CHARACTERIZATION, DISTRIBUTION AND PATHOGENICITY OF BACTERIA ISOLATED FROM CHLOROTIC WHITE SPRUCE (*Picea glauca* (Moench) Voss)

by

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ABSTRACT

Several genera of bacteria were isolated from the foliage, twigs, bark and wood of stems of chlorotic 10 - 12 years old white spruce (Picea glauca (Moench) Voss).

Most of the isolates were nonspore-forming, aerobic or facultative anaerobic rods. Ninety-four percent of all isolates were gram-negative and six percent gram-variable. From 16 isolates, five belonged to the genus Enterobacter (Erwinia), four were Serratia spp., three Pseudomonas spp. and two isolates resembled the genus Pasteurella. Two isolates could not be classified.

The bacteria were found throughout the entire trees, but qualitative and quantitative differences existed between sections of the trees. The number of bacteria varied from 2.3×10^3 to 6.0×10^5 / g.d.w., higher concentrations being present in the fine roots and brown spots of bark.

Inoculations with the selected bacterial isolates, alone or in combination, induced pathological responses on young germinants and rooted cuttings of white and black spruce. Stunting, discoloration, localized swellings, tip necroses of hypocotyl, reduced root growth and needle deformation were induced by dip inoculation of seed and hypocotyl. When the inocula were applied to green cuttings the rooting of cuttings and the number and length of developing roots were significantly reduced.

Re-isolations yielded the original bacterial types used for the inoculations.

The observed pathogenic effects were consistently associated with the Enterobacter isolates and one unknown isolate (U_1). The pathogenicity tests showed that many of the bacteria inhabiting white spruce have the capacity to affect the growth and development of young roots of black and white spruce and thus plausibly to contribute to the chlorosis syndrome of white spruce.

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1. INTRODUCTION

Two white spruce (Picea glauca (Moench) Voss) plantations established in 1967 at Limestone Lake, about 15 miles north of Nipigon, Ontario, exhibited symptoms of an unknown disorder. The most conspicuous symptoms of the disorder were 1) general chlorosis with sparse tree mortality and 2) bark necrosis (brown bark-spotting). Bark necrosis was often accompanied by resinosis. A reduction in the number of feeder roots and poor mycorrhizal development were also observed. Similar appearance of the aforementioned disorder was also observed in black spruce plantations of the same locality (G. Marek, personal communication).

Several factors associated with chlorosis have been sought to explain the disorder. Armillaria mellea (Vahl ex. Fr.) Kummer root rot found on 65% of the most severely chlorotic trees was not considered the causal agent of the disorder (Whitney 1973, unpublished data). Whitney (1977, 1979, unpublished data) considers the drought conditions of 1975 and 1976 and the poor feeder root development to be most important, since drought together with competition by other vegetation for available moisture could cause severe moisture stress on the trees. Navratil (1977, unpublished data) postulated an etiological pattern involving the infection of the root or vascular systems by systemic pathogens (bacteria, virus), leading to poor root development and impaired ability for water absorbtion.

A severe imbalance in the nutrient status of the chlorotic

trees has been observed (Timmer and Morrow, 1977, unpublished data). Nitrogen and calcium content in the needles decreased with increasing chlorosis, and acute deficiency of nitrogen was detected in all chlorotic trees. Tanz (1979) concluded from his fertilization trials on the most severely affected plantations over the 1978 growing season that a soil deficiency of nitrogen, phosphorus, or potassium was not responsible for the disorder.

A preliminary bacteriological study on the chlorotic white spruce trees at Limestone Lake (Navratil and Smith, unpublished data) showed the presence of aerobic and facultative anaerobic bacteria in the bark and wood tissues of both chlorotic and healthy looking trees. A total of 56 different types of bacteria were isolated, based on morphological characteristics of the colonies. Seven types were isolated from apparently healthy bark tissues, 26 from green resin canals in the bark and 23 from brown spots in bark tissues. No attempt was made to characterize and/or identify the bacteria isolates at that time.

The frequent presence of the brown resinous spots in the bark of the chlorotic trees at Limestone Lake, coupled with high concentrations of yellow pigmented gram-negative rods (Enterobacteriaceae) found to be associated with the brown resinous spots in preliminary studies (Navratil and Smith, unpublished data; Mireku, unpublished data), led to the hypothesis that bacteria may play a role in the observed chlorosis of white

spruce.

The possible role of bacteria in inducing chlorosis is little understood and no direct relationship between bacteria and chlorosis has been reported in literature. However, a mixed bacterial flora, commonly present inside trees could interfere with the translocation and availability of nutrients to shoot and needle tissues. The occurrence of discharging bark necroses (weeping necroses) resulting in tree mortality of conifers, especially spruce and fir, has been observed in Europe (Urosevic 1967, 1968; Hasek 1974). These authors concluded that the causal organism of this disease of the bark is a bacterium, probably Erwinia cancerogena.

This study was therefore undertaken to investigate the possible association of bacteria with the observed disorder. The objectives were

a) to provide information on the distribution of bacteria in various parts of white spruce,
b) to characterize and identify as far as possible the bacteria flora in chlorotic and non-chlorotic white spruce and
c) to test the pathogenicity of some of the bacterial isolates on white and black spruce and their possible causal role in chlorosis.

2. REVIEW OF LITERATURE

2.1 Presence of Bacteria in Trees

The presence of bacteria in the wood tissues of living trees is well known. Bacteria have been consistently isolated from discolored wood, wetwood, and soundwood. Their involvement in wood discoloration and wetwood formation in standing trees, and their participation in wood decomposition is well documented. The role of bacteria in discolored wood tissues has been elucidated (McCreary et al.1965, Cosenza et al. 1970, Stankewich et al. 1971, Sharon 1974). Wetwood formation both in softwood and hardwood is believed to be the result of bacterial activity by some (Carter 1945, Clausen and Kaufert 1952, Bourchier 1967, Wilcox and Oldham 1972, Bauch et al. 1975). Other workers assume that wetwood is formed by nonmicrobial means and, once formed, it supports large populations of indigenous bacteria (Knutson 1968).

Bacteria are also frequently found in decayed wood tissues of both softwoods and hardwoods (Aho et al.1974 , Seidler et al. 1972, McCreary et al. 1965). The direct role of bacteria in the wood decomposition process is linked to the primary decomposition of cell walls (Liese and Karnop 1968, Greaves 1971). Certain bacteria are able to produce enzymes which may actively break down the wood cellular structure by attacking the cellulose and lignin compounds (Greaves 1969). Most recently, Tiedeman et al. (1977)

reported that bacteria isolated from living trees of Populus nigra were able to attack pectin, hemicellulose, and holocellulose.

Still other bacteria facilitate wood breakdown by acting as synergists to other microorganisms in the decay process (Henningsson 1967, Greaves 1971). Recently, the role of bacteria populations in the wood decay has acquired new importance. Clostridium spp. (Shigo et al. 1971), some of the bacteria isolated from the stem injuries of Abies concolor (Seidler et al. 1972) and bacteria isolates from decayed portions of white firs (Aho et al. 1974) were shown to be capable of binding atmospheric nitrogen. Thus, bacteria may increase the amount of nitrogen required by decay fungi. Studies by Shigo(1972), Cosenza et al. (1970), Good and Nelson (1962) , and others documented high concentrations of bacteria in the discoloration and initial stages of the wood decay process and elucidated the role of bacteria in the succession of microorganisms associated with wood degradation.

Conversely, bacteria may play an important role in decay resistance through bacteria antagonism. Such bacteria have been called "passive colonizer" by Greaves (1971). They may produce antibiotics and volatile metabolites antagonistic to other members of tree microflora or compete for available nutrients or essential vitamins. The inhibitory effect of volatile bacterial metabolites on several wood-inhabiting fungi has been demonstrated by Moore-Landecker and Stotzky (1972). A gram-negative, rod-shaped bacterium

isolated from a sapwood injury in Norway spruce was found to be antagonistic to Fomes annosus under laboratory conditions (Kallio 1974).

Large populations of indigenous bacteria are known to be present in apparently healthy wood tissues. Such bacteria have been consistently isolated from the soundwood of living coniferous trees (Etheridge and Morin 1967, Bier 1966, Aho and Hutchins 1975, Bacon and Mead 1971, Dangerfield et al. 1978) and deciduous trees (Basham and Taylor 1965, Bacon and Mead 1971, Knutson 1968, Warren 1978, Roth 1950, Filer 1975).

Information on the bacteria flora inhahiting soundwood of living spruce trees is very limited. Etheridge and Morin (1967) isolated a number of unidentified bacteria from the heartwood and soundwood of sound, living black spruce. More detailed studies by Kallio (1973, 1974) and Roll-Hansen et al. (1979) also show the presence of high bacterial populations in wounded Norway spruce trees.

There are even fewer reports on the bacteria content of foliage and buds of deciduous trees (Kenner 1945, Kenner 1950, Leben 1972) and coniferous trees (Leben 1972, Duncan and Razzell 1972, Walsh 1979). Though Duncan and Razzell (1972) observed very low bacterial counts in spruce needles, the number of bacteria isolated from non-chlorotic and chlorotic white spruce needles was 5.5×10^4 and 1.3×10^5 /g dry weight, respectively (Walsh 1979). The bacteria content of the bark of coniferous species has also been in-

vestigated (Duncan and Razzell 1972). Although their results show that the samples of bark examined gave highly variable counts, Klebsiella and Enterobacter species were obtained from all sources.

Though a variety of different bacteria have been isolated from normal and defective wood of living trees, little is known about the identity of the isolates. Most of the identified bacteria species belong to the genera Erwinia (Carter 1945, Bacon and Mead 1971, Knutson 1968, Tiedemann et al. 1977, Aho et al. 1974, Gokhale 1975), Pseudomonas (Chao and Ts'ai 1958, Sheneman and Costilow 1958, Cameron 1970, Cosenza et al.1970, Dowler and Weaver 1974), and Bacillus (Knutson 1968, Gokhale 1975, Cosenza et al. 1970). Other genera include Xanthomonas, Agrobacterium and Acinetobacter (Tiedemann et al. 1977), Clostridium (Ward et al. 1969, Stankewich et al. 1971) and Methanobacterium (Zeikus and Ward 1974). Nitrogen- fixing species of Klebsiella (Seidler et al. 1972, Aho et al. 1974) and Enterobacter (Aho et al. 1974) were also isolated from fungal-decayed portions of white fir. A study by Kallio (1974) shows that a bacterium strain isolated from Norway spruce sapwood showed pleomorphism reminiscent of the Corynebacterium family.

No detailed data on the bacteria species inhabiting white or black spruce have been reported.

2.2 Bacterial Diseases of Forest Trees

Bacteria diseases fall into three general categories: wilting (vascular disease); blights, cankers and leafspots; and hyperplasia (tumefaction) (Westcott 1971). Though bacterial diseases of agricultural and horticultural plants are numerous and well documented, there is limited information on bacterial diseases of deciduous and coniferous forest trees. Only five genera of true bacteria are known to cause diseases of forest trees. These include Pseudomonas, Xanthomonas, Agrobacterium, Erwinia, and Corynebacterium. Vascular diseases of broad-leaf forest trees may be caused by Pseudomonas spp. These bacteria invade the vascular system of the tree causing sudden wilting, shriveling of foliage, stunting, and brown stain of the vascular system (Browne 1968, Spaulding 1961).

Diseases such as necrotic blights, cankers, and leafspots may be caused by different kinds of bacteria. Blights are caused by Erwinia, Pseudomonas spp., or Xanthomonas spp. (Dowson 1957). Blight occurs on many forest tree species including walnut (Juglans spp.), willow (Salix spp.), and mountain ash (Sorbus spp.) (Browne 1968, Tattar 1978, Spaulding 1961). Bacteria are also responsible for a variety of forest tree cankers. Poplar canker has been well studied in Europe and most recently in North America (Hubbes 1977). Sabet and Dowson (1952) reported Pseudomonas syringae var. populea as its causal agent. Recently, Gremmen and de Kam (1974) and

Urosevic (1967) have reported the involvement of Aplanobacter populi and Erwinia cancerogena in the poplar cankers in Europe. Other bacterial diseases of broad-leaf forest trees include ash canker (Pseudomonas savastonoi var. fraxini (Browne) Dowson) found in Europe and the United States, bacterial phloem canker of walnuts (Erwinia rubrifaciens), most common in California (Kado et al. 1977, Schaad and Wilson 1971) and willow canker (Xanthomonas populi subsp. salicis) (Kam De 1977, 1978), found in Europe. Recently, a severe bacterial canker of Corylus avellana caused by Pseudomonas spp., has been reported by Psallidas and Panagopoulos (1979) in Greece.

In contrast to the above bacterial diseases in which the tissues are disintegrated and killed, the hyperplastic diseases induced by bacteria stimulate parenchyma cells to abnomal growth resulting in the formation of galls or tumors on roots, stems, leaves, or flowers. Eucalyptus spp., Juglans spp., Malus spp., Populus spp., Salix spp., are some of the woody hosts (Spaulding 1961, Tattar 1978).

Bacterial diseases of coniferous trees have been less investigated than those of deciduous trees. A bacterial vascular wilt which has caused severe injury to Casuarina equisetifolia and to seedlings of Pinus caribaea in Mauritius and Malaya was described (Browne 1968). Other bacterial diseases of conifers include pine gall (Spaulding 1961) and crown gall found on some North American species of incense cedar (Libocedrus spp.), yew (Taxus spp.) and cypress (Taxodium spp.) (Smith 1970).

Bacterial cankers of conifers are less known than those of hardwoods. The occurrence of discharging necroses has been observed in the bark of conifers, especially spruce and fir in Europe (Urosevic 1967, 1968; Hasek 1974). The disease is reported to occur in the spruce stands between the age of 35 and 80 years. Most conspicuous symptoms include necrotic spots of various sizes and shapes often accompanied by resinosis. The spots may be either scattered in the surface layers of the bark causing no harmful effect to the trees or joined together encircling the stem or branches, and thus leading to death and decay of the parts of the tree above the ringed area. Excess sap may also be observed flowing out through cracks in the bark at the base of the tree. The necrotic spots and exuding slime are reported to contain a very rich microflora, and several strains of bacteria belonging to the genera Erwinia and Pseudomonas have been repeatedly isolated (Urosevic 1968). Their morphological and biochemical characteristics resemble Erwinia cancerogena and Pseudomonas syringae. The pathogenicity of some of the Erwinia isolates has been successfully demonstrated by artificial infection (Urosevic 1967). No similar disease has yet been reported in the USA or Canada, and no information on any bacterial bark diseases of conifers is available from North America.

3. MATERIALS AND METHODS

3.1 Characterization and Distribution of Bacteria

3.1.1 Field Sampling

Location and Description of the Study Area

The study area was located near Limestone Lake, about 12 miles (20 km) north of Nipigon, Ontario (ca. 49^{o} 07' N, 88^{o} W) on a site which had been logged for prime quality white spruce during the 1930's, and was burned by wildfire in 1940 and again in 1948. The study was conducted in a white spruce plantation of about 400 hectares. Planted white spruce ranged in height from about 1.5 m to 4.8 m, and averaged about 2.7 m. Trees were approximately 10-12 years old. Trembling aspen, alder and grasses compete heavily with planted white spruce on this site. The origin of the seedlings and seed source could not be established.

The soil is a sandy Orthic Humo-Ferric Podzol (Clayton et al., 1977) of glaciofluvial origin with thin L and F horizons overlying a thin Ae horizon with few fine and medium roots. Lacustrine parent material lies over a dolomitic limestone bedrock. The area receives an average precipitation of 40 cm between May and September each year, and the average July temperature is 17.0 C. (Env. Canada, 1975).

Sampling in October 1978

In October, 1978, four young white spruce trees, approximately 10 to 12 years old and about 2 to 3 meters high, were selected for the study. Two chlorotic trees (C_1 and C_2) were chosen from the plantation, and two non-chlorotic, apparently healthy trees (H_1 and H_2) were selected from the edge of a natural stand approximately 8 miles (13 km) from the Limestone Lake Plantation.

The trees were cut at ground level, and the stems were bucked into nodal lengths. Yearly height increments were measured and foliage and branches were separated by years. Root systems were excavated to include, as much as possible, all the feeder roots and to prevent wounding of the roots. All tissues were stored at $0^{o}C$ for 2-5 days prior to laboratory analysis.

Sampling in June 1979

In June, 1979, one additional chlorotic white spruce tree was selected from the plantation sampled in 1978, and one additional non-chlorotic tree was sampled from a naturally regenerated stand about 1.2 km away from the chlorotic tree. The same procedure as described above for the 1978 sampling was used.

3.1.2 Laboratory Procedure

Isolation of Bacteria

Trees Sampled in October 1978

After 2-5 days of storage ($0^{o}C$), four subsamples - foliage and twigs, stems, major roots, and fine roots were used for the isolation of bacteria. Foliage and twig subsamples consisted of one- and two- year old twigs and of the current year's (1978) terminal shoots including needles. A 6-cm stem bolt was cut from each tree 0.6 m (2 ft) above ground level. Root segments, 4 cm long, were taken from the tap root and two side roots. Stems and major roots were first shaved with the dull edge of a knife to remove bark scales and to obtain a smooth surface. All fine roots to a maximum of 0.5 cm in diameter were bulked.

All samples were further cut into 2-cm long pieces and washed in tap water for 10 minutes. Approximately half of each sample was used to determine the fresh weight/dry weight ratio of each tissue. The remaining half of each sample was surface sterilized in 1% NaHClO for 3-5 minutes, drained, and rinsed several times with sterile distilled water.

Sterilized samples were blended in sterile water (1:10 w/w) in a Waring Blender for 5 minutes at a low speed to attain a consistent slurry. The suspension was filtered through cheese cloth and successive dilutions

of $1:10^2$ to $1:10^6$ were prepared.

Plates of Knutson's agar medium (Knutson 1968), supplemented with 20 ppm of nystantin (Tuite 1969), were inoculated with 0.2 ml of each dilution. Four replicates per dilution were made. Plates were incubated in the dark at 28^{o}C. Following an incubation period of 48 hr, the number of bacterial colonies was counted with a Quebec colony counter. The plates were observed daily for 7 days to detect subsequent growth. The morphology of colonies was described, and the representative types were transferred onto slants of Knutson medium and incubated for 2 days at 28^{o}C. After the incubation, the slants were maintained under refrigeration for further study.

Trees sampled in May 1979

One chlorotic tree from the plantation and one apparently healthy, non-chlorotic tree from natural stand were used for this study. Each tree was separated into five subsamples: 1979 foliage and twigs, 1977 foliage and twigs, stems, major roots, and fine roots. The same procedure for sterilization and isolation as described for 1978 study was used.

Characterization of the Bacteria

Morphological characteristics

Sixteen representative isolates from the chlorotic and apparently healthy white spruce trees were selected for the characterization. Gram morphology was determined by the method outlined by Skerman (1967). Bacterial cell shape, size, and pattern of flagellation were determined by electron miscroscopic observation of specimens negatively stained with 1.0% PTA (Phosphotungstic acid) at pH 7.0 with a Phillips 300 transmission electron miscroscope. Specimens were prepared for electron microscopy by macerating a pure culture of bacteria in a drop of sterile, distilled water in a ceramic palette to which one drop of phosphotungstic acid was added. An electron miscroscope grid was floated on the surface of the drop for 2 minutes. Excess suspension on the grids was gently blotted off the edge of the grid with a filter paper.

Biochemical characterization of isolates

The Analytical Profile Index procedures (API Manual, 1978) were employed to characterize 16 representative types of bacteria. Ninety four percent of our isolates were gram-negative, only 6% were gram-variable.

The 21 major biochemical tests included on the API strips and 7 additional tests used are listed in Table 4. The procedures followed the

Manual of Analytical Profile Index (API 1978) for the identification of Enterobacteriaceae and other gram-negative bacteria. Inocula were prepared when a loopful of a well-isolated 18-24 hr old colony was suspended in a test tube containing 5 ml of sterile 0.85% saline, pH 5.5-7.0. API 20E strips were inoculated with 5 ml Pasteur pipette. The strips were incubated for 24-48 hours at 37°C. The results of the biochemical tests (Fig. 1) were recorded after 24 hr and 48 hr of incubation on the API result sheet (Fig. 2). Each set of tests was replicated three times.

Interpretation of reactions followed the API 20E Instruction Manual. The bacteria were identified by the API Profile Recognition System (PRS) Computer Service, which is composed of an API Coder and Analytical Profile Index. The API Coder simultaneously considers all 21 biochemical results and transfers them into a seven-digit number by placing them into groups of three and assigning a specific numerical value for each of the positive results. The number is then looked up in the index and the identification determined. An example of a seven-digit number (Profile) for the result of the biochemical test (Fig. 1) is illustrated in Fig. 2. The digit number used in the example was identified by the index as Pseudomonas fluorescens (Fig. 3).

Fig. 1 API 20E strips inoculated with bacteria.

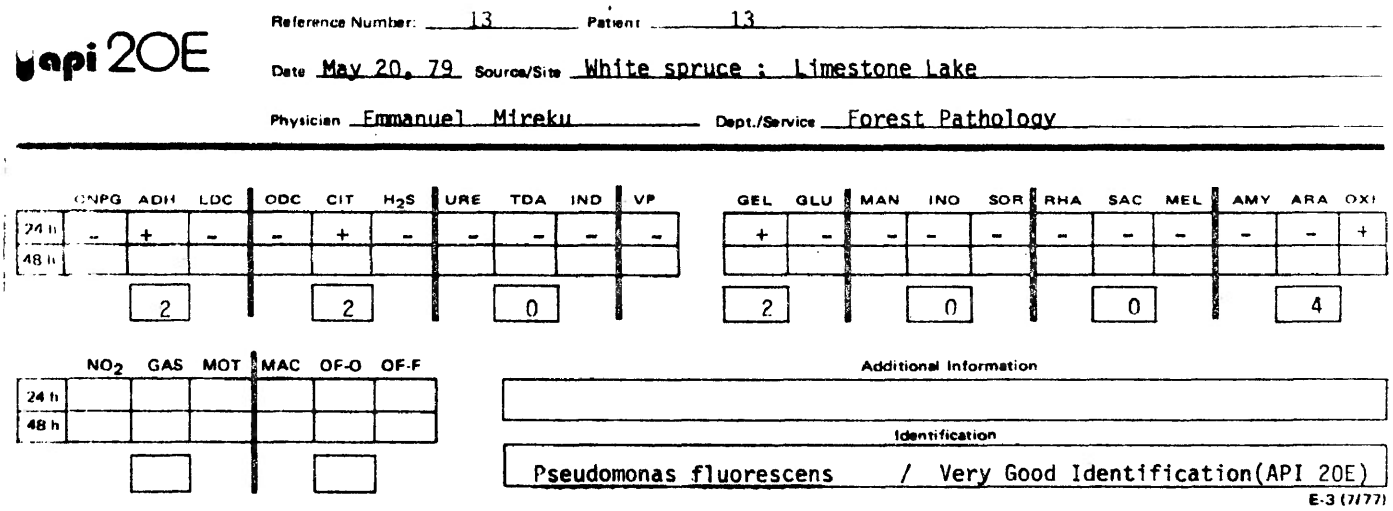


Fig. 2 API result sheet showing results of the biochemical tests (Fig. 1), recorded 24 hours after inoculation.

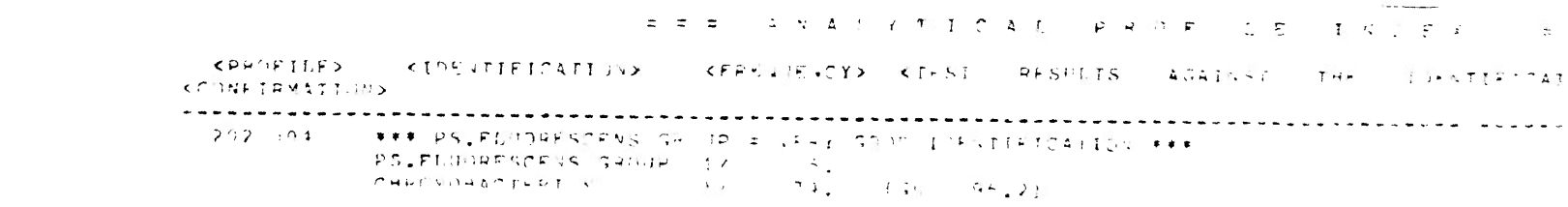


Fig. 3 Identification of the seven-digit number (Fig. 2) by the Profile Recognition Systems.

3.2 PATHOGENICITY TESTS

Tests of (a) seed inoculation, (b) hypocotyl inoculation, and (c) green cutting inoculation were performed with both white and black spruce.

3.2.1 Seed Inoculation

Inoculum preparation

Nine different isolates or mixtures of isolates were used to inoculate white and black spruce seeds. The nine different inocula plus control provided ten treatments (Table 1).

Fresh 1-2-day-old bacteria cultures grown on Knutson agar slants medium were suspended in 5 ml sterile distilled water. Bacterial cell concentration was estimated with a Spencer Haemacytometer soon after suspension was made (Table 1).

Method of seed inoculation

Seeds of white spruce (seed source 3400) and black spruce (seed source 4400) were surface-sterilized in 1% NaHClO for 2 minutes and rinsed several times with sterile distilled water. Seed inoculation was performed by dipping the seeds into the bacterial suspensions. The inocu-

lated seeds were aseptically planted on a 2%, 10-mm thick layer of water agar in deep petri dishes. Twenty-five seeds were included in each petri dish and treatments consisted of four replicates. The controls consisted of the same number of seeds and replicates treated in the same manner except that seeds were dipped in sterile distilled water. The planted dishes were incubated in a growth chamber at 24°C and 24 hours light, and watered once daily with an equal amount of sterile distilled water that was pipetted on the surface of agar with an automatic dispensor. Relative humidity in the growth chamber was maintained at 90%. Treatments were arranged in a complete randomized design. After 3 days of incubation, the seeds were assessed daily and the following data recorded: (a) number of seeds germinated with visible radicle, (b) seeds with no visible radicle, but opening in the seed coat, and (c) seed without opening in the seed coat. Length of hypocotyls was measured after 7 and 14 days of incubation for black and white spruce, respectively.

3.2.2 Hypocotyl Inoculation

In this test, germinating seeds from the previous experiment having 1-5 mm long hypocotyls were used. Seeds with no hypocotyl or hypocotyl less than 1 mm in length were discarded. Hypocotyls were inoculated by dipping the germinated seeds in bacterial suspensions (as described in sec. 3.2.1); bacterial concentration of the inocula is shown in Table 1. Controls were dipped in sterile distilled water. There were 10 treatments

Table 1 Concentrations of bacteria used for seed and hypocotyl inoculation.

Inoculum	Isolates	Concentration (Cells/ml) Seed	Hypocotyl
1	*Pseudomonas* sp.	7.2×10^6	1.1×10^6
2	*Serratia* sp.	10.7×10^6	8.4×10^6
3	*Pseudomonas maltophilia*	1.2×10^6	1.0×10^6
4	*Enterobacter agglomerans*	1.2×10^6	2.4×10^6
5	*Enterobacter agglomerans* + *Serratia rubideae* + *Pseudomonas maltophilia*	2.1×10^6	1.6×10^6
6	*Pasteurella* sp.	1.9×10^6	3.6×10^6
7	*Enterobacter* sp. + *Enterobacter alvei* + *Serratia* sp.	2.2×10^6	3.9×10^6
8	*Enterobacter agglomerans* + *Pasteurella* sp. + *Serratia liquefaciens* + Unknown - 1	3.3×10^6	2.5×10^6
9	All isolates combined	1.9×10^6	1.9×10^6
10	CONTROL (sterile distilled water)	----	----

and 4 replicates per treatment. After inoculation, the seeds were placed back in their respective dishes and incubated in a growth chamber at 90% humidity and 20°C temperature during the day and at 15°C during night with 18 hours light. Symptom development such as wilting, necrosis, swelling of the hypocotyl, stunting, and mortality were recorded every 24 hours. Fourteen days after inoculation, final lengths of the hypocotyls were measured, and a final assessment of the symptoms was made.

Transplanting in the greenhouse

Immediately after the final assessment, surviving seedlings were outplanted in a sterilized sifted mixture (1:1 ratio) of commercial peat moss and vermiculite in Spencer-Lemaire book containers arranged in flats. The planted containers were placed first under an intermittent mist for 7 days, and then on greenhouse benches for another 2 weeks. To achieve maximum growth, the seedlings were transferred to growth chambers with a constant day/night temperature of 22°C and 20 hours light, and mist irrigated once daily with a fox nozzle. As the epicotyls began to elongate, the seedlings were fertilized with 20-20-20 commercial fertilizer (Concentration 3.72 g/l) at two-week intervals. After 10 weeks of growth in a growth chamber, fresh and dry weight of seedlings were determined. Five seedlings from each treatment were processed for re-isolation as described in section 3.1.2.

3.2.3 Inoculation of green cuttings

Young untreated seedlings of white and black spruce (seed source 3400 and 4400, respectively) were raised in the greenhouse for 21 days and later transferred to the growth chamber. When the actively growing healthy epicotyls reached 4 cm or more in length, green cuttings, 4-6 cm long, were severed from the 10-week old seedlings with a sharp razor blade. Needles approximately 1 cm above the base of the cutting were removed to ensure closer contact between the stem and the rooting medium.

Prior to planting, the cuttings were inoculated when the cut surfaces were dipped in bacterial suspensions prepared as previously described. Six isolates which showed the highest virulence in the seed and hypocotyl pathogenicity tests were selected for pathogenicity evaluation on white and black spruce green cuttings. The controls were dipped in sterile distilled water. The inoculated cuttings were planted in a rooting medium consisting of a sterilized mixture of sifted commercial peat moss and vermiculite (1:1 ratio) in a Spencer-Lemaire book container. The inoculated ends of cuttings were approximately 1 cm below the surface of the medium and the medium around the stem was gently packed. The experiment was arranged in a completely randomized block design with five treatments (Inocula E_1, U_1, E_5, $E_4 + E_3 + S_4$ and Control) each containing four replicates. Twenty cuttings were planted in each replicate. Bacterial

suspensions were of the following concentrations: E_1 (1.9 X 10^6), U_1 (13.0 X 10^6), E_5 (23.7 X 10^6) and $E_4 + E_3 + S_4$ (24.4 X 10^6). At the end of 8 weeks, cuttings were carefully uprooted and the following characteristics assessed, number of rooted cuttings, total root length, and number of roots. After assessment, the roots were severed and used for re-isolation.

4.0 RESULTS

4.1 Characterization of the Bacteria

Morphological characteristics

The bacteria isolated from the white spruce trees were classified initially into sixteen groups based on the colony morphology (size, shape, color) and on growth and pigmentation on Knutson agar medium. All the isolates were non-sporing, aerobic or facultative anaerobic and consisted of cells that were medium-sized rods with round or slightly pointed ends, except isolates PS_1 and U_2 that were oval to circular (Table 2). Ninety-four percent of all isolates were gram-negative and six percent gram-variable. Isolates PS_1, PS_2 and U_2 showed no flagellation. The flagella of all other bacteria types were determined electronmicroscopically as being polar (monotrichous or multitrichous) or peritrichous (Table 2). A detailed description of the bacteria types is included in Appendix A. Figures 4 to 10 show the electron micrographs of the representative isolates.

Biochemical identification of bacteria

The results of the biochemical tests of the 16 bacterial isolates are summarized in Table 3. The isolates were tested for 21 biochemical properties provided on the API 20 E strips. Seven additional tests were conducted (Table 3). The bacteria were placed in 5 groups based on the similarity

Table 2 Morphological characteristics of isolated bacteria

Isolate	Colony color	Cell-size (microns)	Cell-shape	Gram test	Flagellation
P_1	off-white	0.6-0.8 X 1.8-2.4	rod		polar
P_2	white	0.7 X 2.0-2.5	rod		polar
P_3	white	0.7-0.9 X 1.2-2.0	rod		polar
S_1	white	0.5-0.7 X 1.0-1.5	rod		peritrichous
S_2	white	0.5-1.0 X 1.0-1.6	rod		peritrichous
S_3	white	0.8 X 1.8-2.6	rod		peritrichous
S_4	pink	0.8-1.1 X 1.8-2.6	rod		peritrichous
PS_1	cream	0.9 X 1.2	oval		none
PS_2	cream	?	rod		none
E_1	yellow	0.7 X 2.2-3.6	rod		peritrichous
E_2	dull-yellow	0.8 X 1.2-3.2	rod		peritrichous
E_3	yellow	0.8-1.0 X 1.5-3.0	rod		peritrichous
E_4	yellow	0.8 X 1.8-3.9	rod		peritrichous
E_5	white	0.9 X 1.7-2.5	rod		peritrichous
U_1	yellow	0.8 X 1.3-2.7	rod		peritrichous
U_2	white	1.6 X 1.6-2.0	cocci	vari-able	none

Table 3

Biochemical characteristics of the bacteria

Types of bacteria / Tests (API 20E)	P_1 - Pseudomonas (Ps) sp.	P_2 - Ps maltophilia	P_3 - Ps maltophilia	S_1 - Serratia (ser.) sp.	S_2 - Serratia sp.	S_3 - Ser. rubideae	S_4 - Ser. liquefaciens	Ps_1 - Pasteurella (?) sp.	Ps_2 - Pasteurella (?) sp.	E_1 - E. agglomerans	E_2 - E. agglomerans	E_3 - E. agglomerans	E_4 - E. alvei	E_5 - Enterobacter (E.) sp.	U_1 - Unknown genera	U_2 - Unknown genera
Betagalactosidase	-	-	-	+	+	+	+	-	+	+	+	+	+	+	+	-
Arginine dihydrolase	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lysine decarboxylase	-	-	-	+	+	+	-	-	-	-	-	-	+	+	-	-
Ornithine decarboxylase	-	-	-	+	+	-	+	+	-	-	-	-	+	+	-	-
Citrate utilization	-	+	+	+	+	-	+	-	-	d	-	-	-	-	-	-
H_2S production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hydrolysis of urea	-	-	-	-	+	+	-	+	+	-	-	-	+	+	-	-
Tryptophane deaminase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indole development	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acetoin production	-	-	-	-	-	d	-	-	-	-	-	-	-	-	-	-
Gelatin liquefaction	-	+	+	+	+	-	+	-	+	d	+	+	-	-	-	-
Acid produced from																
Glucose		-	-	+	+	+	+	+	-	+	+	+	+	+	-	+
Mannitol	-	-	-	+	+	+	+	+	-	+	+	+	d	d	-	+
Inositol	-	-	-	+	+	d	+	-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	+	+	-	+	+	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-	+	d	+	+	+	-	-
Sucrose	-	-	-	+	+	d	+	+	-	+	+	+	-	d	-	+
Melibiose	-	-	-	+	+	+	d	-	-	d	-	-	-	d	-	-
Amygdalin	-	-	-	+	+	+	+	+	-	+	+	+	-	d	-	-
Arabinose	-	-	-	+	+	+	+	-	-	+	+	+	+	+	-	-
Oxidase	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Nitrate reduction	-	-	+	+	+	+	+	-		+	+	+	-	-	-	-
Develop. of gas	+	d	-	o	o	o	o	o	o	o	o	o	o	o	o	o
Mobility	-	+	+	+	+	+	+	-	-	+	+	+	+	+	-	-
Glucose oxidation	+	-	-	+	-	+	-	-	-	+	+	+	+	+	+	-
Glucose fermentation	-	-	-	+	-	+	-	-	-	+	+	+	+	+	+	-
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth on McConkey-agar	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+

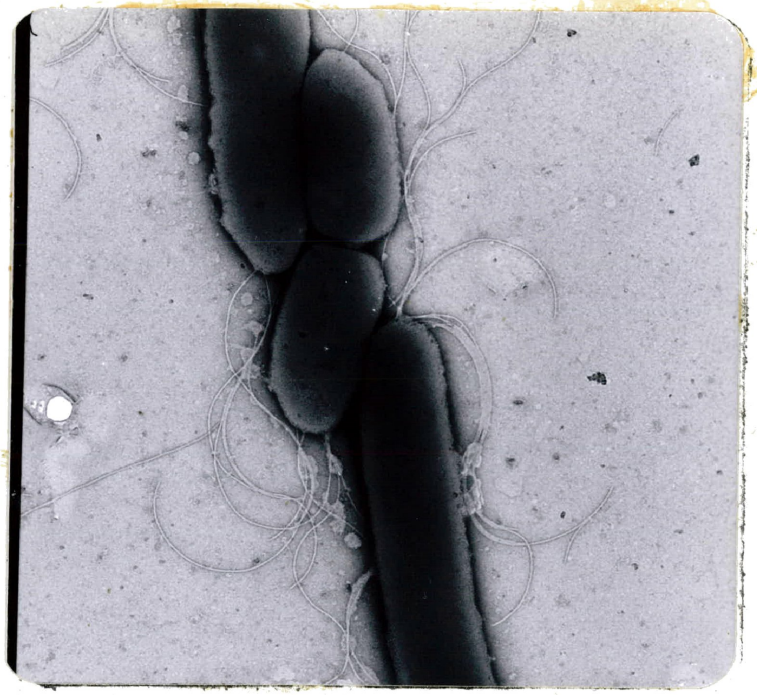
d= variable reaction; o= no data recorded
+= positive reaction; -= negative reaction

of the data obtained from the tests on API 20E strips.

All cultures were catalase positive and neither produced H_2S, nor formed indolepyruvic acid and indole with tryptophane. The transformation of glucose metabolite was not achieved by any of the strains. Variable results were, however, observed in 23 remaining tests.

Group one: Isolates P_1, P_2, and P_3, were classified into this group. Isolate P_1 was oxidase positive while P_2 and P_3 were both oxidase negative on nutrient agar. No diffusible pigment was observed from any of the isolates on the medium. The bacteria were unable to utilize any of the tested carbohydrates. Oxidation and fermentation tests show that isolate P_1 did utilize glucose only under aerobic conditions resulting in acid production. Isolates P_2 and P_3 did not utilize glucose by either method. Citrate was utilized by isolates P_2 and P_3. Two isolates, P_2 and P_3, were strong liquefiers of gelatin. Production of beta-galactosidase, ornithine decarboxylase, lysine decarboxylase, and urease were negative in all cases. Only isolate P_1 produced arginine dihydrolase confirming its proteolytic activity with arginine. Reduction of nitrate to nitrite was verified for isolate P_3. Development of nitrogen was positive for isolate P_1, variable for P_2, and negative for P_3. Good growth on McConkey-agar was observed for isolates P_1 and P_3, whereas P_1 showed no growth.

The morphological and biochemical data as shown in Tables 2 and



Figs. 4 and 5 (Top and below) Electron micrographs of a representative Pseudomonas species. (Magn. 22080 X)

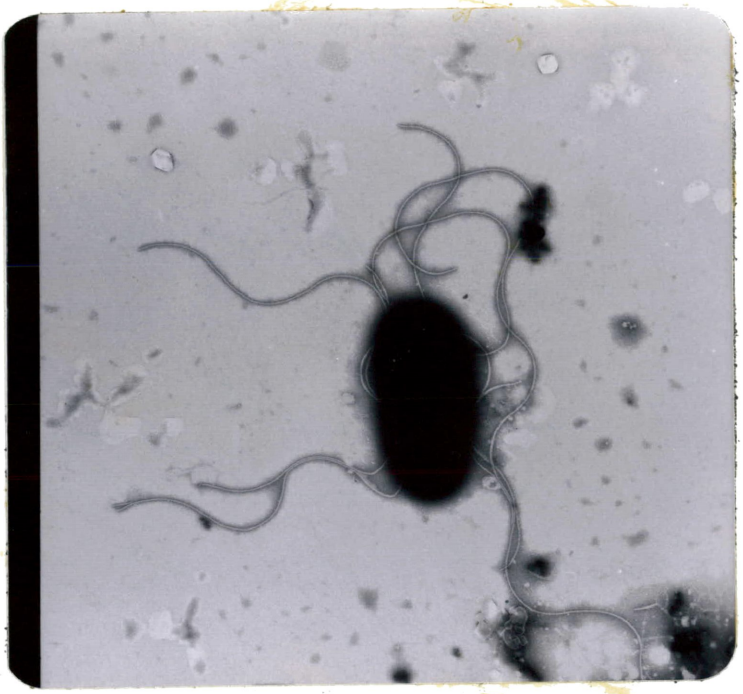


Fig. 6 Electron micrograph of a representative Serratia species. (Magn. 22080 X)

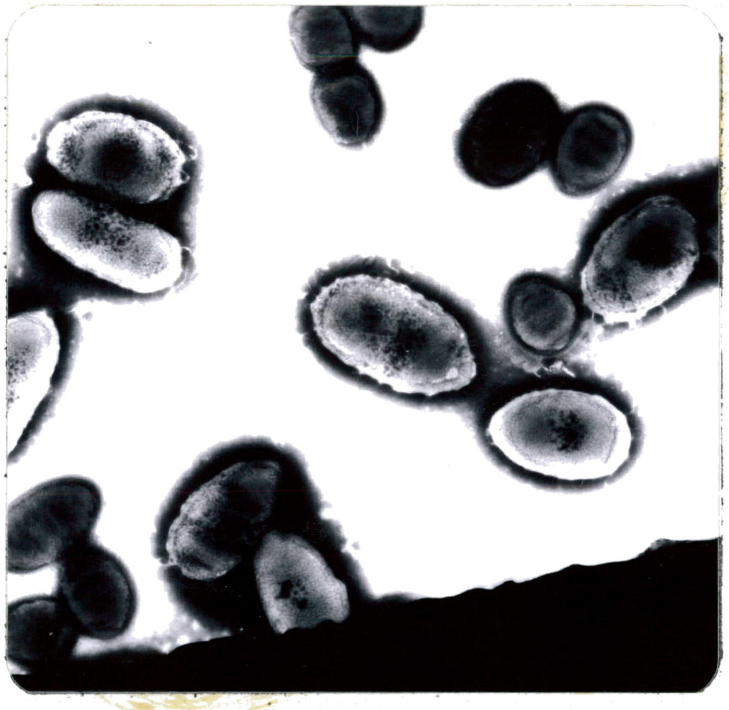
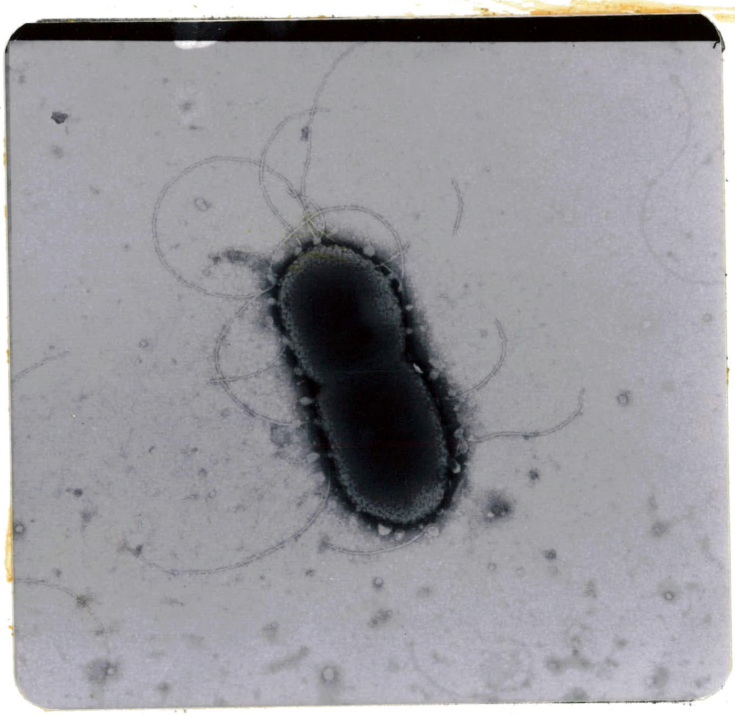


Fig. 7 Electron micrograph of a representative Pasteurella sp. (Magn. 22080 X)



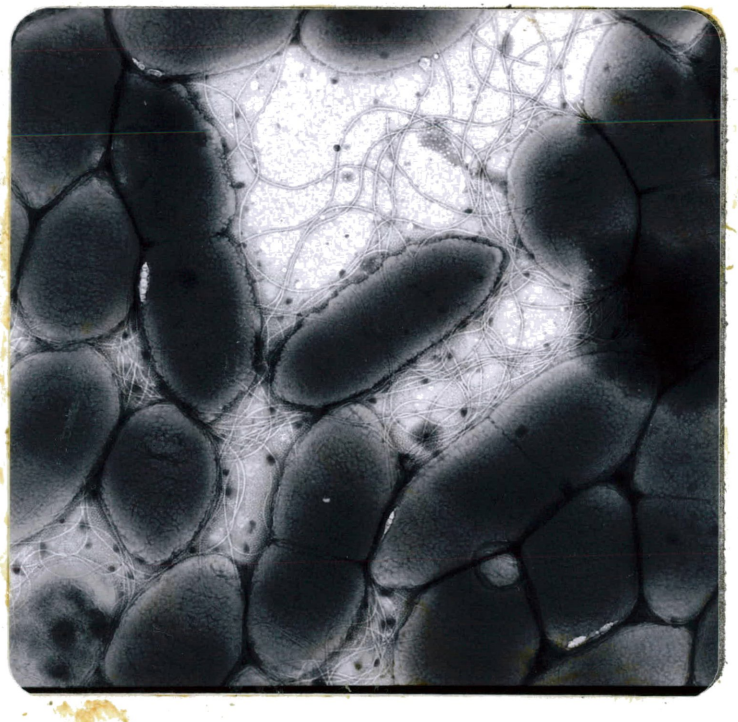


Fig. 8 and 9 (Top and below) Electron micrographs of a representative Enterobacter (Erwinia) species. (Magn. 22080 X)

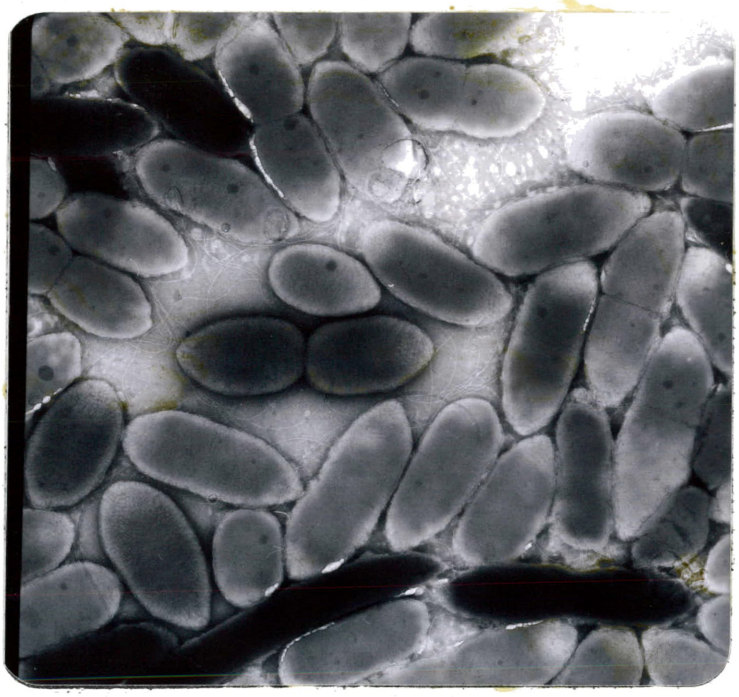


Fig. 10 Electron micrograph of isolate U_1 (Unknown - 1).
(Magn. 13440 X)

3 often allow only a general taxonomical classification of the isolates. The API Profile Recognition System identified isolates P_1, P_2, and P_3 (Group 1) as belonging to the family Pseudomonadaceae. Isolate P_1 was a species of the genus Pseudomonas. Isolates P_2 and P_3 were identified as Pseudomonas maltophilia (P. melanogena) (API Manual 1978).

Group two: This group consists of isolates S_1, S_2, S_3, and S_4. The test of oxidase activity was negative on nutrient agar medium in all cases. The isolates did not produce any diffusible pigment on the medium. The utilization of carbohydrate was very efficient among the strains. All the strains produced acid from glucose without any trace of gas production 18 hours after inoculation. They also produced acid from mannitol, inositol, sucrose, melibiose, amygdalin, and arabinose. Rapid acid production from sorbitol was observed among all strains with the exception of S_3. No isolate was able to produce acid from Rhamnose even after 48 hours of incubation. In a test to determine the type of glucose metabolism, the isolates S_1 and S_3 did produce acid under either aerobic or anaerobic conditions, a result suggesting the presence of an oxidative and fermentative metabolism of glucose. Isolates S_2 and S_4 did not produce acid by either method. Citrate was utilized by all strains except S_3. All isolates showed positive reaction with gelatin, except isolate S_3. Urease was produced by isolate S_2 and S_3. Production of betagalactosidase was positive for all strains. None of the isolates, however, produced arginine dihydrolase. Lysine decarboxylase and ornithine decarboxylase were produced by isolates S_1, S_2, S_3 and S_1, S_2, S_4,

respectively. Reduction of nitrate to nitrite was positive for all the four isolates. Growth occurred with S_1, S_2, and S_3 isolates on McConkey-agar.

This group was assigned to the genus Serratia. Isolates S_3 and S_4 were further classified as S. rubideae and S. liquefaciens, respectively.

Group three: Isolates PS_1 and PS_2 were assigned to this group. PS_2 was oxidase positive while PS_1 showed negative reaction. No diffusible pigment was observed on the medium with both isolates. Production of acid from glucose was evident only from isolate PS_1 without any gas production. Carbohydrates - mannitol, sorbitol, sucrose, and amygdalin were also utilized by isolate PS_1. Isolate PS_2 was unable to utilize any of the tested carbohydrates. Oxidation and fermentation tests showed that neither isolates utilized glucose either aerobically or anaerobically. Both isolates were incapable of utilizing citrate. Gelatin was liquefied by only isolate PS_2. Arginine dihydrolase and lysine decarboxylase were never produced by any of the strains. Betagalactosidase was produced by isolate PS_2 while ornithine decarboxylase was produced by PS_1. Reduction of nitrate to nitrite was negative in both cases. Growth on McConkey-agar was verified by both isolates.

Profile PS_1 was tentatively identified as Pasteurella (?)

(Yersinia enterocolitica) (API Manual 1978), while PS_2 was shown to be either Pasteurella or Flavobacterium.

Group four: Five isolates E_1, E_2, E_3, E_4, and E_5 constitute this group. All the isolates showed a negative test of oxidase activity on nutrient agar. Only isolate E_4 developed colonies that were deeply stained (pink-red) and produced deep red-brown diffusible pigmentation after 48 hours on nutrient agar medium. All the isolates produced acid from glucose without any trace of gas production. Other carbohydrates including mannitol, rhamnose, and arabinose were also positively utilized by all isolates. None of these 5 isolates was able to produce acid from inositol and sorbitol. Isolates E_1, E_2, E_3 and E_5 produced acid from sucrose and amygdalin within 24 hours of incubation. Only E_1 and E_5 produced acid or gas from melibiose. The utilization of citrate could only be verified for strain E_1. In a test to determine the type of glucose metabolism, all the isolates tested produced an acid reaction in an oxidation/fermentation (API OF) glucose medium, suggesting the presence of both aerobic and anaerobic methods of metabolism. Three isolates E_1, E_2, and E_3 were strong gelatin liquefiers. Complete liquefaction was accomplished within 24 hours after inoculation. None of the strains were capable of producing arginine dihydrolase. However, lysine decarboxylase, ornithine decarboxylase and urease production were present in the strains E_4 and E_5. Isolates E_1, E_2, and E_3 gave a positive nitrate reaction. Growth on McConkey-agar was positive in all cases.

According to the characteristics listed in Tables 2 and 3, all the five isolates E_1, E_2, E_3, E_4, and E_5 were placed into the genus Enterobacter (Erwinia). Isolates E_1, E_2, and E_3 were identified as Enterobacter agglomerans (Erwinia herbicola) (Ewing and Eife, 1972), E_4 was Enterobacter alvei (Hafnia-alvei, or Enterobacter hafnia), and E_5 is an unknown species of the genus.

Group five: The classification of isolates U_1 and U_2 under this group was not based on the similarity of the biochemical properties (profiles). They were arbitrarily grouped since they could not be assigned to any genera. Oxidase test was negative for both isolates. No diffusible pigmentation was observed in either case on the medium. Only isolate U_2 produced acid from glucose within 48 hours after inoculation. Isolate U_1 was unable to produce acid from any of the tested carbohydrates. Mannitol and sucrose were partially utilized by U_2 after 48 hours of inoculation. Apart from beta-gelactosidase, which was produced by isolate U_1 no other pectolytic enzymes were produced by either isolates. Nitrate reduction was also negative for both isolates.

4.2 Distribution of Bacteria in Chlorotic and Non-chlorotic Trees

All isolation attempts made from different tree parts produced bacterial colonies on yeast extract peptone agar.

The average total bacteria counts of the 1978 isolations, expressed as the number of bacterial cells per gram oven-dry weight, from the three sections of two chlorotic and two non-chlorotic white spruce trees are listed in Table 4.

Numerous bacteria were isolated from apparently sound, non-chlorotic trees. The roots contained the richest flora, particularly the fine roots. The highest count, 1.3×10^5 per gram dry weight, (/g.d.w.) was obtained from the fine roots, followed by 9.6×10^4/g.d.w. from the large roots. The bacteria concentrations found in the bark and xylem of the stems (5.9×10^3 and 2.5×10^3/g.d.w., respectively) were much lower. The high bacteria counts obtained from the roots could be inflated due to contamination by soil bacteria, though the samples were given a surface sterilization treatment.

Bacterial isolates were more frequent in the chlorotic trees. The overall concentration of bacteria (1.2×10^6/g.d.w. of wood) was almost six times greater than the counts (2.3×10^5/g.d.w. of wood) obtained from the healthy control trees. The bacteria counts were high in all tree sections (Table 4). The highest counts (6.0×10^5/g.d.w. and 1.9×10^5/g.d.w.) were obtained from the brown spots of the stem and the large roots, respectively. Great variations of bacterial concentration were observed among all the sampled trees, most likely reflecting the difference in the concentrations of bacteria present in different rhizo-

Table 4 The average number of bacteria per gram dry weight of the 1978 sampled white spruce trees.

Tree section	Tissue	Bacteria / g. dry weight	
		Chlorotic	Non-chlorotic
Stem	- Bark	1.2×10^4	5.9×10^3
	- Xylem	1.4×10^5	2.5×10^3
	- Brown spots	6.0×10^5	---
Large Roots	- Bark	1.9×10^5	8.1×10^4
	- Xylem	2.2×10^4	1.4×10^4
	- Brown spots	1.1×10^5	---
Fine Roots		1.6×10^5	1.3×10^5

spheres.

The 1979 isolation data from the chlorotic and non-chlorotic trees are summarized in Tables 5, 6 and 7. Bacteria were consistently isolated from all sections of the sampled trees. Table 5 shows the total distribution of the bacteria types in chlorotic and non-chlorotic trees, when the results obtained from all tissues sampled were combined. All the bacteria types isolated from chlorotic trees were also found in the apparently healthy, non-chlorotic trees with the exception of four isolates - S_2, PS_1, PS_2, and U_2. Eight out of 12 bacteria types found in healthy trees were also isolated from the chlorotic trees. P_2, P_3, S_3, and U_1 were present only in the apparently healthy, non-chlorotic trees.

Table 5 Frequency of bacteria isolated from chlorotic and non-chlorotic white spruce trees sampled in 1979.

Type of bacteria[1]	Sample Tree (bact. cells/gm. d.w.)	
	Chlorotic	Non-chlorotic
P_1	2.4×10^2	5.2×10^2
P_2	o	2.4×10^3
P_3	o	3.5×10^3
S_1	1.7×10^3	5.8×10^2
S_2	2.2×10^3	o
S_3	o	1.1×10^5
S_4	1.5×10^4	1.2×10^4
PS_1	3.4×10^3	o
PS_2	2.0×10^3	o
E_1	4.5×10^3	7.1×10^3
E_2	1.2×10^3	3.1×10^3
E_3	1.7×10^4	2.3×10^2
E_4	1.3×10^4	1.8×10^6
E_5	1.9×10^3	2.2×10^3
U_1	o	7.3×10^4
U_2	7.2×10	o

1 Refer to Table 3 for identification of bacterial isolates.

Table 6 Concentration of bacteria in non-chlorotic white spruce tree sampled in 1979. (No. of bacteria/g.d.w. of wood)

Type of bacteria[1]	Tissue used in isolation						
	Needles + twigs (1979)	Needles + branches (1977)	Large roots (bark)	Large roots (xylem)	Stem (bark)	Stem (xylem)	Fine roots
P_1	-	6.8×10	-		4.5×10^2	-	
P_2					2.4×10^3	-	-
P_3			3.2×10^3	2.0×10		3.3×10^2	-
S_1	1.9×10^2			3.9×10^2			
S_2							
S_3		1.2×10^3					1.0×10^5
S_4		1.2×10^4					
PS_1							
PS_2							
E_1	3.9×10^2	6.6×10^3		1.9×10^2			
E_2			2.3×10^3	7.2×10^2		4.6×10	-
E_3						2.3×10^2	-
E_4						-	1.8×10^6
E_5		2.2×10^3			-		-
U_1					7.3×10^4	3.3×10^2	-
U_2							

1 Refer to Table 3 for identification of bacterial isolates.

Table 7 Concentration of bacteria in chlorotic white spruce tree sampled in 1979. (No. of bacteria/g.d.w. of wood)

Type of bacteria[1]	Tissue used in isolation: Needles + twigs (1979)	Needles + branches (1977)	Large roots (bark)	Large roots (xylem)	Stem (bark)	Stem (xylem)	Fine roots
P_1							2.5×10^3
P_2							
P_3							
S_1		1.4×10^3			4.6×10	1.9×10^2	
S_2	2.2×10^3						
S_3							
S_4		1.5×10^4			3.4×10^2		
PS_1	3.4×10^3						
PS_2	2.0×10^3						
E_1		2.8×10^3			2.3×10^2		1.4×10^3
E_2					9.7×10^2	2.6×10^2	
E_3			8.9×10^3	1.1×10^3		5.5×10	6.7×10^3
E_4			7.4×10^2	5.9×10^3	-		6.8×10^3
E_5		1.0×10^3			8.0×0^2	4.9×10	
U_1							
U_2	7.2×10						

1 Refer to Table 3 for identification of bacterial isolates.

In the chlorotic trees, the Erwinia group (E_1 - E_5) was the most dominant. However, the Erwinia populations, with the exception of E_3, found in the healthy non-chlorotic trees exceeded those of chlorotic trees.

The distribution of the bacteria, classified by the type of tissue sampled and the number of bacteria colonies per gram dry weight, are listed in Tables 6 and 7 for non-chlorotic and chlorotic white spruce trees, respectively. The distribution of the bacteria as demonstrated in Tables 6 and 7 does not follow any definite pattern. The concentration of bacteria and type of isolates varied greatly among the sampled trees and tissues used for the isolation. The total bacteria content of the current (1979) foliage were 5.8×10^2 and 7.6×10^3 colonies /g.d.w. for non-chlorotic and chlorotic trees, respectively. The older (1977) needles and branches of the non-chlorotic and chlorotic trees contained 2.2×10^4 and 5.3×10^3 bacteria/g.d.w., respectively. The bark samples from the stem of the non-chlorotic tree gave a high count of 7.5×10^4/g.d.w. while samples of the bark including the brown spots from the stem of the chlorotic tree produced a low population count of 2.4×10^3/g.d.w. Bacteria populations recorded in the xylem of the stems of the non-chlorotic tree averaged 9.3×10^2 bacteria cells /g.d.w. while the same tissue of the chlorotic tree produced 5.5×10^2 bacteria cells /g.d.w. Bacteria content of the large roots was higher in the chlorotic tree (9.7×10^3 and 7.1×10^3 /g.d.w. in the bark and xylem of the roots, respectively) than in the non-

chlorotic tree where 5.5×10^3 and 1.3×10^3 bacteria were present in the bark and xylem of roots, respectively. The fine roots produced the highest bacteria population. Bacteria isolated from the fine roots of the non-chlorotic and chlorotic trees were 1.9×10^6 and 1.7×10^4/g.d.w., respectively.

4.3 Pathogenicity Tests

4.3.1 Seed Inoculation

In general, the bacteria inoculations had more effect on the germination of white spruce than black spruce. The differences between the control and the nine treatments were not statistically significant for black spruce when tested by a Chi-square contingency table. The effect of the bacteria inoculations was more pronounced on the seed of white spruce (Figs. 11 and 12), three (E_1, all isolates combined, PS_1) of the nine treatments showed substantial inhibitory effects. Total germination 14 days after planting were 43, 43, and 45%, respectively, as compared to 58% germination of the control. The difference between the control and treatments (all isolates combined and E_1) were statistically significant at $P = 0.05$.

A stimulatory effect on germination of white spruce seed appeared to be induced by treatment $E_3 + PS_2 + U_1 + S_4$. Total germination in this treatment 10, 11, 12, and 13 days after planting were 61, 31, 44, and 36% respectively, above that of controls (Fig. 12). Statistical analysis using a Chi-square contingency table verified a significant difference

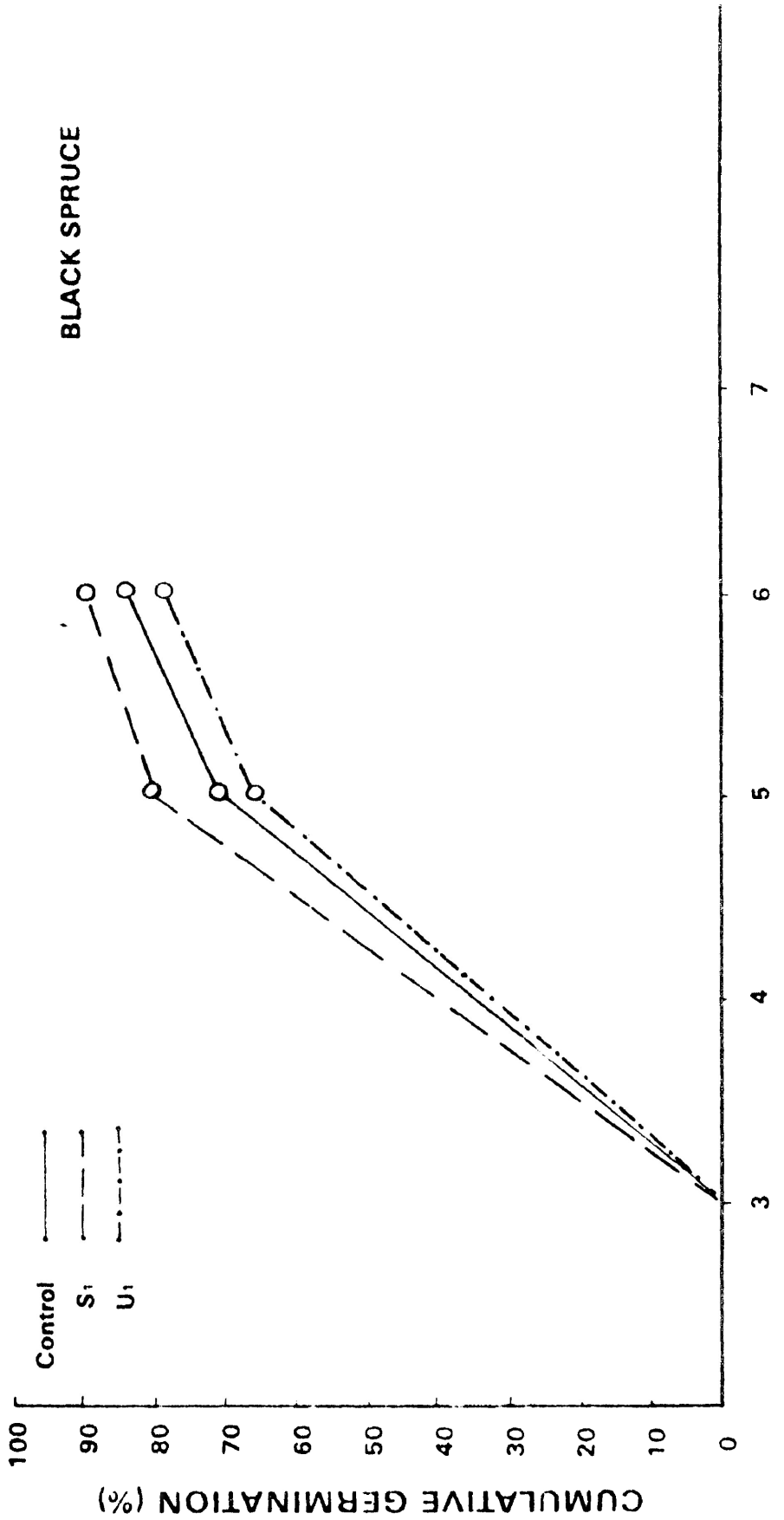


Fig. 11 Cumulative germination of black spruce seed after inoculation.

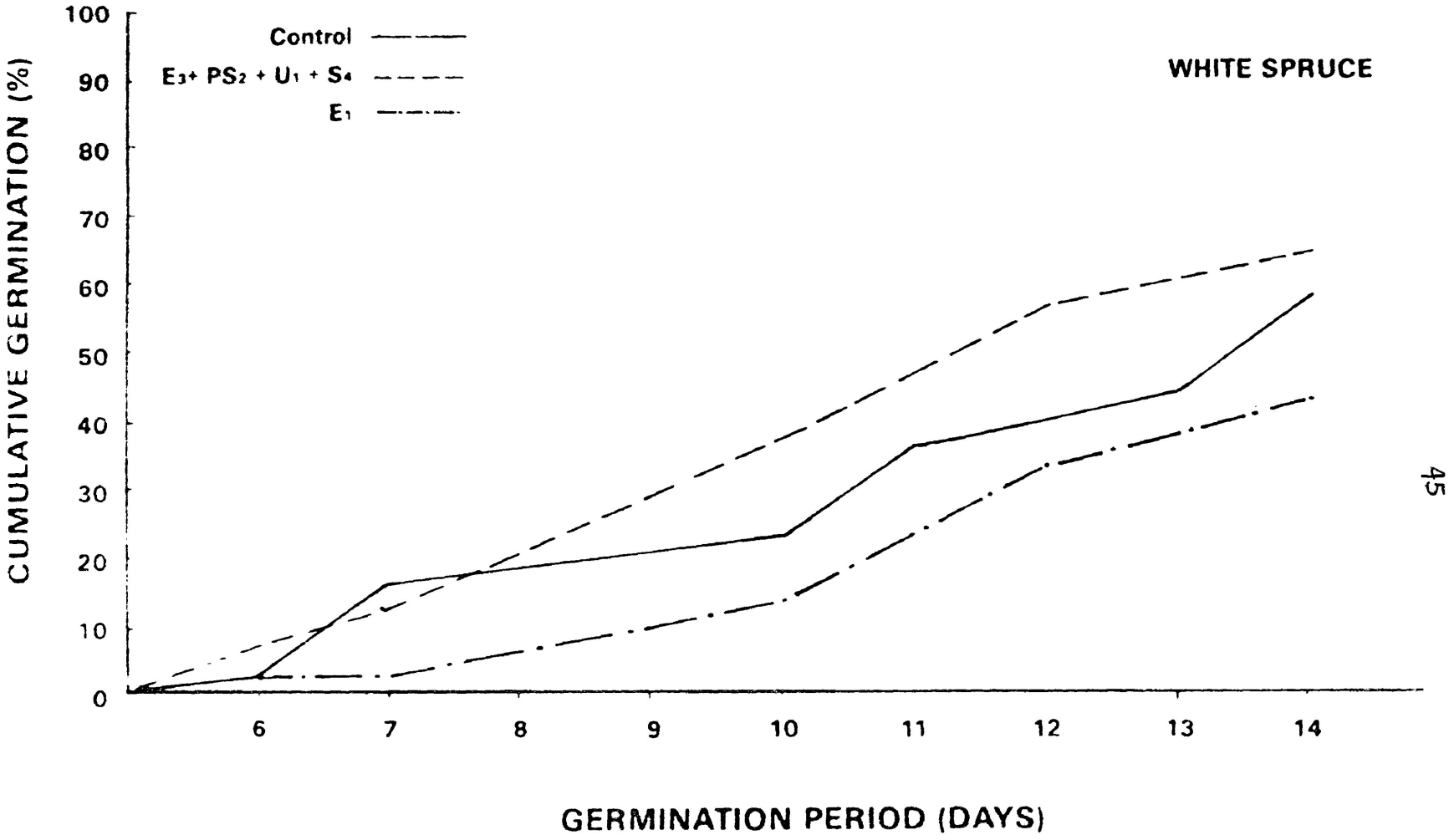


Fig. 12 Cumulative germination of white spruce seed after inoculation.

(χ^2 = 0.05, 0.10, 0.025, 0.025) between treatment $E_3 + PS_2 + U_1 + S_4$ and control for day 10, 11, 12 and 13, respectively.

4.3.2 Hypocotyl Inoculation

All the bacteria inoculations produced various hypocotyl symptoms of various intensities on both black and white spruce within 2 weeks after inoculation, though the incidence of the symptoms was not always significantly higher than the controls. The most conspicuous symptoms were stunting, yellowish-brown superficial discoloration, localized swelling, tip necroses of hypocotyl, and needle deformation (Figs. 13, 14, 15). There was no evidence of yellowing of the cotyledons.

All the inoculations consistently reduced hypocotyl elongation in the black spruce when compared to the control (Fig. 14). Hypocotyls inoculated with treatments (P_1, S_1, P_3, E_1, $E_2 + S_3 + P_2$, PS_1, $E_5 + E_4 + S_2$, $E_3 + PS_2 + U_1 + S_4$, all isolates combined) elongated 16, 23, 16, 22, 15, 6, 26, 12, and 14% less, respectively than the control. Analysis of variance (Table 8), however, revealed no significant differences between treatments.

Both inhibitory and stimulatory effects seemed to be induced by the bacterial inoculations on white spruce germinants. Two treatments (P_1, PS_1) showed a reduction in hypocotyl length, while four treatments (S_1, P_3, $E_2 + S_3 + P_2$, $E_3 + PS_2 + U_1 + S_4$) increased the hypocotyl length

Fig. 13 Stunting effect of bacteria inoculation on germinating white spruce seeds.

Fig. 14 Stunting symptoms induced by the bacteria inoculation on black spruce seedlings. Inoculated seedlings left, uninoculated seedlings right.

Fig. 15 Localized swelling symptom induced by bacteria on black spruce seedlings.

compared to the control. The differences between treatments were statistically significant ($p = 0.005$) (Table 9). Comparing the control against each of the other treatment means (Dunnett two-sided test) showed a significant ($p = 0.05$) difference (stimulatory) between treatment P_3 and the control only.

Hypocotyl and cotyledon symptoms of black and white spruce induced by the bacteria are summarized in Tables 10 and 11, respectively. All the tested bacteria were capable of producing distinct hypocotyl symptoms on the black spruce seedlings. The control showed lower frequency of symptoms. One of the symptoms - localized swelling (Fig. 15), which was present frequently in 5 treatments (P_1, E_1, $E_2 + S_3 + P_2$, PS_1, $E_5 + E_4 + S_2$), was never observed on control seedlings. Average incidence of hypocotyl discoloration was 56% and ranged from 30 to 80% in inoculated seedlings in contrast with 12% average incidence in the control. Treatments P_1, S_1, E_1, $E_2 + S_3 + P_2$, $E_5 + E_4 + S_2$ and $E_3 + PS_2 + U_1 + S_4$ showed high incidence of stunting, ranging from 21 to 42% as compared to only 10% in the control.

In contrast to the results for black spruce, the inoculated white spruce seedlings showed no increase in the frequency of most symptoms with the exception of localized swelling. The average incidence of localized swellings in treated seedlings ranged from 8 to 22% as compared to 7% for the control. In fact, higher frequencies of some symptoms were more apparent in the control than in treated seedlings (Table 11).

Table 8 Analysis of variance of total hypocotyl length of black spruce 14 days after inoculation.

Source of variation	DF	SS	MS	F	P
Treatments	9	90.36	10.04	1.2^{ns}	
Error	30	250.92	8.36		
Total	39	341.28			

Table 9 Analysis of variance of total hypocotyl length of white spruce 14 days after inoculation.

Source of variation	DF	SS	MS	F	P
Treatments	9	354.80	39.42	4.85 **	P(F> 4.85) <<.005
Error	30	243.92	8.13		
Total	39	598.72			

Table 10 Frequency (%) of hypocotyl and cotyledon symptoms on black spruce seedlings.[1]

Tr.	No. seedl.	St.	Dis.	Sw.	Dd. tip.	Rd. Cot. length	Rd. Cot. no.	Cot. deformity
P_1	(65)	21	65	20	11	47	14	18
S_1	(66)	30	73	5	18	56	12	35
P_3	(72)	8	33	4	10	38	18	14
E_1	(67)	23	62	22	10	38	12	40
$E_2 + S_3 + P_2$	(70)	27	60	24	4	56	11	37
PS_1	(60)	7	30	15	3	38	8	18
$E_5 + E_4 + S_2$	(71)	42	80	15	17	49	10	34
$E_3 + PS_2 + U_1 + S_4$	(69)	23	59	6	6	55	16	32
All Isol.	(69)	17	46	12	9	39	12	16
CONTROL	(68)	10	12	0	1	21	9	10

Tr. = treatment; seedl. = seedlings; St. = stunting; Dis. = discoloration; Sw. = swelling; Dd. = dead; Rd. = reduced; Cot. = cotyledon

1 Numbers of seedlings used for each treatment are indicated parenthetically.

Table 11 Frequency (%) of hypocotyl and cotyledon symptoms on white spruce seedlings.[1]

Tr.	No. seedl.	St.	Dis.	Sw.	Dd. tip.	Rd. Cot. length
P_1	(40)	10	53	8	5	35
S_1	(42)	24	54	17	10	52
P_3	(41)	17	39	20	27	48
E_1	(41)	29	58	17	20	60
$E_2 + S_3 + P_2$	(42)	10	74	10	14	62
PS_1	(42)	17	39	20	27	48
$E_5 + E_4 + S_2$	(38)	8	66	5	11	47
$E_3 + PS_2 + U_1 + S_4$	(45)	7	60	22	11	36
All Isol.	(37)	8	54	16	14	41
CONTROL	(41)	27	80	7	22	59

Tr. = treatment; seedl. = seedlings; St. = stunting; Dis. = discoloration; Sw. = swelling; Dd. = dead; Rd. = reduced; Cot. = cotyledon

1 Numbers of seedlings used for each treatment are indicated parenthetically.

4.3.3 Development of Seedlings after Transplanting

The effects of bacteria inoculation on black and white spruce seedlings are summarized in Tables 12 and 13. In most cases the dry weight of roots was higher in control black spruce seedlings than in treated seedlings (Table 12). Conversely, the inoculated white spruce seedlings seemed to produce better root biomass than the control (Table 13). However, in both species, the differences between treatments were not statistically significant.

Inoculation by the bacteria reduced significantly the total root length of black spruce seedlings (Table 14). Fig. 16 illustrates results of Table 14. Means of treatments E_1, $E_5 + E_4 + S_2$, $E_3 + PS_2 + U_1 + S_4$ and P_3, PS_1, for all isolates were found to be significantly different (Dunnett two-sided comparison) from the control at $p = 0.01$ and 0.05, respectively. In these treatments, the reduction of root length was very substantial, ranging from 4 to 43.5% reduction in root length. The opposite was true for white spruce, where at least four treatments seemed to enhance the root development, when compared to the control (Fig. 17). Analysis of variance did not show significant differences between treatments (Table 15). However, great variation between effects of different treatments, for example, the difference between the inoculation by *Serratia* sp. (S_1) and the inoculation combining all isolates was observed.

Table 12 Effect of bacteria inoculation on black spruce seedlings.

Tr	Rt Wgt (mg)		Tot Rt Lgt	St Ht	Mortality[1]	
	Fresh	Dry	(cm)	(cm)	(%)	
P_1	562.75	136.75	57.80	9.43	17	(65)
S_1	634.75	141.25	66.15	9.28	18	(67)
P_3	583.75	126.50	50.67	9.23	12.5	(72)
E_1	522.25	117.25	48.59	8.83	21	(67)
$E_2 + S_3 + P_2$	577.00	126.50	69.90	9.45	14	(70)
PS_1	573.75	123.25	51.95	8.75	12	(60)
$E_5 + E_4 + S_2$	403.50	97.75	41.10	8.48	24[c]	(70)
$E_3 + PS_2 + U_1 + S_4$	525.50	118.75	48.83	9.30	14.5	(69)
ALL ISOLATES	529.00	123.25	54.33	9.40	8	(69)
CONTROL	579.50	140.25	72.68	9.68	12	(68)

c ..... significantly different at $p = 0.05$ (Chi-square test) from control.

Tr = treatment; Rt = Root; Tot = Total; St = stem; Lgt = length: Ht = height

1 Numbers of seedlings used for each treatment are indicated parenthetically.

Table 13 Effect of bacteria inoculation on white spruce seedlings.

Tr	Rt Wgt (mg) Fresh	Rt Wgt (mg) Dry	Tot Rt Lgt (cm)	St Ht (cm)	Mortality[1] (%)
P_1	306.25	58.75	42.30	3.35	15 (39)
S_1	297.00	58.75	43.35	3.18	21[c] (42)
P_3	244.25	44.50	36.43	3.10	29 (41)
E_1	229.25	47.50	36.58	2.60	35 (37)
$E_2 + S_3 + P_2$	269.00	60.75	36.10	2.88	17[b] (42)
PS_1	260.25	52.00	39.00	2.88	44 (43)
$E_5 + E_4 + S_2$	327.25	57.00	33.13	2.88	10[a] (42)
$E_3 + PS_2 + U_1 + S_4$	332.25	59.50	40.80	3.08	15[a] (47)
ALL ISOLATES	235.75	35.50	31.53	2.65	24 (37)
CONTROL	253.50	46.25	34.25	2.48	34 (41)

Significantly different at P = 0.005[a]; 0.01[b]; 0.05[c] from control.

Tr = treatment; Rt = root; St = stem; Lgt = length; Ht = height

1 Numbers of seedlings used for each treatment are indicated parenthetically.

Table 14 Analysis of variance of total root length of inoculated black spruce seedlings 3 months after transplanting.

Source of variation	DF	SS	MS	F	P
Treatments	9	3812.62	423.63	5.49**	P(F> 5.49) << .005
Error	30	2315.10	77.17		
Total	39	6127.72			

Table 15 Analysis of variance of total root length of inoculated white spruce seedlings 3 months after transplanting.

Source of variation	DF	MS	F	P
Treatments	9	558.10	62.01	$.79^{ns}$
Error	30	2350.08	78.34	
Total	39			

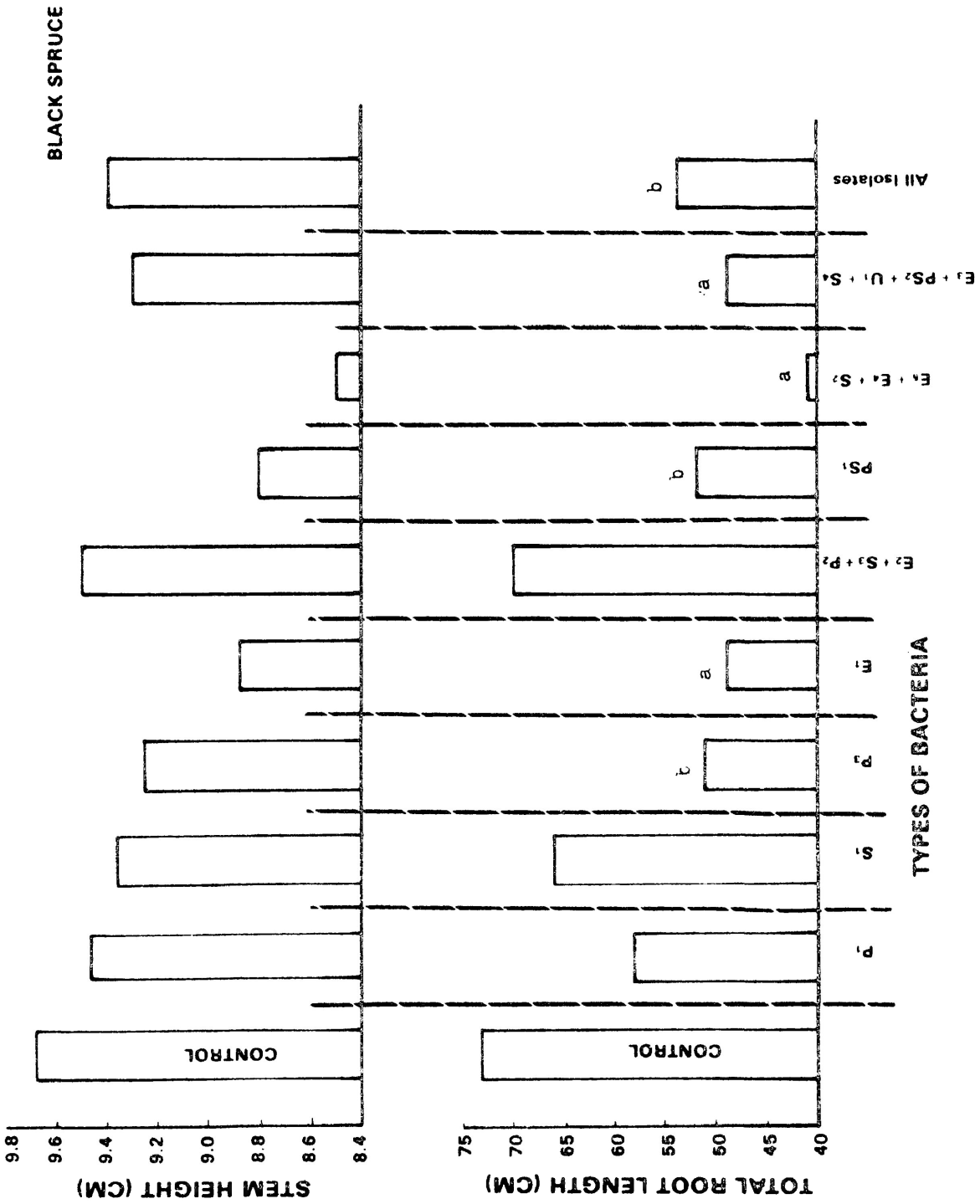


Fig. 16 Mean total root length and stem height of black spruce seedlings 3 months after inoculation with bacteria. Values differ significantly [a] ($P = 0.01$), [b] ($P = 0.05$) from the control value by Duncan's multiple range test. Averages are based on 4 replications.

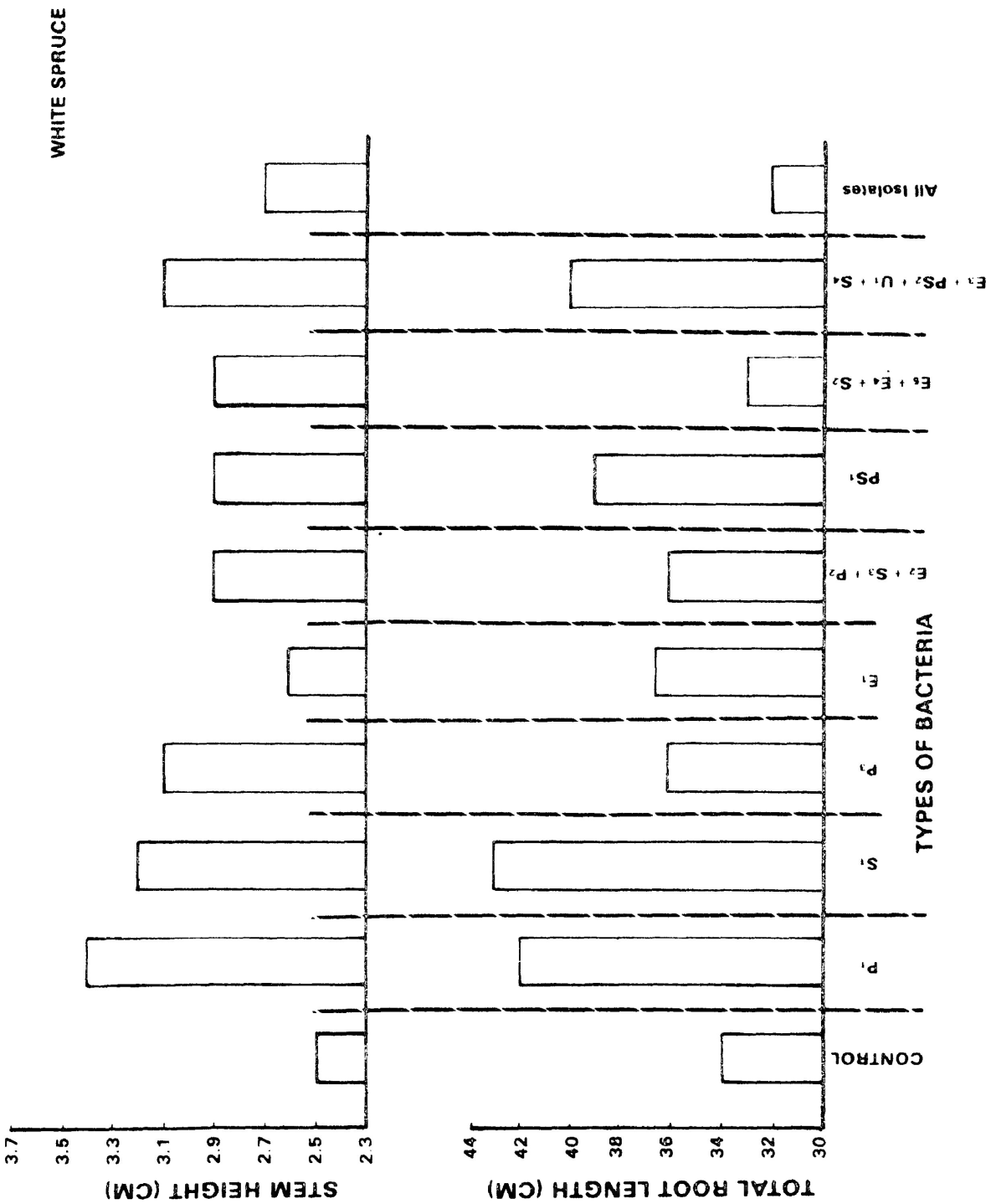


Fig. 17 Mean total root length and stem height of white spruce seedlings 3 months after inoculation with bacteria. Averages are based on 4 replications.

None of the bacteria isolates affected significantly the height growth of both spruce species (Tables 12, 13). Some pattern of effect, however, was shown in the stem height of the black spruce seedlings. In all inoculated treatments, stem heights of the black spruce seedlings were consistently lower than those of the control. Three of the treatments (E_1, PS_1, $E_5 + E_4 + S_2$) produced considerably lower stem heights ranging from 9 to 12% below that of the control. Great differences in the effect of the inoculations existed between treatments. However, these differences were not significant.

Bacteria inoculation seemed to be associated with increased mortality in the treatments of black spruce seedlings (Table 12). Death of seedlings began with a gradual yellowing of the lower needles. The entire seedling faded in color and within 4 weeks needles turned brown. Death occured when all the needles turned brown. The same sequence of symptoms was observed in white spruce seedlings. The highest mortality caused by inoculation with Enterobacter sp. (Erwinia sp.) E_5, E_4, and Serratia sp. (S_2) in the black spruce seedlings was statistically significant ($p = 0.05$) when compared to the control. The opposite trend was observed in white spruce where five inoculations produced significantly lower mortality than that observed in the control. This trend compares well with the similar observations made for seedlings root weights and total lengths.

4.3.4 Green Cutting Inoculation

Results of rooted green cuttings of black and white spruce 8 weeks after inoculation are summarized in Table 16. The results show differences in rooting attributable to both the host species and bacteria inoculations. Black spruce showed very good rooting ability, averaging 84% in control. The rooting ability of white spruce control was about 10% lower.

All the bacteria inoculations reduced the rooting ability of cuttings as compared to the control, though the effect was not significant in all treatments (Table 16). The differences between inoculated and control cuttings were clearly discernible by visual observations (Figs. 18 to 24). Isolate U_1 (Unknown species) reduced the rooting ability of black and white spruce by 23 and 13%, respectively (Table 16); these differences were significant (Chi-square test) at the 99.5 and 95% levels, respectively. The effect of inoculation with one of the Enterobacter (Erwinia) species (E_5) on rooting ability of white spruce cuttings was not statistically different being, 9% below that of the control. However, the same isolate reduced significantly the rooting ability of black spruce cuttings by 11.5%. Conversely, mixed inoculum comprised of Enterobacter hafniae, E. agglomerans, and Serratia liquefaciens ($E_4 + E_3 + S_4$) showed a strong negative effect on the rooting ability of white spruce cuttings; i.e. 24% had fewer roots than the control. However, only a slight reduction was observed for black

Table 16 Percentage of rooted green cuttings of black and white spruce 8 weeks after inoculation.

Inoculum (Treatment)	% Rooted [a] Black spruce	White spruce
E_1	76[ns]	61*
U_1	61**	61*
E_5	72.5*	65[ns]
$E_4 + E_3 + S_4$	77.5[ns]	50**
CONTROL	84	74

[a] Mean of 80 cuttings; significantly different from control at *P = 0.05, **P = 0.005. [ns] not statistically different from control. (Chi-square test).

Fig. 18 Picea mariana control cuttings rooted for 8 weeks in Spencer-Lemaire containers.

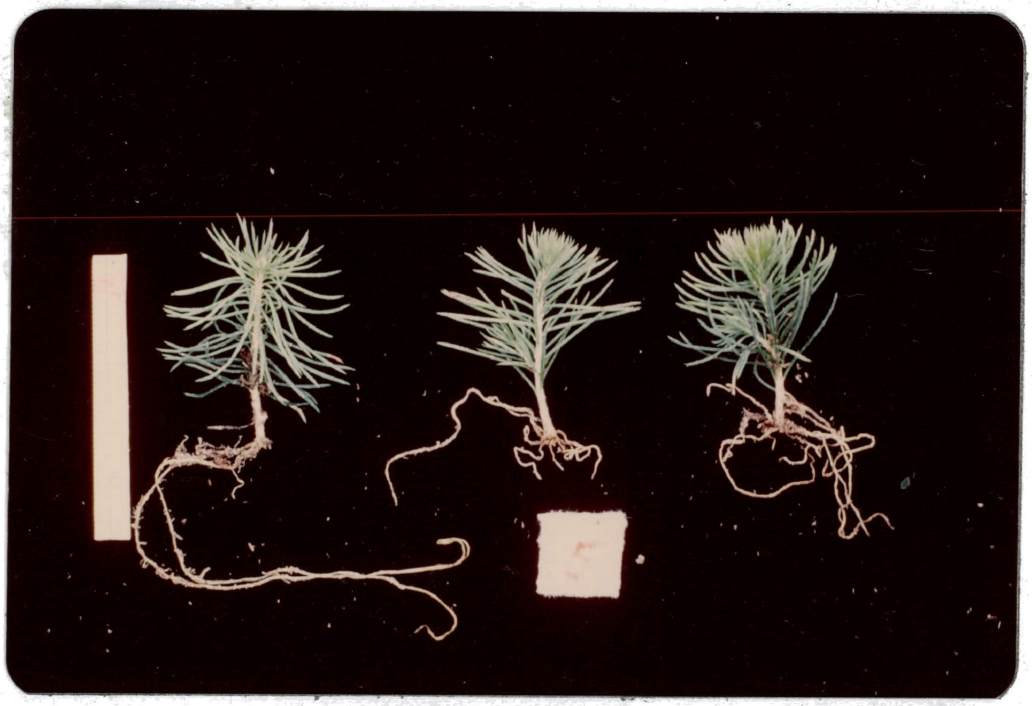


Fig. 19 Picea glauca control cuttings rooted for 8 weeks in Spencer-Lemaire containers.

Fig 20 Picea mariana cuttings inoculated with Erwinia sp. (E_1) and rooted for 8 weeks in Spencer-Lemaire containers.

Fig 21 Picea mariana cuttings inoculated with isolate U_1 and rooted for 8 weeks in Spencer-Lemaire containers.

Fig 22 Picea mariana cuttings inoculated with Enterobacter (Erwinia) sp. and rooted for 8 weeks in Spencer-Lemaire containers.

Fig 23 Picea mariana cuttings inoculated with mixed culture of $E_4 + E_3 + S_4$ and rooted for 8 weeks in Spencer-Lemaire containers

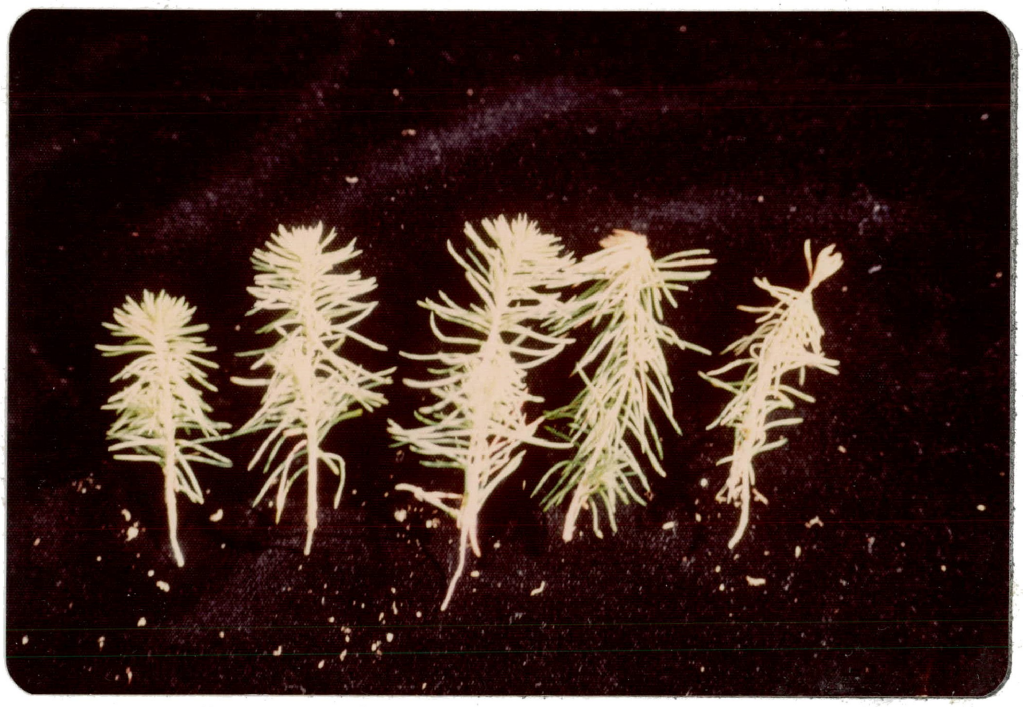


Fig. 24 Picea glauca cuttings inoculated with mixed culture of $E_4 + E_3 + S_4$ and rooted for 8 weeks in Spencer-Lemaire containers

spruce cuttings. A pure culture of E. agglomerans (E. herbicola) also reduced significanly the rooting ability of white spruce as compared to the controls.

The effects of the inoculations on the number and lengths of roots of both white and black spruce formed on cuttings are summarized in Table 17 and Fig. 25. Analysis of the variance of the total root length data is presented in Table 18 for white spruce and in Table 19 for black spruce. Significant differences exist between treatments at $p = 0.025$ and 0.1 for white and black spruce, respectively. In general, all of the inoculations had a negative effect on the root development of inoculated cuttings of both species (Fig. 25). Isolate U_1 (Unknown species) again showed the greatest effect. The root lengths of both white and black spruce cuttings were reduced by 61.6 and 44.5%, respectively, below that of controls, when inoculated by this bacterium (Table 17). Two other treatments, E_1 and a mixed culture of $E_4 + E_3 + S_4$, also affected and reduced significantly total root lengths of white spruce cuttings by 45 and 54.5%, respectively, but had less pronounced effects on black spruce.

An analysis of the variance of root number in Table 17 showed no significant difference between black spruce treatments (results not shown), and the difference between that of white spruce treatments was significant at only the 10% level (Table 20).

Table 17 Root number and total root lengths of black and white spruce 8 weeks after inoculation.

Inoculum	Root length (cm)		Root number	
	white spruce	black spruce	white spruce	black spruce
E_1	2.71*	37.18	2,78*	4.75
U_1	1.90**	24.64*	3.06	3.55
E_5	3.36	40.13	2.63*	5.20
$E_4 + E_3 + S_4$	2.25*	31.77	3.29	4.40
CONTROL	4.95	44.38	4.56	5.48

Means are significantly different from control at * $p = 0.05$, ** $p = 0.01$ (Chi-square test).

Table 18 Analysis of variance of total root length of inoculated white spruce cuttings.

Source of variation	DF	SS	MS	F	P
Blocks	3	4.90	1.63	1.30^{ns}	
Treatments	4	22.93	5.73	4.58**	$P(F > 4.6) << 0.025$
Error	12	15.01	1.25		
Total	19	42.84			

Table 19 Analysis of variance of total root length of inoculated black spruce cuttings.

Source of variation	DF	SS	MS	F	P
Blocks	3	413.36	137.79	1.61^{ns}	
Treatments	4	940.30	235.08	2.74*	$P(F > 2.74) < .10$
Error	12	1028.91	85.74		
Total	19	2382.57			

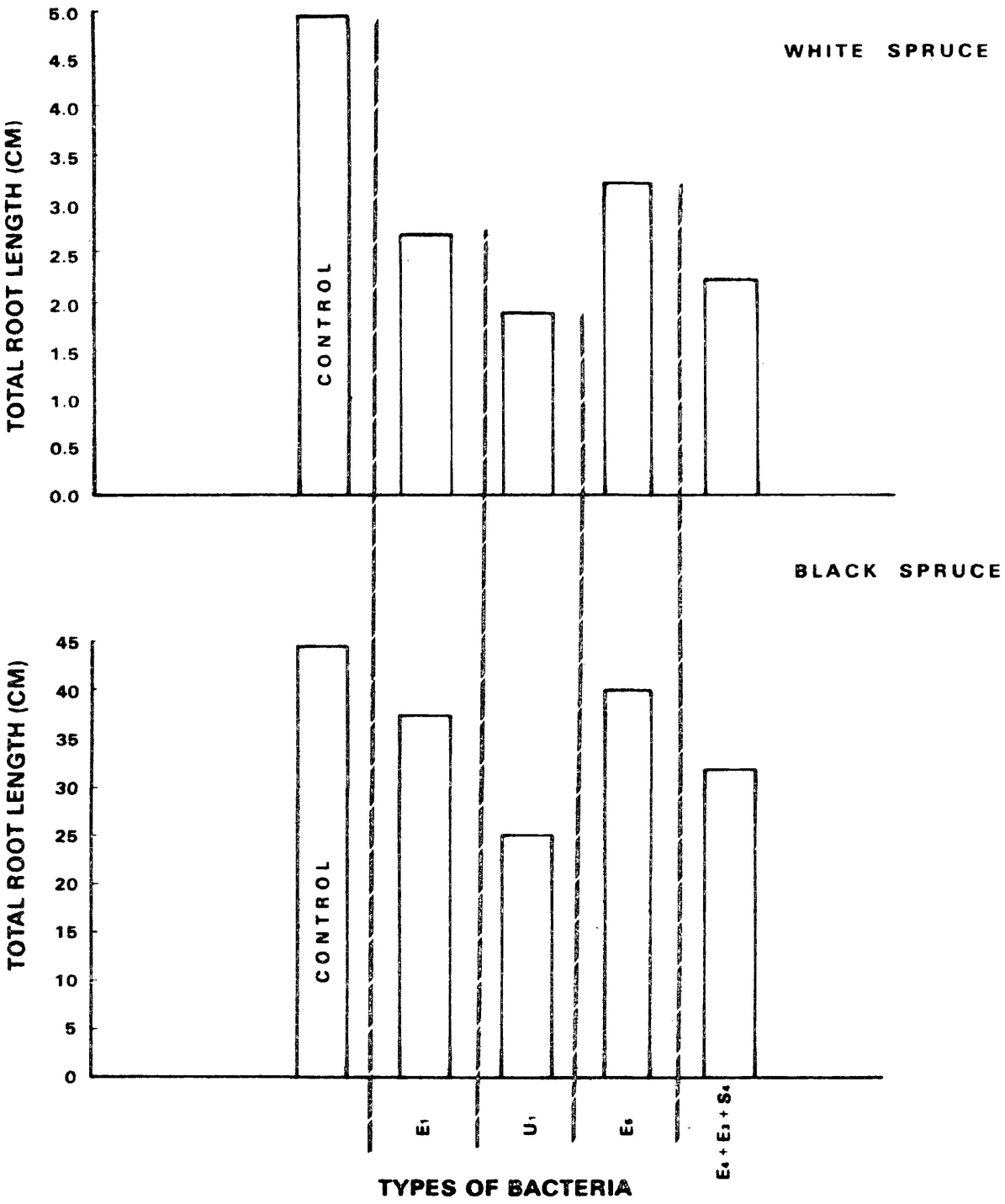


Fig. 25 Total root lengths of black and white spruce 8 weeks after inoculation.

Table 20 Analysis of variance of number of roots formed on white spruce cuttings.

Source of variation	DF	SS	MS	F	P
Blocks	3	1.36	.45	$.65^{ns}$	
Treatment	4	8.48	2.12	3.07^{*}	$P(F > 3.07) < 0.10$
Error	12	8.27	.69		
Total	19	18.11			

4.3.5 Re-isolation of Bacteria

High concentrations of bacteria were isolated from the callus and roots of both inoculated cuttings (in average 1.5×10^7/gm of fresh weight) and control cuttings (in average 3.2×10^5/gm of fresh weight). The original bacteria types used for the inoculations were consistently re-isolated from cuttings of the appropriate treatments in all inoculations of black and white spruce. The identities of the re-isolated bacteria were determined by comparing their cultural, morphological and biochemical characteristics to the original bacteria types. The re-isolated bacteria showed identical properties as the original isolates from pure cultures. In addition to the tested bacteria several other bacteria were isolated. No attempts were made to identify the bacteria isolated in association with the tested types.

Re-isolation attempts were also made from the roots of seedlings (section 4.3.3) showing significant pathological responses. Identities of the

re-isolated types of bacteria were assessed when the morphological characteristics of colonies were compared to those of the original types. By this procedure the bacteria isolates seem to be identical, although this judgement was not verified by biochemical tests.

5.0 DISCUSSION AND CONCLUSION

5.1 Taxonomic Relationship of Isolated Bacteria

The Analytical Profile Index (API Manual, 1978), which provides statistical data derived from the results obtained with API 20E and supplementary tests, was used for the identification of the Enterobacteriaceae and other gram-negative bacteria isolated in our study. The classification system of Bergey's Manual (Buchanan and Gibbon, 1974) was used.

The API data base has been derived mainly from strains of clinical origin, and its applicability to bacteria inhabiting or infecting forest trees has not yet been established. The present study has demonstrated the usefulness of the API 20E and the API numerical identification approach in characterising and identifying Enterobacteriaceae and other gram-negative bacteria from forest trees, at least, to the genus level. Further tests (physiological, biochemical, serological), however, are required in some cases to complete the identification to the species level or higher taxonomic ranks in a few instances.

The taxonomic summary of the 16 representative isolates is presented in Table 3. All the isolates belong to the family Enterobacteriaceae (Bergey's Manual, 1974). Three of the isolates were classified as Pseudomonas Migula,

while 5 of the isolates were shown to belong to the genus Enterobacter (Erwinia) Hormaeche and Edwards. Other genera of the tribe Klebsielleae isolated in this study were Serratia and Pasteurella (?) or Flavobacterium (?).

Two out of the three Pseudomonas Migula isolates were classified predominantly as P. maltophilia (P. melanogena). The genus Pseudomonas, as defined in Bergey's Manual (Buchanan and Gibbons, 1974), consists of a group of aerobic, usually polar (monotrichous, multitrichous) flagellated, gram-negative, asporogenous straight rods with average dimensions of 0.5 to 1 micron by 1.5 to 4 microns. Pseudomonas is a diverse bacterial genus. All members of the genus Pseudomonas are found in soil and water as well as on or within a variety of agricultural plants and forest trees (conifers and deciduous). It is not difficult to speculate on the mode of entry by these bacteria in view of inevitable woundings that occur in forest trees, e.g. storms, animals, insects, nematodes, and man. Many species are tree pathogenic (Hepting, 1971). In other instances, it is not known if their role in trees is beneficial or detrimental to the tree. The presence of Pseudomonas in living trees, often associated with discolored and decayed wood tissues (Cosenza et al. 1970; Chao and Ts'ai, 1958), is said to create suitable conditions for the growth of decay fungi in wood. The association of Pseudomonas and nonhymenomycetes may be mutualistic. Fungi produce organic acids (citric, lactic, and others) (Cochrane 1958) which are readily utilizable by bacteria. Conversely, some Pseudomonas spp. produce thiamine, which is essential for many species of decay fungi (Cochrane, 1958). However, it has not been shown

whether the Pseudomonas isolates recovered in this study can function in the above mentioned roles.

Two of the P. maltophilia isolates (P_2 and P_3) were confirmed by PRS (Profile Recognition System) with a high degree of probability to be similar to typical P. maltophilia. The final identification attained for isolates P_2 and P_3 is, therefore, considered more reliable than that of isolate P_1, which showed a low selectivity identification. To complete the identification of isolate P_1 to species level, further tests (biochemical and serological) will be required, but they are beyond the scope of this study. Thus, the present study, at least in two isolates, represents the first isolation of P. maltophilia (P. melanogena) from within living forest trees. The distribution of Pseudomonas maltophilia in nature seems to be very wide, since the reported sources of known strains have been water, soil, milk, frozen fish, rabbit and blood (Hugh et al. 1961).

Numerically, 31% of the representative isolates from white spruce trees were tentatively considered as Enterobacter Hormaeche and Edwards. Three out of the 5 Enterobacter isolates were classified as E. agglomerans, one as E. hafniae (Hafniae alvei), and the other could only be identified to the genus level, Enterobacter spp. Systematics of the Erwinia herbicola group have recently been revised. According to Ewing and Fife (1972), this group should be reclassified into the genus Enterobacter, and the correct specific epithet is E. agglomerans. The genus Enterobacter (Erwinia) is described in

Bergey's Manual as consisting of a group of usually peritrichously flagellated, gram-negative, asporogenous straight rods with average dimensions of 0.5 - 1.0 by 1.0 - 2.0 microns. The isolates from the white spruce trees have these characteristics. Perhaps the most reliable phenotypic properties exhibited by the E. agglomerans isolates from white spruce trees are their ability to produce acid from mannitol, sucrose, and amygdalin and their inability to decarboxylate arginine, lysine, and ornithine. Aho et al. (1974) observed the same properties exhibited by over 500 of their isolates (E. agglomerans) from the major decays in white fir trees. They also reported the N_2 - fixing ability of this species as determined by the acetylene reduction technique. In this study, however, the N_2 - fixing ability of the isolates (E. agglomerans) from the white spruce trees was not assessed. Nevertheless, it is plausible to assume the E. agglomerans isolates from this study are capable of fixing atmospheric nitrogen, especially where both isolates, ours and those of Aho et al., exhibit almost the same characteristics. Slight variation in reactions to a few of the tests are not unusual, since these isolates were from samples collected from different localities and at different times. Similar variations among isolates have been reported (Shinde and Lukezic 1974, Sands et al. 1970 , Misaghi et al. 1969).

Two isolates E_4 and E_5 are atypical of E. agglomerans because they fail to produce nitrate but they decarboxylate lysine and ornithine and produce urease. On account of this, the two isolates apparently are species

of the genus Enterobacter and most probably are ecotypes or physiotypes of the species E. hafniae Ewing (Hafnia alvei Moller). Members of Enterobacter are prevalent in forest soils and invade the tissues of living plants, causing dry necroses, galls, wilts, and soft rots (Laskin et al. 1977, Skerman 1967). Certain members of the genus produce pectinolytic enzymes; this trait is related to its ability to produce diseases. At present, we do not know whether all our Enterobacter isolates are members of the tree-soil ecosystem, or some possess the above mentioned pathogenic trait. Bacon and Mead (1971) isolated Erwinia from healthy aspen wood tissues. These bacteria, therefore, seem to be an indigenous microflora of aspen heartwood. Carter (1945) consistently isolated Erwinia nimipressuralis from the wetwood of elm in Illinois, however, the role of this bacteria in wetwood formation has not yet been established. Enterobacter (Erwinia) is also known to occur in black cottonwood (Gokhale, 1975), in white fir trees (Aho et al. 1974), in the bark of hemlock, grand fir, douglas fir, and cedar (Duncan and Razzell, 1972) and in many agricultural plants. To our knowledge there is no report in the literature on the presence of Enterobacter in spruce and this study seems to represent the first findings of Enterobacter in living tissues of white spruce trees.

The Profile Recognition System (PRS) showed a high degree of similarity (very good/ excellent) between profiles E_1, E_4, and E_5 and known profiles of E. agglomerans, E. alvei, and Enterobacter sp., respectively. Profiles E_2 and E_3, though identified as E. agglomerans, are less reliable. The

PRS showed good likelihood, but low selectivity for the two profiles. In light of the observations regarding the major differences and similarities between various Enterobacter spp. and the species of Enterobacter isolated from white spruce, it is felt that our isolates may not be distinct from the isolates derived from living trees in other studies.

Members of the genus Serratia Bizio are motile with peritrichous flagella, gram-negative, non-sporing aerobic or facultative anaerobic rods less than 2 microns wide, and grow on ordinary media. Almost all species liquefy gelatin. All species produce acid from glucose; nitrates are reduced to nitrites; and oxidase reaction is negative (Skerman, 1967). Members of this genus are found in water and are common members of the soil ecosystem. The isolate S_1, S_2, S_3 and S_4 from white spruce, identified as Serratia sp., Serratia sp., Serratia rubideae, and Serratia liquefaciens, respectively, all have the above mentioned characteristics. Their relationship to forest trees has not been established, but they are, at the moment, known to be soil saprophytes. Warren and Navratil (unpublished data) have isolated Serratia spp. from aspen wood. The present study , however, represents the first isolation of Serratia spp. from within coniferous trees. The identity of the Serratia genus and of the above named species is considered very reliable because all profiles considered were identified by the API-PRS with a high degree of probability.

According to the API Manual, isolate PS_1 was identified as Pasteurella (Yersinia enterocolitica), while PS_2 was shown to be either Pasteurella (?) or Flavobacterium (?). A further attempt to verify this initial identification was not made. The isolates PS_1 and PS_2 failed to survive on Knutson's media during the process of re-isolation and storage. The profiles of two other isolates, unknown-1 and unknown-2 could not be identified by the API-PRS. Studies made with cultures isolated from clinical specimens have established API 20E as the most complete available system for identification to species level of Enterobacteriaceae (Washington et al. 1971). Our limited study revealed both the usefulness but also the deficiency of this system when applied to bacteria inhabiting tree-soil ecosystem. Two of the isolates could not be identified by the API-PRS and many others were poorly identified. Taxonomically,it is possible that isolate U_1 and U_2 are known species of tree-soil ecosystem which have not been tested and included in API data base or they could be a new species not yet taxonomically classified. In view of this, it is important to point out that one of the unknown isolate (U_1) was shown to be pathogenic on spruce seedlings, and its taxonomic relations need to be clarified in further studies.

5.2 Distribution of Bacteria in White Spruce

The results of the present study show that a number of aerobic and facultative anaerobic bacteria occur in chlorotic as well as in ap-

parently healthy, non-chlorotic white spruce trees. This was shown by the isolation of approximately 16 different types of bacteria. Although bacteria were present in all of the trees, qualitative and quantitative differences existed between healthy and chlorotic trees and between sections of the trees.

It has been well documented that bacteria inhabit the tissues of living trees. Bacteria have been isolated from apparently healthy trembling aspen (Bacon and Mead 1971, Knutson 1968, Warren 1978),from the pith and heartwood of grand fir (Aho and Hutchins 1975), from the pith and heartwood of balsam fir and black spruce (Etheridge and Morin 1967), from douglas fir, western hemlock and alpine fir (Bier 1966), from normal sapwood and heartwood of yellow poplar trees (Roth 1950), from maple tree tapholes (Sheneman and Costilow 1958), and from healthy looking Pinus ponderosa and Alnus tenuifolia (Bacon and Mead 1971). The association of bacteria with root systems (Dangerfield et al 1975, 1978), bark (Duncan and Razzell 1972), and the normal foliage and buds (Kenner 1945, 1950; Leben 1972; Walsh 1979) of forest trees has also been reported.

Examination of the data (Table 4) of the 1978 study shows that bacteria were isolated from all tree parts. Higher concentrations of bacteria were present in the chlorotic trees than in the non-chlorotic trees. The highest bacterial populations in the chlorotic trees were located in

the stem, especially in the brown spots of the bark tissues, where approximately 6.0 X 10^5 bacteria per gram dry weight were isolated. In the non-chlorotic trees, bacteria concentration was lower in all sections when compared to the chlorotic trees. The much higher concentration of bacteria found in the chlorotic trees thus might suggest their involvement in the observed disorder.

In the 1979 study conducted in the summer, considerable variation of bacteria numbers existed among and within the sampled trees. Though the distribution of bacteria does not follow any definite pattern, the results listed in Tables 6 and 7 demonstrate clearly that bacteria populations are more concentrated in the lower portions of the trees than in the upper portions, an observation which is in agreement with the results obtained by Cameron (1970) and Warren and Navratil (unpublished data). Cameron (1970) observed the variation in bacterial numbers when assessing the Pseudomonas content of cherry trees. The fact that bacterial populations tend to decrease as the tree height increases may be the natural phenomenon of bacteria microflora in standing trees. The results of the present study also show fewer bacteria in the upper portion of the trees than in either the middle or lower portion.

An examination of the data (Table 5) does not demonstrate any specific relationship of Enterobacter (Erwinia) spp. to the healthy condition of a tree. Enterobacter spp. are present in all chlorotic as well

as in all non-chlorotic trees. However, P. maltophila (P. melanogena) and Serratia rubideae were present only in the apparently non-chlorotic trees and were predominantly isolated from the lower portions of the trees. Conversely, Serratia sp. (S_2) and Pasteurella or Flavobacterium sp. were present only in the current (1979) needle and twigs of the chlorotic trees.

The presence of P. maltophilia and Serratia rubideae only in the healthy non-chlorotic trees as opposed to Serratia sp. (S_2) and Pasteurella or Flavobacterium species being present only in the chlorotic trees might reflect the differences in substrate conditions occurring in healthy and chlorotic trees in conjunction with the different nutritional and environmental requirements of the aforementioned isolates.

Factors contributing to the higher bacterial population in non-chlorotic trees found in the 1979 study might be due to the method of sampling. Trees of the 1978 and the 1979 studies were sampled from different localities, and therefore rhizosphere effect could be a factor. In addition to the above interpretation, it is apparent from the data obtained from the two studies in the Fall of 1978 and in the Summer of 1979 that seasonal change in bacterial populations within the white spruce trees may occur. Cameron (1970) also observed a seasonal change in the Pseudomonas content of cherry trees. His results show that there is a rapid increase in bacteria content of trees from January to March, a gradual decline through Summer

(April-August), and an increase in the Fall (August-November). The seasonal change in the bacterial content of the white spruce trees may be due to differences in the atmospheric conditions, especially in the temperature, the air humidity, soil water contents and related transpiration and absorption of water. Cameron (1970) also observed a correlation between the numbers of bacteria and rainfall. The increase in available water not only spreads the bacteria as they move through the tree, but also may stimulate an increase in the total number of bacteria.

Our concentration data (Tables 4, 6 and 7) compare well with the total bacterial counts reported from other healthy and infected tree species. Total bacterial populations determined from the early stages of decay in white fir infected with Phellinus chrysoloma and Hericium abietis were 39.3×10^6 and 7.0×10^6 bacterial colonies per ml. of expressed sap, respectively (Aho et al. 1974). Bacterial populations from sound and defective aspen clones ranged from approximately 3.0×10^2 to 10.0×10^6 per gram dry weight in sound clones and 2.4×10^3 to 5.6×10^6 per gram dry weight in defective clones (Warren 1978).

In addition to the observed seasonal changes in bacteria concentration, the data presented in this study may also indicate that some bacteria might be temporarily absent in certain tree tissues. Bacteria such as Acinetobacter and some fluorescent Pseudomonas species which were consistently isolated during the previous and fall study (Navratil and Smith, unpublished data; Mireku, unpublished data) were never found in the trees sam-

pled in the summer. This may reflect the lower population of these bacteria during the summer months or the condition of tree tissues, thus, affecting the efficiency of isolation. The difficulty in isolating P. syringae-like organisms during the summer has already been reported (Dowler and Weaver 1974, Dye 1954, Gardan et al. 1971).

5.3 Pathogenicity of Bacteria Isolates

Very little is known about the bacteria of deciduous and coniferous trees in the Boreal Forest and their role in the trees and in the development of disease.

In this study, many species of bacteria belonging to the genera Pseudomonas Migula, Serratia Bizio, Pasteurella (?) Trevisan and Enterobacter (Erwinia) Hormaeche and Edwards were isolated.

Some species of the genus Pseudomonas are known to cause diseases of forest trees (Hepting 1971), however, no bacteria belonging to the genera Pseudomonas is known to cause diseases in spruce trees. In our study, we could not establish the close association of Pseudomonas species with the chlorotic trees; they were also isolated from control trees. None of our pathogenicity tests, seed inoculation or hypocotyl inoculation, succeeded in inducing repeatedly pathological responses on spruce seedlings. We observed only one significant pathological response associated with this

genus. Pseudomonas maltophilia, reduced root growth when inoculated on the roots of black spruce germinants. The inability of the majority of the Pseudomonas isolates to induce a significant mortality and to reproduce original symptoms (chlorosis or brown spots) on spruce seedlings suggests that the Pseudomonas isolates recovered in this study are not pathogenic to spruce trees.

No consistent pathological phenomena were detected in the inoculation with Serratia sp. alone. This result is in agreement with the known habitat of this genus since Serratia sp. is a common member of water and soil ecosystem and no plant pathogenic species of this genus has been reported.

The inoculations with the unidentified isolate (U_1) revealed some pathogenicity. This isolate reduced significantly both the rooting of cuttings and the subsequent root growth of both black and white spruce. The significance of this finding cannot be assessed at present. Further tests to identify this isolate are warranted.

The association of some Erwinia species with plants and trees as pathogens is well known. They cause necroses, wilting, and soft rot diseases. Recently, Erwinia cancerogena Ur. has been shown to cause bark necroses and resinosis of Picea exelsa L. in Europe (Urosevic 1968). In our study, Enterobacter (Erwinia) species isolated from white spruce

produced pathological responses on young spruce seedlings. These results showed that the Enterobacter species alone or mixed with other bacteria species can cause pathological changes such as superficial brown discoloration, stunting, necroses, and swellings on the hypocotyl of spruce germinants. A high rate of mortality as well as a reduction in root growth and the growth of the above-ground parts of black spruce seedlings was also associated with the inoculation by Enterobacter species.

The Enterobacter spp. produced the most pronounced effects on the root development of germinating seedlings, and their effect on the rooting of vegetatively propagated (green cuttings) black and white spruce was also pronounced. In the latter test, in all cases, the rooting ability and the number of roots formed on the inoculated cuttings of both spruce species were inhibited. The inhibitory effect was more pronounced on white spruce than on black spruce, especially when the inoculum consisted of the combination of Enterobacter and Serratia isolates.

It is clear that chlorosis is a complex problem involving many factors. However, our results of bacteria distribution and pathogenicity studies have demonstrated that involvement of bacteria in this disorder is plausible. Bacteria could play a predisposing role or act as an initiating factor in the sequence of events terminating in chlorosis.

The inoculation tests proved that the bacteria, particularly the isolates belonging to the genus Enterobacter and one Unknown isolate (U_1), can alter, inhibit, or necrotize young developing roots of spruce seedlings. In addition, we demonstrated the inhibitory effects of bacteria on the development of roots formed on vegetatively propagated cuttings. The poor condition of the feeder roots of the chlorotic white spruce trees was reported (Whitney, unreported data). In view of the above results, this might have resulted from bacteria infection, perhaps in combination with severe drought. The reduced efficiency of feeder roots could, in turn, result in poorer nutrient absorption and subsequent chlorosis.

In addition, a mixed bacterial flora, as is commonly found inside trees, could interfere with the water and nutrient economy of the host's tissue under certain circumstances. A severe imbalance in the nutrient status of the chlorotic trees was observed by Timmer (unpublished data), indicating that the trees might have suffered from interference in the uptake or translocation processes. Bacteria could in addition to their effect on roots, block at least partially, the sap flow in xylem and phloem by forming an aggregate of bacterial cells or by changing the viscosity of the sap due to bacterial exudates of polysaccharides (Smith 1970). Bacteria can also release various metabolites such as enzymes, growth regulators and a large number of organic compounds, any of which could interfere with the translocation and availability of nutrients to shoot and needle tissues.

In a few instances, beneficial effects of the bacterial inoculations occured. The significant stimulatory effect of one of the inoculations (E. agglomerans + Pasteurella sp. + Serratia liquefaciens + Unknown-1) on the germination of white spruce seed might suggest that the bacteria exudated substances stimulated seed germination. Polonskaya et al. (1978) observed that metabolites of 42 of the 69 strains of bacteria were capable of exerting a beneficial or an inhibitory effect on the process of germination of pine seeds. The combinations of bacteria (inoculations $E_5 + E_4 + S_2$, and $E_3 + PS_2 + U_1 + S_4$) when inoculated on tne roots of white spruce germinants increased survival after transplanting in the greenhouse. This phenomenon may be similar to the beneficial effects of Rhizobacteria inoculations used in agriculture (Kloepper et al. 1980) to reduce root colonization by deleterious soil bacteria and fungi, though sterile soil was used in this study.

Bacteria can also act as antagonists and exert inhibitory effect on other bacteria and other microorganisms. In forestry, for example, Myxobacteria (Hocking and Cook 1972) has been used to control, at least in part, damping-off and root disease in container-grown coniferous seedlings. In agriculture, the suppression of a virulent strain of E. amylovora by avirulent isolate of E. amylovora, a yellow-Erwinia-like isolate, and Pseudomonas tabaci has been demonstrated (Goodman 1967).

The fact that a saprophytic species, frequently associated with the pathogen in nature, inhibits development of the pathogen in vivo may

explain the observed variation in the distribution of bacteria in chlorotic and control trees. It may also explain the difficulty in relating the distribution of a specific bacteria isolate to the chlorotic condition of the white spruce.

Furthermore, the relationship between bacteria may also be synergistic. Possible synergistic pathogenic interaction occurred in our inoculation between the Enterobacter and Serratia isolates. Similarly, synergistic effect of bacteria has been observed in inoculations of birch and aspen pulpwood (Henningsson 1967).

Several of our bacterial isolates were shown to be distinctly pathogenic. Some isolates of Enterobacter sp. induced significant pathological responses such as a reduced rate of germination of white spruce, reduced root growth of black spruce germinants, increased mortality of black spruce seedlings when transplanted in greenhouse, reduced rooting of black and white spruce cuttings, and reduced length and number of the roots formed on cuttings of white spruce. The unidentified isolate (U_1) reduced significantly rooting and length of roots of black and white spruce cuttings and when inoculated in combination with PS_2 and S_4, it also reduced significantly root length of black spruce germinants. Both Enterobacter sp. and the unidentified bacteria (U_1) were re-isolated and subsequently their identification was verified. This sequence constitutes the evidence of their pathogenicity on spruce seedlings.

Older trees were not inoculated to produce original symptoms observed in the field. Therefore, in this sense, this study did not fulfill completely Koch's postulates. However, the study showed that some of the bacterial isolates from the white spruce trees could produce pathological responses and mortality on young spruce seedlings as described above. Walsh (1979) in her limited attempt to establish the pathogenicity of bacteria isolates from white spruce trees also observed pathological changes such as yellowing of needle tips, inhibition of bud flushing, and mortality of very young spruce seedlings. It appears, therefore, that further pathogenicity tests and a closer study of the relationship between young seedling susceptibility in vitro and tree susceptibility under natural conditions to the bacterial infections are warranted.

By testing the potential for pathogenicity, we have proven that many of the bacteria inhabiting white spruce have the capacity to induce pathological changes on young roots and to interfere in the process of root development of black and white spruce.

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APPENDIX A

DESCRIPTION OF BACTERIA TYPES

APPENDIX A

Description of major Bacteria Types

PSEUDOMONAS sp: Small, white to off-white colonies on agar slants; retains streak pattern; flat and shiny surface; no pigmentation; aerobic; motile by polar flagella, non-sporing, rod shaped; arranged singly; 0.6-0.8 by 1.8-2.4 microns; gram-negative; oxidative.

PSEUDOMONAS maltophilia: White, creamy colonies; shiny surface; large mass with flat surface; does not alter agar color; no pigmentation; aerobic; unicellular, non-sporing; straight, large, rod-shaped cells; with rounded ends; arranged singly; 0.7-0.9 by 1.2-2.5 microns; motile by multitrichous flagella; gram-negative, catalase positive; never fermentative.

SERRATIA sp; White to flesh; large massive colonies; older cultures show characteristic pattern; surface begin wrinkled and flat; shiny regular round colonies; no pigment giving hyaline appearance; aerobic and facultatively anaerobic; over-grows streak pattern; excess moves to base of tube; non-sporing; gram-negative; motile by peritrichous flagella; acid produce from glucose; nitrates reduce to nitrites; give a negative oxidase reaction; gelatin is liquefied by most species; optimum temperature 25-30^{o}c.

APPENDIX A ..

PASTEURELLA sp: Small colonies; creamy; circular and translucent; older cultures colorless; retains streak pattern; flat surface; ovoid to straight (rod-shaped); less than 1 micron wide; non-motile; aerobic; non-sporing; gram - negative; urea is hydrolyzed by most species.

ENTEROBACTER agglomerans; Dull yellow to yellow colonies; mucoid; serrate to entire; raised surface; overgrows streak pattern; shiny; production of yellow and brown pigment; aerobic and facultatively anaerobic; gram-negative; non-sporing; rod-shaped with pointed ends; motile by peritrichous flagella; 0.7-0.9 by 1.5-3.6 microns; acid produce from glucose; gelatin liquefaction is positive; oxidase reaction is negative.

UNKNOWN - 1: Large, yellow colonies; bright and shiny towards centre; outer clear and transparent; older cultures sticky; surface raised; aerobic non-sporing; rod shaped; gram-negative; motile by peritrichous flagella; arranged singly; 0.8 by 1.6-2.0; no acid from carbohydrates.

UNKNOWN - 2: White, very samll circular colonies; raised, very shiny; slow growth and sparse; colonies with smooth edges; aerobic; non-sporing; oval to round; gram-variable; non-motile; 1.6 by 1.6-2.0; acid produced from glucose.

A P P E N D I X B

TABLES OF RESULTS

APPENDIX B-1

Total Hypocotyl Length of Black Spruce 14 days after Inoculation.

	TREATMENT									
	1	2	3	4	5	6	7	8	9	CONTROL
	18.13	12.09	18.85	17.97	15.10	18.46	10.50	15.21	14.66	16.13
	16.50	15.25	14.23	17.80	16.47	18.76	16.90	16.27	18.17	19.52
	14.80	19.85	17.61	13.05	21.40	17.68	10.70	21.85	18.87	22.82
	18.14	15.38	17.12	14.03	15.37	20.93	21.38	17.40	17.95	22.30
Σx_{ij}	67.57	62.48	67.81	62.85	68.34	75.83	59.48	70.73	69.65	80.77
$\bar{x}$	16.89	15.60	16.95	15.71	17.09	18.96	14.87	17.68	17.41	20.19

APPENDIX B-2

Total Hypocotyl Length of White Spruce 14 days after Inoculation.

	TREATMENT									
	1	2	3	4	5	6	7	8	9	CONTROL
	18.60	7.90	5.00	10.70	19.20	16.90	11.00	8.10	8.20	17.20
	19.50	14.00	8.20	13.50	11.50	9.30	21.30	14.80	17.20	10.30
	18.10	11.50	7.20	18.10	8.80	16.30	6.50	16.60	6.20	
	13.70	19.31	12.20	12.00	11.80	13.80	13.60	19.10	11.30	14.00
Σx_{ij}	69.90	52.71	31.30	44.40	60.60	48.80	62.20	60.63	53.30	59.63
$\bar{x}$	17.48	13.18	7.83	11.10	15.15	12.20	15.55	12.13	12.33	11.93

APPENDIX B-3

Total Root Length of Black Spruce 3 months after Inoculation.

	TREATMENT									
	1	2	3	4	5	6	7	8	9	CONTROL
	47.8	59.15	54.88	43.97	61.60	54.78	42.40	50.50	46.60	63.50
	47.2	62.00	47.60	51.80	79.60	57.80	47.20	45.60	59.20	78.60
	78.0	80.40	47.40	60.60	70.80	51.20	48.40	47.00	67.00	76.60
	58.2	62.80	52.80	38.00	67.60	44.00	26.40	52.20	44.50	72.00
Σx_{ij}	231.2	264.60	202.68	194.37	279.60	207.78	164.40	195.30	217.30	290.70
$\bar{x}$	57.80	66.15	50.67	48.59	69.90	51.95	41.10	48.83	54.33	72.68