanning electron microscopy

The cryo-scanning electron microscopy revealed that almost all the cells in the watermark were filled with ice irrespective of the type of cell (Fig. 10 a, b). However, ice was seldom present in the lumina of wood fibers in the sapwood of healthy samples.

The inner tissues of the sapwood (inside the watermark) of the affected tree were also observed. In these samples, there was only a little ice in the lumina of the vessels, although the lumina of wood fibers were filled with ice (Fig. 10 c). Ice in the lumina of wood fibers was rarely observed in the middle to inner parts of the sapwood in healthy samples.

4-3 Discussion

In wilt diseases, vascular plugging often plays an important role in the appearance of initial symptoms and in the ultimate death of affected plants (KOZLOWSKI, 1968). However, there is some debate about the mechanisms of vascular plugging in some wilt diseases of trees (BASHAM, 1969 ; COUTTS, 1976 ; GREGORY, 1977 ; KURODA *et al.*, 1988 ; KURODA, 1989 ; KURODA & YAMADA, 1996).

The ability to conduct water was not observed in the watermark. The contents of the affected ray parenchyma cells in the watermark were yellow to brown. This suggests that there was an accumulation and oxidation of phenolic compounds. A direct detection test of the phenolic compounds in the watermark was not performed in this study ; however, WONG and PREECE (1978 c) reported an increased level of phenolic compounds in infected willow wood. The present anatomical study showed that the parenchyma cells in the watermark caused plasmolysis

and necrosis with the development of the disease. These facts suggest that the watermark in sapwood can be considered to be similar to 'discolored wood'. Discolored wood is the tissue that forms as a kind of defense reaction in sapwood, with similar processes to heartwood formation. The wood is believed to become discolored as follows. When xylem tissues in healthy sapwood are attacked by microorganisms or are mechanically injured, the accumulation level of secondary substances and anti-microbiological substances, such as phenolic compounds, increases. Then, the parenchyma cells gradually die. The color of these tissues will turn brown to black brown with oxidation and polymerization of the secondary substances, then lose the ability to conduct water as in normal heartwood (SHIGO & HILLIS, 1973 ; BAUCH, 1984 ; SHIGO, 1984). Therefore, the non-conductive watermark formation and its expansion in the affected sapwood as the disease progresses may be the reason for the wilting symptoms.

Tyloses were found in some vessels in the tissues free from the watermark. This indicates that these vessels had already lost their water conductivity (ZIMMERMANN & BROWN, 1974). Indeed, water conductivity was not observed in some tissues of the inner sapwood of an affected sample that was free from the watermark (Fig. 7 b-2). Further, the cryo-scanning electron microscopy revealed that there was little ice in the lumina of the vessels, although the lumina of wood fibers were filled with water in these tissues (Fig. 10 c). Therefore, the moisture content of the middle to inner parts of the affected sapwood was higher than in the healthy sapwood, even in the tissues free from the watermark (Fig. 9).

5. Conclusion

This study revealed the pathogens and mechanisms of disease development of the diseases. It presents many important suggestions to elucidate the mechanisms of the disease development of bacterial wilts on other trees.

In Hokkaido, this disease was found only on the local *Salix* species in limited areas of natural forests. European *Salix* species such as *S. alba*, *S. caprea* and *S. purpurea*, which are also affected by the disease, have not been found in the natural forests of Hokkaido. This suggests that this disease and *E. salicis* may be native to Japan. Clarification will require a molecular comparison of European and Japanese strains. Moreover, the bacterium may be found in other Asian countries in the near future. Since *Salix* spp. is widely distributed in Asia, research on the occurrence of this disease in Asia is vital.

The disease has so far occurred only in limited areas in Hokkaido, all of which are located at elevations of above 400 m and with a relatively cool climate. More serious outbreaks were observed in the cool summers of 1993 and 1998 than in the hot summers of 1994 and 1999. Pathogenicity tests produced high infection and rapidly developing symptoms in 1998, when there was a cool summer. On the other hand, the incidence of infection was quite low in pathogenicity tests in 1999, when there was a hot summer. Therefore, climatic conditions may be important factors affecting outbreaks of the disease.

In Europe, the incidence of infection has always been low in pathogenicity tests (ADEGEYE & PREECE, 1978 ; GREMMEN & DE KAM, 1981), whereas the author's tests in 1998 revealed high infection and rapidly developing symptoms. This may be the result of the experimental conditions, not only with regard to climatic conditions but also interaction between the pathogenicity of the bacterium and the susceptibility of the inoculated seedlings.

Little is known about the mechanism of disease transmission. In the Netherlands, the willow weevil, *Cryptorrhynchus lapthi* L., was suspected to be a vector of the bacterium (LINDEIJER, 1932), but this insect has never been associated with the disease in the UK (McCallan, 1938). In addition, the bacterium is suspected to spread via the propagating material, and tree-to-tree spread is rare in the UK (GUVEN *et al.*, 1999). In Hokkaido, the disease has occurred in a natural forest where willows have not been artificially planted. Therefore, the bacterium is suspected to spread by tree-to-tree infection. An exudation of bacterial ooze was observed on the branches and trunks of affected trees during spring and summer, suggesting that the bacterium maybe spread by wind and rain, entering healthy trees via wounds and scars. Transmission by root contact is also suspected, but vector-like insects have not been observed.

There has been conflict from the beginning concerning the description of the causal bacterium. LINDEIJER (1932) in the Netherlands had described it *Pseudomonas saliciperda*. However, GREMMEN and DE KAM (1970) proved that the disease in the UK and the Netherlands was caused by the same bacterium, *E. salicis*. The differences between the Dutch and English strains of *E. salicis* have been studied. A yellow pigment production on autoclaved potato tissues had once been thought to be characteristic of English strains (DOWSON, 1937), and Dutch strains had been reported not to produce it (LINDEIJER, 1932 ; DE KAM, 1976). However, nonpigment-producing strains have also been found in the UK (PREECE *et al.*, 1979). DE KAM (1976) distinguished Dutch strains from English ones by the ability of the latter to utilize galactose, which is lacking in the former. TURNER *et al.* (1992) had the same results, but VERDONCK *et al.* (1987) did not. DE KAM (1976) also noted that nonpigmented strains were unable to utilize raffinose, but GUVEN (1992) did not confirm this findings. Japanese strains produced a yellow pigment and utilized galactose and raffinose. The significance of these differences between the Dutch, English and Japanese strains should be studied in relationship to the epidemiology of the disease.

Several phylogenetic and taxonomical studies of the genus *Erwinia* have been carried out on the basis of the API system (MERGAERT *et al.*, 1984 ; VERDONCK *et al.*, 1987), the analysis of 16S rRNA (KWON *et al.*, 1997) and the analysis of fatty acids (WELLS *et al.*, 1994). Recently, HAUBEN *et al.* (1998) classified several *Erwinia* species, including *E. salicis* ; into a new genus *Breneria* based on the analysis of 16S rDNA, proposing a new name, *Breneria salicis*. However, this name is not widely used at the present time because the results of 16 S rDNA analysis do not provide enough scientific evidence to establish a new genus.

From the physiological point of view, non-conductive watermarks in sapwood can be considered to be similar to discoloured wood. This research revealed that the watermark and the heartwood of *S. sachalinensis* (both affected and healthy trees) had a high level of moisture compared to healthy sapwood. This kind of wood tissue is also known as 'wetwood'. In Japan, some species, such as *Fraxinus mandshurica* var *japonica*, *Ulmus parvifolia* and *Populus maximowiezii*, also have wetwood (wet-heartwood) (YAZAWA & ISHIDA, 1965 ; YAZAWA *et al.*, 1965). The wetwood is responsible for substantial losses of wood and energy and considerable production expenditures in the forest product industry. The consistent mechanism of water accumulation in wetwood has not been elucidated yet ; however, several kinds of bacteria in the xylem tissues are suspected to be a causal factor (HARTLEY *et al.*, 1961 ; WARD & PONG, 1980 ; WARSHAW *et al.*, 1985 ; LEE, 1988). In the case of watermarks, the water accumulation is obviously due to the pathological interaction between *E. salicis* and affected tissues. The precise mechanism of water

accumulation in watermarks should be investigated in the above-mentioned species, as it might provide relevant information to elucidate the mechanisms of wetwood in other trees.

Masses of bacteria were formed in the watermarked vessels. Sometimes, the bacteria were seen in dead ray parenchyma cells in contact with the vessels clogged with the bacteria, but they were never seen in intercellular spaces of the living cells. These facts indicate that *E. salicis* is a vessel-limited, wilt-causing bacterium. In the genus *Erwinia*, *E. stewartii* (in maize) (BRADBURY, 1967) and *E. tracheiphila* (in cucumber) (BRADBURY, 1970 a) are known as wilt-causing bacteria. This study is the first pathological research of wilt disease on woody plants caused by vessel-limited *Erwinia* ever presented.

Histopathological changes in the watermarks, such as plasmolysis and necrosis of parenchyma cells and loss of water conduction, have also been observed in the process of transforming of sapwood into heartwood. However, the consistent mechanism of heartwood formation has not been elucidated yet, and the causal factors of these changes are unknown. The mechanisms of these histopathological changes, including some kinds of toxic reactions caused by the bacterium in the watermark, require study. Many other aspects also remain to be elucidated. For example, the masses of bacteria in the affected tissues must migrate in vessels proximally and distally, but the mechanisms of radial and tangential migration have not been discovered yet. Neither has it been determined why the inner unwatermarked sapwood in the affected wood did not conduct water but contained water in the lumina of wood fibers. Therefore, it is necessary to examine these unclear points in more detail and discover the processes and mechanisms of the inhibition of conductivity and watermark formation in relation to the development of the disease.

Remarks

This pathological study of the two new bacterial diseases of broad-leaved trees in Hokkaido presented many valuable suggestions for pathological research of other bacterial diseases. It is critical to investigate bacterial tree diseases. Because the species composition of natural forests in Hokkaido is very similar to that in Europe, many new bacterial diseases (including some that have been newly found and already reported in Europe) may be found in Hokkaido in the near future.

Developing effective methods for controlling tree diseases is necessary for highly productive forestry. Currently, bacterial cankers on *M. amurensis* are being observed in many places, but the watermark disease of willows has been founds only in limited areas in Hokkaido. Preventing infection and establishing an effective controlling strategy for tree disease are two crucial measures. The periods of infection of these two diseases have been investigated in the field ; however, other ecological aspects, such as climatic factors and the susceptibility of the hosts to the causal pathogens in different places, which affect the incidence or occurrence of those diseases, have been disregarded. Thorough research on the occurrence of these diseases all over Hokkaido would be required to clarify these unknown aspects.

To date, there have been many descriptive and etiological reports of canker and gall diseases of trees, but only few anatomical reports have been presented, besides those on rust diseases (JACKSON & PARKER, 1958 ; JEWELL *et al.*, 1962 ; JEWELL & WALKER, 1966 ; SAKAMOTO & KANEKO, 1992). There have been some reports on the mechanisms of wilting symptoms from the chemical (toxin and extra-cellular polysaccharide produced by pathogenic bacteria) point of view (HUANG & GOODMAN, 1976 ; SJULIN & BEER, 1978 ; GOODMAN & WHITE, 1981 ; SUHAYDA & GOODMAN, 1981).

However, the anatomical and physiological reactions of affected wood tissues, which are useful for establishing effective chemical control methods, such as fungicides or antibiotics injection, have not been carefully studied. By clarifying these kinds of reactions in the two diseases, this study presents two model cases for the study of other diseases.

Tree diseases are a negative factor for wood production. At the some time, they are considered to be indicators of forest stability or a natural regulator for maintaining biodiversity. In general, mono-cultured artificial forests are ecologically unstable. In point of fact, while a major outbreak of the bacterial canker of *M. amurensis* was reported in two mono-cultured plantations at Kawayu and Higashiyama. A major outbreak of this canker has not been observed in natural forests because *M. amurensis* forms a mixed community with other kinds of broad-leaved trees, such as *Fraxinus mandshurica, Phellodendron amurensis, Quercus mongolica* var. *grosseserrata* and *Tilia japonica*. Epidemic tree diseases usually do not occur in a mixed community in a highly-biodiverse, stable natural forest. In the case of the watermark disease of willows, despite the serious dieback caused by the disease in the mountainous area near Mt. Taisetsu, no decline has been observed in the total biomass productivity of willows. One of the reasons is considered to be the high productivity and propagating capability of this tree. There is a possibility that the disease may play a regulating role in maintaining the biomass of willows at the same volume and to make some ecological space for other tree species to propagate, thus increasing the forest's biodiversity. However, to ascertain this hypothesis, biomass production and disease loss of willows in the area should be continuously researched. Studying the ecological aspects of forest pathology in relation to biodiversity is a very interesting facet of forestry.

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北海道において発生した細菌性樹木病害の病理学的研究

坂　本　泰　明[1]

摘　　要

***Pseudomonas syringae* によるイヌエンジュがんしゅ細菌病（bacterial canker of *Maackia amurensis* var. *buergeri*）**

北海道において，イヌエンジュの新病害が発生した。被害は甚大で，病斑部は主幹から細枝にまでおよぶ。罹病木から分離し，宿主に対し病原性を示した細菌を *Pseudomonas syringae* と同定，本病を「イヌエンジュがんしゅ細菌病」と呼称することを提唱した。病徴の進展過程を明らかにするため，解剖観察を行ったところ，がんしゅの形成過程は以下のようであった。まず形成層付近に組織学的異常が現れ，組織の木化が抑制される。そして変形・肥大した柔細胞の増生が引き起こされ，初期病徴である樹皮隆起部が複数形成される。病徴の進展とともに柔細胞はさらに増生を続け，やがて隆起部は裂開し，融合する。以上の過程を経て，縦長のがんしゅとなると考えられた。

***Erwinia salicis*（Day 1924）Chester 1939 によるヤナギ類水紋病（watermark disease of willows）**

北海道において，ヤナギ類水紋病の発生を初めて確認した。本病は英国等でのみ記載されていた，葉枯・萎凋枯死を引き起こす病害である。罹病枝幹の横断面を観察したところ，辺材部に本病名の由来である，赤褐色～黒褐色を呈する弧～円状の着色部（watermark）が確認された。着色部から分離し，ヤナギ類に対し病原性を示した細菌は，*Erwinia salicis* と同定された。罹病木の解剖学的観察および通水機能試験を行ったところ，watermark 内では柔細胞が壊死し，通水機能が失なわれていた。したがって，watermark 部は通水機能を失った discolored wood であり，その辺材部における形成・拡大が，萎凋・枯死の原因であると考えられた。

2000年3月29日受理

(1)　北海道支所保護部

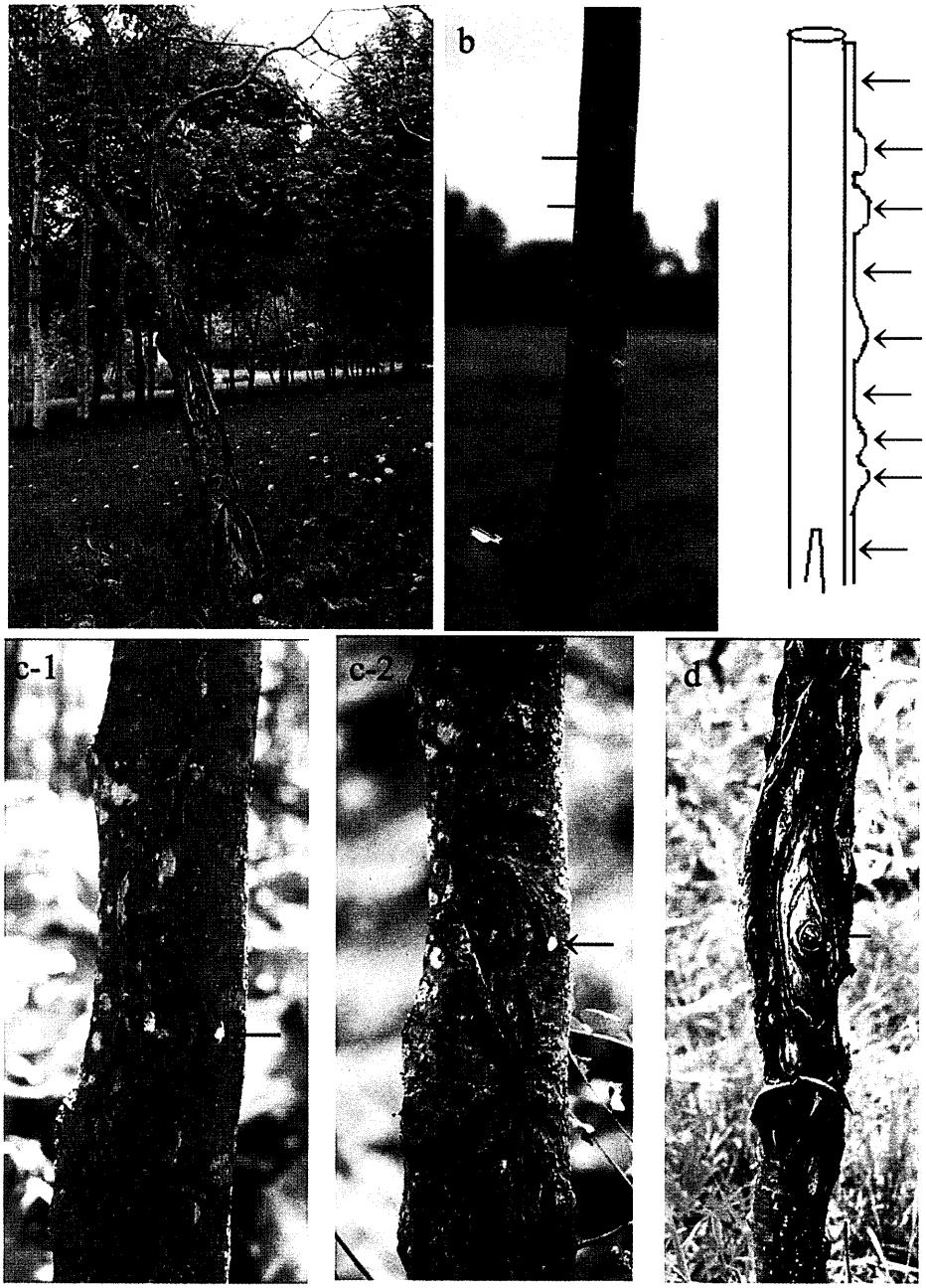

Fig. 1

a : Serious canker of *M. amurensis* var. *buergeri* (photographed in Makkari).

b : A longitudinal series of swellings and slightly swollen bark (SSB) (collected in Teshikaga). Red arrows indicate the swellings ; black arrows indicate the SSB.

c : Development of natural symptoms in Higashiyama stand. Arrows indicate the same site.

1 : Photographed on 17 September 1993.

2 : Photographed on 2 August 1995.

d : A canker that spread from a pruning scar (arrow) (Kawayu).

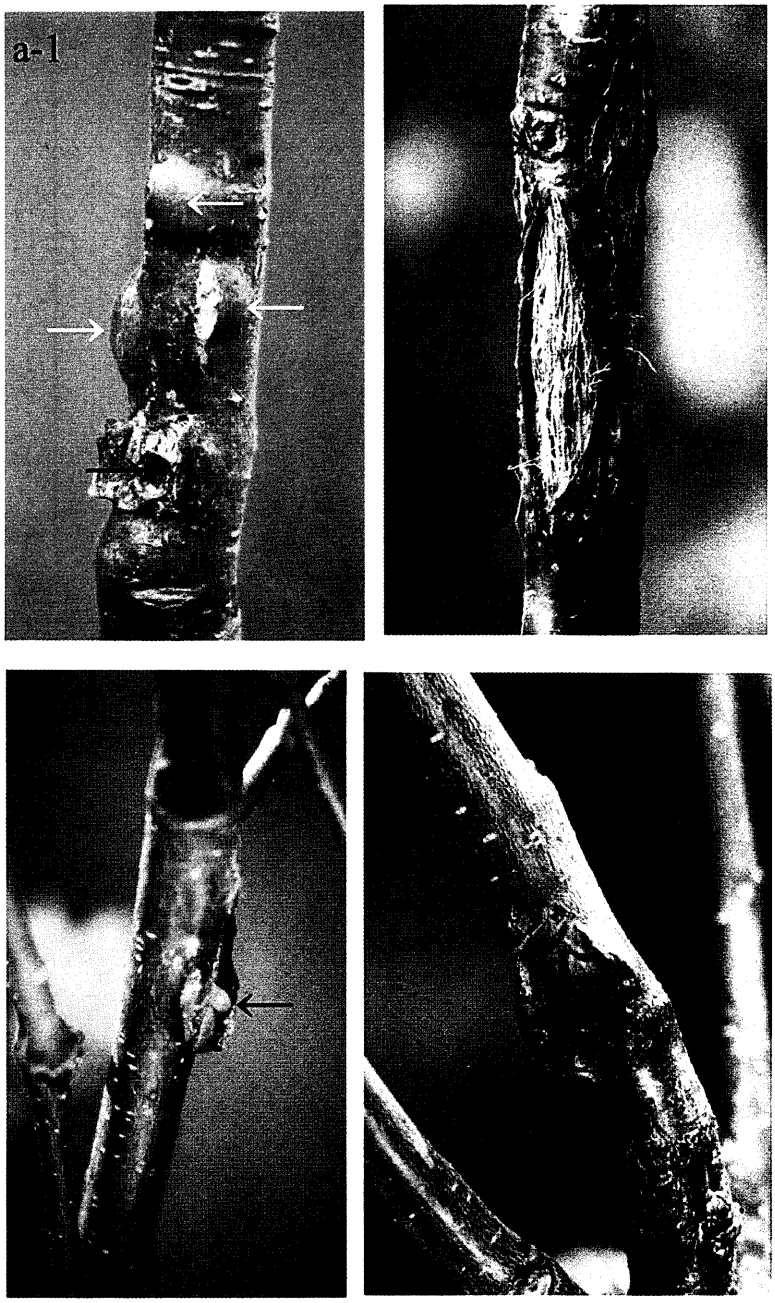

Fig. 2.
a : Symptoms of bacterial canker of *M. amurensis* var. *buergeri* on an inoculated seedling.
1 : Swellings (yellow arrows) which developed after artificial inoculation (date of inoculation : 23 June 1994 ; date of photography : 22 June 1995). Red arrow indicates the inoculation site.
2 : Canker which formed after artificial inoculation (date of inoculation : 15 July 1994 ; date of photography : 31 August 1995). Arrow indicates the inoculation site.
b : Bacterial ooze exuding (arrow) from a cracked swelling after artificial inoculation (date of inoculation : 22 August 1994 ; date of photography : 23 May 1997).
c : A naturally occurring symptom on an uninoculated seedling in the nursery (date of photography : 19 May 1995). The infection may have occurred through a bud-scale scar caused by snow protection supports.

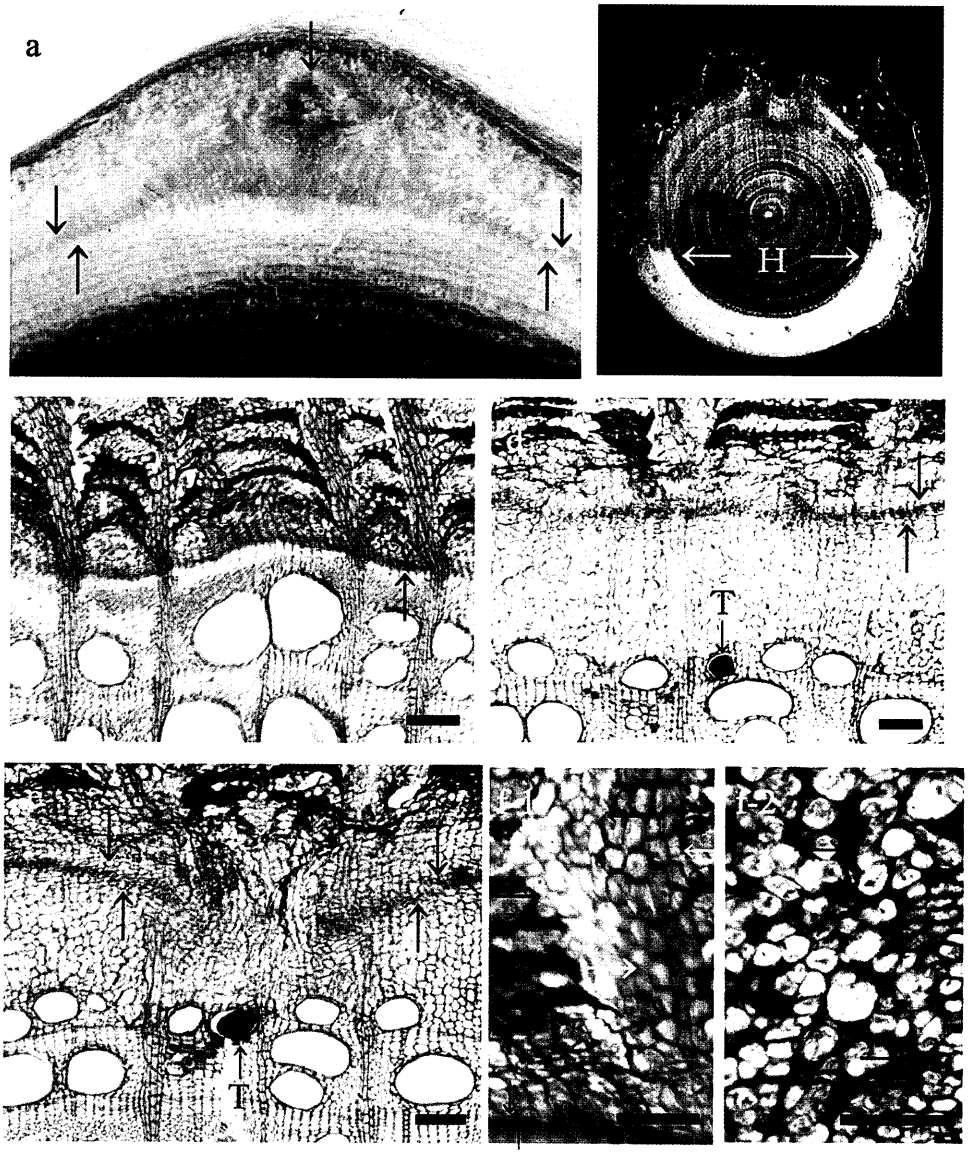

Fig. 3

a : Transverse view of the swelling. Red arrow indicates the stained spot, black arrows indicate the cambial zone. Bar = 1 cm.

b : Transverse view of the canker with exposed wood. Arrows indicate the dead phloem tissues. H = heartwood. Note that part of the exposed wood is dry. Bar = 1 cm.

c : Transverse view of the healthy tissue. Arrows indicate the cambial zone. Bar = 100μm.

d : Transverse view of the SSB far from the swelling. Arrows indicate the cambial zone. T = tylose. Bar = 100μm.

e : Transverse view of the SSB near the swelling. Arrows indicate the cambial zone. T = tylose. Bar = 100μm.

f : The irregular-shaped ray parenchyma cells.

1 : Transverse view of the irregular cells (red arrows). Yellow arrows indicate the normal ray parenchyma cells, black arrows indicate the cambial zone. Bar = 100μm.

2 : Radial view of the irregular cells. Arrows indicate masses of bacteria. Bar = 100μm.

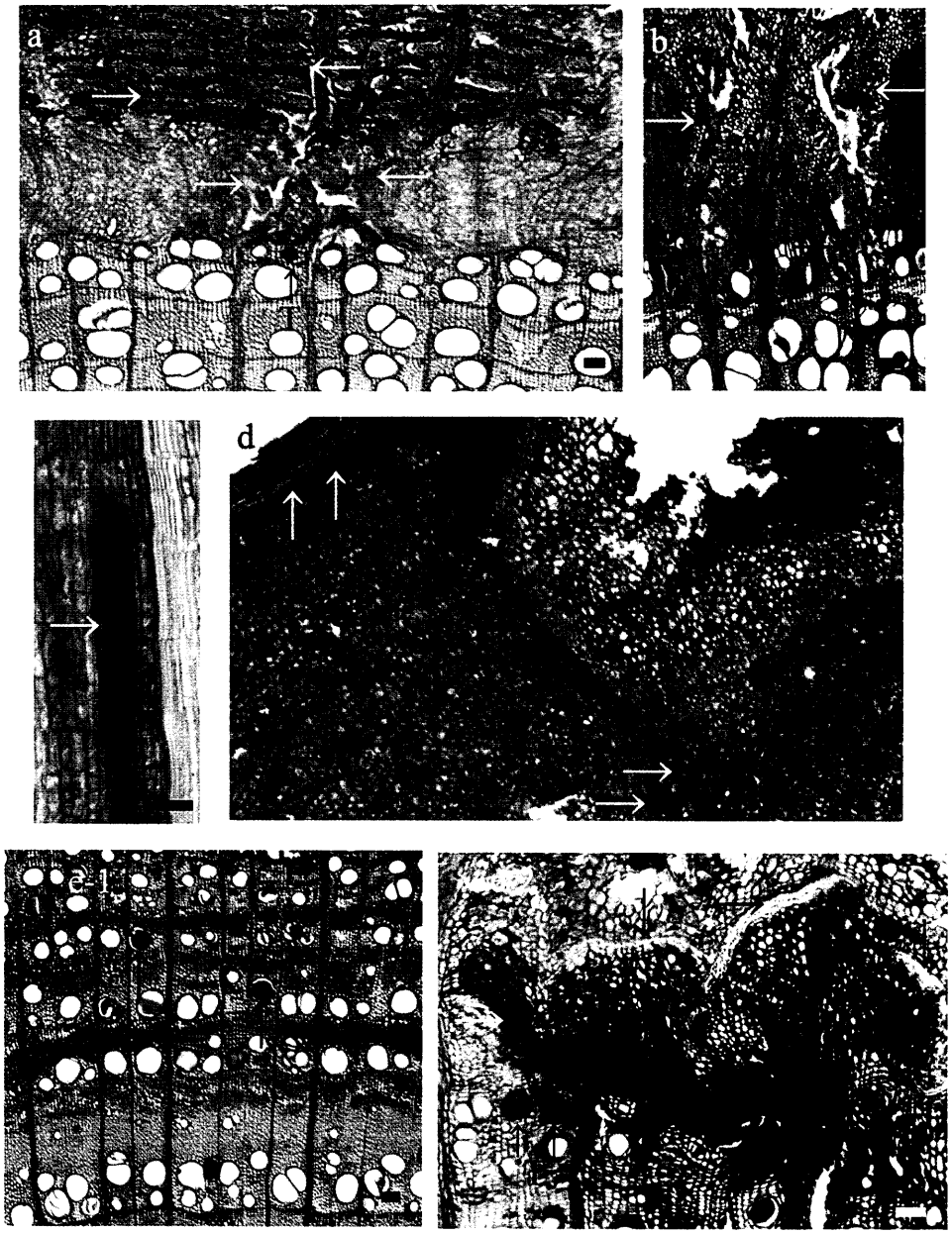

Fig. 4.
a : Transverse view of the swelling. Arrows indicate the bacterial lesions. T = tylose. Bar = 100μm.
b : Transverse view of the swelling. Yellow arrows indicate the bacterial lesions. Note that the tissues degraded near the ray parenchyma cells (black arrows). Bar = 100μm.
c : Radial view of the swelling. Arrow indicates a mass of bacteria in a vessel. Bar = 50μm.
d : Transverse view of the ruptured swellings. White arrows indicate the outer bark, red arrows indicate the wound periderm and yellow arrows indicate bacterial lesion. Bar = 100μm.
e : Transverse view of the margin of canker.
1 : The affected xylem. Arrows indicate the tyloses. Bar = 100μm.
2 : Yellow arrow indicates the bacterial lesion, red arrows indicate the wound periderm. T = tyloses. Bar = 100μm.

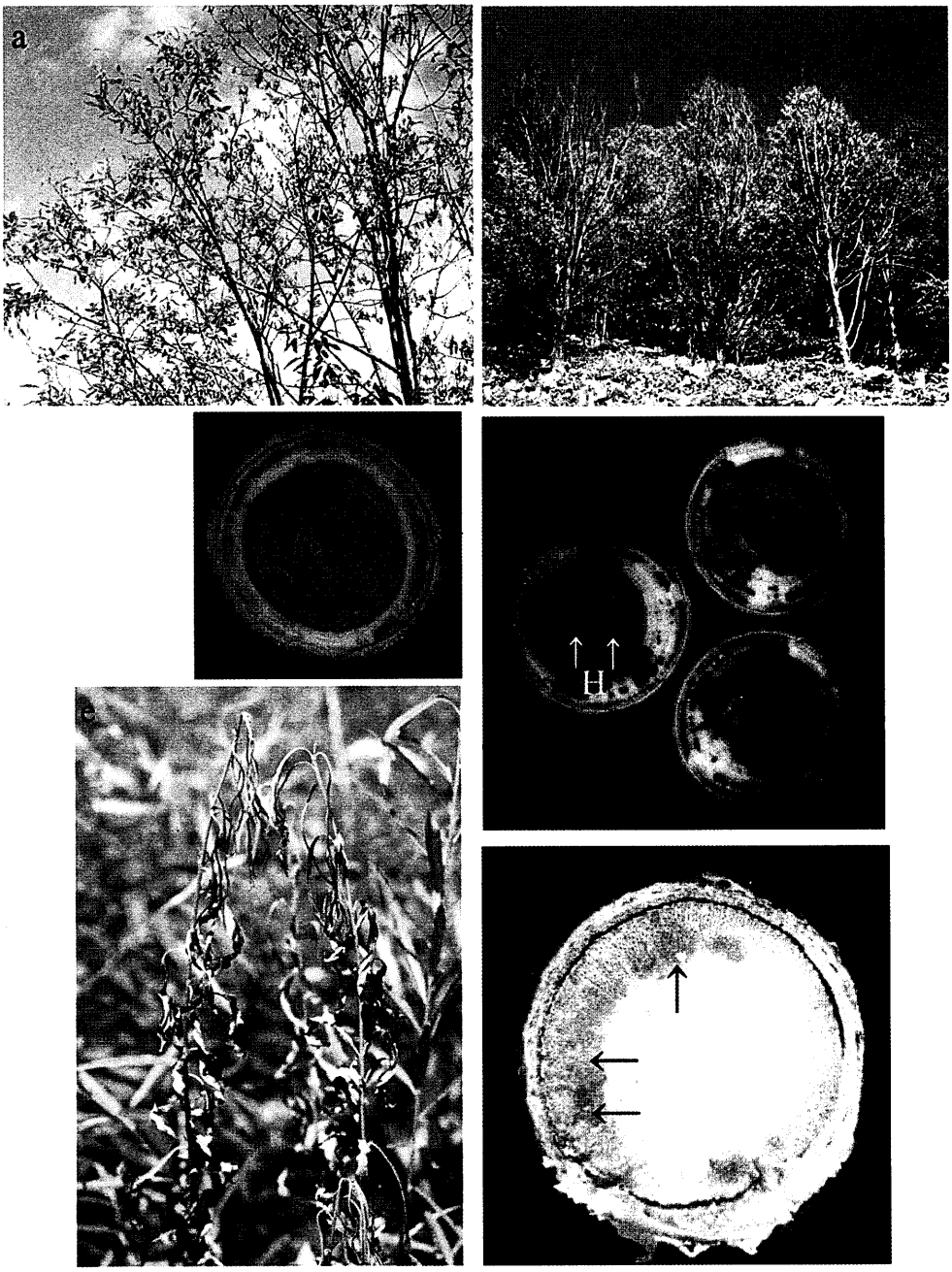

Fig. 5
a : Leaf symptoms in *Salix bakko* (photographed on 10 July 1997 in Kamikawa).
b : Dieback of *S. sachalinensis* (photographed on 18 July 1998 in Kamikawa, in its natural environment).
c : Circumferential watermark stain (arrows) in *S. bakko* (collected in Kamikawa).
d : Serious watermark stain in *S. kinuyanagi* (collected in Kamikawa). H = heartwood.
e : The pathogenic reaction on one-year old branches of *S. sachalinensis* caused by artificial inoculation (inoculated on 1 July 1998, photographed on 15 July 1998).
f : Transverse section of the right shoot in e. Note the watermark (arrows).

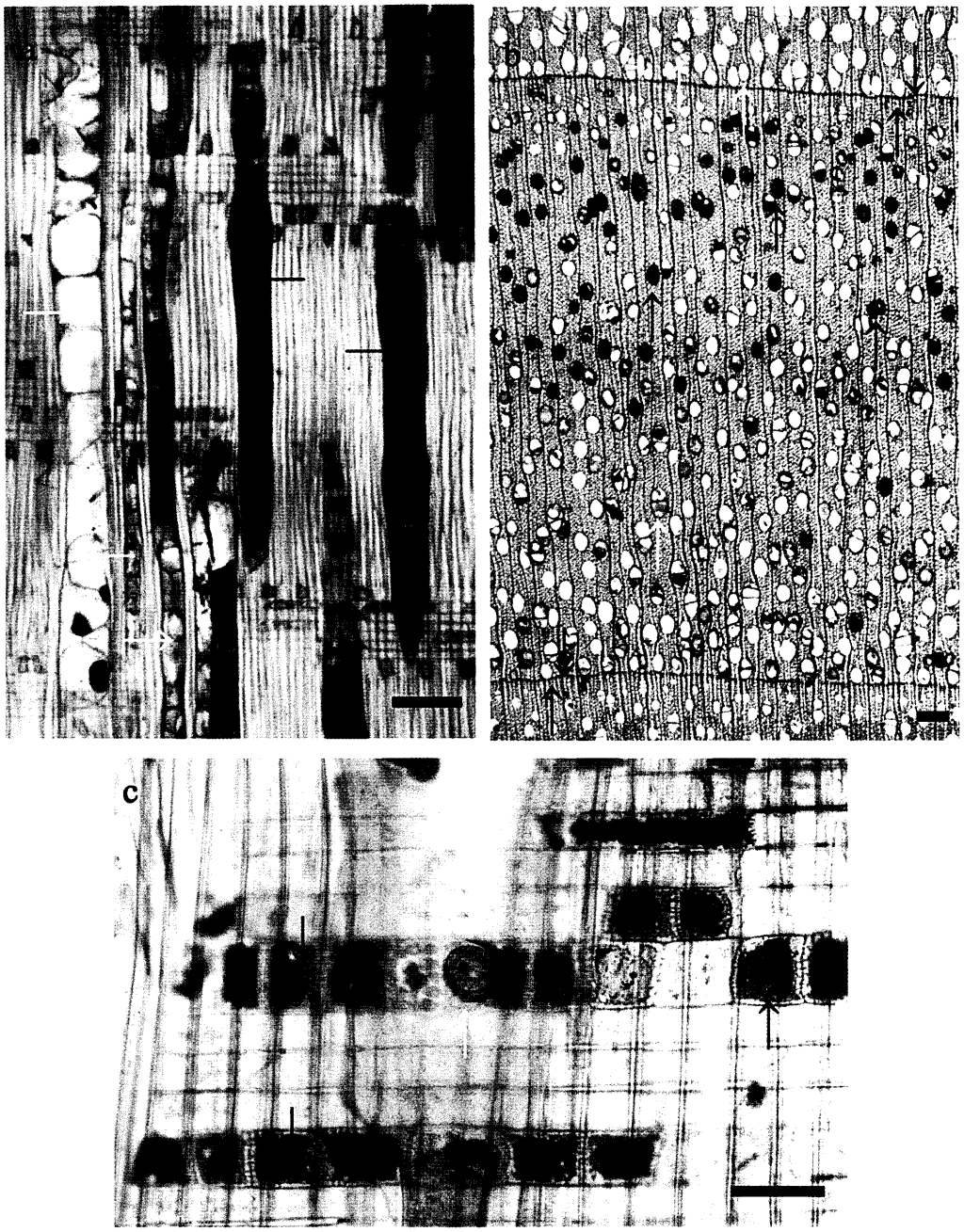

Fig. 6.
Microscopic observations.
a : Radial view of the watermark. Yellow arrows indicate tyloses and red arrows indicate masses of bacteria. Bar = 100μm.
b : Transverse view of the watermark. Yellow arrows indicate tyloses, red arrows indicate masses of bacteria and black arrows indicate the annual rings. Bar = 100μm.
c : Radial view of the ray parenchyma cells in the watermark. Black arrows indicate necrotic cells and the yellow arrow indicates plasmolysised cell. Bar = 50μm.

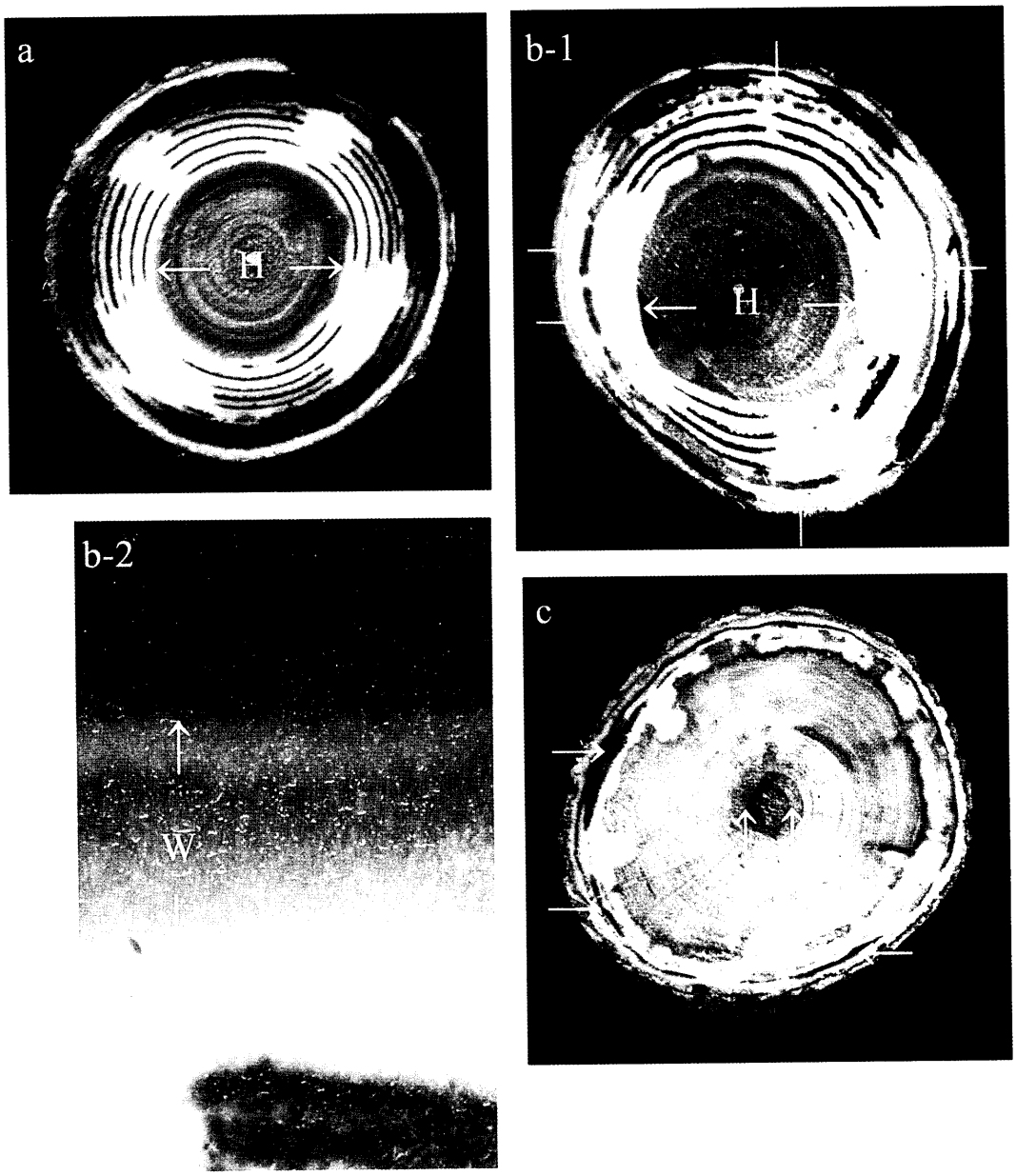

Fig. 7.
Dye-injection test. The stained areas (red) are water conductive.
a : Transverse view of a healthy tree 10 cm above the injection holes. H = heartwood.
b-1 : Transverse view of an affected tree (with approximately 30% branches dead) 10cm above the injection holes. Yellow arrows indicate the watermark (brown), not stained. H = heartwood.
b-2 : Close-up of sapwood of the disk in b-1. The watermark is not stained. W = the watermark.
c : Transverse view of an affected tree (with more than 90% branches dead) 10 cm above the injection holes. White arrows indicate the stained areas. The other brown area is the watermark. H = heartwood.

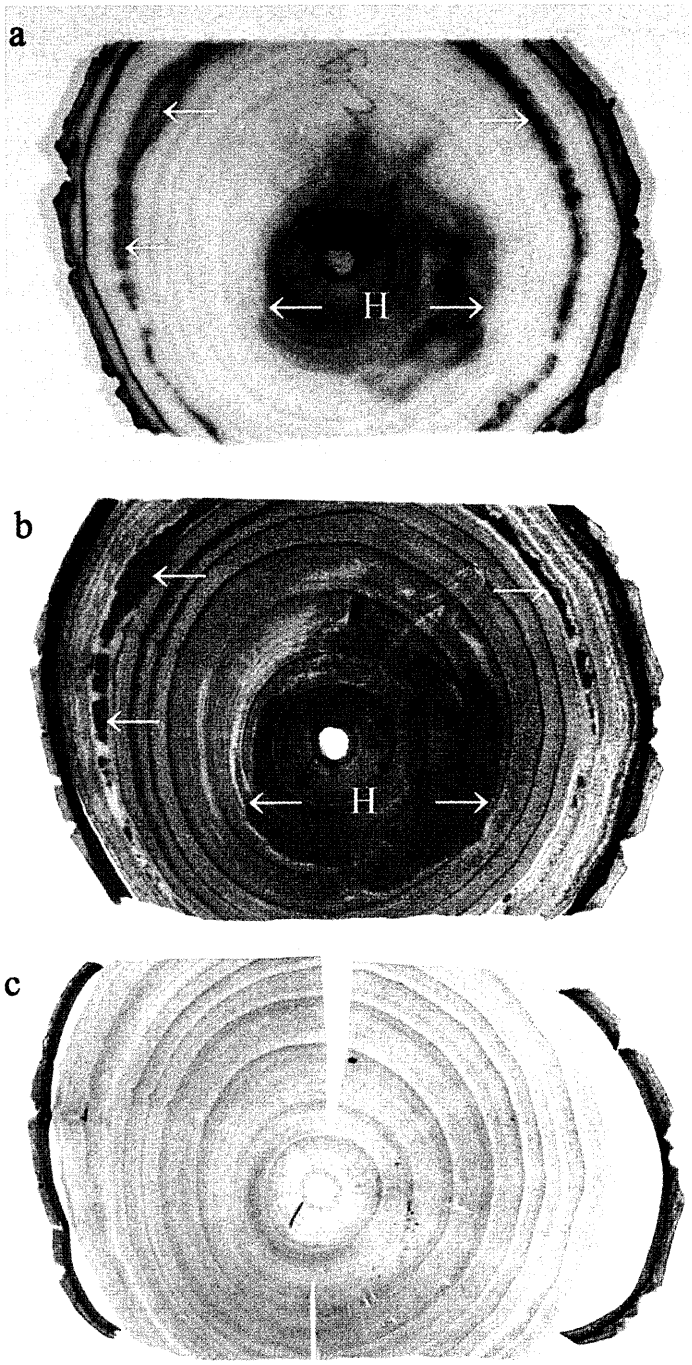

Fig. 8
Soft X-ray photography.
a : A black and white photograph of the wood disk from an affected tree (with more than 90% branches dead) in green state. Arrows indicate the watermark. H = heartwood.
b : A soft X-ray photograph of the disk in a in green state. Note that the dark zone (arrows) corresponds with the watermark in a. H = heartwood.
c : A soft X-ray photograph of the dried disk in a. The dark zone has disappeared.

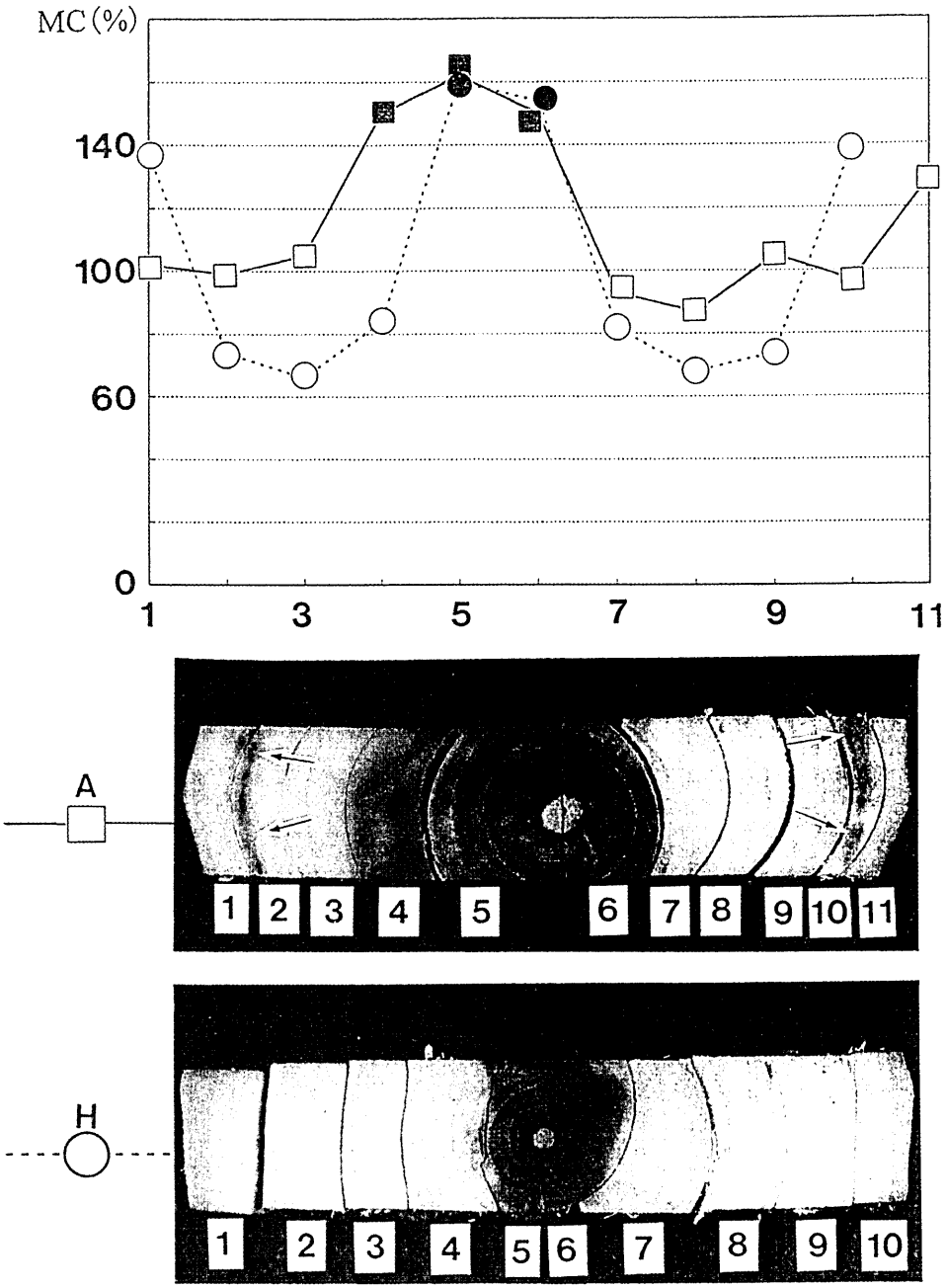

Fig. 9.

Comparison of moisture content between the affected wood disk and the healthy disk. The disks were collected from the specimens which were used in the soft X-ray photography. Arrows indicate the watermark. The black squares and circles indicate the heartwood. A = affected wood. H = healthy wood.

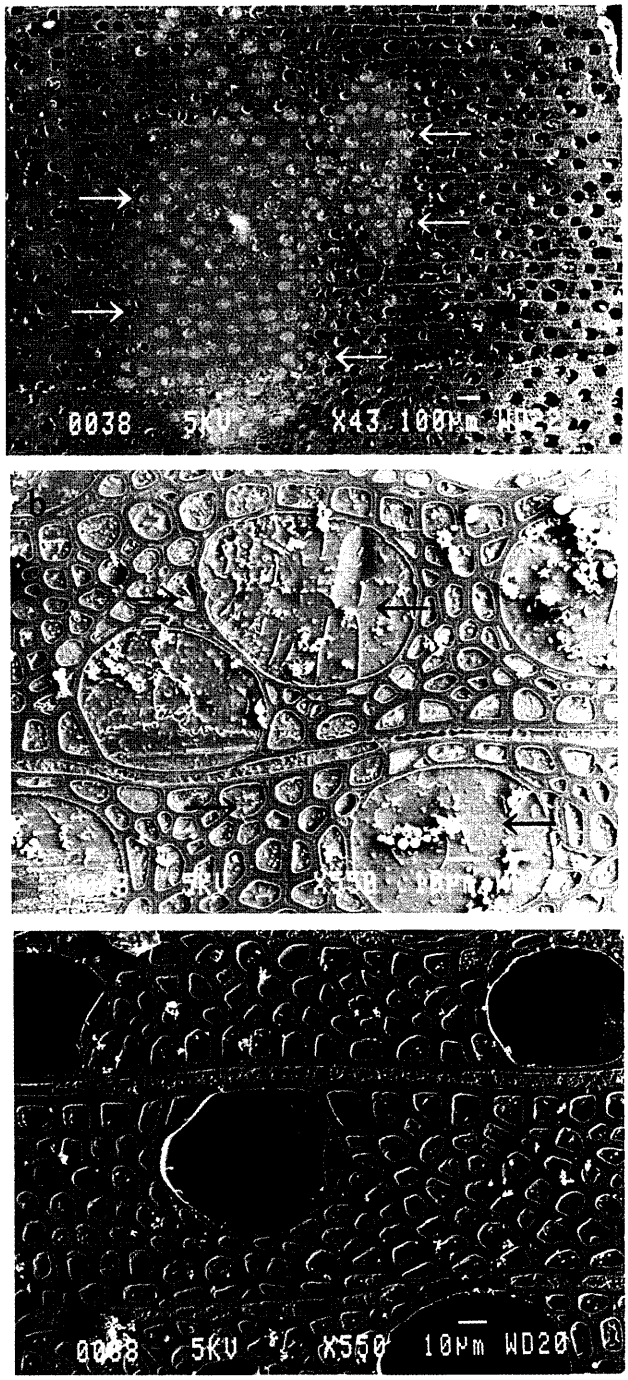

Fig. 10.
Cryo-scanning electron microscopy.
a : A cryo-SEM photograph of the watermark (arrows).
b : Close-up view of cryo-SEM photograph of the watermark. Arrows indicate ice (water in natural condition).
c : A cryo-SEM photograph of the middle to inner layers of the sapwood free from the watermark. Arrows indicate ice.