THE PHYLLOSPHERE OF EUROPEAN LARCH (*LARIX DECIDUA* MILL.) : A MICROECOLOGICAL STUDY OF LEAF INFECTION.

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SUMMARY

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The combination of descriptive field studies with experimental laboratory studies has yielded considerable information about the ecology of the larch phyllosphere. The most important findings are listed below.

Descriptive studies

1. The surface relief of larch leaves was marked by features related to internal leaf tissues, epidermal cells and acellular wax structures.

2. While anticlinal ravines constituted less than 40% of the leaf surface, over 60% of bacterial colonies on the leaf occurred in ravines.

3. The hydrophobic property of larch leaves diminished with increasing age.

4. An estimation of leaf wetness duration was attempted using a lamb-gut recorder and a Hirst wetness recorder. These measurements proved unreliable and difficult to correlate with conditions on the leaf. Both recorders yielded data which correlated strongly with data for number of rainy days and the length of time for which relative humidity was greater than 80%.

5. Leaf leachates contained glucose, fructose, sucrose and at least nine amino acids.

6. Whole seedling leachates were analysed from June until October.

   i. Amino acids and the ions Na, K and Ca were more abundant per gram of seedling in leachates of very young seedlings than in leachates of older seedlings.

ii. Magnesium was abundant in leachates of young seedlings and of very old seedlings.

iii. The carbohydrate content of seedling leachates varied markedly through the growing season with peak quantities apparently related to periods of sunny weather.

7. The preparation of microflora suspensions by homogenizing was shown to be somewhat more effective than single washing of leaves.

8. Microflora population development.

i. Bacterial populations remained constant during June, July and August before rising at an exponential rate to several million per gram fresh weight of leaves in November.

ii. No yeasts were isolated from leaves two-weeks-old or younger, but then the population developed at a more or less constant exponential rate to approximately 1-10 million per gram fresh weight of leaves.

iii. Filamentous fungi were present only as spores on leaves less than 3 months old. Hyphae then developed rapidly colonizing the lower leaf surface more densely than the upper surface.

iv. Microorganism populations developed first on cotyledons, then on lower plumule leaves and finally on upper plumule leaves. The development of bacteria, yeast and fungal populations appeared to be related to leaf age.

9. The numbers of microorganisms on leaves of larch seedlings were similar to the numbers on leaves of larch trees.

10. The filamentous fungi most frequently isolated from larch leaves were common members of the airspora and included: Cladosporium sp., Fusarium sp., Phoma sp., Stemphylium sp., Cephalosporium sp., and Epicoccum sp.

11. The bacterial flora was predominantly Gram-negative rods, motile and pigmented. Most numerous were isolates of Pseudomonas, Flavobacterium and Xanthomonas.

12. The yeast flora included Sporobolomyces roseus, Rhodotorula spp. Cryptococcus spp. and Torulopsis spp.

13. Over 80% of bacterial and yeast isolates from the larch phyllosphere produced lipase.

Experimental studies.

14. Several microorganimms isolated from the larch phyllosphere including motile bacteria and non-motile yeasts were shown to be capable of colonizing growing aseptic seedlings from inoculum on the seedcoat. No migration between seedlings was observed.

15. Artificial inoculations of yeasts and bacteria onto aseptic seedlings resulted in populations which generally declined with increasing time. Mortality in the populations was greater on young leaves than on old leaves.

16. The application of S.roseus inoculum to aseptic seedlings resulted in colonies of the yeast and the production of ballistospores.

The surface wax structures around the S.roseus cells were observed to be destroyed or much reduced up to a distance of 5 μ from the cells.

17. Germination of Cladosporium herbarum spores declined with age, was weakly stimulated by amino acids, and was strongly stimulated by sugars.

18. Leaf leachates of young and old leaves stimulated germination of C.herbarum spores.

19. A leaf isolate (S-100) of Pseudomonas sp. inhibited C.herbarum spore germination when in nutrient-rich solutions. In water it stimulated germination.

20. Sporobolomyces roseus inhibited germination of C.herbarum spores in water but stimulated germination when in nutrient-rich solutions.

21. Inoculation of larch seedlings with C.herbarum resulted in spore germination and hyphal development on old leaves of seedlings sprayed with water or a suspension of bacterium S-100. No hyphae developed on any leaves of seedlings sprayed with a suspension of S.roseus.

22. The growth of Meria laricis in artificial culture was stimulated more by whole larch leaves than by water extract of larch leaves.

23. The germination of M.laricis spores was stimulated by sugars, amino acids and by a pregermination cold treatment ($0^{\circ}C$).

24. Leaf leachates of young and old leaves stimulated M.laricis spore germination.

25. Spore germination of M.laricis was inhibited by a number of bacteria including Bacillus sp. (S-95) and Pseudomonas sp. (S-100).

The inhibition associated with the latter was operative only in nutrient rich solutions.

26. The yeasts S.roseus, Torulopsis sp., and Cryptococcus sp. inhibited M.laricis spore germination in a sugar solution.

27. The microflora of young larch leaves was increased more by the application of bacteria in water. However, the greatest increase was associated with the application of bacteria in a nutrient solution.

28. The application of bacteria (S-100) in Nutrient Broth to larch seedlings resulted in a significant reduction in the damage caused by M.laricis. S-100 did not reduce the damage when applied in water suspension.

29. The application of the yeasts, S.roseus or Torulopsis sp. in sugar solutions to larch seedlings resulted in a significant reduction in the damage caused by M.laricis. S.roseus did not reduce the damage when applied in nutrient-rich solutions.

I certify that this work is my own and has been composed by myself.

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CHAPTER I

INTRODUCTION

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The concept of the phyllosphere originated in the 19th century as an indirect result of the work of Pasteur (1876, 1878). During his investigations he demonstrated that the aerial portions of plants are complex biological communities comprising numerous non-pathogenic bacteria and fungi as well as the host plant tissues. Pasteur and later Hansen (1881, 1882) were primarily concerned with yeasts on fruit and their role in the fermentation process. Burri (1903) and Düggeli (1904) isolated bacteria from the leaves of a wide range of plant species and established the fact that bacterial microfloras were ubiquitous on plant leaves. In 1910, Potter, when discussing epiphyllous bacteria, suggested a second reason for studying plant microfloras when he raised the question, "Are these bacteria at all concerned in the problem of immunity?". After this initial period of interest in plant microfloras, very little work on the subject was published until a revival of interest in the 1950's and 1960's (Table I-1).

TABLE I-1 Number of publications related to the microflora of aerial plant parts. (Pre-1964 data taken from Last and Deighton (1965) and post-1965 data collected by the author)

Dates of publications	pre-1900	1901-1910	1911-1920	1921-1930	1931-1940	1941-1950	1951-1960	1961-1969
Number of publications	4	3	0	1	0	1	15	26

The recent increase in publications has been connected in part with increased interest in leaf decomposition, and what Leben (1965) has called the "fourth dimension" of plant pathology, the role of the non-pathogenic microflora in disease. In addition, observations made by Ruinen (1956, 1965) have indicated that nitrogen-fixing bacteria on leaves may contribute to plant nutrition.

Coincident with the increased interest in the leaf microflora has been the study of nutrient uptake and loss by the leaf in connection with foliar fertilizers and with nutrient cycling respectively. The combination of these studies has led to a great increase in the knowledge of both the biotic and abiotic aspects of the leaf surface.

The establishment of the fact that nutrients are lost from the leaf in considerable quantities (Tukey and Morgan, 1963) and that chemicals can readily be taken in through leaves(Franke, 1967) at once makes possible the existence of an active surface microflora and places it in a position where chemical substances produced by the microflora could influence internal plant physiology as well as leaf pathogens.

Information about the leaf environment may originate from and be of interest to researchers in a wide range of disciplines. It is, therefore, imperative, if confusion is to be avoided, to develop a useful, precise vocabulary easily understood by specialists and non-specialists alike.

The term "phyllosphere" was first applied to the leaf environment by Last (1955a)and Ruinen (1956). They coined the word to parallel the much studied environment of the region surrounding

plant roots. The term "phyllosphere" has gained wide acceptance (di Menna, 1962; Barnes, 1965; Kerling, 1964; Sinha, 1965; Fokkema 1968; Vasantharajan and Bhat, 1968) but some authors (Dickinson, 1965, 1967; Hislop and Cox, 1969) have preferred the term "phylloplane" which was introduced by Kerling (1958) to stress the surface nature of the leaf microflora. As has been found in analogous studies on the rhizosphere, it is difficult, if not impossible, to delimit the zone of influence of the root and it is likewise difficult to define the zone of influence on the leaf. However, while the microflora may be restricted to the surface, the leaf's effect goes beyond its surface, affecting humidity (Yarwood and Hazen, 1944), temperature (Wuenscher, 1970) and bacterial composition of the surrounding air (Smirnoff, 1967). Indeed the microflora may exist in three dimensions, especially in the tropics, where Ruinen observed microbial layers up to 22 μ thick. Thus, the author feels that the habitat in question exists in three-dimensions and that phyllosphere is a more appropriate term than phylloplane for describing the habitat. The phyllosphere should be considered to include the leaf from the outer epidermal cell walls through the cuticle to the water film and/or the air surrounding the leaf. The extent to which the phyllosphere is considered to extend away from the leaf will clearly depend upon the context of the study. For example, a study may be concerned solely with the surface organisms or it may extend to the airspora enclosed by the vegetation canopy.

The leaf surface microbial community has been referred to variously as "epiphytic" (Leben, 1965; Voznyakovskaya, 1962), "epiphyllic" (Ruinen, 1961; Kerling, 1958), "non-parasitic"

(Last and Deighton, 1965; Hislop and Cox, 1969) and "foliage saprophytes" (Bier, 1965). With the exception of "epiphytic" and "epiphyllic", the terms to be applied require knowledge of or an assumption about the mode of nutrition of the organisms. When dealing with living leaves, the terms "non-parasitic" and "saprophyte" could not be properly applied to active microbes on live leaves. The term "non-pathogenic" would perhaps be more appropriate for some organisms but in cases of attenuated infection (Simon, 1960) this definition may require considerable observation and experimentation to establish. "Microbial epiphyte" has been defined by Leben as a member of the microflora that is observed on or is cultured from the plant surface. Thus, "epiphytic" and the more specific term "epiphyllic" do provide general terms based on a criterion "support" which can readily be established by a minimum of observation. The delimiting of microbial epiphytes into units based on function criteria is more difficult and Leben (1965) has suggested the groups "residents" and "casuals". Residents are those organisms which can multiply on healthy plant surfaces while casuals cannot. Multiplication, according to Leben, can be determined by test or inference. Inference must be used with caution, because casuals, while inactive on the leaf, may occur in large numbers or may be active on debris on the plant. Resident as a characteristic is useful in describing an organism and may be an ecologically meaningful concept because the physiological requirements for colonizing a healthy leaf may have features distinct from requirements for other habitats.

The need for a biological understanding of the infection environment is made acute by the ever increasing problems of pesticide pollution and pest resurgence and resistance after three decades of accelerating use of chemical aids to biological production (Moore, 1967). Thus, it is appropriate that crop protection should be a major goal of phyllosphere research.

In addition to species reduction due to pesticides, the economic pressure in crop production has led to severely simplified land areas due to the practice of monoculture on an ever increasing scale, (Johnston et al, 1967 p.236). In 1955, MacArthur established the mathematical validity of the theory that species diversity results in a stable biological community. Elton (1958) documented much observational data which supported the theory for macroorganisms and he concluded that a community that is diverse in species is stable and resistant to invasion by new species. It may be advantageous to examine this conclusion as regards microbial communities (Hairston et al, 1969) and in particular pathogens "invading" the phyllosphere.

The increasing use of chemicals and monoculture in crop production could have catastrophic effects on species diversity and population size in the phyllosphere. It is important, therefore, to determine the role of the phyllosphere community in infection ecology.

The study of the phyllosphere is in a preliminary state and considerable descriptive work is still required to detail the characteristics of the microbial populations. However, it is particularly important in view of the complexity of microbial taxonomy, that precise research goals be defined so that descriptive work will rapidly yield testable hypotheses for experimental investigation.

The above mentioned concepts were paramount in the planning of the present study. It was felt that the species studied should be technically easy to investigate so that rapid progress towards experimentation could be made. The study species should be from a crop in which monoculture and chemical pest control were widely practiced. With these considerations in mind, it was decided to investigate the phyllosphere of European larch (Larix decidua Mill). The practice of monoculture has reached large proportions in conifer cultivation, and in many areas, thousands of acres are planted with a single species (Poole, 1969). In addition, the practice of chemical pest control is being practiced on a large scale in modern forest management (Gilmour and Noorderhauen, 1968). Even more threatening to species diversity is the new practice of chemical eradication of forest undergrowth (Chavasse, 1969). Indeed, these practices of modern forestry may be a threat to species diversity on a very large scale. In addition, very little work has been published (Rishbeth and Meredith, 1957) relating to the phyllosphere of coniferous plants and it was, therefore, considered important to determine if the concepts which have developed from work with angiosperms could be extended to include coniferous hosts1

The production of larch seedlings in forest nursery seedbeds provides a uniform and convenient crop for investigation. The relatively slow growth (approximately 10-20 cm per year) of the seedlings facilitates experimental replication and culture in controlled environment or aseptic conditions. While perhaps difficult to handle, the larch leaves are regularly produced on the main stem and, therefore, are easily aged. The hair-free leaf surface and the deciduous habit are also advantageous.

Young leaves of larch are susceptible to the leaf pathogen Meria laricis Vuill.. Infection results in needle cast disease, which attacks plants of all ages, but is most serious as a nursery disease where it frequently causes severe losses of one- and two-year-old plants (Biggs, 1964). M.laricis has several features which render it favourable for experimentation: it is easily cultured and produces large numbers of spores in culture; its infection biology has been well documented (Peace and Holmes, 1933; Biggs, 1964); and infection is quickly followed by disease symptoms which are easily diagnosed.

The study was planned to begin with a descriptive phase in the natural situation and then an experimental phase in laboratory and controlled environment conditions. The main objectives of the descriptive section were to assess the chemical, physical and microbial components of the phyllosphere environment and to examine environmental factors which might influence microflora development. The descriptive study was to provide field specimens for experimentation. The objectives of the experimental phase of the study were to examine interactions of the pathogen M.laricis with elements of the phyllosphere microflora and to test hypotheses raised by the field study regarding microflora development.

CHAPTER II

THE ECOLOGY OF THE LARCH PHYLLOSPHERE

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INTRODUCTION

A study of the larch phyllosphere was undertaken to determine the nature of the phyllosphere microflora, the factors influencing its development and also to acquire samples of microorganisms for further investigation.

An investigation of a biological system such as the phyllosphere should involve an examination of the total environment. In 1951, the plant ecologist, J. Major, introduced the following equation to emphasize how vegetation is a function of its environment:

$$V = (c,g,r,o,t)$$

V= vegetation, c = climate, g = geological parent material, r = relief, o = organisms and t = time. To make the equation applicable to microbial ecology requires only that the "g" be replaced by "s" which symbolizes the chemical nature of the substrate:

$$\text{Microflora} = (c,s,r,o,t).$$

In addition, the scale at which the environment is studied must be adjusted to be appropriate for microorganisms. Thus, the microflora can be thought of as a function of climate (both macro and micro), substrate, micro-relief, organisms, and time. The study of the larch phyllosphere was designed to assess these five factors.

An attempt was made to measure aspects of the climate on both a macro- and micro-meteorological level. Substrate chemistry and the details of substrate relief were both investigated. Possible influences

by other organisms e.g. insects and pollen grains were examined. The time factor was assessed by examining the development of the microflora on leaves as they aged through the growing season.

THE CHEMISTRY AND RELIEF OF THE LEAF SURFACE

LITERATURE REVIEW

The morphology of leaves influences the amount of inoculum reaching the leaf. Gregory (1952) has demonstrated that in general the rate of spore impaction is greatest on small objects, i.e. the rate of impaction of a spore will be greater on a conifer needle than on a broadleaf. Moreover, the larger the spore the greater is the rate of impaction. Thus, bacteria would be less likely to be impacted than yeast cells or fungal spores.

The leaf surface has a number of topographical features which may be of importance to leaf inhabiting organisms. Commonly, the leaf, especially the abaxial surface, is marked by ridges caused by internal vascular structure. Yeasts (Last, 1955a) have been shown to occur most abundantly along lines parallel to the leaf veins. On Pisum leaves Dickinson (1967) found that yeasts were most numerous over vascular strands. Potter (1910) made leaf prints on agar media and found that the colonies of bacteria which developed mirrored the leaf venation. On many leaves a pattern of depressions occurs overlying the position where the anticlinal cell walls of adjacent epidermal cells abut. di Menna (1959) and Last and Deighton (1965) have reported that yeast-like organisms are concentrated in the depressions. Bacteria are also found most frequently in the depressions (Ruinen, 1961), at least on young leaves. The fact that the anticlinal depressions can be important infection sites for plant pathogenic fungi has been demonstrated by Preece, Barnes and Bayley (1967) and growth of plant

pathogens along the surface depressions has been observed by Johnston (1934) for Erisiphe graminis De Candolle and by Maheshwari and Heldebrand (1967) for Puccinia anthirrhini Diet and Holw.

The size and contours of the anticlinal depressions is determined by the shape of the epidermal cells and by the form of the overlying cuticle. Lange and Schulze (1966) have shown that the depressions on spruce needles are considerably reduced by the differential thickening of the cuticle. The thickness of the cuticle is greater over the anticlinal walls than over the cell lumen and thus the size of the depression is reduced. Lange (1969) has described several different topographies that occur on both outer and inner cuticle surfaces. The fine structure of the cuticle includes the multiple forms of wax excretions that occur on the surface (Juniper, 1960). Amelunxen et al. (1967) have described six basic wax types including scales, sheets, rods, threads and granules. Apparently taxonomically closely related species may vary markedly in the fine structure of the cuticle. Leyton and Juniper (1963) described the wax formation on Pinus sylvestris L. as "tube-like outgrowths frequently branched, usually overlying plate-like secretions of similar material". However, on Pinus radiata D.Don the tubular wax outgrowths are present but there are no plate-like secretions and the underlying wax is smooth or granular (Leyton and Armitage, 1968).

The surface waxes of leaves affect the wetting properties and draining patterns of the leaves which in turn may affect the leaf

microflora. The shapes of the wax formation could possibly affect the retention of spores or cells on the leaf surface.

In addition to the possible physical effects that cuticle topography and leaf wax formations may exert on leaf microbes, they almost certainly have a strong chemical influence. This may be as a source of carbon or as a metabolic inhibitor. They may act directly, as the active compound, or indirectly by influencing the quantity of leaf leachates.

The cuticle is chemically complex and is made up of several layers. On the surface are the wax secretions described above. Below the surface wax is a layer composed of cutin lamellae with fine wax film intrusions (Franke, 1967). Between the wax-cutin layer and the epidermal cell wall is a layer of cutin mixed with cellulose and pectin (Baker et al., 1964). Heinen (1961) reported the isolation of a cutinolytic enzyme from Penicillium spinulosum Thom., so that it is possible for some microorganisms to degrade cutin. Ruinen (1966) reported that two common phyllosphere yeasts, Rhodotorula glutinus (Fres.) Harrison and Cryptococcus laurentii (Kufferath) Skinner could degrade cutin. Her evidence was based partly on microscope observation of cuticle incubated with yeasts in a culture medium containing glucose. The cuticles were isolated from Aloe sp. and, although chemically treated to remove pectin, cellulose and waxes were undoubtedly still not pure cutin. As Ruinen herself states they still contained the basic cellulose framework. In addition, the portion of the cuticle which was apparently most susceptible to degradation was

the anticlinal ridge. This is a portion of the cuticle which is likely to contain both pectin and cellulose in addition to cutin. In addition to the visual assessment of cutin degradation, Ruinen also measured titratable acid following saponification of cultures of Cryptococcus laurentii which included cuticles in a mineral or malt medium. The experiment was carried out without a control culture which contained no cuticle. Without such control it is difficult to accept the conclusion that cutin was being utilized because the amount of acid present after saponification was only slightly less (in the malt medium) or higher (in the mineral medium) at the end of the experiment than at the start. Thus, the utilization by the yeast of some portion of the cuticle was strongly indicated but to establish that cutin is being utilized will require more carefully monitored experiments.

Plant waxes are usually mixtures of long-chain paraffins, alcohols, ketones, esters and acids (Fernandes et al., 1964). The components of wax on leaves can vary considerably from species to species, and these differences may be of taxonomic value (Purdy and Truter, 1961; Baker and Martin, 1967). Watanabe (1953), in a study of leaf waxes of 38 coniferous species, divides the conifers into two groups according to wax type. Group A includes the genera Pinus, Picea, Tsuga, Abies and Chamaecyparis, the wax of which possess a large ester component chiefly of juniperic and sabinic acids. The waxes of Group B do not contain these esters and the group includes the genera Larix, Pseudolarix, Pseudotsuga and Taxus. Isoi (1958) confirmed this grouping and showed that the genus Larix was characterized by a non-estolide type of wax.

The components of leaf waxes may change with the age of the leaf. Radler (1965b)found that young leaves of the sultana vine contained a compound not present in mature leaves. There was also a series of esters present on young leaves whihh are not present on mature leaves. There is apparently a shift from the production of esters to the production of aldehydes in the maturation of leaves.

The quantity of wax on the plant surface may vary considerably with the age of the plant. Fernandes, Baker and Martin (1964) found an increase of 50-100% in the amount of wax per square centimetre during the growth of apples. A seasonal change in wax density was reported for Picea pungens Englem. by von Rudloff (1959). He attained a 40% higher wax yield from leaves of P.pungens during March, April and May than during other months of the year.

The aspects of wax quantity, quality, seasonal and species differences may influence leaf microflora in several ways. Perhaps the most direct and important influence is the occurrence of antifungal agents in the wax. Martin et al (1957) demonstrated that apple mildew (Podosphaera leucotricha (E&E) Salm.) conidia germination was strongly inhibited on apple leaves to which whole leaf wax had been applied. The ether soluble acid extract of the whole wax was shown to be the most effective wax fraction in inhibiting germination of P.leucotricha conidia and Botrytis fabae Sard. Roberts et al. (1961) found that surface waxes of rose cuticle stimulated germination of strawberry mildew spores while the absorbed wax fraction tended to suppress germination.

Some plant wax components can be metabolized by microorganisms. Long chain paraffins (C_{20}-C_{34}) can be used by some microorganisms as their sole source of carbon (Zobell, 1950). The soil bacterium, Micrococcus cerificans has been shown to be able to utilize the plant paraffin, n-nocosane (Hankin and Kolattukudy, 1968). Ursolic acid, a common terpene found in many plant waxes, can be utilized as the sole source of carbon by a Pseudomonas sp. isolated from soil by Hankin and Kolattukudy (1969). It may be inferred that the whole range of plant waxes may be susceptible to microbial decomposition because these compounds, although being continually added to the soil, do not accumulate there in any great quantity (Stevenson, 1966). The activity of a lipase in a number of bacteria has been demonstrated by Sierra (1957).

Organisms known to produce lipase include the following: Pseudomonas fluorescens, Azotobacter sp., Beijerenkia sp., Bacillus cereus, B.mycoides, Rhodotorula graminis di Menna, R. glutinus (Fres) Harrison, Candida bogoriensis Deinema, Mycobacterium phei, Pseudomonas aeruginosa, Micrococcus lysodeikticus, and Streptococcus liquefaciens. The first eight taxons listed above have been reported to occur in the phyllosphere (Burri, 1903; Duggeli, 1904; Deinema and Landheer, 1960; Ruinen, 1961; McBride, 1969). Thus a number of known phyllosphere organisms can utilize the plant waxes and therefore the nature of the leaf microflora may in part be determined by the kind and quantity of leaf waxes. The reported differences of waxes between plant species and in the same species at different ages may influence microflora development.

It has been shown by Radler (1965a) that the water permeability of the cuticle of grape is not affected greatly by the cutin or hard wax fraction but is determined largely by the soft wax fraction. The permeability of the cuticle is an important factor in determining the movement of solutions into and out of the leaf (Franke, 1967). When wax is removed from the leaf the cuticular transpiration increases (Hall and Jones, 1961). Thus, organisms which can degrade leaf waxes may influence the physiological condition of the host. The permeability of the cuticle could be of importance to phyllosphere organisms because it will determine the amount of nutrients reachingthe leaf and could influence the penetration of extracellular microorganism chemicals, which could result in a host reaction.

The wettability of the leaf surface is determined partly by the chemical groups at the leaf surface and the roughness of that surface (van Overbeek, 1956; Leyton and Armitage, 1968). The surface wetting of leaves by water may influence directly and indirectly the microorganisms on the leaf surface. Directly, it may influence the moisture conditions and the deposition or removal of cells and spores because it is one of the major factors affecting the amount of water retained on a leaf surface following rain (Fogg, 1947). Indirectly, it may influence the microflora by affecting the rate of leaching from the leaf (van Overbeek, 1956). Last and Deighton (1965) postulated that as leaves became less hydrophobic with increasing age, more nutrients became available to leaf parasites.

Compounds other than those present in the leaf surface structures are present on the leaf surface. These compounds may come from a number of sources, including the interior of the leaf, rainwater, the airspora

and airborne detritus. Their presence or absence may materially affect microbial development on the leaf surface.

Although the mechanism of foliar penetration of chemicals is a topic of considerable debate, it is clear that large quantities of nutrients can be taken up or lost through the leaf epidermis (Franke, 1967). Both inward and outward penetration of the epidermis by chemicals is of importance to leaf surface ecology. Outgoing chemicals, or leachates, may affect the development of phylloplane microorganisms. Inward moving chemicals produced by phylloplane organisms may in turn affect leaf physiology. Many aspects of the leaching of nutrients from leaves have been reported by Tukey and co-workers in a long series of papers. Both inorganic and organic substances are leached. Sodium and manganese are most readily leached while calcium, magnesium, sulphur, potassium and rubidium also occur in considerable quantities in leachates (Tukey and Tukey, 1962). The ions iron, zinc, phosphorus, and chlorine were also detected in small quantities. In the organic content of leaf leachates, 21 amino acids, 14 organic acids and a number of carbohydrates including four free sugars have been identified (Morgan and Tukey, 1964). According to Tukey and his associates (Tukey, Wittwer and Tukey, 1957; Tukey and Morgan, 1963) the rate of carbohydrate leaching is directly related to light intensity and leaching is increased by toxic chemicals such as oils, by mechanical injury such as surface abrasion, and by climatic factors including frost and high temperatures. Although nutrients are lost from plant foliage throughout the growing season, the quantities increased

greatly at leaf maturity and just before leaf death (Long, Sweet and Tukey, 1956).

It is evident in the literature reporting the chemical composition of rainwater (through fall) under tree canopies, that the natural occurrence of nutrient leaching of leaves is widespread (Mes, 1954; Dalbro, 1955; Will, 1955; Attiwell, 1966; Carlisle et al., 1966, 1967). Carlisle et al. (1967) reported that as much as 453.3 kg/ha/annum of organic matter is present in rain that has passed through a canopy of Quercus petraea (Mattuschka)Leibl. The organic matter included 89.2 kg/ha/annum of soluble carbohydrate.

Seasonal variations in leachate quantity have also been reported. "The factors influencing the quantity of nutrients in the throughfall (leaf senescence, high rainfall, frequency of high winds from the sea, nutrient concentrations in the leaves) all tend to produce autumnal maxima for at least some of the nutrients (Ca and Na). There are also peaks in the spring probably due to concentrations of nutrients in the immature poorly cutinized leaves." (Carlisle et al., 1967). The nutrients contained in throughfall are, of course, lost to the phylloplane organisms but the large quantities of these nutrients indicates the considerable nutrient potential on the leaf surface.

Not all the nutrients contained in throughfall are derived from the canopy. Indeed, incident rainfall also contains appreciable organic matter in solution. While passing through the canopy, rain is enriched in bases and organic matter but may be deprived of inorganic nitrogen (Carlisle et al., 1967). Thus, rainfall may add nutrients to the phyllosphere.

Airborne spores, pollen and dust may also add nutrients to the leaf surface. (Barnes, 1969).

Brown (1922) demonstrated that host nutrients leach out of the leaf into infection droplets and can thus affect the infection process. The comparable situation in the rhizosphere, the effect of root exudates on soil organisms (Shroth and Hildebrandt, 1964), has been extensively studied for both pathogens and non-pathogens. In the phyllosphere, attention has been directed to pathogens. Kovacs and Szeöke (1956), studied the effect of cuticular excretions from a number of plants on the germination of Botrytis cinerea Pers., Ascochyta pisi Lib. and Puccinia triticina Erikss. The behaviour of the fungal spores varied with the concentration of the leachate, with the species of plant from which the leachate originated and with the species of fungus. Both inhibition and stimulation of germination were observed. Generally, B.cinerea was most sensitive and P.triticina least sensitive to the leachates.

Kosuge and Hewitt (1964) reported the stimulation of Botrytis cinerea conidia germination by exudates of grape berries. The exudates also stimulated elongation of germ tubes and formation of appressoria. The exudates, which increased in quantity as the berries matured included fructose, glucose and amino acids. The authors suggested that the nutrients in water on the berry surface may serve as an important energy source for the fungus.

Nutrients from pollen grains falling on the leaf may have marked effects on the phylloplane microflora. Fokkema (1968) reported an increased development of Cladosporium herbarum Link ex Fr. on rye leaves when pollen was applied to the leaves. Barnes (1969) considered

that the development of many microorganisms on clover leaves that he examined was related to pollen grains on the leaves. Chou and Preece (1968) have shown that pollen grains increased the germination of Botrytis cinerea Pers. spores on leaves and fruits of strawberry. The presence of pollen on the fruits led to increased infection.

Thus several nutrient sources provide a variety of compounds which can affect the development of pathogen and non-pathogen elements of the leaf microflora.

THE LARCH LEAF

(a) Leaf position and morphology

The first step made in examining the leaf as a substrate for microorganisms was a survey of leaf form and leaf surface topography. Observations were made on seedlings grown in seedbeds and in the greenhouse. A freezing microtome was used for sectioning the leaves, and staining with Sudan III was used for observation of the cuticle. In addition, a Stereoscan electron microscope was used for examining leaf surface features.

L.decidua germinants normally produced 5-7 cotyledons. The cotyledons were morphologically different from the leaves. In cross-section they were triangular with the abaxial side slightly rounded. They were linear, pointed, and 1-1.5 cm. long. The leaves were linear, soft, pointed and deciduous. They were light green and were keeled below. Leaves were borne singly on young plants and leading shoots. On older plants there were short shoots which bear 30-40 leaves in a short spiral. The leaves varied from 1.5-3.5 cm. in length and 0.8 to 1.2 mm. in width.

Growth of the main stem in the first year varied between 5 cm. and 15 cm. depending on environmental conditions. A number of short side branches may develop at the base of the main stem towards the end of the first growing season. Leaves were formed in the apical meristem and originally were oriented almost vertically. Soon, however, the leaf orientation changed until a near horizontal position was reached. The leaves remained in this horizontal position until leaf fall in the autumn. The portion of the seedling's main stem above the cotyledons is designated as the plumule.

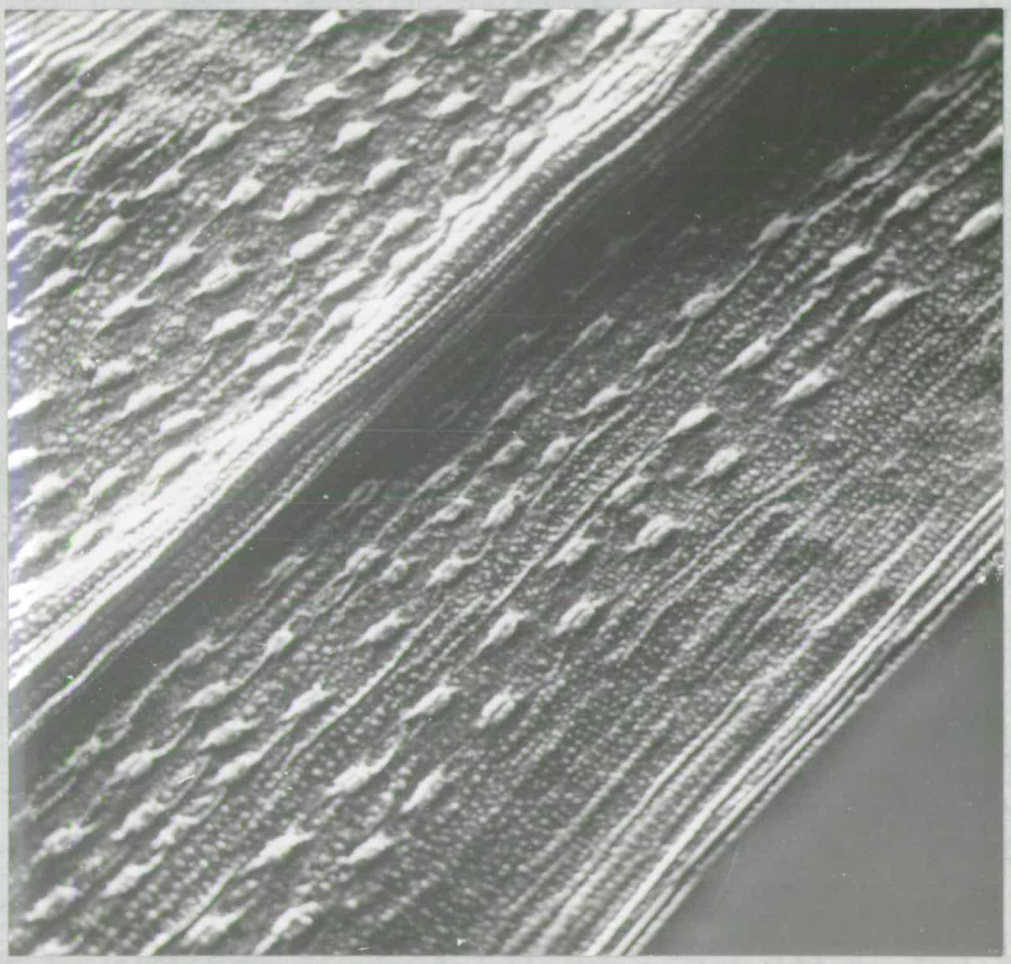
(b) Leaf Surface Relief

The main features of leaf surface relief fell into three convenient size categories: (a) features reflecting internal tissue structures, (b) features reflecting epidermal cell structure, and (c) features of subcellular dimension. The first two categories could be readily observed with standard light microscopy. The latter required the techniques of incident beam electron microscopy.

i. Leaf surface features reflecting tissue structures

The main tissues of the leaf were revealed in a simple cross-section (Figure II-1). The main internal tissues which influence surface features were a single vein running the length of the leaf, the palisade layer, the spongy mesophyll and the resin ducts near the margins of the leaf. The single vein gave the leaf a keeled shape, the abaxial surface having a convex midrib bordered by concave "valleys" running the length of the leaf. The concave "valleys" reflected the location of the spongy mesophyll and contained the stomata which were arranged in longitudinal rows. There was a distinct margin to the leaf which was 50-60 μ wide. This area consisted of 3-4 rows of epidermal cells somewhat narrower but thicker walled than other epidermal cells. These cells overlay two resin ducts, one on either side of the leaf and running the length of the leaf. These features can be seen in Plate II-2. The adaxial surface was simple and convex in cross section reflecting the uniform nature of the palisade layer.

PLATE II-1 LOWER SURFACE OF A LARCH LEAF SHOWING MIDRIB, STOMATAL "VALLEYS" AND LEAF MARGIN. X 140



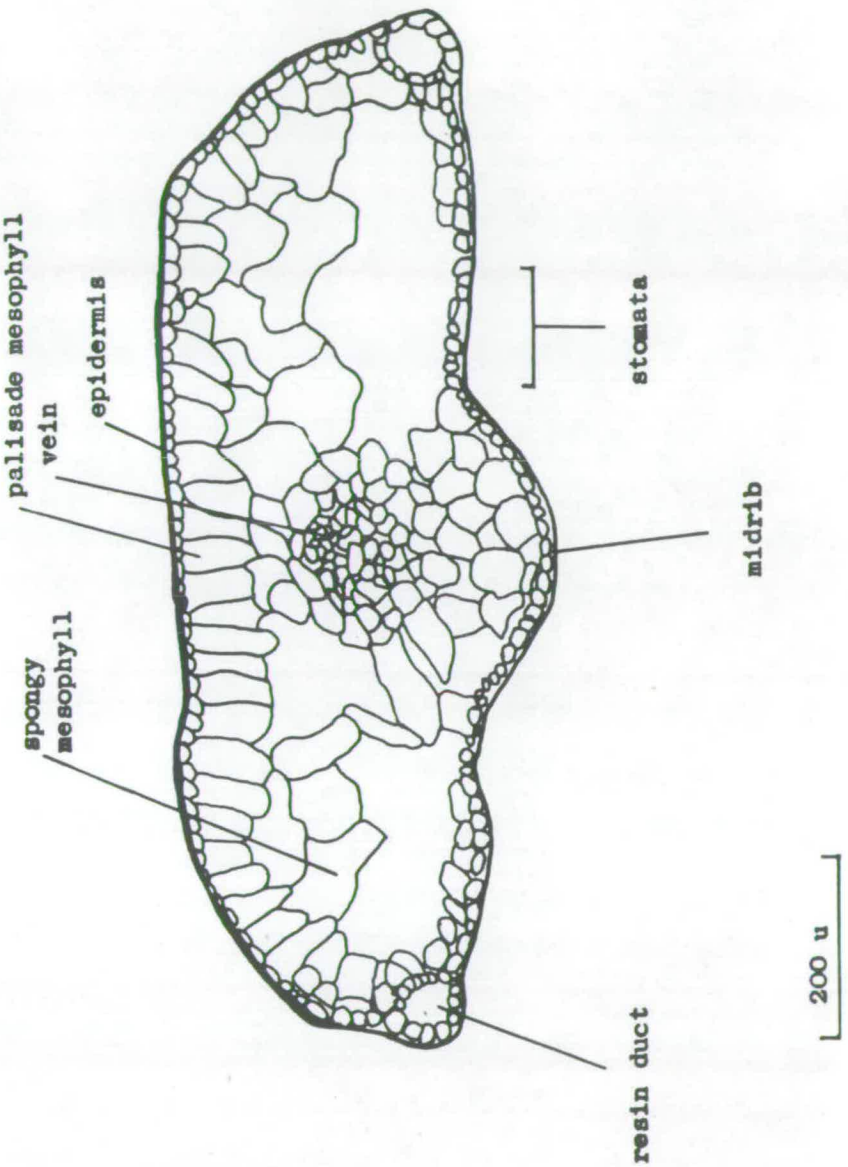


Figure II-1 A leaf of European larch in cross-section revealing internal tissues and related leaf topography.

ii. Leaf surface features reflecting epidermal cell structure

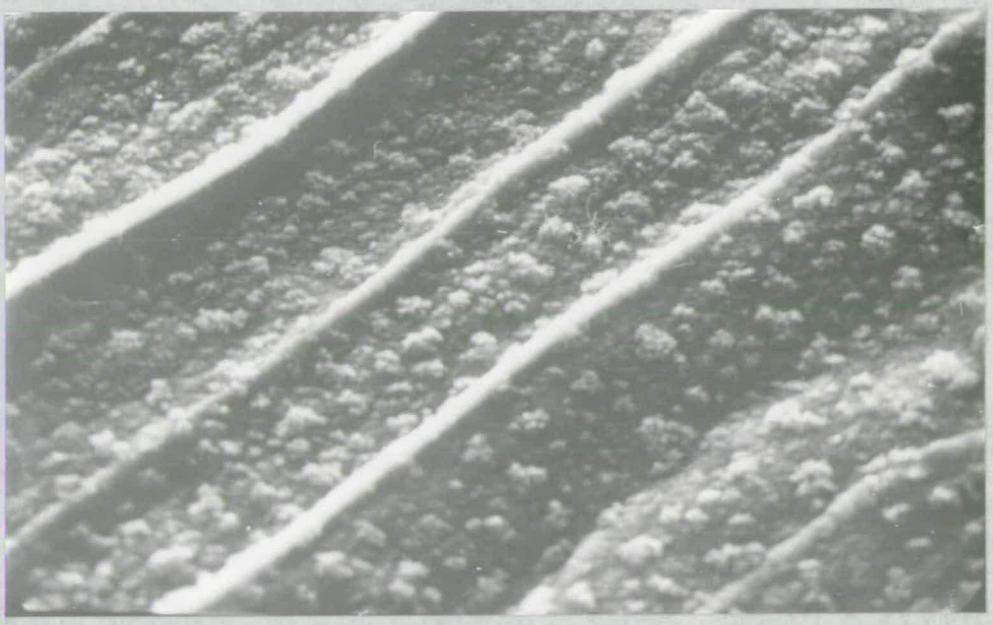
The entire leaf was, of course, enclosed in epidermis. Except for guard cells and their subsidiary cells the epidermal cells were elongated parallel with the long axis of the leaf. Typical epidermal cells varied between 200-250 μ in length and 20-30 μ in width. In cross-section the outer cell wall of the epidermal cells was convex. Thus, where two epidermal cells adjoined, i.e. along the anticlinal cell walls, there was a depression. The entire surface of the leaf was marked by these depressions or ravines. The depth and profile of the ravines were modified by the nature of the overlying cuticle. The cuticle projected rib-like into the ravines so that an isolated cuticle showed the pattern of anticlinal "ravines" by the ribbing on its inner surface. The degree to which the cuticle filled in the anticlinal ravines was different on the adaxial surface of the leaf as compared with the abaxial surface. On the abaxial surface the cuticle was almost uniform in thickness with only thin cuticle ribs in the anticlinal ravines. The ravines were deep, up to 5 μ and were v-shaped at their base. On the upper surface the thickness of the cuticle was variable with the thickest portion being above the anticlinal cell walls. The result of this variable thickness was a dimunition of the anticlinal ravine. The upper surface appeared gently undulating with rounded ridges and rounded depressions. The difference in height between ridge and depression was approximately 2 μ. The anticlinal ravines of the upper and lower leaf surface can be compared in Plates II-2 and II-3.

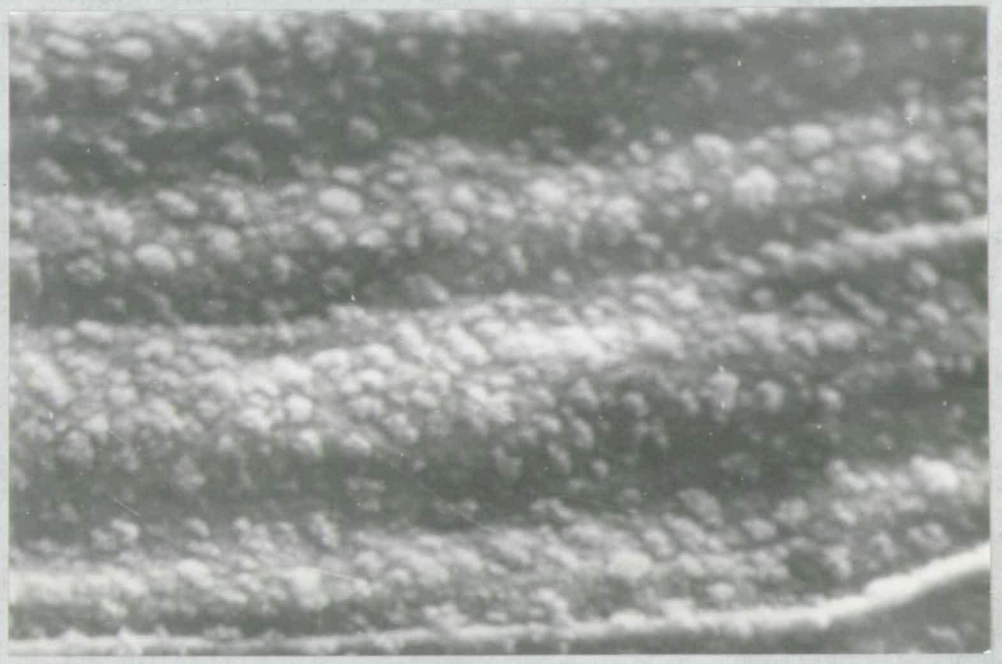
PLATE II-2 ANTICLINAL RAVINES ON THE LOWER SURFACE OF A LARCH LEAF. X 1250

The ravines were v-shaped and up to 5u deep. Note the density of surface wax structures.

PLATE II-3 ANTICLINAL RAVINES ON THE UPPER SURFACE OF A LARCH LEAF. X 1200

The ravines were rounded at the base and up to 2u deep.





The stomata occupied two strips about 300 μ wide on either side of the midrib on the abaxial surface of the leaf. They were arranged in 5 or 6 longitudinal rows on each side of the midrib and ran the length of the leaf. When turgid, the guard cells were crescent shaped and were somewhat sunken below the level of surrounding epidermal cells. The stomatal aperture was approximately 20 μ long by 10 μ wide. The outermost wall of the guard cells was approximately 10-18 μ below the outermost wall of the subsidiary cells (Figure II-2). When viewed in plan view the subsidiary cells overlap the guard cells as shown in Figure II-3.

iii. Leaf surface features of subcellular dimension

Considerable quantities of the wax were observed on the surface of larch leaves. The form of the wax following solidification on the surface resulted in a number of micro-topographical features. Leaves were observed and photographed using a Cambridge Instruments Ltd. Stereoscan Electron Microscope. The leaves were coated with 40% Palladium gold under vacuum prior to observation. The vacuum treatment resulted in some collapse of the epidermal cells, but features such as wax formation appeared to be undisturbed by the process.

Leaves from seedlings grown aseptically in conical flasks were examined so that leaf surfaces totally undisturbed by weathering could be observed. Details of the condition of growth of the aseptic seedlings are given on page 108. Seedlings which had been grown in seedbeds were also examined to determine if the same features were present. This

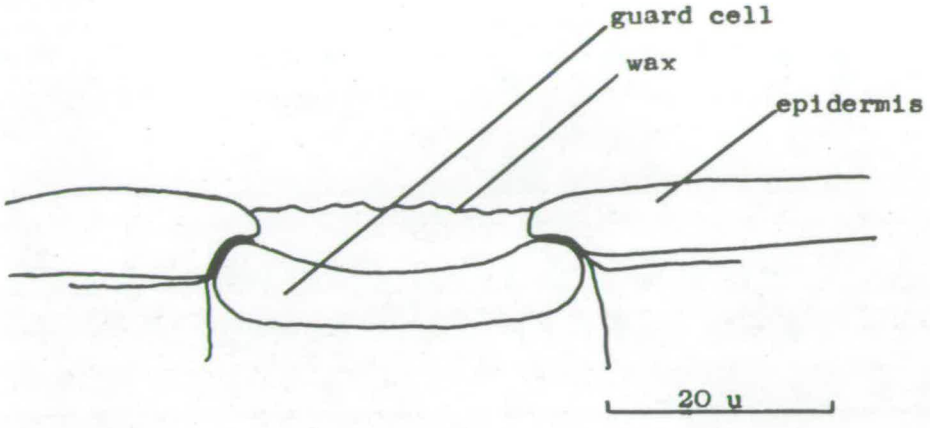


Figure II-2 A cross-section of a stoma showing the guard cells partially sunken below the epidermal cells.

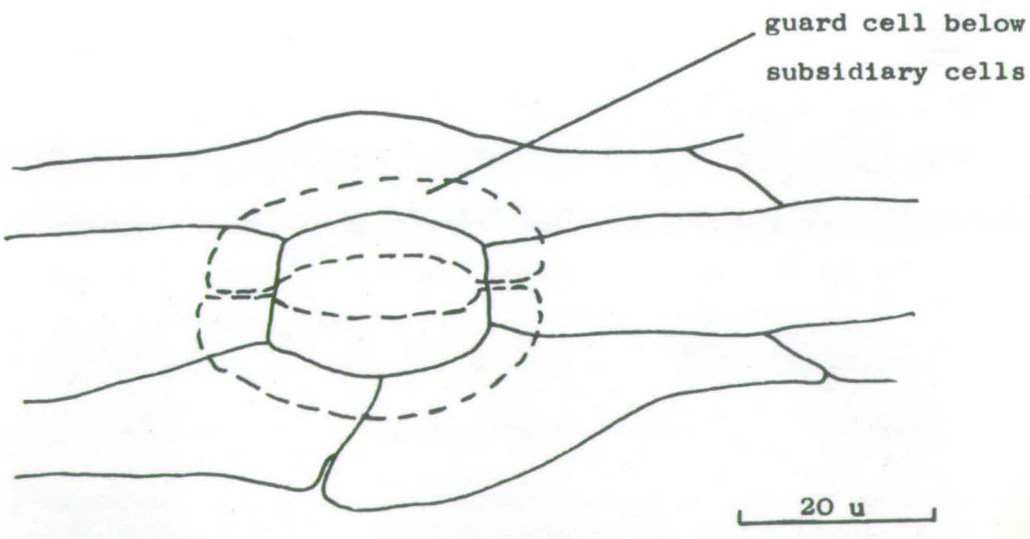


Figure II-3 Surface arrangement of guard cells and subsidiary cells of a larch stoma.

approach proved useful because the wax formation on seedbed material appeared to be weathered and it was difficult to interpret the structure.

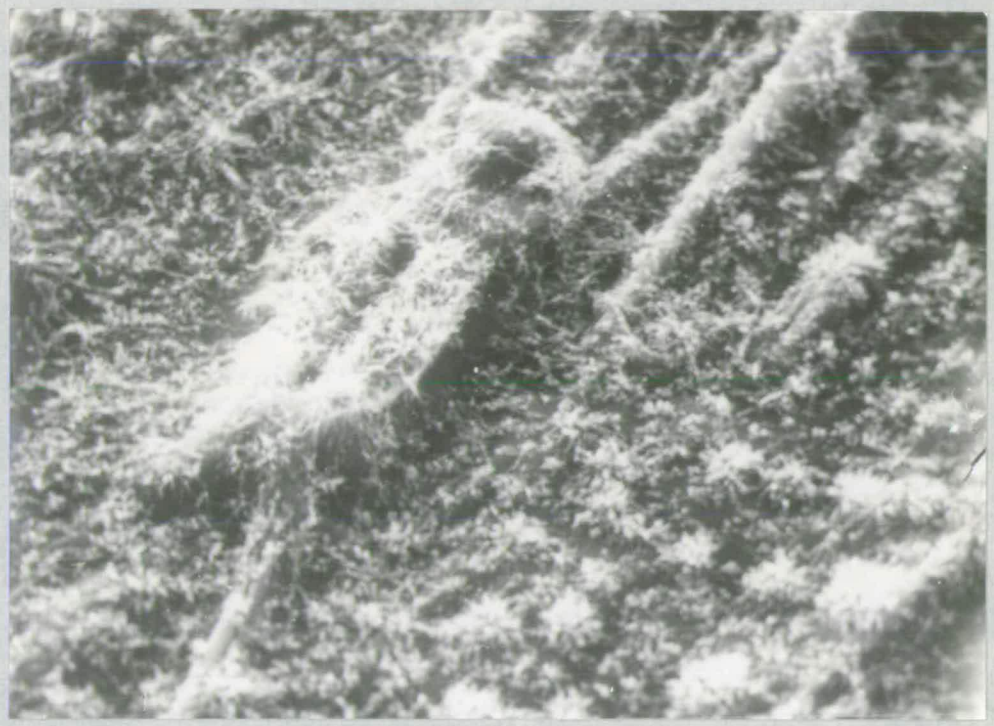
Young leaves which had just reached the fully expanded stage were chosen for observation. The wax appeared to be concentrated in distinct clusters approximately 4 μ in diameter, Plate II-4. The clusters occurred at an average density of 42 ± 4.1 in a 50 μ x 50 μ square. The wax clusters appeared coralloid or granular at magnification of 5,280 and 10,500. They were similar to the structures called "Wachskörnchen" by Amelunxen et al. (1967). The area between the wax clusters was not smooth but was covered by small granular wax particles. The wax formations on the adaxial surface appeared uniform over the entire surface.

The wax patterns on the abaxial surfaces of the leaf varied according to location. The wax in the area between the margin of the leaf and the outermost row of stomata most resembled the wax found on the adaxial surface. Wax clusters averaging 6.0 μ in diameter occurred with a density of 37 + 2.6 per 50 μ x 50 μ square. The clusters were somewhat larger and had less distinct outlines than the similar structures on the adaxial surface. Between the clusters were scattered particles of wax of a coralloid nature.

In the region of the stomata, the wax extrusions were more extensive and of a different form than on other parts of the leaf. The wax did not appear to be concentrated in obvious clusters and the wax was in the form of fine threads about 0.1 μ in diameter and 1-2 μ in length. The wax was most dense around the stomatal aperture (Plate II-5),

PLATE II-4 CORALLOID WAX STRUCTURES OR "WACHSKÖRNCHEN" ON THE LOWER LEAF SURFACE. X 1150

PLATE II-5 CONCENTRATION OF WAX STRUCTURES OR "WACHSFADEN" AROUND A STOMATAL APERTURE. X 2300



where it appeared as a tangled mass of threads. The structure was similar to the wax form called "Wachsfaden" by Amelunxen et al.(1967). Some of the wax around the stomatal aperture originated on the anticlinal wall of the subsidiary cells. It was not clear whether some of the wax originated on the guard cells or not.

The difference in the nature of the wax formation on the cuticle in the stomatal region and on the leaf margin can be seen in Plate II-6. The change from the coralloid-form to the thread-form was abrupt and coincided with the outermost row of stomata.

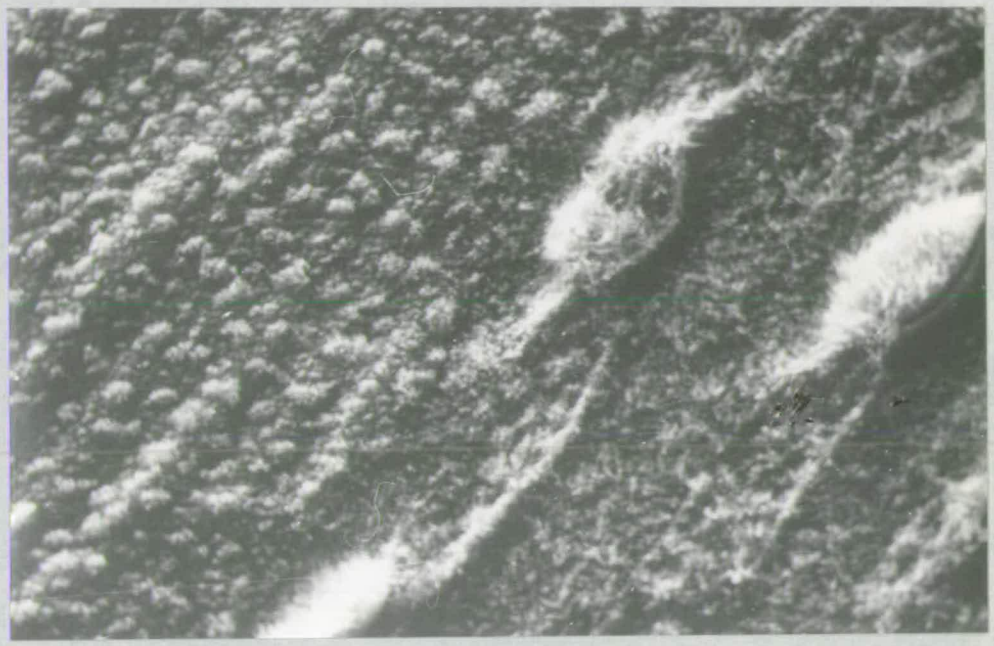
The wax structures on the cuticle overlying the midrib of the abaxial leaf surface were threadlike and arranged in obvious clusters. Because of the cell collapse in this region it was difficult to assess the density of the structures (Plates II-7 and II-8). The thread-like form of the wax and the cluster formation can readily be seen in Plate II-9.

A young fully expanded leaf from the upper plumule of a seedling grown in a nursery seed bed (see p. 46 for details of growing conditions) was examined for surface wax formation.

The same basic formation and patterns were found on the leaves as were found on the leaves from the aseptic seedlings. There were, however, differences which might have been caused by physical and microbial weathering. On the adaxial surface the clusters were not as distinct as they were on the aseptic seedlings (Plate II-10). The wax was, however, still in coralloid or "Wachskörnchen" form (Plate II-11). On the abaxial surface the wax formations were of the same type as described for the aseptic seedlings but the wax threads were short and compact. (Plate II-12).

PLATE II-6 OUTERMOST ROW OF STOMATA SHOWING CHANGE OF WAX STRUCTURE. X 900

PLATE II-7 LARCH LEAF MIDRIB. X 720
Note cell collapse between anticlinal walls.



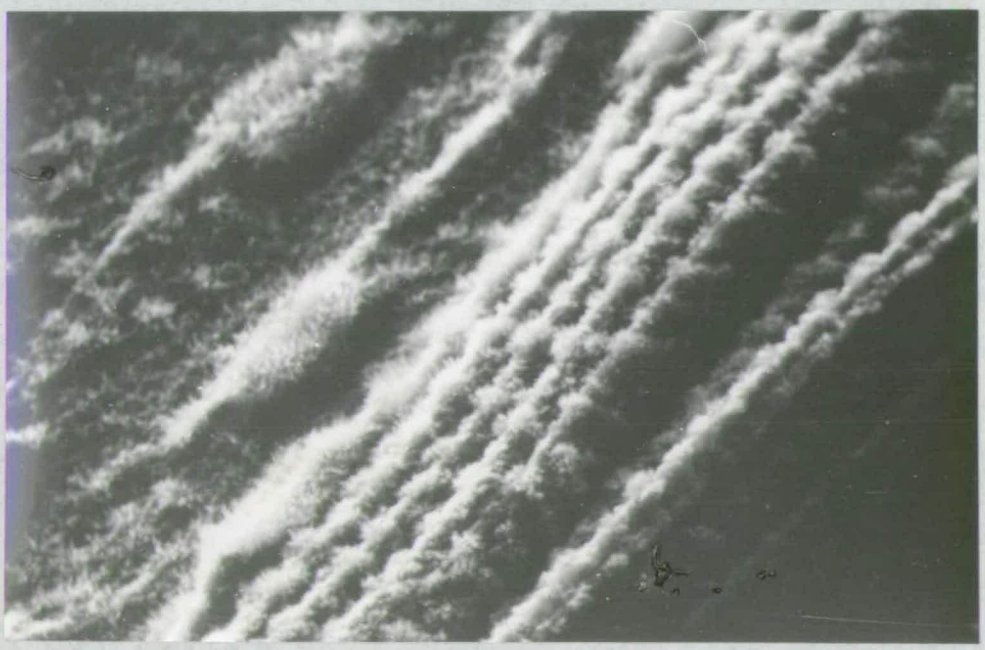


PLATE II-8 WAX FORMATIONS ON THE LARCH LEAF MIDRIB. X 2880

PLATE II-9 CLUSTER OF THREAD-LIKE WAX FORMATIONS ON THE LARCH LEAF MIDRIB. X 12,800

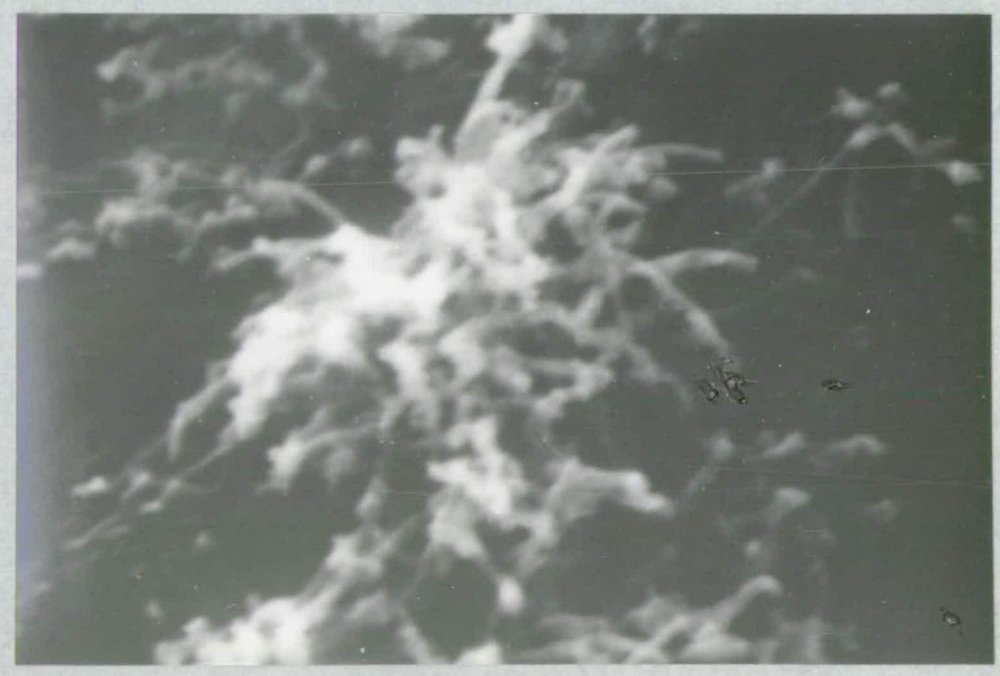


PLATE II-10 COMPACT WAX STRUCTURES ON THE UPPER SURFACE OF A FIELD-GROWN LARCH LEAF. X 1500

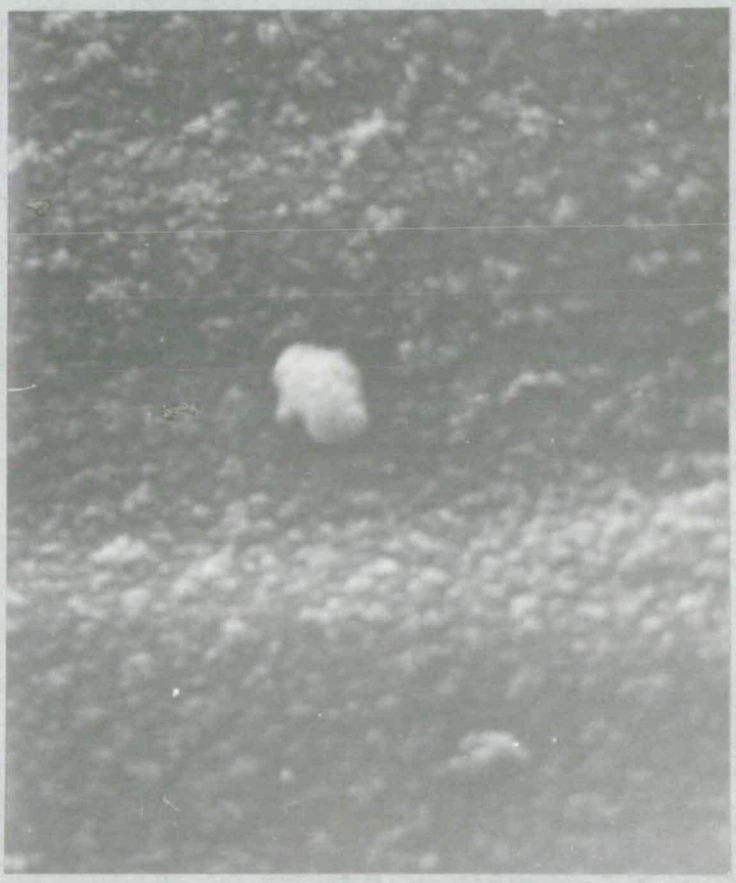
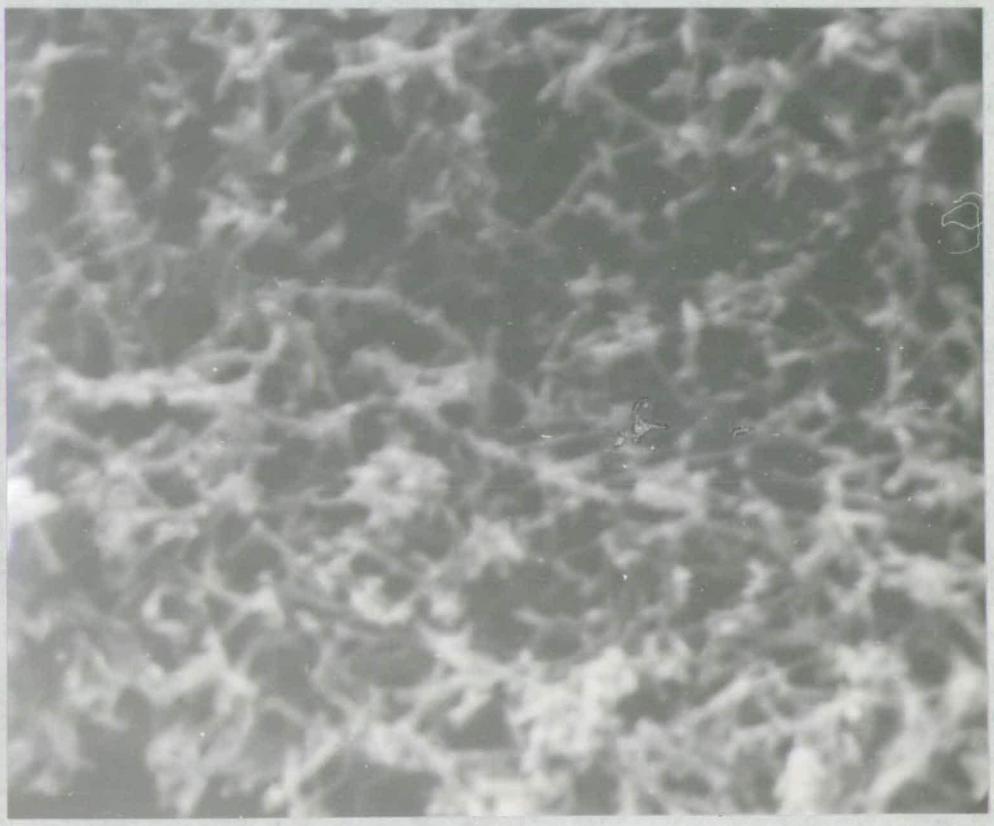


PLATE II-11 CORALLOID WAX STRUCTURES ON THE LOWER SURFACE OF A FIELD-GROWN LARCH LEAF. X 3000

PLATE II-12 CLUSTER OF SHORT, DENSE WAX THREADS ON A FIELD-GROWN LEAF. X 10,450



In many of the stomata the wax appeared to be compacted into a solid plug which wholly or partly blocked the aperture. This condition can be seen in Plates II-13 and II-14.

(c) Chemistry of the larch cuticle

Chemical nature of the larch leaf surface.

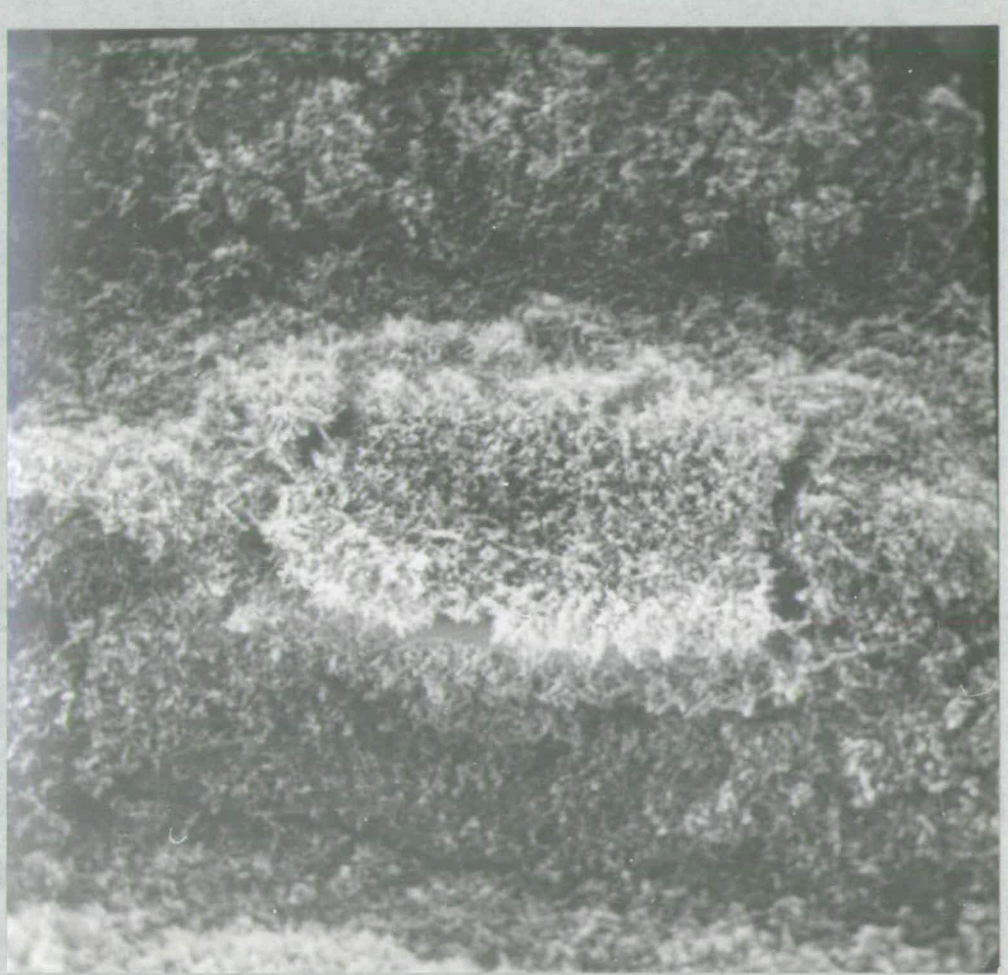
The leaf cuticle may be considered the physical and chemical framework of the phyllosphere. As the investigation of the chemistry of the larch leaf cuticle progressed it became apparent that a detailed study would require a prohibitive amount of time. It is therefore recognized that the results presented here are in many ways incomplete but nevertheless add significant information indicating the composition and complexity of the surface waxes.

The surface waxes of larch leaves were extracted by the method of Radler and Horn (1965) using a series of cold chloroform baths. To determine the effectiveness of this method on larch leaves, 24 g of field-grown larch leaves were immersed in four successive lots of chloroform (100ml). After distilling and drying at 40°C the weight of the four extracts was (1) 64.9 mg, (2) 51.3 mg, (3) 18.9 mg, and (4) 7.1 mg. Thus a total of 5.9 mg of chloroform-extractable wax was isolated per gram of leaves. Over 80% was removed by the first two washes and thus two or perhaps three washes would be sufficient for sampling and would avoid problems of extracting intracellular lipids.

The surface waxes of young and old larch leaves were compared using the chloroform extraction method with three chloroform washes.

PLATE II-13 STOMATAL APERTURES PLUGGED WITH WAX. X 670

PLATE II-14 STOMATAL APERTURE COMPLETELY FILLED WITH WAX.
X 2570



Two grams of young leaves (top 4 cm) and two grams of old leaves (bottom 4 cm) from four-month-old, greenhouse-grown seedlings were used for the analysis. 3.0 mg of chloroform-extractable wax per gram of young leaves and 2.9 mg per gram of old leaves were obtained. Thus, old and young leaves had similar quantities of wax but the greenhouse-grown leaves had less wax per gram than the previously examined field-grown leaves.

The composition of leaf waxes has been shown to change as the leaf ages (Radler, 1965b; von Rudloff, 1959). Therefore, a comparison of the waxes from the young and old larch leaves was carried out using the thin layer chromatographic method of Radler and Horn (1965).

Silica plates which had been activated at 110°C for 30 minutes were used. The solvent used for spotting the extracts (30-100 µg per spot) and running the chromatogram was a mixture of light petroleum (b.p. 60-70°C), ether, and acetic acid (70:30:1.5). The resulting spots were detected by spraying the dried chromatogram with a solution of 5% potassium dichromate in 40% sulphuric acid and heating at 200°C for 10-15 minutes.

A chromatogram showing the comparison of young and old waxes is presented in Plate II-15. Both old and young fractions separated into five distinct spots. Although no determinative tests were made, the spots correspond to the categories isolated from grape leaves (Radler, 1965b; Radler and Horn, 1965). In order of increasing Rf values they were acids, n-alcohols, docosanoic acid, an aldehyde fraction, and a hydrocarbon fraction. Differences between the young and old leaf fractions may have arisen by using an excessive quantity of old leaf extract for spotting the chromatogram with resultant tailing of the spots.

PLATE II-15 THIN LAYER CHROMATOGRAM OF CHLOROFORM EXTRACTABLE LEAF WAX.

Young leaves (left) and old leaves (right). (Silica gel plates activated @ 110°C for 30 minutes. Solvents: light petroleum, ether, acetic acid (70:30:1.5). Spray reagent 5% $K_2Cr_2O_7$ in 40% H_2SO_4.)

In spite of this factor it appears that more hydrocarbon fraction was present on the old leaves than on the young.

A 10 cm column was prepared from 20 g of aluminium oxide (neutral, activity I Merk). Approximately 0.5 g of chloroform-extracted wax from leaves of different ages was refluxed with light petroleum, cooled, and decanted onto the column. The sequence of solvents used was light petroleum, ether (saturated with water), ether plus 1% ethanol, chloroform plus 1% ethanol, and acetic acid. The number of fractions and their weight are summarized in Figure II-4. Two major peaks were apparent in the ether and acetic acid fractions which corresponded to the peaks for esters and free acids in grape wax (Radler, 1965b). Chromatography of the fractions, using the methods described previously, indicated that some of the fractions contained several compounds (Figure II-5).

(d) Surface Wetting Properties

The surface wetting properties of larch leaves were examined to determine if this property changed with age.

Fogg (1947) developed a measurement based on the contact angle of an advancing droplet of water to assess the hydrophobic nature of leaf surfaces. The contact angle is determined both by the chemical groups and by the degree of roughness at the leaf surface. The contact angle is low on easily wetted surfaces and high on hydrophobic surfaces.

The contact angle of larch leaves was measured for a range of leaf ages. Leaves from a four-month-old larch seedling grown in the seedbeds described later in this chapter (see page 46) were used for the

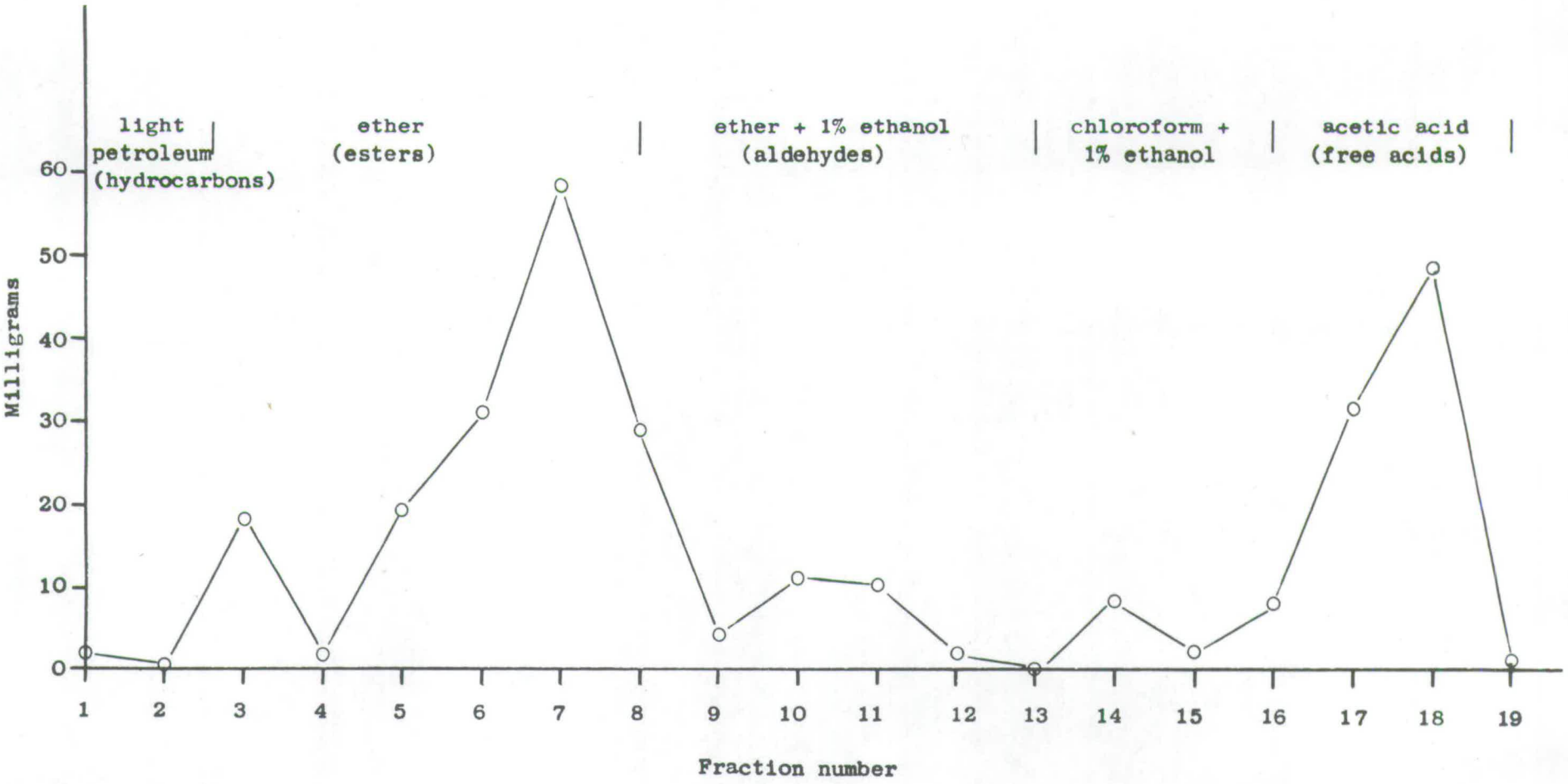


Figure II-4 Fractionation of the light petroleum-soluble part of wax of larch leaves on a column of aluminium oxide (20 g activity grade I). Fraction No.1 was 80ml, fraction No.18 100ml, other fractions 20ml.

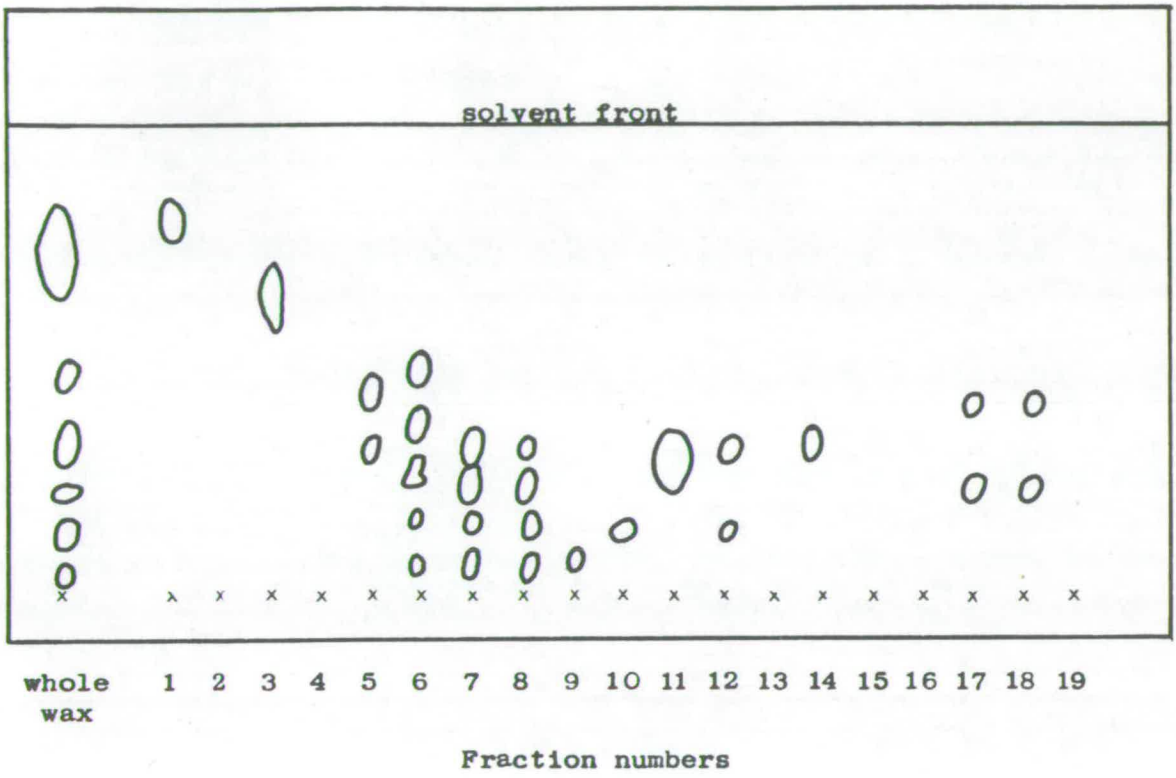


Figure II-5 Thin-layer chromatogram of the fractionated larch leaf wax. Several fractions contained more than one compound. (Silica gel plates activated @ 110°C for 30 minutes. Solvents: light petroleum, ether, acetic acid (70:30:1.5). Spray reagent 5% $K_2Cr_2O_7$ in 40% H_2SO_4).

assessment. The seedling was divided into 6, one-centimeter sections from the cotyledons to the apical meristem. The contact angle was determined on a sample of leaves from each of the six sections, i.e. on leaves from one week to four months old. In addition, a sample of the youngest leaves was gently brushed with a cloth before making the measurements. The contact angle was calculated using the method described by Fogg (1947). Water droplets (1 mm in diameter) were placed with a syringe on the surface of freshly cut leaves Plate II-16. The leaf was held in a horizontal position in the optical axis of a micro-projector (Plate II-17). The projected outline of the water droplet was then traced on paper. The contact angle was measured only for the adaxial leaf surface because the abaxial surface was too irregular in shape for accurate measurements to be made.

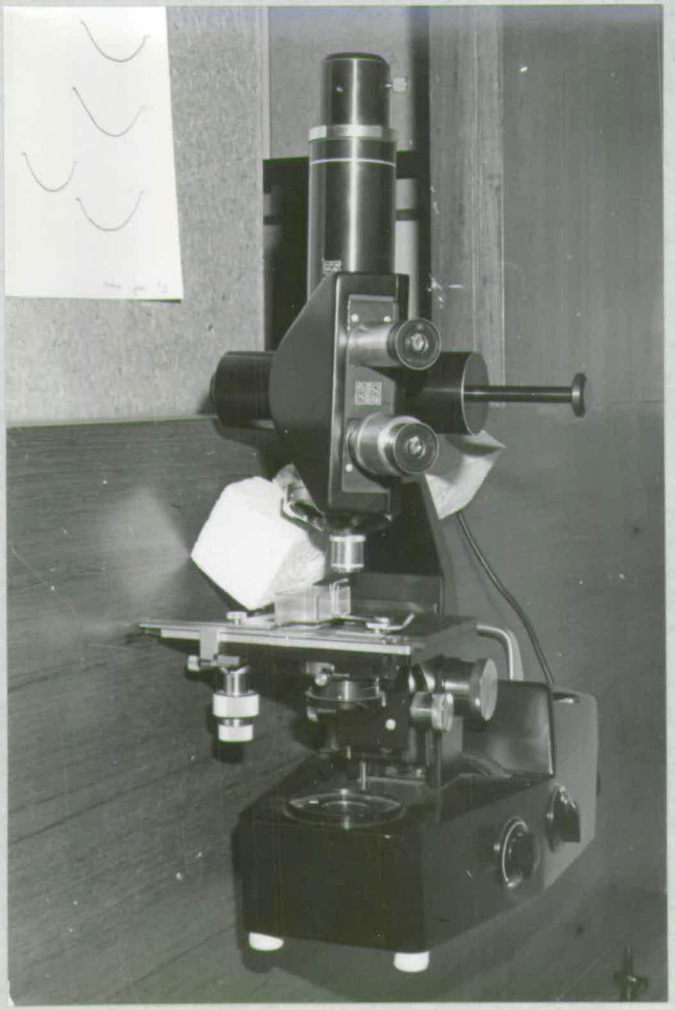
The mean contact angles and the standard deviations of the samples are presented in Table II-1.

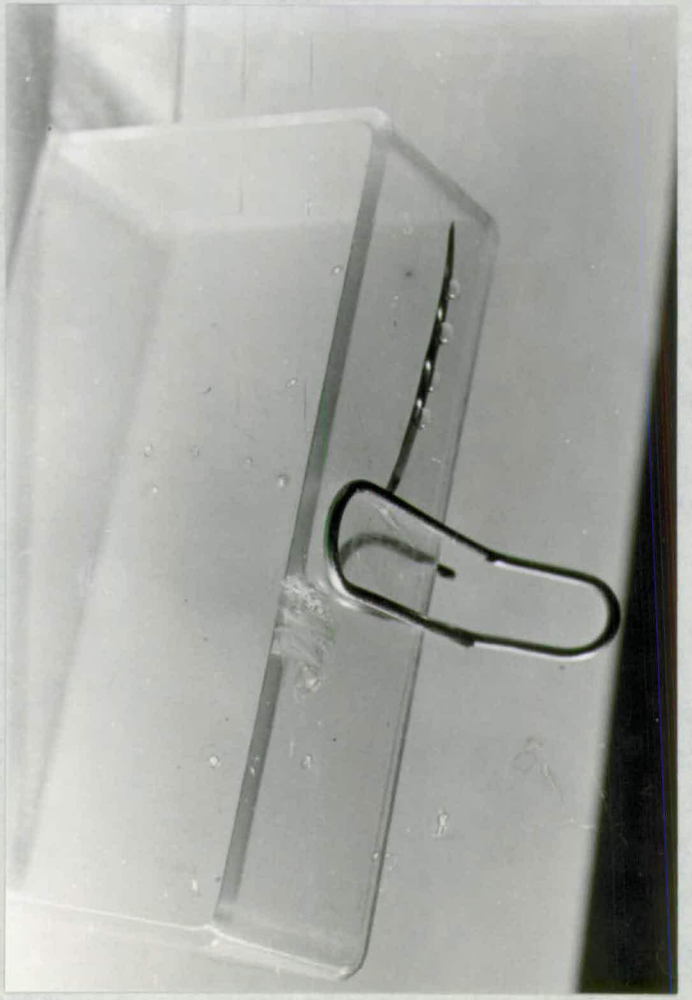
TABLE II-1. Contact angles for different larch leaves (mean and standard deviation).

	Position of leaf as centimeters above cotyledons					
	1	2	3	4	5	6
$\bar{x}$	86.5	84.4	87.1	88.8	88.1	107.8
SD	2.2	1.9	2.5	0.4	0.8	21.2

PLATE II-16 WATER DROPLETS ON LARCH LEAF IN PREPARATION FOR MEASUREMENT OF CONTACT ANGLE.

PLATE II-17 MICROPROJECTOR USED TO PROJECT THE OUTLINE OF WATER DROPLETS FOR MEASUREMENT OF CONTACT ANGLE. The leaf was held horizontally on the microscope stage and the image was projected onto a white surface for drawing.





A general trend of decreasing contact angle (increasing wettability) with rising age was apparent. The high contact angle of the youngest leaves dropped quickly from over 100° to less than 90° in the second youngest age group. The decline was then more gradual. It was also of interest that the youngest leaves showed the greatest variability in contact angle. Brushing markedly decreased the variation on young leaves from a standard deviation of 21° to 0.1° and decreased the contact angle from 107° to 89°, a level similar to older leaves. Thus brushing simulated the natural weathering of the leaf by making it more uniform and more easily wetted than young leaves.

The initial rapid increase in surface wetting of older leaves on the seedlings may be a result of physical weathering of the leaf wax structure. This was seen in photographs comparing protected and weathered larch leaf surfaces (Plates II-4 and II-11). The further gradual increase of surface wetting on ageing leaves may be a result of chemical changes or further weathering and loss of wax from the leaf.

(e) Leaf leachates.

The amino acid and soluble carbohydrates present in leaf leachates were analyzed using thin-layer chromatography. The leachate was collected in such a way as to approximate natural conditions of leaching.

Five hundred, four-month-old seedlings were used in the leachate study. The seedlings were grown in a glasshouse in University of California compost Mix I-C (Baker, 1957) modified with additional

peat to pH 5.0. The seedlings were sprayed with a fine mist of glass-distilled water until the leaves were dripping. One hour later the still wet seedlings were gently tapped with a glass rod knocking the water droplets off the leaves into a large beaker. This procedure was repeated 20 times and was always carried out during daylight. Two samples of 250 ml each were collected in this way.

The samples were filtered immediately on collection through washed Seitz filters. The ion exchange technique of Nykvist (1963) was used to purify the samples. The filtered leaf leachates were passed through a cation exchange column containing 40 g of "Amberlite IR-120 (H+)". The column was adjusted to run at a rate of 0.5 ml/minute. The column was then washed with 300 ml of distilled water to remove anions and non-ionic substances. The cations (including amino-acids) were then eluted with 400 ml of 1N NH_4OH. The two fractions (anions and non-ionic substances, and cations) were reduced to dryness under vacuum at 40°C on a Büchi evaporator. The dry residues were then redissolved in 100 ml of distilled water. Each subsample was then passed through an anion exchange column. The column contained 40 g "Amberlite IRA 410" which had been prepared by washing with 500 ml of 1N Na_2CO_3 and rinsing with distilled water. The column was adjusted to run at a rate of 0.5 ml/minute. After receiving the samples the columns were washed with 300 ml of distilled water before the anions were eluted with 500 ml of 1 N HCl. The neutral and dipolar eluates were prepared for analysis by evaporating to dryness under vacuum at 40°C and redissolving the residue in 5 ml of distilled water.

The carbohydrates present in the neutral portion of the leachates were separated and identified using thin-layer chromatography (Stahl, 1965). The layers were made with Silica Gel G prepared with 0.1N boric acid and activated at 90°C for 30 minutes. Only one-direction techniques were used but several solvent systems were required to identify the sugars.

The solvent systems used were:

1. Benzene-glacial acetic acid-methanol (20:20:60),
2. Butanol-acetone-water (40:50:10)
3. Methylethylketone-acetic acid-methanol (60:20:20).

Water solutions of known sugars were applied to a quantity of 5 ug per spot. The chromatograms were developed by spraying with freshly-prepared anisaldehyde-sulphuric acid (5 ml anisaldehyde in 50 ml glacial acetic acid plus 1 ml of sulphuric acid) and heating at 110°C for 5-10 minutes.

The chromatography of the amino acids present in the leachate was carried out on Silica Gel G layers dried at 90°C for 30 minutes. Several solvent systems were used and because of the complexity of the sample two-dimensional chromatography was required to attain adequate separation of the amino acids. For the two-dimensional chromatograms the first solvent system used was n-butanol-glacial acetic acid-water (80:20:20). When the solvent had run approximately 10 cm. the plates were removed from the tank, the solvent fronts marked and the plates air-dried. After drying, the plates were turned through 90° and run

with the second solvent system, phenol-water (75 g and 25 g). When the solvent front reached 10 cm. the plates were removed from the tank and air-dried. The spots were developed by spraying with Ninhydrin (0.3 g ninhydrin dissolved in 100 ml of n-butanol and mixed with 3 ml glacial acetic acid) and heating at 110°C for 10 minutes.

Solutions of known amino acids in water were applied in quantities of 3 µg per spot.

Developed chromatograms were permanently recorded using a photocopier. Colours were observed and noted immediately after developing as they tended to fade rapidly.

When the dipolar fraction of the leachate was run on a one way chromatogram in n-butanol acetic water (80:20:20) it resolved into 6 to 8 spots which were ninhydrin positive. The spots could not be positively identified with the known run at the same time because of inadequate separation. The sample was also run with the solvent system, phenol:water. This solvent system resulted in four ninhydrin positive spots of which one could be identified positively as aspartic acid because of its Rf and distinct purple colour reaction. The dipolar fraction was then run on a two-way chromatogram utilizing the n-butanol-acetic-water and the phenol-water- solvent systems.

The resulting chromatogram after ninhydrin is shown in Figure II-6. The spots were identified by comparing Rf values for known amino acids in each solvent. These results were then checked by preparing a mixture of the "identified" amino acids and separating the mixture in the same two-way system. The results indicate that the leachate contained

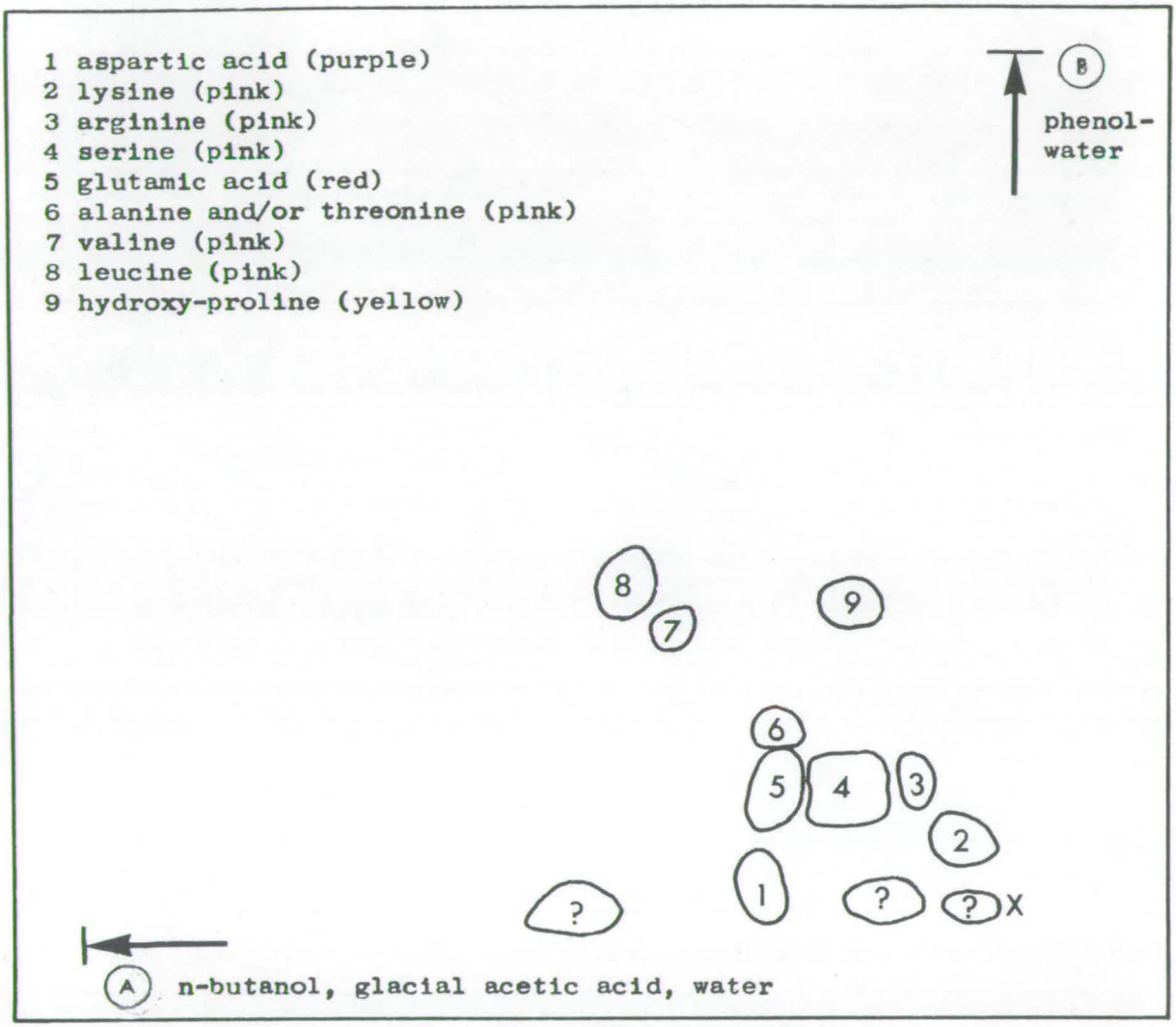


Figure II-6 Thin-layer chromatogram of the dipolar fraction of larch leaf leachate showing nine amino acids. (Silica gel plates activated @ 90°C for 30 minutes. Two solvent systems: A. n-butanol, glacial acetic acid, water (80:20:20): B . phenol-water (75 g + 25 g). Spray reagent was 0.3 g ninhydrin dissolved in 100ml glacial acetic acid. Spots detected after heating @ 110°C for 10 minutes).

the following amino acids: aspartic, lysine, arginine, serine, glutamic, valine, leucine, hydroxy-proline, alanine and/or threonine.

The neutral fraction of the leachate was analyzed for sugars. The first chromatograms run with a n-butanol-acetone-water solvent system indicated a mixture of fructose, glucose and a trace of sucrose. Further use of benzene-glacial acetic acid-methanol and methylethyl ketone-acetic acid-methanol solvent systems supported the identification of these 3 sugars and did not reveal the presence of other sugars.

The two samples of leaf leachate were analyzed along with water blanks which had been subjected to the same purification procedure. The blanks were shown to contain no amino acids or sugars. The two leachate samples were identical in all respects.

The leachate of larch leaves included the major amino acids and sugars reported to occur in Pinus photosynthate (Shiroya et al., 1962).

THE MICROFLORA OF LARCH LEAVES

LITERATURE REVIEW

The general concepts of microorganism communities on aerial parts of plants have been discussed in the Introduction. The following assessment of the literature is not intended to be an orderly chronicling of events in phyllosphere research development or as a review of all published data. These aspects have been covered in reviews by Leben (1965), Last and Deighton (1965), and Sinha (1965). Rather, it is an attempt to assess what generalizations can be made concerning the nature and development of the leaf microflora.

(a) Seasonal trends in numbers.

The interpretation of seasonal trends in phyllosphere populations is made difficult by a complex of factors which have parallel seasonal changes. These factors include the time of exposure to inoculum, physiological changes in the leaf, and macro- and micro-climatic changes.

When the three broad microorganism groups, bacteria, yeasts, and filamentous fungi are considered as units, higher numbers are found towards the end of the growing season than at the beginning. Last (1955a) reported that the number of yeast colonies present on wheat and barley leaves was few until the leaves "had lived half their lives". The numbers of colonies then increased progressively to reach a maximum when the leaves were dead. In 1956, Ruinen reported that the numbers of bacteria increased with leaf age.

Kerling (1958) studied the seasonal development of bacteria, yeasts and fungi on beet leaves which unfurled in May. Although yeasts declined in number in June after an initial high count in May, they showed a steady exponential increase from June to August. There was a parallel increase in numbers of bacteria and fungi. In September she observed a decline in numbers for all groups of organisms except on wilted leaves.

Stout (1960b) obtained lower counts of bacteria in spring than in summer on a number of pasture plant species. Crosse (1963) reported an increase in bacterial numbers on cherry leaves with increasing age of leaf.

Data produced by Dickinson (1967) demonstrates a progressive increase in the number of yeasts and filamentous fungi through the growing season on pea leaves. Similarly, Hislop and Cox (1969) present data for apple leaves which showed an overall increase in bacteria, yeasts and fungi as the leaves aged.

The general trend of increasing numbers of microorganisms as leaves age and as the season progresses appears to be well established by the investigations mentioned above. It is, however, difficult to assess which of many factors are responsible for this increase. Many climatic factors including rainfall, temperature, and relative humidity follow seasonal trends and thus changes in microbial populations may be attributed to them. Climatic changes may be operative on both a micro- and macro-climatic level. The growing plant may change the micro-climate of the first formed leaves. Kerling (1958) considered the changes in numbers that she observed to be correlated with seasonal climatic changes, especially humidity and rainfall.

It is possible that the population changes are related simply to the length of exposure of the leaves to the airspora. Indeed, the airspora shows a seasonal pattern similar to that observed on leaves (Hyde and Williams, 1953; Gregory and Hirst, 1957). Indirectly, leaf microorganism populations may be related to airborne material other than actual microbe propagules. Fokkema (1968) has shown that the increase of Cladosporium on rye leaves was related to the number of pollen grains on the leaves. Thus, the seasonal occurrence of pollen could result in seasonal microflora trends.

The changing physiological condition of a leaf as it ages affords another possible explanation of the seasonal trend in microorganism numbers. Several aspects of the development pattern of phyllosphere populations lend support to this hypothesis. The leaf physiology could influence phyllosphere organisms through changes in leachate quantity. Long, Sweet and Tukey (1956) have shown that leaching increases as a leaf ages. Stout (1960b) sampled two ages of pasture grass on the same date and found numbers of bacteria to be highest on the older leaves. Stout considered the "decisive influence" to be the condition of the leaf and found a lack of correlation between bacterial numbers and moisture and temperature reading. The observed difference in simultaneous samples may indicate that seasonal climatic change was not the dominant factor but it does not separate the time and microclimate factors from leaf ageing.

The occurrence of rapid increase in numbers of filamentous fungi and yeasts coinciding with leaf senescence has been reported by several

researchers and would seem to indicate that leaf age is an important factor. Cladosporium and Alternaria increased rapidly on yellowing leaves of rye (Kerling, 1964). Similarly, Dickinson (1967) reported an abrupt increase in numbers of yeasts and filamentous fungi on pea leaves at the onset of senescence. Dickinson's data is of particular significance since counts derived from leaf washing and culture techniques have been supplemented by direct microscope counts. The leaf washings revealed a low, relatively stable number of fungi on young leaves and a rapid increase on older leaves. The results of direct microscopic examination are even more illuminating in that they revealed that there was no hyphal development on young leaves. The development of hyphae started abruptly and increased rapidly on senescent leaves.

A rapid change in numbers and the change from dormant to active growth are not likely to be the result of macro- or micro-climatic changes which are more likely to be gradual. It may be that some climatic threshold is reached but such a rapid change would seem more likely to be related to a change in leaf physiology.

Another hypothesis for the seasonal trend was given by Ruinen (1961). She described a pattern of development based on a true succession of organisms. The primary colonizers are oligonitrophilic bacteria, the growth of which results in an enriched substrate which can then support algae and yeasts. A continuing sequence of colonizers could result in a seasonal increase in numbers and sudden changes in the microflora.

An interesting phenomenon in the development pattern of leaf microfloras is that the numbers of organisms frequently show an initial peak or high count. This initial peak pattern has been reported for bacteria by Kerling (1958), Stout (1960a), Leben and Daft (1967) and by Hislop and Cox (1969). A similar pattern for fungi has been reported by Kerling (1958), Dickinson (1967) and Hislop and Cox (1969). Only the work of Kerling (1964) reports data in which frequent sampling of young leaves did not reveal this pattern.

The initial peak pattern does not occur constantly with yeasts for which a progressive pattern was observed by Dickinson (1967).

(b) Climatic influences

Although a general trend of increasing numbers with time can be established, population counts are always marked by high variability. Stout (1960b), in comparing soil and foliage population of bacteria said that "the most striking feature of the herbage population is its dramatic fluctuations in response to changing ecological conditions." The dramatic fluctuations could possibly be anticipated if one considered the exposed nature of the phyllosphere. There would appear to be little to mitigate sudden changes in moisture and temperature on the leaf surface. Severe conditions of desiccation would frequently occur. Rain might also be expected to reduce numbers by washing organisms off the leaf. Kerling (1958, 1964) in two separate studies found that declines in leaf populations coincided with heavy rain. Ruinen (1961) considered rain to be responsible for both washing organisms off leaves and for spreading inoculum to leaves. Burri

(1903) found that bacteria multiplied fastest on rainy days. Leben (1965) reported that in experiments with protected seedlings a relative humidity of at least 70% was required for bacteria to freely migrate on the leaf surface. Moreover, Leben and Daft (1967) found that in Puerto Rico high relative humidity was not sufficient for an extensive bacterial microflora to develop and actual free moisture on the leaf surface was necessary for bacterial development. In experiments in Ohio, U.S.A., they found that large populations of bacteria developed only where water droplets had been placed on leaves for 24-72 hours. The final rapid increase of saprophytes on pea leaves reported by Dickinson (1967) coincides with an increase in rainfall and relative humidity.

Temperature may play a role in limiting numbers or types of organisms. Last (1955) isolated three times as many yeast colonies from dead leaves in summer as from dead leaves in winter. di Menna (1959) found fewer Sporobolomyces and Rhodotorula spp. in winter than in summer. The reverse was true of Cryptococcus sp. These seasonal differences may be in part due to temperature differences.

(c) Characteristic Organisms of the Phyllosphere.

(i) Filamentous fungi

Extensive species lists of filamentous fungi occurring in the phyllosphere have been prepared by Kerling (1958, 1964) and by Dickinson (1967). Kerling working with beet, rye and strawberry leaves lists the following genera as dominating the fungal microflora: Cladosporium, Penicillium, Trichoderma, Epicoccum, Aureobasidium, Alternaria,

Cephalosporium,,Fusarium, Stemphylium and Phoma. Dickinson's list of important leaf fungi on pea leaves includes all Kerling's list with the exception of Trichoderma and adds Pleospora, Aspergillus and Ascochyta. With the exception of the last listed genus, these fungi represent the most common elements of the airspora (Gregory and Hirst, 1957; Rishbeth and Meredith, 1957) and/or typical invaders of moribund plant tissues.

The fungal flora may also include what Dickinson calls "unique" species, that is, species with a limited host range. He gives examples from literature for Carex (Metasphaeria), Pinus (Fusicoccum) and Halimione (Ascochytula) and Pisum (Ascochyta). It may be that unique species are the best way of characterizing particular leaf microfloras as the "fidel" species of Braun-Blanquet (1951) are more valuable in characterizing plant communities than the most abundant species.

Nonetheless, species lists would appear to reflect the source of spores and not the ecological suitability of the substrate. Before species lists can attain real significance, some criterion of successful colonization must be established. In referring to successful yeast and bacterial colonizers, Leben terms them "residents" and sets the criterion that a resident must multiply on healthy, living plant parts (Leben, 1965). This criterion, if "grow" were substituted for "multiply" would also serve for filamentous fungi. To study the resident community of leaf fungi will require a method or combination of methods which can determine the criterion "growth". The much used washing-culture technique is unsuitable for this purpose. A specific resident could be studied in the vegetative stage by the technique of immunofluorescence as used to study the leaf-nodule bacterium

Chromobacterium lividum (Bettelheim, Gordon and Taylor, 1969).

Probably the most suitable method for determining the growth criterion would be spore inoculation of leaves which have been protected from the airspora. This method, of course, leaves unanswered the question whether the species would be successful if competing with a normally developed flora.

In the absence of such tests most workers have concentrated on "important fungi" determined by a criterion of abundance.

(ii) Yeasts and Bacteria

When considering the yeast and bacterial elements of a microfloza it is possible to infer more about a species' activity from numbers of the organism washed and cultured from leaves. This is because growth of these organisms, unlike filamentous fungi, results in an increase in the number of individual units and thus in a higher count for that species. Perhaps it is because of this life form that published data about bacteria and yeast species in the phyllosphere show a high degree of unity.

The yeasts commonly found on leaves are representatives of the Cryptococcaceae and Sporobolomycetaceae. Last and Price (1969) listed the following five species as being typical leaf yeasts: Sporobolomyces roseus Kluyver and Van Niel, Rhodotorula glutinis (Fres.) Harrison, R. mucilaginosa (Jörg.)Harrison, Cryptococcus laurentii (Kuff.) Skinner, and Torulopsis ingenosa di Menna. Bullera spp. have been sporadically isolated from leaves (Last, 1955a; di Menna, 1959).

A wide range of bacteria have been isolated from leaf surfaces but all reports are in general agreement as to the dominant forms. The majority of the bacteria are Gram-negative asporogenous rods and include a high percentage of motile and pigmented strains. (Leben, 1965; Last and Deighton, 1965).

Stout (1960a and 1960b) and Voznyakovskaya and Khudyakov (1960) have carried out extensive taxonomic studies of leaf bacteria. The genera most frequently isolated were Pseudomonas, Chromobacterium and Flavobacterium. Achromobacter, Bacillus, and Micrococcus species were also commonly isolated. Small numbers of other groups including Coliform (Geldreich, Kenner and Kabler, 1964) and lactic acid bacteria (Stirling and Whittenbury, 1963) have been found in studies designed to assess only a restricted part of the total microflora.

The similarity between the composition of the phyllosphere flora and that of the rhizosphere has been pointed out by Khudyakov (1961) and Leben (1961, 1964). A number of reports (Voznyakovskaya and Khudyakov, 1960; Leben, 1961) have suggested that the microflora of the seed is the common source of inoculum for the two regions.

The microflora leaves of different plant species show a considerable similarity (Last and Deighton, 1965) while the microflora of leaves is distinct from that of soil (Stout, 1960a, 1960b).

The occurrence of nitrogen-fixing bacteria of the genera Beijerinkia and Azotobacter in the phyllosphere flora of tropical plants has been reported by Ruinen (1956, 1961, 1963).

A study was planned to investigate the phyllosphere microflora of larch leaves. In order to provide uniform material for the study in 1968 and again in 1969, seedbeds of larch were sown following standard forest nursery practice (Edlin, 1964). The aim of the study was to determine the nature of the phyllosphere microflora development of both in terms of numbers of species composition. In addition, certain environmental factors including abiotic and biotic elements were measured in an attempt to assess their influence on the microflora.

The study was designed to precede research into the phytopathological significance of the phyllosphere microflora and to provide cultures of phyllosphere organisms for such research.

The study in 1968 involved intensive and extensive sampling procedures. In 1969 fewer samples were taken and the sampling procedure was changed somewhat in an attempt to answer questions raised by the 1968 study.

METHODS

(a) Experimental Material

The larch seedlings studied during 1968 were grown at the Forestry Commission Nursery at Bush, Midlothian. The nursery is in a farming area south of Edinburgh and is remote from any industrial source of aerial pollution. In the spring of 1968 a seedbed was prepared by the Forestry Commission staff consisting of two square yards of Larix decidua sown at a density of 1000 viable seeds per square yard. Pseudotsuga menziesii (Mirb.) Franco and Picea sitchensis (Bong)Carr. were sown in adjacent seedbeds. The arrangement of the seedbed is shown in Figure II-7.

The seed was sown on April 29, 1968, and was covered with coarse grit to a depth of $^{3}/_{8}$". No chemical sprays were used in the vicinity of the seedlings.

In 1969 a larch seedbed was prepared at the Department of Forestry and Natural Resources nursery area at the University of Edinburgh, King's Buildings site. While the area is not industrial and is adjacent to a golf course and farm lands, it must be assumed that the air contained some domestic air pollution especially from coal smoke. The seedbed was prepared by the same methods as in 1968 and 6 square yards of seedbed were sown with larch on May 6th.

The seed, seedlot 65(4341) from Bad Homburg, Germany, was supplied by the Forestry Commission.

(b) Measurement of Climatic Factors

The preceding discussion of seasonal trends of microflora populations has indicated the importance of moisture in the environment of the phyllosphere. It was therefore considered important to obtain measurements of relative humidity, sunshine, rainfall and dew.

Sunshine and rainfall records were obtained from meteorological stations which were located approximately 400 metres from the plots. In 1968 the data from Bush House (National Grid NT 249639) was used and in 1969 the data from Blackford Hill (National Grid NT 259706) was used.

Because microclimatic differences were likely to affect relative humidity and temperature, these measurements were made in the seedbed. A Casella thermohygrograph was used for this purpose. The thermoghygrograph was placed in a Stevenson screen which was positioned at ground level in the seedbed. The slatted door of the screen was replaced by wire netting to provide improved air circulation over the thermohygrograph.

The standard methods of measuring dew or moisture duration on leaves were reviewed by Wallin (1963). Two methods were used in the study. One, the Hirst wetness recorder (Hirst, 1957), weighs the dew present on a polystyrene cylinder and records the weight by a recording pen. The polystyrene cylinder is mounted above the recorder and with a low level crop such as conifer seedlings the sensing area is about 18" above the plants. Because of this drawback a second method of measuring was also used. A modification of the Wallin-Polhemus (1954) recorder which used lamb-gut as the wetness sensor was

made from the components of a Casella thermograph. The temperature sensing coil was removed and the pen lever attached to a 4" section of dried lamb's gut. The instrument is illustrated in Figure II-8.

Care was taken to position the sensing surfaces so that they were fully exposed to sun, rain and the prevailing wind as this was the case with the seedlings. The position of the recorders in the seedbeds is shown in Figure II-7.

(c) Analysis of seedling leachates

The possible importance of leaf leachates in determining microflora population levels has been discussed in a preceding section. The qualitative analysis of the larch leaf leachates has also been described in a preceding section.

It was thought that some measure of the quantitative aspects of leaf leaching could be useful in understanding leaf microflora development. To this end a method was developed in which a relatively large quantity of leachate could be obtained quickly. Briefly, this method involved removing the seedling from the seedbed with most of its root intact, wrapping the roots in wet cotton and suspending the seedling upside down in a conical flask filled with water. Care was taken not to contaminate the leaves or glass-distilled water with soil particles. After some 8 hours the seedling was removed, blotted dry with filter paper and the aerial portion weighed. The leachate was sterilized by filtration through a washed Seitz filter and stored at $0^{o}C$.

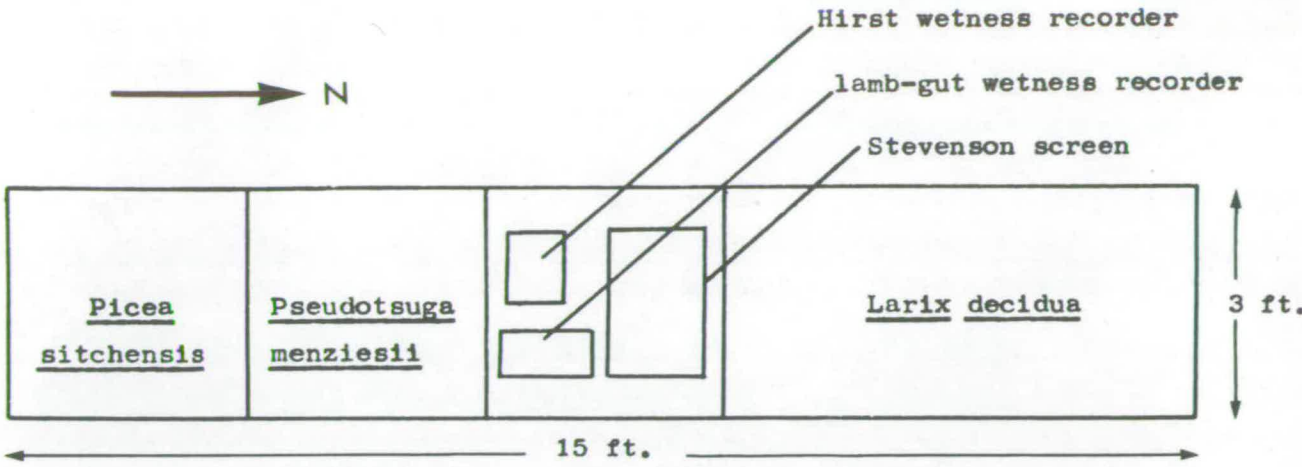


Figure II-7 The arrangement of the seedbed and meteorological equipment used in the 1968 microflora study at Bush, Midlothian.

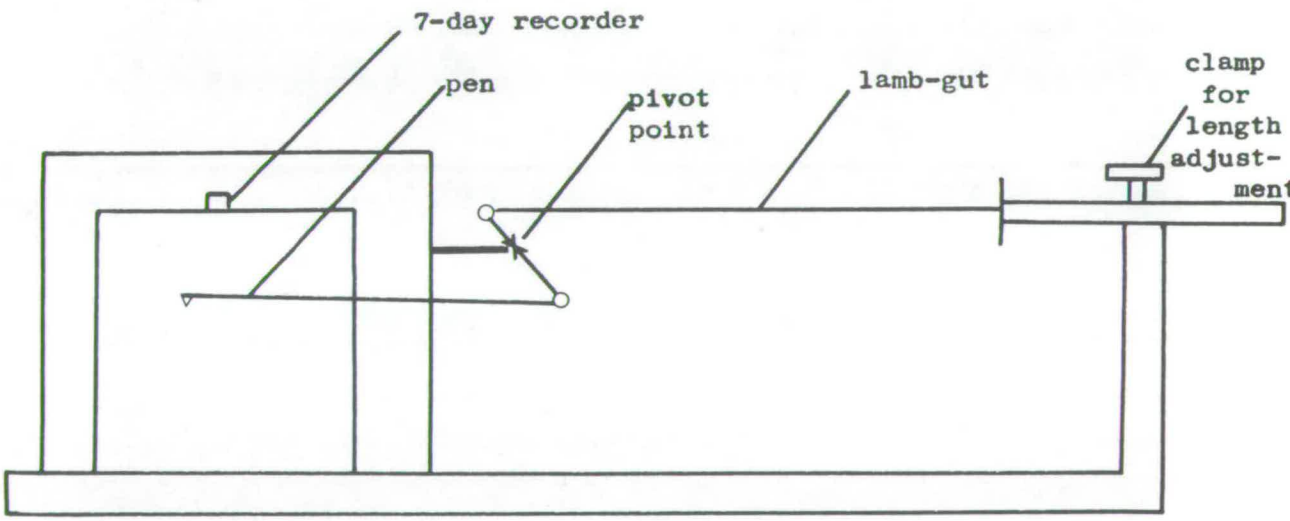


Figure II-8 A drawing of the wetness-duration-recorder which used lamb-gut as the wetness sensor.

The filtered leachate samples were reduced to dryness under vacuum at 40°C on a Büchi evaporator, and the residue taken up in appropriate quantities of glass-distilled water. A series of blank samples which had been treated identically to the leachates but without seedlings paralleled each sampling.

The ions, magnesium, calcium, potassium and sodium were analyzed on a Unicam sp 90 spectrophotometer. The organic portion of the leachate was analyzed as two broad groups, the carbohydrates and the amino acids.

The carbohydrate content of the leachates was assessed by the anthrone colour reaction method of Deriaz (1961) which is specific for sugars, oligosaccharides and fructosans. The reagent and standards were prepared in the following manner:

1. Anthrone reagent: 760 ml of concentrated sulphuric acid (Analar) were added to 330 ml of water. The mixture was cooled to 20°C and made up to one litre. One gram of thiourea and one gram of anthrone were then successively dissolved in the solution. The anthrone reagent was stored at 0°C.

2. Carbohydrate standards: A standard series containing equal grams per litre quantities of sucrose, glucose and fructose were prepared. The mixture was used to simulate the leaf sugar mixture expected in the leachates (Shiroya et al, 1962). The standard series contained 0.1 gram to 0.0001 gram of each sugar or 0.3 gram to 0.0003 gram of total carbohydrate per litre.

The method used for the analysis involved the following procedure:

(i) 2 ml of the test solution (two replicates), 2 ml of the sugar standard (three replicates) and 2 ml of water (as a blank) were pipetted into 1" by 6" boiling tubes;

(ii) 10 ml of the anthrone reagent were added slowly to each tube with cooling in a beaker of tap water;

(iii) the boiling tubes were covered and heated on a water bath at 100°C for 20 minutes;

(iv) the tubes were then cooled in tap water for 10 minutes;

(v) the colour intensity of the solutions was measured at 625 mμ against the reagent blank (O) with an Evans Electroselenium Ltd. Spectra.

The total amino acid concentration in the leachates was analyzed using the ninhydrin colorimetric analysis described by Rosen (1957). Ninhydrin reacts quantitatively with several classes of compounds including α amino acids, imino acids, amino alcohols and primaryd amides. The colour intensity attained with different amino acids varies only over a range of 5% (between lysine and Tyrosine)(Rosen, 1957). The standard series used to relate colour intensity with amino acid content was made up of the amino acids most likely to occur in the leachate. Nykvist's (1963) work with conifer leachates indicated that the most abundant amino acids were alanine, aspartic acid and glutamic acid and thus the standard solution was prepared with equal molar portions of those three amino acids.

The analysis of the leachates would be made much simpler if the organic compounds and the ions could be accurately measured as a mixture. Thus, tests were designed to determine if the accuracy of

any of the analytical methods was influenced by the presence of the substances in mixtures rather than as pure groups. The analyses of a standard series of pure salts, pure amino acids and pure sugars were compared with a parallel series of the three groups mixed. The results of the analyses are given in Figures II-9 and II-10 and in Table II-2.

TABLE II-2 A comparison of methods for measuring ion concentrations in water with and without carbohydrates and amino acids.

	Mg readings		Ca readings		K readings		Na readings	
conc. series	salts only	mixture	salts only	mixture	salts only	mixture	salts only	mixture
1	.3	.3	19	19	90	90	75	76
2	3.5	3.5	34	35	62	62	54	55
3	4.5	4.5	47	47	45	45	41	42
4	6.0	6.0	59	58	33	34	32	33

The estimates of concentration in the mixture showed excellent agreement with the estimates in pure solutions. The error was always less than 5% and it was concluded that the leachates could be analyzed without the separation of substances into salts, amino acids and sugars.

Figure II-9 A comparison of the absorption readings for the colorimetric anthrone assay of carbohydrate content for solutions containing given amounts of carbohydrate in a pure water solution and in a mixture of amino acids, salts and carbohydrates.

Figure II-10 A comparison of the absorption readings for the colorimetric ninhydrin-assay of amino acid content for solutions containing given amounts of amino acid in pure water solution and in a mixture of carbohydrates, salts and amino acids.

(d) Identification and enumeration of microorganisms

The identification and enumeration of microorganisms on leaf surfaces is a task complicated by the wide range of microorganism types and the tendency of some of them to form colonies. Direct microscopic observation of the leaf surface is of restricted use because the majority of the organisms cannot be identified without culturing. Also, direct observation cannot distinguish between viable and non-viable cells. Culture techniques also present serious problems, the restrictive nature of the growth media and the separation of the microflora from the leaf being particularly difficult. For these reasons Dickinson (1967) suggested that a combination of two or more techniques would probably be most successful.

Two basic methods have been developed for direct microscopic examination of the leaf surface: leaf clearing and surface impressions. The leaf clearing method, (especially when chlorine gas is used for clearing), has the advantage that a relatively undisturbed leaf is observed directly. The use of leaf impressions usually results in superior optical conditions but checks must be made to ensure that the impression is removing the majority of the microflora.

The chlorine gas bleaching technique as used by Janes (1962), Daft and Leben (1966) and Preece et al. (1967) was used in the 1968 larch leaf study. The bleaching was carried out in the apparatus shown in Figure II-11. Chlorine gas was generated by adding balls of bleaching powder to concentrated hydrochloric acid.

Most leaves were bleached within 20 minutes. Occasionally older leaves were resistant to bleaching. This was overcome by making scalpel cuts on the edge of the leaf and by extending the length of the chlorine treatment.

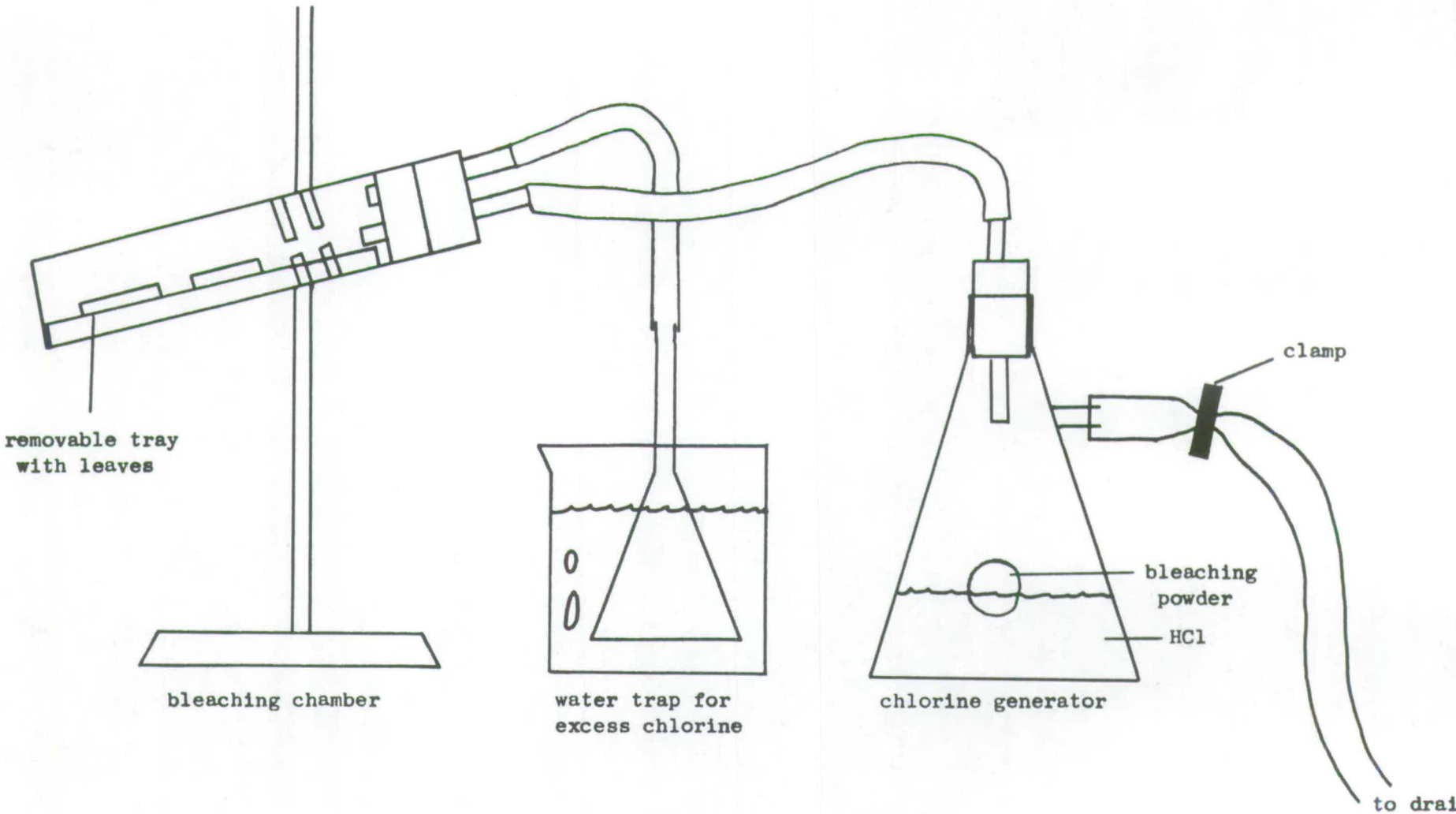


Figure II-11 The apparatus used to bleach larch leaves with chlorine gas.

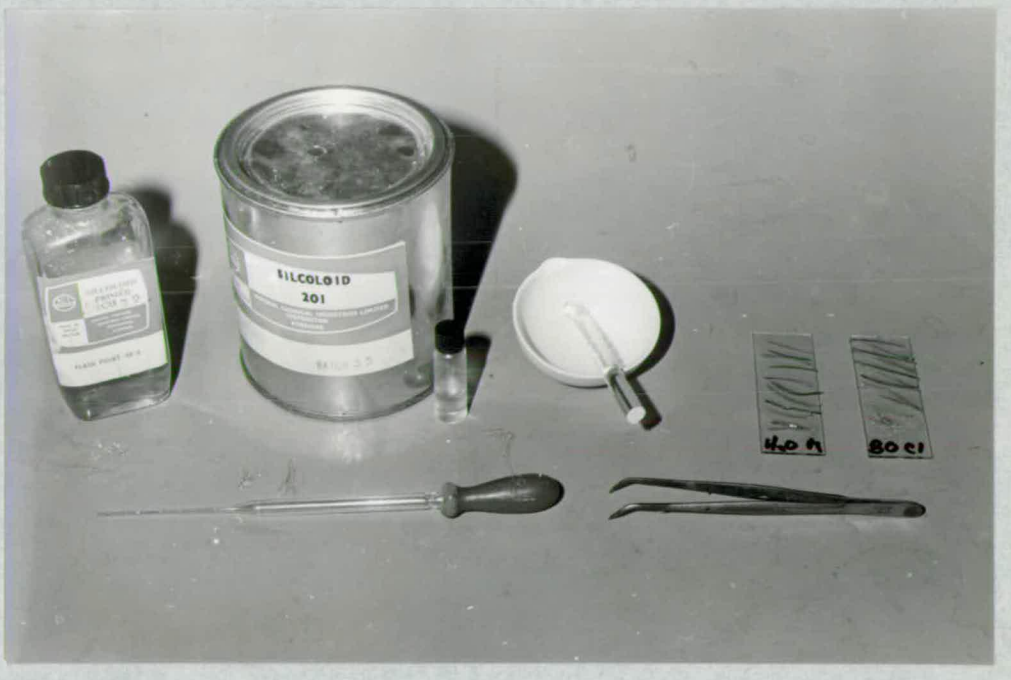
It was found that a slow steady production of chlorine was achieved when the bleaching powder was added as 1" balls made by mixing the powder with water.

After bleaching, the leaves were allowed to dry before staining with dilute Trypan blue. The stain was spread over the leaf with a thin glass rod in the manner described by Daft and Leben (1966), and was allowed to dry before microscopic examination.

The technique of leaf impressions was attempted using nail varnish (Dickinson, 1967) and Collodion (Ruinen, 1961). Poor results were attained probably due to the difficulty of dealing with the small narrow larch leaves. Later in the study a very convenient replica technique was developed utilizing silicone rubbers. Clear Silcoloid 201 was supplied by ICI. After mixing Silcoloid with the curing agent, the liquid was poured onto slides previously treated with Silcoloid primer. The liquid was spread to a thin even layer with a glass rod. The leaves were then pressed gently onto this surface (Plate II-18). After 8 hours hardening the leaves were peeled off and the impression was stained with Trypan blue. Oil immersion lenses could be used directly on the impression without a coverslip.

In order to culture a representative sample of the microflora, the organisms must be removed from a leaf and suspended in a liquid. Some workers (Kerling, 1964; Bier, 1965) have avoided this step by direct plating of leaf fragments on agar media. This method and the allied technique of leaf prints (Potter, 1910; Rusch and Leben, 1968) would be expected to be selective for fast growing organisms and for organisms

PLATE II-18 MATERIALS FOR PREPARING "SILCOLOID" LEAF IMPRESSIONS. The glass slides were first treated with silcoloid primer (left). A mixture of silcoloid 201 and curing agent (small bottle) were mixed for 30 seconds before pouring onto slides. Leaves were then gently pressed into the solidifying liquid.


SILCOLOID
201

easily removed from the leaf. The most frequently used method of isolating microorganisms from leaves is the water shake method. Leaves are shaken in water for various lengths of time, and the resulting suspension is serially diluted and added to agar media for culturing and counting. The shaking procedure is of questionable efficiency in removing the microflora. Different times of shaking have been used by different workers. For example Dickinson (1967) shook leaves for 5 minutes while Hislop and Cox (1969) shook leaves for 4 hours. The efficiency of the shaking procedure can be improved by adding surface wetting agents (Dickinson, 1967). Because the washing technique and factors such as shaking time will favour some organisms compared with others, an alternate method of suspending leaf organisms was sought. To this end, leaf suspensions were prepared by homogenizing leaf samples with a MSE homogenizer for 3 minutes. The resulting suspension was diluted and cultured. Leaf washings were prepared by 10 minutes of vigorous shaking on a wrist action shaker for comparison with the suspensions. The washed leaves were also macerated and the suspension cultured. The results of the trial are given in Table II-3.

TABLE II-3. Recovery of microorganisms using different treatments (numbers of bacteria and yeasts per gram fresh weight of leaves).

Replicate	1	2	3
washed sample 1	3000	3000	3100
macerated sample 1	3700	3000	3200
washed sample 2	1300	1700	1000
macerated sample 2	2100	1800	1800
washed/macerated s.1	35	40	32
washed/macerated s.2	20	22	22

Homogenized suspensions resulted in slightly higher counts than suspensions prepared by shaking. The leaves which were homogenized after washing yielded only slightly more than 1% of the total count.

A 3 minute homogenizing procedure was therefore employed to produce the microflora suspensions for the larch leaf study.

Since this trial was carried out a study by Hislop and Cox (1969) has been published which indicated that no consistent difference was obtained whether shaking or homogenizing was used to prepare the suspension.

The preparation of an unbiased sample of phylloplane organisms in suspension is only the first of many problems. The suspension must then be diluted to the point where only a countable proportion are present and the culture media chosen must allow a wide range of organisms to develop. The problems related to these techniques have been reviewed for fungal counts by Montégut (1960) and for bacterial counts by Jensen (1968).

It was decided to use two broad range media throughout the sampling season; one for the fungal element and one for the bacteria. At intervals additional media were used to assess certain specific groups.

Czapek Dox agar (Oxoid) with 0.75% Difco yeast extract (adjusted to pH 5.0 with lactic acid) was the medium used for plate counts of filamentous fungi (Parkinson and Thomas, 1965). (In the remainder of the text this medium is referred to as CDYE).

A number of media including soil, leaf and yeast extracts were assessed for the number of bacteria and yeast colonies developing

on them. Soil extract agar and larch leaf extract agar both supplemented with 0.25% yeast extract consistently produced the highest counts. Since soil extract agar was more transparent than the leaf extract agar, a property which facilitated the counting of submerged colonies, it was chosen as the medium for the study.

Soil extract agar was prepared in the following manner (Allen, 1957). A soil extract was prepared by autoclaving 2000 gm of loam with 1000 ml tap water and 1g $CaCO_3$ for 30 minutes. The hot mixture was filtered until clear. The filtrate was then made up to 1000 ml before autoclaving in 100 ml aliquots. Two litres of soil extract were prepared at any one time so that the same batch was used throughout a sampling season. 100 ml of soil extract were added to a medium containing 15 g agar, 1.0 g glucose, 0.5 g K_2HPO_4, 10 g yeast extract, and 900 ml of distilled water. The pH of the soil-extract yeast-extract medium (SEYE) was 6.8.

Periodically through the sampling season microflora suspensions were plated with additional media. These media included a medium for the isolation of Azotobacter (Ruinen, 1963) and sodium albuminate agar for the isolation of actinomycetes (Waksman and Fred, 1922).

All morphologically different fungal isolates were established in pure culture and kept on Malt agar (Oxoid) slants at $25^{\circ} \pm 1^{\circ}C$.

The bacteria and yeasts were isolated for stock cultures by a quantitative method. All colonies developing on a plate or a randomly chosen portion of a plate were isolated into pure culture and kept on agar slants containing 0.25% yeast extract (Difco) at $1^{\circ} \pm 1^{\circ}$ C.

Yeasts were classified by the methods of Lodder and van Rij (1952). Bacteria were first classified by the Gram reaction, and Gram-negative organisms were identified by the methods of Park and Holding (1966).

Bergey's Manual of Determinative Bacteriology (Breed et al., 1957) was also used for identification.

(e) Sampling Procedure

The sampling techniques were rigidly standardized to avoid bias in selection of samples and to reduce variation caused by technique variability.

All seedlings for microflora assay, leaching, and height measurements were selected using random numbers. The nearest seedling to a point designated by random numbers on each of two right angle axes was used. The sampling time was set at 10.30am. to 11.00am. to avoid variation due to possible diurnal variation. Samples were taken every 14 days throughout the growing season commencing on June 7th.

Seedlings for microflora assay were cut with a scalpel above soil level and placed in sterile screw top containers.

The seedlings to be used for leachate analysis were prised gently out of the soil and their roots wrapped in moist cotton wool. Care was taken to avoid getting soil on the leaves and to damage the roots as little as possible.

Three samples each consisting of 3 seedlings were taken every fortnight for assay. The leaves were removed from the stem with flamed forceps. The cotyledons were assayed separately from the other leaves. On the ninth (September 26th) sample and thereafter, the seedling foliage was divided into cotyledons, lower plumule (lowest 5cm. of foliage) and the upper plumule (the remainder of the leaves). The foliage samples were placed in vortex baakers which had been plugged with cotton wool, sterilized and weighed. The weight of the sample was then determined to the nearest 0.1 mg.

8 ml of 0.1% yeast extract solution was added to the beaker

before homogenizing on the MSE homogenizer for 3 minutes. The blades of the homogenizer were flamed immediately before the vortex beaker was inserted. The resulting suspension was then diluted appropriately using 0.1% yeast extract solution as the diluting medium. Widemouth, fast delivery pipettes were used in the serial dilutions to avoid blockage.

Dilutions were pipetted into 6 petri plates. SEYE media was added to three of the plates and CDYE media to the other three.

The plates were incubated for 6 days at 22.5°C before counting using a Gallenkamp colony counter. Only plates showing between 30 and 300 colonies on SEYE medium and less than 30 colonies on CDYE medium were counted.

The seedlings used for direct observation and for leaching were treated as described previously in this chapter.

In 1969 a similar procedure was followed except that the lower plumule leaf samples included only the first 5 leaves above the cotyledons and the upper plumule samples included only leaves from the upper centimeter of the seedling. Samples were taken at three to four week intervals.

The numbers of organisms on leaves was usually calculated per gram fresh weight. The surface area of a gram of leaves was calculated for two reasons: (1) so that comparisons of numbers for direct microscope counts and for culture counts could be made; and (2) to determine if a seasonal change in the area to weight ratio was influencing the population curves. The surface area of two-week and four-month-old leaf samples was calculated from simple linear measurements of leaf dimensions. The measurements made no allowance for the curved surface of the leaves.

To provide a convenient reference for sample dates and numbers used in the text, figures and tables the requisite information is listed below.

	1968		1969
S-1	June 7th	S-1	June 19th
2	June 21st	2	July 2nd
3	July 4th	3	August 6th
4	July 17th	4	August 20th
5	August 1st	5	August 28th
6	August 15th	6	September 12th
7	August 29th	7	September 26th
8	September 12th	8	October 11th
9	September 26th	9	November 4th
10	October 10th		
11	October 24th		
12	November 7th		
13	November 21st		

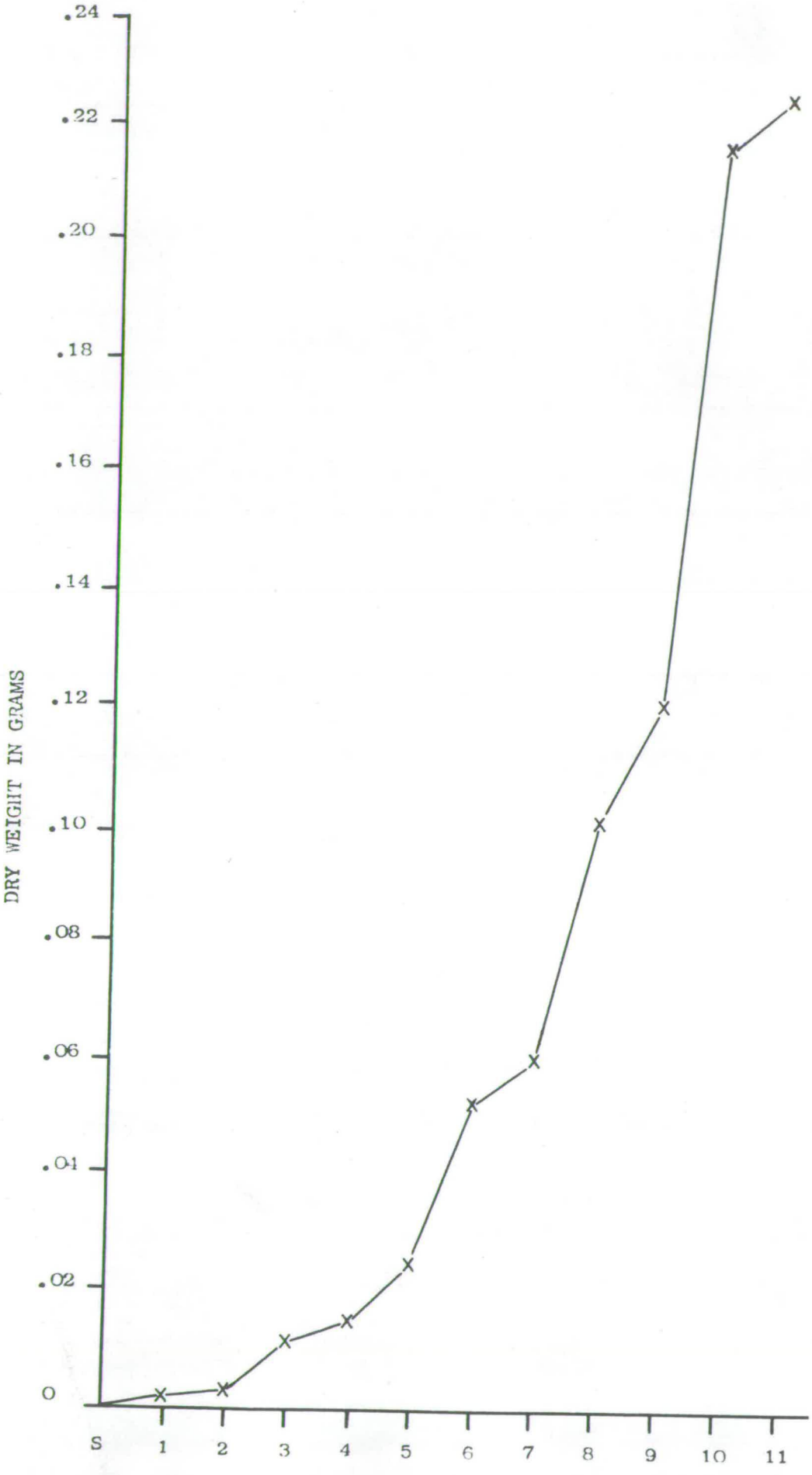


Figure II-12: The dry weight of seedlings during the 1968 growing season(Average of five seedlings).

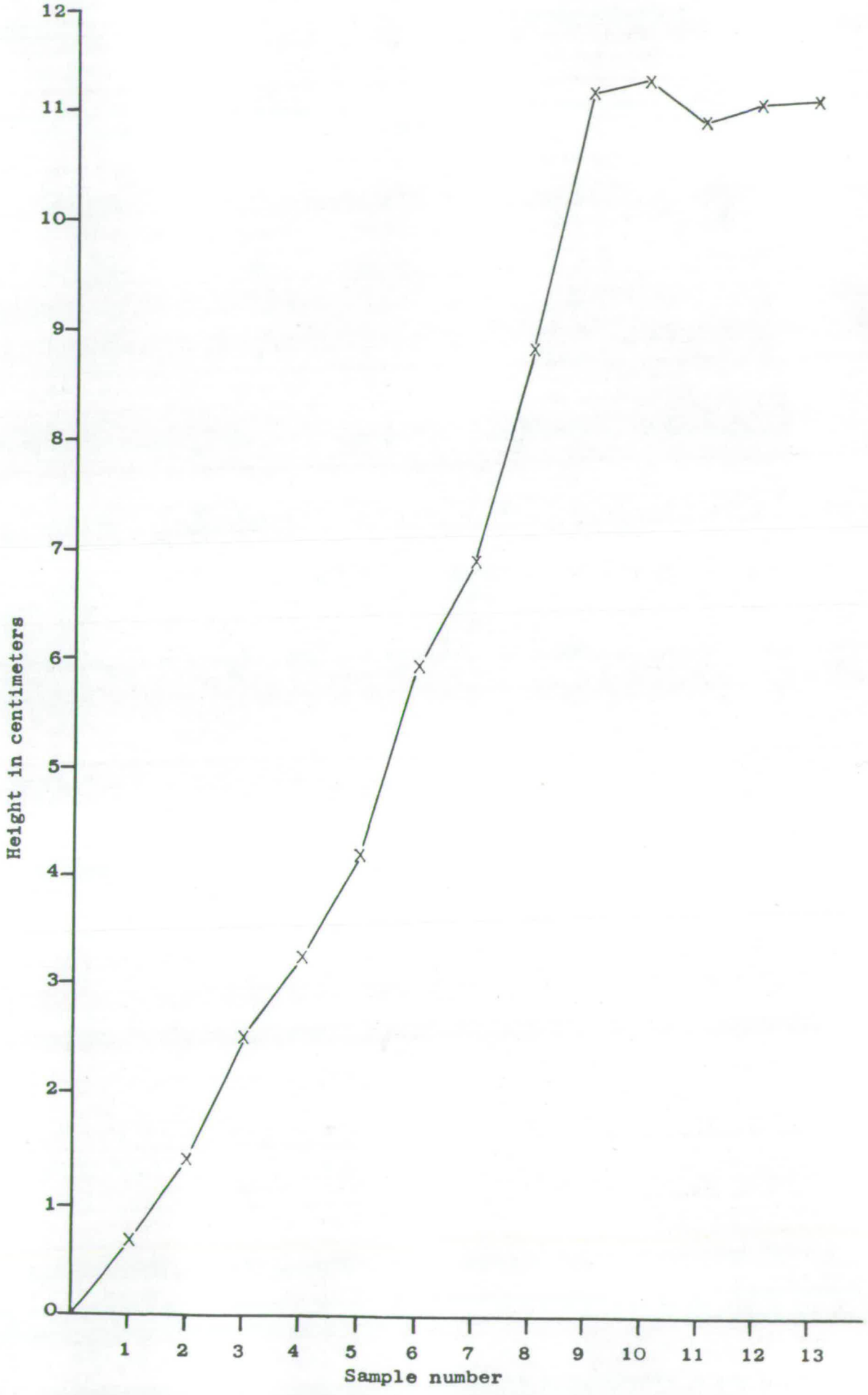


Figure II-12 continued: The growth of larch seedlings in 1968 measured as height in centimeters (average of 5 seedlings).

RESULTS

(a) Seedling Microflora Study 1968

(i) Growth of seedlings.

The larch seed which had been sown on April 29th, began germinating at the end of May. The germinating time was quite uniform and the majority of the germinants appeared in the first week of June. Seeds which germinated after June 10th were removed so that the seedlings in the bed were of uniform age ± 7 days.

The growth of the seedlings was assessed in two ways, height and dry weight above ground. The results are shown in Figure II-12. The dry weight declined after sample 11 (October 24th) because at this stage some leaves had fallen and dry weight was therefore no longer meaningful. Height growth continued steadily from germination until sample 9 (September 26th) with the most rapid growth before samples 8 (September 12th) and sample 9 (September 26th). The dry weight increase follows a Sigmoid curve. Although height growth ceased on or before September 26th, the dry weight increased for another fortnight.

(ii) Climatic measurements.

Data for seedbed temperature, relative humidity, hours of sunshine and duration of wetness were assessed as averages or totals of the recordings for the 14 days between each sample. Because time between sampling periods was constant, totals are as significant as averages and therefore totals were used where it made interpretation easier. Rainfall was assessed on a daily basis so as to emphasize the pattern of rainfall on days immediately preceding sampling.

Temperature

The average daily maximum and minimum temperatures for the 14 days preceding a sample are shown in Figure II-13. From June 1st until September 12th (S-8), the maximum ranges between 17.5°C and 14.5°C and the minimum between 12.8°C and 9.5°C. After September 12th, the maximum daily temperatures declined steadily from 12.5°C before S-9 (September 26th) to 7.5°C before S-13 (November 21st). During this period the minimum shows a general decline to an average of 5°C before S-13. The maximum daily temperature was 22°C and occurred on June 13th. The earliest recorded frost was on November 4th.

Relative Humidity

The setting of the relative humidity recorder in the hygrothermograph required constant calibration. Dry and wet bulb thermometers were used for this purpose and for preparing correction tables. Relative humidity was assessed as total hours between samples in which RH° was above 80% or was below 50%. The results of this assessment are given in Figures II-14 and II-15. The number of hours in which relative humidity was above 80% shows a slight seasonal trend increasing from 200 hours per 14 days in July to over 250 hours per 14 days in September, October and November. The most striking aspect of the figures is the exceptionally low figure of 90 hours for the period preceding S-2 (June 21st).

The seasonal trend for hours of relative humidity below 50% mirrors the seasonal trend for periods above 80%. The most remarkable periods were the long periods of low relative humidity of 84 hours, 54 hours, and 76 hours before S-2 and S-3and S-6 respectively. The daily average of hours of RH° below 50% declined to 1.5 hours before S-4 (July 17th) and

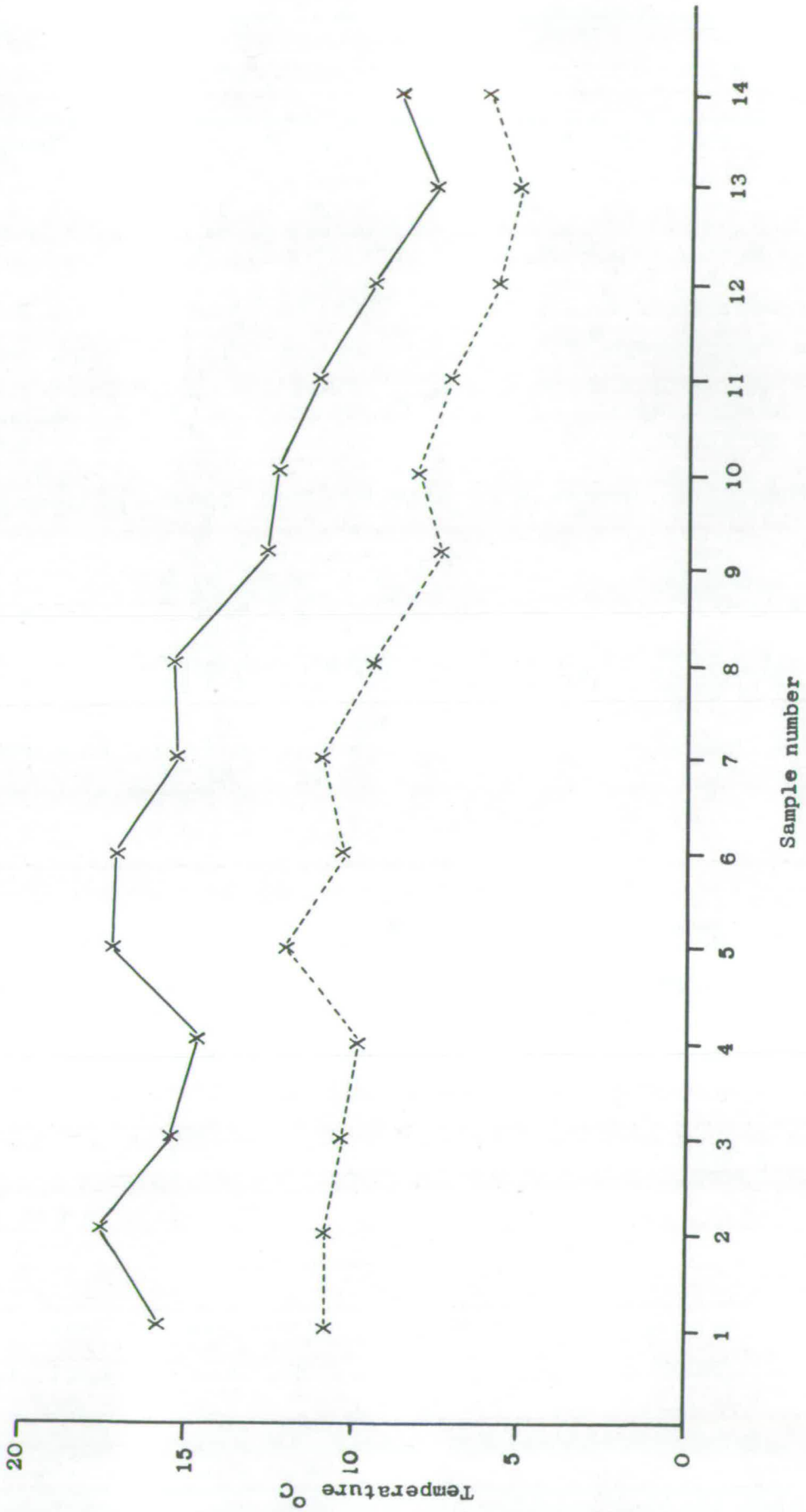


Figure II-13 The average daily maximum and minimum temperatures for the 14 days preceding each sample date of the 1968 microflora study.

Figure II-14 The total number of hours between 1968 sample dates in which relative humidity was greater than 80% (each total is for 14 days except the first which is for 7 days).

Figure II-15 The total number of hours between 1968 sample dates in which relative humidity was less than 50% (each total is for 14 days except the first which is for 7 days).

only consistently remained below 1 hour per day from S-9 (September 26th) onwards.

Hours of sunshine

The hours of sunshine recorded between samples reached peaks of 92.6 hours and 87.5 hours before S-2 and S-6 respectively. A low total of 30.4 hours was recorded before S-4. With the exception of these three points the totals show the expected trend of reduced sunshine as day length shortens from June to November (Figure II-16).

Rainfall

The millimeters of rainfall on days of the growing season are illustrated in the histogram in Figure II-17. Two distinct dry spells occurred in June and at the edd of July and the first half of August. It is noteworthy that heavy falls of rain ($\geq$ 18 mm in one day) occurred one or two days before the samples 3,4,6 and 9 were taken.

Duration of wetness

An attempt was made to evaluate the significance of the wetness recordings by correlation with direct observation of leaf wetting and drying. This proved difficult because of the great differences in the wetting properties of different aged leaves. Both recorders reacted within one minute to wetting by showers. The recording of dryness after wetting was usually within 15 minutes of the observed drying of a glass wetted with a similar amount of water.

The differences in duration of wetness recorded by the two recorders was usually within 10% of the total time recorded but variation was not constant.

Regression analysis showed that the duration of wetness by either recorder was significantly ($p = 0.05$) related to the number of days on which rain fell and to the number of hours during which relative humidity was over 80%.

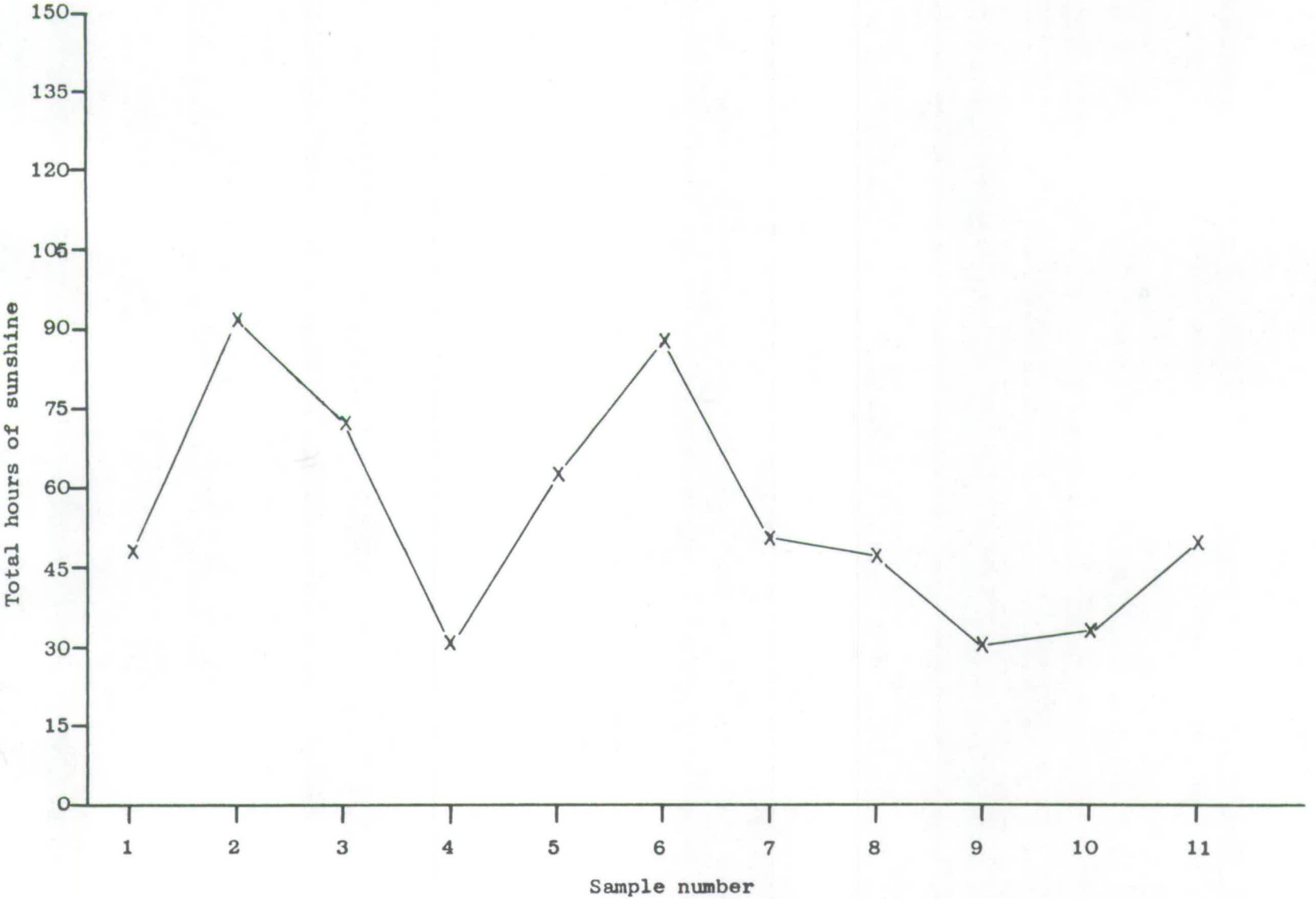


Figure II-16 The total number of hours of sunshine between sample dates in 1968 (all totals are for 14 days).

Figure II-17 The daily rainfall in millimeters during the 1968 microflora study.

The data for duration of wetness between sample dates is given in Figure II-18. The same features as already noted for rainfall and humidity are apparent.

(iii) Analysis of seedling leachates.

It must be remembered that all ages of leaf were being leached in each sample so that while there was an increase in the amount of old leaves present with time there was always a considerable amount of young tissue. The stem may also have lost nutrients into the leachate.

The method of leaching, suspending whole seedlings in water, was highly unnatural and a check was made to determine if the seedlings were becoming waterlogged. After eight hours immersed in the water air bubbles were still present at the stomatal apertures and this observation was taken as an indication that waterlogging had not occurred.

The number of replicates at each sampling date was restricted to two because of the restricted time available for collection and processing. Although two replicates are inadequate for statistical analysis they do provide some information on the variability of leaching. Considerable variation between samples was observed, especially when the seedlings were small.

There were three general sources of variation or error: the precision of the chemical tests, the contamination of samples, and actual variation between samples. The first mentioned source of error was assessed by making repeated measurements of the same sample. The precision of the measurements of the ions and of carbohydrates was very good with an average error of less than 1% of the ions and 1.8% for carbohydrates. The measurement of amino acids was less precise and the error ranged from 1% to 15%. The error resulting from contamination of the samples was checked by comparison with the blanks which were prepared.

Figure II-18 The total number of hours of wetness between 1968 sample dates (i.e. totals of 14 days). Where available, both the records for the Hirst wetness recorder and the lamb-gut recorder are given.

along with each sample. The measurement recorded for the blanks was subtracted from the sample value to give a corrected value. In practice the blank values for carbohydrates, calcium, magnesium and potassium did not exceed 10% of the sample value. Again, the amino acid assessment proved to be the least accurate with the appropriate blank measurement being 50% of the sample measurement in one case. This inaccuracy was probably a result of the very low values recorded for amino acids. By far the largest variation was that which occured between samples, and for this reason the data is presented in such a way as to indicate the value for each of the two samples as well as their mean value.

The results of leachate contents are presented in Figures II-19, II-20, II-21 and II-22. The quantity of ions leached per gram of seedlings was in descending order, sodium, potassium, calcium and magnesium. All four ions were most abundant per gram of seedling during samples 1, 2 and 3. After sample 3, calcium, potassium and sodium were present in very low quantities. Magnesium, however, shows a distinct increase in sample 11 after being low from sample 4 to 10.

The data for the carbohydrate content of leachates is presented in Figure II-23. The carbohydrate content varied markedly throughout the growing season. In spite of the variation it is interesting to note that the replicates for each sample date, although varying in quantity, follow the same general pattern of increase or decrease through the season. No carbohydrate was detected in leachates from very young seedlings (S-1 and S-2).

The amino acid content of the leachates was negligible except in the first sample in which the seedlings had no leaves other than

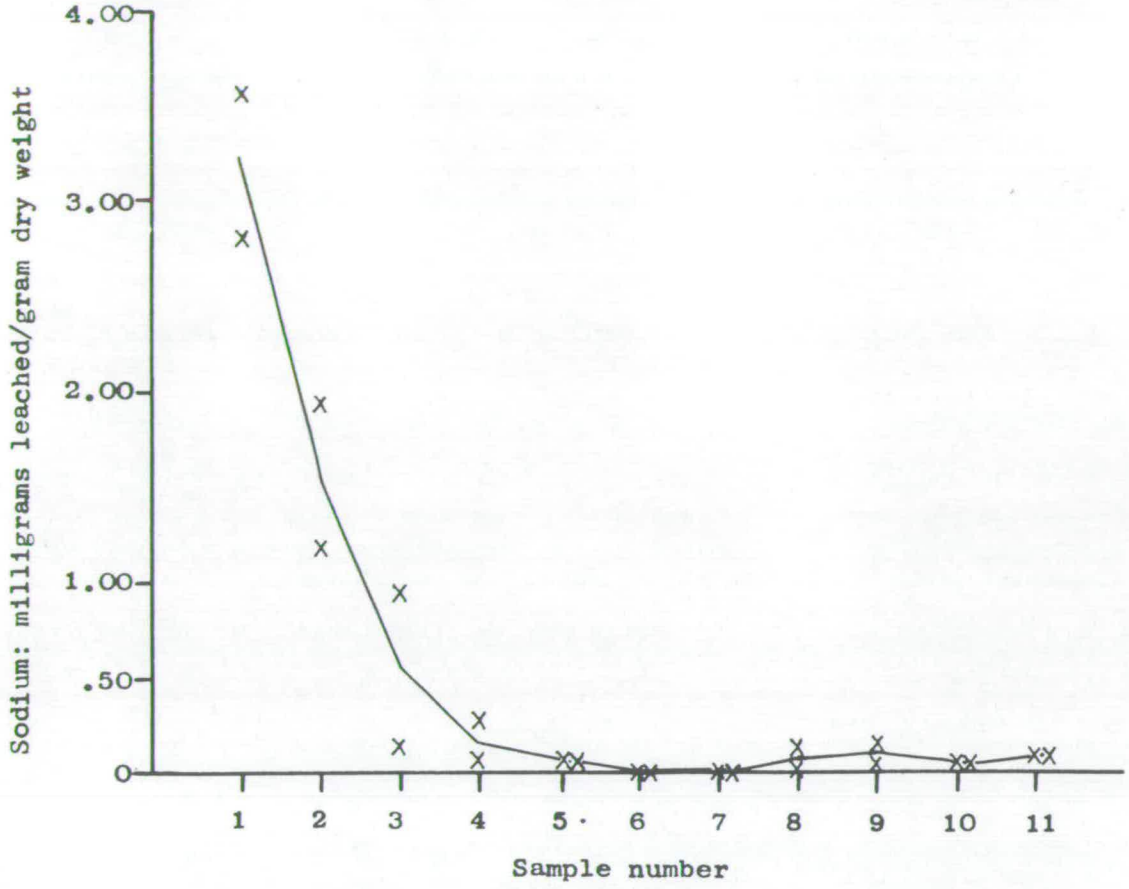


Figure II-19 Sodium ion content of seedling leachates during the 1968 microflora study.

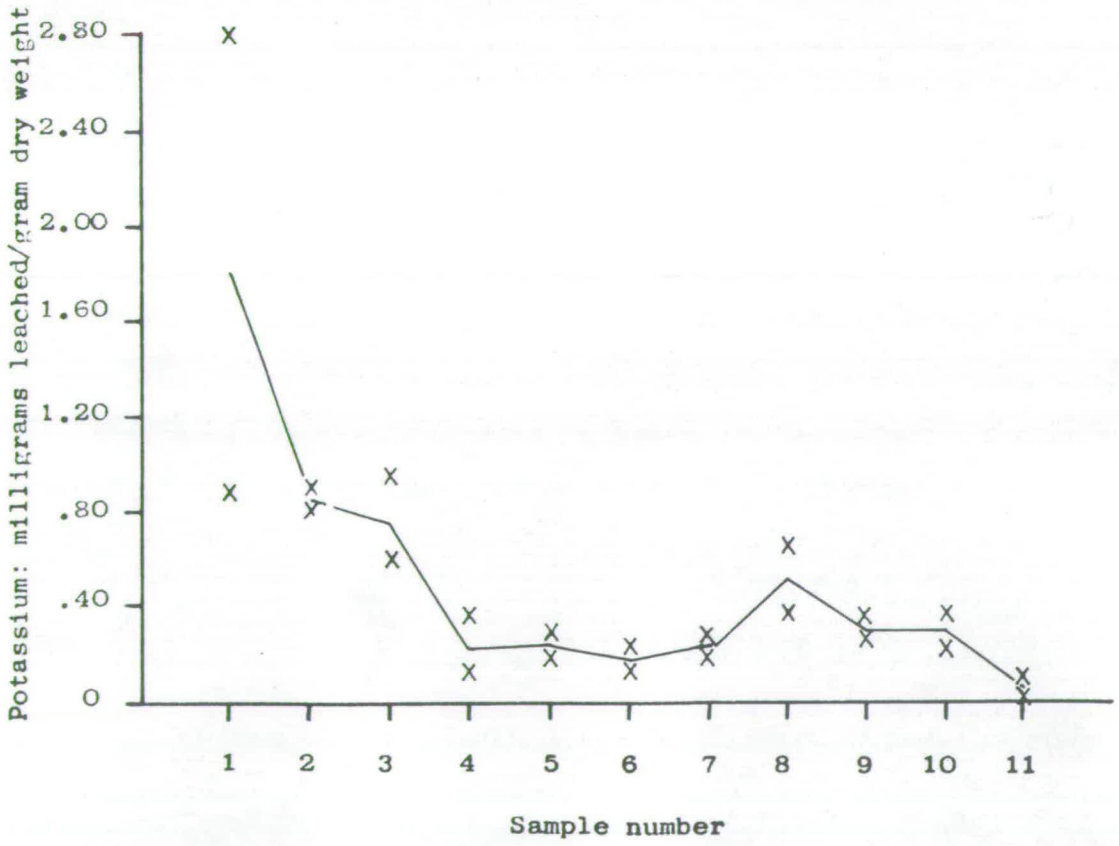


Figure II-20 Potassium ion content of seedling leachates during the 1968 microflora study.

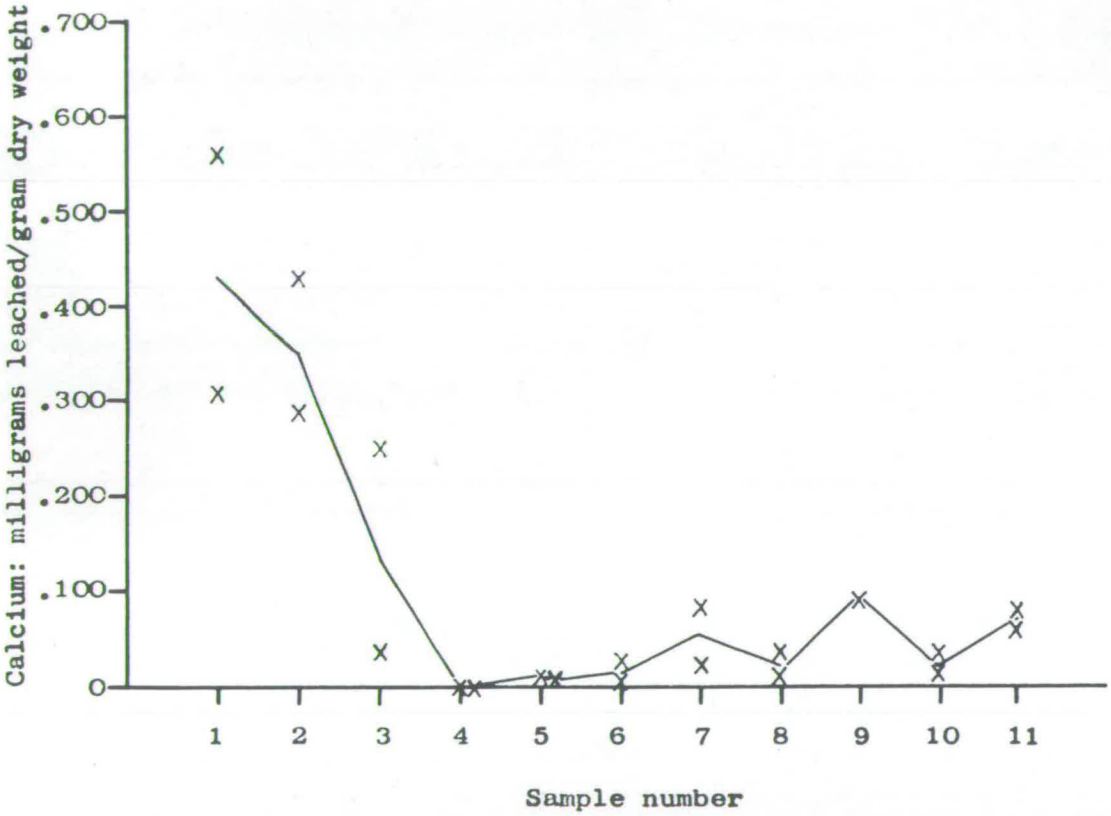


Figure II-21 Calcium ion content of seedling leachates during the 1968 microflora study.

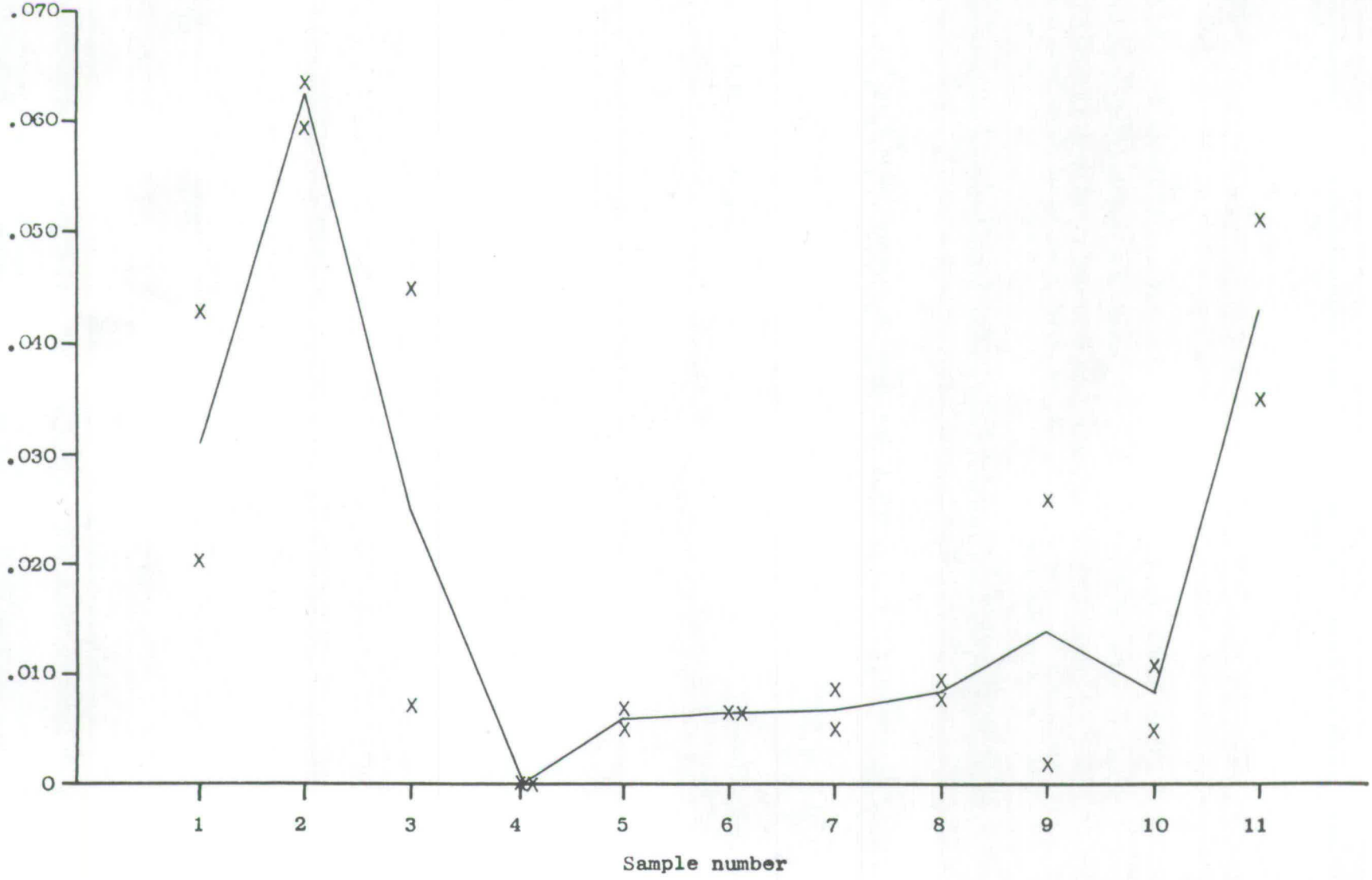


Figure II-22 Magnesium ion content of seedling leachates during the 1968 microflora study.

Figure II-23 Carbohydrate content of seedling leachates during the 1968 microflora study.

cotyledons and a short unopened epicotyl (Figure II-24).

(iv) Microorganism populations

Assessment of the leaf microflora began on the 7th June when only the cotyledons were present. From this date assessments were made fortnightly until leaf fall in November.

Numbers of Microorganisms on Cotyledons

The cotyledons of nine seedlings were sampled on each sampling occasion. For the first four samples the nine seedlings were divided into three replicates which were assessed independently. From the fifth sample onwards the cotyledons of all 9 seedlings were pooled and assessed as one sample. Thus the comparison of the estimates of the three replicates for each of the first four samples provided some indication of the variability in microflora numbers. The means of each replicate together with the overall mean for the total number of bacteria and yeasts and for the number of filamentous fungi are presented in Figures II-25 and II-26. The range of variation was not proportional to the mean in any constant pattern (Table II-4).

The variation between replicates was not large in comparison with seasonal changes in numbers but small differences between samples cannot be considered as significant.

The computation of the relative frequency of bacteria and yeasts occurring on dilution plates allowed the assessment of total numbers to be broken into numbers of yeasts and numbers of bacteria. The development of the yeast and the bacterial populations on the cotyledons is shown in Figures II-27 and II-28.

Bacteria outnumber yeasts on every occasion except one, August 1st.

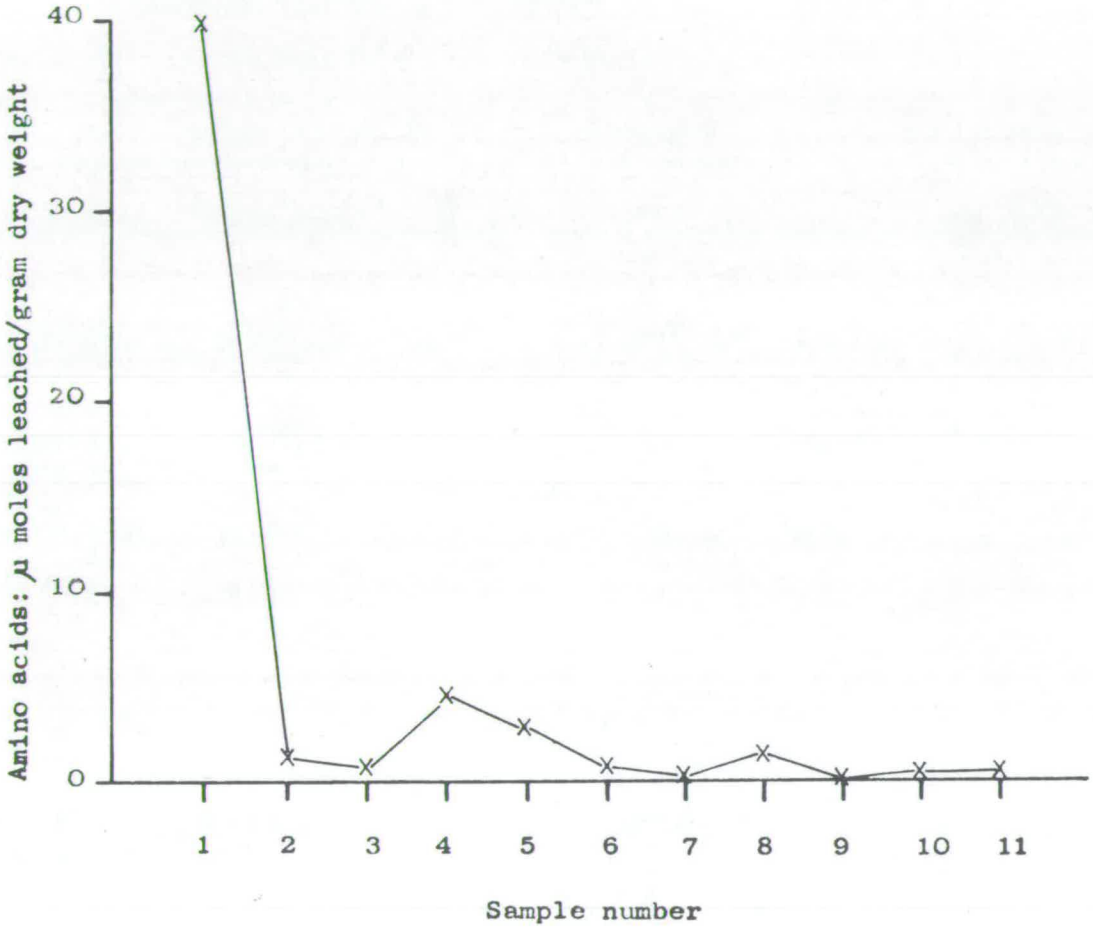


Figure II-24 Amino acid content of seedling leachates during the 1968 microflora study.

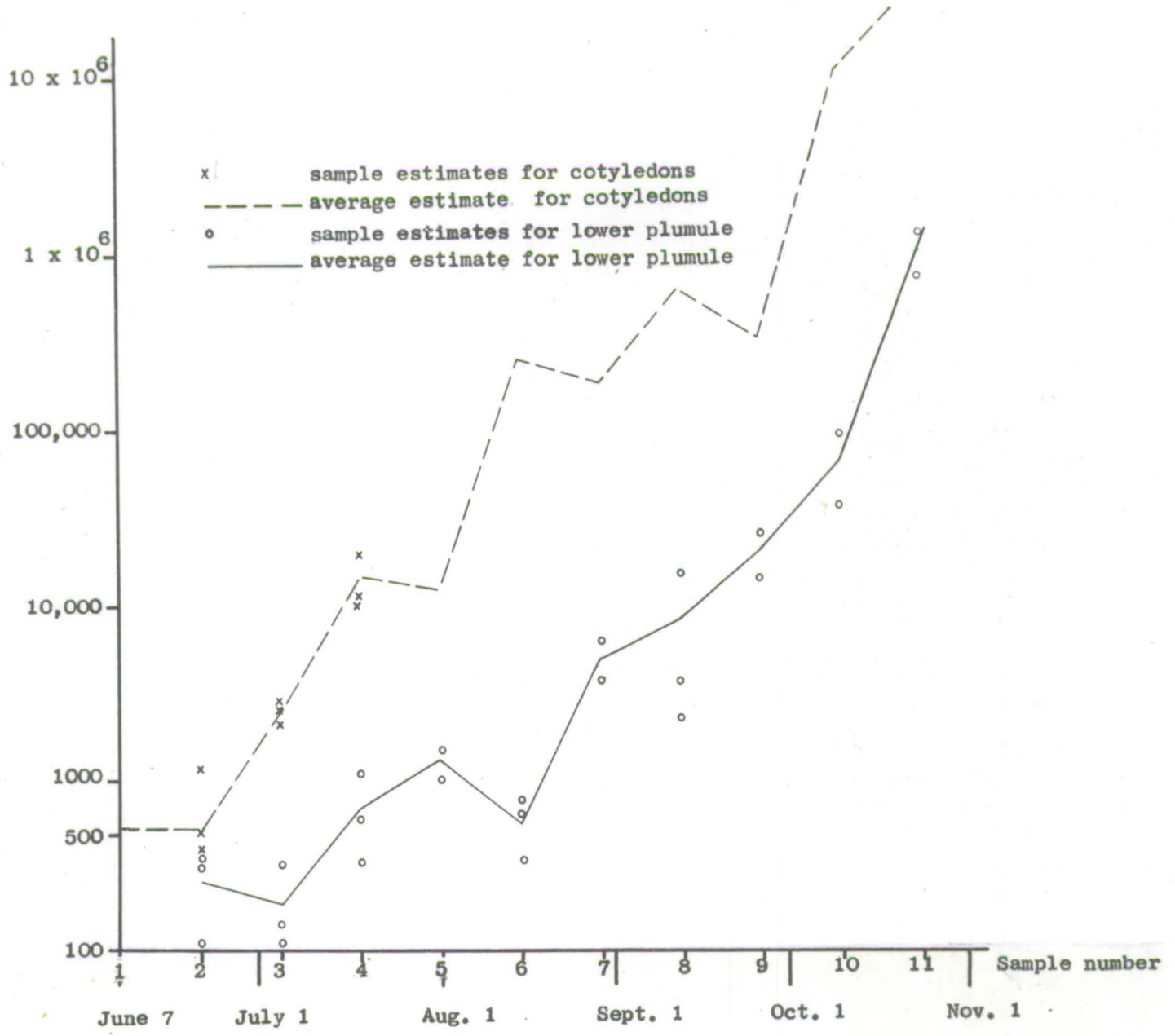


Figure II-25 The sample estimates and overall average number of bacteria and yeasts isolated from cotyledons and lower plumule leaves in 1968.

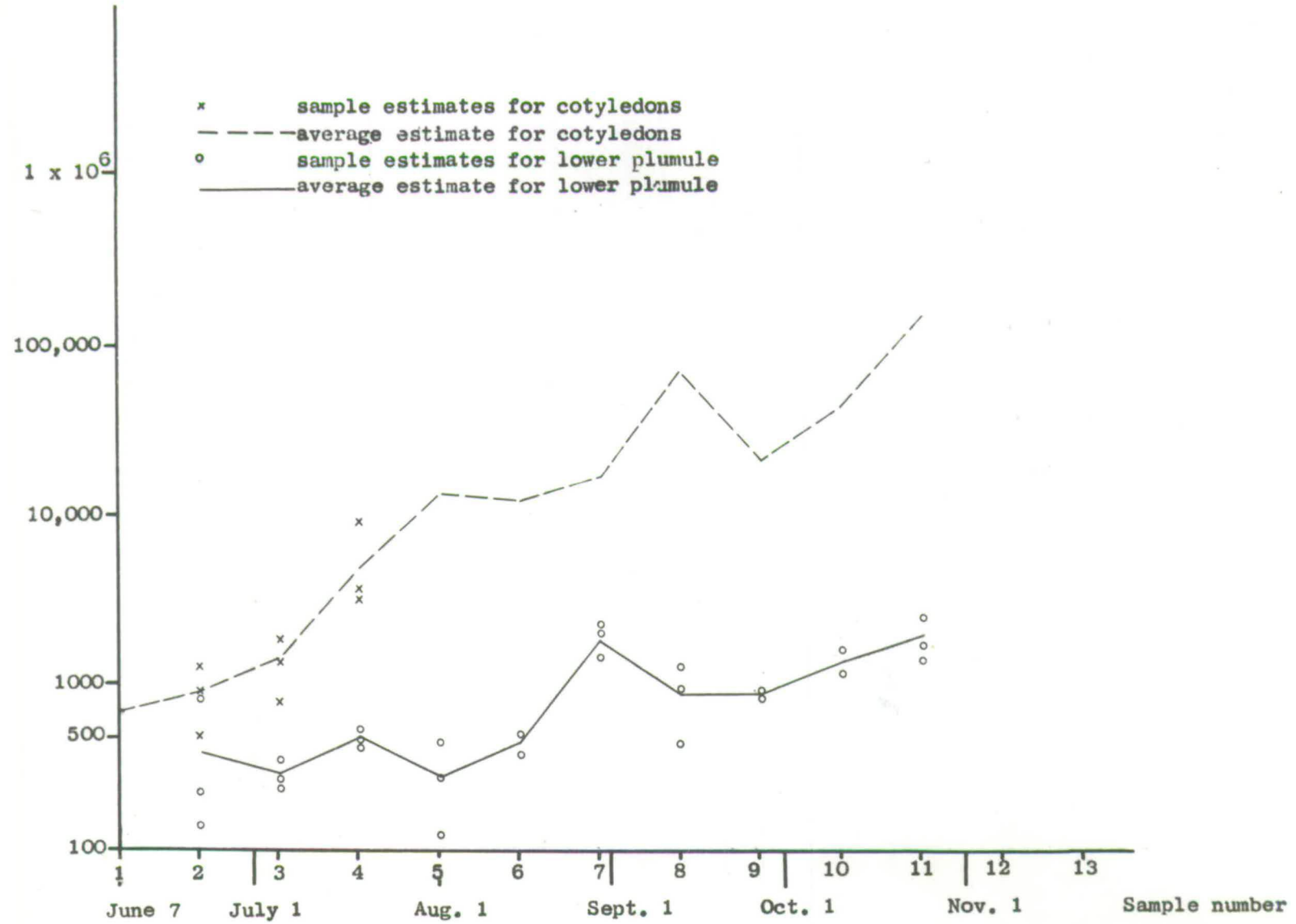


Figure II-26 The sample estimates and overall average number of filamentous fungi isolated from cotyledons and lower plumule leaves in 1968.

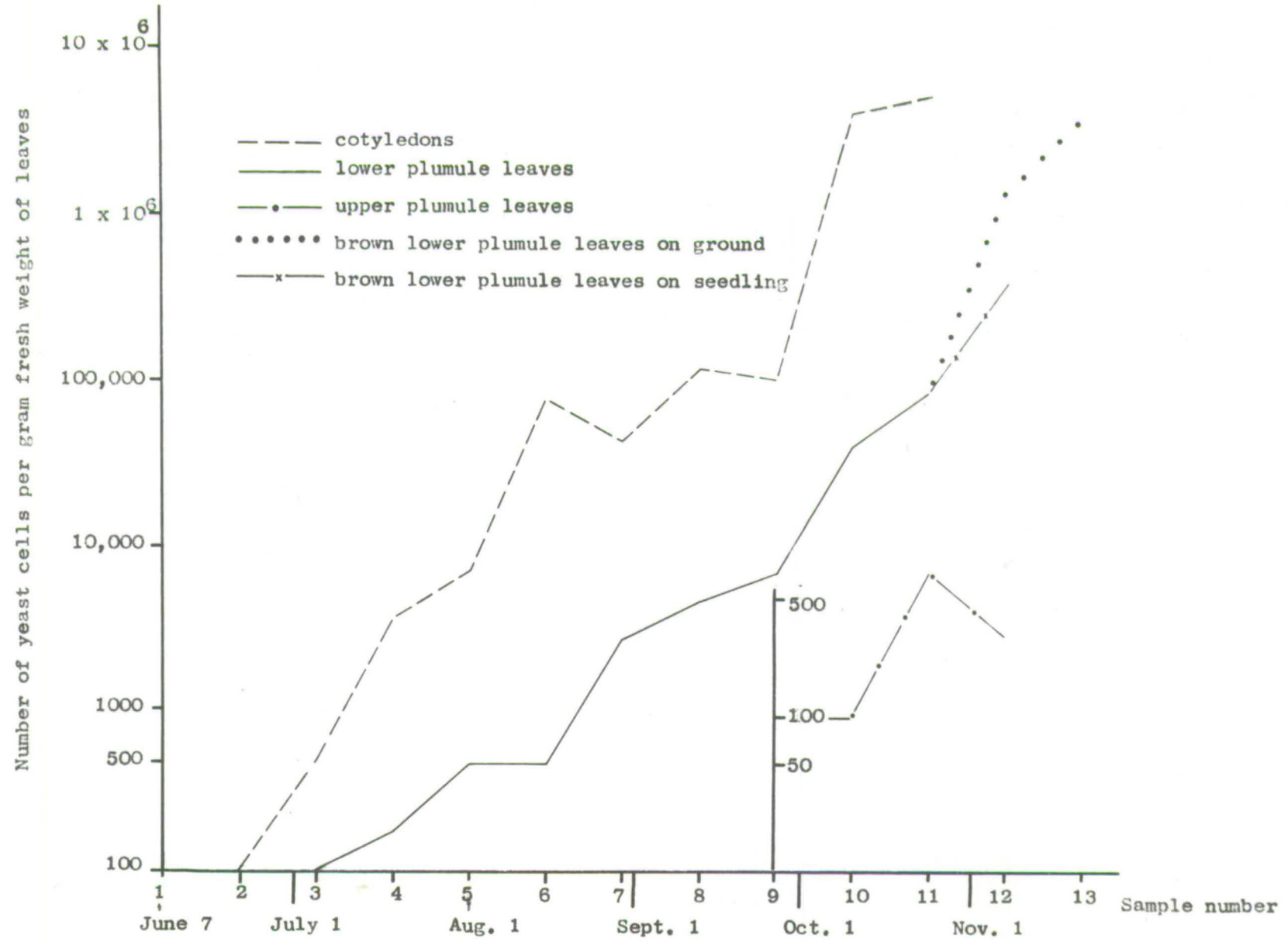


Figure II-27 The development of yeast populations on cotyledons, lower plumule leaves and upper plumule leaves in 1968.

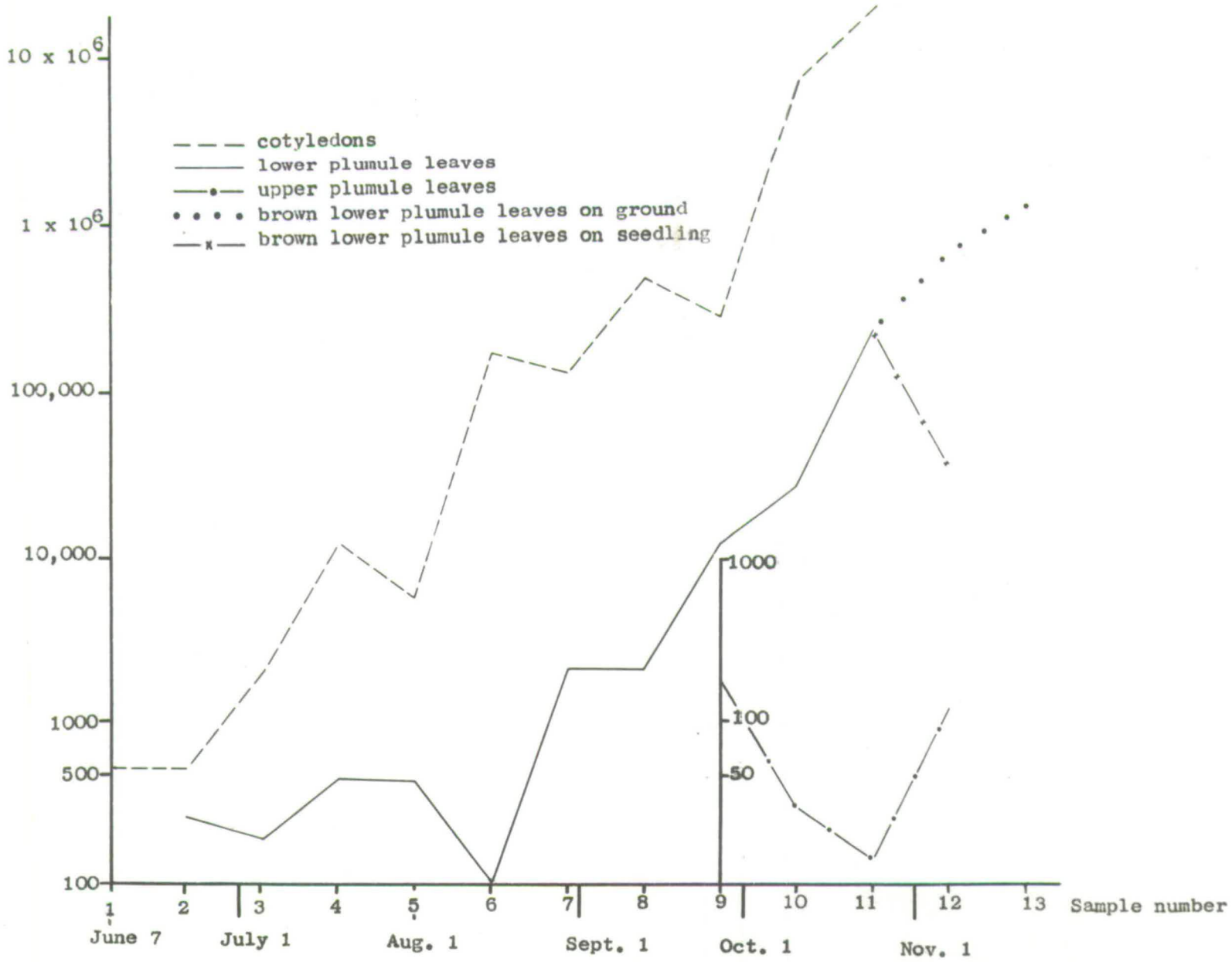


Figure II-28 The development of bacterial populations on cotyledons, lower plumule leaves and upper plumule leaves in 1968.

TABLE II-4 Range of variation between replicates of leaf samples for different groups of microorganisms (the range expressed as a % of the mean)

	Cotyledons		Plumule	
Sample	Bacteria and Yeasts	Filamentous Fungi	Bacteria and Yeasts	Filamentous Fungi
	%	%	%	%
1	96	103.	-	-
2	294	45*	102	178
3	16	62	103	35
4	76	121	141	34
5	-	-	44	111
6	-	-	80	28
7	-	-	43*	36
8	-	-	196	90
9	-	-	44*	10*
10	-	-	73*	44*
11	-	-	46	66
$\bar{x}$	121	83	87	63

* - only two replicates

The seasonal increase in numbers of yeasts and bacteria was exponential and approximated a straight line when the numbers were plotted on a logarithmic scale. The rather slow increase in numbers during the latter half of August and during September was the only sustained departure from the trend of exponential increase. No yeasts were present in the first and second samples.

The numbers of filamentous fungi which were isolated from cotyledons are shown in Figure II-29. There was a trend of increasing numbers with time which closely followed the development of bacteria and yeasts. The numbers of fungi, while similar to the numbers of bacteriaor yeasts during June and July, developed more slowly in August, September and October than the bacteria or yeasts.

Numbers of microorganisms on plumule leaves

The assessment of microorganisms on plumule leaves began on June 21st. In samples 2-8 inclusive all leaves in the upper plumule were included in the foliage sample. In samples 9-12 the plumule foliage was divided into the lower plumule, defined as the first 5cm of epicotyl, and the upper plumule, defined as all leaves above 5 cm on the epicotyl. In all cases the foliage of 3 seedlings was included in each of three replicates which were used to estimate the mean population.

The mean estimate of the total number of bacteria and yeasts are plotted along with the replicate estimates in Figure II-25. The relationship of the range to the mean is shown in Table II-4. It can readily be seen, that while replicate variation was frequently high,

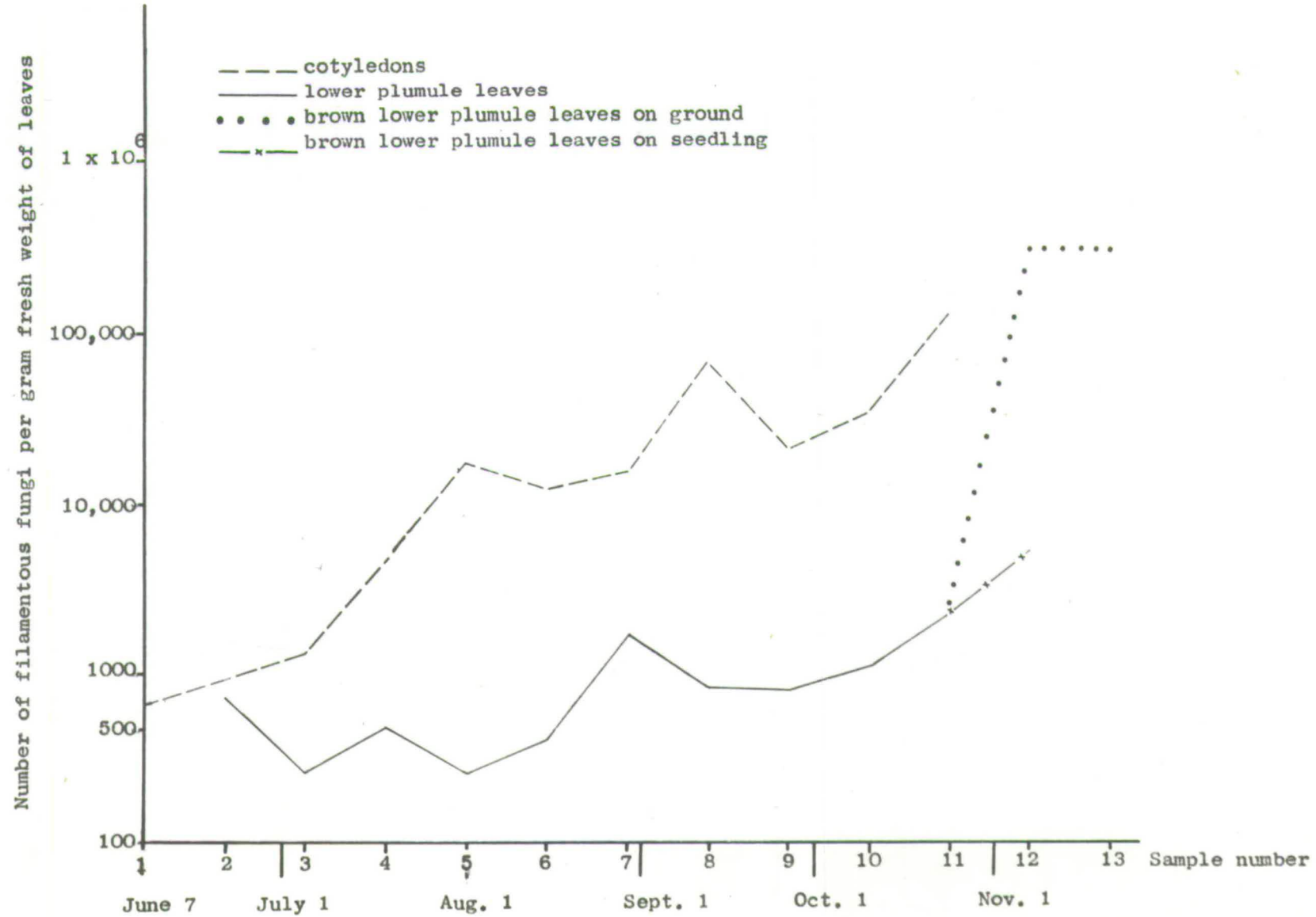


Figure II-29 The number of filamentous fungi isolated from cotyledons and lower plumule leaves in 1968.

the magnitude of seasonal change was even higher. Thus long term seasonal changes can be considered as significant, but caution must be exercised in the interpretation of short term changes in number.

The population development of bacteria, yeasts, and filamentous fungi is shown in Figures II-27, II-28 and II-29.

No yeasts were recorded on the first two plumule samples, but thereafter the number of yeasts developed at an exponential rate. The development curve closely approximates a straight line when numbers of yeasts are plotted on a logarithmic scale.

Bacteria, on the other hand, were present on all samples but their numbers did not begin a sustained increase until late August. During the lag phase from June to mid-August bacterial numbers varied between 171/g and 495/g. The highest replicate estimate indicates a bacterial population of less than 800/g during this period. At the end of August the bacterial population had risen to over 2000/g and the numbers continued to increase at an exponential rate until leaf fall in November.

The number of filamentous fungi obtained from plate counts for the lower plumule show a relatively constant pattern throughout the growing season. The numbers remained below 600/g fr wt of foliage until the end of August. From the end of September until the last green leaf sample on October 24th, the numbers were somewhat higher than earlier in the season, being between 850 and 2000/g. The numbers did not show a steep or sustained increase during any period.

The leaves of the lower plumule began to turn brown, shrivel and fall in November. Because the fresh weight of the browning leaves

decreased by approximately one-third at this time, the numbers of organisms per gram fresh weight does not provide a useful comparison with previous green-leaf samples. To overcome this difficulty, a set number of leaves (50) was used in the samples and the fresh weight of a parallel sample of 50 leaves from the green plumule was used for calculations. Using this method, the bacteria and yeast numbers were calculated for brown leaves in the lower plumule on November 7th and for brown leaves on the ground on November 7th and 21st. The numbers of bacteria, yeasts and filamentous fungi are shown in Figures II-27, II-28 and II-29. The brown leaves still on the stem showed a sharp decrease in numbers of bacteria, whereas yeasts and filamentous fungi more than trebled in number. The brown leaves which had fallen on to the ground showed a 3-fold increase in bacteria while the yeasts increased more than 10-fold and the filamentous fungi more than 100-fold.

A fortnight later, on November 21st, all the lower plumule leaves were on the ground and the numbers of bacteria and yeast had continued to increase in number on the fallen leaves. The filamentous fungi remained constant during the same fortnight.

The upper plumule foliage was sampled for microorganisms five times from the 26th September to the 21st November. At no time were there more than 100 filamentous fungi isolated per gram of foliage. The numbers of yeasts and bacteria remained below 700/g throughout October and the first week of November. The final green-leaf sample made on November 21st had 3000 bacteria and yeasts per gram of foliage.

The number of organisms was calculated per gram fresh weight of foliage. The relationship of fresh weight to surface area was calculated for young and old plumule leaves. For two-week-old leaves, one gram fresh weight had 94.1 ± 1.6 cm^2 of surface area, and for four-month-old leaves 89.1 ± 1.5 cm^2. The change over the growing season was approximately 5%. Thus, if numbers of organisms were related to surface area instead of to fresh weight, the rate of increase with time would be somewhat higher than shown.

Direct observation of bleached leaves was carried out on the light microscope at x 400 and x 1600. Optical conditions were not ideal on any leaves but were best on young leaves. Individual bacteria could be discerned only where optical conditions were excellent. Any attempt to count bacteria and yeast cells was difficult because they frequently occurred in raised colonies of > 50 cells and thus many individuals would be hidden. In spite of such handicaps the technique provided much additional information which supplemented the plate count data in a number of interesting ways. The numbers of fungal spores observed under the microscope did not relate to the numbers of fungi isolated by the plate count technique. The data in Table II-5 compares plate counts and microscope counts for filamentous fungi observed from June, July and the first half of August.

Table II-5

Comparison of estimates for total fungal spores per gram of foliage based on plate counts and direct observation.

		Plate count	Direct count
S-1	cotyledons	710	5,200
S-2	plumule	412	4,000
S-3	plumule	285	60,000-165,000
S-5	plumule	299	13,480,000
S-6	plumule	457	58,000,000

The direct count indicated a rapid and steady increase of fungal spores while plate counts were revealing a relatively static population. These opposing results may be explained by taking into account that direct counts include all spores viable or non-viable. Plate counts would indicate only the number of viable spores, and, indeed, only those which were viable in the environment of the plate count medium.

The counting of fungal spores on the leaves was curtailed after S-6 to allow time to follow closely on the development of hyphae the leaf surface. The length of hyphae per unit area was estimated by whole leaf scanning in S-6 to S-10. By S-11 the hyphae had become so dense that estimates were made by measuring hyphae in randomly chosen microscope fields. In all cases the length of hyphae was measured by

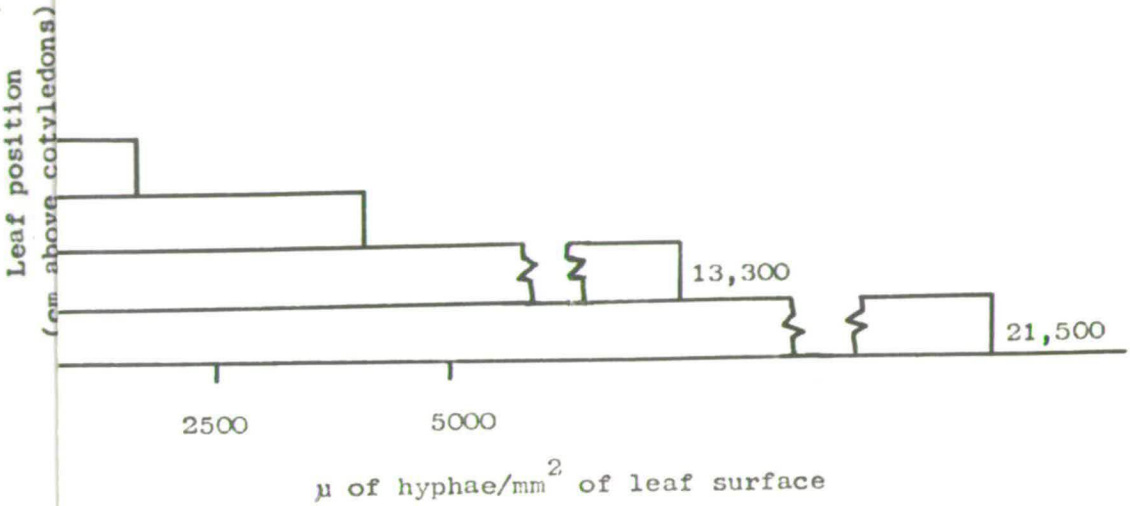
drawing the hyphae with the aid of a camera lucida and measuring with an opisometer.

Extensive hyphal growth was not observed on the leaves until August 15th. Before this date germinating spores had been observed on only one occasion, July 4th. At that time, round spores of two diameter classes, 3-5μ and 8-10μ were observed germinating on 3 of the 5 leaves examined. (A high count of Penicillium sp. was recorded on plate count for the same date). No extensive mycelium resulted from the spores which produced germ tubes between 10μ and 50μ in length.

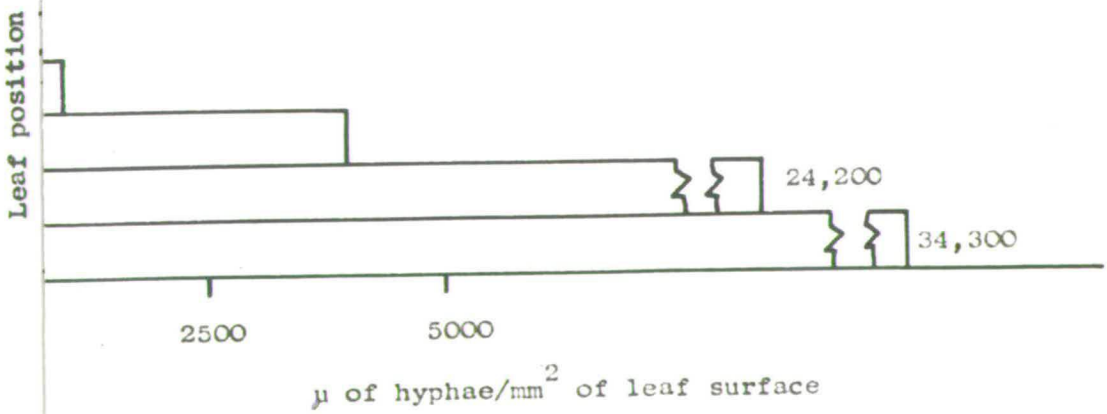
The development of hyphae was first observed sporadically on older leaves on August 15th. By September 12th the hyphal networks were showing a general pattern. To examine this pattern of hyphal development, the seedling was divided vertically into one centimeter sections. Two leaves were prepared for observation, one with the upper surface and one with the lower surface exposed. Because of the time-consuming nature of this work, only two replicate seedlings were examined at each sampling date. A summary of the data is presented in Figures II-30 and II-31.

Three consistent trends were immediately apparent. First, the mycelium was more extensive on the lower surface than on the upper. Second, the mycelium was more extensive near the bottom of the seedling than at the top, and third, the extent of the mycelium at any given position on the seedling increased with increasing time. The latter two patterns were expressed in the figures for both upper and lower leaf surfaces. Thus, hyphal development appears to be related to the age

S-11
October 24



S-11
October 24



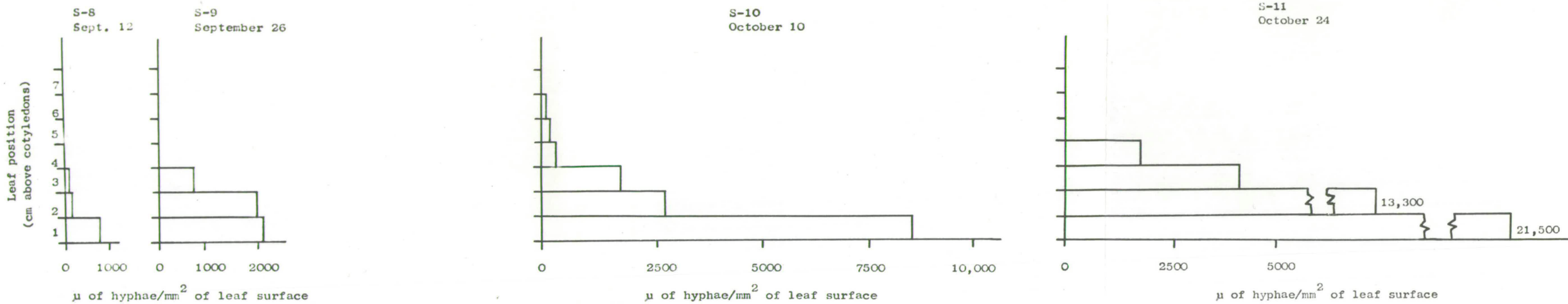


Figure II-30 The development of fungal hyphae on the upper surface of larch leaves in 1968.

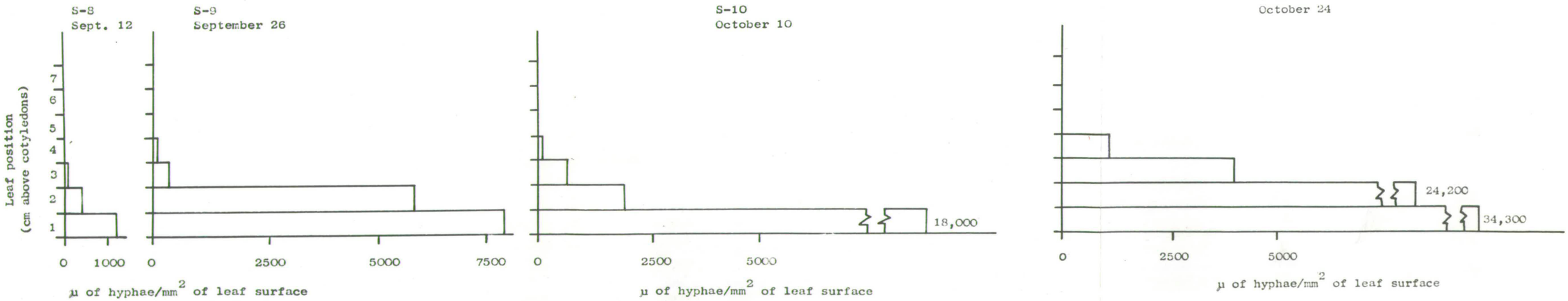


Figure II-31 The development of fungal hyphae on the lower surface of larch leaves in 1968.

of the leaf and this was reflected as a seasonal trend on leaves from a given position and as a position trend on seedlings at a given date.

The identity of the hyphae could sometimes be determined by finding its spore source. Hyphae were observed growing from spores of Cladosporium, Fusarium, Stemphylium and Cephalosporium. Over 80% of the hyphae identified by spore source were identified as Cladosporium sp. Such a figure may, however, be dependent on many factors such as spore resistance to decay, rate of hyphal growth, and ease of observation and identification which would make its use for determining species composition very doubtful. What can be stated is that all the identified fungi do contribute to the colonizing mycelium.

Pollen grains were never observed on the leaves although a conscious search was made for them. Likewise no sign of insect damage or frass was observed.

The bacteria on the leaf surface were often in association with fungal spores or on debris. It was difficult to determine morphology but short rods and short chains of cocci were seen.

The bacterial colonies not associated with spores or debris appeared to be most frequently located in anticlinal depressions. This pattern was established to be statistically significant by the Chi-square test (Table II-6). The hypothesis that bacterial distribution was random was tested by determining the predicted occurrence of colonies in anticlinal areas based on the relative proportions of the anticlinal and non-anticlinal regions. The estimate was made by classifying the area coincident with a cross-hair at 34 randomly chosen stage positions.

This resulted in the prediction that if the colony distribution was random in regards to this feature, 41.2% of the colonies would occur in anticlinal regions. By actual count 60% of the colonies occurred in anticlinal regions on the second sampling date. The difference between expected and observed was significant at $p = 0.01$.

Table II-6

Chi^2 for anticlinal tendency based on Sample 2 data

Total	Expected*	Actual	A-E	$(A-E)^2$	$(A-E)^2_i/E_i$
126	51.9	85	33.1	1095.6	21.1
148	60.9	70	15.1	228.0	3.7
50	20.6	33	12.4	153.8	7.5
174	71.7	112	40.3	1624.1	22.6
			Σ306		54.9**

$\bar{Gx}$ = 61%

* Calculated from the proportion of the leaf surface classed as being over anticlinal cell walls (.412).

(b) Seedling Microflora Study 1969.

The microflora study in 1969 was designed to determine if the development patterns observed in 1968 would be repeated in a second growing season. It was considered important to establish the age of the foliage more exactly than in 1968. For this reason, the sampling procedure was changed so that the lower plumule foliage sample included only the first five true leaves produced by the seedlings. The upper plumule sample was modified so that only the leaves from the top centimeter of the seedlings were included in this category.

The leaves from three seedlings were pooled into one sample of cotyledons, lower plumule and upper plumule.

The frequency of sampling was also reduced from the 1968 level with 1969 intervals ranging from two weeks to four weeks. No attempt was made to assess quantities of seedling leachates. Meteorological data for seedbed temperature and relative humidity, hours of sunshine and duration of wetness were gathered by the same methods as in 1968.

(i) Climatic factors

The climatic data is summarized in Figures II-32, II-33, II-34, II-35 and II-36. As might be expected, the same seasonal trends in temperature, relative humidity and hours of sunshine were observed in 1969 as were observed in 1968. However, the two growing seasons were quantitatively very different. Both the quantity and the pattern of rainfall were very different. In the 168 days of the 1968 study, 543.3 mm of rain were recorded. In the same period in 1969, 260.3 mm were recorded. The quantities recorded for June, however, were greater in 1969 than in 1968. June, 1968 was relatively dry with 11.4 mm of rain, while June 1969 had 46.1 mm.

Figure II-32 The average daily maximum and minimum temperatures for 14 day periods during the 1969 microflora study (cf. 1968 Figure II-13).

Figure II-33 The total number of hours in which relative humidity was greater than 80% during 14 day periods in the 1969 microflora study. (cf. 1968 Figure II-14)

Figure II-34 The total number of hours in which relative humidity was less than 50% during 14 day periods in the 1969 microflora study. (cf. 1968 Figure II-15)

Figure II-35 The total number of hours of sunshine during 14 day periods in the 1969 microflora study.(cf. Figure II-16 for 1968 data)

Figure II-36 The daily rainfall in millimeters during the 1969 microflora study. (cf. Figure II-17 for 1968 data)

The average daily maximum temperature was generally higher in 1969 than in 1968, while the daily minimum was similar in both years. The 1969 season remained warm much longer than 1968 and an abrupt drop in temperature occurred late in October.

To summarise the difference in season, the 1969 growing season was dryer and extended longer into the autumn than the 1968 season. A notable exception was June, which was much wetter in 1969 than in 1968.

(ii) Microorganism populations.

The numbers of bacteria, yeasts and filamentous fungi isolated from cotyledons by the plate count method are summarised in Figures II-37, II-38 and II-39. The numbers of bacteria varied between 500,000/g and 128,000/g from June until mid-September. The highest count during this period was the first count on June 19th. The cotyledons began to turn yellow in September and by October 11th the number of bacteria on the cotyledons had risen abruptly to 3,500,000/g.

No yeasts were isolated from cotyledons on June 19th and July 2nd. After this time their numbers developed rapidly reaching over 20,000,000/g on October 11th.

The numbers of filamentous fungi determined by plate counts for cotyledons declined from an initial count of 12,000/g on June 19th to 5,000/g on July 2nd. The decline was followed by a sustained increase to 70,000/g in July and August. A period of no increase from the end of August to September 12th was followed by a rapid rise to over 2,000,000/g by September 26th.

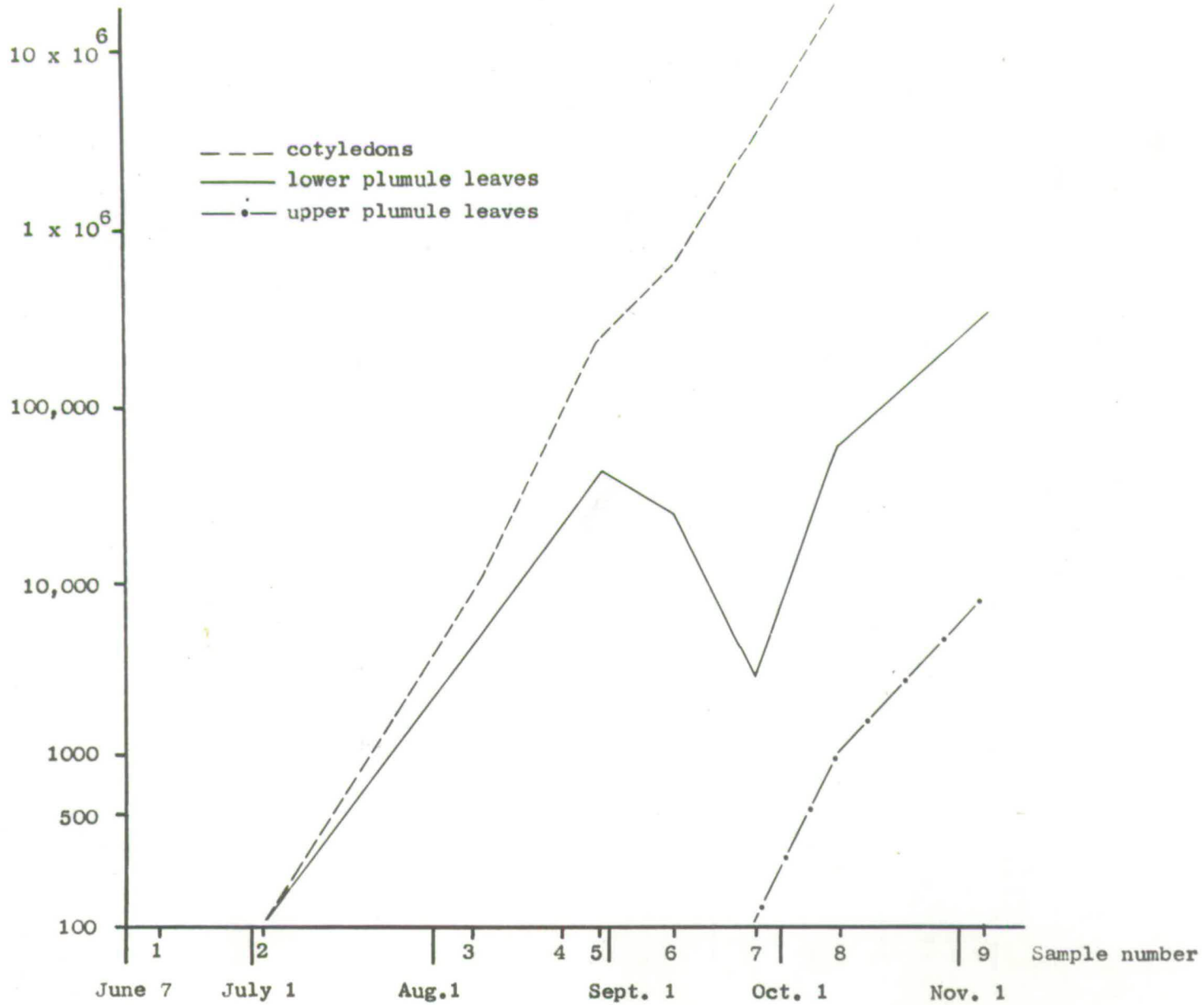


Figure II-37 The development of yeast populations on cotyledons, lower plumule and upper plumule leaves in 1969(cf. 1968 Figure II-27).

Figure II-38 The development of bacterial populations on cotyledons, lower plumule and upper plumule leaves in 1969 (cf. 1968 Figure II-28).

Figure II-39 The number of filamentous fungi isolated from cotyledons, lower plumule and upper plumule leaves in 1969 (cf. 1968 Figure II-29).

The numbers of bacteria, yeasts and filamentous fungi isolated from lower plumule leaves are shown in Figures II-37, II-38 and II-39. Since only the oldest plumule leaves were included in the 1969 samples the results were not directly comparable with the 1968 results.

The numbers of bacteria on lower plumule leaves fluctuated between 5,000/g and 60,000/g with no apparent pattern from June until October 11th. A rapid rise to 1,500,000/g occurred in late October and early November.

No yeasts were isolated from the lower plumule samples taken on June 19th and July 2nd. The yeast population then rose rapidly in July and August to 40,000/g. A decline in the yeast population occurred in September followed by a rapid and steady increase in October.

The numbers of filamentous fungi cultured from the lower plumule leaves fluctuated between 3,000/g and 22,000/g from June until the end of September. A substantial increase to 43,000/g was recorded on November 4th.

The pattern of the development of the three groups of organisms shows some similarities with patterns observed on lower plumule leaves in 1968. The yeast are at first (during June and July) less numerous than the bacteria and then occur in similar numbers as the bacteria for the remainder of the year. There was no sustained increase in bacterial numbers until late October in both 1968 and 1969. Similarily, a large increase in numbers of filamentous fungi occurred in October 1968 and again in October 1969.

The upper plumule sample has no equivalent in the 1968 study and it was made to compare the population size on leaves of a similar stage of development at different times during the season. The seedlings did not

form terminal buds until the end of September and thus until this time the upper plumule samples were made up of newly formed leaves. From October onwards no new leaves were formed, and thus the sample leaves were of increasing age and comparable to the first lower plumule samples. No yeasts were isolated from the upper plumule until October 11th. That is, no yeasts were isolated from leaves less than two weeks old during the entire growing season. The number of yeasts increased steadily during October and early November at a rate similar to that recorded for yeasts on lower plumule leaves in July. The number of bacteria on the upper plumule leaves varied between 340/g and 6,500/g. The numbers remained relatively constant during July and August. The low count of 320/g was recorded in September. The samples taken after bud formation at the end of September indicate a decline in numbers.

In 1969 bleached leaves were examined under the microscope in an attempt to determine the onset of hyphal development. Hyphae were first seen on the oldest leaves on September 12th. A check had been made on August 20th and had revealed no hyphae. No attempt was made to quantify the rate of hyphal development but the pattern appeared to be similar to that observed in 1968. The hyphae developed first and most extensively on the oldest leaves.

It was of considerable interest to determine if the foliage of mature larch trees had a similar microflora to the foliage

of larch seedlings. Samples of the foliage from short shoots of a 10-year-old Larix decidua tree growing near the seedbeds were taken on July 2nd and November 4th. The numbers of yeasts and bacteria isolated from the mature foliage are given in Table II-7 along with the results of corresponding lower plumule seedling samples.

TABLE II-7 Comparison of numbers of bacteria and yeasts on mature and seedling larch.

	July 2nd		November 4th	
	Bacteria/gm	Yeasts/gm	Bacteria/gm	Yeasts/gm
Mature	7,200	0	253,000	364,000
Seedling	2,700	0	1,109,000	350,000

The numbers of yeasts/g showed a remarkable similarity on the two types of foliage. The numbers of bacteria/g differ by factors of 3 and 4 between foliage types. The trend of increasing number with age was present on both types of foliage.

(c) Types of microorganisms occurring in the larch phyllosphere

In addition to the enumeration of the major groups of microorganisms in the phyllosphere, an attempt was made to identify or characterize the main components of each group.

The most numerous components of the filamentous fungi were identified to genus and include Cladosporium sp., Fusarium sp., Phoma sp., Stemphylium sp., Cephalosporium sp., and Epicoccum sp. The above named fungi occurred in almost every foliage sample. Less frequently isolated were species of Penicillium, Botrytis, Aspergillus and an unidentified sterile white mycelium. All genera with readily recognisable spores, i.e. Cladosporium, Fusarium, Stemphylium and Cephalosporium, which were observed during direct microscopy of the leaf surface were subsequently identified in plate count isolates. Because the fungal flora appeared to be in a large part casual it was considered beyond the immediate requirements of this study to identify all isolates or to determine frequencies of the various taxons. However, the most frequent fungus isolated was Cladosporium sp. of which several isolates were kept for further study.

All bacteria and yeasts from randomly selected dilution plates or portions of dilution plates were isolated into pure culture. Although the numbers isolated per sample for identification was small (20-40) the results may be used to give some indication of the importance of each taxon in the microflora.

The bacteria were first divided by their Gram reaction. The Gram-positive bacteria were further characterized only by morphology, e.g. rods, cocci, endospores etc. The Gram-negative bacteria were identified in most cases to genus by the scheme of Park and Holding (1966).

The Gram-negative bacteria frequently accounted for 90% of the total bacteria. Only once, in September, 1969, when a large number of Gram-positive cocci were isolated did the Gram-positive bacteria represent as much as 50% of the total.

The most numerous Gram-negative bacteria were short rods of the genera Pseudomonas, Flavobacterium, and Xanthomonas. The Pseudomonas spp. included a form which produced a fluorescent pigment easily visible under ultraviolet light. The sampling dates on which the above mentioned genera and the Gram-positive groups were isolated are summarised in Tables II-8 and II-9.

Pseudomonas spp. were isolated on every sampling occasion. The genera Flavobacterium and Xanthomonas appear more frequently in the last half of the growing season. The results of 1968 and 1969 were similar.

In addition to the dominant groups mentioned above, the following genera were occasionally isolated: Achromobacter, Agrobacterium, Mycobacterium and Streptomyces. Streptomyces spp. were isolated on SEYE agar on one occasion in 1968 (September) and on one occasion in 1969 (August). Sodium albuminate agar was used on a number of occasions in an attempt to selectively culture actinomycetes but the only successful isolations were made on the same sampling date in September 1968 when the Streptomyces spp. were also isolated on SEYE agar.

TABLE II-8 Occurrence of selected bacteria on different sampling dates in 1968

Sample date	June 7th	June 21st	July 4th	July 17th	Aug. 1st	Aug. 15th	Aug. 29th	Sept. 12th	Sept. 26th	Oct. 10th	Oct. 27th	Nov. 7th
Sample No.	1	2	3	4	5	6	7	8	9	10	11	12
Pseudomonas	*	*	*	*	*	*	*	*	*	*	*	*
Flavobacterium	-	-	-	-	*	*	-	*	*	*	*	*
Xanthomonas	*	-	-	*	*	-	-	*	*	*	*	-
Gram-positive rods	*	*	*	*	*	*	*	-	*	*	*	-
Bacillus	*	-	*	-	*	-	*	-	-	-	-	-
Gram-positive cocci	*	*	-	*	*	-	-	*	*	-	-	*

Also isolated sporadically were: Achromobacter, Agrobacterium, a member of the Enterobacteriaceae, Mycobacterium, and Streptomyces.

TABLE II-9 Occurrence of selected bacteria on different sampling dates in 1969

Sample date	June 19th	July 2nd	Aug. 6th	Aug. 20th	Aug. 28th	Sept. 12th	Sept. 26th	Oct. 11th	Nov. 4th
Sample number	1	2[1]	3	4[1]	5	6	7	8	9
Pseudomonas	*		*		*	*	*	*	*
Flavobacterium	*		-		*	*	*	*	*
Xanthomonas	-		-		-	-	*	-	*
Gram-positive rods	*		*		-	-	-	*	*
Bacillus	-		-		-	-	-	-	*
Gram-positive cocci	-		*		*	-	*	-	-

Also isolated were: Achromobacter, Agrobacterium

[1] no assessment of bacterial groups was made for samples 2 and 4.

Pigmented forms were common among the bacteria. In addition to the fluorescent pigment already mentioned for one group of Pseudomonas spp., a red pigmented form was also isolated. By far the most frequently occurring pigments were the yellows of the Flavobacterium spp. and Xanthomonas spp. The percentages of bacteria producing pigments isolated in all samples in 1968 and 1969 are shown in Table II-10.

TABLE II-10

Proportions of pigmented bacteria present in samples in 1968 and 1969

Sample 1968	% pigmented	Sample 1969	% pigmented
1	8	1	7
2	4	3	0
3	0	5	90
4	8	6	12
5	50	7	33
6	57	8	75
7	0	9	54
8	70		
9	42		
10	60		
11	57		

No Azotobacter sp. were isolated in spite of several attempts to selectively isolate members of that genus in 1968 and 1969.

The yeasts isolated from the leaves were divided almost equally into two groups, the cream coloured and the pink coloured yeasts. The proportions of pink and cream coloured yeasts isolated on different sampling dates in 1968 are presented in Table II-11.

All the pink yeasts were capable of utilizing nitrate as the sole nitrogen source. They all could be assigned to Rhodotorula glutinis (Fres.) Harrison or Sporobolomyces roseus Kluyver et van Niel. The two species can be separated by the fact that only the latter produces ballistospores. Some difficulty was encountered with this characteristic because an isolate which produced spores on one occasion would fail to on another and vice versa. It may be that the trouble arose from mixed cultures or changes occurring during storage.

The cream coloured yeasts belonged to two genera, Cryptococcus and Torulopsis.

Cryptococcus spp. could be readily distinguished from the Torulopsis spp. by the former's ability to form starch. The occurrence of the two genera showed a seasonal pattern. Table II-12 lists the 1968 samples from which isolates of Cryptoccoccus spp. and/or Torulopsis spp. were isolated. Cryptococcus spp. were not isolated before the middle of August. It was then present until leaf fall. Torulopsis spp., on the other hand, occurred consistently through June, July, and August and then occurred less frequently in the Autumn. It must be remembered that the data represents proportions of cream-coloured yeasts, the numbers of which were increasing rapidly throughout the year. Thus, the fact that Torulopsis spp. were less frequently isolated in the Autumn does not indicate that their numbers had declined but only that their proportion relative to Cryptococcus spp. had declined.

TABLE II.11 % occurrence of cream and pink yeasts of different sampling Dates in 1968.

	S-2	3	4	5	6	7	8	9	10	11
cream-coloured yeasts	100	0	67	43	33	91	30	75	64	25
pink-coloured yeasts	0	100	33	57	67	9	70	25	36	75

TABLE II.12 Occurrence of Torulopsis and Cryptococcus in foliage samples

Days	1	2	3	4	5	6	7	8	9	10	11	12
Torulopsis	-	*	*	*	*	*		-		*	-	-
Cryptococcus	-	-	-	-	-	*		*		*	*	*

All bacteria, yeasts and actinomycetes isolated in 1969 were tested for lipase activity. The method of Sierra (1957) was used. Tween 80 was the test substance. Colonies which produced a "halo" of the calcium salt of fatty acids after 14 days were considered to be lipase producers. Over 80% of the isolates produced lipase. In contrast, 50% of a sample of micro-organisms isolated from larch seeds and only 20% of those isolated from the seedbed soil produced lipase.

The organisms which produced lipase included isolates of *Pseudomonas*, *Flavobacterium*, *Xanthomonas*, *Achromobacter*, *Agrobacterium*, Gram-positive cocci, *Streptomyces*, *Torulopsis*, *Cryptococcus* and *Sporobolomyces roseus*.

The leaf microflora was characterized by containing a high proportion of Gram-negative rods. It contained a high proportion of pigmented forms of both bacteria and yeasts. The ability to produce lipase was widespread in the microflora.

DISCUSSION

The analysis of the larch leaf surface revealed a substrate of considerable physical, chemical and microbial complexity. The leaf topographical features are numerous and of a range of sizes which make them potential micro-habitats for yeast and bacterial cells. It was established that bacteria were not randomly distributed on the leaf surface but were most frequently associated with the depressions over anticlinal cell walls. The concentration of bacteria, yeasts and fungal appressoria in this type of microsite has been reported by Last (1955a), Ruinen (1961), Leben and Daft (1964) and Preece et al. (1967). The cause of the concentration of microorganisms in the anticlinal depressions is a matter of some speculation. It is possible that this area might be rich in nutrients. Franke (1967) has found that ectodesmata, which may act as leaching pathways occur in greatest number in this area. Moreover, it is possible that the depressions are the last areas to dry after wetting and thus the water film could concentrate water soluble compounds in the depressions. Such a drying pattern might also draw inoculum into the depressions by the surface tension of the water film. Whatever the cause of the concentration of organisms in the leaf depressions, the area is of considerable interest to plant pathologists. The dense population increases the likelihood of interactions between elements of the microflora. Interactions in this area may be especially significant because of the importance of the depressions as sites for leaf infection (Preece et al., 1967).

The concentration of wax threads around the stomata and the wax plugs which appeared to almost close some stomatal apertures might provide protection from leaf pathogens which infect through the stomata.

It is possible that microsites smaller than the anticlinal depressions provide preferred habitats for microorganisms on leaves. The use of stereoscan electron microscopy in leaf surface ecology research may reveal much important information in this regard. The wax structures observed on larch leaves are of a size which could provide habitats protected from rain.

While not extensive enough to define the substrate the chemical analysis of the leaf surface indicated that the growth requirements for many types of microorganisms were present. In addition to amino acids and sugars present in leaf leachate, the leaf waxes were composed of several fatty acids, esters and paraffins which might provide energy sources for microorganisms.

The quantity of nutrients in seedling leachates varied through the growing season. Amino acids and ions were most abundant in leachates of very young seedlings while carbohydrates were detected only in leachates of seedlings one month or more old. The quantity of these substances in the leachates may reflect the internal content of the seedling. A low concentration of soluble carbohydrates and a high concentration of amino acids would be expected in young seedlings (Webster, 1959).

Magnesium was the only ion to increase in quantity in leachates of seedlings at the end of the growing season. The increase of magnesium may be associated with the breakdown of chlorophyll in senescent leaves.

The quantity of carbohydrates in the leachates varied considerably during the growing season. The high carbohydrate

quantities recorded on July 4th and August 1st follow long sunny periods while the low quantities recorded on July 17th and August 29th follow periods of cloudy weather. Thus, carbohydrate leaching may have been related to duration of sunshine as was reported by Tukey, Wittwer and Tukey (1957) and Tukey and Morgan (1963) for several plant species.

In this regard it is of interest that the bacterial flora in the rhizosphere of wheat has been reported to have decreased when the light intensity for the plant was reduced, (Rouatt and Katznelson, 1960).

The high levels of carbohydrate in leachates of seedlings at the end of the growing season may be due to increased leaching from senescent leaves. Long, Sweet and Tukey (1956) reported a large increase in leaching just before the death of leaves. The quantity and quality of leaf leachates appear to be influenced by weather and by the age of the leaf.

The interpretation of the leachates of seedbed seedlings is complicated by a number of factors. Firstly, the method of whole seedling leaching, while essential for practical reasons, would tend to mask changes due to the age of leaves. Also, field-grown seedlings may have nutrients derived from spores, pollen and detritus on their leaf surfaces. In addition, an active microflora would be expected to transfer the soluble leachate substances into insoluble compounds and thus could influence leachate measurements. For these reasons it may be best to confine leachate analysis to more controlled conditions where a comparison of age could be done with the exclusion of other factors.

The larch phyllosphere study provided population data for bacteria, yeasts, and filamentous fungi for a variety of foliage types from seedlings of different ages and for two growing seasons. An assessment and comparison of the data may provide useful hypotheses concerning the critical factors governing phyllosphere populations, their development and their significance to the plant.

The growth of populations of many unicellular microorganisms can be expressed by the logistics equation:

$$\frac{dN}{dt} = kN - \left(\frac{k}{G} N^2\right)$$ where N is the number of individuals, G the maximum population size possible, and k is the growth rate constant. The growth rate of bacteria and yeasts of the types found in the phyllosphere have been shown to vary between 10 and 100 divisions per day under optimum conditions (Brock, 1966). Thus under optimum conditions a single bacterium or yeast would be expected to increase to at least a million cells in a matter of days and the population development would follow a sigmoid curve.

It may not be valid to look for population patterns when dealing with large heterogeneous groups such as yeasts and bacteria. However, it is obvious that the microorganisms on the larch leaves did not increase at anywhere near the maximum rate possible for these type of organisms. It may be of value, therefore, to examine the range of environmental factors which may have prevented the biotic potential for increase from being realized.

The pattern of increasing numbers of bacteria and yeasts on ageing larch leaves was established by the observations in 1968 and 1969. By the end of the growing season a gram of leaves supported upwards of a million microorganism cells. If the carrying capacity of the leaf substrate was similar throughout the growing season it must be determined why the development of microorganisms proceeded at a level which was less than the potential. On the other hand, if the substrate has changed through the growing season the nature and rate of that change may be the most important factor restricting the development. The nutrient status of the leaf as a substrate may change in response to autonomous physiological change in the seedling. It may be changed by the actions of the microorganisms on the leaf, and/or by physical weathering.

In considering the carrying capacity of larch leaves it is of interest to compare the total numbers of bacteria and yeasts on the leaves at the end of the 1968 and 1969 growing seasons. The ratio of the highest and the lowest numbers of bacteria and yeasts on each type of leaf was calculated. The numbers which occurred on the 140th day after germination were used to calculate the ratio for the cotyledons and on the 154th day after germination for the lower plumule leaves. The ratio numbers of yeasts on cotyledons in 1968 and 1969 was 5 and of bacteria 1.6. The ratio of numbers of yeasts on the lower plumule was 2.25 and of bacteria 1.7 . If the numbers of bacteria and yeasts are summed together the ratio of the sums of 1968 and 1969 on cotyledons was 2.1 and on lower plumule leaves 1.05.

Moreover, the ratio of the microorganism numbers on leaves from a mature tree and from seedlings in 1969 was 5.0 for bacteria and 1.0 for yeasts.

The low value of the ratios indicate that the numbers of microorganisms at the end of the growing season are very similar from year to year and also on leaves from seedlings and mature trees. The ratios appear small in comparison to the seasonal differences in numbers which change by factors of 10^5 or 10^6. The similarity of final populations after the two growing seasons, which had very different weather conditions and on seedlings grown in two different locations, could indicate that the numbers are determined by a fundamental limit to the carrying capacity. That is, the number of yeasts and bacteria reach a maximum number determined by either the available nutrients or some other factor such as space. If there was a maximum carrying capacity which was reached by the end of the growing season, it would be important to know what factor or complex of factors limited the development rate.

The pattern of yeast development appeared to be basically similar in 1968 and 1969. The yeast development on cotyledons approximated to a logarithmic rate rising from zero to several million in the course of the growing season. The development pattern on lower plumule samples in 1968 cannot be directly compared with 1969 or with the cotyledons because the 1968 foliage samples included leaves of a wide range of ages. However, in 1968 and 1969 the numbers of yeasts on the lower plumule foliage remained low during June before rising at a steady logarithmic rate.

In 1969 the steady increase of yeast cells was interrupted by a period of decline during September. The rate of increase on leaves of a defined age was remarkably similar. For example, the yeast population on cotyledons in 1968 reached 10,000/g 44 days after germination and in 1969 after 37 days. On the lower plumule samples in 1969, 10,000 yeasts/g was reached 43 days after the leaves emerged. As stated above, the lower plumule leaves in 1969 were not comparable but the samples of upper plumule leaves in 1969 were of a defined age following the formation of apical buds. From the date of apical bud formation it was 40 days until the yeasts numbered 10,000/g. Thus the rate of yeast development on the newly exposed leaves and cotyledons was very similar. The similarity between rates in 1968 and 1969 is especially interesting because of the very different weather in the two years. In addition, the similar rate observed on upper plumule foliage in 1969 occurred in October when temperature, humidity and sunshine were very different than in June and July, the months in which the rates on cotyledons and lower/plumule leaves were observed. Thus the development rate of yeasts does not appear to have been directly limited by climatic factors which differed greatly from year to year and from spring to autumn.

The rate of inoculum arriving on the leaf may be a factor limiting the development rate of yeasts. Yeast cells are not motile and it must be assumed since they do not occur on newly formed leaves that they reach the leaf by deposition from the airspora. If yeast cells could spread over the entire leaf surface the rate of incoming inoculum

would not seriously affect the development of yeast numbers. However, if yeasts were restricted to certain microsites on the leaf or were restricted to low density the development of a spatially contiguous population could be made impossible. If this were the case, a yeast inoculum would only be able to increase in number to exploit a small area. Thus even if each colony were developing at the biotic potential, the rate of development for the whole leaf would be dependent on the amount of inoculum. Thus the logistics equation could be modified to

$$\frac{dN}{dt} = I + I\left(KN - \frac{k}{G} N^2\right)$$ where I is the rate of inoculum arriving on the leaf.

The density of the airspora is not likely to have remained constant during the growing season. Indeed, Last (1955b) and Hamilton (1959) have reported that the number of yeast cells in the airspora varies with weather and with time of year. The peak periods are in late summer and in humid conditions. The airspora factor can be examined by comparing development rates of different leaf types during the same period of time. To make the comparisons valid it must be assumed that inoculum deposition rates are similar on the leaves compared. This assumption is relatively safe with cotyledons and lower plumule leaves because of their proximity to each other and their similar length of exposure to weathering etc. The comparison of upper plumule leaves with lower plumule leaves would seem less easily justified partly because of a five centimeter difference in position and especially

because of differences in surface wetting properties which may affect spore deposition. The similarity of the logarithmic rate of development on the physiologically different cotyledons and lower plumule leaves in June, July and August in 1969 suggests that airspora may determine population development. However, the decline in numbers of yeasts on lower plumule leaves in September occurs while the numbers on the cotyledons continue to increase rapidly. Thus it is not likely that airspora was a limiting factor during this time. It is also interesting that no yeasts were recorded on upper plumule leaves until October even though fungal spores, presumably from the airspora, were isolated in fairly large numbers from these leaves.

The possibility that changes in the substrate determine the rate of development must now be considered. As stated previously, changes in the substrate might be autonomous changes reflecting host physiology or they may be successional changes brought about by interaction of the host and the microflora. The substrate might also be altered by physical weathering and by the accummulation of spores, pollen or airborne detritus. The comments made concerning the density of the airspora can be applied to the last mentioned factor. Airborne particles would be expected to influence all leaves at the same time. Moreover, in contrast to the condition reported by Fokkema (1968) no large accumulations of pollen grains were observed on the larch leaves. The three types of leaf samples, cotyledons, lower plumule and upper plumule were in juvenile and senescent stages at different times of the growing season. No exact

measure was made of senescence but cotyledons turned yellow and then brown while the lower plumule leaves were still green. The upper plumule leaves were still green when sampling was stopped and when the leaves of the lower plumule had fallen to the ground. Thus, if the physiological age of the leaf was determining the development pattern, the pattern would be expected to appear first on cotyledons and then on leaves of the lower plumule and finally on the leaves of the upper plumule. In both 1968 and 1969 the sequence of development fits this hypothesis. The development on lower plumule leaves parallels the development on the cotyledons but was somewhat later. Moreover, although the start of development on upper plumule leaves was three months later than on lower plumule leaves, the development followed the same basic pattern.

The development of yeast populations on larch leaves would appear to be determined to a large part by factors related to the age of the leaf. This conclusion agrees with other studies on yeasts in the phyllosphere by Last (1955à), Ruinen (1961) and Dickinson (1967).

Similar considerations can be applied to the development of bacterial populations as have been applied to yeast populations. The pattern of development was first observed on cotyledons and then on lower plumule leaves. The bacterial populations on different types of leaves do not usually show similar changes on the same sample dates. There were two exceptions to this rule. The first, the decline in numbers after the first sample in 1969, will be discussed later. The second concerns a marked decline in bacterial numbers on all three foliage types on September 12th, 1969. The sample was taken after

two days of heavy rain and may indicate that rain can remove bacteria from leaves as suggested by di Menna (1959) and Ruinen (1961).

The bacteria in the phyllosphere differ from the yeasts in that many are motile and thus can migrate on leaves and seedlings (Leben, 1965). It has been reported by Leben and Daft (1966) that a few inocula present on the seed can multiply to millions and migrate over the entire plant. The capacity for colonizing leaves by this method may be reflected in the observation that bacteria were present on all larch leaves, even the most juvenile.

While the final numbers of bacteria on cotyledons or lower plumule leaves was similar in 1968 and 1969, the numbers during the first half of the growing season were much higher than in 1968. The ratio of numbers of bacteria in the first samples of 1969 and 1968 was 10^4 on the cotyledons and 50.0 on the lower plumule. The differences between 1968 and 1969 may be due to different weather or the different location of the seedbeds. The seedbed in 1969 was more exposed to wind than was the seedbed in 1968. Bacterial inoculum in the airspora is thought to be mainly derived from soil particles (Pady and Kelly 1953) and thus a windy site might be expected to have higher numbers of bacteria. Moreover, more soil would become airborne in the dry summer of 1969 than in the wet summer of 1968. That the high numbers of bacteria were due to a high inoculum density was supported by the constantly high numbers of bacteria isolated from the upper plumule leaves.

Another possible explanation of the high bacterial numbers in 1969 is thecolonization of the seedling from the seed. The seeds germinated in June, which was the only period of the 1969 season wetter than the 1968 season. During germination, the wet weather may have

provided good conditions for bacteria to migrate and develop. The high numbers could then have been self-perpetuating by the continuous colonization of the newly formed leaves.

Unlike yeasts, bacteria did not increase rapidly on young leaves. The pattern of development showed an initial lag period followed by a period of rapid increase. The lag phase was shorter on the cotyledons than on the lower plumule leaves. The upper plumule leaves never showed a period of rapid development even on samples taken in November. The different lengths of the lag phases on the different types of leaves supports the hypothesis that the physiological condition of the leaf determines the microflora development.

The filamentous fungi were examined in 1968 by both direct observation and culture methods. A comparison of the data collected by the two methods reveals the inadequacy of each. On young leaves the direct observation method revealed rapidly increasing spore counts while the culture method indicated a relatively stable population. It is possible that the culture technique gave a true picture of the number of viable spores while the direct count represented the total number of viable and non-viable spores.

Similarly, the rapid development of hyphae on larch leaves was seen to occur by direct observation at a time when cultural methods did not show a rapid increase in numbers of fungi. Warcup (1960) has shown that such results are not unexpected because the majority of the fungal colonies counted in cultural methods are derived from spores. It is thus likely that the rapid increase in numbers observed with the culture technique for brown cotyledons and lower plumule leaves, which had fallen to the ground, was due to sporulation of the fungi.

Hyphal development was consistently more extensive on the lower surface of the leaf than on the upper surface. This pattern may reflect a difference in nutrients on the two sides or it may indicate that the lower surface was more protected from rain, drying or other environmental factors.

Germinating spores were observed on only one occasion on young leaves on which hyphae had not developed. Therefore, it is possible that fungal development was restricted by an inhibition of spore germination and/or a failure to produce hyphae.

Hyphal development on the leaf surface showed a direct relationship to leaf age. No hyphae developed on leaves less than two months old but then the mycelium developed rapidly. The relationship with age was apparent on the different aged leaves of each seedling and on leaves at a given position on seedlings through the growing season. This pattern of hyphal development indicates that leaf age or physiology and not climatic or other external factors limit the rate of fungal growth.

The patterns of development for yeasts, bacteria and filamentous fungi all indicate that the age of the leaf is the most important factor limiting the rate of development of the microflora on larch leaves. The sudden start of hyphal development and the sudden increase in bacterial numbers may reflect a rapid change in leaf condition. However, it is also possible that the change is not sudden but that a gradual change had passdd a threshold level below which development was impossible. For example, a germinating fungal spore may require a certain minimum concentration of available nutrients before it can successfully produce

hyphae. This minimum level may be reached by a slow change in leaf physiology, but when it is reached fungal development would begin rapidly.

Last and Deighton (1965) postulated that the change may be related to increased leaching from the ageing leaves or to the change to less hydrophobic conditions on the leaf surface. The hydrophobic nature of the leaf can be reduced by physical weathering (Hall and Jones, 1961). The ability of the majority of larch leaf colonizers to decompose lipids may suggest that microbial weathering of the leaf surface also occurs. Because leaf wax is extruded only by young leaves (Hall and Jones, 1961) physical and microbial weathering would be most damaging on old leaves where the surface wax would not be restored. Thus, the interaction of host physiology, microbial decomposition and physical weathering might lead to a sudden deterioration of the leaf surface.

Recently, Bailey (1969) has reported that phytoalexin production in Pisum leaves decreased as the leaves underwent senescence. He postulated that the decline of phytoalexin production may allow the development of an extensive microflora on the senescent leaf.

The "initial peak" pattern reported by several authors and described in the introduction of this chapter was observed for both bacteria and fungi in 1968 and 1969. It occurred on both cotyledons and lower plumule leaves, the only exception being fungal counts on cotyledons in 1968. Yeasts did not occur in the microflora at this time and do not show the pattern. The frequency of the occurrence of this pattern on

several plant species, in different years and in different locations would indicate that it is a phenomenon related to the age of the leaves and not to weather or airspora. The occurrence of high initial numbers on larch leaves might be related to the maximum amino acid quantity found in leachates of the very young seedling. However, while nutrients could explain the high numbers of bacteria, they could not account for the high numbers of fungi which were shown by direct observation to exist as dormant spores. Differences in surface wetting properties of the young leaf surface may be a factor. The rough surface topography of the wax structures on young leaves became flattened as the leaves were exposed to weathering and the leaf surface became more easily wetted as the leaf aged. The rough,hydrophobic surface of the very young leaf may provide a more protected site for microorganisms than the surface of somewhat older leaves.

The filamentous fungi isolated from larch leaves included the same important genera reported to occur on leaves of pea, rye and strawberry plants (Kerling, 1969; Dickinson, 1967). As has been pointed out by Last and Deighton (1965), they are the same genera of fungi which form the majority of the airspora. Moreover, the same genera have been reported as being dominant on tree bark (Garner, 1967). The similarity of fungal isolates from leaves to the airspora is not surprising because direct observation of the leaf surface indicated that the fungi were present mainly as spores. In addition, Warcup (1960) has reported that the majority of colonies isolated by the dilution plate method arise from spores.

Aureobasidium pullulans (deBary) Arn. was infrequently isolated from larch leaves whereas many studies have indicated that it is commonly an important leaf colonizer (see Last and Deighton, 1965). Without further studies it is impossible to determine if this difference was due to the nature of larch leaves, to the location of the study or to the techniques used in the study.

The majority of the hyphae on the leaf surface which could be identified by spore source belonged to the two genera, Cladosporium and Fusarium. However, an intensive study involving inoculation or immunofluorescence techniques would be required to establish the true composition of the mycelium on senescent leaves.

The bacterial flora of larch leaves does not appear to reflect bacterial composition of the airspora. Although the bacterial airspora has not been studied in great detail, what studies have been done indicate that it is usually dominated by Gram-positive rods, Bacillaceae and Micrococcaceae. Moreover, Gram-negative rods such as Flavobacterium, Achromobacter and Pseudomonas constitute less than 5% of the bacteria in the airspora (Flemming, 1908; Zobell, 1942; Pady and Kelly, 1953). Pady and Kelly (1953) postulated that the airspora is composed mainly of soil forms carried into the air by wind. Thus, the phyllosphere is dominated by those groups of bacteria which are scarce in the airspora. Indeed, phyllosphere bacteria most resemble rhizosphere bacteria. This similarity has been demonstrated for the bacteria in the rhizosphere and phyllosphere of pasture plants by Stout (1960a and b). The dominant genera on larch leaves, Pseudomonas, Flavobacterium and Xanthomonas, are

the same genera reported to occur more frequently in the rhizosphere than in plant-free soil (Holding, 1960; Rouatt and Katznelson, 1961). Thus the bacterial flora of the phyllosphere appears to differ from the airspora surrounding it in a manner similar to the manner in which the rhizosphere flora differs from the soil flora.

The dominance of Gram-negative rods near plants may be a result of growth stimulation in response to organic forms of nitrogen present in plant exudates. Such a response has been shown for rhizosphere bacteria by Holding (1960).

The bacterial flora of larch leaves contained a high percentage of chromogenic bacteria. This characteristic of phyllosphere floras has been reported for many plant species (Last and Deighton, 1965; Leben, 1965). The occurrence of pigmented forms in the larch phyllosphere was not restricted to bacteria but was also true for yeasts and fungal spores. The latter two groups contained a high proportion of red and dark brown pigments respectively. Any speculation that pigmentation has a survival value related to light exposure would appear to be unjustified because pigmented forms are also found in high numbers in the rhizosphere.

The yeasts isolated from the larch phyllosphere belong to the genera Cryptococcus, Torulopsis, Rhodotorula and Sporobolomyces and are similar to the yeasts which dominate the phyllosphere of other plant species (Last and Price, 1969). The change in dominance from Torulopsis spp. to Cryptococcus spp. observed in the late summer of 1968 may be related to temperature optima for the two genera since di Menna (1959) has also reported that Cryptococcus spp. increased during the winter when other yeasts declined in number.

A high proportion of the bacteria and yeasts isolated from the larch phyllosphere were able to produce lipase. Ruinen (1963) reported a similar finding regarding yeasts isolated from leaves in the tropics. She suggested that the lipolytic activity of phyllosphere organisms may result in the breakdown of the leaf cuticle. The fact that leaf yeasts are usually restricted to leaves and do not occur in abundance in the soil (di Menna, 1959, 1962) prompts the speculation that the leaf provides a unique habitat for these organisms. The widespread occurrence of lipase in both bacteria and yeasts of the phyllosphere may indicate that leaf waxes or cutin provide a nutrient source available to these organisms.

CHAPTER III STUDIES ON THE ESTABLISHMENT AND DEVELOPMENT OF THE LARCH PHYLLOSPHERE MICROFLORA

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INTRODUCTION

In order to assess the relative importance of a number of factors which may have coincident trends it is necessary to isolate the factors for individual study. Düggeli (1904) was the first researcher to use such an approach in connection with leaf microfloras. By isolating emerging seedlings from the airspora, he was able to establish that bacterial populations on plants increased by multiplication and not solely by additions from the airspora. More recently, in studies by Khudyakov (1961), Mundt et al (1962), Woznyakovskaya (1962), Leben (1961, 1963, 1964 and 1965), Leben and Daft (1966), and Vasantharajan and Bhat (1968), isolator or aseptic seedling techniques have been used to establish the capacity of several species of bacteria and yeasts to migrate and multiply on leaf surfaces in humid conditions.

A series of experiments using aseptically grown larch seedlings was undertaken to investigate several aspects of larch phyllosphere ecology.

Firstly, experiments were designed to exclude soil and air microfloras as sources of inoculum, in order to establish if certain organisms could migrate over the leaf surface of larch seedlings.

Secondly, experiments were designed to test the hypotheses formulated from observations of natural larch leaf microfloras, that the age of larch leaves was the most important factor determining the size of the leaf microflora. Thus,attempts were made to separate leaf age from other environmental influences such as seasonal weather trends, length of exposure to inoculum, and weathering of the leaf surface.

In addition, leaves which had been protected from physical weathering were examined to determine if elements of the microflora brought about changes in the leaf surface.

ASEPTIC SEEDLING CULTURE

Because of their slow growth rate and compact form, it was practicable to grow larch seedlings in totally aseptic conditions rather than partially aseptic conditions such as attained by Leben (1961) when growing cucumber plants in plastic film isolators. The method developed for conifer mycorrhizal studies by Marx and Zak (1965) was followed. The seedlings were grown in one litre, wide-mouth Erlenmyer flasks fitted with cotton wool plugs and containing a mixture of 400 ml of "Verifloo" vermiculite and 50 ml of fine peat (Plate III-1). To this mixture was added 300 ml of a nutrient solution (Olson, 1944) which contained:

0.40 g ammonium dihydrogen orthophosphate,

0.40 g potassium nitrate,

0.45 g calcium nitrate hydrated,

0.35 g magnesium sulphate,

0.30 g ammonium nitrate,

0.03 g ferric citrate,

0.003 g boric acid,

0.002 g manganous chloride,

0.0002 g zinc sulphate

per litre of tap water.

After autoclaving at 120°C for 45 minutes this rooting medium had a stable pH of 4.5.

PLATE III-1 ASEPTIC SEEDLING CULTURE.

One litre flasks containing larch seedlings in vermiculite-peat mixture.

Seeds were surface sterilized by immersing them in 30% hydrogen peroxide for 60 minutes (Trappe, 1961). Immersion periods of less than 30 minutes resulted in poor sterilization, while periods over 90 minutes caused damage to the embryo.

The surface sterilized seeds were pressed into the surface of Czapek-Dox agar plates and incubated at 22.5°C. The seeds germinated between four and ten days after the sterilizing treatment. Germinating seeds were examined through a x 40 stereo microscope for contaminants. Seeds with no visible contaminants were planted using flamed forceps in the sterilized flasks.

All procedures involved in aseptic seedling culture were carried out in glove boxes. Periodically during the growth of the seedlings, samples of the seedling and rooting medium were planted onto Czapek-Dox agar to test for contamination. In practice approximately 20% of the flasks became contaminated during the course of experiments. All contaminated flasks were discarded.

Aseptic seedlings were grown in a controlled environment room under approximately 1500 foot-candles of light, with 16°C, 18 hour days and 10.5°C, 6 hour nights. The temperatures were derived from the mean daily maximum and minimum temperatures recorded for July and August in the seedbed used in the 1968 microflora study. The flasks were positioned in the controlled environment room according to random numbers. The positions were re-randomized every ten days to minimize variation due to bench effect.

INOCULATION OF ASEPTIC SEEDLINGS

Suspensions of test organisms were prepared by removing cells from agar plates with a wire loop and suspending them in water. After shaking, the suspensions were centrifuged (3000 RCF), the liquid decanted, and the cells resuspended in water. The washing procedure was repeated before the final water suspension was prepared.

In microflora migration experiments the seedcoat of the germinating seed was immersed in the suspension before planting.

In experiments concerned with the size of microflora populations on different aged leaves, the inoculum was not applied until the seedlings were several months old. It was, therefore, necessary to develop an inoculation technique which would distribute the inoculum evenly over the seedling. "Spore fall" techniques were unsatisfactory because the top of the seedlings stopped the inoculum from reaching the bottom leaves. Attempts to apply the inoculum by spraying water suspensions were unsuccessful because of the hydrophobic leaf surface. Therefore, trials with surface wetting agents were carried out. Teepol was found to be damaging to the seedlings, the young leaves of which turned brown 4-5 days after the application of a 1% Teepol solution. Tween 80 was used by Dickinson (1967) to improve the efficiency of washing techniques for removing leaf microfloras. Applications of 1% Tween 80 solutions to seedlings resulted in no visible damage. To assess the effect of Tween 80 on the test organisms, these were streaked onto SEYE agar plates containing 10%, 1%. 0.1% and 0% Tween 80. No difference in growth rate or form was observed with any of the organisms on the different media. Therefore, an inoculation technique was developed utilizing Tween 80 as a surface wetting agent.

After the centrifuge washing described above, the final cell suspension was made in water containing 0.1% (v:v) Tween 80. This suspension was then sprayed onto the seedlings with a fine misting nozzle. The fine mist was produced by a jet of air from a small compressor with sterile cotton wool filters attached to the intake and outlet hoses. The nozzle was thrust through the cotton plug of the aseptic seedling flask and the seedling was sprayed until all leaves were dripping (approximately 12 seconds). The entire procedure was carried out in a glove box. For the microflora migration studies only a qualitative assessment of the presence or absence of microorganisms was required. Leaves were removed from the seedlings with flamed forceps and plated directly onto agar plates. Records were made of the types of organisms growing out from the leaves.

In experiments requiring a quantitative assessment of the microflora, a small sample consisting of two or three leaves was removed from the seedling with flamed forceps and placed in one-ounce universal vials. After the addition of 10 mlof a 0.1% yeast extract solution the vials were vigorously shaken for 5 minutes on a wrist action shaker. The resulting suspension was then serially diluted and added to SEYE agar plates for culturing and counting (as described in Chapter II).

The surface area of the leaves in each sample was calculated from length and width measurements and microorganism numbers were calculated per cm^2.

MICROFLORA MIGRATION STUDIES

An experiment was designed to determine if different elements of the larch leaf microflora could migrate from the seedcoat to (1) other parts of the growing seedling, (2) the rooting medium, and (3) other seedlings growing in the same flask.

Three seeds which had been treated with different microflora suspensions were planted in each flask in the pattern of an equilateral triangle. A fourth seed was planted equidistant from the other three seeds (i.e. the centre of the flask).

The test organisms were isolated from larch leaves during the 1968 microflora study. They included isolates of Torulopsis sp., Cryptococcus sp., Sporobolomyces roseus, Bacillus mycoides, Pseudomonas sp. (S-100) and a yellow, motile, gram-negative rod (S-109). The organisms were chosen to represent a wide range of microorganism types and because each type could be readily distinguished in culture from all the others. Thus, S.roseus and S-109 could be distinguished by their pigmentation. B.mycoides was distinguished by its rhizoid colony form. S-100 could be differentiated from the yeasts by a microscope check of cell size. The Cryptococcus sp. colonies were mucoid and could be distinguished by that character from Torulopsis sp. This distinction was, however, always checked by testing for starch formation, a characteristic of Cryptococcus, on starch-free agar (Lodder and van Rij, 1952).

The microflora treated seeds plus control seeds (no microflora) were replicated in such a way that all possible combinations occurred

at least once in the experiment. Thus, each organism type was presented with the opportunity to migrate to a seedling inoculated with the other types of organisms or to a control. The treatment combinations are summarized in Table III-1.

TABLE III-1. The pattern of treatment planting

Points of triangle			Centre of flask
1	2	3	4
C	S	T	Nil
S-100	B	S-109	Nil
C	S	T	S-109
C	S	T	S-100
C	S	T	B

C = Cryptococcus sp., T = Torulopsis sp., S = S. roseus,
B = B. mycoides, nil = control

The seedlings were grown in the controlled environment chamber for 50 days by which time the average seedling height was approximately 5 cm. There was no overlapping of one seedlings' leaves with other seedlings. Condensation was always present on the inside of the flasks indicating a water saturated atmosphere around the seedlings.

Three types of foliage were sampled from each seedling (1) cotyledons, (2) leaves from the bottom centimeter of the plumule, (3) leaves from the top centimeter of the plumule. A sample of the rooting medium was also taken.

A number of the flasks had become contaminated and were not assessed in the results.

There was no instance of an organism having migrated from the inoculated seedling to any other seedling in the flask. However, in every case the inoculated organisms were found to be present in the rooting medium.

The results of the foliage assessment wre presented in Table III-2.

TABLE III-2. The percentage occurrence of microorganisms on the three leaf types (100% = 11 leaves).

	Crypto-coccus	Torulopsis	S.roseus	B.mycoides	S-100	S-109
cotyledons	0	73	73	64	18	64
lower plumule	0	100	73	0	0	64
upper plumule	0	36	0	0	0	18

A range of success in colonizing the seedling leaves was apparent. Cryptococcus sp. was apparently unable to maintain itself on the seedlings although it was present in the rooting medium. Torulopsis sp. was the most successful colonizer of leaves of all ages.

There appeared to be no relationship between the motile habit and success in colonizing the leaves. This may indicate that colonization occurs by cell multiplication at the growing tip of the seedling as suggested for yeasts by Voznyakovskaya (1962).

The most common contaminants of the flasks were sporing fungi. When a flask was contaminated, the contaminant was invariably isolated from all foliage samples taken from the flask. Thus, it seems likely that air currents carried the spores to all parts of the flask. It is interesting in this regard that S.roseus, which was shown to forcibly discharge spores in culture, was not apparently able to colonize upper plumule leaves or other seedlings in the same flask. It is possible that conditions in the flask were not suitable for spore formation or discharge.

The colonization did not appear to be uniform over the length of the leaves and frequently colonies developed only from the tips of the leaves. However, direct microscopic examination of the leaf surface would be required to determine if there was a definite pattern of colonization.

A number of untreated aseptic seedlings were grown alongside the experimental plants. There did not appear to be any differences in leaf form or colour between treated and untreated seedlings.

RELATIONSHIP OF LEAF AGE AND MICROFLORA SIZE

The interpretation of the data concerning microflora populations collected during the field studies of 1968 and 1969 (Chapter II) led to the hypothesis that the age of the leaf was important in determining the size of the leaf microflora. Under natural conditions the age of a leaf is combined with such factors as duration of exposure to inoculum, weathering of the leaf surface, and seasonal changes in weather. By growing seedlings in aseptic conditions in cotton plugged flasks, the influence of the single factor, leaf age, could be examined for its effect on an artificially applied microflora.

An experiment was carried out on 5-month-old aseptic seedlings in an attempt to determine if different aged leaves supported different sized populations of yeasts and bacteria. Using the techniques described previously, the following treatments were applied:

i. S.roseus in 1% Tween 80,

ii. S-100 and S-109 in Tween 80,

iii. a mixture of i and ii.

Each treatment was replicated in three flasks each of which contained three seedlings.

Eight hours after the inoculum had been applied, the leaf surfaces appeared dry and the first assessment of microflora populations was made. Two areas of foliage were sampled: the bottom one-centimeter and the top one-centimeter of the plumule designated at the time of inoculation. Further assessments were made 6, 13 and 27 days after inoculation. The results of these assessments are summarized in Figures III-1, III-2 and III-3.

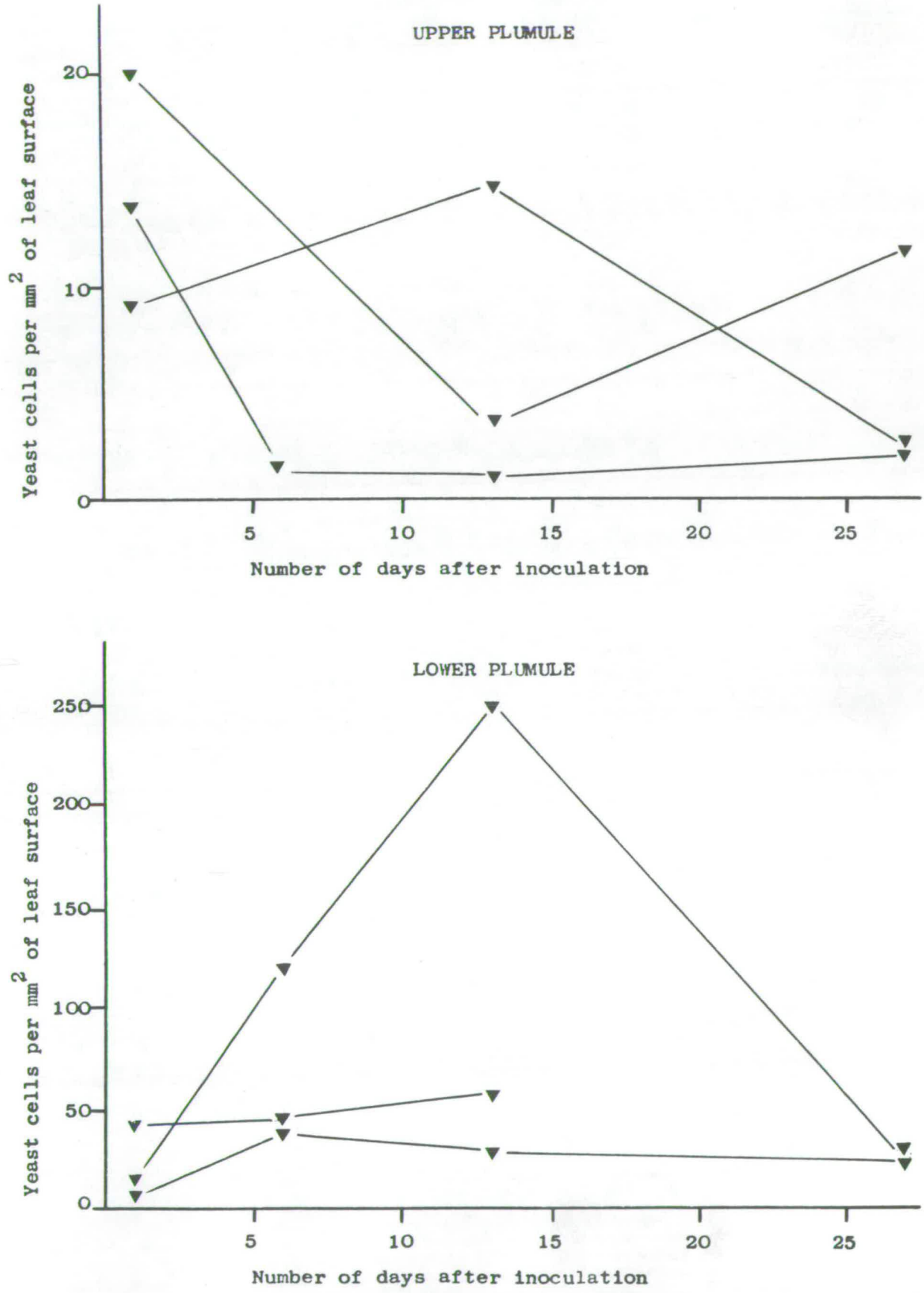


Figure III-1 Populations of S. roseus on leaves of aseptically-grown seedlings. Estimates for three replicates are given except when treatments were contaminated.

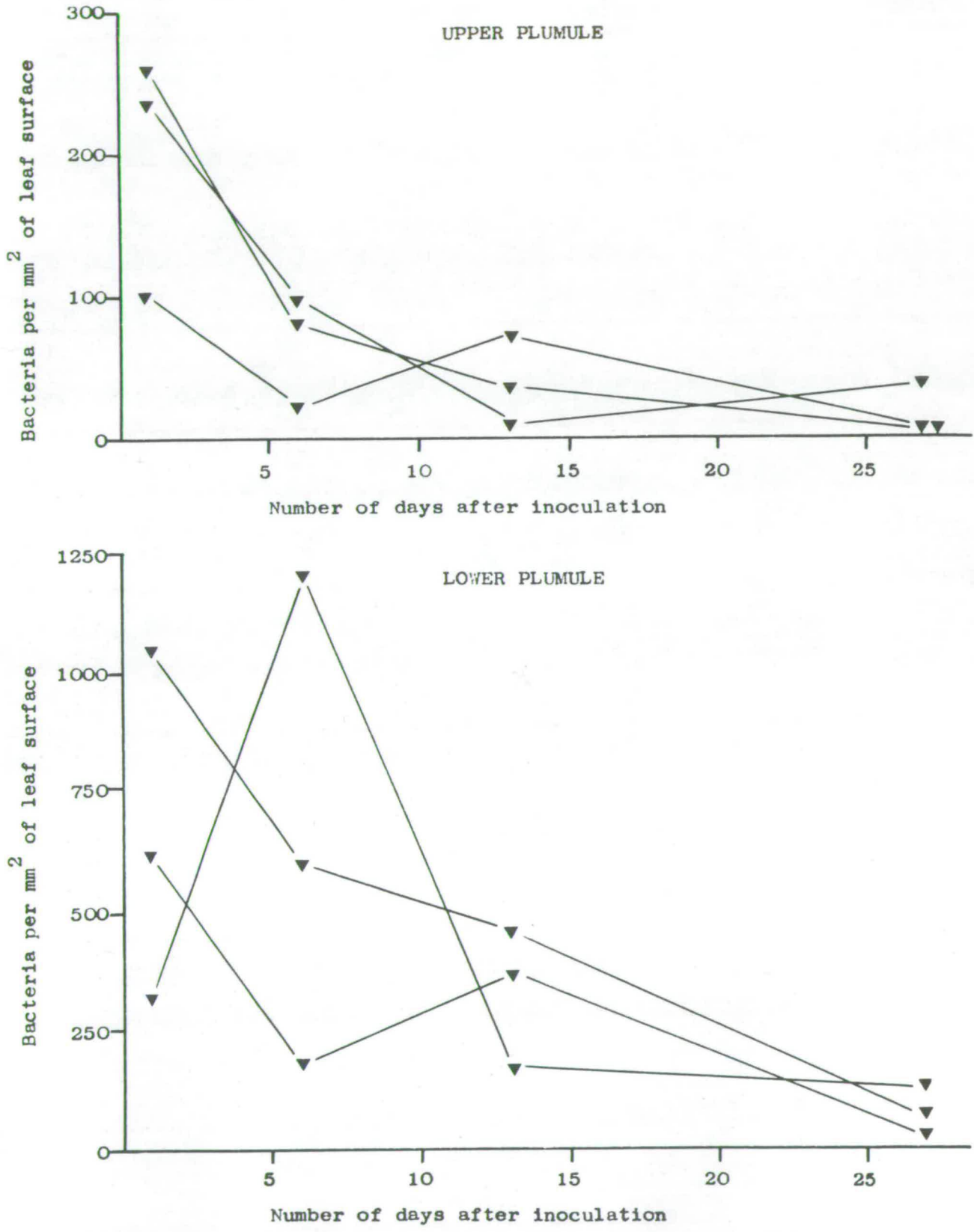


Figure III-2 Populations of bacteria on leaves of aseptically-grown seedlings. Estimates for three replicates are given.

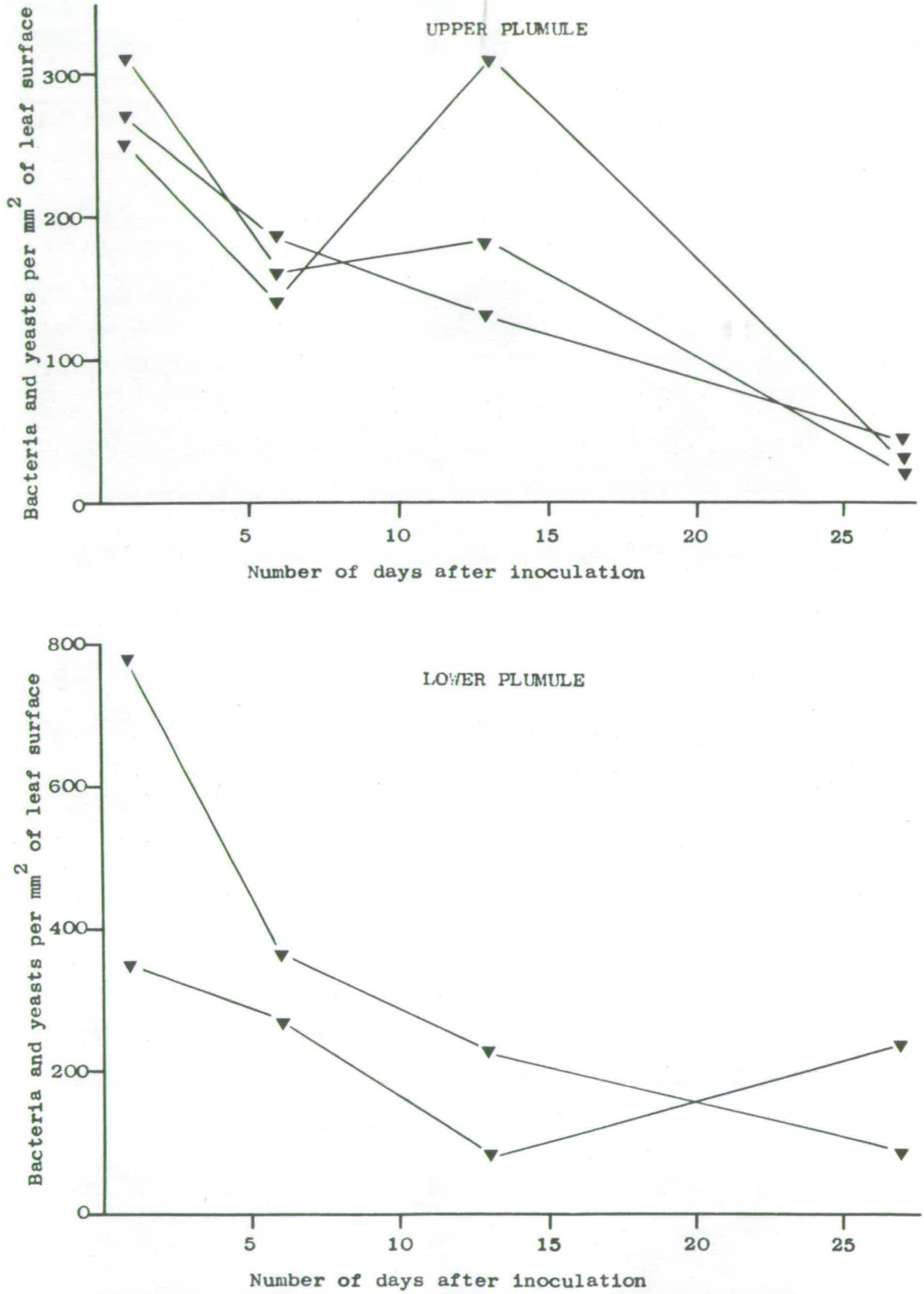


Figure III-3 Populations of S. roseus and bacteria on leaves of aseptically-grown seedlings. Estimates for three replicates are given except when treatments were contaminated.

The initial assessment revealed that the bacterial populations were two to three times greater on lower plumule leaves than on upper plumule leaves. Initial yeast populations were similar on the two ages of foliage. The initial populations showed a relatively small variation between replicates. With the exception of the S.roseus treatment in which numbers were very low, the ratio of the maximum and minimum values was never more than 3.5.

It is not known if the differences observed in the initial populations was a result of the inoculation technique or of the properties of the leaves. In the successive population assessments there was a general trend of decreasing numbers with increasing time. The change in the populations in the three treatments can be compared using the ratio of the final population over the initial population. These ratios, calculated from the mean of the replicates, are presented in Table III-3.

TABLE III-3. The ratio of average final population to average initial population.

	Lower plumule	Upper plumule
S. roseus	1.3	.28
S-100 and S-109	0.09	.02
S. roseus, S-100 and S-109	0.33	.13

Two patterns emerge from a comparison of the ratios. One, the ratio was always smaller on the upper plumule than on the lower plumule. Two, the yeast treatment had the largest ratio of any treatment for both upper and lower plumules. Thus, it could be concluded that mortality was more severe in the upper plumule than in the lower plumule and that a higher percentage of yeast cells survive than do bacterial cells. Indeed, yeast populations on the lower plumule showed a slight average increase with time. However, it is not a valid comparison between yeast and bacteria because their initial populations were very different.

The mixture of bacteria and S.roseus resulted in population levels intermediate between the yeast treatment and the bacterial treatment. However, no breakdown of the population numbers into numbers of bacteria and numbers of yeasts was possible because yeasts accounted for less than 10% of the colonies developing on dilution plates. The results therefore, were unreliable due to the low numbers of yeast colonies.

Under these conditions of aseptic seedling culture, the artificially inoculated leaf microfloras were unable to increase in number. Leben and Daft (1967) have concluded that free surface water is required to permit the formation of significant bacterial populations. Thus, although the seedlings were in a saturated environment, the lack of free surface water may have restricted microflora development. Nevertheless, survival of bacteria and yeasts varied with leaf age. The higher percentage survival on

old leaves may indicate that they provided a more amenable environment than the young leaves. This difference must be concerned with the ageing process in the leaf because external ageing factors such as weathering had been excluded.

There did not appear to be any differences in size and condition between seedlings in different treatments.

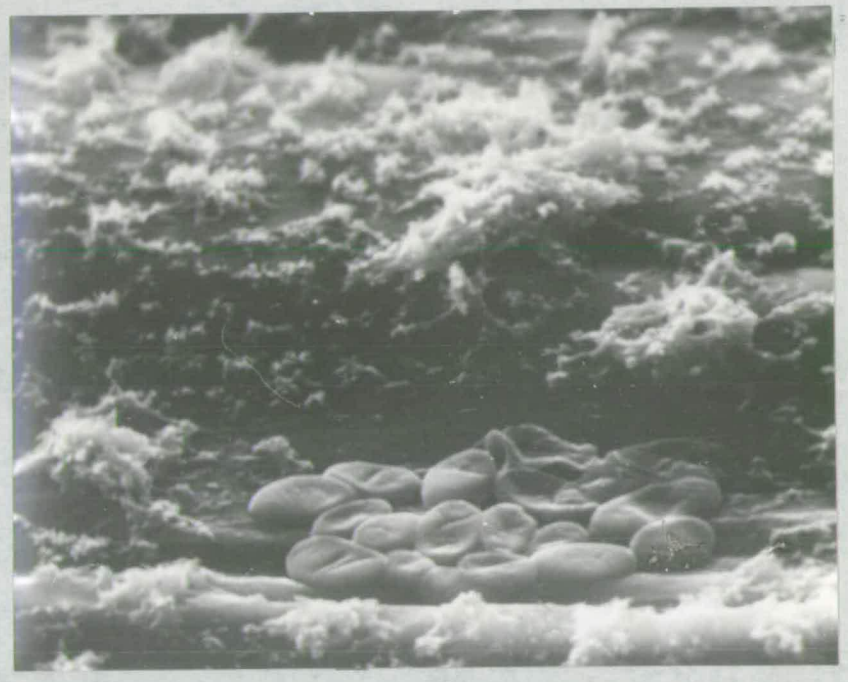
EXAMINATION OF SURFACE WAXES OF LEAVES INOCULATED WITH S.ROSEUS

Samples of leaves from the S.roseus treatment of the previous experiment were examined with the stereoscan electron microscope five weeks after inoculation.

The examination resulted in several interesting observations. The S.roseus cells occurred in widely spaced colonies over the leaf surface. The existence of colonies would indicate active multiplication of S.roseus on the leaf surface (Plate III-2). In addition, a single S.roseus cell was observed with what appeared to be a mature ballistospore (Plate III-4). Again, this observation would support the conclusion that active yeast multiplication occurs on the leaf surface. In addition to this evidence of yeast activity, the area of the leaf surface immediately surrounding yeast cells was devoid of the coralloid wax structures (see page 25) which uniformly covered all other areas of the leaf surface (Plates III-2, III-3, III-4 and III-5). This "wax-free" area extended up to 5 μ beyond the yeast cells and consistently occurred around all yeast cells observed. The leaf surface was closely examined to determine if clear areas existed without yeast cells, but none was found. Thus, it seems probable that the wax structures were destroyed and perhaps utilized by the yeast cells. The portion of the cuticle exposed by the "wax-free" area appeared smooth and there was no indication that its structure had been altered.

PLATE III-2 *S. ROSEUS* COLONY ON A LARCH LEAF. X 3000
Note the wax-free zone around the colony.

PLATE III-3 *S. ROSEUS* CELLS ON THE LARCH LEAF SURFACE. X 3200



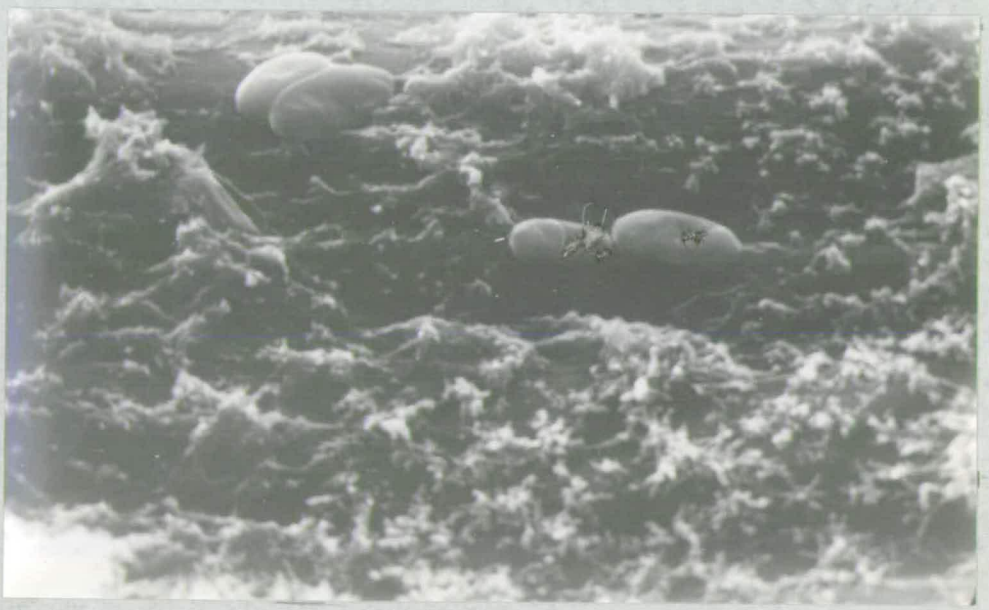


PLATE III-4 CELL OF *S. ROSEUS* PRODUCING A BALLISTOSPORE ON THE LARCH LEAF SURFACE. X 15,000
The area immediately surrounding the cells is devoid of surface wax structures.

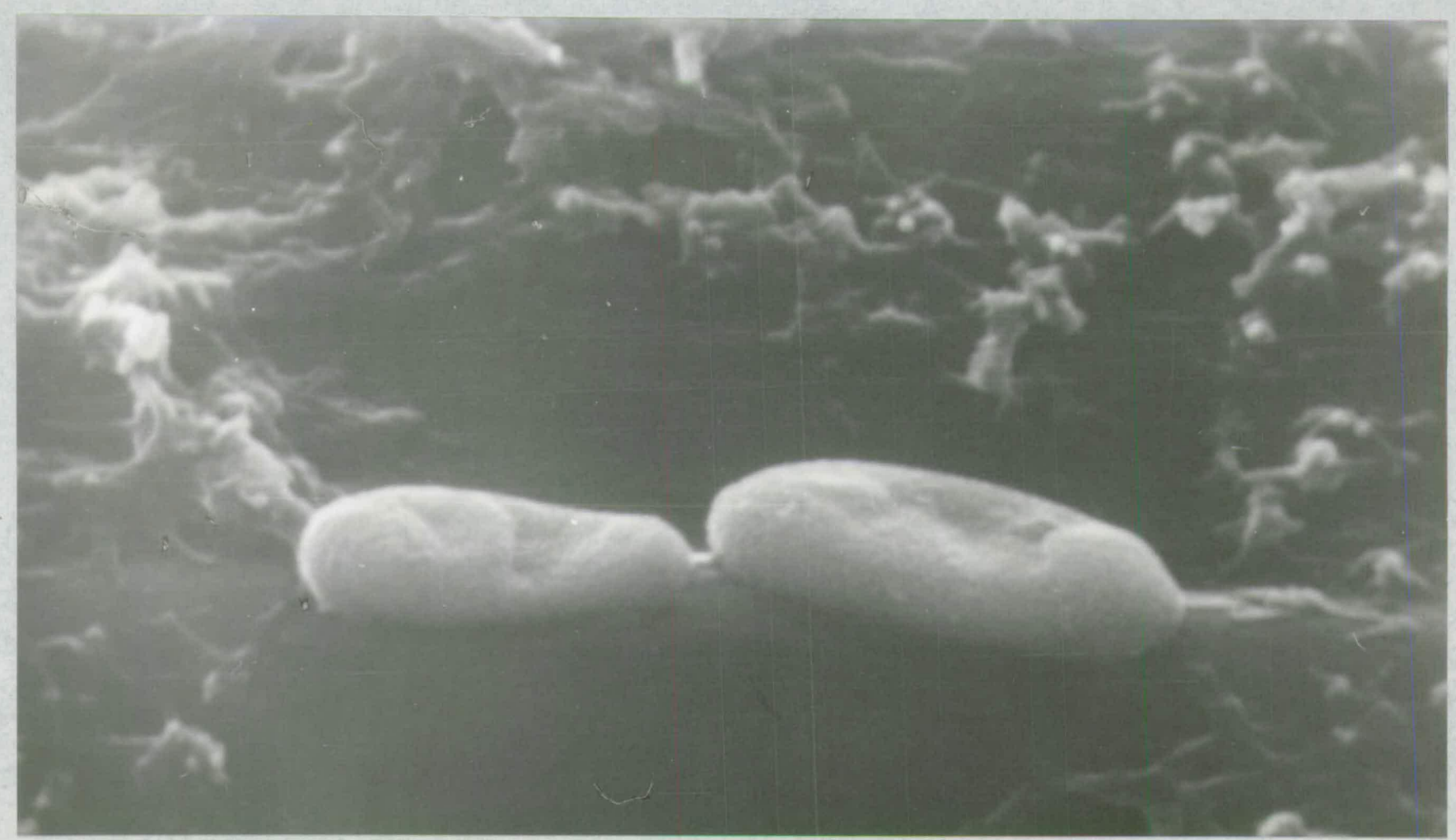
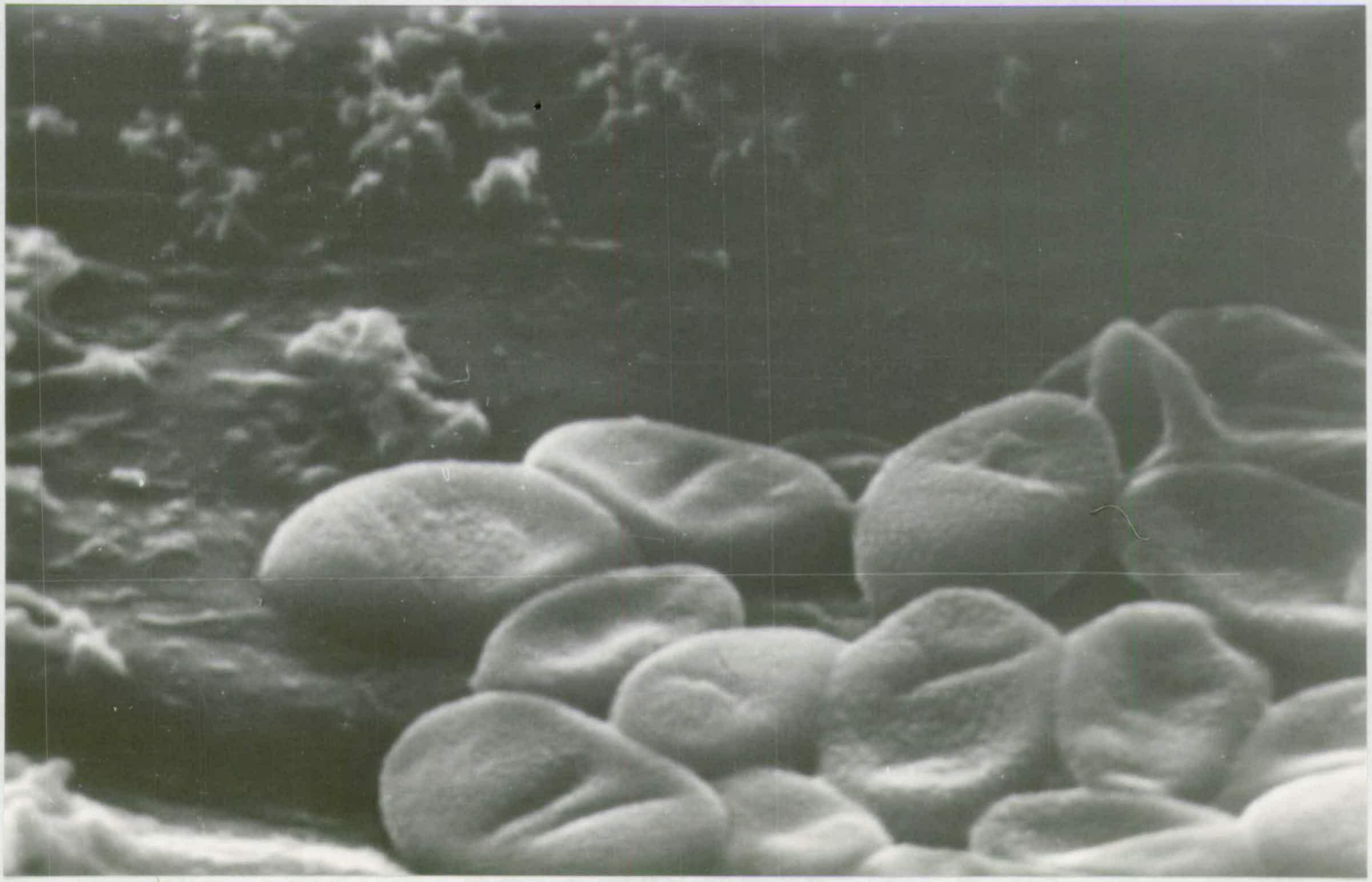


PLATE III-5 SMOOTH "WAX-FREE" ZONE AROUND THE MARGIN OF AN *S. ROSEUS* COLONY ON A LARCH LEAF. X 12,000



DISCUSSION

The experimental approach of isolating certain phyllosphere factors for close examination has provided some additional information which may contribute to an understanding of phyllosphere ecology.

The experiments demonstrated the ability of leaf yeasts to colonize the growing larch seedling when the sole source of inoculum was the seedcoat. It was also shown that this form of colonization was not restricted to motile bacteria, but was also possible for leaf yeasts. The seedcoat has been shown to be a possible source of the phyllosphere microflora by Burri (1903), Düggeli (1904), Voznyakovskaya and Khudyakov (1960) and Leben (1961). Although the seedcoat is one possible source of inoculum, the soil (Leben, 1964) and the air (Last and Deighton, 1965) also contain at least some cells of most phyllosphere species, even if these species do not predominate in those habitats. Thus, the basic question of why the microflora species composition differs from the air and soil microflora remains unanswered. The continued examination of species to categorize them as leaf residents or casuals could lead to an understanding of the physiological nature of leaf residents. The failure of Cryptococcus sp. to survive on the young larch seedlings compared to the extensive colonization by Torulopsis sp. coincides with the field data which indicated Torulopsis sp. was the most abundant of the two genera on young seedlings in the field.

Di Menna (1962) concluded that competition for nutrients was the most important factor determining the species composition of yeast

populations in the phyllosphere. In this regard, the high percentage of larch leaf isolates which were shown to produce lipase and the destruction of leaf waxes *in situ* by *S.roseus* may indicate a very important adaptation to the specialized habitat of the phyllosphere. Moreover, Ruinen (1966) has demonstrated that some leaf yeasts may produce cutinase. Thus, the ability to utilize the leaf surface structures may be an important physiological characteristic of phyllosphere residents. However, as some common soil organisms are also able to utilize plant waxes, it is obvious that other characteristics are important in determining the species composition of leaf microflora.

Under the conditions of aseptic seedling culture the old seedling leaves apparently were a more suitable substrate for microorganisms than young leaves. Thus, it would be important to the understanding of leaf microflora population development to know more about the physiological changes associated with ageing leaves. The succession of microorganisms on leaves may be a result of autonomous host changes, but in addition the alteration of the substrate by microorganisms may also be important. The latter could be a very important factor if the destruction of leaf waxes associated with *S.roseus* occurred in the field. Damage to the leaf surface could result in increased transpiration (Hall and Jones, 1961) and leaching of nutrients (Tukey and Morgan, 1963) which in turn could lead to a succession of microorganisms.

The pattern of microflora development is most probably the result of many factors working in combination. However, more experiments designed to examine in isolation the different factors of the leaf environment must be carried out if the role of the leaf microflora in disease, nutrition and ageing is to be understood and if the microflora is to be controlled to enhance or suppress these roles.

CHAPTER IV

MICROORGANISM INTERACTIONS IN THE LARCH PHYLLOSPHERE

CHAPTER IV MICROORGANISM INTERACTIONS IN THE LARCH PHYLLOSPHERE

INTRODUCTION

One of the main objectives in the present study of the larch leaf phyllosphere was to investigate interactional phenomena so as to attempt understanding of the possible role of epiphyllic organisms in microbiological control of leaf pathogens. A pathogen spore arriving at the leaf surface becomes part of the chemical and microbiological complex of the phyllosphere. If it is to germinate and colonize the leaf, it must compete successfully with other leaf microorganisms for its requirements, e.g. nutrients, water, and space. While a great deal of effort has been directed towards controlling pathogens by making the leaf chemically toxic, scant attention has been given to the possible role of microorganism interactions in controlling leaf pathogens.

The research into microbiological control of plant disease has been reviewed by Wood and Tveit (1955) and Krstic (1956). Leben (1965) and Sinha (1965) have reviewed recent work concerning microbiological control of leaf pathogens. It is, therefore, not the purpose of this introduction to review the literature but rather to illustrate the different approaches which have been used to control leaf pathogens by microbiological methods.

Attempts to enhance the "protective" role of saprophytes on leaves using crude mixtures of inoculum and nutrients have been carried out by Davuidov (1951) and Bier (1965). Davuidov was able to reduce infection of powdery mildews by spraying plants with a manure infusion which contained lytic bacteria. Bier used a more natural source of inoculum when he applied aqueous suspensions of homogenized leaves to control Melampsora occidentalis Jacks infection of poplar leaves. Both authors concluded that the resulting reduction in disease was due to the increased numbers of non-pathogens on the leaves.

Mixtures of selected microorganisms have been used by Bamberg (1930), Wood (1951) and Newhook (1951a,b) to control leaf pathogens. Bamberg 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 (1930) and Johnson (1931) showed that some of the bacteria were antagonistic to Ustilago zeae (Beckm.)Ung. in artificial culture. When applied to the corn plants the antagonistic bacteria 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 also demonstrated that cell free filtrates of the bacterial cultures were ineffective in controlling the disease.

Wood (1951) and Newhook (1951a,b) studied the antagonism of some soil microorganisms towards 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 depression 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 saprophytes 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.

More recently, Leben and Daft (1965), Leben et al. (1965) and McBride (1969) have used specific bacteria isolated from the host phyllosphere and have applied them in a defined nutrient suspension to control leaf pathogens. Leben and his coworkers isolated a gram-negative short rod bacterium from cucumber leaves and found that suspensions of the bacterium reduced the development of cucumber anthracnose lesions (Colletotrichum lagenarium (Pass.) Ell and Halst.). Control of the disease was improved by applying the bacterium in a nutrient suspension and in moist conditions. Later, it was found that the bacterium was ineffective in field tests because it failed to remain viable on the leaf under field conditions.

McBride (1969) isolated three species of Bacillus from leaves of Douglas fir and was able to reduce Melampsora medusae Thüm. infection by applying the bacteria in nutrient suspensions to Douglas fir leaves. The bacteria were ineffective in controlling the

pathogen when applied in water suspensions. The application of a mixed suspension of the three species resulted in better control of the pathogen than any of the species applied singly.

In the present study a series of experiments was carried out to examine the effect of phyllosphere nutrients and microorganisms on the development of two larch leaf fungi, M.laricis and C.herbarum.

M.laricis was selected for study because it is the pathogen causing leaf cast disease of young larch leaves. Peace and Holmes (1933) and later Biggs (1964) have previously examined some aspects of M.laricis spore germination and infection. Only the imperfect stage of the fungus is known and it spreads and infects by means of conidiospores. The spores infect by germ tubes which enter the leaf through stomata. The mycelium is restricted to the leaf where clusters of conidiophores are formed. The presence of the distinctive two-celled conidia projecting through the stomata can be readily observed under the microscope and can be taken as proof of M.laricis infection.

M.laricis can infect several species of larch but L.decidua is the most susceptible host. Only leaves aged up to four weeks old are infected.

A period of at least forty-eight hours during which relative humidity is greater than 95% is required for infection. The diseased leaves turn brown approximately two to three weeks after infection.

C.herbarum was selected for study because it appeared to occupy a distinctly different niche in the larch phyllosphere than did M.laricis. C.herbarum was a common fungus observed on senescent leaves of larch seedlings during the 1968 microflora study. It is apparently ubiquitous on plant foliage and has been isolated from plants as diverse as pea (Dickinson, 1967), rye (Kerling, 1964) and heather (Peace, 1962). It is considered a common saprophyte on dead plant material (Last and Deighton, 1965) and has been reported as occurring in the first stages of conifer litter decomposition (Ward, 1952). Although normally saprophytic it can become parasitic on plants weakened by other factors (Gäumann,1950). Campana and Rosinski (1962) cited reports of its pathogenicity on several conifers including species of Picea and Pinus in Russia, Pinus rigida Mill. in German, Pinus strobus L. in Yugoslavia, and Thuja orientalis L. in China.

SPORE GERMINATION EXPERIMENTS

The examination of phyllosphere environmental factors which might influence fungal development *in vitro* was directed mainly towards their effect on spore germination. The emphasis was placed on spore germination for a number of reasons. These reasons include the following:

(1) field observations indicated that the majority of spores on larch leaves remained ungerminated for long periods;

(2) *M. laricis* does not colonize the leaf surface but enters the leaf through stomata soon after germination (Biggs, 1964);

(3) many factors can be controlled and studied in a short time in spore germination experiments.

A multitude of nutritional requirements for spore germination exists among the fungi, varying from complete independence to complete dependence on the environment (Sussman and Halvorson, 1966). As has been discussed previously (page 19), a variety of leaf chemicals may influence spore germination. Thus, a number of experiments were undertaken to assess the effect of sugars, amino acids and natural leaf leachates on the germination of *C.herbarum* and *M. laricis* spores. In addition, a number of phyllosphere microorganisms were tested for interactions with spore germination.

In order to be able to concentrate effort on the nutrient and microorganism factors, only one isolate of each fungus was studied. Spore density was kept constant throughout the study by adjusting spore suspensions to a given opacity. In a similar manner, the density of microorganism suspensions was kept constant in successive experiments.

GENERAL METHODS

The *in vitro* spore germination experiments were carried out in Van Tieghem cells. It was felt that the Van Tieghem cell (water droplet) technique more closely paralleled the natural germination conditions of spores on leaves than did other standard methods such as germination on agar films. The cells were made up of a glass ring 12 mm inside diameter by 3 mm high attached to a microscope slide and covered with a cover slip. The cells were sealed by a vaseline film on the edges of the glass ring.

Two methods of preparing the cells were compared. Firstly, the spore suspension was added as a hanging drop on the cover slip. Secondly, the spore suspension was placed at the base of the cell on the microscope slide. There appeared to be no difference in germination of *M. laricis* and *C. herbarum* spores between these two methods (Table IV-1) and the latter method was adopted for future experiments because of the relative ease of setting up the cells.

TABLE IV-1: Comparison of germination in Van Tieghem Cells with hanging drops or basal drops.

	Hanging drop	Basal drop
C. herbarum	92%	95%
	90%	90%
	90%	94%
M. laricis	32%	34%
	38%	32%
	30%	34%

This method also overcame the problem reported by Peace and Holmes (1933) of irregularly shaped hanging drops influencing the germination of M.laricis spores.

O.1 ml. of germinating medium was placed in each cell. This volume gave a high surface: volume ratio and thus reduced the risk of low oxygen potentials developing in the medium.

The cells were placed over water in vaseline sealed staining pots to reduce any loss by evaporation and incubated @ 20±1 C.

The criterion for spore germination was the development of a germ tube at least as long as the spore was wide. This criterion avoids inaccuracies due to germ tubes being hidden from view and thus not being counted. Counts were made by positioning the cell according to random numbers describing the position of the stage micrometers. The first 50 spores encountered moving clockwise from the top of the field were taken as comprising one observation. The cell would then be moved and a second count taken.(6 counts/cell) .

The time of observation was determined in the first experiment as the time after which no marked change in percentage germination occurred. Random checks in all treatments of all experiments were made after this arbitrary time interval, to establish if any change had occurred.

The spore germination experiments were designed as Randomized Blocks with a number of observations in each experimental unit. The basic experimental unit was a Van Tieghem cell and each treatment was replicated once in each block (staining pot). In this design the Analysis of Variance assesses variation from the following sources:

i. Between observations in the same experimental unit (Van Tieghem cell),

ii. between blocks (staining pots),

iii. between treatments,

iv. residual.

In practice, no significant variation was found in the first two categories and the variation was pooled and analysed as in a Completely Randomized Design (Cochran and Cox, 1957).

The statistical procedures used were those of Snedecor and Cochran (1967). In general, standard analysis of variance was used to assess the amount and source of variation. In the case of data in percentages the arcsin (angular) transformation was applied. All tests of difference between treatments were then carried out on transformed means which were not retranslated to per cents. The mean of untransformed data was always used in the figures and the text.

Analysis of variance tables for each experiment are presented in Appendix I.

CLADOSPORIUM HERBARUM EXPERIMENTS

Isolation and culture of C.herbarum.

The specimen of C.herbarum used in the study was isolated in the course of sampling the leaf microflora of larch seedlings at the Bush Forest Nursery, Midlothian, Scotland. The isolation was made by macerating leaf tissue and after serial dilution, plating samples onto Czapek-Dox yeast extract agar. After incubation at 22.5°C for 5 days a colony of C.herbarum was subcultured into pure culture.

Special techniques used in C.herbarum experiments.

C.herbarum produces a dry turf of erect conidiophores when grown on Czapek-Dox-yeast extract agar. This growth habit allows for ready harvesting of spores by gently brushing the turf with a flamed wire. Spores collected in this manner were not contaminated by nutrients from the agar. Spores were collected by this method and suspended in sterile distilled water. Density of inoculum was kept constant by adjusting the opacity of the spore suspension to 25 at 525 mμ on a Spectrophotometer (water blank equals 0). This opacity was equivalent to 11,000 ± 540 spores/ml.

Effect of spore age on germination.

A colony of C.herbarum contains spores of many different ages, from the newly formed spore on the growing edge of the colony to spores several weeks old near the colony centre. Therefore,

different aged spores could be harvested at different distances from the growing margin. The germination of spores of different ages was tested in sterile distilled water. The growing edge of a culture was marked and dated so that age could be determined. When the first culture grew to within 2 cm. of the edge of the agar a second culture was started. Spores of age greater than 40 days, 32-28 days, 17-13 days and less than 10 days were used. The results are summarized in Table IV-2.

TABLE IV-2. Effect of spore age on the germination of C. herbarum spores in sterile distilled water.

spore age (days)	40	32-28	17-13	10
average germination	0.0%	1.0%	53.3%	100.0%

There was no significant (P = 0.05) difference in germination between spores 32-28 days old and spores greater than 40 days old. The differences between all other age groups were significant (P=0.01). Thus, spore germination in water appears to fall off rapidly with increasing age. This pattern of ageing is useful in germination studies because a high or low germinating spore crop can be selected depending on the nature of the experiment.

The effect of nutrients on germination.

An experiment was designed to test the response of C.herbarum spores to external nutrient sources. Solutions of simple sugars and amino acids which occur in larch leaf leachates were prepared

(page 35). Spore germination was assessed at various concentrations of nutrients and compared with a control of distilled water. The sugar solution contained equal molar quantities of glucose, fructose and sucrose. The amino acid solution contained equal molar quantities of alanine, aspartic acid and glutamic acid. The treatments were:

i. distilled water control

ii. 5 μ moles sugar,

iii. 50 μ moles sugar,

iv. 500 μ moles sugar,

v. 5 μ moles amino acids,

vi. 50 μ moles amino acids,

vii. 500 μ moles amino acids,

viii. 500 μ moles sugars and 500 μ moles amino acids.

A suspension of 20-24 day-old spores was used as inoculum. The results of the experiment are summarised in Figure IV-1.

In the case of the sugar solution there was a consistent increase in germination with increasing sugar concentration up to 500 μ moles. At 5 μ moles of sugars the spore germination was the same as the water control (8.5%) while at 50 μ moles germination had increased to 19.3% and at 500 μ moles to 49.0%.

Conversely, the amino acid solution stimulated germination most strongly at the lowest concentration tested (5 μ moles). The increase in germination was of a much smaller magnitude with amino acids than with sugars.

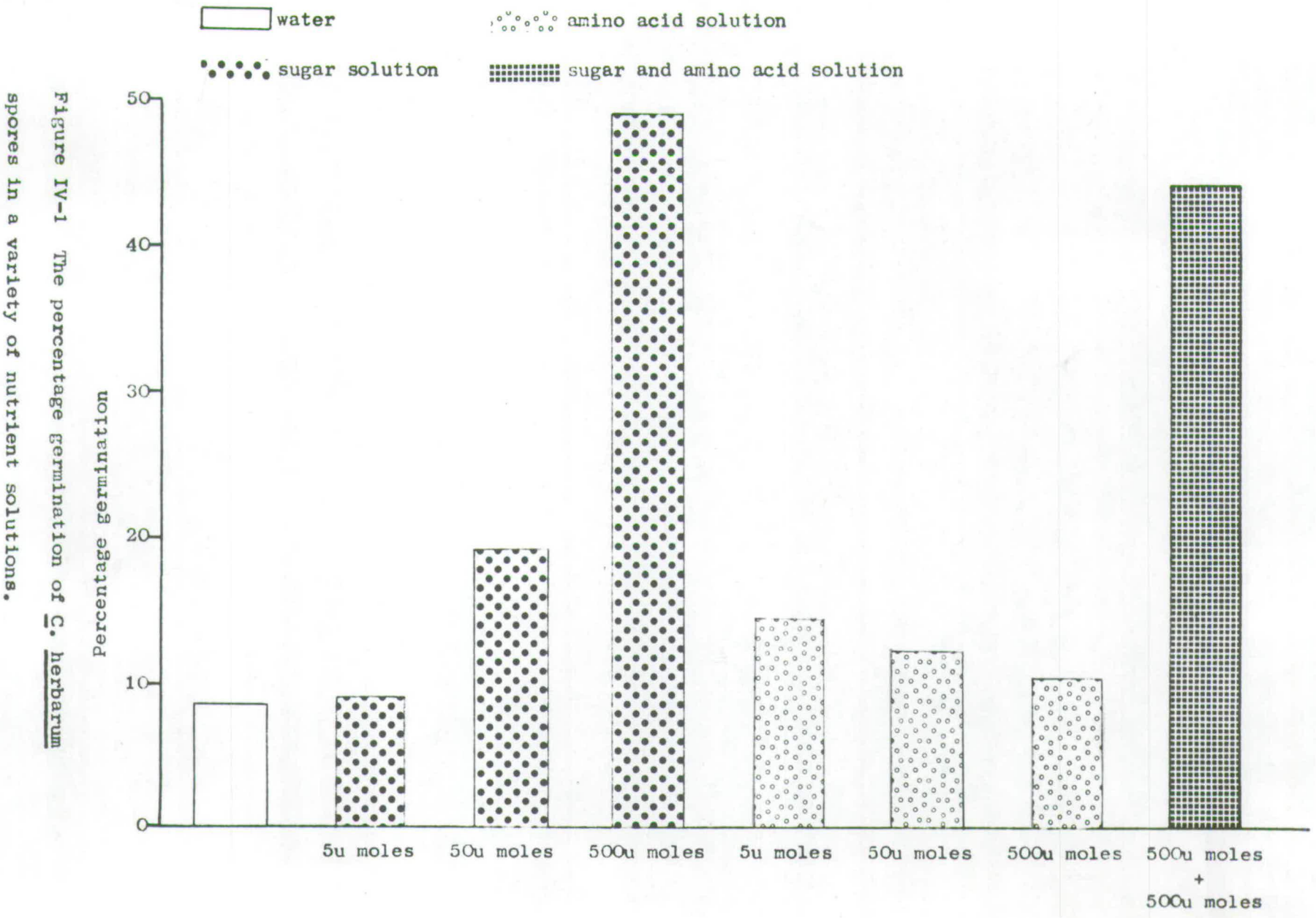


Figure IV-1 The percentage germination of C. herbarum spores in a variety of nutrient solutions.

The treatment with a mixture of sugars and amino acids at a concentration of 500 μ moles of sugar and 500 μ moles of amino acid gave a somewhat lower germination than pure sugars at 500 μ moles, but a much higher germination than pure amino acids.

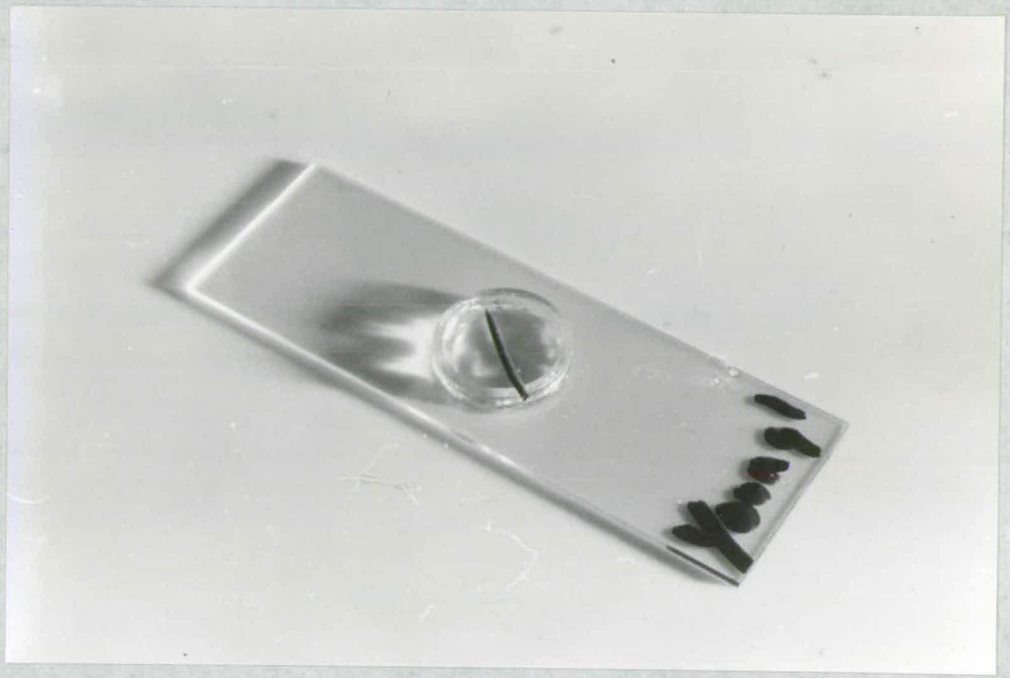
The quantities of nutrients available on the leaf surface, especially carbohydrates, could markedly affect the germination of C.herbarum. In this regard it should be noted that sources other than the host leaf may provide nutrients for spores on leaves. Fokkema (1968) has shown that C.herbarum activity on leaves of rye is related to the number of pollen grains present. Nutrients are likely to leach from pollen grains, fungal spores and detritus on the leaf.

It is clear from the results of the preceding experiment that germination of young spores can proceed independently of external nutrients, whereas older spores become dependent on external nutrients.

Effect of leaves on germination.

The growth pattern of C.herbarum on larch leaves could possibly be a result of one or more mechanisms; increased nutrient leaching with age, change of chemical inhibitor with age, or exposure of inner tissues due to damage. To assess these possible mechanisms, Van Tieghem cells were prepared with spores in distilled water into which leaves were immersed (Plate IV-1). This technique imitates the natural situation in which nutrients or chemical inhibitors are leached into water films on the leaf surface.

PLATE IV-1 VAN TIEGHEM CELL USED FOR SPORE GERMINATION TRIALS. In this case a larch leaf is immersed in the germinating solution.



In this experiment it was necessary initially to use leaves which had no microflora, otherwise the difference in microflora quantity and quality on leaves of different ages could interfere with the results. Therefore, leaves from aseptically grown seedlings (page 108) were used. It must be realized therefore, that these leaves have been protected from the possibility of microbial and physical weathering. The leaves were taken from three-month-old seedlings.

The following treatments were used:

i. young leaves - less than one month-old,

ii. old leaves - two and a half-months-old,

iii. old leaves which had been rubbed with a cloth,

iv. old leaves which had been repeatedly cut through the epidermis with a scalpel,

v. 500 μ moles of sugar (equal molar quantities of sucrose, glucose and fructose).

The results of this experiment are presented in Figure IV-2.

Germination was significantly increased compared to the water control by old leaves ($P = 0.05$), old rubbed leaves and young leaves ($P = 0.01$). Old leaves which had been rubbed stimulated germination more than unrubbed leaves, but damaged leaves showed an inhibition of germination when compared to old and rubbed leaves ($P = 0.01$). Indeed, spores with damaged leaves had a slightly lower average germination than spores in water. The sugar solution compared with water, increased germination by 50% while the greatest increase with a leaf treatment (old rubbed leaves) was 27%.

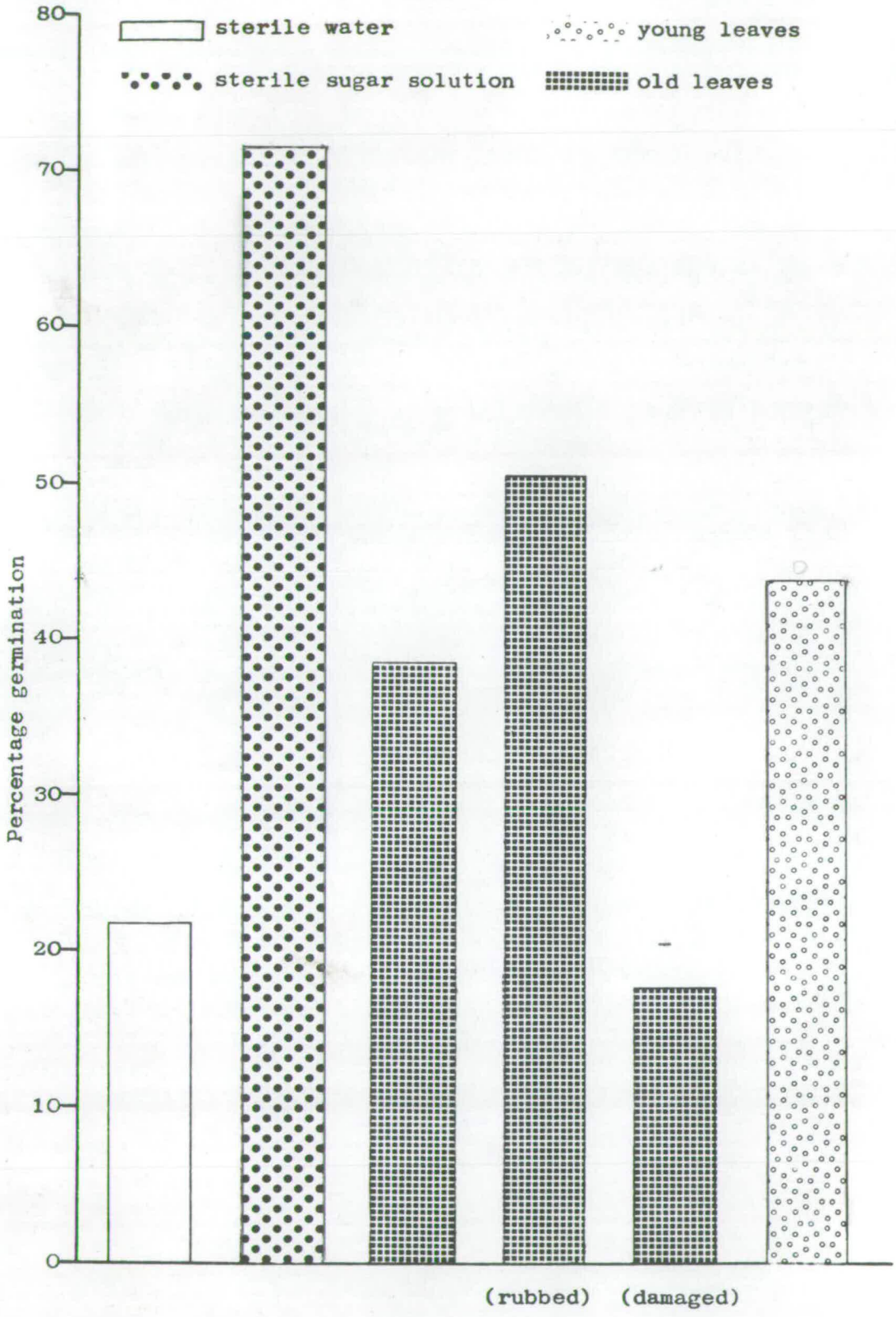


Figure IV-2 The percentage germination of C. herbarum spores in solutions containing aseptic larch leaves.

It appears that a substance or substances which leach from young and old leaves stimulate the germination of C.herbarum spores. The increased stimulation associated with leaves which had been rubbed may reflect an increase in leaching due to cuticle damage. However, leaves which have had the epidermis damaged and inner tissues exposed inhibit germination.

The leaves used in this experiment had not been exposed to physical weathering of the cuticle. It may be that rubbing the leaves with a cloth caused changes in the cuticle similar to those naturally caused by wind-driven rain and abrasion with other leaves and soil. Hall and Jones (1961), reported that leaf wax of clovers was removed by brushing against other leaves or the soil and that this effect could be simulated by brushing with a camel-hair brush. Surface waxes of clover leaves are extruded only while the leaf is growing (Hall and Jones, 1961). Therefore, wax lost by surface weathering may be replaced on young, growing leaves but not on older leaves. If the same were true for larch leaves it might be possible to explain in part the growth pattern of C.herbarum on larch leaves.

Spore germination with different aged leaves

Because of the difference in stimulation of spore germination by different-aged aseptic leaves, a second experiment was carried out to determine if field-grown leaves produced similar effects. The leaves for this experiment were taken from four-month-old larch seedlings used in the 1969 field microflora study (page 46).

The experimental methods were the same as in the previous experiment and the treatments were:

i. water control,

ii. young leaves-less than 4 weeks old,

iii. young leaves as in (ii) but rubbed with a cloth,

iv. old green leaves (3-4 months old),

v. old brown leaves (3-4 months old leaves still on the stem),

vi. brown leaves from the ground beneath the seedlings.

The leaves had a naturally developed microflora and therefore the young leaves would have a small population of bacteria and no yeasts. The old leaves would have a large population of both bacteria and yeasts as well as a surface mycelium. The microflora on brown leaves on the ground may have included some species of the soil microflora. The amount of germination and the length of germ tubes were recorded after 40 hours incubation. These results are summarized in Figure IV-3. All leaf treatments resulted in significant ($p = 0.01$) changes in spore germination compared to the water control. Young rubbed leaves stimulated germination to a greater extent than young unrubbed leaves. The greatest stimulation was attained with old leaves; both green and brown old leaves giving similar results. Conversely, brown leaves taken from the ground beneath the seedlings resulted in a marked reduction in germination.

Germ tube growth was increased by all leaf treatments. Old leaves resulted in the greatest stimulation of germ tube growth (cf. spore germination), but old green leaves resulted in an average germ tube length twice the length of those associated with old brown leaves.

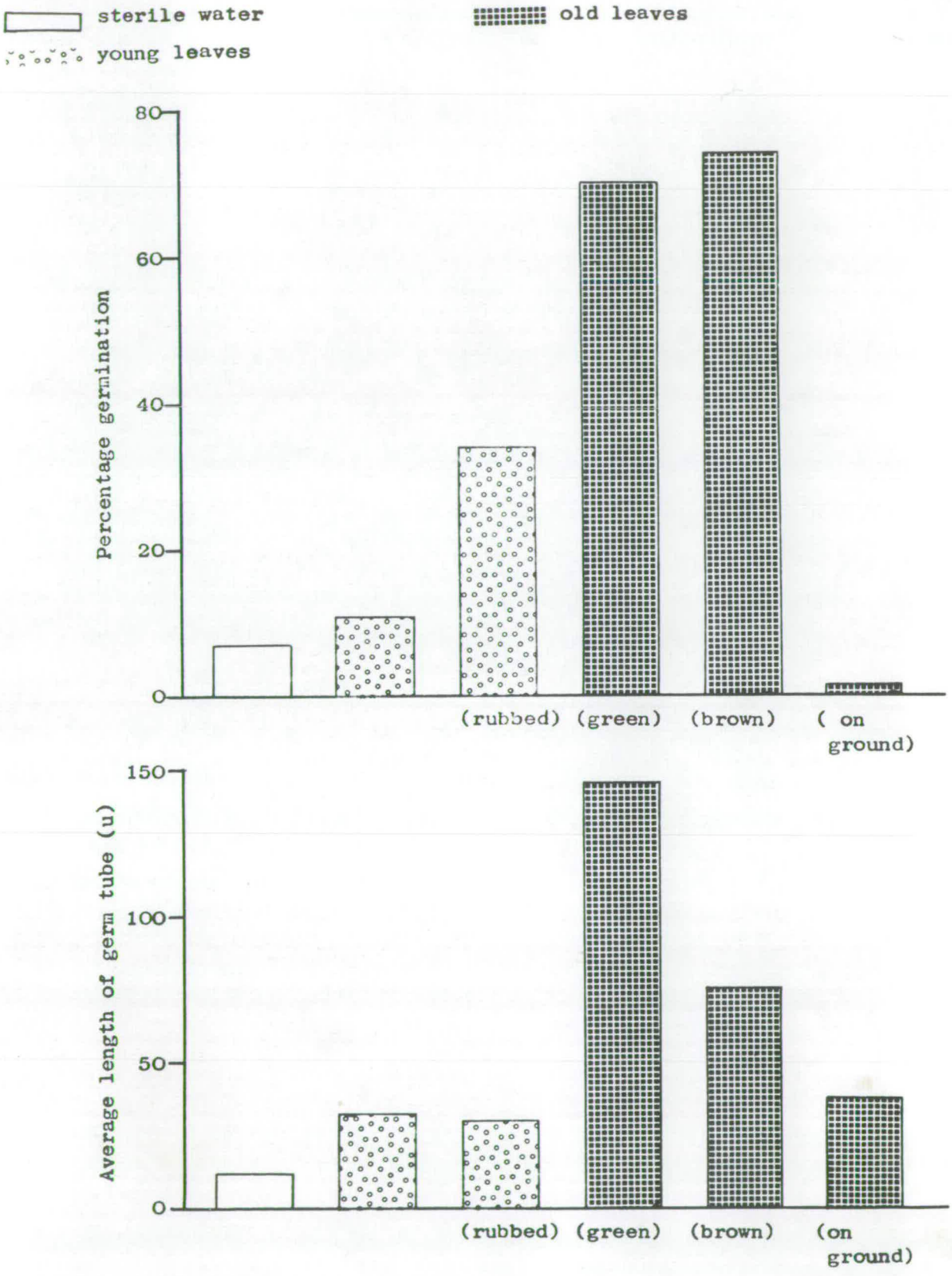


Figure IV-3 The percentage germination of C. herbarum spores and germ tube growth after 40 hours in solutions containing field-grown larch leaves.

It is also of interest that while rubbing increased spore germination associated with young leaves, it did not increase germ tube growth which was similar on both rubbed and unrubbed young leaves. Brown leaves from the ground also stimulated germ tube growth compared to the water control.

The different leaf treatments resulted in different microfloras in the germination medium. Young leaves in Van Tieghem cells developed a considerable population of motile rod bacteria and some bacteria colonized the germ tubes. The colonized germ tubes were often amber coloured and irregular in shape compared with the colourless straight germ tubes in the water control. In contrast, only a few bacteria developed in association with young rubbed leaves. Bacteria and yeasts developed in both old green and old brown leaf treatments. The colonies of bacteria and yeasts did not grow intermingled, but remained spatially separate. There was no colonization of germ tubes by bacteria or yeasts in the old leaf treatments. Very few bacteria were observed in the treatment with brown leaves from the ground.

The similarity of spore germination obtained with field-grown leaves and with aseptically-grown leaves would indicate that leaf age, independent of physical or microbial weathering, results in changes in the leaf which influence spore germination. Moreover,

old leaves, which support an active fungal development in the field were shown to produce a greater stimulation of spore germination and germ tube/growth than did the young leaves, which do not support fungal growth in the field.

If rubbing increased germination by releasing more nutrients to the medium, it might be expected that germ tube growth would also be increased. As this was not the case, it is possible that rubbing either removed a germination inhibitor or released a growth inhibitor.

The inhibition of spore germination and lack of yeasts or bacteria in the germinating media associated with brown leaves from the ground indicated that an antimicrobial effect resulting, perhaps, from colonization of the leaves by soil organisms, e.g. Actinomycetes, was present.

Effect of bacteria on germination

The possibility of the inhibition of spore germination by phyllosphere microorganisms was investigated using the Van Tieghem cell technique. Seven bacteria which had been isolated from larch seedlings were chosen for the experiment. The bacteria represented the most frequently isolated types of bacteria but also included the infrequently isolated Bacillus mycoides because of its reported inhibition of a leaf pathogen (McBride, 1969).

The bacteria were grown for 24 hours in shake culture of soil-extract-yeast extract (SEYE). Each Van Tieghem cell was made

up with 5 parts sterile distilled water, 1 part spore suspension and 1 part sterile SEYE or bacterial suspension. Young spores, approximately 10 days old, were used so that the high potential germination would give a wide range over which to observe inhibitory effects.

The treatments were:

i. control sterile SEYE,

ii. B.mycoides in SEYE,

iii. S-12 (Gram positive rod) in SEYE,

iv. S-79 (Flavobacterium sp.) in SEYE,

v. S-92 (Pseudomonas sp) in SEYE,

vi. S-95 (Bacillus sp.) in SEYE,

vii. S-100 (Pseudomonas sp.) in SEYE,

viii. S-117 (cream coloured Gram negative rod) in SEYE.

The results of the experiment are summarized in Figure IV-4.

The germination was very consistent in the control and in treatments S-12, S-79, S-92, S-95 and S-117. The percentage germination of the 18 replicates included in these treatments varied from 91.9% to 100%. Germination in the presence of B.mycoides was variable and ranged from 54.6% to 97.7%. Because of the obvious heterogeneity of variance between the control and the B.mycoides treatment the LSD would be an inappropriate test of statistical significance. For this reason the control and B.mycoides treatment were compared by the t-test, using Cochran's technique for unequal variance (Snedecor and Cochran p.114, 1967). The result of this test indicated no significant difference between the B.mycoides treatment and the control. There

Figure IV-4 The effect of phyllosphere bacteria on the germination of C. herbarum spores in SEYE solution.

was complete inhibition of spore germination by the S-100 treatment, i.e. no germination occurred. The inhibition was significant at $p = 0.01$. Two replicates of the S-100 treatment were observed again at 48 hours and 96 hours but the percentage germination remained below 1%. The possibility that the observed fungistasis in treatment S-100 would be reduced by the addition of nutrients was examined in the third replicate of the S-100 treatment. A solution containing equal molar quantities of glucose, fructose, and sucrose was added to the cell to give a concentration of 500 μ moles of sugar. The germination remained below 1% when re-examined 24 and 72 hours later.

In treatments S-117 and S-79, there was a distinct clustering of the bacteria around the germ tubes (Plates IV-2, IV-3, IV-4). No lysis was observed. There was no similar clumping around the spore and it may be that nutrients are leached more readily from the germ tubes or that the cell wall of the germ tube provides an attractant which the spore wall lacks.

Effect of bacteria and yeasts on germination

The preceding experiment was carried out with [illegible] media containing considerable quantities of sugars, amino acids and vitamins. Water films on larch leaves probably contain low levels of nutrient. Therefore, an experiment was designed to examine the inhibitory effect of S-100 on spore germination in distilled water and in a simple sugar solution. The effect of the yeast, Sporobolomyces roseus, on spore germination was also assessed.

PLATE IV-2 GERMINATING SPORES OF *CLADOSPORIUM HERBARUM* IN A SOLUTION CONTAINING NO MOTILE BACTERIA. X 1000

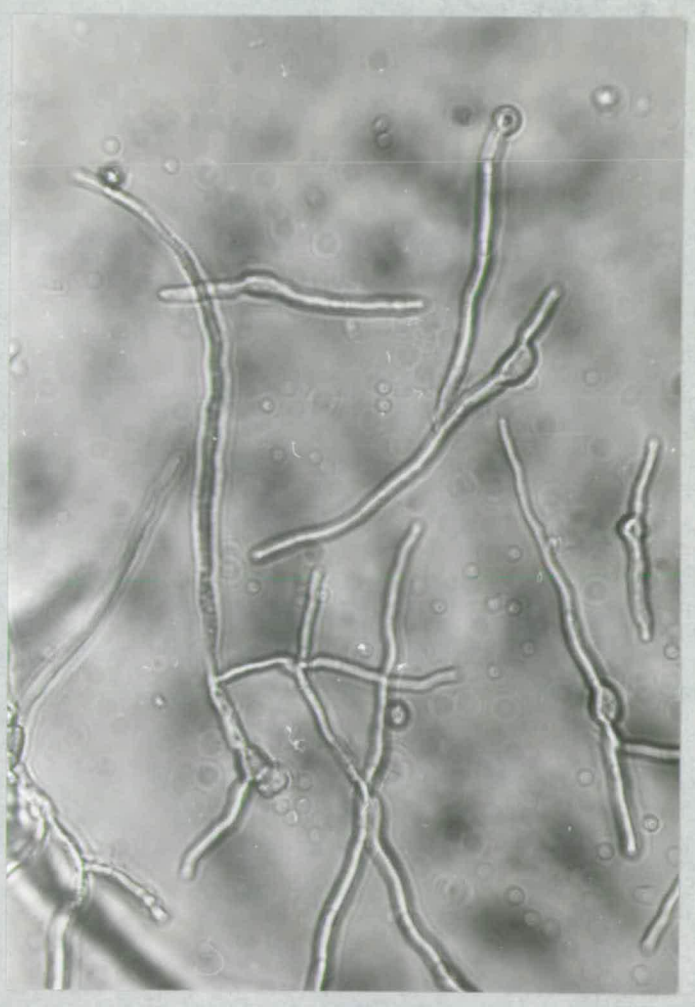
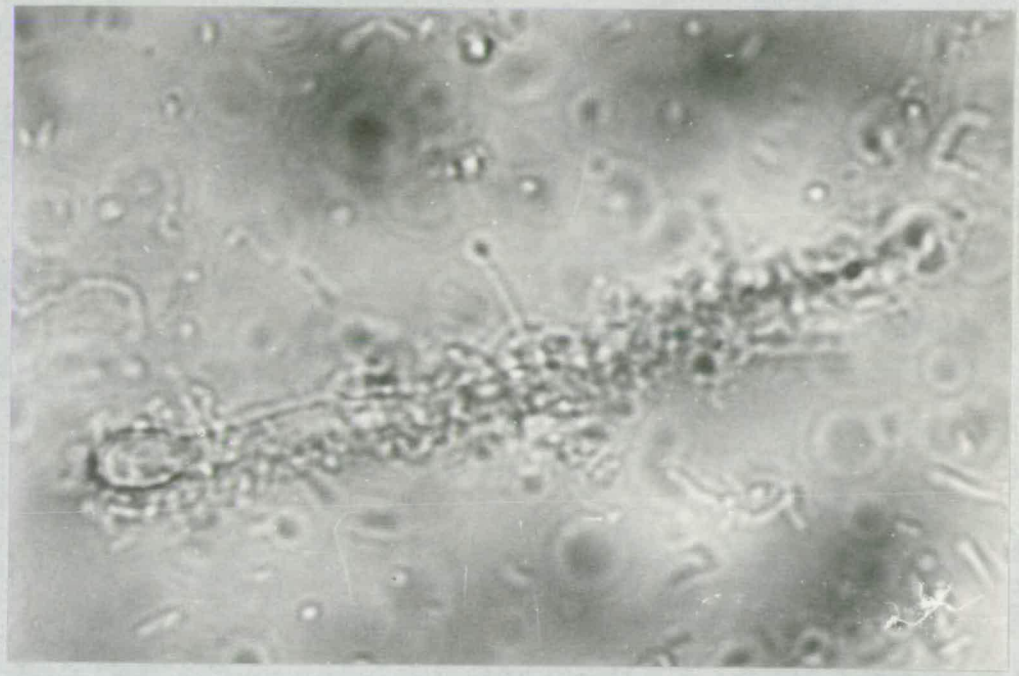
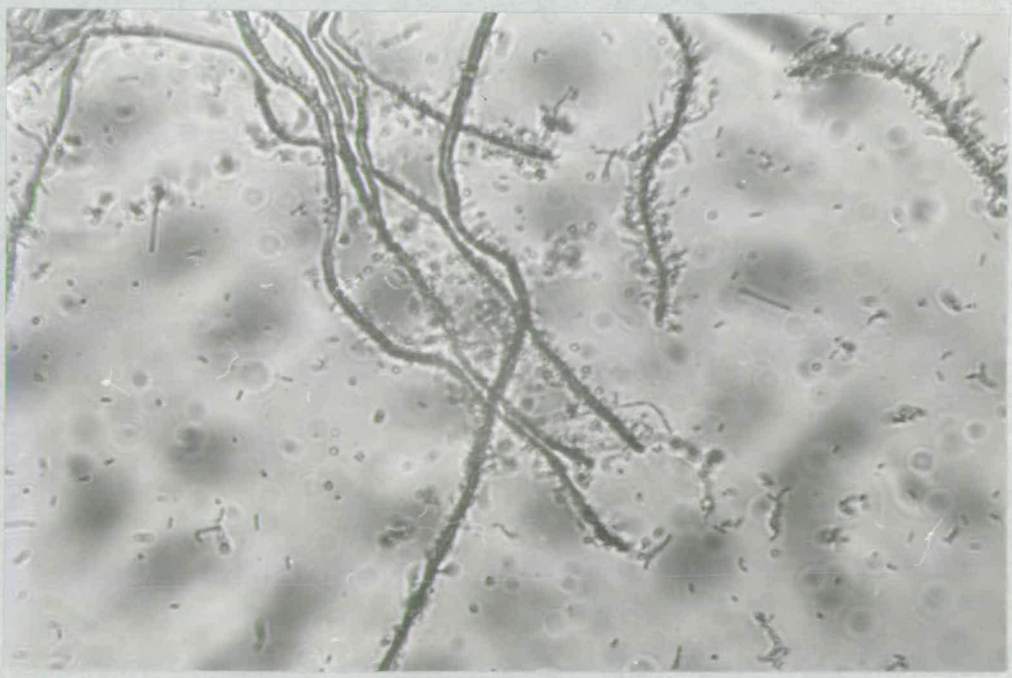


PLATE IV-3 MOTILE BACTERIA CLUSTERING AROUND THE GERM TUBE OF A CLADOSPORIUM HERBARUM SPORE. X 2100

PLATE IV-4 MOTILE BACTERIA CLUSTERING AROUND ACTIVELY GROWING HYPHAE OF CLADOSPORIUM HERBARUM. X 980





The bacterium and yeasts were grown on SEYE agar for 48 hours and 96 hours respectively. A wire loop was used to remove the organisms from the surface of the culture and suspend them in sterile distilled water. The sugar solution was the same as used in previous experiments. The spores were approximately 20 days old.

The cells were made up with 5 parts water or sugar solution, 1 part spore suspension and one part S-100 or S.roseus suspension. The treatments were:

i. control-sterile distilled water,

ii. 250 μ moles of sugar,

iii. S-100 in water,

iv. S-100 in 250 μ moles of sugar,

v. S.roseus in water,

vi. S.roseus in 250 μ moles of sugar,

vii. S-100 and S.roseus in water,

viii. S-100 and S.roseus in 250 μ moles of sugar.

The results of this experiment are presented in Figure IV-5.

The experiment revealed an interesting interaction between nutrients and germination inhibition. When in distilled water the bacteria did not cause a significant reduction in germination, but in the sugar solution a significant ($p = 0.01$) reduction occurred. This reduction was significant when compared to the sugar control and to the water control. The presence of bacteria in the bacteria-yeast-sugar treatment significantly ($p = 0.01$) reduced germination compared

Figure IV-5 The effect of different combinations of bacteria, yeasts and nutrients on C. herbarum spore germination.

to the yeast-sugar treatment. Thus, in both cases where bacteria occurred in a sugar solution, germination was reduced. Where bacteria were in water, no significant reduction occurred. The comparison of bacteria-yeast-water treatment with yeast-water does not give additional information because both had 0% germination.

S.roseus in water completely suppressed spore germination. With the addition of the sugar solution (yeast-sugar treatment) the suppression disappeared and a stimulation of germination occurred. The yeast-sugar treatment germination was significantly ($p = 0.01$) higher than the sugar treatment germination. Similarly, when yeasts were present (bacteria-yeast-sugar treatment) an increase in germination was observed in comparison to the bacteria-sugar treatment. Thus, in both cases where S.roseus occurred in a sugar solution, germination was increased.

Spore germination with S.roseus in different nutrient solutions.

An additional experiment was designed to examine further the interaction of S.roseus and nutrient level. Yeast suspensions prepared with unwashed cells growing on nutrient-rich media may contain quantities of extracellular nutrients. Therefore, in this experiment, yeast cells were washed by suspending them in water, centrifuging and pouring off the supernatant. This process was repeated twice before the final water suspension was prepared.

With this sole modification, i.e. washed yeast cells, the same methods were used as in the previous experiment. The following treatments were used:

i. water control,

ii. water plus S.roseus,

iii. 5000 μ moles of sugars,

iv. 500 μ moles of sugars,

v. 50 μ moles of sugars,

vi, vii and viii as in iii, iv and v respectively plus S.roseus,

ix, x and xi, as in iii, iv and v respectively plus 0.1% yeast extract,

xii, xiii and xiv as in ix, x, xi respectively plus S.roseus.

No further change in germination was noted after 40 hours and the results are summarized in Figure IV-6. Since all levels of sterile sugars showed 100% germination, they are shown as one treatment. The same was true of sugars with yeast extract. Similarly, in treatments including S.roseus with sugars, concentration did not influence percentage germination, which in any case was never more than 2%. These results have therefore been combined.

S.roseus, in conjunction with all sugar concentrations tested, resulted in total suppression of germination. The suppression was completely eliminated by the addition of 0.1% yeast extract.

If unwashed yeast cells can be considered to contribute a similar factor to the germinating medium as yeast extract, the results of this experiment corroborate the results of the previous experiment. Thus, S.roseus, in the presence of sugars, inhibited spore germination but S. roseus in the presence of sugars plus yeast extract did not inhibit spore germination. Without S.roseus both sugars, and sugars plus yeast extract stimulated spore germination.

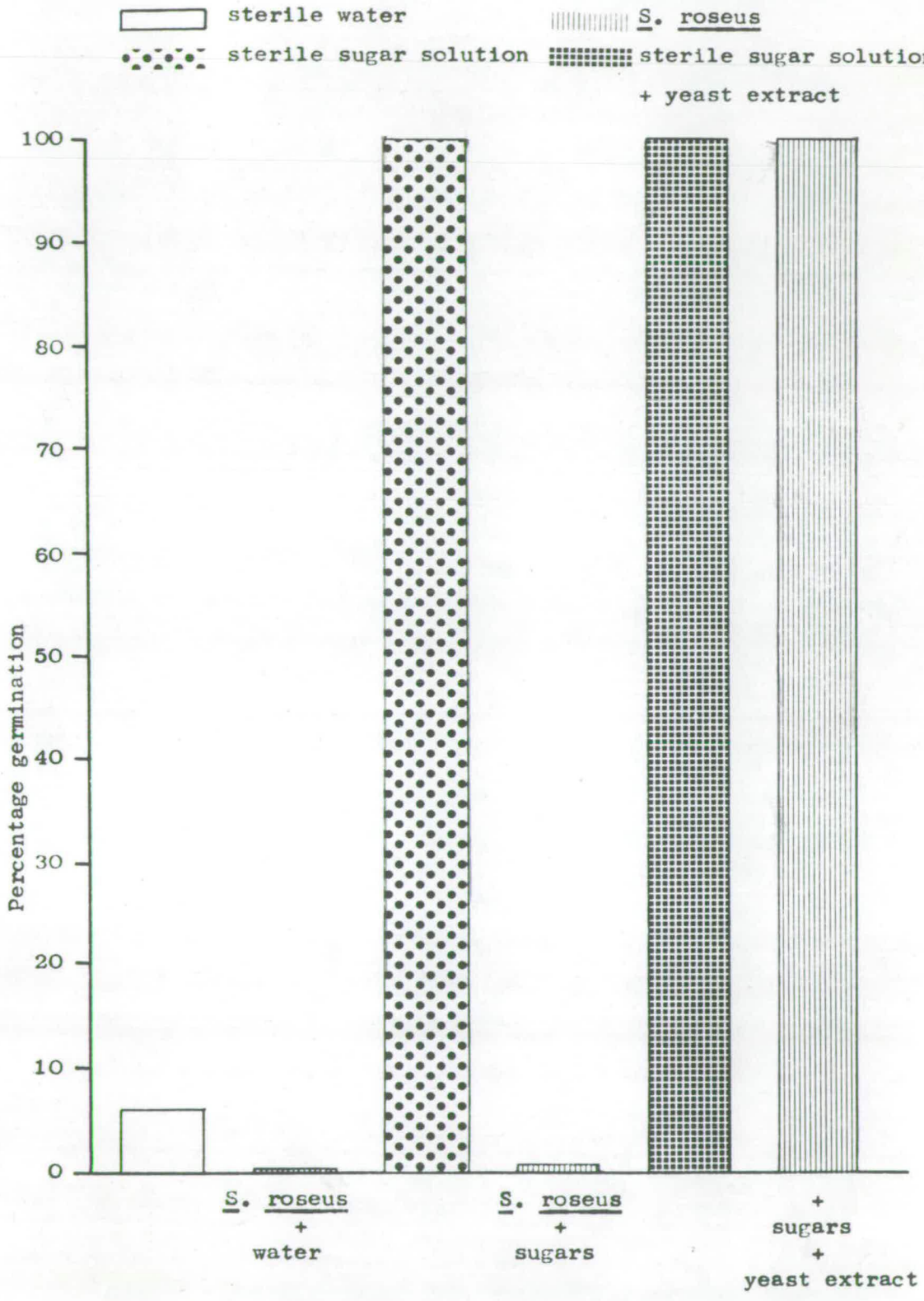


Figure IV-6 The effect of S. roseus on C. herbarum spore germination in different nutrient solutions.

Interaction of leaves and microbial inhibition of germination.

In the preceding series of experiments it has been shown that certain bacteria and S.roseus have effects on the germination of C.herbarum spores which were dependent on the nutrient content of the germinating medium. Spore germination was stimulated by larch leaves which have been shown (Chapter II) to lose nutrients by leaching. It was, therefore, of interest to examine the interaction of leaf leachates with bacteria or S.roseus and their combined effect on spore germination.

An experiment was carried out in which the germination of spores could be compared when no leaves, young leaves, or old leaves were present, either in sterile conditions, with S.roseus or with the bacterium S-100. The experimental procedures were the same as described previously. The suspensions of S.roseus and S-100 were prepared by centrifuge washing of water suspensions. The leaves were taken from aseptic seedlings and were less than one-month-old for young leaf treatments and two-and-a-half-months-old for old leaf treatments.

The treatments were (3 replicates):

i. water control,

ii. water plus S-100,

iii. water plus S.roseus,

iv. young leaf,

v. young leaf plus S-100,

vi. young leaf plus S.roseus,

vii. old leaf,

viii. old leaf plus S-100,

ix. old leaf plus S.roseus.

The percentage germination increased in the S-100 with water and S-100 with young leaf treatments in the period between 24 hours and 48 hours. Germination in all other treatments during this period remained constant. No increase was observed after 48 hours and the data for that time was used for analysis.

For clarity, the results of the S-100 treatments and S.roseus treatments were analyzed separately and are presented in Figures IV-7 and IV-8 respectively. The results within treatments were very uniform, perhaps due to the use of aseptically grown leaves and of washed cell suspensions. All treatment differences were significant at $p = 0.01$ with two exceptions, i.e. sterile water control/sterile young leaf treatment ($p = 0.05$) and sterile young leaf treatments/S-100-young leaf treatment (not significant).

The results confirmed the observation of previous aseptic leaf treatments. Young and old leaves stimulatedspore germination, with old leaves having the greatest effect.

In water, the bacterium S-100 resulted in 66.7% germination compared to only 6.7% in the sterile water control. When S-100 was present with young leaves the germination was 15.7%, not significantly greater than the 13.0% recorded for young leaves in sterile water. Conversely, the combination of S-100 with old leaves reduced germination to 17.7% from the 41.3% recorded for spores with old leaves in sterile water and the 66.7% recorded for spores with S-100 in water.

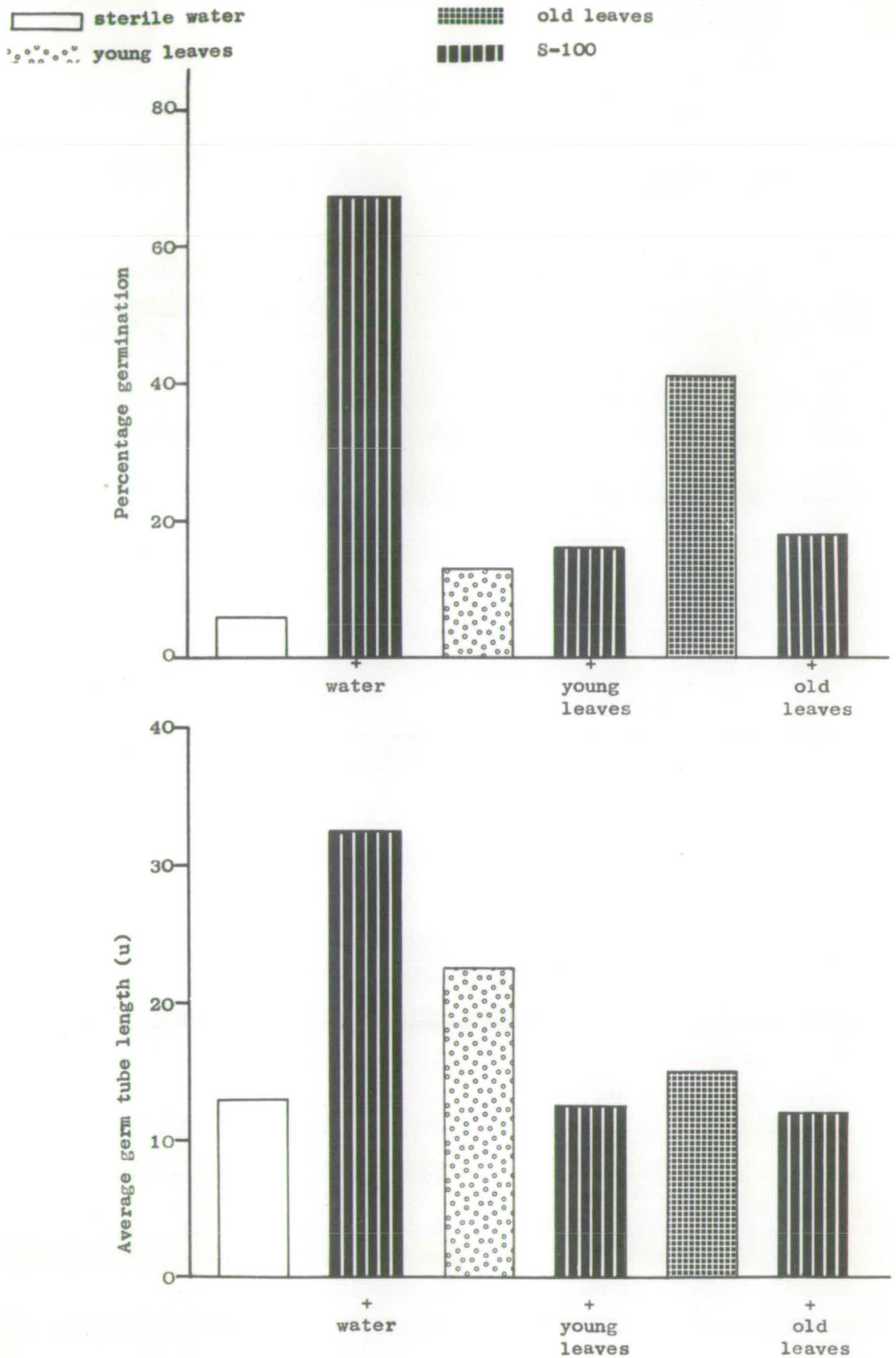


Figure IV-7 The interaction of bacterium S-100 with leaf leachates and C. herbarum spore germination and germ tube growth at 48 hours.

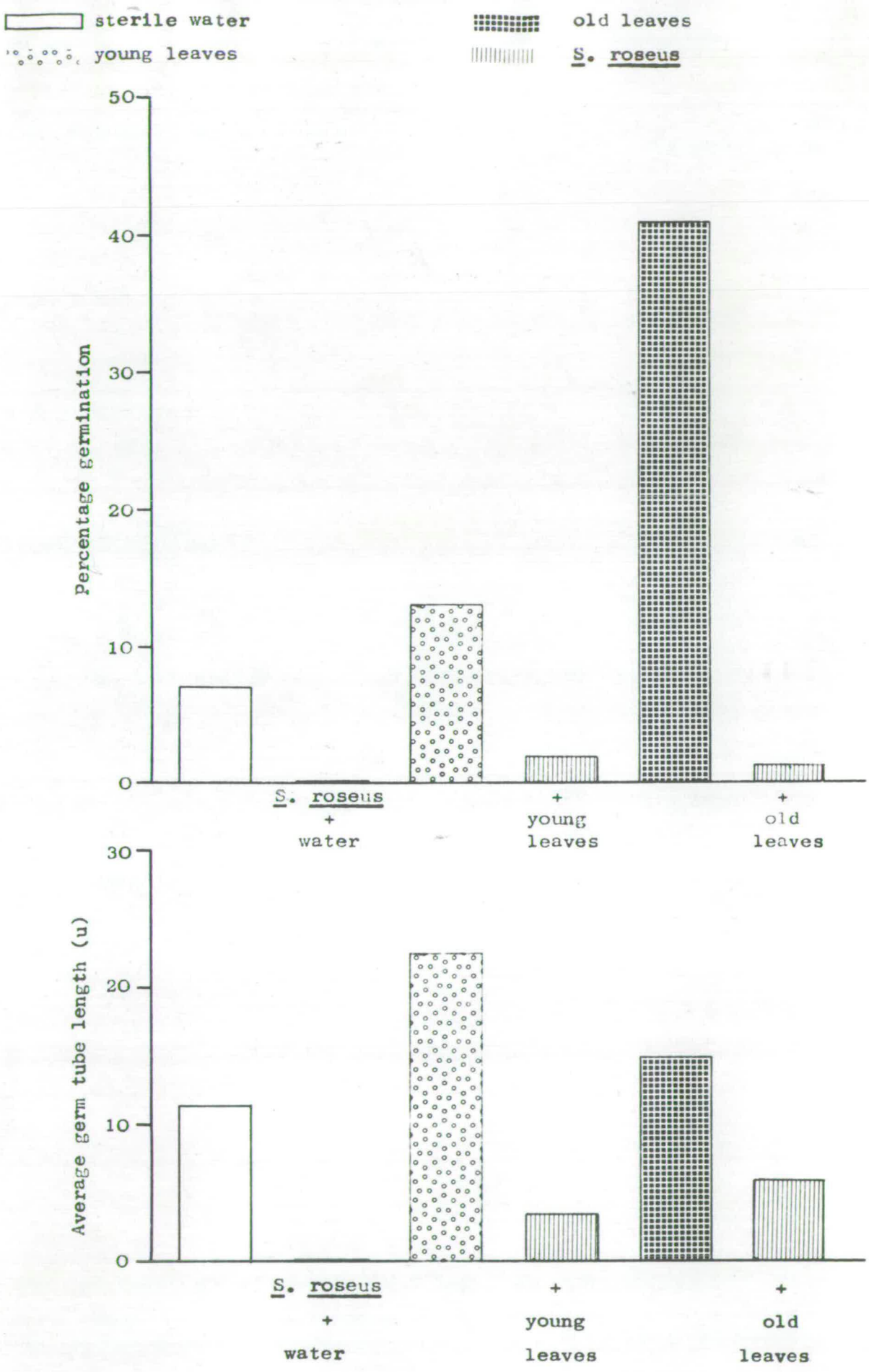


Figure IV-8 The interaction of S. roseus with leaf leachates and C. herbarum spore germination and germ tube growth at 24 hours.

The average length of germ tubes was also determined after 48 hours. The results are presented in Figure IV-7. S-100 in water resulted in the longest germ tubes while the spores in S-100 with young or old leaves developed germ tubes somewhat shorter than spores in the respective sterile treatments.

All treatments which included S.roseus showed a reduction in germination compared with the respective sterile controls. There was no germination in the water plus S.roseus control and less than 3% germination when either young or old leaves were combined with S.roseus. Germ tube elongation (Figure IV-8) was also reduced in all S.roseus treatments compared to the sterile controls.

The results of this experiment comparing the interaction of S-100 and S.roseus with leaf leachates and their effect on C.herbarum spore germination confirmed the results of previous experiments. If old leaves can be assumed to lose more nutrients than young leaves then the effects on spore germination were similar to those obtained using pure chemical additions to the germinating medium. That is, S-100 stimulated spore germination in low nutrient conditions but inhibited germination in high nutrient conditions. Leachates of old leaves apparently provided conditions for inhibition with S-100 while leachates of young leaves did not. The delay of over 24 hours before S-100 in water stimulated germination of C.herbarum may indicate that a change occurred in the germinating medium perhaps due to a loss of nutrients from dead cells.

MERIA LARICIS EXPERIMENTS

Isolation and culture of M.laricis.

A sample of two year old L.decidua seedlings infected with M.laricis was collected in polythene bags at Ben Reid's Nursery, Aberdeen. It was kindly sent to Edinburgh by Dr. J.S. Murray of the Forestry Department, University of Aberdeen.

Two methods were used in attempts to isolate M.laricis from the infected leaves; spore suspensions and surface sterilized leaves. M.laricis spore suspensions were readily obtained by shaking infected leaves in water. Portions of the spore suspensions were placed on Czapek-Dox agar and incubated at 22.5°C. Microscopic examination after 48 hours and 96 hours indicated a slow germination of M. laricis spores and the petri plates were overgrown by bacteria and other fungi before M.laricis could be isolated.

In the second method, segments of infected leaves were surface sterilized in 0.1% mercuric chloride in water for three minutes. The segments were then rinsed three times in sterile water before plating onto Czapek-Dox agar. This method followed by repeated subculturing resulted in several pure cultures of M.laricis.

Considerable variation in culture features were observed among the isolates. The colour ranged from white through pink and tan to yellow green. Some isolates had aerial mycelium while others were entirely recumbent. Abundance of spores, media discolouration and dryness of the mycelium were other features which varied among the isolates. All isolates could be assigned to one or other of the

two strains described by Peace and Holmes (1933). It was more difficult to assign cultures to the four categories of Biggs (1964) because of intermediate forms. Thus the following strains were recognized:

(a) Peace and Holmes strain a, tan or pink becoming greenish black after two months, deliquescent surface with abundant spores and little or no discolouration of the medium;

(b) Peace and Holmes strain b, yellowish green mycelium, dry surface with few spores and a marked discolouration of the medium to brown or black.

Both Biggs and Peace and Holmes consider the strains to be aggregates containing a /mixture of nuclear types.

To avoid being enmeshed in variations due to strain differences and to ensure an ample supply of spores, an isolate of strain (a) was selected for further study.

Biggs (1964) reported maximum growth of M.laricis in Czapek-Dox agar with yeast-extract and pH 4-6 at 15-20°C. Even under these conditions growth is slow with colonies attaining less than 30 mm. diameter in three weeks. An attempt was made to increase growth by the addition of nutrient supplements. An experiment was designed with the following treatments (media):

i. Control. Czapek-Dox agar +0.7% yeast,

ii. as in (i) + 1% water soluble extract of L.decidua leaves. (filtrate of 250 g. of leaves autoclaved for 20 minutes in 1000 ml H_2 + 2.0 g KH_2PO_4).

iii. as in (i) + 1% L.decidua leaves.

All media were autoclaved at 120°C for 15 minutes and their pH was adjusted to 5.0 with lactic acid before being poured into petri plates. Each treatment had seven replicates.

The plates were inoculated with 5 mm diameter portions of mycelium from the margin of a 21 day old culture of strain (a) growing on Czapek-Dox-yeast-extract agar.

The diameters of the resulting colonies were measured 4,10,14,17 and 23 days after inoculation. The plates were incubated @ 20±1 C.

The results of the experiment are summarized in Figure IV-9. The F-test of the analysis of variance of growth after 23 days rejected the null hypothesis that there was no difference between treatments at p = 0.01. The computation of the Least Significant Difference (LSD) at P = 0.05 showed no significant difference between Czapek-Dox yeast-extract and leaf-extract. There was, however, a significant difference at P = 0.01 between the larch leaf media and the other two media.

Thus, at the concentrations used there was no stimulation of M. laricis growth by water soluble extracts of larch leaves. The addition of whole leaves markedly increased growth.

Because the concentration of the various leaf nutrient supplements had been chosen arbitrarily a second experiment was designed to assess the effect of different concentrations. The treatments were prepared as in the preceding experiment and were:

i. Czapek-Dox agar + 0.7% yeast-extract + 1% leaf-extract,

ii. as in (i) but with 2% leaf-extract,

iii. as in (i) but with 4% leaf-extract,

iv. Czapek-Dox agar + larch leaves in a concentration series.

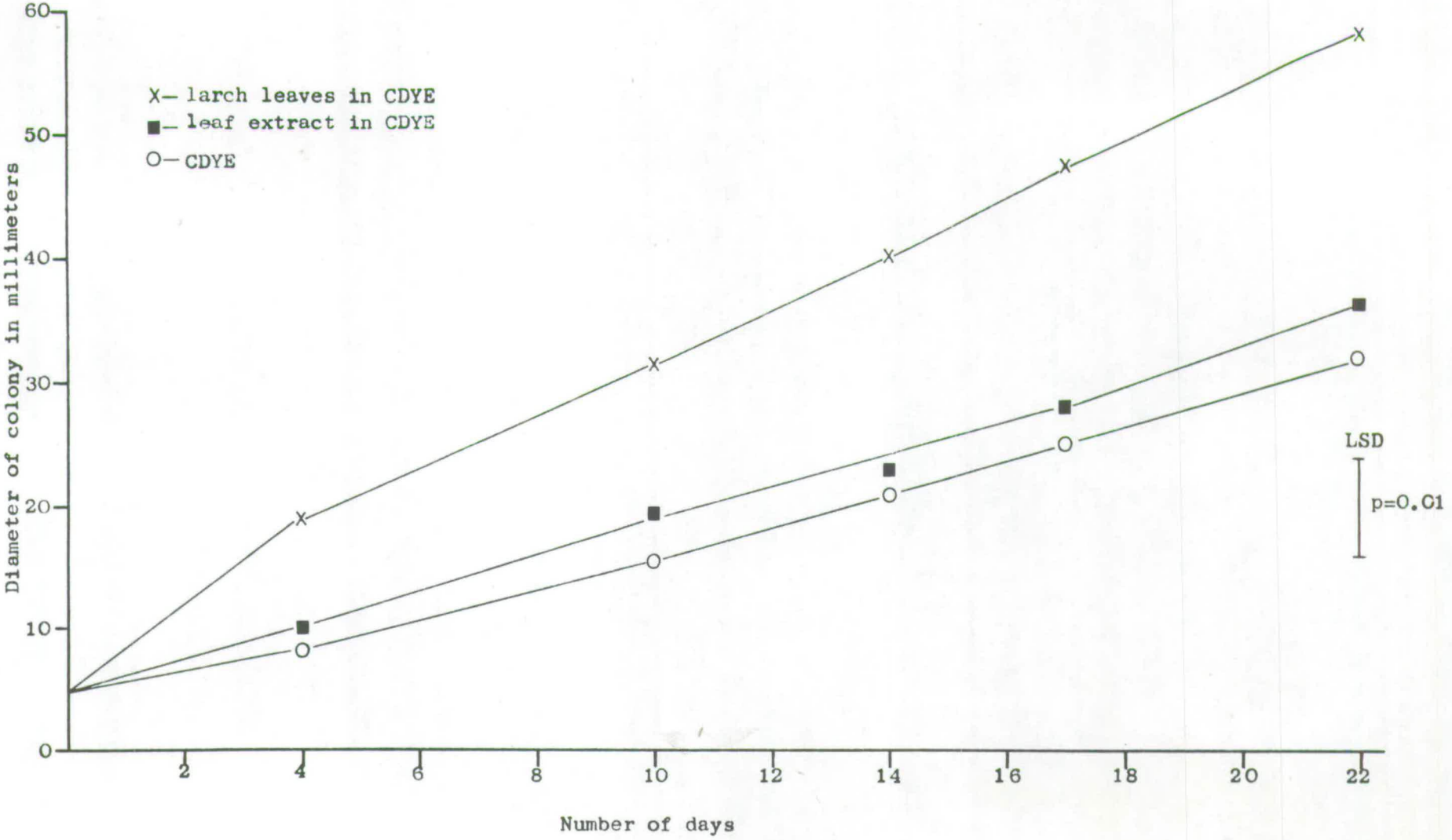


Figure IV-9 The rate of M. laricis colony growth on media containing larch leaves or larch leaf extract.

When the media for treatment (iv) was poured an uneven distribution of leaves resulted. One plate had only 3 needles while another plate had over 100. Before inoculating, the plates were ranked from 1 to 12 in descending order of concentration of leaves.

Inoculations and measurements were carried out as in the preceding experiment. Diameters were measured 6,9,13,19 and 27 days after inoculation.

An analysis of variance of the whole experiment could not be carried out due to the variability in larch leaf media (treatment four). Therefore, the leaf-extract treatments were assessed separately by analysis of variance. The average colony diameter after 27 days was 46.7 mm. in 1% leaf extract, 46.2 mm. in 2% leaf extract and 49.4 mm. in 4% leaf extract. The analysis of variance resulted in the acceptance of the null hypothesis, that there was no difference between treatments. Thus, the concentration of leaf-extract did not significantly affect the growth of M.laricis.

The results of the larch leaf treatment were analyzed using the Spearman rank correlation coefficient (Snedecor and Cochran, 1967). The petri plates, as mentioned above, were ranked from 1 to 12 according to the quantity of leaves present in each plate. The plates were then ranked by diameter of the colony after 27 days. The ranks are summarized in Table IV-3.

TABLE IV-3: Ranks of larch leaf quantity and growth of M.laricis.

Quantity of leaves-rank	1	2	3	4	5	6	7	8	9	10	11	12
Diameter of colony in mm.	59	54	54	55	47	48	45	44	44	42	41	40
Diameter of colony-rank	1	3.5	3.5	2	5	6	7	8.5	8.5	10	11	12

$r_s = .968$ $r_{.01} = .684$

The rank correlation coefficient is significant at p = 0.01. Thus the increased growth of M.laricis appears to be closely related to the quantity of larch leaves in the medium. The higher ranks of leaf quantity showed greater growth than any of the leaf extract treatments.

The growth rates were consistent between the first and second experiments. From these results it appears that the water soluble leaf extract does not add to the nutrients already available to the fungus in the Czapek-Dox-yeast-extract medium. The addition of whole leaves, however, did apparently provide an additional source of nutrient which the fungus can utilize for more rapid growth. The additional nutrient may be one or a combination of a large number of water insoluble leaf components.

The increased growth of M.laricis in the leaf media did not sufficiently increase spore production to make this media desirable for further studies on spore germination and infection. Therefore, culturing of M. laricis was continued on Czapek-Dox-yeast-extract agar with a pH of 5.0.

During the course of this work it was observed that cultures grown in the light on the laboratory bench top were more heavily sporing than cultures grown at 20.0°C in a dark incubator. The bench temperature varied within the range 16°C-20°C. It was not determined if temperature, temperature fluctuation, light or a myriad of other bench effects was the cause of this heavy spore production.

Special techniques used in M.laricis experiments.

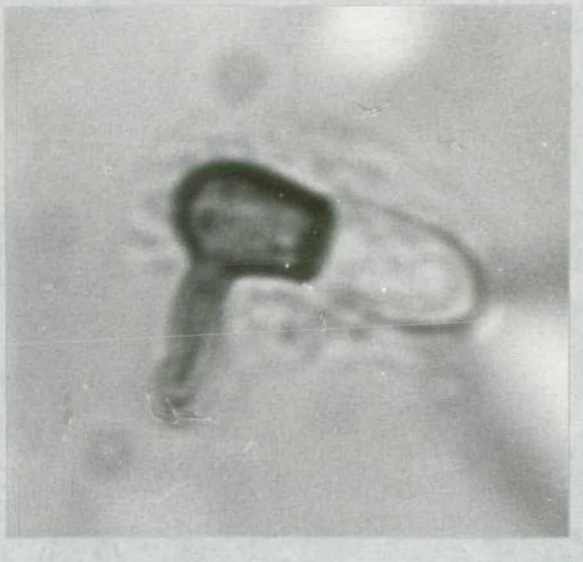
Cultures for spore production were grown in 1 oz. screw top vials on 2.5% Agar slants of Czapek-Dox-yeast Extract (CDYE) adjusted to pH 5.0 with lactic acid. After 3 to 4 weeks incubation on the bench top, spores were harvested by adding 5 ml sterile distilled water and vigorously shaking the flask for 2 minutes. The hard, 2.5% agar remained firm during the shaking process and a debris-free spore suspension was readily obtained. The spore suspension did undoubtedly contain some nutrients leached from the medium.

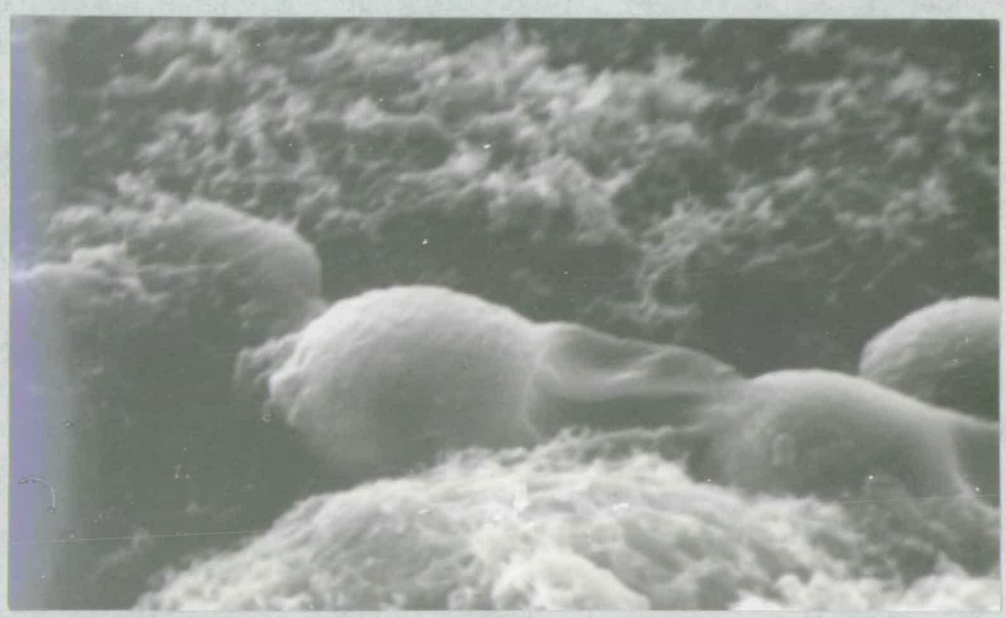
The spore density was standardized by adjusting the spore suspensions to a constant opacity (25 at 525 mμ compared to a water blank). This opacity represented 750 ± 103 spores/ml.

Meria spores vary in size from 6 μ x 2.5 μ to 10 μ x 4 μ with the mean size 9 μ x 3 μ. Spores may be one or two celled when released from the sterigmata. In any case, a wall was formed at the constriction of the cell before germination began. The first stage of germination was a swelling of one cell accompanied by a darkening in colour and a thickening of the cell wall (Plate IV-5).

PLATE IV-5 GERMINATING SPORE OF MERIA LARICIS. X 4500
Note the dark pigmentation of the cell producing the germ tube.

PLATE IV-6 GERMINATING SPORE OF MERIA LARICIS ON A LARCH LEAF. X 7500
Note the germ tube (upper left) and the collapse of the cell which has not produced a germ tube (centre).





The second cell may remain unchanged, become flaccid, or collapse completely (Plate IV-6). The significance of the pigmentation is not known. A small peg-like projection then develops on the side of the swollen cell. This may develop into a short hypha or in some cases microconidia are produced from it. It is thought (Peace and Holmes, 1933) that these microconidia are produced under poor nutrient conditions. The criterion used for germination counts was the development of a germ tube at least as long as the cell was wide.

Effect of nutrients and cold treatments on spore germination.

Preliminary germination trials in water had shown low (less than 15%) germination and it was decided to assess different methods of increasing germination. Biggs (1964) had observed that spore germination could be increased two-fold by exposing spores to a temperature of 0°C for periods up to 48 hours prior to germination at 20°C. A factorial experiment was designed to assess three nutrient treatments; water, amino acids and carbohydrates, and three lengths of 0°C treatment; 0, 12 and 48 hours.

The treatments were:

i. distilled water,

ii. 500 µ moles sugar (equal molar portions of glucose, fructose and sucrose),

iii. 500 µ moles amino acid (equal molar portions of alanine, aspartic acid and glutamic acid),

iv. no 0°C treatment,

v. 12 hours at 0°C,

vi. 48 hours at 0°C.

Some germination had occurred after 12 hours but the percentage germination continued to increase in all treatments at 24 hours and 48 hour observations. Final counts were made after 60 hours. The results of the final counts are summarised in Table IV-4.

TABLE IV-4: Factorial experiment with 0°C treatments and nutrient treatments. Average percentage germination after 60 hours.

Time at 0°C	0	12	48	Simple $\bar{x}$
Water	8.6%	17.6%	15.6%	13.9%
Amino acids	9.6%	11.4%	69.0%	30.0%
Sugars	29.4%	37.8%	61.8%	43.0%
Simple $\bar{x}$	15.9%	22.3%	48.8%	

The analysis of variance indicated that the nutrient treatments, the 0°C treatments and their interactions show significant variation. By observing the simple effects it can be seen that additional nutrients, especially sugars, and the 0°C treatments increase spore germination. A number of interesting trends can be observed. Firstly, with no 0°C treatment, sugars increased germination three-fold compared to the water treatment. Secondly, the 12 hour and 48 hour 0°C treatment increased germination in all treatments but was most effective in treatments containing amino acids or sugars. Thirdly, the amino acid treatments did not stimulate germination of spores with only 12 hours

or zero hours at 0°C but did significantly stimulate germination of spores with 48 hours at 0°C.

The production of microconidia by the germinating spores was observed only in the water treatments.

The effect on spore germination of longer periods at 0°C can be assessed by using data collected during later experiments. In this way it is possible to compare the germination in sugar solutions of spores kept at 0°C for 72, 96 and 108 hours with germination in water of spores kept at 0°C for 72 and 96 hours. It should be pointed out that because these results are from experiments carried out at different dates, precise statistical comparison would be invalid. The data is graphed along with some results from Table IV-4 in Figure IV-10.

The results indicated a continuing increase in percentage germination with increasing length of 0°C treatment when spores were in the sugar solution. When spores were germinated in water, however, a maximum percentage germination was attained between 12 and 48 hours at 0°C. If the cool treatment was continued the spore germination began to decline.

These trends may indicate that the cold treatments affected spore germination by influencing spore permeability, increasing the degree to which compounds could pass into or out of the spore. Thus, a spore in the sugar solution could increase its nutrient content with a resultant increase in germination, whereas a spore in water may lose nutrients to the environment with a resultant decrease in germination.

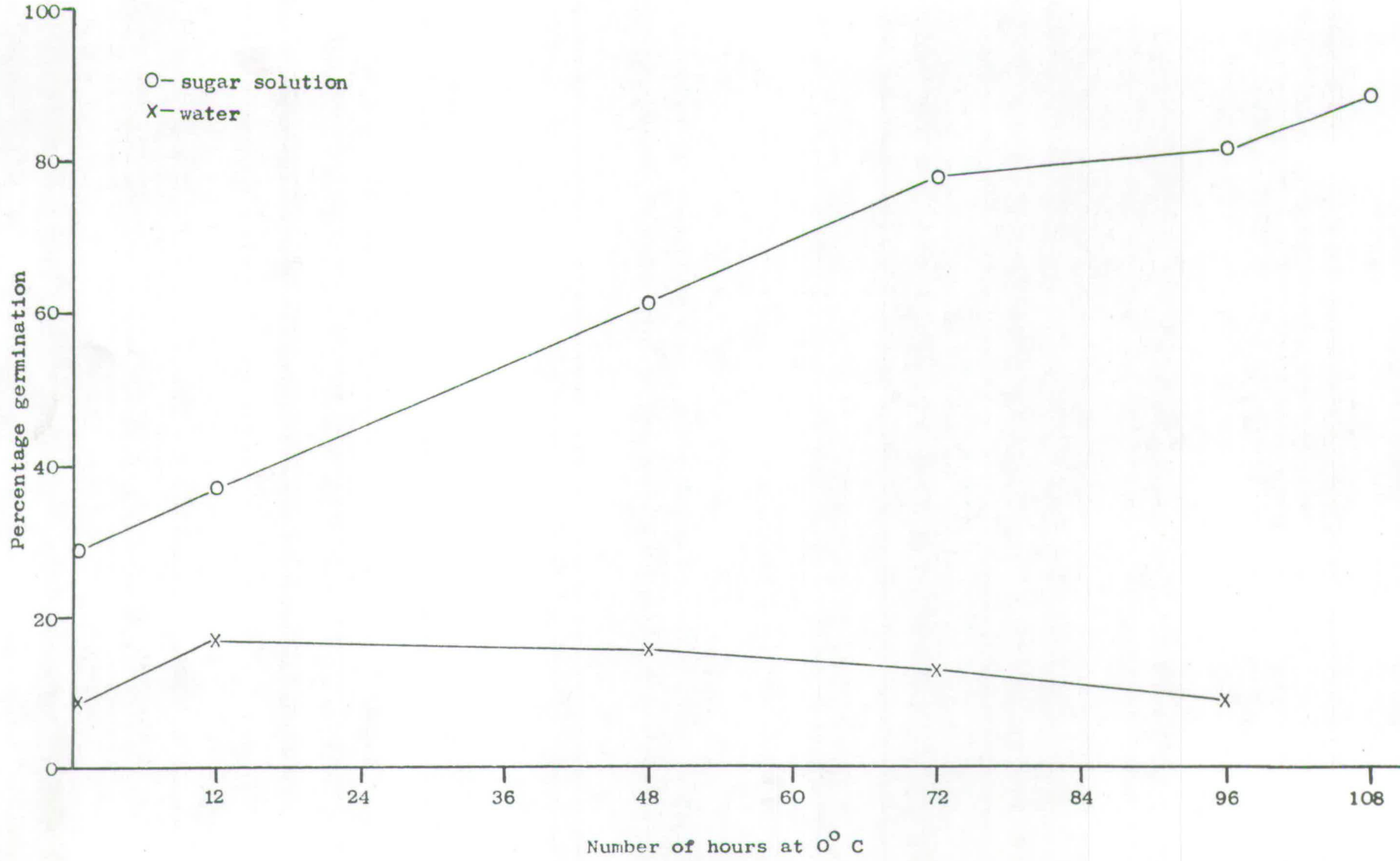


Figure IV-10 The effect of different lengths of 0° C treatments on M. laricis spore germination in water or sugar solution.

Effect of leaves on germination

It has been demonstrated in the preceding experiment that M.laricis spores are capable of responding to nutrients in their environment. The conditions in which the responses were demonstrated were very artificial and it was now considered desirable to assess spore germination in response to actual leaf leachates. Leaves of two ages and variously treated leaves were removed from a seedling and inserted into the germination liquid of a Van Tieghem cell. To assess the effect of leaf leachate and leaf age etc. and to avoid interference from leaf microfloras, leaves from aseptically grown seedlings were used. Leaf weathering was imitated by rubbing some leaves with a cloth. Other leaves were artificially damaged by repeating cutting of the epidermis with a scalpel. The leaves were taken from a three month old seedling. The spore suspension was kept at $0^{\circ}C$ for 96 hours prior to the germination trials at $20^{\circ}C$. The treatments, each replicated three times, were:

i. water control,

ii. young leaves less than one month old,

iii. old leaves two and a half months old,

iv. young leaves which had been rubbed with a cloth,

v. old leaves which had been rubbed with a cloth,

vi. young leaves damaged by scalpel cuts,

vii. old leaves damaged by scalpel cuts.

The results are summarized in Table IV-5.

TABLE IV-5: Percentage germination of M.laricis spores in leaf treatments (mean % germination; mean and variance of transformed data.

	H_2O	Young	Old	Young rubbed	Young damaged	Old rubbed	Old damaged
$\bar{x}$	13.9	23.6	24.3	34.8	33.2	32.9	24.8
$\bar{x}$ transformed	21.6	28.8	29.4	36.1	35.1	34.9	29.5
variance	8.9	38.1	12.1	3.3	9.9	2.6	3.8

The variances of the treatments showed a wide range with the variance for the young leaf treatment being much larger than for any other treatment. In addition, one replicate in each of the three old-leaf treatments was damaged and had to be discarded resulting in unequal observations in treatments. These two factors, unequal variance and unequal sample size, made it inadvisable to use the standard analysis of variance. Therefore, the variance for each treatment was calculated separately and individual treatment comparisons were tested for significance using the t-test (Snedecor and Cochran, 1967).

All leaf treatments resulted in a significantly ($p = 0.05$) higher percentage germination of M.laricis spores than in water control. Old and young leaves stimulated germination to about the same degree.

The rubbing treatment significantly (p = 0105) increased germination over unrubbed leaves in both young and old leaf treatments. However, in the damaged leaf treatments, spore germination was only increased by damaged young leaves. Damaged old leaves resulted in no change compared to undamaged old leaves.

The high variance of 38.1 for unrubbed young leaves sharply contrasted with a low variance of 3.3 for rubbed young leaves. The three replicates of the unrubbed young leaves were analyzed by an analysis of variance as a Completely Randomized Design and the difference between replicate means was significant (p = 0.01). Thus, young leaves showed a significant difference between each other in their effect on spore germination. The rubbed leaves, however, showed a much more uniform effect on germination. The rubbing treatment may have eliminated the factor or factors causing variability by removing surface wax or by making the leaf cuticles more permeable.

Effect of bacteria on germination in SEYE

The seven phyllosphere bacteria that were assessed for their effect on C.herbarum spore germination were used in a test of the germination of M.laricis spores. The bacteria were grown in shake cultures of soil-extract-yeast-extract (SEYE) for 24 hours. M.laricis spores were kept at $0^{\circ}C$ for 48 hours prior to the trials. Each Van Tieghem Cell was made up of 5 parts sterile distilled water, 1 part spore suspension and 1 part sterile SEYE or bacterial suspension. The treatments were:

i. control, sterile SEYE,

ii. B.mycoides in SEYE,

iii. S-12 (Gram-positive rod) in SEYE,

iv. S-79 (*Flavobacterium* sp.) in SEYE,

v. S-92 (*Pseudomonas* sp.) in SEYE,

vi. S-95 (*Bacillus* sp.) in SEYE,

vii. S-100 (*Pseudomonas* sp.) in SEYE,

viii. S-117 (cream coloured Gram-negative rod) in SEYE.

Treatment iii, with S-12, became contaminated and was not included in the results or analysis. The results of the other treatments are summarized in Figure IV-11.

All treatments containing bacteria showed a significantly ($p = 0.01$) lower germination than the sterile SEYE control. The greatest reduction in germination was caused by S-95 (*Bacillus* sp.) and S-100. In the bacterial treatments the non-germinating thin-walled cell of the spore sometimes collapsed or disappeared altogether. The germ tubes were often heavily colonized by bacteria. The darkened, thick-walled cell of germinating spores was never seen to be collapsed or colonized by bacteria. The spores in the treatment with S-95 appeared heavily vacuolated.

M.laricis spore germination was affected by all bacteria tested. The mechanism of inhibition may be connected with the visible changes observed in some spores, that is, heavy vacuolation and cell collapse.

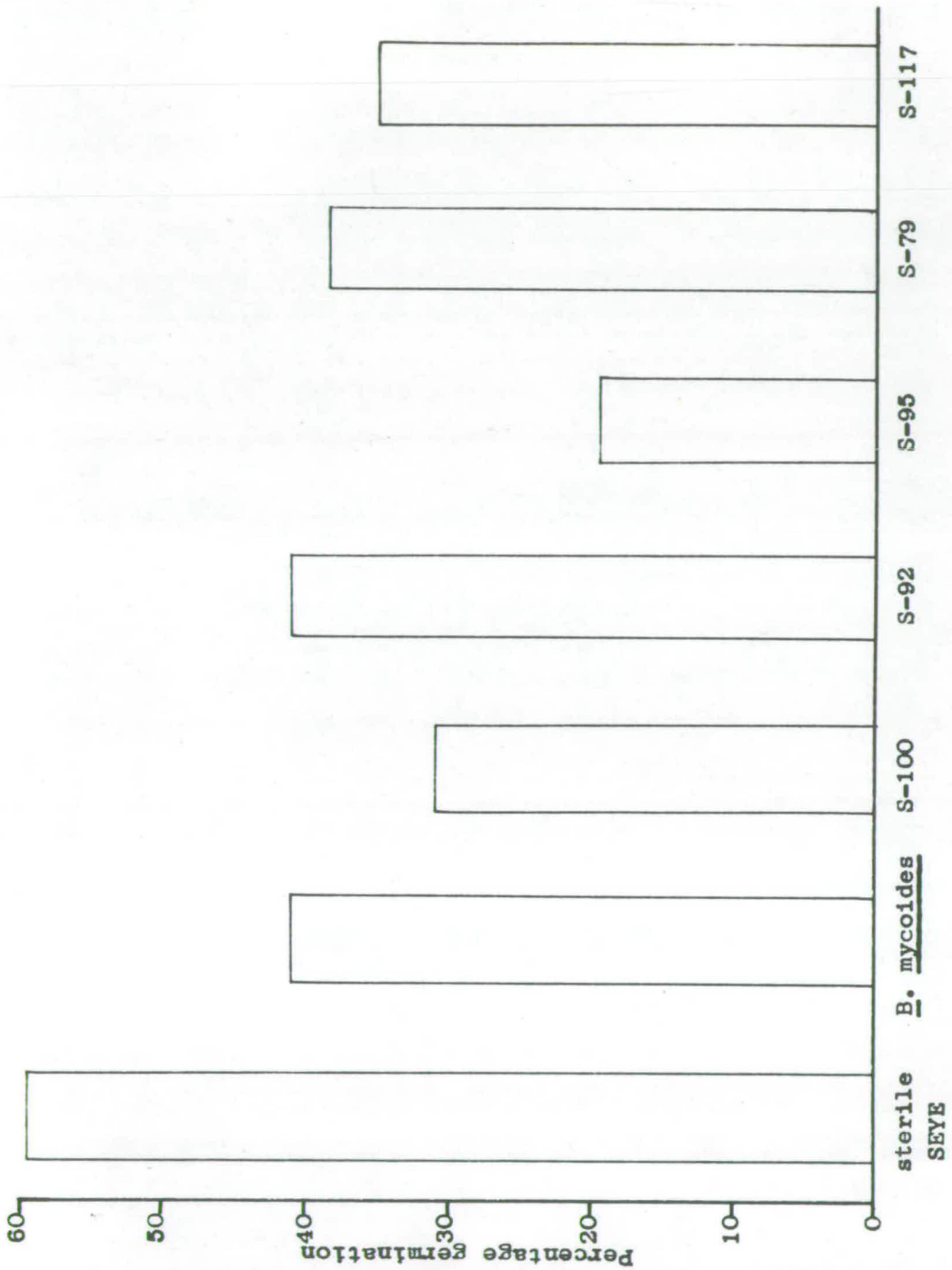


Figure IV-11 The effect of phyllosphere bacteria on the germination of M. laricis spores in SEYE solution.

Effect of bacteria on germination in a sugar solution.

The inhibitory effect of bacteria on M.laricis spore germination was observed in a relatively rich nutrient solution (SEYE). The inhibition mechanisms may not be operative in conditions of low nutrient. An experiment was designed to assess germination inhibition in a solution containing 50 millimoles of sugar. The sugar solution contained equal molar quantities of fructose, glucose and sucrose. The bacteria were grown on SEYE agar. Bacteria were removed from the agar with a wire loop and suspended in sterile distilled water. The M.laricis spore suspension was prepared in the usual way and kept at $0^{\circ}C$ for 48 hours. Some nutrients would be present in the spore suspension because of the method of spore harvest. Also, the bacterial suspension may have added nutrients from leaching and in the case of B.mycoides the low growth form may have led to agar media being disturbed when collecting the bacteria. The treatments were:

i. Control 50 millimoles sugar solution,

ii. B.mycoides in 50 millimoles sugar solution,

iii. S-100 in 50 millimoles sugar solution,

iv. S-95 in 50 millimoles sugar solution.

The results of this experiment are summarised in Figure IV-12. S-100 shows no inhibition or stimulation of spore germination. Both B.mycoides and S-95 significantly reduced spore germination ($p = 0.01$) with B.mycoides having the most marked effect.

It can be seen that the inhibition demonstrated by S-100 in SEYE did not apparently operate in the low nutrient conditions of this experiment. The inhibition by B.mycoides and S-95, however, was still

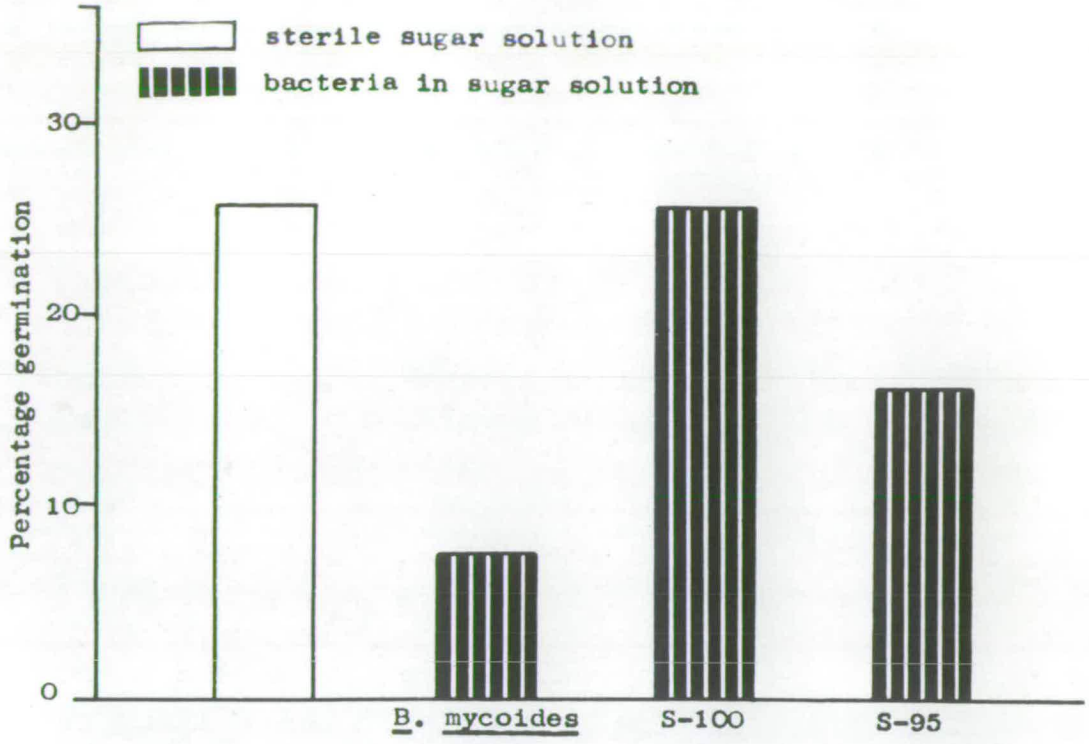


Figure IV-12 The effect of phyllosphere bacteria on the germination of M. laricis spores in a sugar solution.

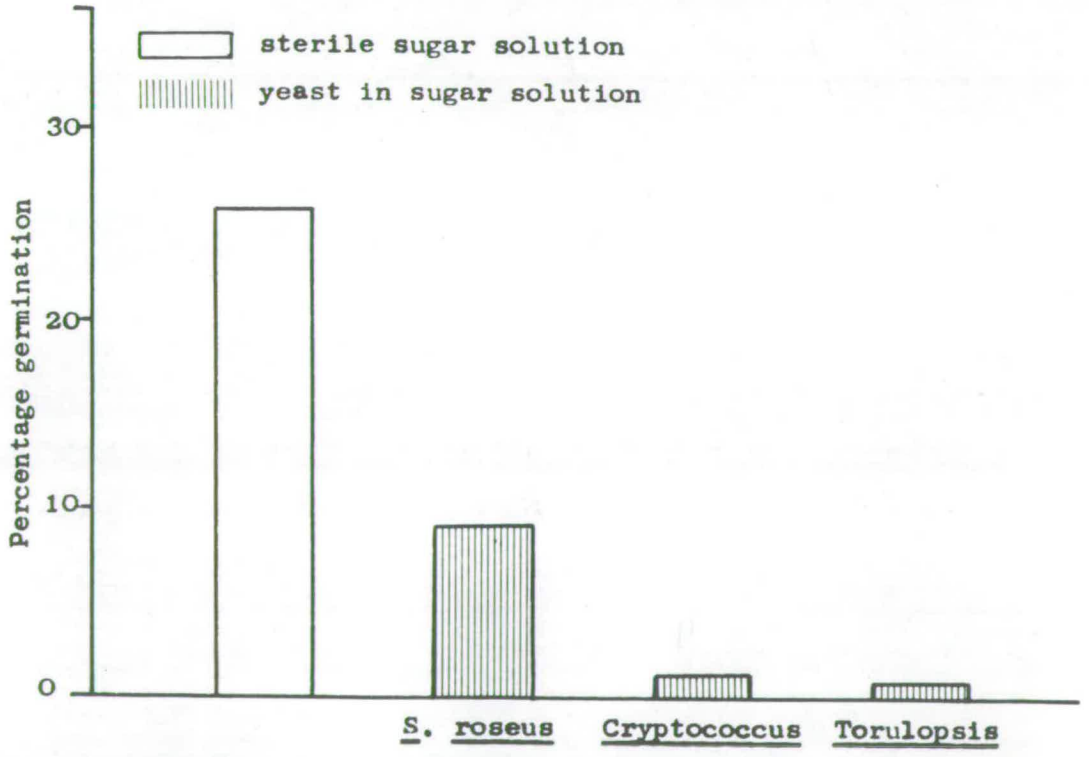


Figure IV-13 The effect of three phyllosphere yeasts on the germination of M. laricis spores in a sugar solution.

apparent. It would be improper to compare the relative inhibition caused by B.mycoides and S-95 in this experiment because the difficulty in preparing a media-free suspension of the former.

Effect of yeasts on germination in a sugar solution.

A parallel experiment was performed to test the effect of phylloplane yeasts on M.laricis spore germination. The treatments were prepared by the methods used in previous experiments and were:

i. Control 50 millimoles sugar solution,

ii. S.roseus in 50 millimoles sugar solution,

iii. Cryptococcus sp. (S-101) in 50 millimoles sugar solution,

iv. Torulopsis sp. (S-41) in 50 millimoles sugar solution.

The results of this experiment are presented in Figure IV-13. The germination of spores in all three yeast suspensions was significantly lower than in the sterile control ($P = 0.01$). Germination in Cryptococcus sp. and Torulopsis sp. suspensions was significantly lower than germination in the S.roseus suspension ($P = 0.01$).

Spore germination with S.roseus in water and in sugar solution.

The possibility of an interaction between fungistasis caused by microorganisms and nutrient level has been indicated in C.herbarum germination tests and in tests with bacteria M.laricis. An experiment was designed to examine the effect of S.roseus on M.laricis spore germination at high and low nutrient levels. The nutrient provided was 500 millimoles of sugar made up of equal molar quantities of glucose, fructose and sucrose. The spores were stored at $0^{\circ}C$ for 72 hours before the experiment started. The treatments were:

i. Control sterile distilled water,

ii. Control sterile 500 millimoles sugar solution,

iii. S.roseus in distilled water,

iv. S.roseus in 500 millimoles of sugar solution,

An interaction between fungistasis caused by S.roseus and nutrient level was apparent (Figure IV-14). In distilled water the presence of S.roseus stimulated germination from an average of 13.8% in the water control to an average of 38.7% in the yeast-water treatment. In the sugar solution the presence of S.roseus inhibited germination from an average of 76.0% in the sterile sugar solution to 41.3% in the yeast-sugar treatment. Both the stimulation associated with S.roseus in water and the inhibition associated with S.roseus in the sugar solution were significant at $p = 0.01$.

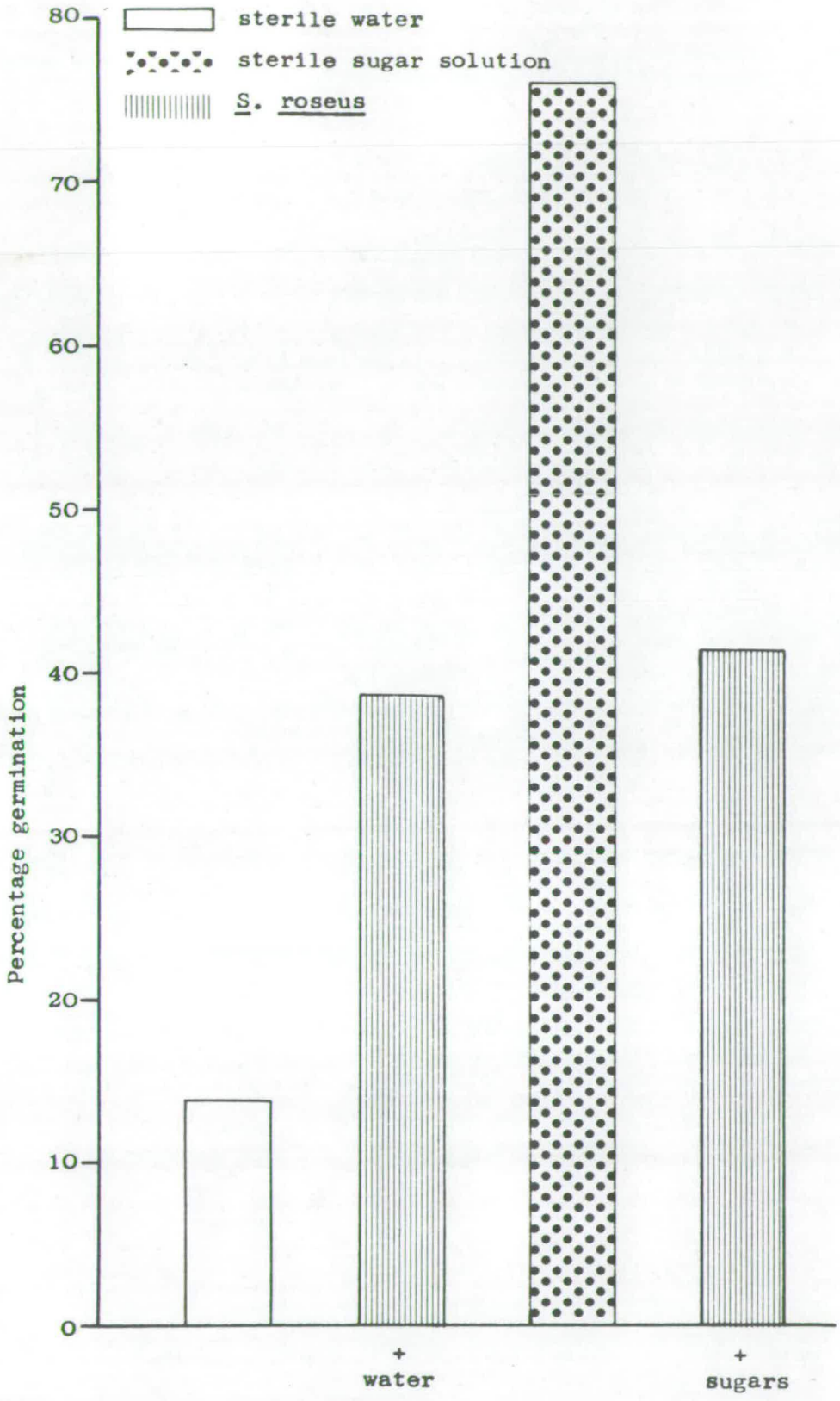


Figure IV-14 Spore germination of M. laricis with S. roseus in water and in a sugar solution.

INOCULATION EXPERIMENTS

In the initial phase of studying spore germination, *in vitro* methods were preferable to *in vivo* methods because of the relative ease with which conditions can be controlled in the former. Difficulties of observation of spores *in vivo* would greatly restrict the number of replicates and variables which could be studied. It would, however, be inadvisable to allow the initial *in vitro* phase to become the final objective. Hypotheses developed from *in vitro* observations were tested *in vivo* by incubation studies on larch seedlings.

CLADOSPORIUM HERBARUM ON LARCH LEAVES

Methods

The effects of S.roseus and the bacterium S-100 have been well established for in vitro conditions in Van Tieghem cells. It was now considered of importance to determine if similar phenomena occurred in vivo on the leaf surface.

A technique was required in which mycelium-free leaves could be treated and inoculated with C.herbarum spores. The condition of the spores and hyphal development would then have to be assessed by direct microscopic observation.

The silicone leaf stripping technique described in Chapter II (page 53) was used on 3-month-old, greenhouse-grown seedlings. In contrast to field-grown seedlings of similar age, no mycelium was observed on any of the seedling leaves. The difference between field and greenhouse seedlings may have been due to many factors including low humidity and reduced airspora in the greenhouse. The greenhouse-seedlings provided non-sterile butmycelium-free leaf surface for inoculation experiments.

Three, five-inch greenhouse pots each containing four, three-month-old seedlings were selected for an experiment to determine the effect of S-100 and S.roseus on C.herbarum development. C.herbarum spore suspensions, S-100 and S.roseus cell suspensions were prepared in the same manner as in the spore germination experiments. The following treatments were applied to the seedlings with a hand atomizer until all leaves were dripping.

i. water control,

ii. S.roseus in water,

iii. S-100 in water.

The seedlings were then allowed to dry before being thoroughly sprayed with the C.herbarum spore suspension.

The inoculated seedlings were then enclosed in wetted polythene bags and placed in a growth chamber. The growth chamber was scheduled to provide 18 hour, 16°C days and 6 hour, 11°C nights. The temperatures were determined from the average maximum and minimum temperatures recorded in the larch seedbed for July and August during the 1968 field microflora study.

After three days the polythene bags were removed. When the leaves had dried, a sample was taken for preparation of silicone strips. A pair of leaves from each of three positions, lowest centimeter, middle and top centimeter of the plumule were removed. For each pair of leaves a strip was prepared from an upper and lower surface. After staining with dilute acid fuchsin the percentage spore germination was determined for the first 200 spores encountered. The procedure was repeated 20 days after inoculation and the length of hyphae per square centimeter was determined. The same method as described in Chapter II for measuring hyphae with a camera lucida and an opisometer was used. The results are summarized in Table IV-6.

Results.

TABLE IV-6: C.herbarum spore germination and hyphal development on leaves of larch seedlings.

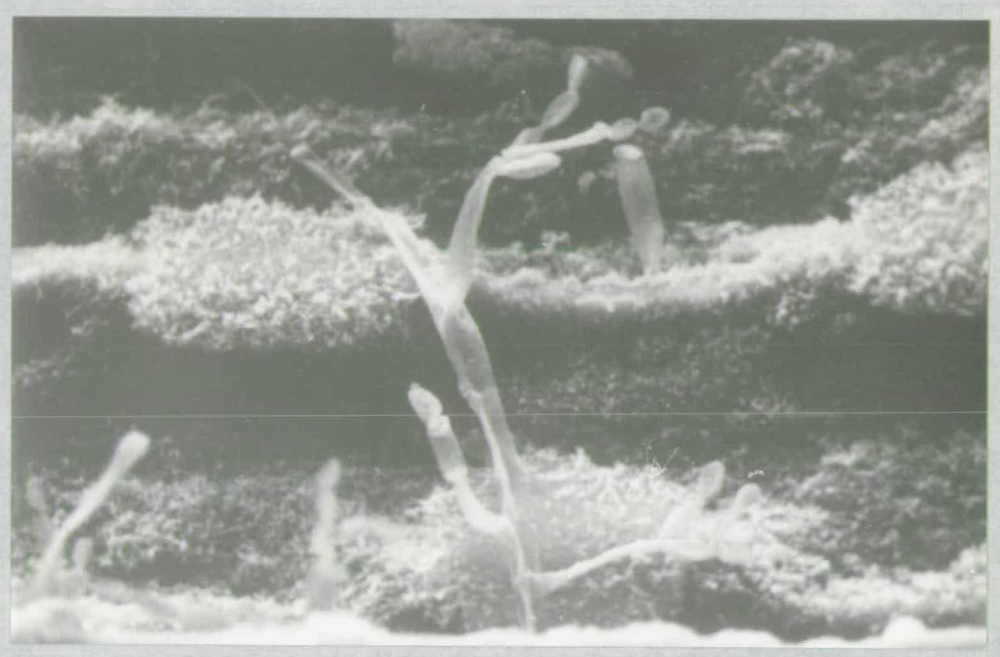
		Position of leaf					
		Bottom		Middle		Top	
Treatment	Surface .	%	u/cm^2	%	u/cm^2	%	u/cm^2
Water	lower	36.5	659	42	132	0	0
	upper	32.0	233	22.5	47	0	0
S-100	lower	30.0	343	20.0	180	0	0
	upper	24.0	210	29.0	82	0	0
S.roseus	lower	1.5	0	0	0	0	0
	upper	0	0	0	0	0	0

Spore germination was greatest on bottom and middle leaves treated with water & S-100. No germination was observed on any leaves from the tops of the plumule. On leaves treated with S.roseus the only germination observed was on the lower surface of the bottom leaf and was only 1.5%.

Hyphae on the leaves of seedlings treated with water & S-100 developed in a pattern similar to that observed on seedlings grown in the field in 1968 (p. 46). The mycelium developed most extensively on the lower surface of the oldest leaves. No hyphae were observed on the top leaves in any treatment. The S.roseus treatment resulted in complete inhibition of hyphal development on all leaves sampled. Conidia were produced on a number of the oldest leaves (Plate IV-7 and IV-8).

PLATE IV-7 CLADOSPORIUM HERBARUM ON THE LOWER SURFACE OF A LARCH LEAF. X 1350

PLATE IV-8 CONIDIOPHORE OF CLADOSPORIUM HERBARUM BEARING CONIDIA ON A LARCH LEAF. X 2200



Although the experiment involved only a relatively small number of observations, the results are of interest for a number of reasons. Firstly, on control seedlings sprayed with water the hyphae developed in a manner which closely followed the pattern observed on field-grown seedlings in 1968. This similarity might suggest that the conditions of inoculation approximated the natural field situation. Secondly, S.roseus inhibited spore germination on the leaf surface in an exactly similar manner to that observed under low nutrient conditions in vitro. Thirdly, the S-100 treatment, which only inhibited spore germination under high nutrient conditions in vitro did not affect spore germination on larch leaves.

Since inoculation experiments were extremely time consuming it was decided to concentrate on the economically more important leaf fungus M.laricis in further studies.

MERIA LARICIS ON LARCH LEAVES

Methods

Artificial inoculation of larch with M.laricis has been attempted by Peace and Holmes (1933) and more recently by Biggs (1964). Peace and Holmes used spore suspensions prepared by macerating diseased leaves in water. Plants inoculated with the spore suspension were kept under humid conditions until symptoms developed in about 14 days. They normally attained 80-100% infection by this method. However, the technique is unsuitable for phylloplane interaction studies because macerating the leaves leads to a spore suspension in undefined nutrient and with too much debris for centrifuge washing of the spores. Biggs (1964) used spores suspensions prepared from M.laricis agar cultures and attained inconsistent infection results. He also was troubled by high temperatures (30°C) which killed spores and by "Botrytis damage" to plants kept in humid conditions. If the interactions of phylloplane organisms with M.laricis infection were to be studied successfully, a procedure which yielded a consistently high level of infection and in which spores could be applied in water or a defined nutrient suspension was required.

The conditions of the experiments should also closely approximate natural field conditions. Biggs (1964) determined that the majority of natural infections occurred when a combination of relative humidity over 90% and temperature above 10°C occurred for at least 48 hours.

An infection procedure was developed which satisfied these requirements. It consistently resulted in 100% infection of untreated seedlings although there was frequently considerable variation in the amount of discoloured foliage on different seedlings. The procedure used in all M.laricis infection experiments was as follows:

1. M. laricis strain "a" was grown on Czapek-Dox agar slants (2.5% agar) with 0.25% yeast extract in one ounce universal vials. The cultures were grown on the laboratory bench in natural light and in temperatures varying from 16°C to 20°C.

2. Spore suspensions were prepared by adding sterile distilled water to four-week-old cultures and shaking on a wrist-action shaker for three minutes. The resulting spore suspension was then centrifuged at 3,500 R.C.F. for ten minutes. After decanting the supernatant the spores were resuspended in sterile distilled water.

3. The spore suspension was stored at 0°C for 48 hours and then applied to the seedlings with an "Atomist" atomizer. The atomizer, originally designed to be operated by a hand pump, was attached to a small, electric compressor by tubing containing sterile cotton wool as a filter. The modified atomizer gave a steady drenching spray. Upper and lower surfaces of the leaves were sprayed until dripping.

4. L.decidua seedlings were grown in 5" diameter pots in the greenhouse. Before inoculation moist cotton was placed around the rim of the pot. After inoculation the pot was covered with a wetted polythene bag.

5. The seedlings were then placed in a controlled environment room with 16°C, 18 hour days and 11°C, 6 hour night.

6. After 4 days the polythene bags were removed. Disease symptoms developed on the leaves approximately 15 days after inoculation.

The amount of infection was assessed by counting the number of brown needles on each seedling. To verify that browning of the leaves was associated with M.laricis, a number of leaves from each treatment were mounted in Trypan blue. The presence of M.laricis conidia emerging from the stomata was taken as conclusive evidence of infection.

The infection experiments involved treatment of seedlings with different bacteria and yeast suspensions. The suspensions used in the experiments were prepared by the same procedures as used in the spore germination experiments and were applied to the seedlings with an atomizer in the same manner described above for M.laricis spore suspensions. The microflora or nutrient suspensions were sprayed 24 hours before M.laricis was applied. During this time the seedlings were covered by wetted polythene bags. Prior to M.laricis application the bags were removed and the seedlings allowed to dry. After M.laricis application the bags were replaced. After a further four days the bags were removed. When the seedlings were dry, several juvenile leaves were removed from the seedlings with flamed forceps for assessment of the microflora population. The samples were placed in sterile one ounce universal vials, weighed and then vigorously shaken with 10 ml of 0.1% yeast extract solution for five minutes. The resulting suspensions were serially diluted and incubated in the SEYE agar at 25°C for six days.

The microflora suspension was prepared by shaking rather than by homogenizing because of the convenience of the former method when dealing with a large number of samples.

It was originally planned to design all infection experiments as Randomized Complete Block Designs with each treatment replicated in three pots with a number of seedlings in each plot. Two factors arose which made this procedure impracticable or undesirable. These were (1) uneven sample size caused by death of seedlings due to root rot, and (2) the variation in infection intensity from seedling to seedling. Consequently the experiments were analyzed as Completely Randomized Designs and differences between pairs of treatments were examined with the t-test, using treatment variances rather than the pooled variance.

Results

Effect of a mixture of bacteria in SEYE

A mixture of bacteria which had been shown to inhibit M.laricis spore germination in vitro was used in the first attempt to alter the leaf microflora and examine its effect on M.laricis development. Three bacteria, Bacillus mycoides, Bacillus S-95 and S-100, were selected. To test the effect of the bacteria with a minimum of treatments the bacteria were applied as a mixture which was prepared from pure cultures immediately prior to application to the seedlings. Approximately 20 two-month-old seedlings were used in each treatment. The treatments were:

i. sterile water control,

ii. B.mycoides, Bacillus S-95 and S-100 in water,

iii. sterile SEYE,

iv. B.mycoides, Bacillus S-92, and S-100 in SEYE.

In addition, sterile water was applied to some seedlings to which no M.laricis spores were applied. This was done in order to assess the effect of the spores on microflora numbers.

The treatments had marked effects on the size of the leaf microfloras. The average number of bacteria and yeast colonies per gram of foliage is given in Table IV-7.

TABLE IV-7: The average number of colonies of bacteria and yeast isolated per gram of foliage.

Water	water + M. laricis	Bacteria + water	SEYE	Bacteria + SEYE
1,300	2,700	25,000	61,000	70,000

The addition of M.laricis spores to the water control seedlings resulted in a two-fold increase in the microflora compared with the water control seedlings to which no spores were applied. The application of bacteria in water increased the microflora twenty-fold while sterile nutrients raised the number by over forty times. The combination of bacteria in nutrients resulted in the largest microflora. Thus nutrients appeared to be more effective than inoculum in increasing the microflora, while the combination of inoculum and nutrients was the most effective of all treatments.

The amount of leaf damage by M.laricis was assessed 20 days after inoculation. The average number of diseased leaves per seedling in the four treatments is shown in Figure IV-15. An average of 15 leaves per seedling became diseased in the water control. The

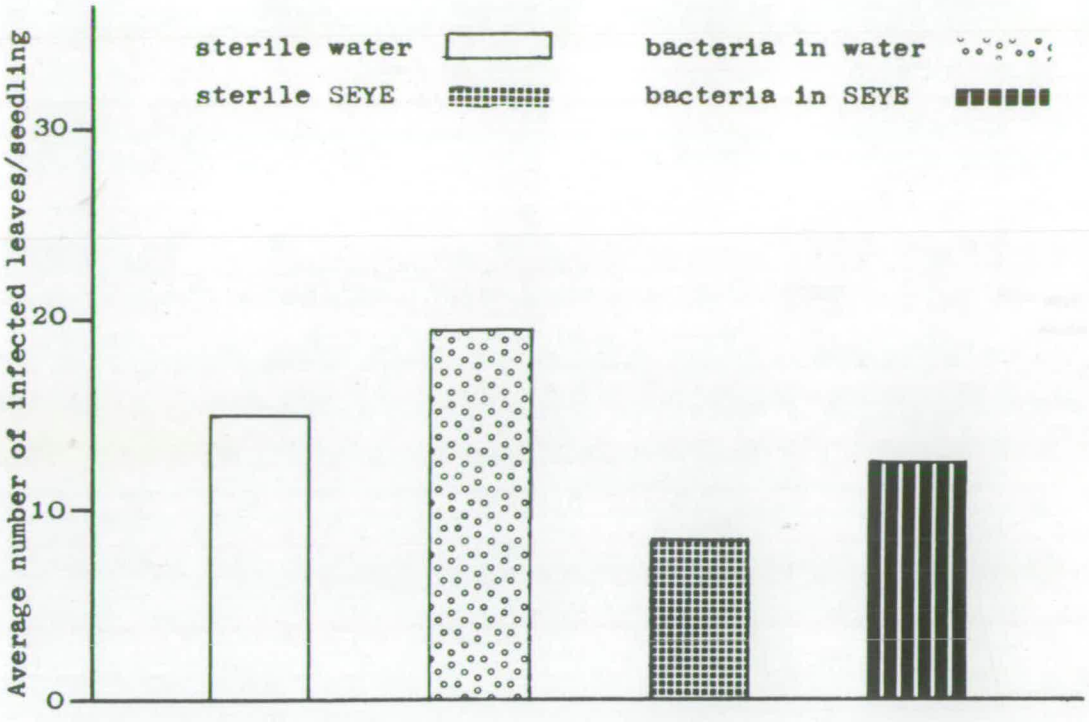


Figure IV-15 The average number of leaves infected with M. laricis on seedlings treated with bacteria and SEYE solution.

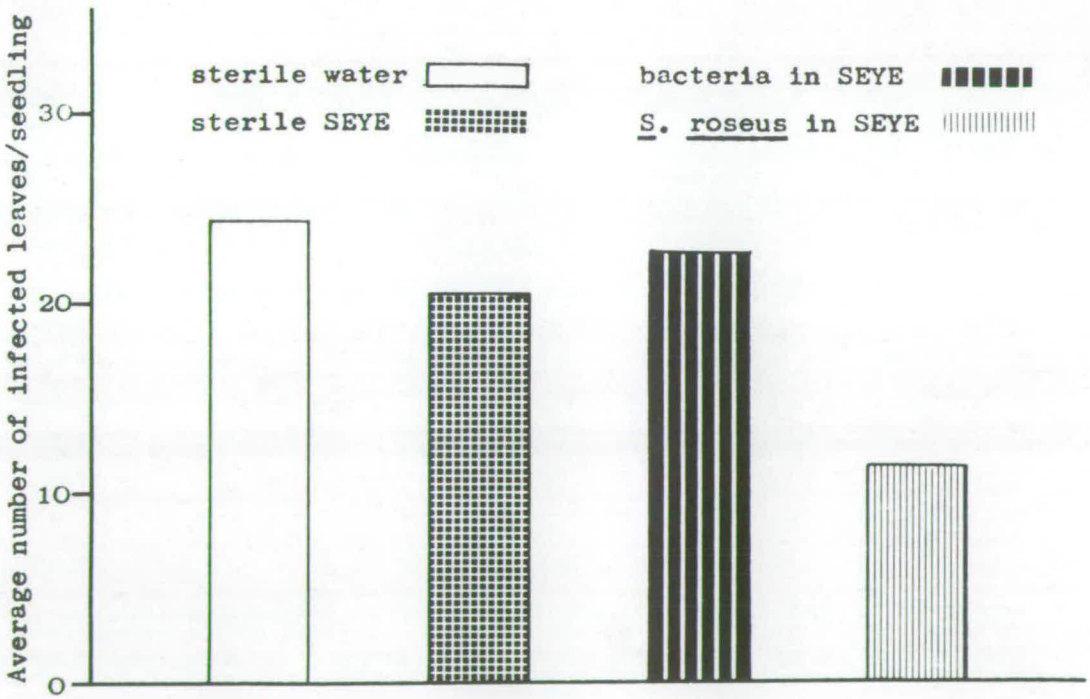


Figure IV-16 The levels of disease caused by M. laricis on seedlings treated with bacteria (S-100) or S. roseus in SEYE solution.

aqueous suspension of bacteria resulted in an increase in disease to 19.4 leaves per seedling, which was significant at p = 0.05. The two treatments which resulted in the highest microflora number, i.e. sterile SEYE and bacteria in SEYE reduced infection compared to the water control. However, only the reduction associated with sterile SEYE was statistically significant (p = 0.01). The mechanism of disease reduction associated with sterile SEYE was not determined. It may have acted as a chemical inhibitor or it may have stimulated the natural microflora of the leaves to an inhibitory level. If the latter case were so then the normal flora would appear to be more effective than the three selected bacteria in controlling M.laricis. Similarly, the mechanism by which bacteria in water caused an increase in disease is not known. The bacteria may have provided nutrients which stimulated spore germination or fungal growth.

Effect of S-100 and S.roseus in SEYE.

A second experiment using different microorganisms was carried out since the results of bacterial treatments were inconclusive. The procedure was the same as previously used, but bacterium S-100 and S.roseus were the test organisms. Thus the treatments were:

i. sterile water control,

ii. sterile SEYE,

iii. S-100 in SEYE,

iv. S.roseus in SEYE.

A total of seven four-month-old seedlings were used in each treatment.

The microflora assessment data is summarized in Table IV-8.

TABLE IV-8: The average number of bacteria and yeasts isolated per gram of foliage.

	H_2O	SEYE	SEYE+ S-100	SEYE + S.roseus
Bacteria	5,700	50,000	5,400,000	-
Yeasts	200	20,000	-	4,500,000

The proportions of bacteria and yeasts were determined by making microscope mounts of all colonies occurring onpportions of dilution plates. No yeasts were found in dilution plates of the bacterial treatment and no bacteria were found in dilution plates of the yeast treatment. This observation does not mean that bacteria or yeast did not occur in the microflora but rather that they were in such a small proportion as not to occur at the high dilution levels.

Although the sterile SEYE treatment resulted in approximately the same size of microflora as in the previous experiment, the S-100 in SEYE treatment resulted in a microflora almost 100 times as large as the previous bacteria in SEYE treatment.

The number of yeasts in the microflora was increased 100 times by the sterile SEYE treatment. S.roseus in SEYE increased the yeast count from 200 per gram to 4.5 million per gram.

The average level of disease development in the four treatments is summarized in Figure IV-16. Infection was more severe than in the previous experiment. The level of disease was similar in the water

control, sterile SEYE and S-100 in SEYE treatments. The S.roseus treatment resulted in a marked reduction in disease, but due to the small number of seedlings and large variance, the reduction was not significant at p = 0.05.

In the first inoculation experiment the bacterial treatment included two species of Bacillus and S-100. The Bacillus spp. occurred only infrequently in the natural larch microflora while S-100 was very common. The application of the three bacteria in SEYE resulted in only 70,000 bacteria/gram whereas when S-100 was applied by itself in SEYE the microflora increased to 5,400,000 bacteria/gram. Although no firm conclusions can be drawn from results of the two different experiments, it would appear that the two Bacillus spp. inhibited the development of the artificial microflora.

Effect of S-100 in Nutrient Broth.

As the in vitro spore germination trials proceeded it became apparent that bacteria required high nutrient conditions in order to inhibit the germination of M.laricis spores. The variability of infection intensity encountered among seedlings within a treatment indicated that a large number of seedlings would have to be used in each treatment if statistical significance was to be established. A third infection experiment was, therefore, carried out repeating the bacterial treatment with Nutrient Broth and using 18 to 20 seedlings per treatment. Oxoid Nutrient Broth contains beef extract and peptone as well as yeast extract and was a much richer nitrogen and carbon source than soil extract with 0.1% glucose and 0.1% yeast extract.

The treatments were:

i. sterile water control,

ii. 1% nutrient broth (Oxoid),

iii. S-100 in 1% Nutrient Broth.

The numbers of organisms isolated from foliage in the three treatments are summarised in Table IV-9.

TABLE IV-9: The average number of bacteria and yeasts isolated per gram of foliage.

H_2O	Nutrient Broth	S-100 + Nutrient Broth
1,670	90,600	31,000,000

The use of sterile nutrient broth stimulated the natural seedling microflora 1.5 times more than the sterile SEYE treatment of the previous experiment. Moreover, the application of S-100 in nutrient broth increased the microflora population 6 times more than had S-100 in SEYE. The population of 31 million bacteria per gram attained with S-100 in nutrient broth was higher than any population recorded in the field study.

The average numbers of infected leaves per seedling for the treatments are summarized in Figure IV-17. Sterile nutrient broth significantly reduced infection compared to the water control ($p = 0.01$). Moreover, S-100 in nutrient broth resulted in a significantly lower level of infection than either the water control or the sterile nutrient ($p = 0.01$).

The results are interesting in that a nutrient rich medium was required for disease reduction just as a nutrient rich medium was required for bacterial inhibition of spore germination *in vitro*. The reduction of disease associated with the sterile nutrient treatment may

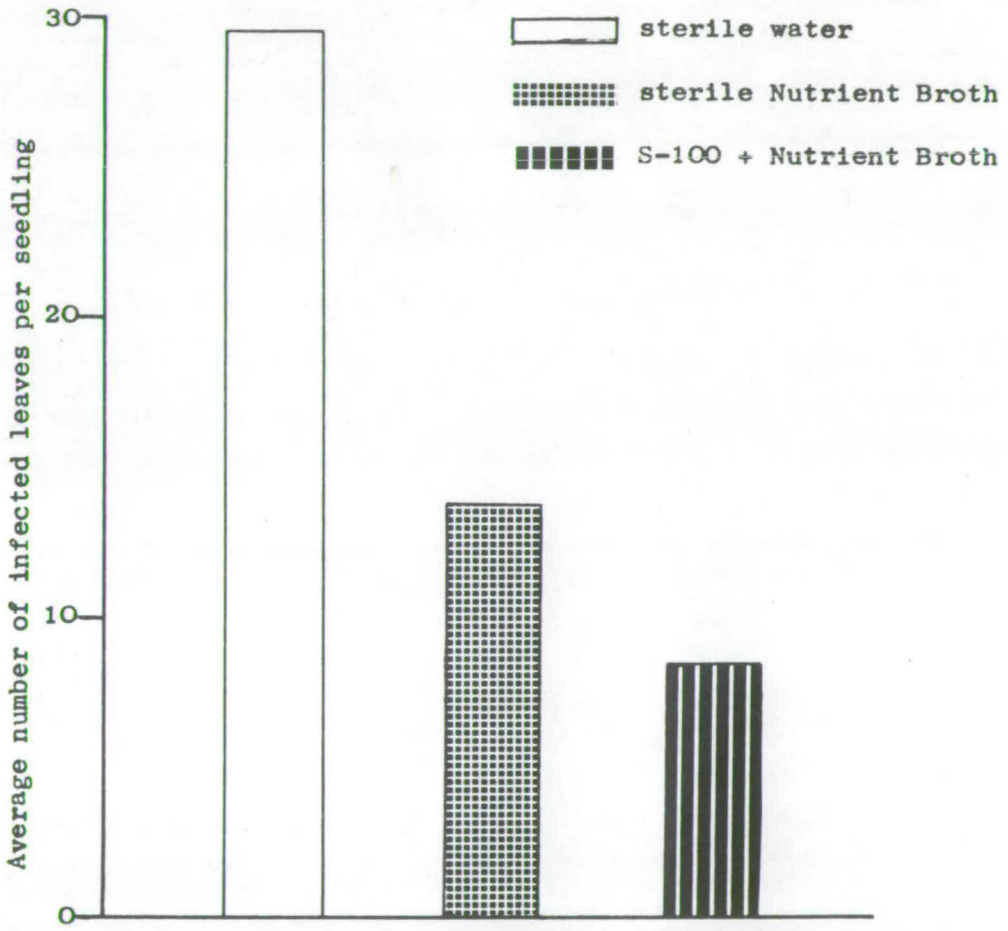


Figure IV-17 The levels of disease caused by M. laricis on seedlings treated with bacteria (S-100) and/or Nutrient Broth.

be a result of a chemical inhibition or of an inhibitory effect from the increased microflora. The latter explanation appears most plausible because previous experiments with M.laricis spores indicated that external nutrients stimulated spore germination.

Bacteria and yeasts in a number of different treatments.

A fourth inoculation experiment was designed to replicate the earlier trial with S.roseus, to examine the effect of other leaf yeasts, and to investigate further the nature of the inhibition of M.laricis by bacteria. The bacteria and yeast treatments were run simultaneously to provide comparisons of effect and so that labour and seedlings could be reduced by using the same control for all treatments. However, to make the presentation of data more lucid the bacteria and yeast sections will be described separately.

Each treatment included a total of 12 to 24 seedlings contained in three pots. The experiment included the following treatments:

a) Controls

i. sterile water,

ii. sterile 1% glucose solution,

iii. sterile 1% nutrient broth,

b) Bacterial treatments

iv. cell-free culture medium of S-100,

v. S-100 in water,

vi. S-100 in nutrient broth,

vii. cell-free culture medium of B.mycoides,

viii. B.mycoides in water,

ix. B.mycoides in nutrient broth,

c) Yeast treatments

x. S.roseus in 1% glucose solution,

xi. Torulopsis sp. in 1% glucose solution,

xii. Cryptococcus sp. in 1% glucose solution.

Bacteria were grown in 1% nutrient broth shake culture for 24 hours. One half of the cultures were then centrifuged at 3,000 R.C.F. for 10 minutes. The resulting supernatant was decanted and applied as the cell-free culture medium of the bacteria. The precipitated bacteria were resuspended in sterile water and applied as the bacteria in water treatments. The remaining half of the cultures were applied as the bacteria in nutrient broth treatments.

Yeast treatments were prepared by removing yeast cells from 48 hour SEYE agar cultures with a wire loop and suspending them in sterile 1% glucose solutions. The suspensions were then standardized by adjusting the opacity (25 at 525 mμ compared to 1% glucose blank).

The standard inoculation procedure was followed. Unfortunately the microflora assessment results were spoiled by a faulty batch of media and thus no estimate of the microflora populations was obtained.

The intensity of disease development is summarized in Figure IV-18. The number of diseased leaves on each seedling is shown so that the range of disease intensity in each treatment can be seen.

Firstly, a comparison of the sterile controls indicates that infection was similar in sterile water and sterile nutrient broth. However, the sterile glucose control resulted in a disease level significantly lower ($p = 0.01$) than the sterile water control.

Figure IV-18 The number of diseased leaves (M. laricis) on seedlings treated with different nutrients, bacteria, or yeasts.

The bacterial treatments had a wide range of effects on disease level. Both B.mycoides and S-100 in nutrient broth resulted in a significant reduction in disease ($p = 0.01$) compared to sterile water and sterile nutrient broth controls. The same bacteria in water did not significantly reduce disease compared to the sterile water control. The cell-free culture media of both bacteria reduced infection compared to the sterile nutrient control, but only the reduction associated with the S-100 medium was significant ($p = 0.01$).

Thus, the disease of larch leaves caused by M.laricis was effectively reduced by the application of either S-100 or B.mycoides in nutrient broth. An inhibitory factor was present in the cell-free culture media but none was evident when the bacteria wase applied in water without nutrients.

Two of the yeast treatments also resulted in a reduction of infection by M.laricis. The application of S.roseus or Torulopsis sp. significantly reduced the number of diseased leaves compared to the water and glucose controls ($p = 0.01$). Cryptococcus sp. did not markedly alter the disease intensity compared to the water and glucose controls.

It is difficult to interpret the results when no information is available on the size of the microflora on leaves in the different treatments. From in vitro tests on spore germination, it would be expected that glucose would stimulate M.laricis. However, it may have stimulated the natural microflora resulting in less severe infection.

The interaction between bacteria, nutrient and M.laricis development observed in this experiment parallels the interaction observed in in vitro spore germination trials. The inhibitory effect of both bacteria was only observed when the nutrient level was high.

The inhibition of M. laricis by cell-free culture media compared with sterile nutrient broth could indicate that a bacterial metabolite was a factor in the inhibition. Moreover, the requirement of high nutrient level for bacterial inhibition would indicate that actively metabolizing bacteria were required for inhibition and this also supports the hypothesis that an antagonistic metabolite was involved.

Effect of S.roseus in different nutrient solutions

It has been observed in vitro tests that S.roseus inhibited M.laricis spore germination at a low nutrient level but not at high nutrient level. It was therefore of interest to determine if a similar interaction with nutrient level occurred on the leaf itself. To examine this possible interaction an inoculation experiment was set up in which S.roseus was applied to seedlings in a number of different nutrient media.

The experimental methods were the same as used for yeast treatments in the previous experiment. Three pots containing a total of 13-19 seedlings were used for each treatment. The treatments were:

i. sterile water control,

ii. sterile sugar control (a total of 1000 μ moles of sugars made up by equal molar quantities of glucose, fructose and sucrose),

iii. sterile 1% yeast extract,

iv. sterile 1.3% nutrient broth,

v. S.roseus in water,

vi. *S.roseus* in 1000 μ moles of sugar solution,

vii. *S.roseus* in 1% yeast extract,

viii. *S.roseus* in 1.3% nutrient broth,

The microflora populations of treated leaves were assessed and the results are summarised in Table IV-10.

TABLE IV-10: The average number of bacteria and yeasts isolated per gram of foliage.

	H_2O	sugar solution	Yeast Extract	Nutrient Broth
sterile control	150	2,800	1,120,000	25,000
control solution plus *S.roseus*	300,000	3,000,000	16,000,000	13,000,000

The seedlings used in this experiment had a low natural microflora as was shown by the low number isolated from the sterile water control. The very high population associated with the yeast extract treatment was so much higher than the other sterile controls that its validity is questionable. The inclusion of sugars in the *S.roseus* application increased the population 10 fold compared with the *S.roseus* water suspension. However, the addition of yeast extract or nutrient broth with *S.roseus* resulted in by far the highest microflora populations.

The extent of disease development on individual seedlings in the four treatments is summarised in Figure IV-19. The four sterile treatments resulted in different levels of disease. Compared to the water control the sugar solution did not significantly reduce the amount of disease. On the other hand, yeast extract significantly ($p = 0.01$)

Figure IV-19 The number of diseased leaves (M. laricis) on seedlings treated with S. roseus in a variety of nutrient solutions.

reduced the disease level, and nutrient broth ($p = 0.01$) raised it. The reduction of disease associated with yeast extract may be connected with the high microflora population attained with that treatment.

The effects of S.roseus treatments on disease development showed an interaction with nutrient type. S.roseus in water resulted in a somewhat lower level of disease than the sterile water control. This reduction was significant only at $p = 0.1$. When S.roseus was applied in the sugar solution a marked reduction in disease occurred which was highly significant ($p = 0.005$) when compared with the sterile sugar treatment. However, in yeast extract S.roseus did not reduce infection below the level of sterile yeast extract. Similarly in nutrient broth S.roseus did not reduce the disease level. Indeed, it was associated with the highest level in the entire experiment.

Thus, the effect of S.roseus on M.laricis on larch leaves has an interaction with nutrient type similar to that observed for the spore germination of C.herbarum and M.laricis. It is possible that the mechanism of inhibition operates by competition for certain nutrients on the leaf surface. The postulated competition results in inhibition of M.laricis only when certain nutrients are in limited supply. Thus, if this hypothesis is correct, nutrient broth provides the nutrients which release M.laricis from the restrictive competition of S.roseus.

All seedlings used 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 treatments other than defoliation caused by M.laricis.

DISCUSSION

Interactions of the phyllosphere microflora with the spores of filamentous fungi have been examined in some detail. The approach of combining *in vitro* spore germination experiments with *in vivo* inoculations proved fruitful. The *in vitro* methods made possible the examination of a wider range of factors and a more precise examination of those factors than was possible with *in vivo* methods. The *in vivo* experiments, on the other hand, more closely approximated field conditions and thus extended the hypotheses towards the natural situation.

Fungistasis of spores of *C.herbarum* and *M.laricis* was observed to be associated with several microorganisms isolated from the phyllosphere. All the observed fungistatic phenomena associated with microorganisms showed marked interaction with the nutrient level. An examination of the interactions of germination inhibition and nutrients may indicate the nature of the mechanisms involved.

The fungistasis associated with bacteria was operative only in conditions of high nutrients. The bacterium S-100 inhibited spore germination of both *C.herbarum* and *M.laricis* when in germinating media containing soil extract, glucose, and yeast extract. The inhibition of *C.herbarum* spore germination by S-100 also occurred in a medium containing a mixture of sugars. However, in water solution, S-100 did not inhibit germination and in some cases its presence led to increased germination. The interaction of nutrients with *C.herbarum* spore germination was also observed in a somewhat more natural medium containing leaf leachates. With young leaves, S-100 increased

germination whereas with old leaves, which would be expected to leach more nutrient than young leaves, the presence of S-100 inhibited germination.

A similar pattern was observed in the reduction of M.laricis development on larch seedlings. However, even richer nutrient solutions were required for inhibition on leaves than in Van Tieghem cells. Bacteria applied to leaves in water or SEYE resulted in higher levels of disease than the same solutions without bacteria. However, when the bacteria were applied in nutrient broth they resulted in decreased levels of infection. Thus, it can be concluded that the bacteria tested required a concentrated nutrient solution in order to inhibit the germination and/or development of C.herbarum and M. laricis.

The reason for the nutrient requirement is obscured by the multiplicity of effects the nutrient could have. It is unlikely that the nutrient itself was inhibitory because sterile nutrients in vitro stimulated germination. In vivo, however, the results with "sterile" nutrient treatments sometimes caused some reduction in disease level. The inconsistent results may be explained by the fact that the seedlings had a microflora present which responded to sterile nutrient treatments and may have influenced the development of M.laricis. In the two instances where cell-free culture media were tested they reduced infection compared to sterile nutrient but not as much as bacteria in nutrient.

Thus, it would seem likely that the bacteria produced extracellular substances which rendered inhibitory the culture medium in which they were grown. It has been demonstrated that some leaf bacteria of the genus Pseudomonas produce antibiotics (Lewis, 1929). Bacterial

populations in water may have been too small or too inactive to produce sufficient inhibitory substances to reduce spore germination.

The inhibition of spore germination which was associated with yeasts was operative only in water or carbohydrate solutions. The inhibition was annulled by the addition of other nutrients, e.g. yeast extract. It has been demonstrated that some leaf yeasts (Torulopsis and Sporobolomyces) produce antifungal substances (Waksman, 1941; Yamasaki et al., 1951) and the inhibition may have been a result of a chemical antagonism which inhibited spore germination only in conditions of low nutrient. However, it is also possible that the inhibition arose from the competition of yeasts and fungi for nutrients in the environment. Ko and Lockwood (1967) have demonstrated that many fungal spores rapidly lose nutrients by leaching. If the leached nutrients accummulated around the spores, the spores could germinate. On the other hand, if the nutrients were immediately absorbed by yeast cells in the environment the spores could be deprived of nutrients which might be essential for germination. In the rich nutrient environment the activity of the yeasts would perhaps not reduce the nutrient level around the spores below the level required for germination. It is, of course, possible that two or more different mechanisms are operating to inhibit spore germination.

The general characteristics of the microbial fungistasis were consistent in both in vitro and in vivo experiments. The principle of using competitive or antagonistic interactions to control fungi on leaf surfaces has been established for the specific conditions of the

experiments. It must be emphasized that these conditions vary considerably from the conditions associated with the fungi on seedling leaves in the field. The addition of nutrients and large quantities of inoculum resulted in highly artificial populations. Moreover, the extended periods of high humidity when the leaf populations were protected from desiccation, rain washing, and strong light would not normally be encountered in the field. Although these conditions are ones which would probably be favourable for the pathogen, it is the balance between pathogen and microflora which must be examined under field conditions before predictions about disease control can be made.

The effect of the different treatments on microflora populations is of interest in determining factors governing the size of natural populations. As McBride (1969) found, the bacterial population was increased more by application of sterile nutrients than by the application of bacteria in water. Thus, nutrients were apparently more limiting in the larch phyllosphere than was inoculum. The combination of inoculum and nutrients was, however, more effective than either of the factors applied individually.

A similar result was observed with yeast inoculations although yeast inoculum in water appeared to be more effective than bacterial inoculum in increasing numbers. The highest populations of yeasts were attained with inoculum in a soil-extract yeast-extract solution.

C.herbarum and M.laricis have been shown to interact with both nutrient and microbiological aspects of the phyllosphere. The two fungi occupy different ecological niches on the larch leaf. M.laricis is pathogenic and infects only young leaves whereas C.herbarum is usually considered non-pathogenic and colonized old, senescent

leaves. This restriction in habit was coincident with age of leaf and size of leaf microflora and occurred even though inoculum existed on leaves of all ages. It is not probable that climatic factors restrict the niches because both fungi may be active at any time during the growing season on any age of plant which has leaves of the appropriate age.

The spore germination of both fungi was stimulated in a similar manner by external nutrients. In addition, the spore germination of both fungi was stimulated more by leachates of old leaves than by leachates of young leaves. However, the presence of damaged leaves, i.e. internal tissues exposed, did not stimulate C.herbarum germination and while young damaged leaves stimulated M.laricis germination, old damaged leaves did not. Thus, it may be some aspect of the internal leaf condition which changed with age and restricted the fungi to their specific niche.

The germination experiments provided information which may reveal adaptions of the fungi which suit them to these niches. Both fungi have a brown pigment in their spores superficially resembling the pigment melanin which Potgieter and Alexander (1966) have postulated makes fungi resistant to microbial lysis. The colonization of germinating C.herbarum and M.laricis spores was restricted to the non-pigmented portion of the germ tubes. Thus it would appear that the spores wre adapted to resist microbial attack. The spore germination of both fungi was influenced in a similar way by the presence of certain bacteria and yeasts. However, the spores of M.laricis were affected by more of the bacterial types tested than were the spores

of C.herbarum. Thus, the fungus which was most successful on leaves with a high microbial population appeared to be resistant to a wider range of microorganisms than was the fungus which was successful only on leaves with a low microbial population.

The nature of spore germination may suggest further adaptions to the two niches. The slow germination of M. laricis spores accompanied by the apparent concentration of spore content into one cell may place a much lower demand on phyllosphere nutrients than the more rapid germination and germ tube growth of C.herbarum spores. These characteristics may be adaptions to the particular environments of their respective niche. M.laricis develops in a phyllosphere with a small microflora and small quantities of nutrient. C.herbarum, on the other hand, develops in a phyllosphere with a large microflora and where large quantities of nutrients may become available at leaf senescence.

CHAPTER V

DISCUSSION

CHAPTER V DISCUSSION

A number of observations and experiments in the present study and in the published literature indicate that there exists in the phyllosphere a phenomenon in many ways parallel to the widespread fungistasis which occurs in soil.

On young larch leaves the majority of fungal spores remain ungerminated (see Ch. II). Similar observations have been made for young leaves of pea (Dickinson, 1967) and clover (Barnes, 1969). One of the characteristics of soil fungistasis is its annulment by nutrients, especially complex mixtures of organic substances such as plant extracts and root exudates (Lockwood, 1964). Amino acids have been reported as being ineffective in annulling fungistasis in the soil when applied without other types of nutrients (Chinn and Ledingham, 1957). This report parallels the observations made in the present study, where it was found that mixtures of amino acid caused a very small stimulation of C.herbarum germination in comparison with a mixture of sugars (see Ch. IV). In the phyllosphere an increase in fungal activity was observed by Fokkema (1967) when she added pollen grains to leaves of rye inoculated with C.herbarum. Barnes (1969) also observed increased fungal activity in the vicinity of pollen grains on clover leaves. In the present study the leachates from larch leaves were shown to stimulate the germination of C.herbarum and M.laricis spores. Old leaves caused a greater stimulation than young leaves (see Ch.IV). This evidence, in addition to observations (Tukey, Wittwer and Tukey, 1957) that leaf leachates increase in quantity as the leaf begins senescence, could be used to support the

hypothesis that the sudden increase of fungal activity associated with senescence is a result of a nutrient annulment of phyllosphere fungistasis.

Fungistasis in the soil is thought to be microbiological in origin because the effect is removed by sterilizing and can be restored by inoculating sterilized soil with microorganisms. The mechanism of soil fungistasis has been a subject of considerable debate. Dobbs et al.(1960) postulated that chemicals produced by microorganisms were the inhibitory mechanism, whereas Lockwood (1964) and Ko and Lockwood (1967) proposed an explanation based on competition for nutrients. This latter explanation suggests that microorganisms compete with spores for nutrients in the environment including those nutrients leached from the fungal spores. When nutrients are at a low level their utilization ~~of the nutrients~~ by the microorganisms keeps the nutrients in the vicinity of the spores at a level below the spore's requirement for germination. When surplus nutrients are available, the fungistasis pressure is released and spores germinate.

In the present study, experiments have revealed an interaction between the phyllosphere microflora and spores of two common leaf fungi which suggest that one or all of those mechanisms may operate in the phyllosphere (see Ch.IV). A bacterium (S-100) was observed to inhibit spore germination only when in rich nutrient conditions. It is suggested that the inhibition resulted from antibiotic or staling agents (or other metabolic products of the bacterium) which curtailed spore germination even though the nutrient conditions of sterile controls were conducive to germination. Conversely, the inhibition of spore germination associated with S.roseus was only present in low

nutrient conditions and was annulled by the addition of nutrients. The inhibition associated with S.roseus may result from competition for nutrients or it may be the result of a chemical inhibitor operative only in low nutrient conditions which do not themselves strongly stimulate spore germination. Whatever the mechanisms of microbial fungistasis may be, the two types associated with S.roseus and the bacterial isolate, S-100, were shown to function not only in vitro but also on the leaf surface itself.

In the limited experiments of the present study, two main types of microorganism interactions which prevented fungal spore germination were observed. Indeed, a high percentage of the phyllosphere isolates tested were shown to have some inhibitory effect on the leaf fungi, C.herbarum and M.laricis. It is likely that these examples are but a few of the interactions which could occur between phyllosphere microorganisms. It is important to determine to what extent these interactions occur on the leaf surface in natural conditions. In inoculation experiments, very large artificially developed leaf microfloras were required to demonstrate significant microbiological control of M.laricis by phyllosphere microorganisms. Moreover, to establish statistically significant control in experiments it was necessary to maximize inoculum level and environment conditions for both pathogens and non-pathogen. Indeed, the aim of such experimental techniques is to reduce variation. However, in the natural situation variation is the rule; variation in inoculum, variation in microflora and variation in the physical environment. The possible protective role of leaf microfloras in natural conditions must be considered in the context of the natural variation of these factors.

The observation that leaf microflora populations concentrate in the anticlinal depressions, an important infection site for some leaf pathogens (Preece et al., 1967), increases the likelihood of interactions occurring between microorganisms.

Inhibition associated with S.roseus under low nutrient conditions would seem more likely to occur in the impoverished nutrient conditions which appear to exist for fungal spores on young leaves than would the inhibition associated with the bacterium, S-100. However, the high nutrient levels required for fungal inhibition by S-100 may be encountered on the leaf surface in microsites near insect damage, pollen grains or debris. At present it is not known if such interactions occur under natural conditions and if they contribute to the natural balance of disease resistance and susceptibility.

The manipulation of the natural phyllosphere microflora to provide microbiological control of leaf pathogens would be highly desirable if it led to crop protection without the ecosystem disruption accompanied by so many forms of chemical control. However, many problems and possible hazards would be involved with attempts to manipulate phyllosphere populations.

To consider the possible hazards first, it is possible that leaf microorganisms if increased in number might themselves damage the host. Leaf wax utilization by organisms such as S.roseus could cause serious damage. In addition, some leaf microorganisms may have synergistic effects on fungal pathogens. Bald and Solberg (1960) demonstrated an example of such a synergistic effect on lilies in which Pseudomonas sp. stimulated the scale tip rot pathogen, Fusarium

oxysporum Schlecht.. The application of large quantities of microflora inoculum may adversely affect microorganism populations on other substrates such as the soil, or decomposing litter. While theoretically possible and requiring close observation, radical alteration of microfloras is not likely to be a practical problem because most substrates have an established and frequently specific microflora. This point has been demonstrated by the work of Gremmen (1957) and Kendrick and Burges (1962) in litter decomposition studies. As regards the leaf bacterial flora, Stout (1960b) has shown that the leaf and soil floras remain taxonomically distinct despite close physical proximity. di Menna (1962) has shown a similar distinction between the yeast microfloras of leaves and soil. Moreover, while microorganism communities may have rapid successional changes of species they are frequently resistant to the introduction of additional species (Brock, 1966, p.162).

The resistance of microorganism communities to new species may reduce the hazards but also present the most difficult problem involved with manipulation of phyllosphere populations. The natural population level of microorganisms on larch leaves has been shown to be governed by one or more limiting factors. In any attempt to manipulate the size of the population, the limiting factors must be adjusted appropriately. In the inoculation studies described in Chapter IV the application of bacterial inoculum to leaves did not cause as great an increase in leaf bacteria as did sterile nutrients. Thus, it would seem to be necessary to alter the nutrient status on leaves in order to substantially increase the bacterial microflora. Similar results were found with

yeast populations which have been shown (see Ch.II) to be limited in numbers by a factor associated with leaf age. Thus, if practical microbiological control utilizing by phyllosphere bacteria or yeasts is to be developed, future research must attempt to understand and find ways to control the factors limiting microorganism populations.

Crop-culture techniques must also be evaluated for their effect on the microflora. Foliar fertilizers or even soil treatments (Last, 1955a) may influence the nutrients and crop microclimate and thereby alter the microflora of the phyllosphere. Such treatments may well have secondary beneficial effects if they resulted in a microflora which had enhanced protective qualities. In a similar manner the microbiological antagonistic or competitive forces in the phyllosphere could be reduced by crop-culture techniques such as non-specific pesticides, monoculture or weed control. The reduction of such forms of natural resistance might lead to a resurgence of disease when the applied chemical resistance diminished. Also, the upgrading of a non-virulent pathogen to a virulent form might be possible on a leaf on which antagonistic and competitive forces had been reduced.

An example of a disease situation which may be influenced by crop culture techniques was suggested by Wood (1951). He observed that lettuce plants grown in depressions were more resistant to Botrytis cinerea Pers. than were plants grown on raised soil. He concluded that this resistance was a result of rapid colonization of the plants in the depressions by soil saprophytes antagonistic to B. cinerea. Horsfall and Lukens (1966) have suggested that the spraying of captan in orchards controls apple scab (Venturia inequalis (Cke)Wint.) but

it may also result in an increase of apple mildew (Podosphaera leucotricha (E. + E.)Salm.). In addition, it has been suggested that the increase in blossom blight of peach associated with the application of some fungicides may have resulted from a reduction in the normal antagonistic microflora (Anon 1949).

On the other hand, crop culture techniques may encourage or discourage the development of attenuate pathogens in the phyllosphere. In such a way populations of cuticle damaging yeasts like S.roseus might either be controlled or else stimulated to cause serious injury to the leaf. The destruction of the leaf cuticle could lead to increased transpiration and nutrient leaching resulting in an accelerated leaf senescence and crop reduction.

The acceptance of the roles of phyllosphere microorganisms as antagonists, competitors or attenuate pathogens demands a broad and detailed evaluation of crop condition and crop production associated with various management techniques.

Thus, if a pesticide is to be used to control a pathogen, appropriate observations should be made on the populations of other pathogens and saprophytes. The continuing high production of the crop, is of course, the absolute measure of a successful technique. Such measurements may well reveal that crop production is increased after fungicide application even in cases where the expected pathogen did not materialize. Careful and extensive observation of field situations of this type may lead to an assessment of the importance of attenuated infection and/or natural microbiological control of pathogens in the phyllosphere of field grown crops.

Future development of crop production methods must consider the consequence of new techniques on the phyllosphere microflora in order that beneficial functions of that microflora are enhanced and detrimental functions are suppressed.

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APPENDIX I.

APPENDIX I

The statistical procedures used were those of Snedecor and Cochran (1967). In general, standard analysis of variance was used to assess the amount and source of variation. In the case of percentage data the arcsin (angular) transformation was applied. All tests of difference between treatments were carried out on transformed means which were not retranslated to per-cents. The mean of the untransformed data was always used in figures and text.

Significant F values are indicated as * for $p = 0.05$ and as ** for $p = 0.01$. No-significant-difference between means is indicated by underlining the ranked means of the treatments, e.g. $\underline{x_1 \quad x_2} \quad x_3$. x_1 is not significantly different than x_2 but both x_1 and x_2 are significantly different than x_3.

SPORE GERMINATION EXPERIMENTS

CLADOSPORIUM HERBARUM EXPERIMENTS

Effect of spore age on germination

Anova

Source of variation	df	Sums of Squares	Mean Square	F
Treatment	3	32,410.1	10,803.4	1,367.5**
Error	20	159.4	7.9	
Total	23	32,569.5		

Least Significant Difference

at p = 0.05 is 3.36
at p = 0.01 is 4.58

Ranked means of transformed data

40 days old	32-28 days old	17-13 days old	10 days old	
0	3.21	46.9	90.0	p = 0.01

Effect of nutrients on germination

Anova

Source of variation	df	Sums of Squares	Mean Square	F
Treatment	7	10,356.1	1,479.4	160.0**
Error	88	810.1	9.2	
Total	95	11,166.2		

Least Significant Difference

at p = 0.05 is 2.44
at p = 0.01 is 3.26

Ranked means of transformed data

water	5µmoles sugars	500 µmoles amino acids	50µmoles amino acids	5µmoles amino acids	50µmoles sugars	500µmoles sugars + 500 µmoles amino acids	500µmoles sugars	
16.8	16.9	18.9	20.4	22.2	26.1	42.1	44.4	p = 0.01

Effects of leaves on germination

Anova

Source of variation	df	Sums of Squares	Mean Square	F
Treatments	5	26,931.5	5382.7	238.9**
Error	12	270.4	22.5	
Total	17	27,201.9		

Least Significant Difference

at p = 0.05 is 8.49
at p = 0.01 is 12.11

Ranked means of transformed data

Old damaged leaves	water control	old leaves	young leaves	rubbed leaves	sugar solution	
24.1	27.7	38.0	41.4	45.2	57.7	p = 0.05

Spore germination with different aged leaves

Anova

Source of variation	df	Sums of Squares	Mean Square	F
Treatments	5	33,083.9	6616.8	516.9**
Error	66	848.0	12.8	
Total	71	33,931.9		

Least Significant Difference

at p = 0.05 is 2.8
at p = 0.01 is 3.7

Ranked means of transformed data

Brown leaves from ground	water	young leaves	young rubbed leaves	old green leaves	old brown leaves	
2.9	14.7	18.2	35.9	57.9	59.8	p = 0.01

Effect of bacteria on germination

Anova

Source of variation	df	Sums of Squares	Mean Square	F
Treatments	7	18,655.2	2,665.0	34.8**
Error	16	1,224.4	76.5	
Total	23	19,879.6		

Least Significant Difference (excluding *B.mycoides* treatment because $S_1 \neq S_2$)

at $p = 0.05$ is 15.3
at $p = 0.01$ is 21.0

Ranked means of transformed data

S-100	S-12	S-117	S-92	S-95	S-79	control
0.0	81.9	83.7	84.4	85.1	85.9	86.0

$p = 0.01$

comparison of control and *B.mycoides* by t-test

$$t = (\bar{x}_1 - \bar{x}_2)/S_{\bar{x} - \bar{x}_2} = 2.29$$

$t(p=0.05 \quad df = 2) = 4.30$

Therefore, control and *B.mycoides* treatment are not significantly different

Effect of bacteria and yeasts on germination

Anova

Source of variation	df	Sums of Squares	Mean Square	F
Treatments	7	16,850.8	2407.3	302.4**
Error	64	509.5	7.96	
Total	71	17,360.3		

There was unequal sample size between sugar and non-sugar treatments. Therefore, three Least Significant Difference values were calculated.

(i) Comparisons between sugar treatments

Least Significant Difference at p = 0.05 is 3.24
at p = 0.01 is 4.31

(ii) Comparisons between non-sugar treatments

Least Significant Difference at p = 0.05 is 2.30
at p = 0.01 is 3.05

(iii) Comparisons between sugar and non-sugar treatments

Least Significant Difference at p = 0.05 is 2.82
at p = 0.01 is 3.75

Ranked means of transformed data

water + S.roseus	S-100 + S.roseus	sugar + S-100	sugar + S-100 + S.roseus	water + S-100	Sterile water	sugars
0	0	23.9	30.2	30.7	32.6	34.2

Interactions of leaves and microbial inhibition of germination

(i) Controls and S-100 treatments

Anova

Source of variation	df	Sum of Squares	Mean Square	F
Treatments	5	6,581.8	1,316.3	63.3**
Error	30	623.0	20.8	
Total	35	7,204.8		

Least Significant Difference

at p = 0.05 is 5.3
at p = 0.01 is 7.1

Ranked means of transformed data

water	young leaves	young leaves + S-100	old leaves + S-100	old leaves	water + S-100
14.7	20.9	23.3	24.7	39.8	54.8

p = 0.01

(ii) Controls and S. roseus treatments

Anova

Source of variation	df	Sums of Squares	Mean Square	F
Treatments	5	6115.9	1223.2	48.3**
Error	30	759.0	25.3	
Total	35	6874.9		

Least Significant Difference

at p = 0.05 is 5.9
at p = 0.01 is 7.9

Ranked means of transformed data

water + S. roseus	old leaves + S. roseus	young leaves + S. roseus	water	young leaves	old leaves	
0	6.0	7.0	14.7	20.9	39.8	p = 0.05
						p = 0.01

MERIA LARICIS EXPERIMENTS

Isolation and culture of M.laricis

(i) Ammended media

Anova

Source of variation	df	Sums of Squares	Mean Square	F
Treatment	2	25,402	12,701	760.5**
Error	12	200	16.7	
Total	14	26,602		

Least Significant Difference

at p = 0.05 is 5.66
at p = 0.01 . is 7.94

Ranked means

CDYE	Leaf extract	Larch leaves	
32.6	36.6	56.6	p = 0.01

(ii) Different concentrations of leaf extract

Anova

Source of variation	df	Sums of Squares	Mean Square	F
Treatments	2	12	6	0.25 N.S.
Error	18	427	23.7	
Total	20	439		

Effect of nutrients and cold treatments on spore germination

Anova

Source of Error	df	Sums of Squares	Mean Square	F
Treatments	(8)	(17,494.7)	(2,186.8)	(180.7**)
A (time)	2	7,546.1	3,773.0	311.8**
B (nutrients)	2	5,589.5	2,794.7	231.0**
AB	4	4,359.1	1,089.8	90.1**
Error	81	980.4	12.1	
Total	89	18,475.0		

Least Significant Difference

at p = 0.05 is 3.1
at p = 0.01 is 4.1

Ranked means of transformed data

Cold	0	0	12	48	12	0	12	48	48	
Nutrient	water	amino acids	amino acids	water	water	sugars	sugars	sugars	amino acids	
	16.8	17.5	19.4	23.1	24.7	32.8	37.9	51.9	56.4	p = 0.05
										p = 0.01

Effect of leaves on germination

(i) t-tests used to compare treatments because of unequal variance and unequal sample size.

Treatment comparisons	t value	df	table(0.05)
water : young leaves	4.5	35	2.03
water : old leaves	6.5	29	2.04
young leaves: young rubbed leaves	4.8	35	2.03
young leaves: young damaged leaves	3.9	35	2.03
young rubbed leaves:young damaged leaves	1.1	35	2.03
old leaves : young leaves	0.33	29	2.04
old leaves : old rubbed leaves	5.0	23	2.07
old leaves : old damaged leaves	0.08	23	2.07

(ii) Analysis of young leaf replicates

Anova

Source of variation	df	Sums of Squares	Mean Square	F
Treatments	2	473.4	236.7	73.51**
Error	15	48.3	3.2	
Total	17	521.7		

Effect of bacteria on germination in SEYE

Anova

Source of variation	df	Sums of Squares	Mean Square	F
Treatments	6	2,887.5	481.2	12.73**
Replicates	7	122.1	17.4	.46 N.S.
Residual Error	42	1,587.7	37.8	
Total	55	4,597.3		

Least Significant Difference

at p = 0.05 is 6.22
at p = 0.01 is 8.33

Ranked means of transformed data

Control	B.mycoides	S-92	S-100	S-117	S-79	S-95	
50.5	40.6	40.0	38.0	36.6	31.2	25.8	
							p = 0.05
							p = 0.01

Effect of bacteria on germination in a sugar solution; and

Effect of yeast on germination in a sugar solution

The two experiments were run simultaneously with sterile controls being used for both yeast and bacterial treatment comparisons. Therefore, the two experiments were analyzed as one.

Anova

Source of variation	df	Sums of Squares	Mean Square	F
Treatments	6	4885.2	814.2	76.1**
Error	35	376.5	10.7	
Total	41	5261.7		

Least Significant Difference

at p = 0.05 is 3.75
at p = 0.01 is 5.04

Bacteria Experiment

Ranked means of transformed data

B.mycoides	S-95	S-100	sterile sugar
15.6	23.8	30.4	30.4

p = 0.01

Yeast Experiment

Ranked means of transformed data

Torulopsis	Cryptococcus	S.roseus	sterile sugar
2.7	3.3	21.8	30.4

p = 0.01

Spore germination with S.roseus in water and in sugar solution

Anova

Source of variation	df	Sums of Squares	Mean Square	F
Treatments	3	12,147.4	4049.1	77.0**
Error	68	3,575.1	52.6	
Total	71	15,722.5		

Least Significant Difference

at p = 0.05 is 4.8
at p = 0.01 is 6.4

Ranked means of transformed data

water	S.roseus + water	S.roseus + sugars	sugars	
21.7	38.5	39.8	66.2	p = 0.01

INOCULATION EXPERIMENTS

MERIA LARICIS ON LARCH SEEDLINGS

Effect of a mixture of bacteria in SEYE

The differences between treatments were compared by a series of t-tests because of unequal sample size and uneven variance.

	water	bacteria + water	SEYE	bacteria + SEYE
n	23	20	20	20
$\bar{x}$	15.0	19.4	8.2	12.4
s_i^2	23.1	64.8	15.6	31.3

Treatment Comparison	t value	level of significance
Water:bacteria + water	2.09	0.05
SEYE:bacteria + SEYE	2.73	0.01
water:SEYE	5.15	0.01
bacteria + water:bacteria + SEYE	3.18	0.01
water:bacteria + SEYE	1.62	N.S.

Effect of S-100 and S. roseus in SEYE

Anova

Source of variation	df	Sums of Squares	Mean Square	F
Treatments	3	674	225	5.24 N.S.
Error	24	1031	42.9	
Total	27	1705		

Effect of S-100 in Nutrient Broth

Anova

Source of Variation	df	Sums of Squares	Mean Square,	F
Treatments	2	4,190	2,095	23.1**
Error	53	4,809	90.7	
Total	55	8,999		

Because variance was heterogeneous and sample size varied between treatments the pooled variance was not used and comparisons were made individually between pairs of treatments by the t-test.

	t-value	level of significance
water:Nutrient Broth	4.38	p = 0.01
Nutrient Broth:Bacteria + Nutrient Broth	3.25	p = 0.01
water:Bacteria + Nutrient Broth	6.21	p = 0.01

Bacteria and yeasts in a number of different treatments

Anova

Source of Variance	df	Sums of Squares	Mean Square	F
Treatments	11	8,269	751.7	37.2**
Error	197	3,974	20,2	
Total	208	12,243		

Treatment comparisons were made with the t-test because of unequal sample size.

	t-value	level of significance
water:S.roseus	6.58	p = 0.01
glucose:S.roseus	6.22	p = 0.01
glucose:Torulopsis	5.06	p = 0.01
glucose:Cryptococcus	2.69	N.S.
water:water + S-100	1.47	N.S.
Nutrient Broth:S-100 Cell Free medium	4.1	p = 0.01
Nutrient Broth:S-100 + Nutrient Broth	7.20	p = 0.01
S-100 Cell Free medium:S-100 + Nutrient Broth	2.71	p = 0.05
water:water + B.mycoides	1.43	N.S.
Nutrient Broth:B.mycoides Cell Free medium	1.30	N.S.
Nutrient Broth:B.mycoides + Nutrient Broth	7.05	p = 0.01
water:glucose	3.7	p = 0.01
water:Nutrient Broth	1.03	N.S.

Effect of S.roseus in different nutrient solutions

Anova

Source of variation	df	Sums of Squares	Mean Square	F
Treatments	7	2019	288.4	24.2**
Error	119	1420	11.9	
Total	126	3439		

Treatment comparisons were made with the t-test because of unequal sample size.

	t-value	level of significance
water:S.roseus + water	1.91	p = 0.10
sugars:S.roseus + sugars	3.27	p = 0.005
yeast extract:S.roseus + yeast extract	.92	N.S.
Nutrient Broth:S.roseus + Nutrient Broth	4.6	p = 0.001
water:sugars	1.13	N.S.
water:yeast extract	2.88	p = 0.01
water:Nutrient Broth	5.40	p = 0.001

ABSTRACT OF THESIS

Name of Candidate Richard Phillips McBride

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Degree Ph.D *Date* June, 1970

Title of Thesis THE PHYLLOSPHERE OF EUROPEAN LARCH (LARIX DECIDUA MILL.): A MICRO-ECOLOGICAL STUDY OF LEAF INFECTION.

The ecology of the phyllosphere of European larch was studied with special reference to interactions between leaf pathogens and the non-pathogenic microflora. Examination of the larch leaf surface revealed surface relief features related to internal leaf tissues, epidermal cells and subcellular wax structures. Chemical assessment of the leaf surface indicated the presence of a large number of wax components as well as leaf surface water containing sugars and amino acids.

The microflora of larch seedlings grown in a forest nursery was assessed over two growing seasons. The microflora was composed of bacteria, yeasts and filamentous fungi. The microflora population increased with increasing leaf age, and hyphal development was restricted to old leaves. By the time of leaf fall in October, the microflora of one gram fresh weight of 4-month-old leaves consisted of approximately $1x10^6$ bacteria, $9x10^5$ yeast cells and $1x10^5$ filamentous fungi propagules.

Several leaf yeasts and bacteria were shown to be able to colonize growing aseptic seedlings from inoculum placed on the seedcoat. The yeast, Sporobolomyces roseus was shown to be associated with surface wax damage. This association was demonstrated by inoculation of aseptic seedlings and subsequent examination with a stereo-scan electron microscope.

The larch leaf pathogen, Meria laricis and the common leaf saprophyte, Cladosporium herbarum were examined in spore germination and inoculation experiments for interactions with a variety of phyllosphere factors. In vitro spore germination of both species was stimulated by nutrients, particularly sugars. Larch leaves, especially old leaves, immersed in the germinating liquid stimulated germination. Spore germination of both species was inhibited by a number of leaf bacteria and yeasts. The inhibitions interacted with nutrient level. The yeast Sporobolomyces roseus inhibited germination only in low nutrient conditions whereas a bacterium (isolate S-100, Pseudomonas sp.) inhibited germination only in rich nutrient conditions. The same inhibitions were shown to be active in inoculations of larch seedlings. The damage done by the pathogen M. laricis to greenhouse-grown seedlings was significantly reduced by spraying the plants with bacteria in nutrient broth or S. roseus in a sugar solution.

Fungistasis in the phyllosphere was described and compared with soil fungistasis.

The significance of the leaf microflora to techniques of plant

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culture as regards attenuate infection by yeasts and microflora based immunity to leaf pathogens is discussed.