A MICROBIOLOGICAL CONTROL OF *MELAMPSORA MEDUSAE* THUM. RUST ON *PSEUDOTSUGA MENZIESII* (Mirb.) Franco SEEDLINGS

by

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ABSTRACT

Saprophytic fungi and bacteria were recovered from healthy foliage of Douglas fir (Pseudotsuga menziesii (Mirb.) Franco). The populations of microorganisms on foliage were variable and changed through the growing season.

Three species of the bacteria isolated from Douglas fir foliage were used in attempts to control the development of the rust, Melampsora medusae Thum., on Douglas fir seedlings in the greenhouse. Effective control was obtained when Bacillus cereus Frankland and Frankland and B. mycoides Flugge were applied to the seedlings in pure cultures of nutrient broth. The most effective control was obtained from the application of a mixture of these two species and a third unidentified species of Bacillus in nutrient broth. Less effective control resulted from the application of bacteria suspended in water and cell free filtrates of the bacterial cultures.

The numbers of bacteria on the foliage of Douglas fir were estimated using a modification of the soil dilution and bacterial plate count technique. The bacterial populations on foliage of Douglas fir in the greenhouse and in the field were increased by the application of bacteria in nutrient broth and sterile nutrient broth.

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INTRODUCTION

Frequently immature plant tissues are highly susceptible to parasitic microorganisms that are unable to infect more mature tissues of the same plant (9). Presumably this susceptibility could be eliminated only by chemical control methods because natural resistance was thought to depend on the development of anatomical and physiological defenses. Bier (2,3,4,5) and others (6,7,15,16,21,22,23,24) have shown that aerial portions of plants are complex biological communities comprised of numerous saprophytic bacteria and fungi as well as the host plant tissues. Furthermore, Bier (2,3,4,5) has demonstrated that the interactional phenomena related to these saprophytic microorganisms may provide a natural form of defense against infection and/or disease development. It has been reported by Ruinen (24) and Last (15) that immature leaves had considerably lower populations of saprophytes than mature leaves. Thus, if the relatively low microorganism populations of immature tissues could be supplemented by the application of saprophytes, perhaps natural resistance could be improved. The present study was undertaken to investigate this concept in regard to the development of the rust, Melampsora medusae Thum., on the immature foliage of Douglas fir (Pseudotsuga menziesii (Mirb.) Franco).

The aecial stage of M. medusae occurs in May and June on immature needles of Douglas fir. The uredial and telial stages develop on leaves of trembling aspen (Populus tremuloides Michx.) from June until autumn defoliation. Molnar and Sivak (18) have investigated the life history of the rust pathogen. Ziller (29,30) has presented detailed studies on the host range and nomenclature of the rust fungus.

LITERATURE REVIEW

Leaf microfloras

The existence of non-parasitic microfloras on aerial plant structures has been known since the studies of Pasteur (21,22). These studies were concerned with the distribution of yeasts on fruits and suggested that physiological changes of the ripening fruit influenced the number of yeast microorganisms occurring on its surface. Burri (6) and Duggeli (7) isolated bacteria from the leaves of a wide range of plant species and established the fact that bacterial microfloras were ubiquitous on plant leaves. Their bacterial counts varied from 10^4 to 10^9 bacteria per gram fresh weight of leaf.

Last (15), after studying the seasonal incidence of the yeast, Sporobolomyces, on wheat and barley leaves, concluded that the two main factors influencing the numbers of yeast cells were the age of the leaf and atmospheric humidity. The results of his investigation showed that the yeast population remained low through the first half of the leaf's life and then increased rapidly.

Ruinen (24) studied the populations of microorganisms on leaves in Indonesia and Surinam. She found distinct differences in the species composition of the microfloras on (a) leaves of different plant species; (b) leaves of different ages; (c) leaves at different heights; and (d) leaves with different exposures to rain and to sunlight. Furthermore, the results of her studies indicated a definite succession in the populations during growth and senescence of the leaf. The succession began with bacteria, some of which were present on even the youngest leaves. After this juven-

ile stage the most rapid increase in the population began. This increase coincided with the most active period of photosynthesis and transpiration of the host and resulted in a mixture of filamentous fungi, yeasts, and bacteria. The bacteria at this time were the major component. The decline of leaf activity correlated with a change to a predominantly fungal microflora.

The succession in the microflora apparently reflects a change in the quantity and quality of nutrients on the leaf surface. Last (15) found that the populations of Sporobolomyces were significantly higher on fertilized plants than on unfertilized plants. Tukey et al. (25) reported that large quantities of nutrients were leached out of the leaf into water films on the leaf surface, with more nutrients being leached from fertilized plants than from unfertilized plants. The quantities lost in this manner increased when the leaf's activity began to decline at the start of leaf senescence. Thus, the amount of nutrients on the leaf and size of the microflora appear to be closely connected with the physiological condition of the host plant.

Biological control of disease using foliage saprophytes

The possibility that foliage saprophytes influence the development of parasitic microorganisms on plants has received only scant attention from plant pathologists. In 1910, when discussing epiphyllous bacteria, Potter (23) raised the question, "Are these bacteria at all concerned in the problem of immunity?" Fifty-five years later, in a review of the research on non-parasitic leaf microfloras, Last and Deighton (16) ask, "Do saprophytes play an important part in controlling the incidence of disease?" As the similarity of the two questions suggests, the knowledge

of this aspect of plant pathology has not developed rapidly!

The fact that certain leaf saprophytes produce antifungal substances in vitro has been demonstrated by many workers (8,13,14, and others). These studies on artificial media at constant humidity and temperature reveal little about the natural interactions between saprophytes. Only in a limited number of cases has the effect of saprophytes on parasites been studied on host material.

Bamberg (1) used saprophytes in an attempt to control the infection of corn plants by the smut, Ustilago zeae. Bacteria were isolated from corn plants which had failed to become infected after inoculation with virulent strains of the smut. Bamberg (1) and Johnson (11) showed that some of the bacteria were antagonistic to Ustilago zeae in artificial culture. The antagonistic bacteria when applied to the corn plants significantly reduced infection by the smut. The reduced infection was evident when the bacteria were applied 3 days before, simultaneously with, or 3 days after the smut inoculum. Bamberg (1) also demonstrated that cell free filtrates of the bacterial cultures were ineffective in controlling the disease.

Wood (27,28) and Newhook (19,20) studied the antagonism of some soil microorganisms toward Botrytis cinerea Pers. on lettuce leaves. Their studies were initiated after it was observed that lettuce seedlings grown in soil depressions were resistant to the disease. It was suggested that this resistance was a result of rapid colonization of the plants in the depressions by soil saprophytes. Several bacteria, actinomycetes and fungi antagonistic to B. cinerea were isolated from the soil. Control of the disease was achieved when the lettuce leaves were inoculated with the sapro-

phytes before or simultaneously with B. cinerea. The best control was obtained by spraying the plants with suspensions of the antagonistic saprophytes in a 1% glucose solution.

The effect of bark and leaf microfloras on certain tree pathogens has been studied by Bier (2,3,4,5). The results of his work have indicated the protective value of the natural microflora. The application of microflora suspensions to watered greenwood cuttings of black cottonwood and willow prevented the development of Hypoxylon canker. The microflora suspensions were not effective in preventing canker development when the bark turgor of the cuttings was lowered. When the natural microflora was reduced by surface-sterilizing the cuttings, cankers developed at all turgor levels subsequent to inoculation with the pathogen.

Bier (3) has also studied the microfloras of poplar leaves and their effect on the development of the rust, Melampsora occidentalis Jacks. Microflora suspensions were found to inhibit germination of uredospores in vitro. They also prevented the development of the rust on leaves of poplar cuttings growing in the greenhouse. When microflora suspensions were applied to poplar leaves in the field the development of the rust was significantly reduced. Furthermore, Bier (3) showed that the microflora composition of poplar leaves was altered for several months following the application of microflora suspensions.

METHODS

1. Recovery of leaf microfloras

The healthy foliage of Douglas fir to be sampled for microorganisms was collected in the field, placed in sterile test tubes, and taken immediately to the laboratory. Five grams of foliage were placed in a sterile metal-capped flask containing 100 ml. of sterile distilled water and shaken on a reciprocating shaker for six hours. Two types of agar media were used to culture the microorganisms, 5% malt extract and 2% agar, and 0.8% nutrient broth and 2% agar. Ten ml. portions of the microflora suspensions were added to cool agar media (44.5°C.) which were poured into petri dishes. Filamentous fungi, yeasts and a few species of bacteria were recovered in the malt extract medium. Bacteria were the only organisms recovered in the nutrient broth medium.

2. The pathogen: collection, storage and germination of teliospores

Leaves bearing telia were collected in May of 1964 and 1965 from the forest floor beneath aspen groves that had been heavily infected with *M. medusae* the summer prior to the collections. Teliospores from two locations, Botanie Valley near Lytton, B.C. and Williams Lake, B.C., were used in inoculation experiments. The infected leaves were air-dried and stored in kraft paper bags at room temperature until required for inoculations.

Teliospore germination was induced by soaking the aspen leaves for one hour in distilled water at room temperature. The leaves were then placed in moist petri dishes in constant temperature chambers until the teliospores germinated. Teliospore germination was tested at temperatures

from 5°C. to 35°C. The optimum temperature for germination was 10°C. for the Botanie Valley material and 20°C. for the Williams Lake material. The distinct colour change from the black of the teliospores to the golden brown of the promycelia and basidiospores facilitated a determination of the amount of germination.

Unlike the teliospores used by Molnar and Sivak (18), those used in this study retained their capacity to germinate for only a few months. Even when the spores had been stored at 0°C. the germination was considered to be too low by the end of September to be used in inoculation studies. The period required for the teliospores to germinate at the optimum temperature increased from ten hours in June to 48 hours in September. The increasing period for germination was accompanied by a decrease in the amount of germination.

3. The host

The Douglas fir seedlings for the inoculation experiments were grown in 5 1/2" diameter pots from seed collected in 1959 at Duncan, B.C. The seed was stratified before planting to obtain even germination and uniformly aged seedlings. One month old seedlings proved to be the most susceptible to the rust. At this age both the cotyledons and the epicotyl became infected. The rust was never observed to develop on foliage over five weeks old.

4. The disease

Inoculation of Douglas fir seedlings with basidiospores was carried out using the technique described by Ziller (30). This technique is illustrated in Figure 1 and consisted of the following steps:

Figure 1. The method of inoculation: from left to right; aspen leaves bearing germinating teliospores placed on wire screens above the pot of seedlings, moist cotton placed on the aspen leaves, polyethylene bag placed over the pot.

(i) nets on wire frames were placed above the pots of Douglas fir seedlings,

(ii) the aspen leaves with germinating teliospores were placed (telia facing down) on the nets and moist absorbent cotton was placed over the leaves,

(iii) a sterile hand atomizer was employed to spray the seedlings with sterile distilled water,

(iv) polyethylene bags were placed over the pots,

(v) after four days of inoculation the leaves, nets, cotton and bags were removed.

The intensity of disease was assessed by the number of pycnia which developed on the host. The pycnia (Fig. 2) could be counted easily because the pycnial droplets remained distinct even in heavy infections. The aecia (Fig. 3) were more difficult to count because in heavy infections the individual aecia did not remain distinct but merged with adjacent aecia. Frequently, mortality occurred in heavily infected seedlings before aecia were able to develop. The number of aecia may be a less direct measure of infection and disease development than pycnia because fertilization must take place before their development. For these reasons the number of pycnia was considered to be the most useful criterion of disease intensity. The number of pycnia was assessed in two ways; the percentage of seedlings on which pycnia developed and the average number of pycnia per diseased seedling calculated when the total number of pycnia was at a maximum.

5. Preparation of treatments

The bacteria were grown in liquid cultures of 0.8% Difco Nutrient Broth for a period of six days on a reciprocating shaker at room tempera-

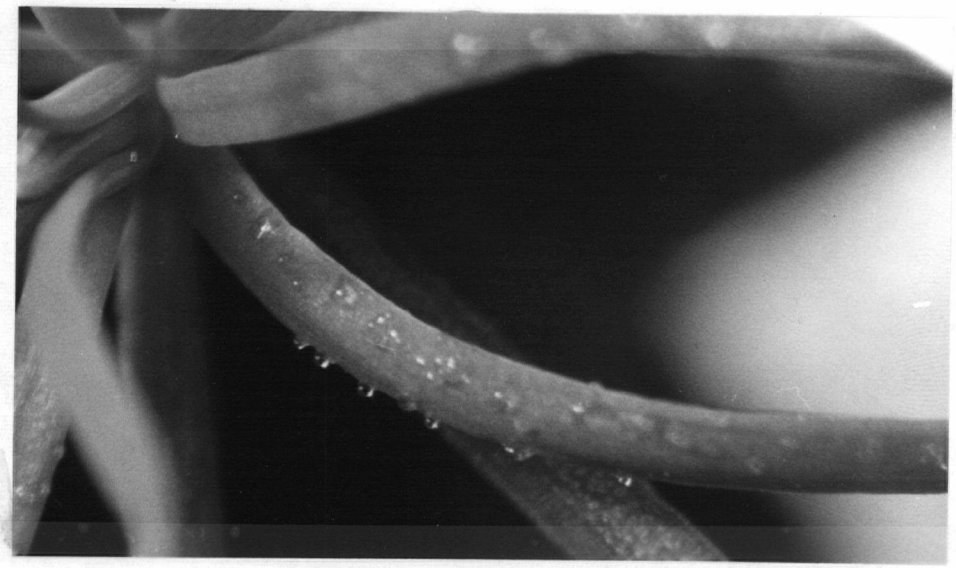

Figure 2. Pycnial droplets of Melampsora medusae on a Douglas fir seedling.

Figure 3. Aecia of Melampsora medusae on a Douglas fir seedling.

ture. The cell free controls were prepared by filtering one half of the bacterial suspension through a Millipore filter with 0.45μ pore size. When more than one species were applied in a treatment the bacteria were cultured individually in nutrient broth and mixed immediately prior to Millipore filtering and/or application to the seedlings.

In experiments where bacteria were applied as water suspensions, the bacteria were cultured as described above. The cultures were then centrifuged at approximately 3000 r.p.m. When the bacteria had precipitated, the nutrient solution was decanted and the bacteria resuspended in sterile distilled water. This water suspension was then centrifuged and the procedure repeated three times. The final suspension was applied to the seedlings.

A small sterilized atomizer was employed to apply the material in all experiments. Approximately five ml. of material were applied per seedling. The seedlings treated with suspensions were incubated for 24 hours before inoculation with rust spores was started. Freeze-dried samples of the three bacteria (Bacillus cereus Frankland and Frankland, B. mycoides Flugge, and Bacillus sp.) used in the inoculation experiments are deposited at the Forest Pathology Laboratory, U.B.C. B. cereus and B. mycoides were identified by Dr. J. Basaraba of Acadia University, Nova Scotia. The Bacillus sp. was tentatively identified by the author as a strain of B. megaterium de Bary.

6. Estimation of bacterial populations

Field Experiment

The bacterial populations of foliage of Douglas fir trees were estimated using a modification of the soil dilution technique (12). To ensure

that samples contained approximately the same total surface area of foliage, only the distal 1.5 cm. of the needles were used. Thirty such needle portions were placed in a sterile test tube containing 10 ml. sterile distilled water that was shaken for 15 minutes on a wrist action shaker. The bacterial population of the water was then estimated using the soil dilution and plate count method (12). Difco nutrient agar was the culture medium used in the plate counts. This medium was selective for bacteria and therefore the fungal components of the microflora were not studied in this experiment. The foliage samples were always taken at 8:00 a.m.

Greenhouse Experiments

The experimental procedures were the same as those used for the field experiment except for the size of the foliage sample. Because of the uniform size of the needles and the small amount of foliage on the seedlings, five whole needles were used in each sample. The foliage sampling was made 48 hours after the nets, aspen leaves, cotton and bags used in the inoculations were removed.

RESULTS

Observations on foliage microfloras of Douglas fir

The foliage of several Douglas fir trees on the University of B.C. Endowment Lands was sampled to determine the types of microorganisms present. The foliage sampled ranged from three year old needles to those enclosed by the bud scales. Microorganisms were recovered from all samples. The microorganisms most frequently isolated were:

fungi: Aureobasidium pullulans (de Bary) Arnaud, Epicoccum sp. and an unidentified pink yeast

bacteria: Bacillus cereus, B. mycoides, Bacillus sp. and an unidentified yellow pigmented bacterium.

The microfloras of Douglas fir needles were found to vary widely in the numbers and the types of microorganisms. After sampling over an entire growing season the following trends became evident:

1. Young needles (1-2 months old) appeared to have microfloras more restricted in numbers and species, and bacteria appeared to form the major portion of these microfloras.

2. Mixtures of fungi and bacteria were present on the older needles (3 months to 3 years old) and there appeared to be an increase in the incidence of fungi compared to the incidence of bacteria as the age of the needles increased.

Inoculation experiments

The bacteria, Bacillus cereus, B. mycoides and Bacillus sp., were chosen for use in the biological control experiments because they appeared

to be among the first colonizers of immature Douglas fir foliage.

The first inoculation experiment included the following treatments:

(i) sterile distilled water,

(ii) Bacillus cereus, B. mycoides and Bacillus sp. in sterile distilled water,

(iii) sterile nutrient broth,

(iv) Bacillus cereus, B. mycoides and Bacillus sp. in nutrient broth,

(v) a cell free filtrate of (iv).

Teliospores from the Williams Lake collection of aspen leaves were used for the inoculation. Four pots, each containing 18, two-month-old Douglas fir seedlings, were used in each experiment.

The results of the first inoculation experiment are summarized in Table 1. Compared to the sterile distilled water control in which 75% of the seedlings became diseased, all treatments resulted in a significant reduction (at the 1% level of probability) in the percentage of seedlings diseased (Figure 4).

The application of bacteria in nutrient broth resulted in the most effective control of the rust with only 1.4% of the seedlings becoming diseased. This treatment also had the greatest effect on the number of bacteria recovered from seedling foliage, the number increasing to 400 times that recovered from foliage sprayed with sterile distilled water.

The applications of bacteria in water and of sterile nutrient broth provided less effective control of the rust disease with respectively 36% and 39% of the seedlings becoming diseased. Four times as many bacteria were recovered from foliage of seedlings treated with bacteria in water than that treated with sterile distilled water. The application of sterile

TABLE 1

The effect of water, nutrient broth, cell free filtrate and bacterial treatments on the development of Melampsora medusae on the foliage of Douglas fir seedlings

Treatment	No. of seedlings	Average no. of bacteria per needle seven days after treatment	% of seedlings diseased	Average no. of pycnia per diseased seedling
Sterile water	72	1,500	75.0	89.6
Bacteria in water	72	6,500	36.1	95.1
Sterile nutrient broth	72	64,000	38.9	96.4
Cell free filtrate	72	60,000	30.5	71.0
Bacteria in nutrient broth	72	328,000	1.4	46.0

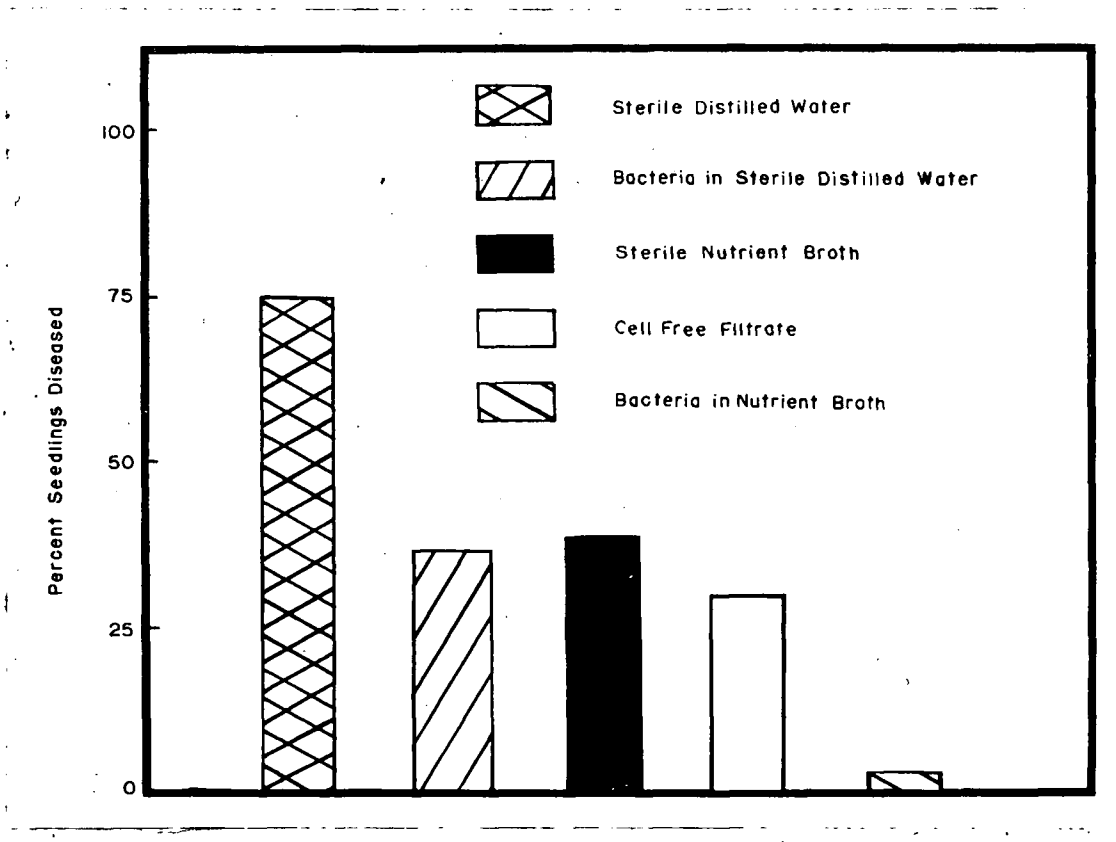

Figure 4. A comparison of the percent seedlings diseased in the first inoculation experiment.

nutrient broth increased the number of bacteria recovered from the foliage to 40 times that recovered from foliage treated with sterile distilled water.

The cell free filtrate was not as effective as bacteria in nutrient broth but was more effective than sterile nutrient broth in controlling the rust disease. The effect of the cell free filtrate on the number of bacteria recovered from the foliage was similar to that resulting from the application of sterile nutrient broth.

The effect of the nutritional and bacterial elements on the development of M. medusae was examined in a second experiment in which teliospores on aspen leaves from Botanie Valley were used as inoculum. Four pots, each containing 16, one-month-old Douglas fir seedlings, were used in each treatment. The treatments were:

(i) sterile nutrient broth,

(ii) Bacillus cereus, B. mycoides, and Bacillus sp. in nutrient broth,

(iii) cell free filtrate of (ii) (Millipore filtered).

The results of this experiment are summarized in Table 2. As in the first experiment, application of bacteria in nutrient broth provided the most effective control of the rust disease with only 12.5% of the seedlings in that treatment becoming diseased compared to 70% in the sterile nutrient broth treatment and 58% in the cell free filtrate treatment (Figure 5). The effect of the treatments on the numbers of bacteria recovered from the foliage seven days after the treatments were applied was of the same magnitude as in the previous experiment (Table 2). Approximately eight times as many bacteria were recovered from foliage treated with bacteria in nutrient broth as from foliage treated with the cell free filtrate or

TABLE 2

The effect of nutrient broth, cell free filtrate, and bacterial treatments on the development of Melampsora medusae and the number of bacteria on the foliage of Douglas fir seedlings

Treatment	No. of seedlings	Average no. of bacteria per needle seven days after treatment	% of seedlings diseased	Average no. of pycnia per diseased seedling
Sterile nutrient broth	64	65,500	70.3	138.6
Bacteria in nutrient broth	64	508,000	12.5	31.5
Cell free filtrate	64	60,500	57.8	130.5

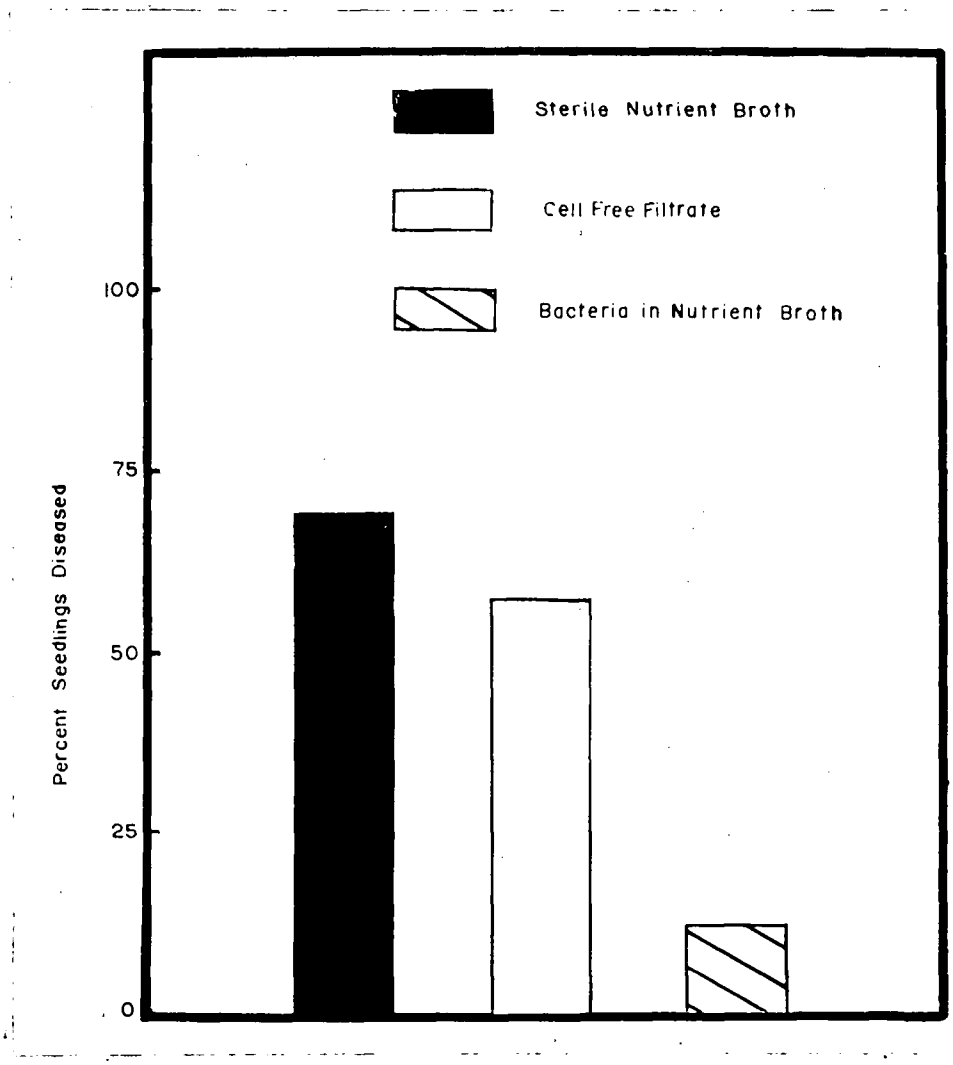

Figure 5. A comparison of the percent seedlings diseased in the second inoculation experiment.

sterile nutrient broth.

As mentioned previously the bacterial treatments in the preceding experiments were mixtures of three species of bacteria, Bacillus cereus, B. mycoides and Bacillus sp. It was considered of interest to determine if each of the species would be effective in controlling the rust when applied individually. Therefore, the species of bacteria were applied individually as pure cultures in nutrient broth. A mixture of the three species of bacteria similar to the bacterial treatments of the previous experiments was also applied. The control treatment in each case was a cell free filtrate of the bacterial treatment. Thus the following treatments were applied:

(i) Bacillus cereus in nutrient broth,

(ii) cell free filtrate of (i),

(iii) Bacillus mycoides in nutrient broth,

(iv) cell free filtrate of (iii),

(v) Bacillus sp. in nutrient broth,

(vi) cell free filtrate of (v),

(vii) a mixture of (i), (iii) and (v),

(viii) cell free filtrate of (vii).

Teliospores on aspen leaves from the Botanie Valley collection were used for the inoculum in this experiment. Three pots, each containing 12, three-week-old Douglas fir seedlings, were used for each treatment.

The results of this experiment are summarized in Table 3. Compared to their cell free filtrates the Bacillus mycoides treatment reduced the percentage of diseased seedlings from 94.4% to 55.5%, the Bacillus cereus treatment from 97.2% to 27.8%, and mixture of bacteria treatment from

TABLE 3

The effect of pure cultures in nutrient broth of *Bacillus cereus*, *B. mycoides*, *Bacillus* sp., and of a mixture of these bacteria on the development of *Melampsora medusae* on the foliage of Douglas fir seedlings

Treatment	No. of seedlings	Average no. of bacteria per needle seven days after treatment	% of seedlings diseased	Average no. of pycnia per diseased seedling
Bacillus cereus in nutrient	36	416,000	27.8	11.2
Cell free filtrate of *B. cereus* culture	36	25,000	97.2	140.4
Bacillus mycoides in nutrient	36	400,000	55.5	53.9
Cell free filtrate of *B. mycoides* culture	36	33,000	94.4	129.8
Bacillus sp. in nutrient	36	250,000	69.4	75.2
Cell free filtrate of *Bacillus* sp. culture	36	32,000	91.7	326.4

- continued

TABLE 3, cont'd.

Treatment	No. of seedlings	Average no. of bacteria per needle seven days after treatment	% of seedlings diseased	Average no. of pycnia per diseased seedling
B. cereus, *Bacillus* sp. and *B. mycoides* in nutrient	36	420,000	5.5	15.5
Cell free filtrate of *B. cereus*, *B. mycoides* and *Bacillus* sp. cultures	36	26,000	94.4	217.7

94.4% to 5.5% (Fig. 6). There was no significant difference in the percentages of diseased seedlings within the four different cell free filtrate treatments (ii, iv, vi, viii). The application of the unidentified species, Bacillus sp., did not significantly reduce the percentage of diseased seedlings.

As shown before the treatments which were most effective in controlling the rust were also most effective in increasing the number of bacteria recovered from the seedling foliage.

All seedlings in the inoculation experiments were grown in the greenhouse for six months after inoculation. During this time there were no signs of damage resulting from any of the spray treatments.

The analyses of variance of the results of the preceding experiments are tabulated in Appendix 1.

Field study

The numbers of bacteria recovered from the foliage of seedlings used in the inoculation experiments indicated that the high bacterial population resulting from the spray treatments persisted for at least four weeks under greenhouse conditions. Field experiments for the control of M. medusae were impossible at this time because no area near the University had a history of chronic M. medusae infection. It was considered of interest, however, to determine if the microflora of immature Douglas fir foliage could be altered under field conditions.

Buds of 8-year-old Douglas firs growing in a nursery at the University of British Columbia were used in the experiment. Buds which broke dormancy at the same time and which had similar exposure to sun and rain were chosen for the experiment. Three treatments were applied:

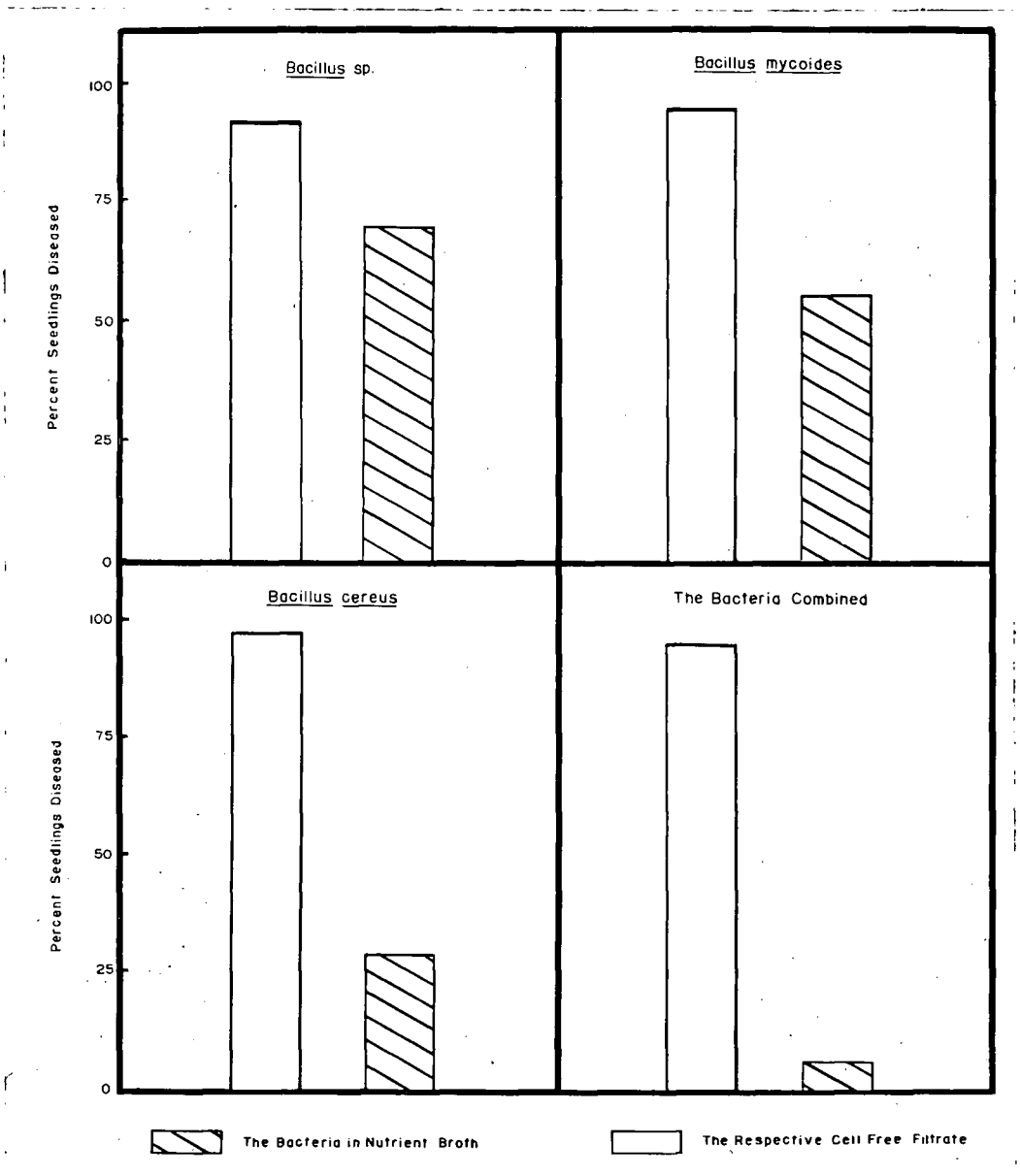

Figure 6. A comparison of the effects of pure cultures on three species of bacteria and a mixture of the three species on the development of Melampsora medusae.

(i) sterile distilled water,

(ii) Bacillus cereus, B. mycoides and Bacillus sp. in sterile distilled water,

(iii) Bacillus cereus, B. mycoides and Bacillus sp. in nutrient broth.

The buds were treated as soon as the needles had broken free from the bud scales. The number of bacteria recovered from the needles was determined one day before the treatments were applied and one, two, four, seven, twelve and fifteen days after the treatments were applied.

During the first five days temperatures were moderate and the skies were generally overcast. From the sixth day until the termination of the experiment on the 15th day there was an increase in daytime temperatures and the hours of sunshine. The results of this experiment are given in Figure 7. The application of bacteria in nutrient broth resulted in a greatly increased bacterial population for 4 to 6 days. The application of bacteria in water caused a much smaller increase which lasted for only 2 to 3 days. Coinciding with the change to warm sunny weather the bacterial populations of needles of all treatments, including the water control, declined. The influence of weather conditions appeared to have an important influence on the microflora populations. Further field studies in an area with a history of chronic M. medusae infection are required to determine if the weather conditions suitable for infection are also suitable for the maintenance of a large microflora.

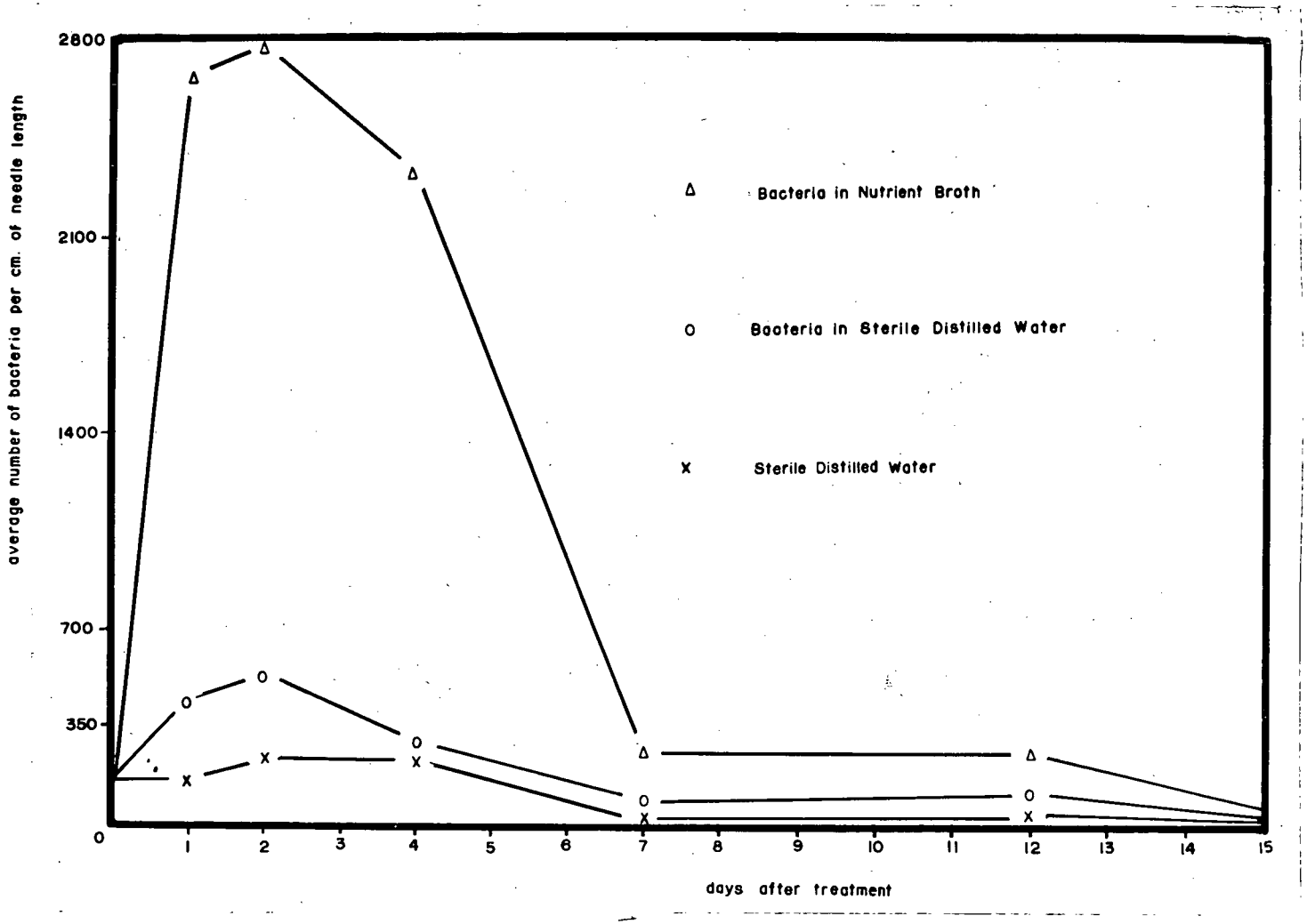

Figure 7. The effect of water and bacterial treatments on the number of bacteria recovered from Douglas fir foliage in the field.

DISCUSSION

Healthy needles of Douglas fir proved to be complex biological communities consisting of the plant tissues and microfloras of saprophytic bacteria and fungi. Immature needles had considerably fewer microorganisms in their microfloras than mature foliage. Control of the rust, M. medusae, was obtained when the microfloras of immature needles were supplemented by suspensions of bacteria isolated from Douglas fir foliage. Thus, one factor in the natural susceptibility of immature foliage to this rust may be the lack of sufficiently developed microfloras.

Because common soil organisms such as Bacillus cereus and Aureobasidium pullulans occurred as part of foliage microfloras, a microflora may be partially dependent for its formation on dust-borne inoculum from the soil. Consequently, the microflora composition and level of rust resistance may in part be dependent on factors related to soil microbiology.

The microfloras of needles of the same age and from the same tree were found to be variable and, therefore, genetically identical host material may have varying degrees of resistance to disease. Part of the variation of microfloras may be a result of microclimatic factors. A recent study (26) has correlated the incidence of white pine blister rust with microclimatic conditions. In considering the effect of the microclimate, attention should be given to both the development of the host's microflora and the development of the pathogen.

Attempts to alter the microfloras of the needles of Douglas fir seedlings in the greenhouse indicated that nutrition was an important factor influencing the number of bacteria in the microfloras. When sterile

nutrient broth was applied to the foliage the number of bacteria increased to approximately 40 times the number of bacteria on untreated foliage. The application of bacteria suspended in sterile distilled water to foliage increased the number of bacteria to only four times that on untreated needles. The greatest increase in the number of bacteria on foliage was attained when bacteria were applied in nutrient broth suspension.

Attempts to alter microfloras of Douglas fir foliage under field conditions indicated the possible importance of the influence of weather on microflora. A decline of the numbers of bacteria recovered from both control and treated foliage coincided with a change to hot dry weather. The influence of weather on the development of microfloras may account in part for variation in the severity of disease in nature.

Inoculation experiments were carried out on Douglas fir seedlings in the greenhouse to determine if bacteria from the natural microfloras of Douglas fir foliage would be effective in controlling the disease caused by M. medusae. Significant levels of rust control were achieved by individual applications of sterile nutrient broth, bacteria in water, three species of bacteria in nutrient broth, and cell free filtrates of three species of bacteria in nutrient broth. Two species of bacteria, Bacillus cereus and B. mycoides provided significant control of the rust when applied individually as pure cultures in nutrient broth. The most effective control was attained when three species of bacteria, Bacillus cereus, B. mycoides and Bacillus sp. were applied as a mixture in nutrient broth. The degree of rust control attained by all treatments appeared to be related to the effect the treatment had on the total number of bacteria on the foliage. The treatments which resulted in the greatest increase in the number of

bacteria on the foliage provided the most effective control of the rust, and the treatments which resulted in a relatively low number of bacteria on the foliage did not provide effective control. Therefore, the bacteria appeared to be an active agent in the control of the rust. However, the manner in which the bacteria controlled the rust is not known. The cell free filtrates of bacteria in nutrient broth cultures were more effective than sterile nutrient broth in controlling the rust. This indicated that some bacterial metabolite may have been inhibitory to the pathogen. Several species of the genus Bacillus are known to produce antibiotics (17). It is possible that interactional phenomena of the bacteria on the foliage resulted in an inhibition of basidiospore germination similar to the inhibition of germination of uredospores of Melampsora occidentialis Jacks by microfloral organisms as reported by Bier (5). However, the control could also have occurred later in the infection process or perhaps after infection had taken place.

The three Bacillus species used in the inoculation experiments appeared to be suitable for the biological control of M. medusae. They occurred frequently on immature foliage of Douglas fir and therefore have demonstrated their ability to become established on susceptible tissues. Resistant endospores are produced by these bacteria thus enabling them to persist over periods of unfavorable environmental conditions. The application of bacteria to susceptible foliage resulted in rust control in the greenhouse. Further studies are required to investigate the feasibility of this control method under field conditions.

The use of organisms from the natural microflora of the host tissues to achieve one form of biological control of a plant disease could

have advantages over chemical control methods. One advantage would be in avoiding the use of toxic sprays which could destroy beneficial as well as pathogenic microorganisms. Because the organisms used in the control occur naturally on the host there are no foreign substances introduced as there are in chemical control methods. There is also the possibility that the microorganisms and, therefore, the protection would become self-perpetuating. The results of the present study indicate that no damage is done to the plant by the altered microflora, whereas, toxic chemical sprays often result in damage to young plant tissues (10).

SUMMARY

The needles of Douglas fir were found to have natural microfloras of saprophytic fungi and bacteria. As the foliage matured the microfloras increased in numbers of organisms present and changed in species composition. Attempts to increase the size of the microflora artificially were successful and indicated the importance of nutrition, inoculum, and weather as factors controlling the microflora size. The microflora of immature needles included saprophytic bacteria which were effective in controlling the rust, Melampsora medusae. The most effective control was obtained when three species of bacteria, Bacillus cereus, B. mycoides and Bacillus sp. were applied as a mixed suspension in nutrient broth. Two species, B. cereus and B. mycoides provided effective control of the rust when applied as pure cultures in nutrient broth. Cell free filtrates of bacterial cultures provided a small degree of rust control indicating the possible production of a bacterial metabolite which inhibited rust development. Sterile nutrient broth treatments resulted in partial control of the rust. Apparently this control was due to the increased number of bacteria in the microflora which followed the application of additional nutrition. None of the treatments had any harmful effect on the seedlings.

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APPENDIX

Summary tables of analyses of variance and Duncan's New Multiple Range Tests of the results of the inoculation experiments

A. The effect of water, nutrient broth, cell free filtrate and bacterial treatments on the development of Melampsora medusae on the foliage of Douglas fir.

(i) Analysis of Variance

Source of variation	d.f.	Sum of squares	Mean square	Calculated F	Tabled F at 5%	Tabled F at 1%
Treatments	4	115,535.5	28,883.9	128.3**	3.26	5.41
Block	3	2,414.7	804.9	3.6*	3.49	5.95
Error	12	2,701.3	225.1			
Total	19	120,651.5				

(ii) Duncan's New Multiple Range Test of treatment means (5% level)

Sterile distilled water	Sterile nutrient broth	Bacteria in water	Cell free filtrate
75.0	38.9	36.1	30.5

Bacteria in nutrient broth
1.9

B. The effect of nutrient broth, cell free filtrate and bacteria in nutrient broth treatments on the development of Melampsora medusae on the foliage of Douglas fir.

(i) Analysis of Variance

Source of variation	d.f.	Sum of squares	Mean square	Calculated F	Tabled F at 5%	Tabled F at 1%
Treatments	2	4,181.6	2,090.8	105.2**	5.14	10.92
Block	3	29.8	9.9	.50	4.76	9.78
Error	6	119.2	19.9			
Total	11	4,330.6				

(ii) Duncan's New Multiple Range Test of treatment means (1% level)

Sterile nutrient broth	Cell free filtrate	Bacteria in nutrient broth
70.3	57.8	12.5

C. The effect of pure cultures of Bacillus cereus, B. mycoides, and Bacillus sp. and a mixture of the three species on the development of Melampsora medusae.

(i) Analysis of Variance

Source of variation	d.f.	Sum of squares	Mean square	Calculated F	Tabled F at 5%	Tabled F at 1%
Treatments	7	22,254.3	3,179.2	15.3**	2.77	4.28
Block	2	1,406.9	703.5	3.4	3.74	6.51
Error	14	2,903.3	207.4			
Total	23	26,564.5				

(ii) Duncan's New Multiple Range Test of treatment means (5% level)

Cell free filtrate of Bacillus cereus	Cell free filtrate of a mixture of Bacillus cereus, B. mycoides, and Bacillus sp.	Cell free filtrate of Bacillus mycoides	Cell free filtrate of Bacillus sp.	Bacillus sp. in nutrient broth
97.2	94.4	94.4	91.7	69.4

Bacillus mycoides in nutrient broth	*Bacillus cereus* in nutrient broth	A mixture of *Bacillus cereus*, *B. mycoides* and *Bacillus* sp. in nutrient broth
55.5	27.8	5.5

Footnote
* Significant at the 5% level.
** Significant at the 1% level.