A PRELIMINARY INVESTIGATION OF THE DISEASES OF WILLOWS (*SALIX* SPP.)

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FRONTISPIECE

Willow showing evidence of dieback.

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CHAPTER 1

INTRODUCTION

Willows belong to the Salicaceae, a family of woody plants containing two genera, Salix, (willows) and Populus, (poplars). The genus includes both shrubs and trees varying in height from a few inches to 120 ft., and occurs most abundantly in cold or temperate climates of the Northern Hemisphere. Australasia and the Malaysian Archipelago are the only areas to which the genus is not indigenous.

There is now general agreement as to the number of Salix species. Lawrence (1951) and Clapham, Tutin and Warburg (1962) suggested that there are about 300 distinguishable species. Determination of species is often difficult since the dioecious nature of the genus leads to extensive cross-fertilisation and consequently many hybrid forms.

Willows were introduced into New Zealand by the early European settlers, (van Kraayenoord, 1968a). Several species and hybrids have become naturalised and are usually found growing by riverbanks or in other moist situations.

The use of willows and poplars to control erosion on the steep country and highly erodible soils frequently found in

New Zealand, was first implemented during the early days of settlement of the Colony, (van Kraayenoord, 1968a). Willows now play a significant role in erosion control, particularly in the South Island catchments, the central North Island pumice country and the East Coast North Island papa country. It is estimated that $ 850,000 is spent yearly on willow planting along the main rivers of New Zealand (van Kraayenoord, 1968b). In the past, the most frequently planted species have been S. fragilis L, the common crack willow, S. vitellina (L) Stokes, the golden willow and the S. cinerea L - S. caprea L complex, the goat willows or sallows. At present interest is focussed on such species as S. alba L, S. purpurea L, and S. discolor Muhl. (van Kraayenoord 1968b).

With the extensive use of willows in riverbank and erosion control in New Zealand, the disease factor could play a significant role, especially in those areas where planting of a single species has been adopted.

A study of the literature reveals only two papers in which investigations have been specifically directed at diseases of willows in New Zealand. Murray (1926) reported and described three fungal pathogens of S. fragilis and S. babylonica L., in Nelson: Marssonina salicicola (Bres.) Magn. on S. fragilis and S. babylonica, causing anthracnose of young stems and twigs; Macrophoma salicis Dearn. & Barth., on S. fragilis; and Gloesporium capreae Allesch. on S. fragilis, the latter two fungi being leaf parasites. Jenkins and

Grodinsky (1943) subsequently found that Gloeosporium capreae was typical of the genus Sphaceloma and re-named it Sphaceloma murrayae Jenkins & Grodinsky.

Dingley (1969) lists the following pathogens of willows in New Zealand:- Armillaria mellea Fr., Macrophoma salicis, Marssonina salicicola, Nectria ochroleuca Berk., Physalospora miyabeana Fukushi, Sphaceloma murrayae, Trametes versicolor Lloyd, Stereum purpureum Fr., Valsa sordida Nitschke (con.stat. Cytospora chrysosperma Fr.), and Pseudomonas syringae van Hall.

OBJECTS OF THE INVESTIGATION

The main object of this investigation was to study stem and leaf diseases of Salix spp. incited by micro-organisms and may be summarised this:-

(1) To carry out a survey of the extent and severity of the disease factor in New Zealand.

(2) To isolate, study in culture and prove the pathogenicity of organisms considered important in the death and dieback of willows.

(3) To describe and identify the causal organisms.

(4) To determine if possible, factors influencing the spread and degree of infection of the diseases.

CHAPTER 2

MATERIALS AND METHODS

Isolation

Isolations were carried out for two reasons:-

1. To fulfil "Kochs Postulates" for proof of pathogenicity.
2. To obtain isolates from material collected during the survey for taxonomic studies.

Surface Disinfection of Diseased Material

Diseased leaf material was washed in running water, placed in 70% ethyl alcohol for a few seconds - this acting as a wetting agent - and removed to a 1:4 solution of "Janola" and water for four minutes. ("Janola" is a commercial bleach and thus a source of sodium hypochlorite). Following four washings with sterile water the pieces of leaf tissue were placed on B.B.L. potato dextrose agar (PDA) in Petri-dishes using flamed forceps.

Diseased woody tissue was treated in a similar manner with "Janola" or was placed in a concentrated solution of 100 volume hyrdrogen peroxide for 15 minutes. A flamed scalpel was then used to cut away surface tissue and small

pieces of the underlying, infected tissue were placed on Petri-dishes containing PDA, using flamed forceps. From the results obtained, "Janola" and hydrogen peroxide were equally efficient surface disinfectants.

Where a bacterial pathogen was suspected, infected tissue was surface disinfected with 100 volume hydrogen peroxide for 15 minutes, and subsequently macerated in sterile water. Using a sterilised platinum wire loop, the resulting suspension was streaked onto PDA and B.B.L. plate count agar in Petri-dishes.

Plates were incubated at 24°C until growth became apparent at which stage, discrete colonies or parts of colonies were transferred to fresh media.

Source of Collections

During the investigation, some 50 isolates of known or suspected pathogenic micro-organisms were collected from many parts of New Zealand (Table 2.1). 31 isolates were selected for further study.

Preservation and Maintenance of Collections

Isolates were maintained by periodic transfer to PDA or plate count agar slopes in test-tubes. Some isolates on agar plates were preserved by freeze drying, as was some fresh material. This provided a ready source of material for morphological studies and for sectioning. Leaf material was dried in a plant press. Representative collections of fungal pathogens and cultures of Erwinia salicis have been deposited

in the Herbarium, Plant Diseases Division, D.S.I.R., Auckland.

Microtome Sectioning Methods

A freezing microtome was used to section material for morphological studies.

Material to be sectioned was cut into pieces up to 3mm square and soaked in water for at least 30 minutes. The piece was placed on the freezing stage in a drop of water and held in position with a needle until frozen. Satisfactory sections were cut at 15µm thickness.

Microscope Methods

Morphological characteristics of fungi were studied by placing material to be examined in a drop of lactophenol cotton blue, (Peacock, 1940), on a slide.

Slide preparations were made semi-permanent by ringing with nail varnish or by mounting stained material in glycerine-jelly (Peacock, 1940).

All measurements were made with an ocular micrometer calibrated with a stage micrometer, and using lactophenol cotton blue as the mountant. Measurements of fructifications were made under the low power of the microscope.

In studies to determine the range of spore size, 50 spores were measured in random fields.

Photographs

All photomicrographs were taken by the writer with a standard 35mm camera attachment, using Ilford micronegative film.

Photographs were taken with a 35mm camera with a close-up lens where required, using Ilford Pan F film.

SURVEY OF THE DISEASE FACTOR

The survey and inspection of diseased trees extended from February 1970 to May 1971. Areas included in the survey were:-

(a) The South Canterbury, North Canterbury and Otago Catchment Board areas in the South Island, and

(b) The Manawatu and Southern Hawke's Bay Catchment Board areas in the North Island.

Because of its proximity to the laboratory, the area surrounding the Selwyn River received the greatest attention. The remaining areas were visited for short periods only. The survey concentrated on stem and leaf pathogens. Table 2.1 shows details of collection date, locality, host, part of the plant affected and accession number for each isolate.

Table 2.1 List of Collections

Organism	Label	Location/Date	Host/Symptoms
Cytospora chrysosperma	L.C.2	College nursery 29.9.70	S. daphnoides, S. discolor, S. ulzio Young cuttings girdled and killed.
Cytospora chrysosperma	P.N.15	Massey nursery 17.4.70	S. purpurea Black lesions on stems.
Cytospora chrysosperma	S.1	Selwyn R. 23.2.70	S. fragilis Large branch - girdled.
Cytospora chrysosperma	R.2	Rakaia R. 14.4.70	S. fragilis Girdled branch.
Cytospora chrysosperma	S.4	Selwyn R. 19.2.70	S. fragilis Girdled branch.
Cytospora chrysosperma	C.F.9	Selwyn R. 14.4.70	S. fragilis Girdled branch.
Cytospora chrysosperma	P.N.1	Manawatu R. 27.1.71	S. vitellina Lesions on stems.
Cytospora chrysosperma	T.7	Opihi R. Timaru 9.3.71	S. fragilis Girdled branch.
Cytospora chrysosperma	O.3	Mt. Aspiring Stn. Otago 21.4.71	S. vitellina Dieback of nursery stock.
Cytospora chrysosperma	O.5	Millers Flat, S. Otago 22.4.71	S. fragilis Girdled branch.
Cytospora chrysosperma	O.7	Mt. Aspiring Stn. Otago 21.4.71	S. pendulina Dieback of nursery stock.

Table 2.1 (contd)

Organism	Label	Location/Date	Host/Symptoms
Marssonina salicicola	CH.1	Christchurch 11.9.70	*S. babylonica* Anthracnose of twigs.
Marssonina salicicola	CH.2	Christchurch 11.5.71	*S. babylonica* Anthracnose of branches.
Marssonina salicicola	P.N.3	Massey nursery 26.1.71	*S. babylonica* Anthracnose of twigs.
Sphaceloma murrayae	S.R.3	Selwyn R. 19.2.70	*S. fragilis* Leaf Spot.
Sphaceloma murrayae	P.N.16	Massey nursery 17.4.70	*S. fragilis* Leaf spot.
Sphaceloma murrayae	CH.5	Christchurch 6.3.71	*S. fragilis* Leaf spot.
Sphaceloma murrayae	O.11	Millers Flat, S. Otago	*S. fragilis* Leaf spot.
Melampsora epitea	R.10-28	Massey nursery 26.1.71	*Salix* spp. Leaf rust.
Melampsora epitea	R.3	Wanstead, S. Hawke's Bay 27.1.71	*S. discolor* and *S. epitea* Leaf rust.
Melampsora epitea	R.4	Massey nursery 17.4.70	*S. cinerea* Leaf rust.
Melampsora epitea	R.5	Christchurch 21.5.71	*S. cinerea* Leaf rust.
Erwinia salicis	C.3	Cadrona Valley, Otago 22.4.71	*S. fragilis* Black weeping wound on branches.
Erwinia salicis	C.2	Cadrona Valley, Otago 22.4.71	*S. fragilis* Black weeping wound on branches.

Table 2.1 (contd)

Organism	Label	Location/Date	Host/Symptoms
Erwinia salicis	M.F.2	Millers Flat, Otago 22.4.71	*S. fragilis* Black weeping wound on branches.
Erwinia salicis	S.12	Selwyn R. 30.4.71	*S. fragilis*
Erwinia salicis	T.3	Opihi R. Timaru 10.3.71	*S. fragilis*
Erwinia salicis	T.5	Opihi R. Timaru 10.3.71	*S. fragilis*

Other recognised pathogens identified on willows but which were not studied further were: *Stereum purpureum* on *Salix* nursery stock in the Manawatu in April 1970 and to a lesser extent in January 1971; and *Agrobacterium tumafaciens* Conn. on *Salix* nursery stock, both in the Manawatu and Southern Hawke's Bay.

Further organisms isolated from willows which were not suspected of being pathogens included: *Phoma sp.*, *Alternaria tenuis* Nees, *Ulocladium sp.*, *Cephalosporium sp.* and *Stemphylium sp.* from leaves; and *Fusarium sp.* and *Phomachora sp.* from woody tissue.

CHAPTER 3

CYTOSPORA CHRYSOSPERMA

INTRODUCTION

Cytospora chrysosperma Fr. is the imperfect stage of the Ascomycete, *Valsa sordida* Nitschke (1870). The relationship between the two organisms had been suspected for some years before it was proved in cultural experiments by Schreiner (1931).

C. chrysosperma was first described in 1823 as a saprophyte of dead poplars, (Fries 1823). It was not recognised as a pathogen until Long (1918) reported that it caused disease on eleven species and varieties of poplars and three species of willows in the southwestern states of the U.S.A. Hubert (1920) reported *C. chrysosperma* as a wound parasite on poorly growing specimens of two species of willows in the northwestern states of the U.S.A., and Moss (1922) reported that it caused disease on poplars in Ontario, Canada. Grove (1923) noted that *C. chrysosperma* caused a serious disease of poplars in England, but Day (1924) considered the disease to be of secondary occurrence in trees weakened by *Erwinia salicis* (Day) Chester.

Because of the severity of the disease and its widespread occurrence, several workers have studied factors influencing the development and severity of infection by C. chrysosperma. These studies have been confined mainly to poplars.

Schreiner (1931) conducted pathogenicity trials and cultural studies of C. chrysosperma and Valsa sordida on poplars in the U.S.A. and concluded that host vigour and severity of the wound were important factors influencing the degree of infection. Christensen (1940) considered C. chrysosperma to be among the most common bark inhabiting fungi found in the U.S.A. He also suggested "that the fungus (C. chrysosperma) often is not responsible for the injury with which it is associated."

Butin (1955), working in Germany, noted that successful inoculations of cuttings of Populus deltoides Marsh with C. chrysosperma only occurred if their moisture content was below a certain level, while in the U.S.A., Wright (1957) found that susceptibility of shelter-belt P. deltoides was due to low soil moisture conditions. Bloomberg (1962) in Canada found a negative correlation between bark moisture content and Cytospora canker growth. He suggested that as poplar cuttings would lose moisture during storage and for a period following planting, infection by C. chrysosperma was likely until root growth balanced transpiration. Bloomberg also suggested that providing soil moisture and relative humidity were sufficiently low, rooted cuttings could be attacked by the fungus. Stahl (1967) investigating an outbreak of the disease on poplars

around Canberra and areas of New South Wales Australia reported: "that the initial attack always occurs on the north side of the tree". He ascribed this to localised heating by solar radiation and suggested an association between late flushing, high spring temperatures and infection by C. chrysosperma. Stahl suggested that under conditions of much higher than normal spring temperatures, those poplars without leaf protection apparently lost their natural resistance to the disease. This, he postulated, was due to a blocking or inactivation of the phytoalexin mechanism due to abnormally high tissue temperatures.

In New Zealand, C. chrysosperma was first recorded by Birch (1937) as a pathogen of Populus nigra L. but of rare economic significance. Lancaster (1955) recorded C. chrysosperma as a relatively unimportant parasite of willows and poplars in New Zealand, and Weston (1956) wrote of C. chrysosperma causing dieback of stools and cuttings of poplar nursery stock. Gilmour (1966) recorded C. chrysosperma as: "a wound pathogen causing canker and dieback of (willow) stools and cuttings". He stated that the disease was of no great significance as little use was made of willows in forestry in New Zealand. Dingley (1969) reported C. chrysosperma to be a common pathogen of Salix spp. and Populus spp.

Figure 3.1 *Salix* cutting naturally infected with *Cytospora chrysosperma*.

DESCRIPTION OF THE DISEASE ON WILLOWS

Cytospora chrysosperma produces lesions or cankers on the trunks and large limbs of affected trees. The area invaded by the fungus is usually of fairly regular outline (Figure 3.1) and in the earlier stages is recognisable by the characteristic purplish-brown discolouration. Small branches and twigs show a general necrosis and are usually killed without the formation of a definite canker.

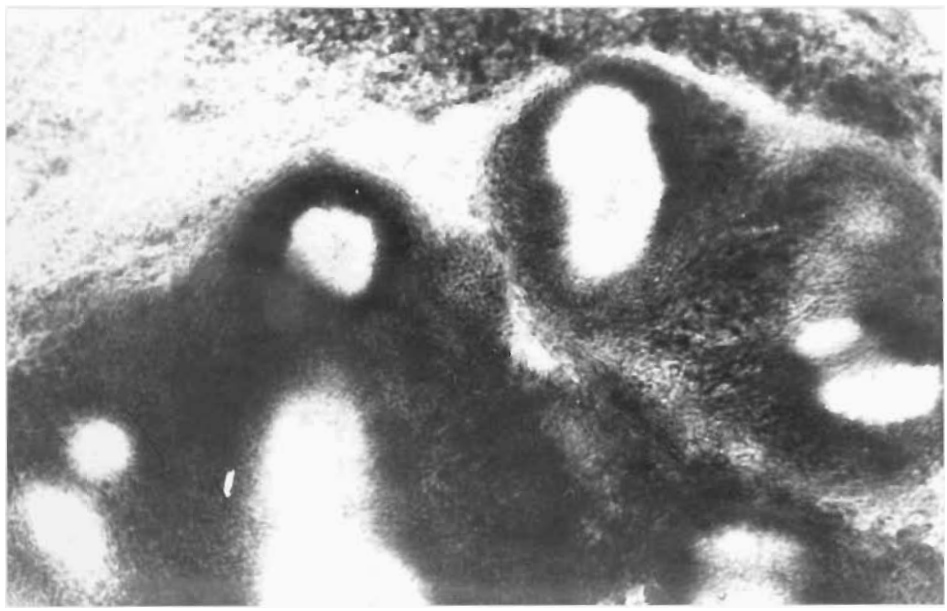
Field observations suggest that severity of the disease depends upon the vigour of the host. A vigorous host plant may grow sufficiently during infection to cover and seal in the fungus. Less vigorous hosts may exhibit an easily recognisable scar in the shape of the previous canker, or the infected limb may be girdled and killed. The bark of girdled branches cracks with drying, becomes loose and ultimately sloughs off.

Under conditions of high relative humidity and warm temperatures, pycnidia in infected areas produce characteristic spore horns (Figure 3.2). These appear as red to yellow twisted threads up to 1cm long, which are soft and sticky when first formed but become brittle on drying.

Entry of the fungus is mainly through wounds, dead twig tips or leaf abscission scars. Occasionally the disease may occur although no point of entry is apparent. This is probably due to the perennial mycelium reported by Schreiner (1931).

Figure 3.2 Spore tendrils of *Cytospora chrysosperma* on naturally infected willow.

Figure 3.3 T.S. *Salix* bark showing pycnidia of *Cytospora chrysosperma*.



OCCURRENCE

The disease is widespread in New Zealand and was recorded in all areas visited during the course of the survey. It was particularly prevalent where summer conditions were hot and dry, such as on the Canterbury plains and throughout Central Otago. The incidence of disease was considerably less in areas with a high summer rainfall. The disease was also common on nursery stock and newly-planted cuttings in their first summer season.

Cuttings and poles planted in shingle or sandy riverbeds, appeared particularly susceptible during dry periods when the water table was low.

Wounds due to burning render willows particularly susceptible to extensive damage by C. chrysosperma.

DESCRIPTION AND TAXONOMY OF THE PATHOGEN

Cytospora chrysosperma Fr. (Figure 3.4)

Syst. mycol. 2 : 542, 1823

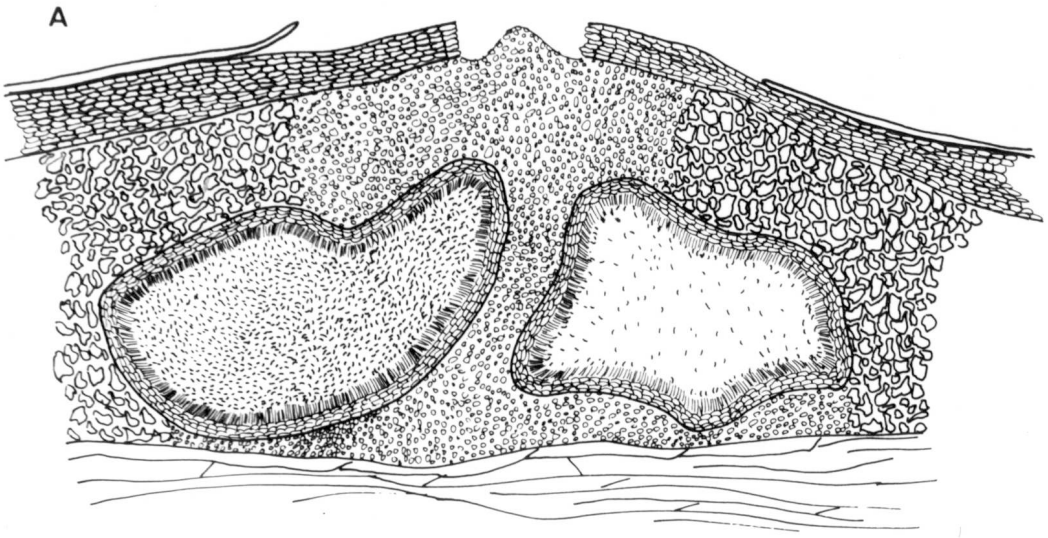
(stat.conid. of Valsa sordida Nitschke, 1870)

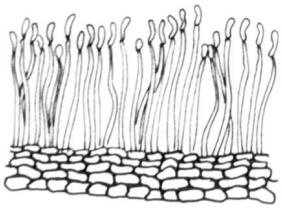
Fructifications lignicolous, scattered or gregarious in small groups and possessing a common ostiole, subepidermal, becoming erumpent at maturity, depressed - globose, up to 2mm wide, cinereous to black. Pycnidia represented by loculi

Figure 3.4 A. T.S. bark of Salix, showing pycnidia of Cytospora chrysosperma (x 200)

B. Conidiophores with conidia (x 600)

C. Conidia (x 2000)


A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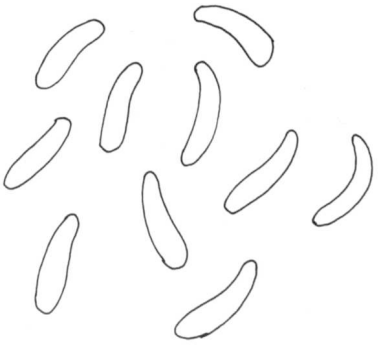

C

embedded in the stroma, irregular in size and shape. Conidiophores simple, filiform, hyaline, 9 - 14 x 1µm, arising from pigmented, pseudoparenchymatous stromatic tissue. Conidia aseptate, allantoid, thin-walled, hyaline, produced singly from apex of conidiophore, 3.5-5 x 1µm., sometimes extruded through ostiole in a long, yellow to red spore horn. Habitat: living and dead wood of Salix and Populus. N.Z. Host range: S. fragilis, S. vitellina, S.purpurea, S. discolor, S. ulzio L, S. pendulina L, S. babylonica, S. viminalis L.

The collections of Cytospora examined during the study agree closely with the description of Cytospora chrysosperma published by Grove (1923, 1935) and Schreiner (1931), except that Grove recorded the species on Populus only. Grove based his taxonomy of the genus Cytospora on spore morphology, colour of spore tendrils and particularly host range. On this basis, he listed seven species of Cytospora infecting Salix only, a further three species infecting Populus only and Cytospora ambiens Sacc. common to both genera. However, Kern (1957) reported the range in spore size from one pycnidium of Cytospora overlapped those from virtually all described species. Furthermore, Kern stated that many species of Cytospora "do not represent clear entities in the classical sense but rather subjective groups or strains without sharp limits". Hubbes (1960) in his taxonomic study of the Valsaceae considered Cytospora chrysosperma, C. germanica Sacc. and C. salicis to be synonyms of C. ambiens, the imperfect stage of

Valsa ambiens (Pers.) Fr. 1849. He also stated that "the existence of many distinct strains and transitions in characters led to a wide conception of species".

If Hubbes' findings are accepted, the correct name for the fungus is *Valsa ambiens*, the first name applied to the perfect stage. However, as Dennis (1960) pointed out, the genus *Valsa* is in urgent need of revision. At the present time it is considered preferable to retain the established and widely used names *Valsa sordida* for the perfect stage and *Cytospora chrysosperma* for the imperfect stage rather than introduce two further names that are possibly not correct.

CHAPTER 4

CYTOSPORA CHRYSOSPERMA - EXPERIMENTAL

CULTURAL STUDIES

Cultural studies were carried out to delimit optimum conditions for growth and sporulation of Cytospora chrysosperma. A knowledge of optimum conditions for mycelial growth was necessary if maximum symptom expression was to be obtained in pathogenicity studies.

FACTORS AFFECTING MYCELIAL GROWTH

Nutrient medium, temperature and pH were considered to be the factors most likely to affect growth. To determine the optimum combination of nutrient medium and temperature an experiment of factorial design was used; pH was studied separately.

Colony diameter on agar was considered by Brancato and Golding (1953) to be a reliable method of assessing fungal growth. Cochrane (1958) considered the accuracy of this method to be doubtful. However, Pirt (1967) found it reliable where the depth of agar was standardised at approximately 5mm, (equivalent to 35ml in a 9cm Petri-dish). He showed that growth rate was characteristically linear on solid media and

established the expression:

$$r = K_r t + r_o$$

where r = colony radius at time t

r_o = colony radius at time zero

K_r = constant, radial growth rate.

In the present studies, it was considered that colony diameter would give a reliable measure of the effects of pH and temperature. However, when comparing growth on different nutrient media, measurement of colony diameter after a given time was considered inadequate, since growth form and staling varied with medium (Hawker, 1950). Results of this experiment are therefore given in the form of a graph showing growth rate.

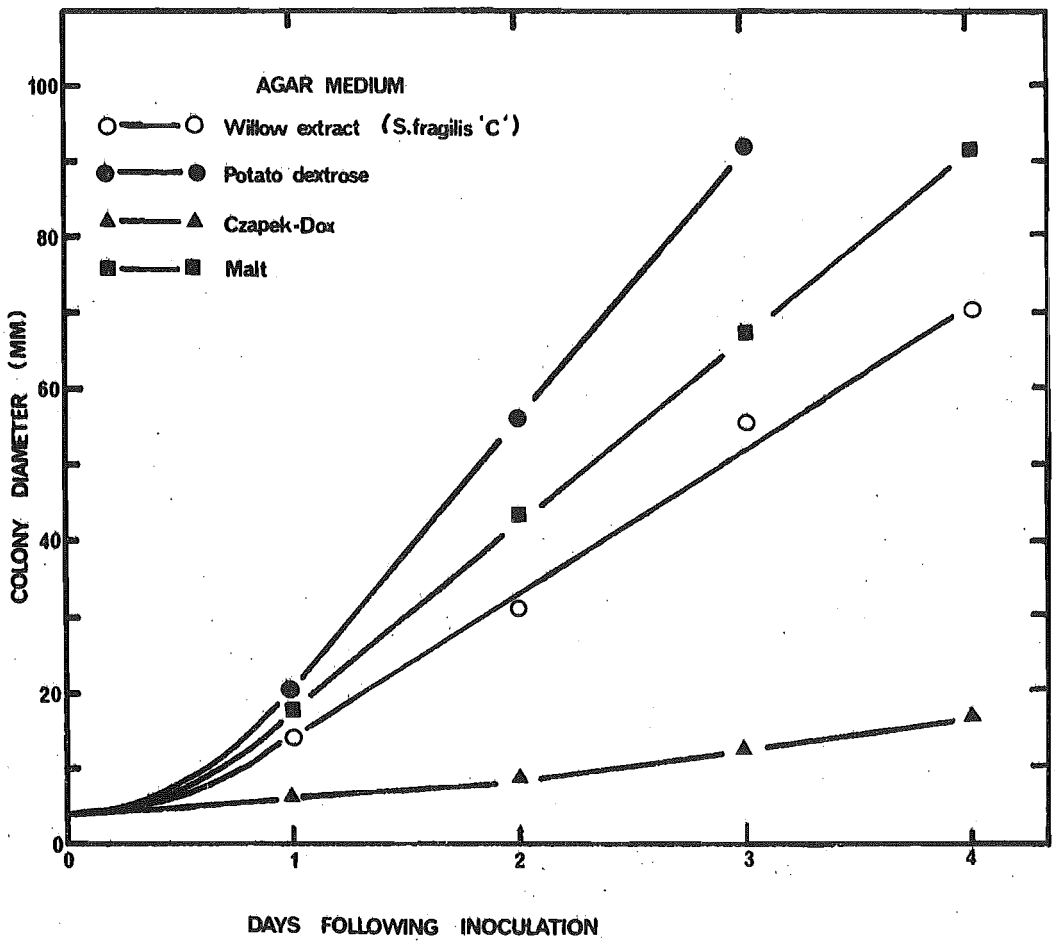
In all mycelial growth experiments plates were inoculated with mycelium from the margin of an actively growing colony on PDA. Blocks of agar 2mm square were cut and transferred with a flamed scalpel and inverted on the test media.

Each treatment was replicated six times. Colony diameter was taken as the average of two measurements at right angles and measurements were made at daily intervals following inoculation.

Effect of Nutrient Medium on Growth

Growth was measured initially on three commonly used media; malt agar, PDA and Czapek-Dox agar (Harrigan and McCance, 1966). Malt agar and PDA were prepared from B.B.L. powders. In a later experiment nine different willow wood

Figure 4.1 Effect of different media on growth rate of Cytospora chrysosperma S.1.


AGAR MEDIUM
Willow extract (S.fragilis 'C')
Potato dextrose
Czapek-Dox
Malt
COLONY DIAMETER (MM)
100
80
60
40
20
0
0
1
2
3
4
DAYS FOLLOWING INOCULATION

decoction agars were used (Appendix 2). Agar plates were inoculated with mycelium from isolate S.1 and incubated at 24°C.

The growth rate of Cytospora chrysosperma on PDA, Czapek-Dox and malt media is shown in figure 4.1. S. fragilis 'C' decoction medium is included for comparison. The effects of willow decoction media on growth are shown in table 4.1.

Table 4.1 Effect on decoction media from different willow species and varieties on growth of Cytospora chrysosperma S.1 at 24°C.

Willow species or variety	Colony diameter (mm)Day 3	Duncans Test 5% level	Colony diameter (mm)Day 4.	Duncans Test 5% level
S. discolor 'B'	62.3	a	79.7	a
S. fragilis 'C'	55.5	cd	70.7	b
S. matsudana	58.5	b	70.5	b
S. vitellina 'Westhaven'	53.3	d	68.2	c
S. incana	54.2	cd	66.3	d
S. ulzio 1	53.0	d	65.7	d
S. fragilis 'M'	53.3	d	64.0	e
S. daphnoides 'G'	44.8	e	55.5	f
S. purpurea 'Booth'	44.2	e	54.5	f

Growth form and colony appearance of Cytospora chrysosperma on each medium was:

1. Malt agar - white, turning brown except for the marginal mycelium remaining white, outline regular, little aerial mycelium.

2. Potato dextrose agar - white, slightly floccose, outline irregular.

3. Czapek-Dox agar - white, floccose mycelium, outline irregular.

4. Willow wood extract agar - white, turning brown except for marginal mycelium, outline slightly irregular, little aerial mycelium.

The results showed that rate of growth was highest on plant extract agars (i.e. all except Czapek-Dox agar). The rapid growth rate on these media minimised the effect of staling.

Significant differences in growth were obtained on the different willow wood decoction agars, but these could not be correlated with the tree or shrub habit of the species and varieties tested.

Effect of Incubation Temperature on Growth

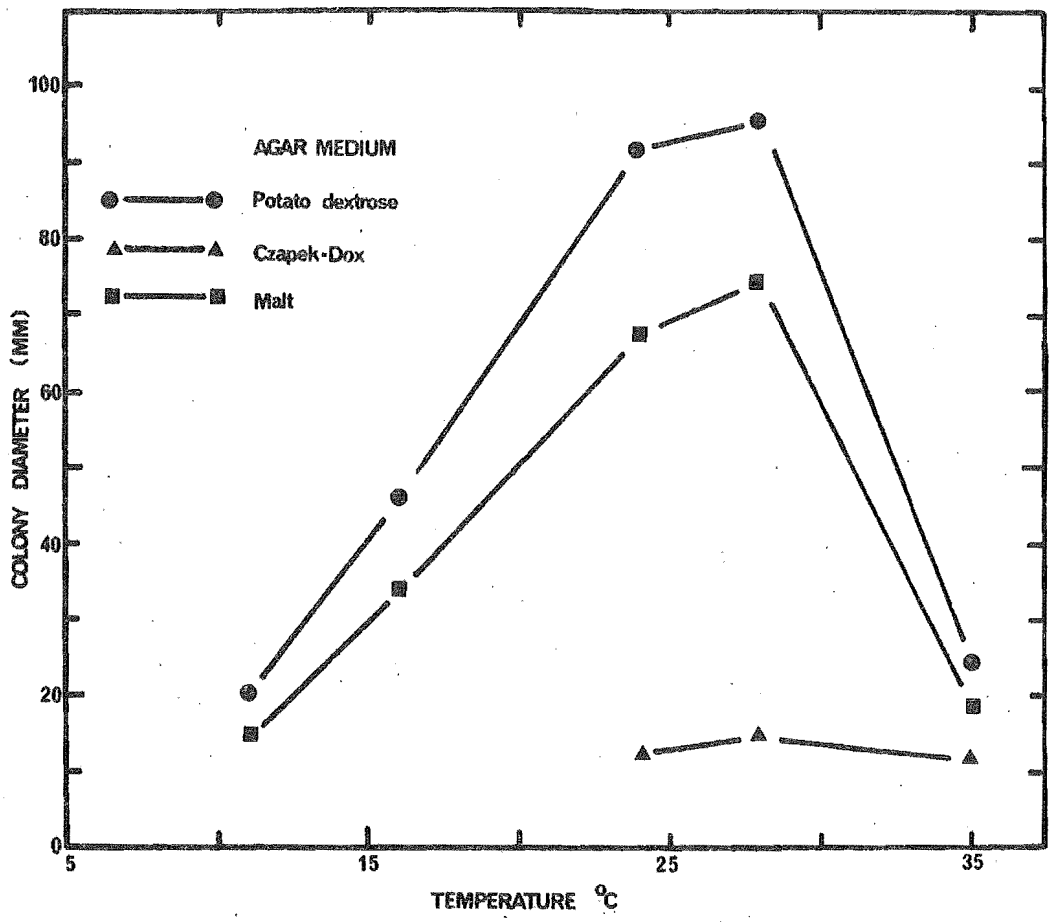
Inoculated PDA, malt and Czapek-Dox agar plates were incubated at six temperatures:

4°, 11°, 16°, 24°, 28° and 35°C.

Plates were inoculated with mycelium from the edge of an actively growing colony of isolate S.1.

The results are shown in figure 4.2. Optimum temperature for this isolate ranged from 24° - 28°C on all media. A marked reduction in growth occurred on either side of the optimum range, with no growth occurring at 4°C.

Figure 4.2 Effect of incubation temperature on growth of *Cytospora chrysosperma* S.1.


AGAR MEDIUM
Potato dextrose
Czapek-Dox
Malt
COLONY DIAMETER (MM)
100
80
60
40
20
0
5
15
25
35
TEMPERATURE °C

Effect of Initial pH of Medium on Growth

A series of six pH levels of PDA were prepared:

4.8, 5.2, 5.6, 6.0, 6.5 and 7.0

Because autoclaving tends to alter pH of agar media, it was adjusted under aseptic conditions after sterilisation. The medium was held in liquid form in a water bath at 56°C. Sterile normal hydrochloric acid or sterile normal sodium hydroxide was added to give the required pH. The pH was measured with a "Radiometer" glass electrode meter. Plates were inoculated with mycelium from isolate S.1. and incubated at 24°C. Each treatment was replicated three times.

Table 4.2 Effect of pH of medium on growth of Cytospora chrysosperma S.1. at 24°C.

pH	4.8	5.2	5.6	6.0	6.5	7.0
Colony diameter 2 days	54.2	52.6	55.3	54.8	52.1	56.7

The results are shown in table 4.2. No significant differences in growth rate occurred at the different pH levels. This could be expected since many fungi have a wide range of insensitivity to pH on the acid side of neutrality, (Brancato and Golding, 1953).

FACTORS AFFECTING SPORULATION

Effect of Temperature on Sporulation

Petri-dishes of PDA inoculated with isolate S.1 were incubated at four temperatures: 11°, 16°, 24° and 28°C. Each treatment was replicated twice.

Table 4.3 Effect of temperature on sporulation of *Cytospora chrysosperma* S.1 on PDA.

Temperature °C	Sporulation after 20 days
11°	-
16°	-
24°	+++
28°	++++

-	no stroma
+++	10-15 stroma/plate
++++	15-20 stroma/plate

The results are shown in table 4.3. At 24° and 28°C fructifications appeared after approximately 14 days.

DISCUSSION

Media and temperature were the factors most affecting growth of *Cytospora chrysosperma* in culture. Culturing on PDA at 24° to 28°C gave optimum conditions for mycelial growth. Optimum temperature for sporulation was within the same range. The fungus exhibited a wide range of tolerance to acid pH.

On willow decoction agars significant differences in growth occurred between treatments, but no varietal pattern emerged.

PATHOGENICITY STUDIES

Method of Inoculation

Preliminary small scale inoculation trials were carried out to obtain proof of pathogenicity as required by "Koch's postulates", and to determine a reliable method of inoculation.

As the fungus had been found infecting only woody tissue of *Salix spp.* wound inoculation with mycelium was adopted. Bloomberg (1962) obtained satisfactory results with this method although Stahl (1966) successfully inoculated wounds with a spore suspension.

Cuttings for inoculation were obtained from the Lincoln College Nursery (appendix 1) and grown in potted soil in the glasshouse. Inoculations were carried out in a glasshouse maintained at 22°C.

Inoculum from isolate S.1 was prepared by placing small squares (5mm x 5mm) of sterilised, single strand, fine nylon mesh around the margins of an actively growing colony on PDA in a Petri-dish (figure 4.4). The colony was allowed to grow until the margin was 5mm beyond the nylon mesh. The nylon was then removed from the surface of the medium, the mycelium interwoven with the nylon strands. This provided mycelial inoculum free from adhering nutrient medium.

Effective inoculation was achieved by excising a flap of surface disinfected bark with a flamed scalpel. This

Figure 4.5 Method of inoculation of willow cuttings with Cytospora chrysosperma S.1. using the nylon mesh method.

Figure 4.6 Distinctive lesion produced on a willow cutting inoculated with Cytospora chrysosperma S.1. in comparison with uninoculated control.


10
20
30
40
50
60
70
80
90
100
100 MILLIMETRES

This allowed the inoculum to be placed under the bark in direct contact with the woody tissue of the host, (figure 4.5). The wound was sealed by binding tightly with plastic insulation tape.

Three branches on each plant were inoculated. A fourth branch acting as a control was treated in the same manner but without the addition of mycelial inoculum.

Table 4.4 Appearance of lesions on glasshouse grown plants of S. fragilis 'C' and S. vitellina Westhaven, inoculated with Cytospora chrysosperma S.1.

Plant	S.fragilis 'C' No.1		S.fragilis 'C' No.2		S.fragilis 'C' No.3		S.vitellina Westhaven	
	inoc.	cont.	inoc.	cont.	inoc.	cont.	inoc.	cont.
No. of lesions after 7 days	1	-	1	-	2	-	2	-
No. of lesions after 14 days	3	-	3	-	2	-	3	-

Successful inoculation was indicated by the appearance of the characteristic lesion associated with Cytospora chrysosperma on willows. This was visible above or below the plastic tape. Recordings were made on the seventh and fourteenth day after inoculation. The results are shown in table 4.4.

Wound inoculation proved satisfactory in producing infection, although in only one instance was a severe lesion produced. This would suggest that glasshouse conditions of

warm temperature and high humidity (95 - 100%) prevented maximum expression of the disease.

EFFECT OF BARK MOISTURE ON DEVELOPMENT OF CYTOSPORA CHRYSOSPERMA.

Bloomberg (1962) working with Populus trichocarpa considered that bark moisture content was negatively correlated with Cytospora canker growth. He found that inoculated cuttings kept under dry conditions decreased in bark moisture and that cankers developed. When these dry cuttings were placed in water soon after cankers had developed, they increased in bark moisture while canker growth decreased and finally stopped. Cuttings kept in water throughout failed to develop severe cankers. Filer (1967) studied the effects of climate and bark turgidity on infection of 2 to 3 year-old Populus deltoides plantations by Cytospora. The study extended over three seasons and he concluded that there appeared to be no simple, direct relationship between relative bark turgidity and severity of infection. Stahl (1967) stated that dehydration could not be correlated with infection by Cytospora. but that high temperatures were necessary to obtain infection.

Cuttings from 7 month-old stools of S. fragilis 'C' were taken from the Lincoln College Nursery in March, 1971. In the laboratory they were pruned so that only the basal 30cm remained. Inoculation of these cuttings with isolate

S.1 was carried out with nylon mesh squares, as described earlier in this chapter. Cuttings were placed upright in boiling tubes in the laboratory and subjected to the following initial treatments:

Treatment A1 - 12 inoculated cuttings, bases in water, exposed to the air.

Treatment A2 - 12 inoculated cuttings, bases not in water, tops in plastic bags sealed beneath point of inoculation.

Treatment B3 - 12 inoculated cuttings, bases in water, exposed to the air.

Treatment B4 - 12 inoculated cuttings, bases in water, tops in plastic bags sealed beneath point of inoculation.

Controls were maintained with all treatments. Temperatures in the laboratory ranged from 14° to 18°C and relative humidity remained at approximately 65% throughout the experiment.

After eight days, six cuttings from each of treatments A1 and A2 which had been kept dry were placed with their bases in water. Similarly, six cuttings from each of treatments B3 and B4 which had been kept in water were removed to dry tubes. Lesions which appeared were measured daily.

Table 4.5 Effect of different moisture regimes on lesion length in S. fragilis 'C' inoculated with Cytospora chrysosperma S.1. Bark moistures as a percentage of dry weight are given in parentheses.

Initial treatment	Days following inoculation - Lesion length in mms.				
	6	7	8	Subsequent treatment	14
A1 dry exposed (148%)	26.9	36.3	47.0 (75%)	5. wet exposed	109.7 (115%)
				6. dry exposed	87.5 (39%)
A2 dry in bags (148%)	22.2	25.7	30.3 (80%)	7. dry in bags	83.0 (40%)
				8. wet in bags	49.2 (125%)
B3 wet exposed (148%)	1.8	5.8	7.7 (135%)	9. dry exposed	35.8 (75%)
				10. wet exposed	13.7 (132%)
B4 wet in bags (148%)	0.0	0.0	0.0 (148%)	11. wet in bags	-
				12. dry in bags	-
Duncans Test non-significant groups at 5% level	A1 to A2			5 to 7	
	B3 to B4	B3 to B4	B3 to B4	6 to 8	
				8 to 10	

During the eight days of the initial treatments, canker growth was most pronounced on cuttings not in water. They finally died, presumably because dehydration was too far

advanced for fungal growth to be checked. Similarly, death occurred if cuttings were kept dry throughout the entire experiment. Cuttings with their bases in water for the initial eight days showed limited signs of cankering. When removed from water cuttings which had had their bases in water, but without plastic bags, developed severe cankers and ultimately died. Cuttings in water and with plastic bags failed to develop cankers during initial and subsequent treatments. The use of plastic bags did not result in significant differences between treatments kept dry during the initial eight days. No cankers appeared on the controls.

HOST SUSCEPTIBILITY TO *CYTOSPORA CHRYSOSPERMA*

Cuttings from 7 month-old stools were taken from the nursery in March 1971. The cuttings were pruned so that only the basal 30 cm remained, and were saturated by immersion in water for 36 hours. Species and varieties tested were:

S. fragilis 'M' Manawatu clone; *S. discolor* 'B'; *S. daphnoides* 'G'; *S. incana*; *S. matsudana*; *S. purpurea* 'Booth'; *S. vitellina* 'Westhaven'; *S. ulzio* 1; and *S. fragilis* 'C' Canterbury clone.

Inoculation was carried out as in previous experiments using the nylon mesh method. Cuttings were exposed on the laboratory bench so that rapid dehydration could be achieved and consequently maximum expression of the disease obtained. Treatments were replicated eight times. Cuttings were

examined daily and lesion length measured where applicable.

Table 4.6 Lesion length in different species and varieties of *Salix* inoculated with *Cytospora chrysosperma* S.1

	DAY 5		DAY 6		DAY 7	
	Length mm	Duncans Test 5% Level	Length mm	Duncans Test 5% Level	Length mm	Duncans Test 5% Level
S.vitellina 'Westhaven'	38.5	a	64.1	a	79.3	a
S. incana	31.1	ab	50.6	b	61.8	b
S.ulzio 1	25.3	bc	41.4	bc	54.4	bc
S.fragilis 'M'	24.6	bc	41.0	bc	52.4	bc
S.purpurea 'Booth'	15.4	cd	32.6	c	45.1	cd
S.daphnoides 'G'	6.5	de	31.3	c	43.0	cd
S.fragilis 'C'	12.1	def	29.9	c	34.5	d
S.matsudana	2.6	ef	6.5	d	7.5	e
S.discolor 'B'	0.0	f	3.0	d	6.6	e

The results (table 4.6) show *S. vitellina* 'Westhaven' to be significantly more susceptible to *C. chrysosperma* than any of the other species and varieties tested. The least susceptible species were *S. matsudana* and *S. discolor* 'B' which, up to the seventh day after inoculation showed only slight signs of cankering. A significant difference

occurred between the clones S. fragilis 'M' and S. fragilis 'C'. If these two clones originated from common stock this would suggest natural selection within the Canterbury clone of those trees more resistant to attack by Cytospora chrysosperma.

DISCUSSION

Experimental results indicate a negative correlation between bark moisture content and Cytospora canker growth. Under similar conditions of drying and moisture loss, S. vitellina 'Westhaven' was more susceptible to the disease than other species and varieties tested. The most resistant species were S. matsudana and S. discolor 'B'.

It has been shown by Butin (1957) and Gibbs (1957) that moisture content of a number of poplar varieties falls to a minimum during winter. In addition, dehydration of cuttings is likely to occur in storage and during shipment (Bloomberg, 1962). Since cuttings are made during the winter, these factors could lead to a reduction in mark moisture content to the point where infection is likely to occur. Predisposition to infection by C. chrysosperma would be even more marked if cuttings when outplanted failed to rapidly develop a root system to balance transpiration loss.

The results show that established cuttings can also be attacked by the fungus. At high relative humidities with

water readily available to the root system, cankers are rarely severe.

Optimum temperature for the growth of C. chrysosperma in culture is greater than 24°C. Cuttings held at temperatures in excess of 24°C suffer appreciable moisture loss when exposed to the atmosphere. Any effect of temperature on canker growth could therefore be attributed partly to its effect on dessication of the cuttings and partly to its effect on growth of the fungus.

The results suggest that it should be possible to minimise attack by C. chrysosperma by selection of the more resistant willow varieties and adoption of cultural practices aimed at producing healthy cuttings. Maintaining bark moisture at a high level during storage and transit should reduce the incidence of infected cuttings. Dipping the ends of cuttings in a fungicide or sealing compound may prevent entry of the fungus. Planting of cuttings so that no more than 7cm remains above the surface, may help reduce dessication of the bark and lessen predisposition to infection.

CHAPTER 5

MELAMPSORA EPITEA

INTRODUCTION

The rust genus Melampsora is regarded as intermediate in character between the families Melampsoraceae and Pucciniaceae. Most species in the genus are macrocyclic; generally heteroecious when teleutospores occur on woody plants and autoecious when on herbaceous plants, (Wilson & Henderson, 1966).

Seven species of Melampsora are recognised as parasitic on Salix in Britain; M. allii-fragilis Kleb., M. amygdalinae Kleb., M. capraearum Thuem, M. epitea Thuem, M. larici-pentandrae Kleb., M. ribesii-viminalis Kleb., and M. salicis-albae Kleb.,(Wilson & Henderson, 1966). M. epitea is regarded as a collective species comprising various races and race groups (Jorstad, 1940; Wilson & Henderson, 1966). Previously these races were regarded as distinct species and were separated on the basis of their differing aecidial hosts, although they were morphologically indistinguishable. Within M. epitea two varieties are recognised; var. epitea and var. reticulatae (A. Blytt) Jorst. Var. reticulatae differs from all races of var. epitea in its larger uredospores and

paraphyses, and is the only race found infecting Salix reticulata. Races of M. epitea var. epitea are generally identified by their aecidial hosts; Saxifraga, Euonymus, Larix, Ribes and some genera of the Orchidaceae. Thus while morphologically indistinguishable each race has a distinctive host specialisation pattern (Wilson & Henderson, 1966).

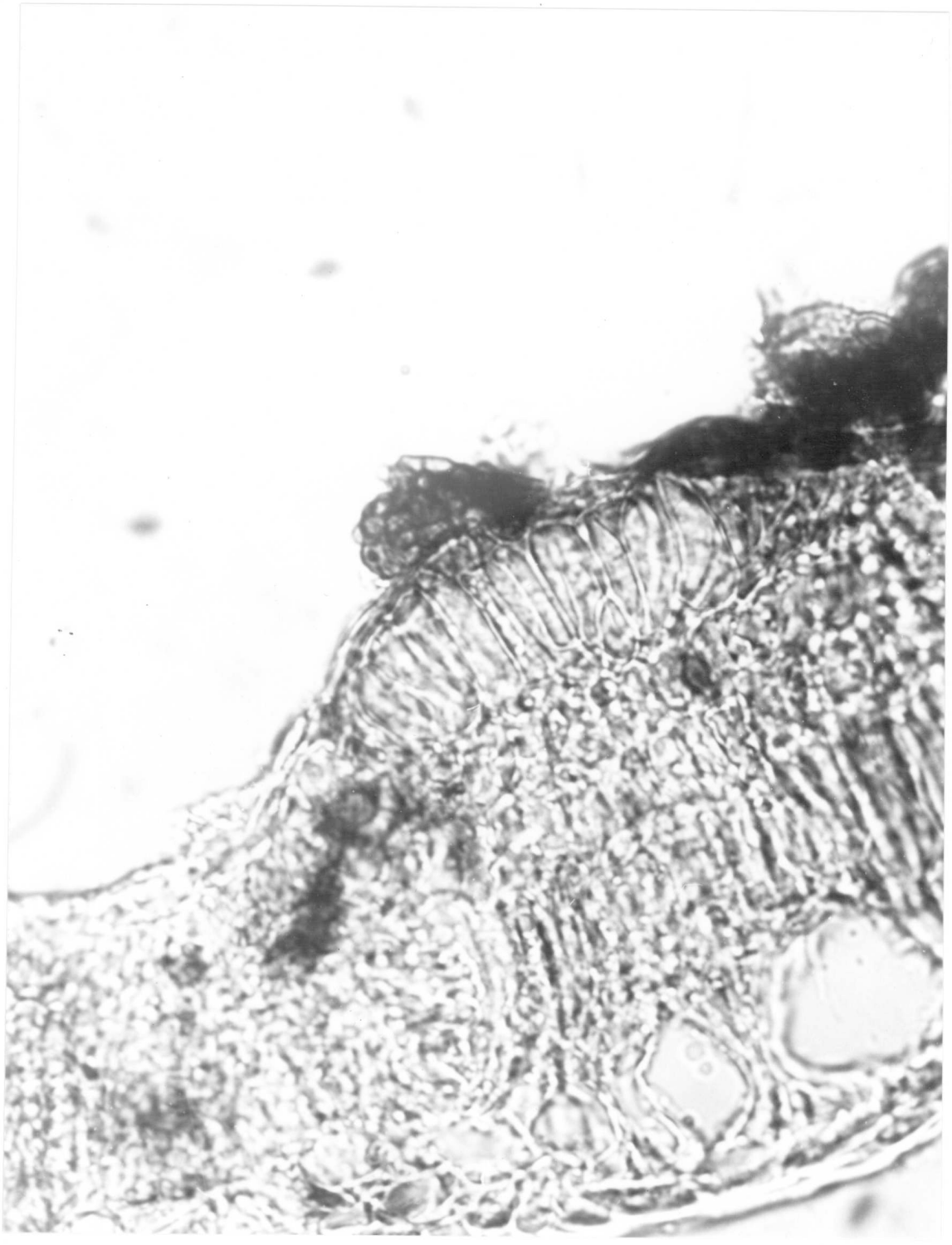
Melampsora epitea var. epitea is recorded overseas on the following species of willow; Salix alba, S. arbuscula L, S. atrocinerea L, S. aurita L, S. calodendron L, S. caprea L, S. cinerea, S. daphnoides, S. fragilis, S. glauca L., S. hastata L, S. herbacea L, S. lapponum L, S. myrsinites L, S. nigricans Sm., S. phylicifolia L, S. polaris L, S. sadleri Sm., S. triandra L, S. viminalis L, S. xerophila Flod., and a number of hybrid species.

M. epitea has not been recorded in New Zealand, although a rust of willows has been recognised for some time. (C.W.S. van Kraayenoord, pers. comm.).

DESCRIPTION OF THE DISEASE

The disease affects only the leaves of willows. It appears in late spring and early summer as scattered yellow uredosori on the undersurface of leaves. Mature leaves become heavily infected as the season progresses, and mature uredosori turn orange. The disease leads to early abscission of affected leaves. Heavily infected trees may be

Figure 5.1 T.S. leaf of *Salix cinerea* showing subepidermal teleutosorus (x 65).



completely defoliated by early January, with no further growth occurring until the following spring. This leads to a reduction in growth but not the death of trees.

Site does not appear to infect severity of infection.

OCCURRENCE

The disease is widespread in New Zealand, particularly in the North Island (C.W.S. van Kraayenoord, pers.comm.). During the survey it was encountered in all areas expect those south of Christchurch. This pattern of distribution may be accounted for by the relative scarcity of susceptible host species in the southern part of the South Island.

MATERIALS AND METHODS

Infected leaves collected during the survey were placed between clean sheets of absorbent paper in a plant press.

Uredosori were removed with a sharp needle and placed in a drop of lactophenol cotton blue (Peacock, 1940) on a slide. Uredosori were forcibly disrupted by pressing the coverslip with a needle, and the slide warmed gently for 30 seconds.

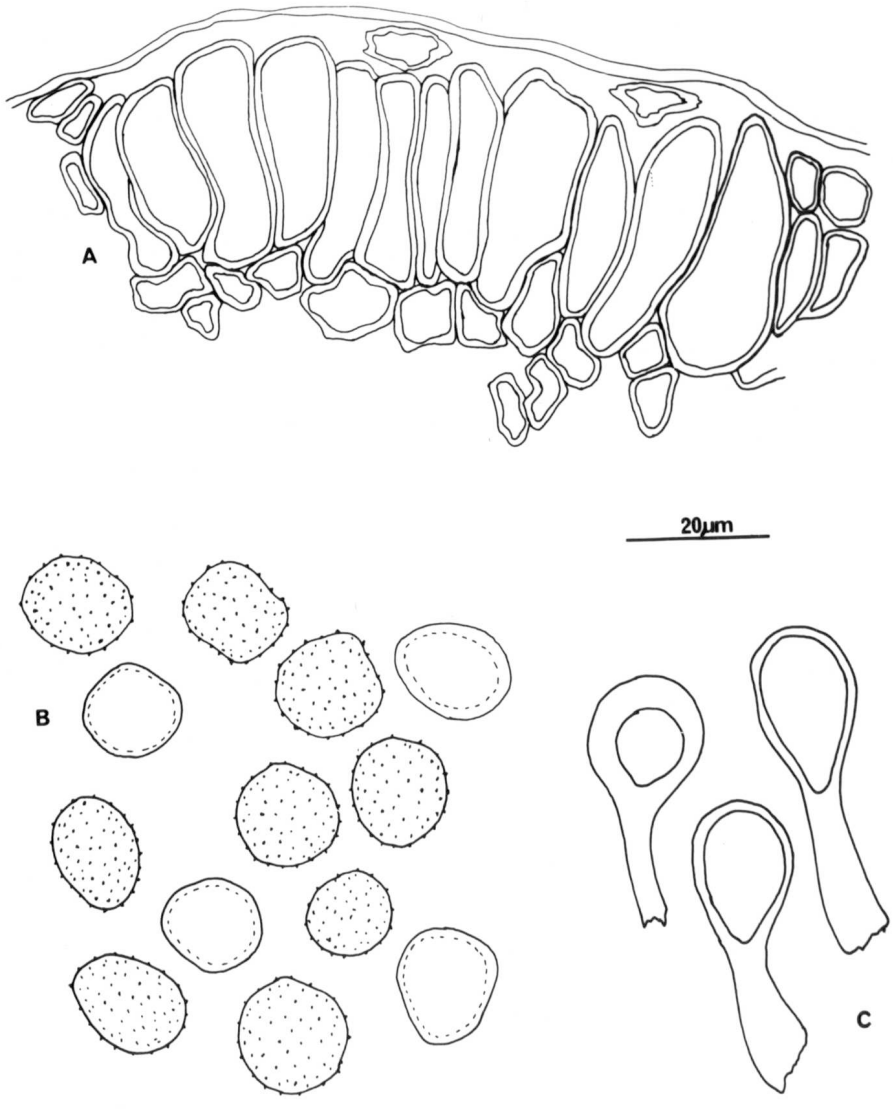
To induce formation of teleutospores, leaves were placed on the surface of moist soil in plastic pots. Pots were sealed in plastic bags to prevent dehydration and placed in a refrigerator ($4^{\circ}C$) for five weeks.

Figure 5.2 Melampsora epitea

A. Subepidermal teleutosorus

B. Uredospores

C. Paraphyses


A
20µm
B
C

DESCRIPTION AND TAXONOMY OF THE PATHOGEN

Melampsora epitea Thuem., (figures 5.1 and 5.2)
Mitth. Forstl. Versuchsw. Oesterr., 2:38, 1879

Spermogonia and Aecidia: not found in New Zealand.

Uredosori: hypophyllous occasionally amphigenous, orange-yellow, surrounded by ruptured epidermis, rounded, oval or slightly elongated, 0.5 - 1.0mm wide with numerous capitate or clavate paraphyses 30-65 x 13-27µm, wall hyaline, 1.5µm thick, stalk 3.5-7µm wide; uredospores ellipsoid or globoid, 10-20µm diam., wall aculeate-verrucose, 1.0-2.2µm thick, with indistinct germ pores.

Teleutosori: subepidermal, amphigenous but mostly hypophyllous, minute, 0.25-0.5mm diam., blackish when mature, teleutospores prismatic, rounded at both ends or slightly tapering towards the unthickened apex, pale brown, 25-50 x 5-15µm, wall 2µm thick.

Host Range in New Zealand

Uredospores on Salix caprea, S. caprea x viminalis, S. cinerea, S. cinerea var. oleifolia Sm., S. discolor, S. hippophaefolia Thuill., S. medemii L, S. nigra L, S. nigricans, S. phylicifolia L, S. pontederana L, S. silesiaca L.

Figure 5.3 *Melampsora epitea*, Uredospore size. See Appendix 3 for designation of collections.

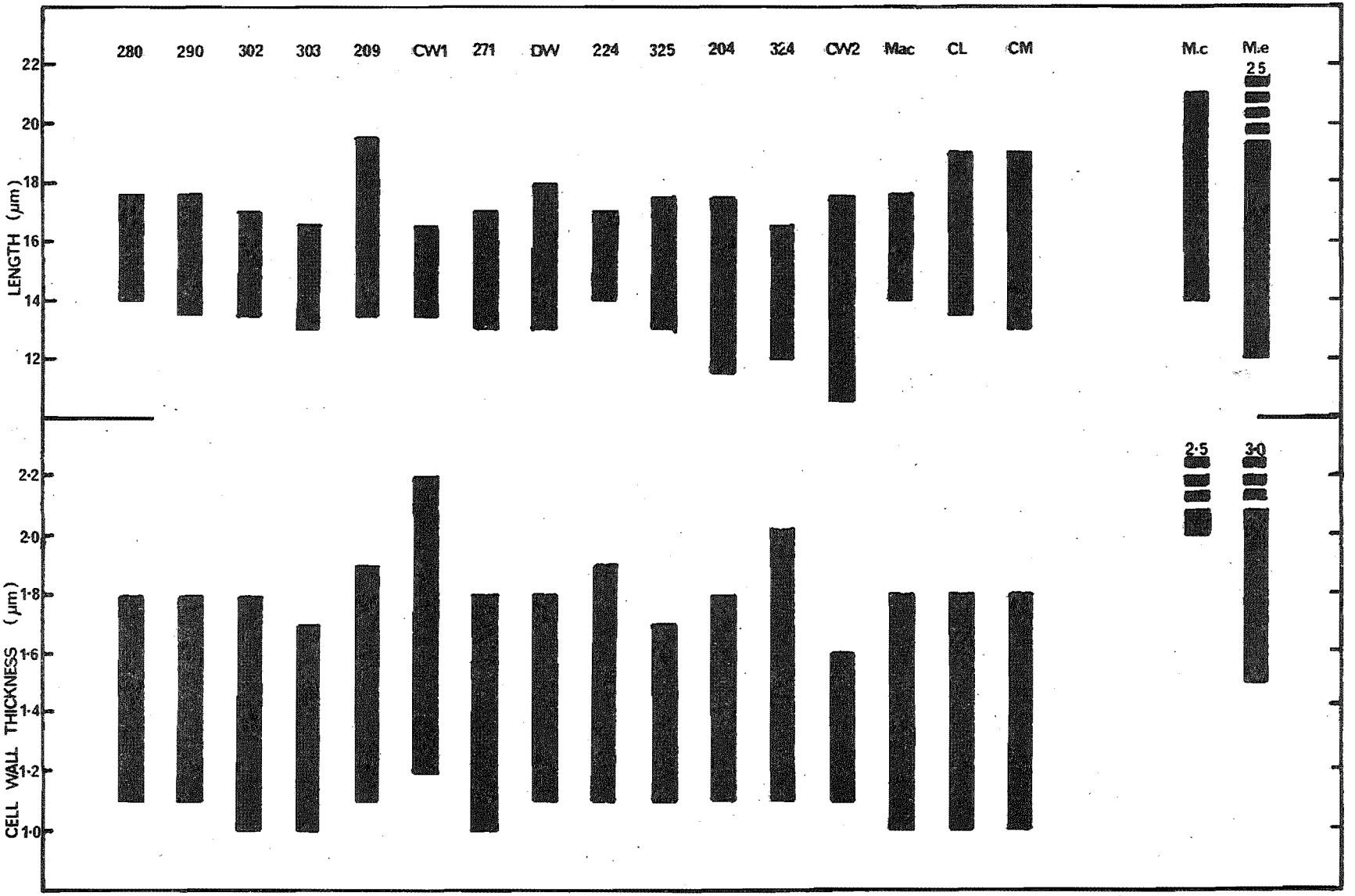
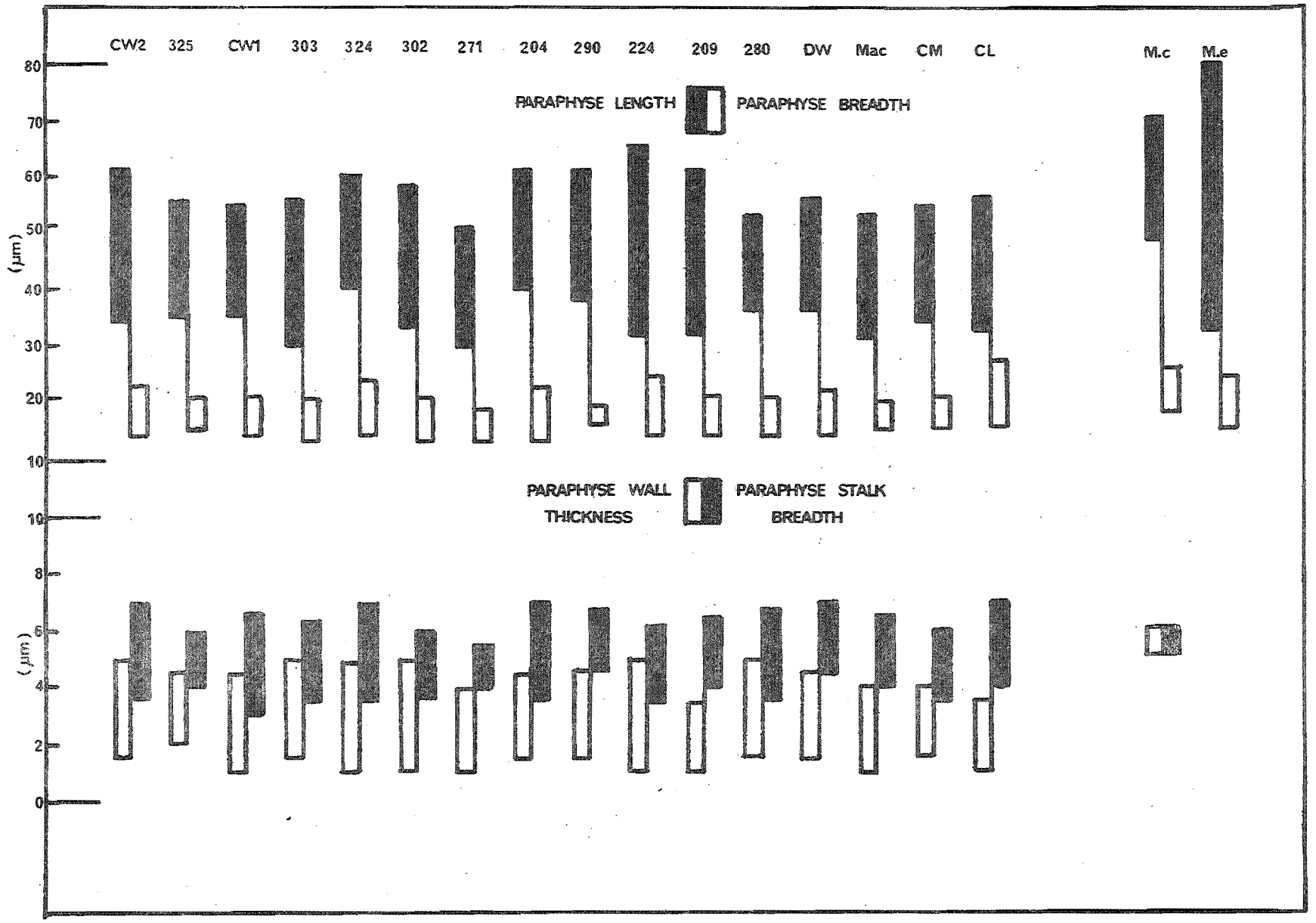

280
290
302
303
209
CW1
271
DW
224
325
204
324
CW2
Mac
CL
CM
M.c
M.e
LENGTH (μm)
22
20
18
16
14
12
2·5
CELL WALL THICKNESS (μm)
2·2
2·0
1·8
1·6
1·4
1·2
1·0
2·5
3·0

Figure 5.4 *Melampsora epitea*, Paraphysis size. See Appendix 3 for designation of collections.


CW2
325
CW1
303
324
302
271
204
290
224
209
280
DW
Mac
CM
CL
M.c
M.e
PARAPHYSE LENGTH
PARAPHYSE BREADTH
PARAPHYSE WALL THICKNESS
PARAPHYSE STALK BREADTH
(µm)
80
70
60
50
40
30
20
10
10
8
6
4
2
0
(µm)

Teleutospores on S. cinerea

The collections examined during this study agree closely with the description of Melampsora epitea published by Jorstad (1940, 1951 and 1953) and Wilson and Henderson (1966).

Host range of the uredo stage suggests that the rust is either M. capraearum or M. epitea var. epitea. Uredospore and paraphysis measurements of New Zealand collections (figures 5.3 and 5.4) are within the range given for M. epitea by Wilson and Henderson (1966), but are smaller than those given for M. capraearum. In addition, Wilson and Henderson (1966) describe teleutospores of M. capraearum as having walls up to 10µm thick at the apex, whereas teleutospores examined during the present study had walls of uniform thickness with no apical thickening, a characteristic of M. epitea (Wilson & Henderson, 1966).

Teleutospore formation was induced in only a single collection of S. cinerea from the Lincoln area. Jorstad (1940) found the teleuto stage of M. epitea was rarely formed in nature and considered that the fungus was able to over-winter in the uredo stage as perennial mycelium in buds of Salix spp.

The aecidial stage of M. epitea has not been found in New Zealand and the rust may be independent of alternate hosts in this country.

Figure 5.5 Host susceptibility to *Melampsora epitea*.

S.caprea 'Lane Poole'

S.caprea 'F'

S.caprea x viminalis 'Balana'

S.caprea x viminalis 'SM'

S.caprea x viminalis 'Hybrida'

S.cinerea 'M'

S.cinerea 'W'

S.cinerea tricolor 'Mac'

S.cinerea oleifolia 'NCCB'

S.discolor 'B'

S.discolor 'WCB'

S.discolor 'MCB'

S.aurita phillicifolia

S.hippophaefolia

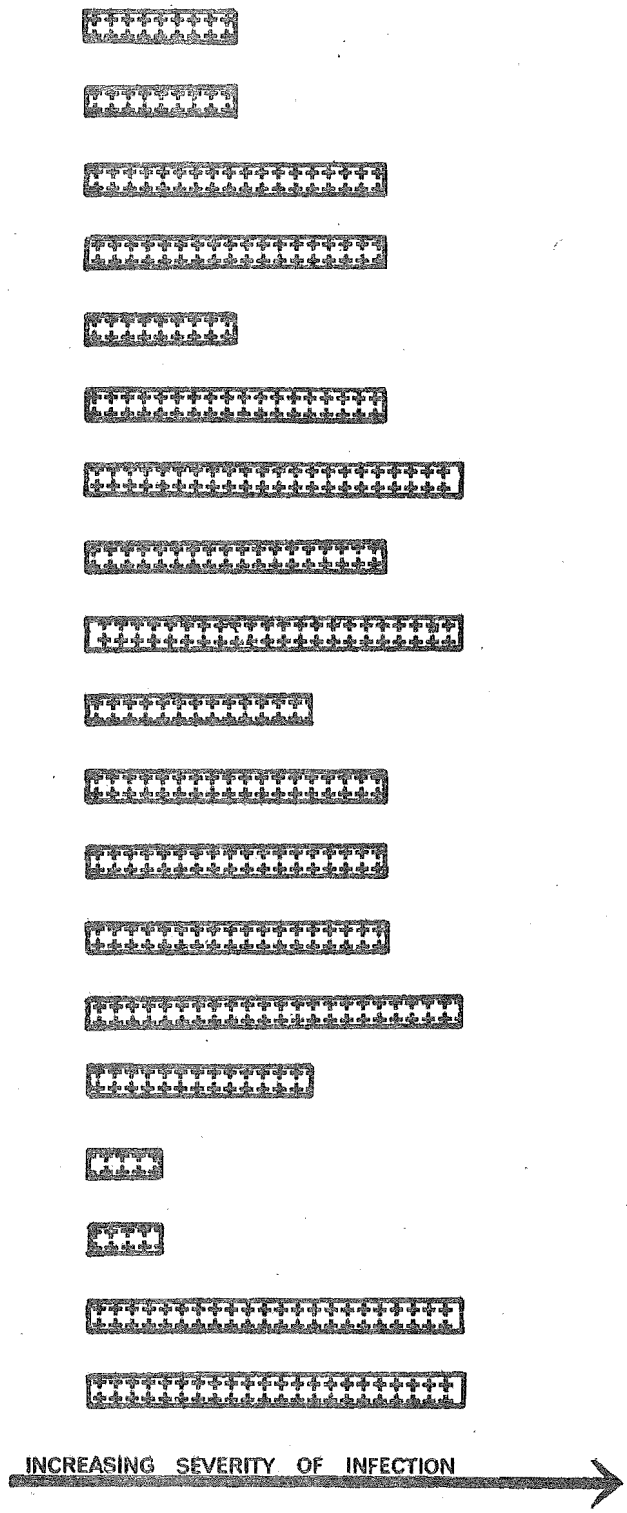
S.nigricans

S.medemii

S.pontederana

S.sileslaca

S.nigra 'SCCB'



The full citation and abbreviated synonymy of M. epitea as given by Jorstad (1953) is:

Melampsora epitea Thuem.

Mitt. Forstl. Versuchsw. Oesterr. 2:38, 40. 1879.

Synonyms:

M. abieti-capraearum Tub. 1905

M. alpina Jule, 1894.

M. arctica Rostr. 1888.

M. larici-epitea Kleb., 1899.

M. repentis Plowr., 1891.

M. ribesii-epitea Kleb., 1913.

M. ribesii-purpureae Kleb., in Pringsh. 1901.

HOST SUSCEPTIBILITY

A survey of the severity of Melampsora epitea on different species and varieties of willows was conducted in late January 1971 at the Plant Materials Centre Nursery, Palmerston North. Visual observation of the extent of disease on naturally infected willow stools was based on a 0-5 scale. Uninfected stools were scored 0, and heavily infected stools 5, the latter being completely defoliated.

The results are given as a histogram (figure 5.5) showing severity of infection. Host range suggests that M. epitea infects only osier type willows; tree willows are apparently resistant. Salix cinerea and its varieties,

S. hippophaefolia, S. silesiaca and S. nigra were the most severely infected species.

DISCUSSION

A rust infecting the leaves of Salix spp. in New Zealand is described. Morphological studies and host range show the fungus to be Melampsora epitea var. epitea as described by Jorstad (1940, 1951 & 1953) and Wilson and Henderson (1966).

A survey of host susceptibility indicates that M. epitea is pathogenic only on osier types of willow. Where rapid establishment of willows is required, the use of resistant species and varieties is suggested.

CHAPTER 6

ERWINIA SALICIS

INTRODUCTION

Day (1924) isolated a bacterium from Salix coerulea Sm. the cricket-bat willow, in Britain which caused dieback and staining of the wood. He called the disease the watermark disease and gave a brief description of the causal organism which he named Bacterium salicis Day. The bacterium was also isolated from Salix alba and he noted that S. fragilis was similarly affected.

A fuller description of Bacterium salicis was published by Dowson (1937) and the species was later transferred to Erwinia by Chester (1939).

Lindeijer (1932) investigating a similar disease of varieties of S. alba in Holland, isolated the causal organism and named it Pseudomonas saliciperda Lindeijer.

Metcalfe (1940) in Britain isolated Erwinia salicis from Salix coerulea, S. alba, S. russeliana L and S. cinerea L. He also isolated three other bacteria from old lesions, but failed to establish their pathogenicity.

Following a reinvestigation of the disease in Holland,

Gremmen and de Kam (1970) regarded Pseudomonas saliciperda as a synonym of E. salicis. The two species differed mainly in number and attachment of flagella, but Gremmen and de Kam considered that such differences were the result of the different staining techniques employed.

Day (1924), Dowson (1937) and Lindeijer (1932) all proved the pathogenicity of their isolates by successful inoculation of willows. Both Day and Dowson inoculated plants by puncturing shoots artificially, but Lindeijer showed that the organism could be transmitted on the legs and body of the weevil Cryptorrhynchus lapathi L. However, Callan (1939) in Britain, was unable to demonstrate transmission of E. salicis by Cryptorrhynchus lapathi.

A disease of willows now recognised as being caused by E. salicis was first observed in the South Canterbury area of New Zealand in 1961 (A. Evans, pers.comm.). The causal organism was not isolated and while the disease was not described, it became known locally as "black spot of willow". E. salicis has not previously been recorded in New Zealand.

DESCRIPTION OF THE DISEASE

The symptoms and pathological anatomy of the disease in Salix coerulea in Britain, have been adequately described by Dowson (1937) and Metcalfe (1940 and 1941). Salix coerulea is a relatively uncommon species in New Zealand and the disease was found only on S. fragilis during the survey.

Figure 6.1 Characteristic "black spot" on willow naturally infected with *Erwinia salicis*.

Figure 6.2 Bark cut away from above "black spot" to show underlying stained wood.

Erwinia salicis invades wood of branches more than 2-3 years old. The first sign of the disease is the exudation of a thin, nearly colourless, sticky fluid. This exudate may appear through cracks, insect wounds or wounds due to other causes on affected branches or trunks. Ultimately, the exudate stains the exterior of the bark a dark brown colour producing a characteristic "black spot".

In severely affected branches sudden wilting of foliage with the margins of wilted leaves rapidly turning brown is a characteristic cymptom. This is often followed by dieback of the entire branch.

When bark is peeled back from an infected area, a dark brown staining of the underlying cambial tissue is evident extending considerably further than is indicated by external symptoms. The bacterium appears to spread with ease up and down the branch, but lateral spread is slow. Lesion growth and confluence of adjacent lesions leads to girdling of branches, resulting in dieback and stagheading of infected trees. The tree may lay down healthy tissue over infected areas although the symptoms may reappear at a later date.

The means of infection and spread of the disease from tree to tree are unknown in New Zealand, although it is very probable that insects are involved. Carriage by birds or opossums is also possible.

Infected branches are frequently invaded by Cytospora chrysosperma which hastens death.

OCCURRENCE

The disease is widespread in the South Canterbury and Otago areas of New Zealand, but has not been observed north of the Waimakariri R., North Canterbury. The incidence of disease appears greater on wet sites although it may also infect trees growing in dry situations.

TAXONOMY OF THE ORGANISM

The morphology, physiology and biochemistry of two isolates of Erwinia salicis, T.3 and C.2 were tested. Spectrometric studies included a further isolate 3.A.

EXPERIMENTAL METHODS

Media and methods used for examining cultural, physiological and biochemical characters of isolates were largely the same as those employed by Dye (1962). Incubation was at 24°C unless otherwise stated. Isolates were maintained on glucose yeast carbonate agar, (GYCA, glucose, 5g; yeast extract (Difco) 5g; calcium carbonate precip., 40g; agar (Davis, N.Z.), 15g; water 1l) at 40°C. All media were steam sterilised at 121°C for 15 minutes unless otherwise stated.

Prior to tests fresh sub-cultures were made on GYCA.

After 1-4 days incubation, cells from these cultures were dispersed in sterile water in 50ml bottles to give a turbid suspension. A 2mm loop was used to inoculate both solid and liquid media.

Each experiment was replicated four times.

Microscopical Characters

1. Morphology, Gram Reaction and Capsule Staining. Air dried films were prepared from colonies grown on GYCA slopes for 24 hours. Hucker's (1957), modification of the Gram stain was used. Capsule staining was by the method of Harrigan and McCance (1966); films were subjected to Leifson's flagella stain with methylene blue as a counterstain to show capsules.

2. Motility and Flagella Staining

One loop of each isolate was placed in 5ml of sterile water and incubated at 24°C for 1 hour. Motility was determined by the hanging drop slide technique. Flagella were stained using the method of Rhodes (1958).

Cultural Characters

Four media were used for observation of cultural characters: PDA, nutrient agar, GYCA, and potato wedges. Cultures were incubated for 48 hours. Fluorescence was determined by placing cultures under a U.V. light source, wave-length 265μm.

Physiological and Biochemical Characters

1. Oxygen Requirements

The ability of isolates to grow anaerobically was tested by culturing in an anaerobic jar.

2. Carbohydrate Utilisation

The ability of isolates to utilise carbohydrates was studied using the purple broth base method (Difco), (beef extract (Difco), 1g; protcose peptone (Difco), 10g; bromcresol purple, 0.015g; distilled water 1l). Carbohydrate solutions were sterilised with ethylene oxide (Judge & Pelczar, 1955), and added aseptically to the purple broth base to give a final carbohydrate concentration of 0.5%. Media were incubated for 3 days prior to inoculation to test for sterility. Cultures were examined for growth and acid or gas production after 3, 7, 14 and 21 days. The carbon sources tested were adonitol, arabinose, dextrose, dulcitol, fructose, erythritol, galactose, glucose, glycerol, inulin, maltose, mannose, mannitol, raffinose, rhamnose, salicin and sucrose.

3. Hydrolysis of Starch

Starch hydrolysis was tested by the layer plate method described by Harrigan and McCance (1966). Plates were prepared by pouring 10ml of nutrient agar into each plate, allowing it to set and then overlaying this with 5ml of starch agar, (soluble starch, 0.2g; nutrient agar 100ml). Duplicate plates were spot inoculated and after 4 days two colonies of each culture were wiped from the agar surface

which was then flooded with dilute iodine solution.

4. Nitrate Reduction

Nitrate reduction was tested by the method of Harrigan and McCance (1966), the broth used consisting of: beef extract (Difco), 3g; peptone (Difco), 5g; distilled water 1l; KNO_3, 1g. Cultures were incubated and tested for nitrite with sulphanilic acid and dimethyl alpha-naphthylamine, (Wilson & Miles, 1964) after 7 and 14 days. Nitrate persistence was determined by the use of diphenylamine (Wilson & Miles, 1964) and the presence of gas in Durham tubes revealed production of nitrogen. Production of ammonia was assumed if the above tests were negative.

5. Indole Production

To test for production of indole, the isolates were inoculated into duplicate tubes of peptone water, (tryptone (Difco), 10g; NaCl, 5g; distilled water 1l.) After incubation for 5 days cultures were tested with Kovac's indole reagent (Harrigan & McCance, 1966).

6. Catalase Production

Catalase production was examined by removing a loopful of a colony incubated for 24 hours on GYCA. This was emulsified with a drop of 10 vol. hydrogen peroxide on a slide and examined for the formation of gas bubbles.

7. Oxidase Production

To test for oxidase production the filter paper method described by Harrigan and McCance (1966) was used. To a

piece of Whatman No. 1 filter paper was added a few drops of tetramethyl-p-phenylenediamine hydrochloride reagent. Some bacterial growth was smeared onto the impregnated filter paper and the formation of a purple colouration within 5-10 sec. regarded as a positive reaction.

8. Acetoin Production

Production of acetoin was measured by culturing in yeast extract-salts (YS) broth, ($NH_4H_2PO_4$, 0.5g; K_2HPO_4, 0.5g; distilled water 1l; glucose, 5g. The presence of acetylmethylcarbinol (acetoin) was tested for after 2 and 5 days with alpha-naphthol in absolute alcohol and potassium hydroxide as described by Dye (1962). A rose colouration indicated a positive result.

9. Hydrolysis of Aesculin

Aesculin hydrolysis was tested for, using YS broth with: aesculin 1g; ferric ammonium citrate, 0.5g/l (Dye, 1962). Results were recorded in daylight and under U.V. light (265μm) after 3 and 7 days. Absence of fluorescence indicated a positive result.

10. Pigmentation

The three isolates, T.3, 3.A and C.2 were grown on GYCA for 5 days. Cells were then scraped off with a glass slide and the pigment extracted by the method of Starr and Stephens (1964) as outlined below:

(i) Add 50ml absolute methanol per gram of moist cells.

(ii) Warm methanol to extract pigment. Allow the liquid to cool and centrifuge.

(iii) To the supernatent add KOH-methanol to a final concentration of 3% KOH. Heat gently, (40°C for 5 minutes).

(iv) Add 2 vols diethyl ether, then sufficient water to separate the two layers. Shake gently in a separatory funnel.

(v) Drain off hypophase and if still coloured partition frequently against fresh diethyl ether.

(vi) Dehydrate hypophase with Na_2SO_4 and evaporate to dryness in vacuo with a Bucci evaporator.

(vii) Purification by adsorption of pigment onto columns of magnesia-Celite, 1 to 1 by weight.

(viii) Elute column with absolute methanol and carry out spectrometric analysis, (Beckman recording spectrophotometer, model DB).

RESULTS

Microscopical Characters

The organisms from both isolates, (T.3 and C.2) were Gram-negative, anaerobic, non-sporing, capsulate rods, 0.5 - 0.8 x 0.8 - 1.8µm, occurring singly. Both isolates were motile with up to 7 peritrichous flagella.

Physiological and Biochemical Characters

No fermentative metabolism was shown by either isolate. However, several of the carbohydrates and related carbon sources were oxidised within 7 days (table 6.1). Neither isolate showed evidence of starch hydrolysis, production of indole or production of oxidase. Positive results were recorded for hydrolysis of aesculin, acetoin production and catalase production. Both isolates showed reduction of nitrate to nitrite, with no persistence of nitrate or production of ammonia (table 6.2).

Pigmentation of Cultural Characters

When grown on PDA, nutrient agar and GYCA, isolates produced circular colonies with slightly uneven margins. A yellow water soluble pigment was produced by both isolates; production was dependant on the medium.

Table 6.1 Production of acid from carbohydrates and related carbon sources by isolates T.3 and C.2. Relevant data for *Erwinia salicis* from two sources is included for comparison.

Table 6.1

Organism	T.3	C.2	*E. salicis* Bradbury (1967)	*E. salicis* Dye (1968)
Medium	Purple broth base		*	**
arabinose	+	+	-	-
rhamnose	+	+	-	-
glucose	+	+	+	.
sucrose	+	+	+	.
maltose	+	+	+	.
mannose	+	+	+	+
glycerol	+	+	+	+
galactose	+	+	+	.
mannitol	+	+	+	+
calicin	+	+	+	+
fructose	+	+	-	.
inulin	-	-	-	.
dextrin	-	-	-	.
erythritol	-	-	-	.
adonitol	-	-	-	.
dulcitol	-	-	-	.
raffinose	-	-	+	+

* Medium not given

** Medium C of Dye (1968)

\+ = 75-100% replicates positive

\- = 0- 25% replicates positive

. = not tested

Table 6.2 Some biochemical characters of isolates T.3 and C.2. Relevant data for *Erwinia salicis* from two sources is included for comparison.

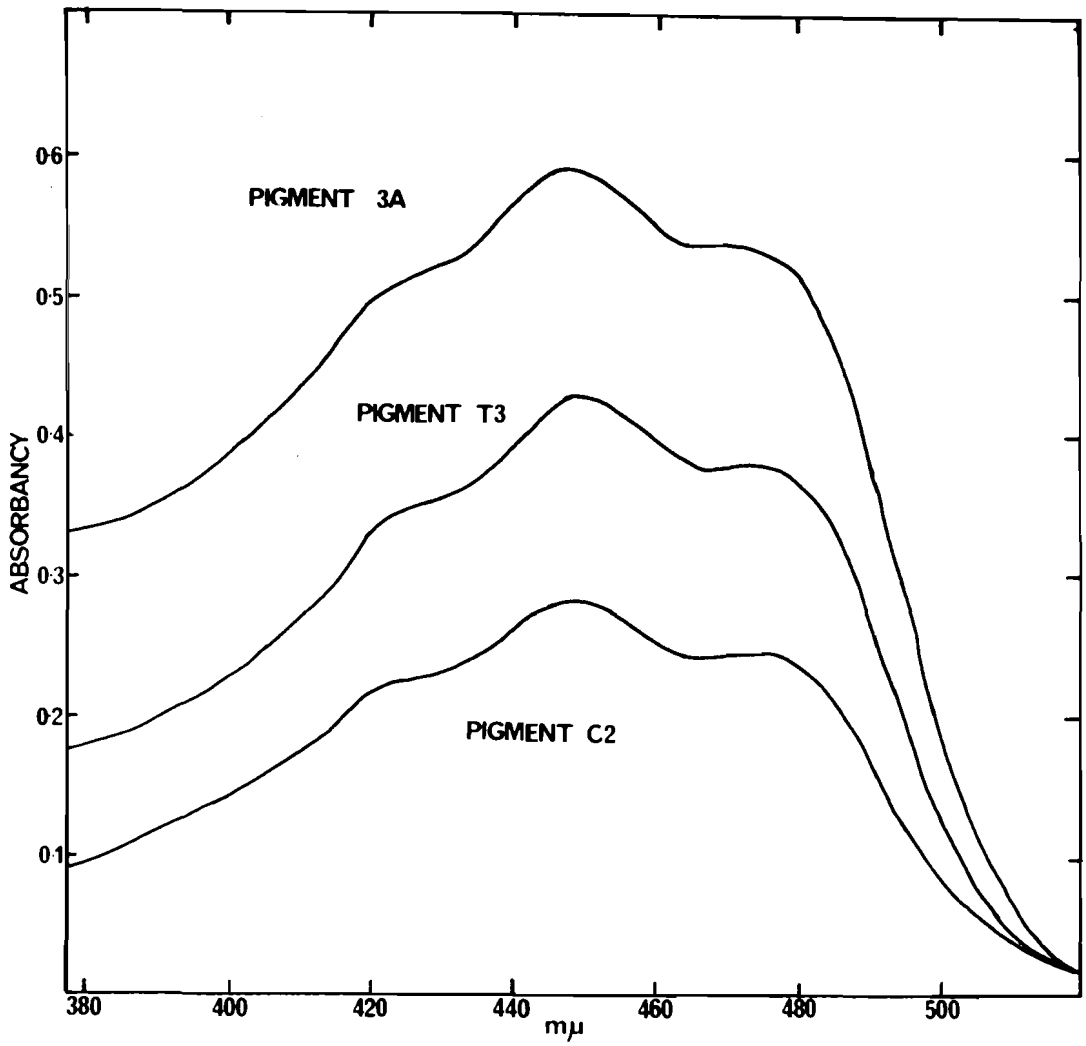
Organism	T.3	C.2	*E. salicis* Bradbury (1967)	*E. salicis* Dye (1968)
Nitrate reduction	+	+	+	-
Production of:				
ammonia	-	-	-	-
acetoin	+	+	.	+
indole	-	-	-	-
catalase	+	+	.	-
oxidase	-	-	.	-
Hydrolysis of:				
starch	-	-	-	-
aesculin	+	+	.	+
Fluorescence	-	-	-	-

\+ = 75-100% replicates positive

\- = 0-25% replicates positive

. = information not given

Figure 6.3 Comparison of spectrometric analysis of pigment of three isolates of *Erwinia salicis*.


PIGMENT 3A
PIGMENT T3
PIGMENT C2
ABSORBANCY
0·6
0·5
0·4
0·3
0·2
0·1
380
400
420
440
460
480
500
mμ

Nutrient agar	-	pale yellow pigment
PDA	-	pale yellow pigment
GYCA	-	bright yellow pigment

On sterile potato wedges a bright yellow pigment was produced which diffused over the surface. Neither isolate produced a fluorescent pigment on any of the media.

Spectrometric analysis of the yellow pigment in absolute methanol showed three characteristic peaks at 425, 450 and 476μm common to all three isolates (figure 6.3).

DISCUSSION

Isolates examined during the study agree closely with Erwinia salicis as described by Dowson (1937), Burkholder (1957), Bradbury (1967), Dye (1968) and Gremmen and de Kam (1970).

Dye (1968, 1969a, b & c) in his taxonomic study of the genus Erwinia, accepted the proposal of Martinec and Kocur (1963) that E. salicis be regarded as a variety of Erwinia amylovora (Burrill) Winslow et al., and listed the organism as E. amylovora var. salicis (Day) Martinec and Kocur.

Subsequent workers (Jansen, 1969) and Gremmen and de Kam, 1970) have not accepted this relegation of E. salicis to varietal rank.

The full citation and abbreviated synonymy of *Erwinia salicis* is:

Erwinia salicis (Day) Chester,
in Bergey et al. Manual of Det. Bact.
5th ed. 406. 1939.

Synonyms:

Bacterium salicis Day, 1924.

Pseudomonas saliciperda Lindeijer, 1932.

Phytomonas salicis (Day) Magrou, 1937.

Pseudobacterium salicis (Day) Krasil'nikov, 1949.

Erwinia amylovora var. *salicis* (Day) Martinec & Kocur, 1963.

PATHOGENICITY

METHODS

Isolation

Two methods of isolation from naturally infected material were used. Exudate from an active lesion was removed with a flamed loop and mixed with sterile water in a 20ml bottle, before streaking onto GYCA plates. Bark was removed from a lesion and pieces of stained cambial tissue from the lesion margin cut with a flamed scalpel. Thin sections of this tissue were placed in sterile water in 20ml bottles and macerated aseptically. The resulting suspension

was streaked on GYCA plates.

Cultures for Inoculation

Cultures used for inoculation were grown on GYCA plates for 4 days at 24°C. One loopful of cells from each culture to be tested for pathogenicity was placed in sterile water in 20ml bottles, and incubated for 1 hour at 24°C.

Method of Inoculation

Sites on the host chosen for inoculation were surface sterilised by swabbing with cotton-wool soaked in absolute alcohol. When these had dried, sterile 5cc hypodermic syringes were used to inject the bacterial suspensions into cambial tissue of the host. The point of inoculation was covered with a cotton-wool swab soaked in sterile water and this was covered with a layer of clear plastic film, ("Glad-Wrap").

INOCULATION

Healthy poles of S. fragilis were obtained in April 1971 from the Selwyn R. where the disease had not been observed. These were standardised at approximately 1.0m in length and 7cm in diameter and grown in a nutrient solution ($NaNO_3$, 0.5g; KCl, 0.1g; $MgSO_4.7H_2O$, 0.1g; K_2KPO_4, 0.3g; $FeSO_4.7H_2O$, trace; water 10 litres) in the glasshouse, temperature, 22°C, R.H. 95-100%). Three of these were

inoculated in three places in April 1971, each with a different isolate; T.3, C.2 and an unidentified saprophytic bacterium. At the end of 6 weeks recognisable lesions with an exudation of fluid from the point of inoculation were evident on poles inoculated with isolates T.3 and C.2. No lesions appeared on poles inoculated with the unidentified saprophyte or on the controls.

Twelve weeks after inoculation a sudden wilting of the foliage of poles inoculated with isolates T.3 and C.2 was observed. The margins of the wilted leaves turned brown and the leaves ultimately withered, but remained attached for approximately three weeks.

When the first bark symptoms appeared, the bark was excised from inoculation wounds and characteristic staining of the underlying cambial tissue was evident. The causal organism was re-isolated from cambial tissue by the method described earlier in this chapter. The organism isolated from the pole inoculated with T.3 and designated 3.A and the organism from the pole inoculated with C.2, 2.A. Isolates 3.A and 2.A both produced a characteristic yellow pigment on autoclaved potato wedges.

Having proved the pathogenicity of isolates T.3 and C.2, a further experiment was conducted in which ten healthy poles of S. fragilis were inoculated with isolates T.3, C.2, 3.A and 2.A. Each pole was inoculated with a single isolate in three places, and controls were inoculated with sterile water in a

similar manner. Recognisable lesions were evident after six weeks on inoculated poles. Results were recorded as positive or negative and are shown in table 6.3.

Table 6.3 Appearance of lesions on healthy S. fragilis poles inoculated with isolates of Erwinia salicis.

Isolate	No. of Inoculations	No. of Lesions
T.3	6	6
C.2	6	4
3.A	6	2
2.A	6	2
Control	6	0

Discussion of Inoculation Experiments

Signs and symptoms of disease on poles inoculated with isolates of Erwinia salicis are comparable with those seen in the field. Bark exudate appeared after 6 weeks, although foliar symptoms were not apparent for at least 12-14 weeks.

Reasons for the failure of some inoculations are not clear, although frequent sub-culturing of isolates may have resulted in a reduction in virulence of the organism.

DISCUSSION

A bacterial disease of *Salix fragilis* in New Zealand is described. Cultural, morphological, physiological and biochemical characters were studied to determine the identity of the causal bacterium. Suitable isolation and inoculation techniques were developed and pathogenicity of the organism proved.

The results of the study show the organism to be *Erwinia salicis*.

CHAPTER 7

CONCLUSIONS AND SUMMARY

1. A survey of stem and leaf pathogens of willows was carried out. Seven pathogens were recognised, of which three were investigated further.

2. *Cytospora chrysosperma*, a fungal pathogen of the wood of willows is described. *C. chrysosperma* was found to be widespread throughout areas of the South Island visited during this study, but relatively uncommon in the North Island.

3. Pathogenicity of *C. chrysosperma* was demonstrated using a nylon mesh square method of inoculation. Host susceptibility of nine different species and varieties of willows to *C. chrysosperma* was studied. The most resistant species were *Salix matsudana* and *S. discolor* B.

4. Bark moisture of *Salix* cuttings inoculated with *C. chrysosperma* was shown to be negatively correlated with severity of the disease.

5. *Melampsora epitea*, a fungal pathogen of leaves of osier types of willow is described. *M. epitea* was particularly prevalent in the Manawatu and Hawke's Bay

region, where osier types of willow are common, but was uncommon in the South Island.

A survey of the species and varieties of Salix infected by M. epitea and the severity of the disease was carried out at the Plant Materials Centre, Palmerston North.

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APPENDIX 1

Establishment of Nursery of Different Species and Varieties of Willows.

One hundred cuttings of each of the following species of willow were forwarded from the National Plants Materials Centre, Palmerston North: *Salix discolor* B, *S. daphnoides* G, *S. incana*, *S. matsudana*, *S. purpurea* Booth, *S. vitellina* Westhaven, *S. ulzio* 1. A further 100 cuttings of each of the Manawatu and Canterbury clones of *S. fragilis* were obtained.

Cuttings were rooted in sawdust and planted out on the research farm, Lincoln College, in Early September 1970. Planting was through rows of black polythene at 18" centres, and cuttings were trickle irrigated through the summer.

APPENDIX 2

Method of Preparation of Willow Decoction Agar.

Branches of not less than 1cm diam. were cut into short lengths. Two hundred grams of this material was boiled in distilled water for 2 hours. The decoction was decanted and 5g glucose (analar) and 15g Agar (Davis, N.Z.) added. Distilled water was added to 1 litre and the decoction agar autoclaved at 121°C for 15 minutes.

Final pH of willow decoction agars ranged from 5.2 - 5.3.

APPENDIX 3

Identity of *Salix spp.* infected with *Melampsora epitea*. All specimens obtained from Plant Material Centre Nursery, Palmerston North, unless otherwise stated.

204	*S. aurita* var. *philicifolia*	
209	*S. cinerea* 'M'	
224	*S. hippophaefolia*	
271	*S. silesiaca*	
280	*S. discolor* 'WCB'	
290	*S. cinerea* 'W'	
302	*S. caprea* x *viminalis* 'Balana'	
303	*S. caprea* x *viminalis* 'SM'	
324	*S. cinerea* var. *tricolor* 'Mac'	
325	*S. nigra* 'SCCB'	
CW1	*S. cinerea*	near Wanstead
CW2	*S. cinerea*	near Wanstead
DW	*S. discolor*	near Wanstead
CM	*S. cinerea*	near Foxton
CL	*S. cinerea*	Lincoln
Mac	*S. cinerea* var. *tricolor*	near Wanstead