Studies of the phylloplane microflora of lettuce and its interactions with pesticides and *Sclerotinia sclerotiorum*

by

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ABSTRACT

M.Sc. JULIEN MERCIER Plant Science
Plant Pathology

STUDIES OF THE PHYLLOPLANE MICROFLORA OF LETTUCE AND ITS INTERACTIONS WITH PESTICIDES AND SCLEROTINIA SCLEROTIORUM

Populations and components of the microflora of lettuce leaves were determined over two growing seasons. Effects of a fungicide (maneb) and an insecticide (carbaryl) on this microflora were studied. Only maneb was found to cause a reduction in the fungal populations. Bacterial populations remained unaffected by pesticide applications.

Fungi isolated from lettuce leaves were screened for their ability to antagonize ascospores of *Sclerotinia sclerotiorum*, the causal agent of lettuce drop. The most inhibitory isolates were tested for disease control in a controlled environment. Three isolates caused a significant reduction in the number of infected plants. Interactions between these antagonists and the pathogen on the phylloplane were studied by Scanning Electron Microscopy

RESUME

M.Sc. JULIEN MERCIER Phytotechnie
Phytopathologie

ETUDES DE LA MICROFLORE DES FEUILLES DE LAITUE ET DE SES INTERACTIONS AVEC DES PESTICIDES ET LE SCLEROTINIA SCLEROTIORUM

La composition de la flore microbienne des feuilles de laitue, de même que sa population, furent évaluées durant deux étés. Les effets d'un fongicide (manèbe) et d'un insecticide (carbaryl) sur cette microflore ont été étudiés. Seulement manèbe s'avéra nuisible aux populations de champignons, les populations bactériennes demeurant insensibles aux applications de pesticides.

Une sélection fut faite parmi des champignons isolés de feuilles de laitue pour déterminer leur pouvoir antagoniste envers les ascospores de <u>Sclerotinia sclerotiorum</u>, cause de l'affaissement sclérotique de la laitue. Les souches démontrant un haut niveau d'inhibition furent testées en conditions contrôlées pour évaluer leurs potentiels comme moyens de lutte de la maladie. Trois souches réduisirent le nombre de plantes infectées de façon significative. Les intéractions sur la surface des feuilles entre ces trois antagonistes et le pathogène furent observées à l'aide du microscope électronique à balayage.

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GENERAL INTRODUCTION

The microbial community on the phylloplane has been intensively studied during the last twenty years, especially after the publication of a report by Ruinen (1961). These microorganisms, which, most often, are not pathogenic on plants, are nevertheless in contact with chemicals used for crop protection. To date, the effects of some fungicides on the microflora have been studied (Andrews, 1981). While other environmental factors affecting these microorganisms are somewhat better understood, information on effects of insecticides is still lacking.

At sufficiently high population levels, these microorganisms can antagonize plant pathogens and reduce disease severity (Blakeman and Fokkema, 1982). These population levels must usually be increased artificially by application of spores and/or nutrients in order to obtain a significant reduction in infection. To date, many fungi and bacteria have been shown to be antagonistic to fungal plant pathogens. There are also reports of successful disease control under field conditions. Sclerotinia sclerotiorum is an important pathogen of many vegetables, as well as crops such as rapeseed and sunflower. This pathogen has been difficult to control with chemicals for various reasons. The use of biocontrol agents may offer an alternative approach to the control of diseases caused by S. sclerotiorum.

The objectives of this project were:

1-To define the microflora of lettuce (Lactuca sativa L.) leaves and the effects of two pesticides on it.

2-To identify phylloplane fungi exibiting antagonism to Sclerotinia sclerotiorum, the causal agent of lettuce drop.

LITERATURE REVIEW

A-Phylloplane antagonism

1)Introduction

The interactions existing between different microorganisms may be potentially useful if they can be used for the control of plant diseases. There are many reports of antagonism on the phylloplane but relatively few lead to practical disease control (Blakeman and Fokkema, 1982; Dubos and Bulit, 1981; Fokkema, 1976; Kranz, 1981; Skidmore, 1976a). Biocontrol of pathogens in the soil and rhizosphere appears to be more advanced than on the aerial part of plants. This is due in part to the availability and effectiveness of foliar fungicides (Blakeman and Fokkema, 1982). Also, the soil offers a more stable environment for the growth and survival of the biocontrol agents. Leaf surfaces are dry most of the time, poor in nutrients (Blakeman, 1978), and exposed to temperature fluctuations and ultra-violet radiation. These conditions limit the choice of future biocontrol agents. Both bacteria and fungi have been used experimentally. They have been isolated from the phylloplane, disease lesions, and soil (Blakeman and Fokkema, 1982). Antagonistic action has been of three different types: competition for nutrients, production of antibiotics and parasitism. A biocontrol agent may also act indirectly by triggering the host defense mechanisms (Blakeman and Fokkema, 1982).

2)Competition for nutrients

Antagonists competing for nutrients would generally be expected to affect necrotrophic pathogens. Phylloplane microbes would appear to be the best suited organisms to act in that fashion since they are adapted to this environment. The antagonists act by depleting nutrients which may be lost from the leaf (mainly simple sugars and amino acids) or which may be exogeneous, such as pollen, aphid honeydew, and dead petals (Blakeman, 1978). This will result in reduced growth of the pathogen due to a nutrient shortage and thus a reduction in its ability to infect. Fokkema _et al_. (1983) demonstrated that, although infections by _Cochliobolus sativus_ and _Septoria nodorum_ were stimulated by the presence of aphid honeydew, such stimulation is never observed in the field due to rapid removal of this nutrient source by the saprophytes. Similarly, the presence of an abundant microflora reduced the stimulatory effect of pollen on _Drechslera sorokiana_ on rye leaves (Fokkema, 1973) and on _Phoma betae_ on sugar-beet leaves (Warren, 1972b). Yeasts and bacteria with a high amino acid uptake were the microflora components most inhibitory to _Botrytis cinerea_ (Brodie and Blakeman, 1976). Species of _Colletotrichum_ were reported to form shorter germ tubes and appressoria more rapidly in the presence of bacterial competitors (Blakeman and Brodie, 1977; Blakeman and Parbery, 1977; Lenne and Parbery, 1976). The production of appressoria, which in these cases is a response to a hostile environment, was reduced if nutrients were given to the spores.

3)Antibiotics

Several cases of phylloplane antagonism were shown to involve the production of antibiotic substances. The soil-borne fungus Trichoderma was reported to produce a number of volatile and non-volatile antibiotics (Dennis and Webster, 1971a, b; Tronsmo and Dennis, 1978). This helps to explain why this fungus has been so widely used for biocontrol experiments on aerial plant surfaces. Trichoderma species were used with some success to control strawberry fruit rots (Tronsmo and Dennis, 1977), apple dry rot (Tronsmo and Raa, 1977; Tronsmo and Ystaas, 1980) and grey mold and excoriosis on grape (Dubos et al., 1978).

Several species of Bacillus were able to attack a number of fungal pathogens with antibiotics. The antibiotics were either heat-labile or heat-resistant and caused either lysis of germ tubes (Doherty and Preece, 1978; Morgan, 1963) or simply prevented spore germination and germ tube growth (Baker et al., 1983). Species of Bacillus, which have the advantage of being spore-formers, were used successfully for preventing infections of stored fruits (Sing and Deverall, 1984; Pusey and Wilson, 1984), leaf scars (Swinburne et al., 1975), rust diseases (Baker et al., 1983; Doherty and Preece, 1978; McBride, 1969; Morgan, 1963) and tobacco brown-spot (Flavel and Spurr, 1977). In all these cases, the antagonistic action seemed to be due to antibiotics. Other bacteria such as pseudomonads also inhibited plant pathogens with antibiotics (Austin et al., 1977; Rotem et al, 1976). The ability to produce antibiotic substances is probably widespread among other species of antagonists. Although workers have not always been able to demonstrate antibiotic production, they have often suggested this possibility (Andrews _et al._, 1983; Fokkema and Lorbeer, 1974).

4)Parasitism

Parasitism on the pathogen's spores and mycelium is another possible mode of antagonistic action. Relatively unspecialized soil and phylloplane fungi such as _Trichoderma_ spp. (Dennis and Webster, 1971c; Tronsmo and Raa, 1977), _Cladosporium_ sp. and _Alternaria_ sp. (Omar and Heather, 1979) can establish a parasitic relationship under certain conditions. This can greatly improve their efficiency as biocontrol agents. Some parasitic antagonists can attack pathogens established in the plant (Sharma and Heather, 1983; Swendsrud and Calpouzos, 1972). Many cases of phylloplane parasitism studied have involved attacks on rusts and powdery mildews by rather more specialised fungi (Jarvis and Slingsby, 1977; Koc _et al._, 1981; Sharma and Heather, 1983; Spencer, 1980; Swendsrud and Calpouzos, 1972). There is also a case of _Xanthomonas_ parasitic on rust (Pon _et al._, 1954). Much more research will be needed before the use of these hyperparasites as biocontrol agents can be of practical use (Kranz, 1981). They often survive poorly on the leaf surface (Spencer, 1980; Swendrud and Calpouzos, 1972) and can be slow growing (Tsuneda and Skoropad, 1978). In nature, their populations are slow to build up and often reach a significant level only at the end of the growing season. Meanwhile the pathogens they attack have had ample time to cause damage (Kranz, 1981).

Sundheim (1982), who attempted to control powdery mildew on cucumber with _Ampelomyces quisqualis_, reported that while the hyperparasite was found to parasitize the pathogen, it did not reduce disease incidence. A certain level of parasitism seems to be required in order for the hyperparasites to maintain themselves. In natural situations, they might play an important role in regulating levels of diseases by reducing the production of pathogenic inoculum (Blakeman and Fokkema, 1982).

5)Selection methods

Attention must be given to the selection of potential biocontrol agents. Ideally, the selection should be done directly in the environment in which the antagonists are expected to perform. However, time and space limitations make screening on plants rather difficult. For example, Leben (1964) found only one bacterial isolate out of 230 to be antagonistic to anthracnose of cucumber. The candidate antagonists can be tested in petri dishes, glass slides, cellophane membranes, leaf discs and detached leaves. The degree of antagonism can be evaluated by the reduction in germination of the pathogen's spores, the reduction of germ tube length, the reduction of the necrotic area on the leaf or in lesion number, and by the inhibition zone on agar plates. Cytological observations permit the observation of abnormal cytoplasm and mycelium and parasitism (Austin et al., 1977; Baker et al., 1983; Morgan, 1963; Omar and Heather, 1979).

The degree of inhibition of spore germination and germ tube growth appear to be reliable estimates of antagonism as they seem to be linked to a reduction in disease levels (Andrews et al., 1983; Austin et al., 1977; Blakeman and Fraser, 1971; Dickinson and Skidmore, 1976; Doherti and Preece, 1978; Flavel and Spurr, 1977; Fokkema and Lorbeer, 1974; Fokkema and Van der Meulen, 1976; Sleesman and Leben, 1976; Spencer, 1980; Van den Heuvel, 1969). Often, however, antagonists that inhibited germ tube growth had little effect on the percentage of spore germination, thus these parameters should not be used alone for screening purposes (Brame and Flood, 1983; Fokkema, 1973; Fokkema and Lorbeer, 1974; Spencer, 1980; Van den Heuvel, 1969). The use of inhibition zones on agar is also questionable. Fokkema (1973) found in vivo results had no relations with tests performed in petri dishes. Van den Heuvel (1969) found that isolates of Aureobasidium pullulans effective in reducing plant infection had no inhibitory effect on the pathogen in culture media. On the other hand, a Penicillium species producing an inhibition zone on agar was not effective as a biocontrol agent when tested on plants (Bhatt and Vaughan, 1962).

Time span between the application of the antagonist and the pathogen often appears to be critical (Brame and Flood, 1983; Omar and Heather, 1979; Pace and Campbell, 1974; Spencer, 1980; Van den Heuvel, 1969), with antagonism usually being stronger with longer time spans. This permits the antagonists to grow and develop and use nutrients and/or produce inhibitors. The addition of nutrients to the antagonist inoculum can improve its effectiveness (Bashi and Fokkema, 1977; Leben and Daft, 1965; McBride and Hayes, 1979b). Nutrients help in the establishment of the biocontrol agent and stimulate its growth.

6)Field experimentation

Field testing of selected antagonists has yielded variable results. An effective bacterial antagonist under laboratory conditions failed to control disease in the field due to its inability to withstand desiccation (Leben et al., 1965; Sleesman and Leben, 1976). Trichoderma pseudokoningii, antagonist of Botrytis cinerea on apple, was not effective in controlling infections under cool weather (Tronsmo and Raa, 1977). Successful results were obtained later with a strain capable of growing at lower temperatures (Tronsmo and Ystaas, 1980). Spraying of Trichoderma viride could control grey mold up to 70 % and excoriosis up to 79 % on grape in the field (Dubos et al., 1978). The antagonist reduced the Botrytis inoculum by colonizing the senescent flower parts and thus prevented the spreading of diseased areas.

Cladosporium sp. and Aureobasidium pullulans were used to control blossom blight and green fruit rot of strawberry in the field (Bhatt and Vaughan, 1962). A higher yield of fruits was obtained but with the same percentage of rot. Newhook (1957) demonstrated the importance of phylloplane fungi in colonizing dead flower parts in tomato and thus protecting the fruits against Botrytis cinerea. Wood (1951) and Newhook (1951) showed that any manipulation favouring a rapid colonization by saprophytes of wounds and frost lesions on lettuce leaves helped in reducing the incidence of grey mold. The importance of saprophytes was emphasized when an increase of Rhizopus damage was recorded on strawberry fruits sprayed with fungicides having little effect on mucoraceous fungi but which presumably reduced saprophyte populations (Jordan, 1973).

Spraying field-grown wheat with a mixture of yeast cells and nutrients permitted the yeast population to reach about 10^4 cells/cm^2 in 15 days (Fokkema, den Houter et al., 1979). With such a dense yeast population, leaves were less susceptible to infection by Cochliobolus sativus and Septoria nodorum. As untreated leaves became more densely colonized with naturally-occurring yeasts over time, differences in susceptibility between treated and untreated leaves disapeared. Since there was no colonization of the younger leaves from yeast populations on older leaves, spraying permitted an earlier establishment of an antagonistic population on young leaves. When the natural yeast population of rye leaves was drastically reduced by fungicide applications, infection by Cochliobulus sativus was increased (Fokkema et al., 1975).

It thus appears that the saprophytic microflora on plants may play an important role in limiting disease development. In order to establish a biological control program, it is necessary to understand the biology of these microbes colonizing the leaf surface. In particular, the major components of the phylloplane microflora, the succession of species on the phylloplane, and the factors affecting these population must be well defined.

B-Phylloplane microflora

1)Introduction

Ruinen (1961) was the first to attract attention to the microbiology of the external surface of the leaf, which she called the "phyllosphere". The tropical plants she worked with had a diverse community of fungi, bacteria, algae and lichens. Since that time, numerous reports have been published on the composition of the bacterial and/or fungal microflora of various herbaceous and woody plants, including conifers, in both temperate and tropical regions. In temperate climates, a limited number of taxa are almost always associated with the phylloplane environment.

2)Fungal flora

Fungi are mainly represented by pigmented forms. Cladosporium spp. and Aureobasidium pullulans (de Bary)Arnaud, along with red and white yeasts such as Sporobolomyces roseus Kluyver et van Niel, Rhodotorula spp., Cryptococcus laurentii (Kufferath) Skinner, Bullera spp., Tilletiopsis spp. and Torulopsis spp., are the dominating fungi. Also consistently reported but less important are Alternaria alternata (Fries)Keissler, Botrytis cinerea Pers., Cephalosporium spp., Epicoccum purpurascens Ehrenberg ex Schlechtendal, Fusarium spp., Penicillium spp., Phoma spp., Stemphyllium botryosum Wallroth and some sterile mycelium forms. The taxa mentioned above form nearly the totality of the mycoflora of potato (Bainbridge and Dickinson, 1972; Hollomon, 1967), pea (Dickinson, 1967), barley (Diem, 1967, 1974; Dickinson, 1973a), wheat (Flannigan and Campbell, 1977), rapeseed (Tsuneda and Skoropad, 1978b), beet (Warren, 1972b), tobacco (Ducommun and Corbaz, 1982; Norse, 1972) and apple (Andrews and Kenerley, 1978; Hislop and Cox, 1969; Pennicook and Newhook, 1981). Newhook (1951), as part of a biocontrol study, isolated fungi from dead tissues of field-grown lettuce. He reported Fusarium flocciferum, Phoma spp. (mainly P. eupyrena), Cephalosporium sp. and Cladosporium sp. as being the most common saprophytes found.

Non-crop plants studied had much the same fungal components. Bracken fern (Godfrey, 1974), Phragmites communis (Apinis et al., 1972), Typha latifolia (Pugh and Mulder, 1971), Halimione portulacoides (Dickinson, 1965), Hippophae rhamnoides (Lindsey and Pugh, 1976), Salsola kali (Pugh and Williams, 1968), Norway maple (Breeze and Dix, 1981), trembling aspen (Wildman and Parkinson, 1979), larch (McBride and Hayes, 1977), and Norway spruce (Collins and Hayes, 1976) have been some of the species examined.

Most of the fungi found on the leaf surface are air-borne. *Cladosporium*, *Alternaria*, *Sporobolomyces* and *Tilletiopsis* propagules are major components of the air spora (Gregory and Hirst, 1957; Last, 1955b). A number of soil-borne fungi can also be found on leaves close to the ground (Lamb and Brown, 1970; Mishra and Tewari, 1969). Buds can also contribute to the microflora. Pennycook and Newhook (1981) found buds of apple to be colonized by a community distinct from the one of expanded leaves. Fungal propagules isolated from the flag leaf of wheat occured in a successional manner (Flannigan and Campbell, 1977). At first, *Aureobasidium pullulans* and *Cladosporium* were the only molds isolated. *Alternaria alternata*, although appearing early, became important only toward the end when it accounted for about 20 % of the propagules. *E. purpurascens* appeared only when the flag leaf had turned yellow.

A large proportion of the propagules deposited on the leaf surface seem to remain inactive (Irvine *et al.*, 1978). Diem (1974) found *Cladosporium* to be the only mold to colonize barley leaves to a significant extent. Dickinson (1967), working with pea, concluded that only yeasts grew actively on green leaves, the filamentous fungi becoming active only at senescence. Yeasts accounted for half or more of the propagules found on Norway maple leaves (Irvine *et al.*, 1978) and their biomass was always more important than that of filamentous fungi (Breeze and Dix, 1981). *Sporobolomyces*, along with other yeasts, are the first fungi to colonize leaves (Dickinson and Wallace, 1976) and their populations usually increase as the season advances (Flannigan and

Campbell, 1977; Last, 1955a; Lindsey and Pugh, 1976; McBride and Hayes, 1977; Pugh and Mulder, 1971). Generally, both mycelial growth and species diversity increase as leaves age (Bainbridge and Dickinson, 1972; Breeze and Dix, 1981; Flannigan and Campbell 1977, Hislop and Cox, 1969; McBride and Hayes 1977, Pugh and Mulder 1971, Wildman and Parkinson 1979). Godfrey (1974) reports an increase of the filamentous forms such as Alternaria and Epicoccum at the end of the summer, Aureobasidium pullulans and Cladosporium herbarum being the only ones growing on green leaves with the yeasts. Collins and Hayes (1976) attributed the lack of an increase in fungal activity on spruce needles at the end of the season to the lack of leaf senescence in this plant.

3)Bacterial flora

Phylloplane bacteria are often closely related to the rhizosphere bacterial flora. Indeed, some phylloplane bacteria colonizing plants were shown to migrate there from seed and soil (Fryda and Otta, 1978; Leben, 1965; Leben and Daft, 1966). Very large differences have been reported in the literature with respect to the composition of the bacterial flora. While 90 % of the bacteria isolated from larch needles were Gram negative and belonged to Pseudomonas, Flavobacterium and Xanthomonas (McBride and Hayes, 1977), Bacillus, Brevibacterium and Micrococcus, which are Gram positive, were the most common on Norway spruce needles (Collins and Hayes, 1976). On Lolium perenne, the genera Chromobacterium, Corynebacterium, Pseudomonas and Xanthomonas were the most common (Dickinson et al., 1975). Yoshimura (1982) classified 70 % of his isolates from pine into Flavobacterium, Bacillus, Coryneform bacteria and a pink chromagen, and found only a few pseudomonads.

Hossell and Baker (1979) reported that Pseudomonas and Flavobacterium each comprised nearly one quarter of the bacteria isolated from the aquatic plant Ranunculus penicillatus, and were followed in importance by several minor groups such as Acinotobacter, Moraxella, Xanthomonas, Aeromonas, Cytophaga, Alcaligenes and Agrobacterium. Climates and types of soil and vegetation can explain some of these differences. The identification of bacteria can be a problem and is also very time consuming. It thus depends on the amount of resources and time that microbial ecologists are willing to spend on taxonomic work (Billing 1976).

Some phylloplane bacteria are plant pathogens having some kind of resident phase on the plant (Hirano and Upper, 1983; Leben et al., 1970). They can also be active as ice nuclei (Lindow et al., 1978). Providing there are favourable conditions for growth, bacterial populations increase steadily during the growing season (Dickinson et al., 1975; McBride and Hayes, 1977). Collins and Hayes (1976) observed a drop in the population in June and attributed it to the extremely dry weather. Long periods of dry weather can be particularly harmful to bacteria (Hirano and Upper, 1983). Leben (1965) showed the importance of relative humidity in the migration of bacteria to the upper part of the plant. At relative humidities greater than 90 %, a seed-borne isolate densely colonized cucumber seedlings , while at 70-80 % R.H. colonization was patchy, and at 30-40 % R.H. colonization was scattered and the seed-borne isolate absent. Fryda and Otta (1978) showed similar results on colonization and relative humidity.

4)Population and distribution

Distribution of microorganisms on leaves is not uniform but, instead, mostly patchy. SEM observations of Sporobolomyces roseus indicated that it aggregates in colonies covered with mucilage (Bashi and Fokkema, 1976). Bacteria also have clumped distributions on leaves (Hossell and Baker, 1979). Leben (1965) observed that bacteria occurred mainly in the depressions formed by the juncture of the anticlinal wall of epidermal cells of plants. On corn, bacteria were associated with the leaf margins (Leben and Daft, 1966). More fungi were found to colonize the leaf tips of Phragmites communis than the blade (Apinis et al., 1972). Differences in the colonization between the upper and lower surfaces of leaves were also reported.

Populations of microorganisms are most often expressed in number of propagules or colony-forming units (c.f.u.) per cm^2 or gram of leaves. These figures can range from zero to as high as 10^4 c.f.u./cm^2. Both direct and indirect methods are used to evaluate the populations on leaves. The direct methods involve the observation of leaves or leaf impressions using light microscopy or scanning electron microscopy. These methods enable one to actually count the real number of propagules on the leaf surface and see their distribution. On the other hand, direct observation is very labourious and, with light microscopy, often difficult due to lack of depth of field and the opacity of the material. Furthermore, it is almost impossible to identify the propagules and assess their viability.

Indirect methods involve the washing or grinding of known surface areas or leaf weights, followed by isolation on a medium, usually with a dilution series. Care must be taken that nearly all the propagules are removed and that no major increase or loss of propagules occurs during the process (Hirano and Upper, 1983). Results obtained by both types of methods can be very different. For example, on Norway maple leaves collected on the same date, there was an average of 3358 fungal propagules/cm^2 by direct observation versus 213 c.f.u./cm^2 obtained by the leaf washing method (Irvine et al., 1978). Andrews and Kenerley (1978) also had higher populations on apple leaves with direct observations. Hossell and Baker (1979) found similar bacterial numbers on young leaves with both methods, but on older leaves, the direct counts were much higher.

Variations in bacterial populations can be enormous between individual plants or plots and it is often better to express them in logarithms (Hirano et al., 1982). In trees, certain microbial populations were negatively correlated with height (Andrews et al., 1980; Wildman and Parkinson, 1979). The position of the leaf within the canopy was also a determining factor (Andrews et al., 1980). On herbaceous plants, microclimatic conditions on lower leaves are often more favourable and permit more microbial growth (Dickinson and O'Donnell, 1977; Norse, 1972). The associated vegetation can be important as it can supply propagules to the plant. On barley, higher populations of bacteria and fungi were found in a plot in the middle of a barley field than one away from other vegetation (Diem, 1967).

5)Effects of the microflora on the plant.

In addition to protecting plants from infections, the phylloplane microflora may have other effects on the plant (Fokkema, 1981). In fact, adverse effects have been reported. For example, Botrytis cinerea and yeasts (Cryptococcus laurentii and Sporobolomyces spp.) were able to degrade leaf cutin in vitro (Dickinson and Macnamara, 1983). However, other filamentous phylloplane fungi were not stimulated when cutin was added to their culture medium. McBride (1972) observed that waxes were removed around S. roseus colonies on larch needles. The removal of waxes from the leaf could make the plant more susceptible to pathogens. Furthermore, it would increase leaf wettability and nutrient loss, which could cause a premature senescence. Whether the phylloplane microflora hastens senescence and possibly reduces yields is controversial. Among several species of phylloplane fungi actually growing on the surface of barley leaves, two fungi, Stemphylium botryosum and Alternaria alternata, caused the formation of spots and accelerated the breakdown of chlorophyll (Skidmore and Dickinson, 1973). On the other hand, Fokkema, Kastelein and Post (1979) could not show any effects of phylloplane fungi on chlorophyll content and senescence in wheat leaves.

To determine whether phylloplane fungi could act as nutrient sinks, Frossard et al. (1983) tested the uptake of ^{14}C from wheat by Sporobolomyces roseus and Cladosporium cladosporioides. They could not detect any significant increase in leaching when the fungi were present on the leaves. Rabbinge et al. (1984) did not obtain any reduction in yield from winter wheat colonized with a well developed microflora. During a summer with a high infestation of Septoria , higher yields were obtained from plots where the fungal populations had been stimulated.

There is the possibility that certain phylloplane fungi act as weak pathogens. Epicoccum purpurascens was observed developing appressoria and penetrating underlying epidermal cells (Mansfield et al., 1975). Isolates of Alternaria alternata, C. cladosporioides and C. herbarum were able to penetrate through stomata in bean leaves (O'Donnell and Dickinson, 1980). However, this process was slow and under conditions very favourable to fungal growth. Also, only certain isolates of A. alternata caused necrosis and none were able to invade the mesophyll cells.

6)Factors affecting the microflora

a)Plant species:

Although the phylloplane microflora is composed of a rather limited number of taxa, some differences do exist between plant species with respect to the dominant and minor components of the microflora. Structural and chemical differences between plants can explain these variations. Lamb and Brown (1970) found striking differences when comparing the microflora of a species of eucalyptus with the microflora of willow and paspalum. The fungi dominating on eucalyptus were not present on the two others.

Lilac leaves were shown to produce a compound that inhibited the germination of A. alternata and B. cinerea conidia (Godfrey and Clements, 1978). Inhibitors (gallic acid and three unknown compounds, probably phenolics) produced by Norway maple prevented colonization by filamentous fungi (Irvine et al., 1978). Decline in the production of inhibitors in the late summer was associated with an increase in fungal activity.

b)Environmental factors:

Available moisture and nutrients are the two most important factors affecting microbial growth on leaves. Phylloplane fungi must be able to survive long periods of dessication and make the best of the short periods of moisture availability. Park (1982) compared several phylloplane fungi with various field, storage and soil fungi for their ability to grow at low water potential and to survive dessication. He found that while the soil fungi needed the highest water potential in order to grow, the three other groups had more or less similar requirements, with the storage fungi being able to grow at the lowest minimum water potential. However, the ability of hyphae to grow under low water availability was not associated with the ability to withstand dessication. Hyphal tips of all the phylloplane fungi tested could survive 14 days of drying and resume growth in less than one hour. This indicates that they can survive dessication with very little loss of biomass. Hyphal tips of storage and soil fungi survived less than a week but often the colonies could resume growth from conidia or other structures in the older part of the colony. The ability of many phylloplane fungi to form secondary spores rapidly may be of great survival value (Skidmore, 1976b).

Dickinson and O'Donnell (1977) observed a spore germination rate of 100 % and a fairly extensive colonization of leaves after 31 days by Cladosporium cladosporioides and Alternaria alternata when the relative humidity was maintained at 97 %. Growth and germination were very limited at 70-80 % R.H.. Germination of conidia of A. alternata was reduced in relative humidities of less than 100 % at 5° and 10° C. However, at temperatures of 15°, 25° and 35°, germination occured without being much affected by relative humidities as low as 90 %.

Last (1955a), found that high populations of Sporobolomyces on spring wheat occurred only when the canopy was dense enough to increase the level of humidity. Populations of S. roseus steadily increased under dew conditions or 95 % R.H. but declined at 65 % R.H., while they were maintained at 75 % R.H. (Bashi and Fokkema, 1977). Low relative humidity levels were as detrimental to well established populations as to poorly developed ones.

Rodger and Blakeman (1984), in a study of nutrient uptake in sycamore maple trees, concluded that the microbial populations were limited in the early part of the growing season more by a lack of moisture than by any other factor. However, later in the season, population growth was controlled by the availability of amino acids. Bashi and Fokkema (1977) attributed the plateau in the population of *S. roseus* to nutrient depletion. Thus, it appears that under favourable conditions, microbial populations expand until nutrient supplies are exhausted.

In low-nutrient or nutrient-free situations, some fungi can remain active due to the reserves they carry in their spores. This has been shown for *Epicoccum* and *Stemphyllium* (Breeze and Dix, 1981) and *Alternaria* (Dickinson and Bottomley, 1980). *Cladosporium*, which has much smaller spores, has very low levels of activity under the same conditions. *C. herbarum* conidia responded positively to the higher concentrations of sugar and leachates from old leaves (McBride and Hayes, 1979a). Old leaves usually lose more nutrients as they become more wettable (Blakeman, 1973). Growth of phylloplane fungi was also associated with the presence of pollen (Fokkema, 1968; Norse, 1972, Warren, 1972a). Aphid honeydew is another strong stimulant of microbial populations (Breeze and Dix, 1981; Rabbinge *et al*., 1984). Aphid control caused a greater reduction of the microflora than fungicide applications (Rabbinge *et al*., 1984). Infection of the leaf can favour saprophytic growth as it makes more nutrients available and permits the establishment of secondary invaders (Collins, 1982; Hayes, 1982; Last, 1970; Stadelmann and Schwinn, 1976).

Environmental pollution can affect the microflora, although some microorganisms appear to be much more sensitive than others (Bewley, 1979). Sporobolomyces roseus could not be detected on hawthorn leaves 2 km around a smelting complex while Aureobasidium pullulans did not seem to be affected (Bewley and Campbell, 1980).

c)Pesticide sprays:

It is likely that pesticide sprays have negative effects on saprophytic organisms. Studies in this field, involving the comparison of microflora composition and populations between sprayed and unsprayed plants, have been performed. Most of the knowledge acquired so far in this area is on the effects of fungicides on the mycoflora. Very little is known about the effects of insecticides (Andrews, 1981; Hislop, 1976). Also, few workers took account of the bacterial flora in their studies (Dickinson, 1973b).

Effects of each fungicide vary greatly. Usually, non-specific protectants such as captafol, captan and zineb reduced the population of most fungi significantly (Bainbridge and Dickinson, 1972; Dickinson, 1973a; Dickinson and Wallace, 1976; Warren, 1974). Bacterial numbers were apparently unaffected by captofol (Bainbridge and Dickinson, 1972). Maneb had no or only slight effects on filamentous fungi (Bainbridge and Dickinson, 1972). The population of yeasts and A. pullulans were affected but were able to recover afterwards. Ducommun and Corbaz (1982) hypothesized that maneb mainly affects sporulation since the greatest reductions in population were observed with the spore fall technique.

Systemics such as metalaxyl used as a seed treatment had only minor effects (Ducommun and Corbaz, 1982). Benomyl effects were variable. In general, Cladosporium, Sporobolomyces, and A. pullulans were sensitive (Dickinson and Wallace, 1976; Jenkyn and Prew, 1973; McKenzie, 1971; Warren, 1974). No reductions were seen in the numbers of Alternaria, Epicoccum, Fusarium and Stemphylium (McKenzie, 1971).

Kuthubutheen and Pugh (1978) studied the effects of fungicides on several phylloplane fungi in culture. Captan and dicloran had only a fungistatic effect since spores treated with these fungicides were found to be viable after washing. So, it is possible that spores could survive spraying and take part in the recolonization of the leaf afterwards. On the other hand, thiram and verdasan were found to have a fungicidal effect on these same organisms.

C-Sclerotinia diseases

Lettuce drop occurs in most lettuce growing areas and can be caused by either Sclerotinia sclerotiorum (Lib.) de Bary (Sclerotiniaceae, Ascomycotina) or S. minor Jagger. The first species produces large sclerotia which may germinate carpogenically or myceliogenically, while the latter is a small sclerotial type which infects plants directly by mycelium produced in the soil (Willets and Wong, 1980). Both species are present in Québec, although S. sclerotiorum seems more widespread (Reeleder and Charboneau, 1986).

Air-borne ascospores are forcibly released by the apothecia, which are an important source of inoculum in most susceptible crop and weed species (Abawi and Grogan, 1974; Newton and Sequeira, 1972; Purdy, 1979). Apothecia can be produced from sclerotia throughout the growing season providing there is sufficient moisture on the soil surface, and usually they are found under the canopy of the crop or in weed stands (Abawi and Grogan, 1975; Newton and Sequeira, 1972). Ascospores deposited on plants can survive for a few days and will germinate within a few hours when free moisture becomes available (Abawi *et al.*, 1975; Caesar and Pearson, 1983; Newton and Sequeira, 1972; Purdy, 1979; Sing and Sing, 1984). Abawi and Grogan (1975) report an optimum temperature of 25°C for ascospore germination, while it was good at 20° and poor at 5°, 10° and 30°C.

Between 48 and 72 hours of leaf wetness are required for ascospores to successfully cause infection (Abawi and Grogan, 1979). Sutton and Deverall (1983) were able to show direct infection of leaves by ascospores under laboratory conditions. However, in most cases, a food base seems to be required for infection to occur at any significant level (Abawi *et al.*, 1975; Newton and Sequeira, 1972; Purdy, 1958; Sitepu and Wallace, 1984). The food base usually occurs as senescent plant material or wounds which are colonized by the fungus prior to infection. At this point, the fungus can then penetrate the host directly by mechanical pressure with simple or multiple appressoria (Abawi *et al.*, 1975; Purdy, 1958; Tariq and Jeffries, 1984). Direct penetration without formation of appressoria was reported (Sutton and Deverall, 1983) as well as penetration through stomata (Jones, 1976). In lettuce, a rapid expansion of the infected area follows, killing the plant in a few days (Abawi and Grogan, 1979).

The possibility of biological control of ascospore infection has been investigated. Using beans, Trutmann *et al.* (1982) were able to successfully establish parasitism of *S. sclerotiorum* hyphae by the hyperparasite Coniothyrium minitans but no significant reduction of disease was obtained. Sitepu and Wallace (1984) obtained inhibition of ascospore germination by Fusarium lateritium on various culture media. With young lettuce plants in contact with dead organic matter, they obtained an infection rate of 33.3 % on plants sprayed with macroconidia of F. lateritium and 100 % for the control. On pollen-treated tobacco leaves, a reduced level of ascospore germination as the leaves grew older was attributed to inhibition by phylloplane fungi stimulated by the presence of pollen (Hartill, 1975). There were no differences in germination between younger and older pollen-free leaves.

SECTION I: Effects of pesticides on the phylloplane microflora of lettuce

INTRODUCTION

The purpose of this study was to evaluate the effects of a pest and disease control programme on the phylloplane microflora of lettuce. To date, several fungicides have been shown to affect saprophytes on various plants. This subject has been extensively reviewed (Andrews, 1981; Dickinson, 1973b; Hislop, 1976).

Biological disease control on the phylloplane is now being considered as an interesting alternative to fungicides (Blakeman and Fokkema, 1982). Naturally occurring saprophytes on the leaf surface can antagonize pathogens and reduce infection levels. Under field situations, Fokkema et al. (1983) showed that a well-developed microflora prevented stimulation of Cochliobolus sativus and Septoria nodorum by aphid honeydew. Cochliobolus sativus caused greater degrees of necrosis on rye plants when microflora populations had been reduced by benomyl applications (Fokkema et al., 1975). Newhook (1951) and Wood (1951) showed that naturally occuRring saprophytes on lettuce could be inhibitory to Botrytis cinerea. For these reasons, the elimination of these potentially useful microbial populations might not be desirable.

An insecticide (carbaryl) and a fungicide (maneb) were tested in the field during two summers to determine their effects on the lettuce microflora. Population levels and composition of the fungal and bacterial microflora were evaluated during this time.

MATERIALS AND METHODS

A-Field plots

Lettuce, cv. Ithaca, was seeded in the plant pathology field at Macdonald College using a randomized complete block design with six replicates. Plots of 2 m x 2 m, each consisting of four rows, were separated from the neighbouring plots by either three guard rows or 0.5 m of row. The gradient was oriented toward the north, in the direction of the highway. North of the field were plots of garlic and onions, to the west were plots of sunflower. To the south and east were uncultivated areas. Rows were oriented east-west. Overhead irrigation was used to provide moisture during dry periods.

There were three different treatments in 1983: a single application of carbaryl (Sevin, 85 % WP) at a rate of 3.25 kg/ha (1.3 g/plot), a single application of maneb (Maneb, 80 % WP) at a rate of 2.2 kg/ha (0.88 g/plot) and an untreated control (see Table I). In 1984, a fourth treatment consisting of multiple applications of maneb (at the same rate as the single application) was added. In 1983, the field was sown on May 25 and sampled on July 11, 18, 25 and August 2 with pesticides applied on July 14. In 1984, the field sown on May 28 was sampled on July 3, 12, 17 and 25. Pesticides were applied on July 9 and, for the multiple application of maneb, additional treatments were made on July 13 and 21. Applications were made with a compressed air sprayer (Chapin no. 133).

TABLE I: Sampling schedules

Date	1983			1984		
		rain (mm)	max Temp. (°C)		rain	max Temp.
June 29					---	26.4
30					1.2	26.5
July 01					---	26.4
02					---	29.8
03		---	29.5	sampling 1	---	28.0
04		---	32.5		4.0	27.7
05		10.0	31.7		---	25.2
06		2.2	23.2		---	27.5
07		---	23.3		1.0	27.5
08		---	23.5		---	22.4
09		0.4	24.9	spraying	---	26.4
10		---	23.0		---	27.4
11	sampling 1	4.4	23.0		18.2	27.6
12		1.6	30.6	sampling 2	19.3	27.8
13		---	29.6	spraying*	2.4	26.7
14	spraying	---	30.1		2.0	27.2
15		6.5	29.7		---	27.0
16		---	28.4		12.2	26.2
17		---	27.1	sampling 3	---	25.1
18	sampling 2	---	27.2		1.0	23.8
19		---	29.4		---	25.0
20		22.2	28.4		---	26.0
21		4.2	26.0	spraying*	---	28.0
22		---	27.1		---	29.0
23		---	27.1		---	28.7
24		---	25.0		---	28.2
25	sampling 3	---	27.7	sampling 4	---	23.8
26		---	27.7			
27		---	29.5	total	61.3	
28		---	27.8			
29		0.8	25.8			
30		6.0	29.5			
31		---	26.4			
Aug. 01		10.5	28.2			
02	sampling 4	3.0	25.3			
	total	71.8				

* multiple applications of maneb only

B-Sampling

On each sampling date, three plants were selected at random in each plot and placed in separate plastic bags in a cooler until processing. Five discs (1 cm diameter) were cut, using a sterile cork borer, from the third, fourth or fifth leaf. Discs from each plot were combined together for further processing. Bacteria were isolated on nutrient agar by the leaf washing method whereas fungi were isolated on malt extract agar with 100 p.p.m. of tetracycline, using the three methods described below. Three replicate plates were prepared per plot sample for each of the following methods:

1-Leaf washings: Five leaf discs were placed in 125 ml flasks containing 25 ml of sterile phosphate buffer, pH 7.0, and shaken for 1 h at 200 r.p.m.. Then, 0.25 ml of the suspension was pipetted onto each plate and spread over the surface.

2-Leaf imprints: One leaf disc was placed on each plate and removed after 12 or 24 h. In 1984, only the 24 h period was used.

3-Washed discs: Discs used for the leaf washings were washed twice more in 50 ml of sterile water with a drop of Tween 80 for 10 min at 200 r.p.m.. This was done to ensure that most of the detachable propagules on leaves were recovered. A disc was placed in each plate. This method was used in 1984 only.

C-Composition of the microflora

Three randomly selected colonies of filamentous fungi were identified from each plate treated with leaf washings. For the other methods, identification was attempted for all the colonies present. Exposing the cultures to near ultra-violet light in order to stimulate sporulation was often necessary for identification.

Bacteria were purified by streaking and subculturing onto nutrient agar, then classified into groups on the basis of results of Gram and oxidase tests. From each original plate, three colonies were selected at random and streaked onto fresh plates. The oxidase reaction was determined by smearing bacteria on filter paper previously treated with a solution of 1 % tetramethyl-p-phenylenediamine dihydrochloride (Smilbert and Krieg, 1981). The Gram staining was performed according to the Hucker method (Smilbert and Krieg, 1981). A number of Gram positive strains were subjected to the heat test to determine their spore-forming ability (Smilbert and Krieg, 1981). Gram negative strains were further characterized by the test for oxidation-fermentation of glucose (Smilbert and Krieg, 1981) and various biochemical tests were performed using API 20E kits (Analytab Products, Plainview, N.Y.).

Populations of filamentous fungi and bacteria were estimated by counting the total number of colonies obtained for each replicate plate prepared during the leaf washing procedures. Yeast presence was noted but their populations were not included in the population estimates as their colonies often coalesced. Multiplying the number of colonies obtained per plot by 4.24628 transforms the data into populations per cm^2 of leaf surface. This index was obtained as follows: the area of each disc (0.785 cm^2) x 2 sides x 5 discs gives a total area of 7.85 cm^2 for each flask of 25 ml. Since 0.25 ml of suspension is used for each plate and there are 3 plates/plot, the total agar surface is equivalent to 0.23552 cm^2 of leaf surface. To make 1 cm^2, this has to be multiplied by 4.24628. An analysis of variance of the log-transformed population data was performed along with Duncan's multiple range test.

RESULTS

A-Fungal flora

In 1983, fungal populations were negatively affected by maneb (see Figure 1). The effect of this fungicide was detected on the second sampling by a reduced population level of 70 cfu/cm^2 while it was at 126 cfu/cm^2 for the control. This reduction was not dramatic, however, and population levels were comparable to the control and carbaryl treatment in the third and fourth sampling. Carbaryl did not cause any reduction in populations. The control and carbaryl-treated plants had very stable population levels at around 125 cfu/cm^2 for the two first samplings. They started to increase slightly at the third sampling to reach a significantly higher level on the fourth at about 230-280 cfu/cm^2.

In 1984, a single application of maneb did not seem to have any effect on the fungal population (see Figure 2). The population levels in the control and maneb (single application) treatments were similar on the four sampling dates. The second and third applications of maneb in the multiple applications treatment, however, caused a significant reduction in the number of colonies isolated. The reduction was especially dramatic on the fourth sampling with 10 cfu/cm^2 for the multiple application treatment versus 62 cfu/cm^2 for the control.

Populations in carbaryl-treated plots were always equal or higher than in the control plots. It could not be demonstrated statistically, however, that there was an actual stimulation due to this insecticide. The total population mean of 66 cfu/cm^2 increased significantly to about 140 cfu/cm^2 during the second and third sampling dates (see Table II). Populations levels were significantly lower on the fourth date. Raw population data and details on statistical analysis are given in the appendix III. Tranformation of the data was necessary since population counts have a Poisson distribution and a normal distribution was needed for the analysis of variance. The data approached normality after log-transformation (see skewness values in Appendix III).

There were from 150 to 220 fungal isolates identified for each sampling method on each date (see Tables III and IV). Since no difference in the composition of the mycoflora could be detected for any treatment, these results were combined together. The mycoflora of lettuce is apparently composed of a number of soil-borne and air-borne types. The proximity of lettuce leaves to the ground may explain why soil-borne fungi were so common. _Phoma_ (mainly _P. medicaginis_ Malbr. and Roum.) and _Fusarium_ (mainly _F. equiseti_ (Corda) Sacc.) were the filamentous fungi most consistently recovered by the three methods. The occurrence of other genera varied, depending upon the date of sampling and the isolation method used. Yeasts, _Aureobasidium pullulans_, and _Cladosporium_ spp. were major components with the leaf washing method. Yeasts were represented mainly by _Rhodotorula glutinis_ and _Bullera_ sp.. _Alternaria_ spp. was present on each date but was more often isolated from leaf imprints and washed discs. Soil-borne fungi such as _Gliocladium_, _Myrothecium_, _Trichoderma_ and Mucoraceous fungi occurred sporadically. White sterile mycelium was present in small numbers in 1984. _Fusarium_, _Phoma_, _Alternaria_, _A. pullulans_ and _Cladosporium_ were the fungi most commonly isolated from washed discs.

Figure 1 - 1983 Fungal populations

Populations of filamentous fungi expressed as log of colony forming units per cm^2 of leaf surface (cfu/cm^2).

Date 1: 11/07/83 Date 2: 18/07/83

Date 3: 25/07/83 Date 4: 2/08/83

U: untreated control M: maneb C: carbaryl

Means with the same letter are not significantly different according to Duncan's Multiple Range Test, p = 0.05

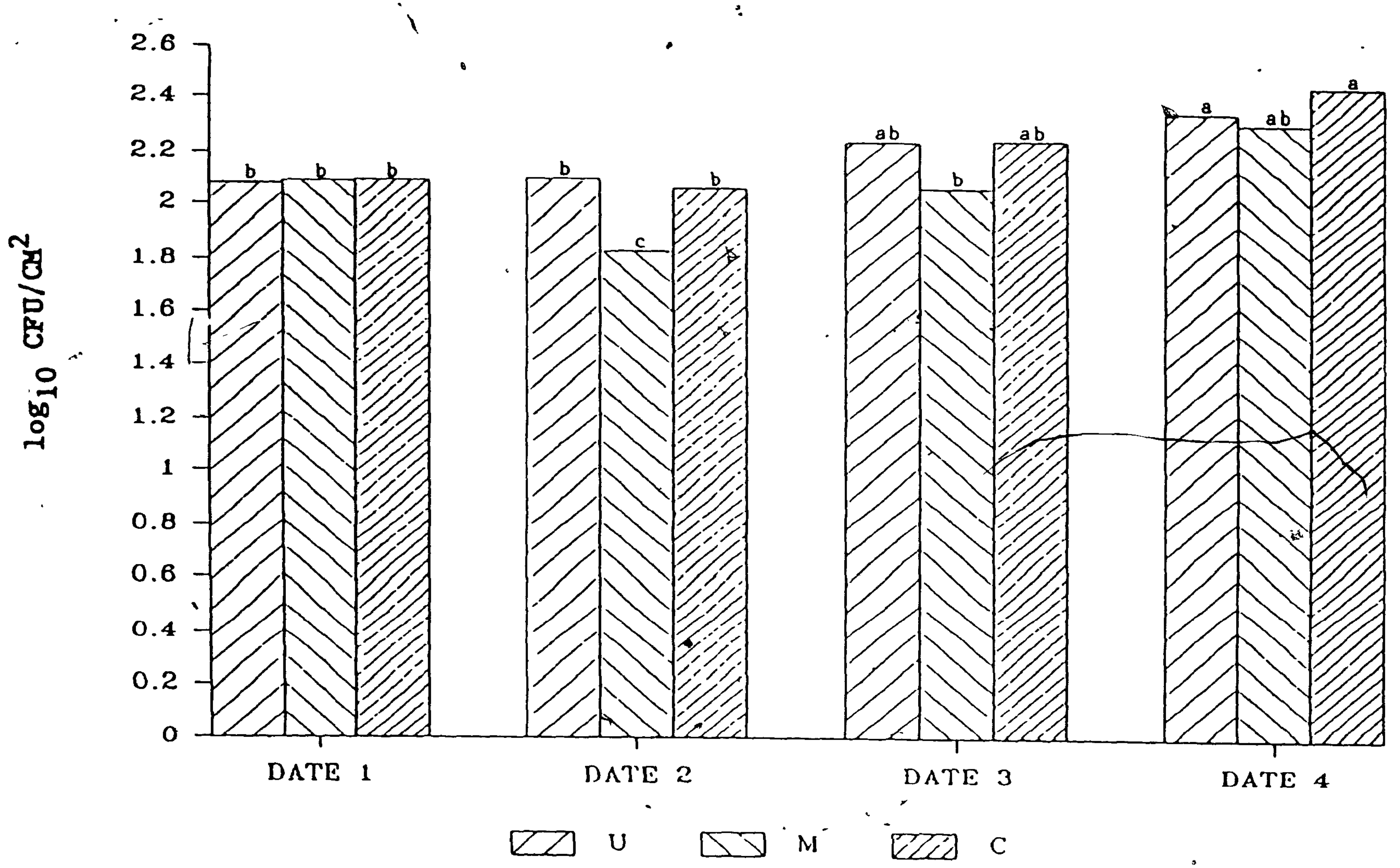

log$_{10}$ CFU/CM^2
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
b
b
b
b
c
b
ab
b
ab
a
ab
a
DATE 1
DATE 2
DATE 3
DATE 4
U
M
C

Figure 2 - 1984 Fungal populations

Populations of filamentous fungi expressed as log of colony forming units per cm^2 of leaf surface (cfu/cm^2).

Date 1: 3/07/84 Date 2: 12/07/84

Date 3: 17/07/84 Date 4: 25/07/84

U: untreated control M: maneb C: carbaryl MA: multiple applications of maneb

Means with the same letter are not significantly different according to Duncan's Multiple Range Test, $p = 0.05$

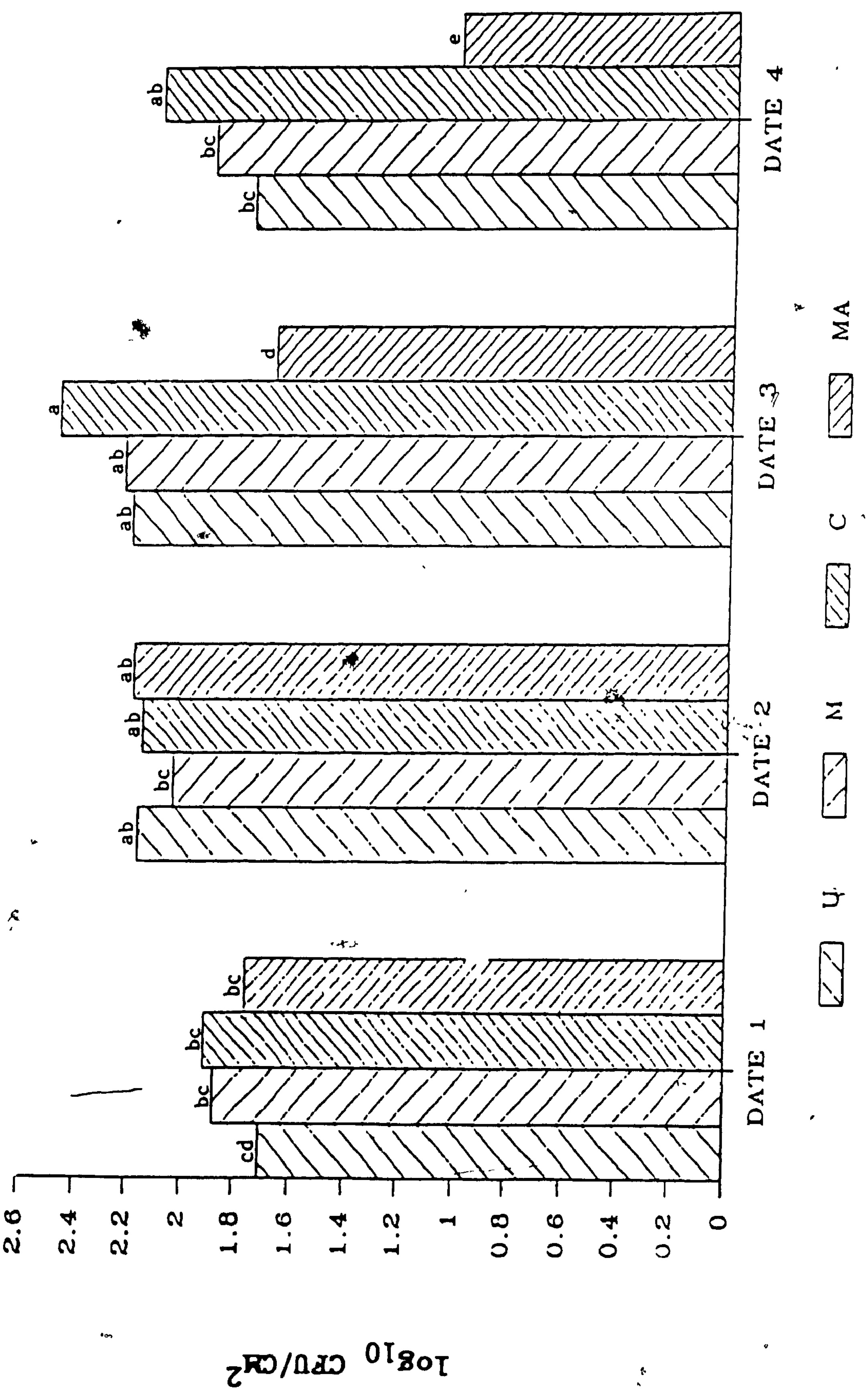

log10 CFU/CM2
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
cd
bc
bc
bc
DATE 1
ab
bc
ab
ab
DATE 2
ab
ab
a
d
DATE 3
bc
bc
ab
e
DATE 4
U
M
C
MA

TABLE II : Total populations (expressed as colony forming units per cm^2)

1983

Sampling date (day/month/year)	Filamentous fungi	Bacteria
11/07/83	122 bc*	669 b
18/07/83	100 c	623 b
25/07/83	153 b	660 b
2/08/83	236 a	1254 a

1984

Sampling date	Filamentous fungi	Bacteria
3/07/84	66 b	381 b
12/07/84	140 a	537 b
17/07/84	146 a	1823 a
25/07/84	53 b	536 b

* For each year and each class of microorganism, means followed by the same letter are not significantly different according to Duncan's Multiple Range Test, $p = 0.05$

TABLE III: Mycoflora composition, 1983

	11/07/83[a]			18/07/83			25/07/83			2/08/83		
Fungus recovered	L.W.	L.I. 12h	L.I. 24h	L.W.	L.I. 12h	L.I. 24h	L.W.	L.I. 12h	L.I. 24h	L.W.	L.I. 12h	L.I. 24h
Alternaria spp.	1.3[b]	4.8	4.1	0.0	14.6	14.7	0.6	3.0	2.8	0.6	0.9	1.9
Aureobasidium pullulans	7.2	0.0	0.0	14.8	2.1	4.2	1.2	1.0	0.9	3.8	0.9	0.9
Botrytis cinerea	0.0	0.0	2.4	1.9	0.0	0.0	0.0	0.0	0.9	0.0	4.7	0.9
Cladosporium spp.	21.1	1.6	0.8	22.6	0.0	0.0	0.0	0.0	0.0	0.6	0.0	0.0
Epicoccum purpurascens	2.0	1.6	3.3	1.3	18.8	12.6	0.0	4.0	1.9	0.0	0.0	0.0
Fusarium spp.	19.7	21.8	12.2	15.5	16.7	16.8	16.1	34.3	20.6	15.6	36.8	34.9
Gliocladium spp.	2.0	8.9	16.3	1.3	3.1	1.1	1.2	5.1	11.2	2.5	2.8	0.0
Myrothecium sp.	4.6	8.1	4.9	0.0	0.0	0.0	10.6	2.0	0.9	13.1	0.0	7.5
Phoma spp.	41.4	33.1	28.5	38.7	32.3	44.2	59.6	38.4	34.6	56.9	31.1	34.0
Mucorales	0.7	8.1	13.0	0.6	4.2	2.1	0.0	4.0	20.6	0.0	10.4	7.5
Trichoderma spp.	0.0	4.0	6.5	0.0	1.0	1.1	3.7	3.0	4.7	0.6	4.7	11.3
Penicillium spp.	0.0	0.8	0.0	1.9	1.0	0.0	0.6	0.0	0.0	1.9	0.9	0.0
Unknown	0.0	7.3	8.1	1.3	6.3	3.2	6.2	5.1	0.9	3.1	4.7	0.0
Other	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.3	1.9	0.9
Sterile mycelium	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

a: day/month/year
b: percentage of the total fungi isolated, for each isolation method and date
L.W. Leaf washings
L.I. Leaf imprints

TABLE IV: Mycoflora composition, 1984

Fungus recovered	3/07/84[a]			12/07/84			17/07/84			25/07/84		
	L.W.	L.I.	W.D.	L.W.	L.I.	W.D.	L.W.	L.I.	W.D.	L.W.	L.I.	W.D.
Alternaria spp.	3.7[b]	8.8	17.2	1.0	5.5	4.5	1.4	3.3	5.2	5.8	23.3	21.7
Aureobasidium pullulans	9.0	13.1	8.6	5.8	5.5	3.7	1.4	1.3	0.7	14.6	6.2	0.9
Botrytis cinerea	1.6	0.7	4.3	0.0	0.6	0.0	0.0	1.3	0.7	0.6	1.4	0.0
Cladosporium spp.	31.7	7.3	6.9	8.2	1.8	2.2	13.0	1.3	1.5	23.4	2.1	11.3
Epicoccum purpurascens	1.6	3.6	2.6	0.5	0.6	0.0	0.5	0.0	0.0	0.6	4.1	1.7
Fusarium spp.	14.8	33.6	19.8	9.1	38.8	31.3	16.8	39.1	40.0	4.1	25.3	17.4
Gliocladium spp.	0.0	0.7	0.0	1.0	3.6	3.0	0.0	1.3	0.7	1.8	1.4	0.0
Myrothecium sp.	2.1	2.2	2.6	0.5	0.0	1.5	4.3	4.0	7.4	0.0	0.0	0.0
Phoma spp.	25.4	19.7	33.6	66.8	36.4	47.0	59.6	34.4	38.5	45.6	28.8	35.7
Mucorales	1.1	2.2	0.0	0.5	2.4	0.0	0.0	5.3	0.7	0.0	0.0	0.0
Trichoderma spp.	0.5	0.7	0.0	0.5	0.0	1.5	0.0	0.0	0.0	0.0	0.7	0.0
Penicillium spp.	0.5	0.0	0.0	0.0	0.6	0.0	0.5	2.0	0.0	0.0	0.7	0.9
Unknown	5.8	5.1	0.9	2.9	3.6	2.2	1.9	4.0	1.5	2.3	4.1	7.0
Other	0.5	0.7	2.6	0.0	0.0	0.7	0.0	2.0	2.2	1.2	0.7	1.7
Sterile mycelium	1.6	1.5	0.9	3.4	0.6	2.2	0.5	0.7	0.7	0.0	1.4	1.7

a:day/month/year
b:percentage of the total fungi isolated, for each isolation method and date
L.W. Leaf washings
L.I. Leaf imprints
W.D. Washed discs

B-Bacterial flora

Bacterial populations did not seem to be affected by any of the pesticide treatments (see Figures 3 and 4). The population levels were quite stable in 1983 with a mean between 623 and 670 cfu/cm^2 for the three first sampling dates (see Table II). Only on the fourth date was a significant increase observed (1254 cfu/cm^2). In 1984, population levels were about 380 to 540 cfu/cm^2 except on the third date when they peaked to an average of 1823 cfu/cm^2 and then dropped back again on the fourth sampling date (see Table II).

The composition of the bacterial community varied considerably for each sampling date (see Table V). Gram positive or Gram negative (oxidase negative and positive) types dominated on different dates. Actinomycetes were always present on each date with variable importance. There were no differences observed in the proportion of each group for the different chemical treatments. Among the Gram positive strains, 25 out of 39 were able to form spores and were thus classified as Bacillus. 133 strains of Gram negative bacteria were found to be non-fermentative, with only 31 capable of fermenting glucose. Only 14 out of 78 were pigmented. Isolate numbers and detailed results on biochemical tests are given in Appendix IV. These results did not permit the classification of the isolates into taxonomic groups.

Figure 3 - 1983 Bacterial populations

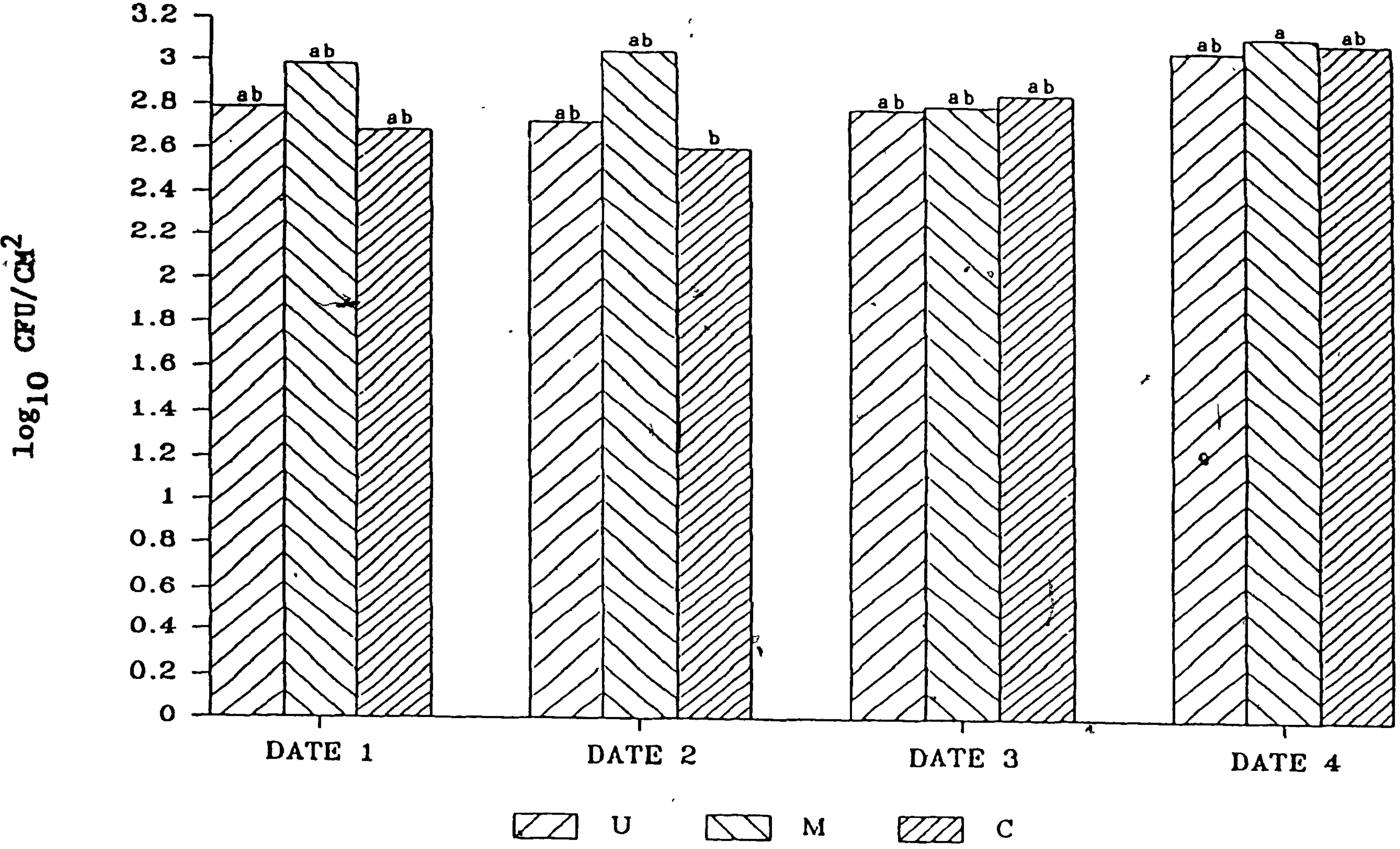
Populations of bacteria expressed as log of colony forming units per cm^2 of leaf surface (cfu/cm^2).

Date 1: 11/07/83 Date 2: 18/07/83

Date 3: 25/07/83 Date 4: 2/08/83

U: untreated control M: maneb C: carbaryl

Means with the same letter are not significantly different according to Duncan's Multiple Range Test, $p = 0.05$



3.2
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2.8
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0
$\log_{10}$ CFU/CM^2
ab
ab
ab
ab
ab
b
ab
ab
ab
ab
a
ab
DATE 1
DATE 2
DATE 3
DATE 4
U
M
C

Figure 4 - 1984 Bacterial populations

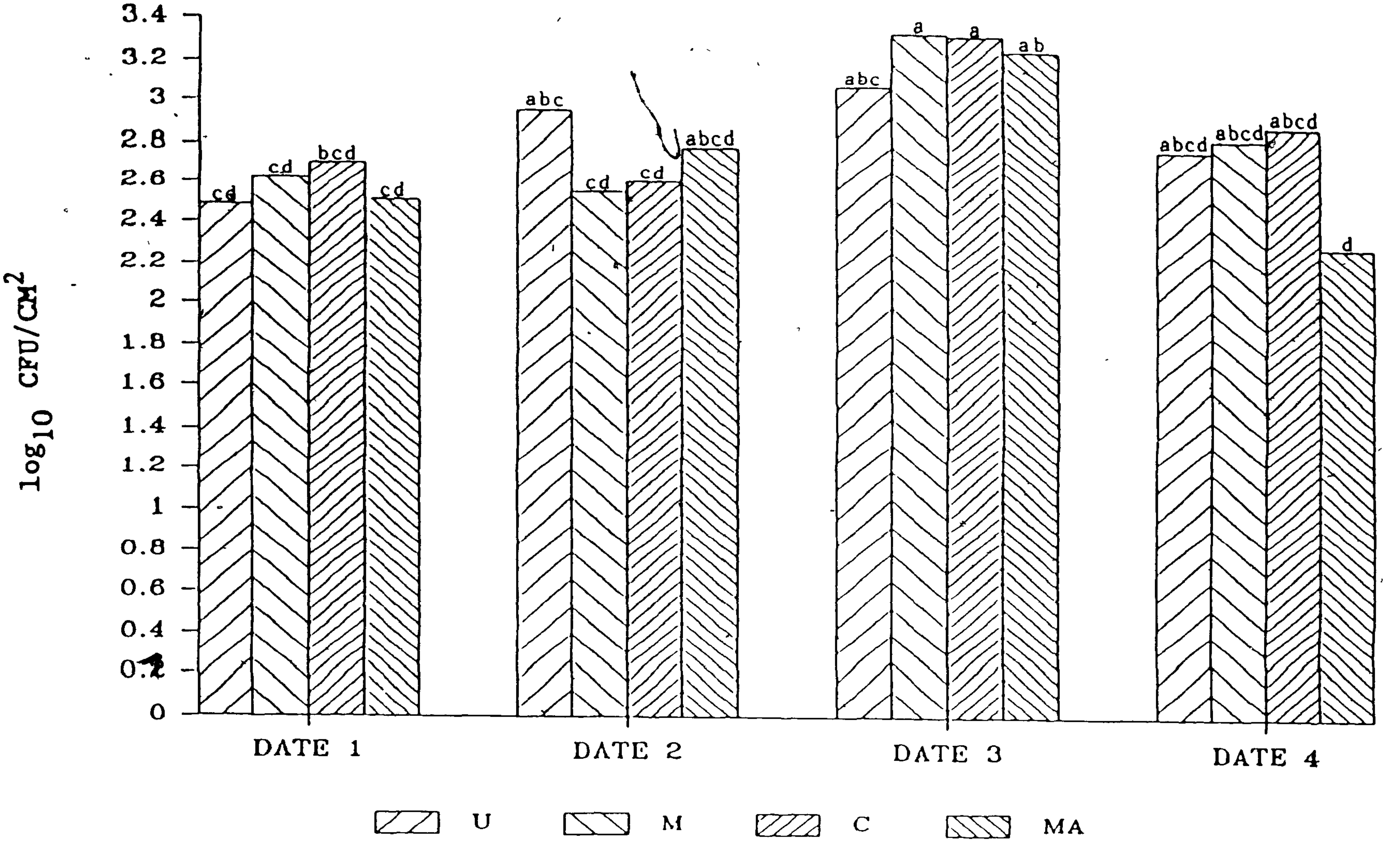
Populations of bacteria expressed as log of colony forming units per cm^2 of leaf surface (cfu/cm^2).

Date 1: 3/07/84 Date 2: 12/07/84

Date 3: 17/07/84 Date 4: 25/07/84

U: untreated control M: maneb C: carbaryl MA: multiple applications of maneb

Means with the same letter are not significantly different according to Duncan´s Multiple Range Test, $p = 0.05$



3.4
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TABLE V: Composition of the bacterial flora

Group	1983			
	11/07/83[a]	18/07/83	25/07/83	2/08/83
Actinomycetes	10.3[b]	14.6	8.3	2.0
Gram positive	27.9	44.3	18.0	11.9
Gram negative/oxidase negative	33.9	30.4	29.3	56.2
Gram negative/oxidase positive	27.9	10.8	44.4	29.9
	1984			
	3/07/84	12/07/84	17/07/84	25/07/84
Actinomycetes	37.1	9.2	14.2	14.2
Gram positive	49.3	41.7	32.7	32.7
Gram negative/oxidase negative	9.8	24.3	38.1	38.1
Gram negative/oxidase positive	3.9	24.8	15.0	15.0

a: day/month/year

b: percentage of the total bacteria isolated for each date

DISCUSSION

There was no consistent trend in either summer with respect to changes in population over time. However, increases or decreases in the fungal and bacterial population levels usually occurred at about the same time. Increases were observed on the fourth sampling in 1983 and in the second and third samplings in 1984, all of which were made after several rainy days. The decrease observed at the fourth sampling in 1984 was after an extremely dry and hot week (see Table 1). It seems, therefore, that climatic conditions are probably the main factors involved in affecting the microflora. Bacteria are sensitive to periods of dryness and their populations are favoured by rainy weather (Hirano and Upper, 1983).

Maneb appears to have an effect on the fungal populations on lettuce leaves. The importance of this effect, however, depends mainly on climatic conditions and the number of applications made. In 1984, when climatic conditions favoured population increase, a single application of maneb had no apparent effect on the populations. It is possible that the absence of effect is due to a rapid recolonization of leaves. Microorganisms killed could be replaced over a short period of time if environmental conditions are favourable. Any amount of soil splashed on leaves could restore the population levels of soil-borne microorganisms. Kuthubutheen and Pugh (1978) found spores of phylloplane fungi treated with captan or dicloran to be viable after washing. Periods of rain could thus restore the viability of phylloplane fungal spores and cancel the effect of the pesticide. In 1983, the recovery in the population observed after an initial reduction due to maneb application could have similar causes.

In 1984, repeated applications of maneb caused population levels to fall progressively. The reduction was especially great on the last sampling which occurred after a hot and dry spell. This type of weather did not permit washing or degradation of the fungicide and subsequent recolonization of the leaves.

Overall, the results obtained agree well with those of Bainbridge and Dickinson (1972) with regards to the microflora of potato. They found filamentous fungi to be slightly affected by maneb. They also found that the bacterial flora were not affected. Ducommun and Corbaz (1982) also studied the effect of maneb on the microflora of tobacco but used different techniques to evaluate the populations. They found the greatest reduction was with the spore fall technique, which indicates that it was mainly sporulation that was inhibited.

The effects of this fungicide appear to be quite unspecific since it caused no obvious change in the microflora composition. Shifts in microflora compositions have been reported, such as an increase in the importance of white yeasts (Warren, 1974,) or the selective elimination of certain taxa (Andrews and Kenerley, 1978; Fokkema et al., 1975; McKenzie, 1971; Warren, 1974).

Carbaryl did not have any effect on the bacterial or fungal flora. It might even have caused a stimulation of the microflora but no statistically significant effects were observed. No particular component of the microflora appeared to be stimulated or negatively affected. Almost nothing is known about possible effects of insecticides on phylloplane microorganisms (Andrews, 1981). This is unfortunate since destruction or stimulation of the microflora could have an influence on disease incidence. This study appears to be the first one dealing directly with the effects of insecticides on phylloplane microflora. However, they are many other insecticides currently in use and it cannot be assumed that they are all harmless to the microflora.

When looking at the composition of the microflora over the different sampling dates, it is not possible to find any definite succession pattern. The microflora appears to be formed of a mix of air-borne and soil-borne taxa which fluctuate rapidly in composition. Generally, the microflora constituents are similar to those Newhook (1951) isolated from dead lettuce tissues. Among the fungi, Phoma and Fusarium were clearly the dominating types. These fungi are often reported to be present on plants but they are rarely reported as important parts of the microflora. Various Phoma species were present on beet seed (Byford and Gambogi, 1985). Norse (1972) found Phoma sporulating on the surface of tobacco leaves. Since Phoma and Fusarium were isolated from washed discs it can be assumed that either their spores adhere strongly to the leaf surface or they exibit some form of growth on the surface. Lettuce leaves disappear rapidly when they reach senescence and these fungi could play an important role in their decomposition.

The other major fungal and bacterial groups fluctuated greatly in importance from one sampling to another. Propagules are probably deposited on leaves in rapid successive waves and the arrival of the contituents likely depends on the current climatic conditions. The importance of certain genera in the air-spora has been shown to depend largely on climatic conditions (Last, 1955a). Heavy rains can wash the leaves and splash soil on the lower leaves. Periods of dryness can kill taxa less resistant to desiccation. Overall, only a few fungal genera were found in significant numbers (see Tables III and IV). The bacterial flora was found to be very variable for each date. Unpigmented and non-fermentative types dominated in the Gram negative groups while the Gram positive group appeared to have a large proportion of spore-formers.

The indirect methods used to evaluate the microflora composition and population appear to be the most advantageous for this kind of study, since it considers only the viable propagules on leaves. Direct techniques have an advantage in obtaining information on colonization but have many drawbacks for such quantitative studies. Since microbial populations are often log-normally distributed (Hirano _et al_., 1982; Parbery _et al_., 1981) measurements of the colonization of a large number of leaf samples would be necessary in order to detect a difference between the treatments. Also, differentiation between the different taxa and assessment of their viability might be a problem. In temperate climates, the extent of colonization often remains low on leaves until they reach senescence, so most fungi are probably present in an inactive state for much of the season. Since the variability between the individual plots remains high, an increase in the number of replicates would have been desirable to evaluate treatments with smaller effects such as carbaryl, which may have actually stimulated microbial populations. A culture medium which restricts growth of yeasts would have been useful in evaluating their populations. Employment of a selective culture medium could have been helpful in separating bacteria into taxonomic groups.

SECTION II: Phylloplane interactions with S. sclerotiorum.

INTRODUCTION

Lettuce drop may be caused by Sclerotinia sclerotiorum (Lib.) de Bary or S. minor Jagger. While S. minor is most often reported as the causal agent of drop, it appears that in Quebec most infections are caused by S. sclerotiorum (Reeleder and Charbonneau, 1986). Newton and Sequeira (1972) have demonstrated the importance of ascosporic inoculum in infection of lettuce by S. sclerotiorum. Ascospores are discharged from apothecia located either within or outside fields (Abawi and Grogan, 1975; Newton and Sequeira, 1972). The use of antagonistic microorganisms to prevent ascospore infection thus becomes a possible approach to the control of this disease. Biocontrol of several diseases with phylloplane microorganisms has been investigated and shown to have some promise (Blakeman and Fokkema, 1982).

Wood (1951) and Newhook (1951) were the first to use saprophytic fungi for disease control in lettuce. A number of isolates were found to be effective in varying degrees against Botrytis cinerea. Sitepu and Wallace (1984) successfully used Fusarium lateritium to reduce incidence of drop in a controlled environment. Field experiments were carried on to control S. sclerotiorum on bean with Coniothyrium minitans (Trutmann et al., 1982). Parasitism of the hyphae was established but no significant reduction of disease levels were observed. In the present study, a collection of fungi isolated from lettuce leaves were tested on leaf discs as an assay for their antagonistic action against S. sclerotiorum ascospores. The most inhibitory isolates were then tested for control of lettuce drop in a controlled environment. Observations of fungal colonization of leaves and interaction of antagonistic fungi with ascospores were made with a scanning electron microscope.

MATERIALS AND METHODS

A-Production of Sclerotinia ascospores

A single spore isolate obtained from infected lettuce plants found in the muck soil area near St-Patrice-de-Sherrington was used for these experiments. Sclerotia were produced in flasks containing autoclaved carrot or celery. The flasks were kept on a laboratory bench. Mature sclerotia were washed in a sieve, dried at room temperature, and were stored at room temperature until use. To induce germination, the sclerotia were first soaked in water for 12 hours at 10°C, and then washed for 2 hours in running tap water. The sclerotia were surface sterilized for 2 min. in 10 % Javex and rinsed twice with deionized water (D. McKenzie, Agriculture Canada, personal communication). The sclerotia were aseptically transfered to 625 ml glass jars containing approximately 200 ml of either moist sand or 1 % water agar and incubated at 10°C in the dark (Henson and Valleau, 1940). When stipes started to appear, fluorescent light was supplied. Ascospores were harvested from mature apothecia by aspirating them onto a Millipore filter held in a funnel connected to a vacuum pump (Hunter *et al.*, 1982). They were stored on the filters in a dessicator at 4°C.

B-Growth conditions of the experimental host

Lettuce plants (*Lactuca sativa* L.) cv. Ithaca were grown in a mixture containing 2 parts sterilized Ste-Clothilde muck soil to 1 part vermiculite. Plants were grown in 130 mm plastic pots in a growth chamber or on a growth bench. Fertilizer (20-20-20) was applied biweekly. Temperature was maintained at 20°C. Photoperiods were of 14 hours with a light intensity of 300 microEinstein/m^2s.

C-Pathogenicity of Sclerotinia ascospores on lettuce

Pathogenicity of ascospores of S. sclerotiorum was demonstrated by exposing mature plants (8 weeks old) to discharging apothecia or by spraying them with a spore suspension. The apothecia were placed on moist sand in small plastic cups located under the foliage of the plants but on the soil surface, in either a growth chamber at 20°C or in a dew chamber at 22°C with, in both cases, a 14 h photoperiod. The plants inoculated in the growth chamber were placed in plastic bags after one week to promote infection. Plants in the dew chamber were left there until signs of infection started to appear, which took about 4-6 days. In the second method of inoculation, a fresh suspension of 10^6 ascospores/ml, with 0.01 % Tween 80, was sprayed on the foliage until runoff. The plants were placed in the dew chamber in the dark at 22°C until infection was observed.

D-Germ tube inhibition tests

Antagonistic properties of phylloplane fungi were evaluated by determining their ability to reduce germ tube growth of ascospores on leaf discs. Leaf discs were cut from healthy outer leaves of plants grown in a growth chamber. They were washed and surface sterilized according to the ethanol treatment method of Spurr (1979) and plated on 0.8% water agar. Fungi used as candidate antagonists were isolated from field grown lettuce leaves using the leaf washing and the leaf imprint methods for a phylloplane microflora study, as previously described in Section I. Representative genera were selected, purified and cultured in petri plates containing malt agar (Difco). Near ultra-violet light was provided as needed to stimulate sporulation. The spore suspensions were obtained by flooding one to two week-old plates with 0.01 M phosphate buffer (pH 6.0) and then rubbing the colony surface gently with a glass rod. The suspensions were passed through a double layer of cheese cloth to remove large mycelial fragments. To remove products from the culture medium, the suspensions were centrifuged twice at 7000 rpm for 20 min and resuspended in the same buffer. Spore populations were determined with a haemocytometer and adjusted to 2.3 x 10^6 spores/cm^2 for filamentous fungi and 1.1 x 10^7 cell/cm^2 for yeasts. Then, 0.4 ml of the suspension was applied to the leaf discs, prepared as described above, and having an area of 9.08 cm2. This resulted in propagule concentrations of 10^5/cm^2 for filamentous fungi and 5 x 10^5/cm^2 for yeasts. The spore suspensions were applied to discs with a pipette and the excess of moisture was allowed to dry out before the plate covers were put in place. The discs were incubated in the dark at 22°C for 24 h. At this point, an ascospore suspension (10^7 spores/ml) was sprayed on the discs, which were incubated for a further 24 h in the same conditions. Control treatments consisted of discs treated only with ascospores. The germinating ascospores were removed by applying a piece of scotch tape on the disc surface and mounted on slides in lactophenol with cotton blue. One hundred germ tubes were measured for each treatment, using a camera lucida. Inhibition of growth by the phylloplane fungi was evaluated by comparing germ tube elongation in the presence of candidate antagonists with elongation in control treatments. Data were compared using the Student's t test.

E-Biocontrol of lettuce drop

The six fungi most inhibitory on leaf discs were tested on mature plants (8 weeks old) for the control of drop in the dew chamber. Each antagonist was tested individually on batches of 15 plants with 15 untreated plants serving as controls. Antagonist spore suspensions were obtained as before, except that distilled water was used rather than buffer and washing of the suspension was not carried out. Tween 80 (0.01 %) was added as a wetting agent. Spore concentrations were determined with a haemocytometer, then adjusted to various concentrations (see Table VIII) and suspensions were then sprayed on plants until runoff. The plants were immediately placed in a dew chamber at 22°C and held in the dark for 24 h. Subsequently, all treated and control plants were sprayed with an ascospore suspension of 10^6 spores/ml as described in section C, above. Plants were returned to the dew chamber and scored for infection one week later. Plants were rated as either infected or non-infected. The experiment was repeated once for each antagonist. Results were analyzed using the Fisher exact test (Daniel, 1978).

F-Scanning electron microscopy

The growth of Alternaria alternata, Epicoccum purpurascens and Trichoderma viride on the leaf surface and their interactions with S. sclerotiorum were studied using scanning electron microscopy. Plants were inoculated with the antagonists and the pathogen as described in section E, above. Pieces of leaves at different ages were fixed at several time intervals with a modified procedure from Brown and Brotzman (1976). Samples were fixed in 2 % osmium tetroxide (OsO_4) containing

Kodak Photo-flo 200 (0.2 %) at 4°C for 24 h and then treated in a saturated solution of thiocarbohydrazide for 1 h, followed by 1 h in 2 % OsO_4. The samples were dehydrated through a series of ethanol and amyl acetate solutions. They were then critical point dried and coated with gold before observation with a Cambridge Stereoscan 600 microscope.

RESULTS

A-Pathogenicity of ascospore inoculum on lettuce

All plants inoculated in the dew chamber, whether by ascospore suspension or by discharging apothecia, showed signs of infection after 6 days (see Table VI). All infections started on basal leaves. Once a leaf was infected, the pathogen progressed rapidly to the stem giving the appearance that plants were infected from the crown, stem or root areas. In plants inoculated in the growth chamber, infections were also mainly on basal leaves except for three where infection started on the head. Since placing plants in plastic bags may wound the foliage, thus making them more susceptible to infection, the use of the dew chamber was preferred to induce disease development.

B-Germ tube inhibition test

Nearly all ascospores germinated on discs after 24 h, with germ tube average lengths ranging from 6 to 13 micrometres in the control treatments. Germ tubes were significantly shorter for all but two of the fungi tested when compared with the control (see Table VII). Since these tests were made in several batches with a control for each, it is not possible to compare them on the basis of the absolute germ tube length but only by their percentage of inhibition. The isolate of Myrothecium verrucaria caused a significant stimulation of the germ tube growth on discs. It is likely that this fungus caused the senescence of the discs, making them more favourable to growth of S. sclerotiorum.

TABLE VI: Ascospore infection of lettuce

method of inoculation[a]	infected plants[b]
apothecia + plastic bags	11/12
apothecia + dew chamber	6/6
spore suspension + dew chamber	5/5

a: see text for details of inoculation procedures
b: rated six days after inoculation

TABLE VII: Inhibition of germ-tube growth on leaf discs

Fungal isolate	% inhibition	t
Alternaria alternata	60.6	9.2865**
Fusarium sp. isolate "A"	45.8	7.0742**
Cladosporium cladosporioides	38.8	6.8674**
Epicoccum purpurascens	38.3	5.3677**
Trichoderma viride	34.2	5.1400**
Red yeast isolate "A"	32.6	5.1462**
White yeast isolate "A"	32.5	5.3264**
Aureobasidium pullulans	28.3	4.046 **
Fusarium sp. isolate "B"	23.0	3.1421**
Trichoderma sp.	21.3	2.8837**
Red yeast isolate "B"	17.1	2.5132**
White yeast isolate "B"	- 7.6	0.8616
Myrothecium verrucaria	- 22.0	2.3525**

** significant at the 0.01 level according to the Student's t test.

C-Control of lettuce drop

Three of the six antagonists, Alternaria alternata, Trichoderma viride and Epicoccum purpurascens, were effective in protecting plants against lettuce drop. The degree of protection, although significant, was variable for each test (see Table VIII). All the filamentous fungi were successful in colonizing the senescent foliage and the dead leaf tips, as sporulation by antagonists could be observed with the naked eye after 5 days. Only the Fusarium isolate appeared to attack lettuce, causing a rapid rotting of the plants in the dew chamber. Cladosporium cladosporioides, despite its development on senescent leaves, could not bring about any significant reduction in disease incidence, nor could the red yeast isolate. It appeared, however, that there was a reduction in the degree of damage, but this was difficult to quantify, since all plants showing disease symptoms were rated "infected" regardless of the degree of rot present. The white yeast isolate (used as a negative control) did not have any effect on the disease.

TABLE VIII: Biocontrol of lettuce drop

Antagonist (spores/ml)	test	no. of infected plants		% control
		treated	control	
A. alternata	1st	10/18	18/18	44.4**
(10^6)	2nd	1/15	15/15	93.3**
C. cladosporioides	1st	10/15	14/15	26.7
($5x10^6$)	2nd	12/15	15/15	20.0
E. purpurascens	1st	6/15	13/15	46.7**
(10^6)	2nd	9/15	15/15	40.0**
T. viride	1st	9/15	15/15	40.0**
($7x10^7$)	2nd	6/15	15/15	60.0**
Red yeast iso."A"	1st	13/15	13/15	0.0
(10^8)	2nd	15/15	15/15	0.0
White yeast iso."B"	1st	15/15	15/15	0.0
(10^8)	2nd	15/15	15/15	0.0
Fusarium iso."A" ($4x10^6$)		pathogenic to lettuce		

** significant at the 0.01 level according to the Fisher exact test

D-Scanning electron microscopy

Ascospores germinated on the leaf surface, usually within 24 h. After germination, the pathogen exhibited little activity on green leaves except some germ tube elongation. However, on senescent leaves, simple appressorium-like structures were formed after 48 h, connecting the germ tubes and the leaf epidermis (see Fig 5-1). These were the only infective structures observed. In only one case, was penetration observed to occur through a stomate (see Fig 5-3).

Conidia of Epicoccum purpurascens germinated and grew extensively on green and senescent leaves (see Figs 5-2 and 5-4). This fungus did not seem to have any direct interactions with ascospores, as it never grew towards them nor did it appear to have contact with them (see Figs 5-2 and 5-4). Ascospores were not affected by the presence of E. purpurascens on green leaves as they germinated normally (see Fig 5-4). However, on senescent leaves, when there was a greater biomass of antagonist hyphae present, germination of the ascospores appeared to be inhibited (see Fig 5-2).

Trichoderma viride had no activity on green leaves. Its conidia remained ungerminated for as long as 80 h. On the other hand, this fungus grew extremely well on dead tissues, sporulating within 72 h (see Fig 5-8). The ungerminated conidia did not appear to have any effect on ascospore germination but Trichoderma hyphae were seen surrounding and in contact with ascospores, suggesting a parasitic relationship (see Fig 5-8).

Alternaria alternata colonized both senescent and green leaf surfaces extremely well (see Fig 5-5). Its hyphae were often observed to be apparently growing towards ascospores and to contact them (see Fig 5-6 and 5-7). Although the contact appears to be very close, it was difficult to determine whether the ascospores were really being parasitized.

FIGURE 5

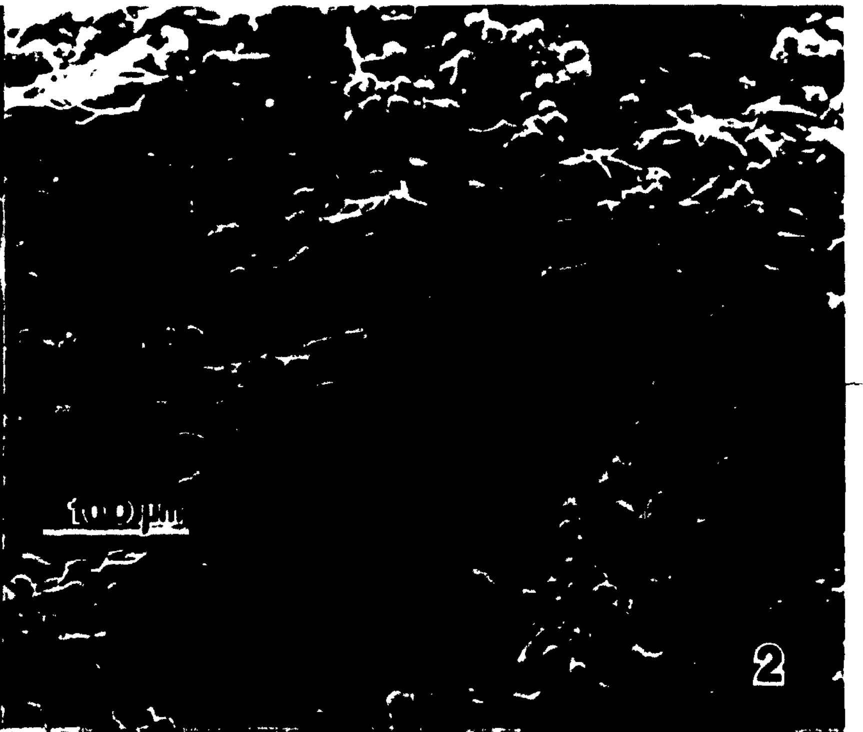
1-Ascospore with appressorium-like structure (arrow), 48 h after application of suspension (x 2200).

2-Epiccocum purpurascens on a senescent leaf with ungerminated ascospores, at 60 h and 36 h respectively (x 210).

3-Ascospore with appressorium-like structure (arrow) penetrating through a stomate, at 48 h (x 2200).

4-E. purpurascens with germinating ascospores on a healthy leaf, at 44 h and 20 h respectively (x 450).


10 µm
1


100 µm
2


10 µm
3

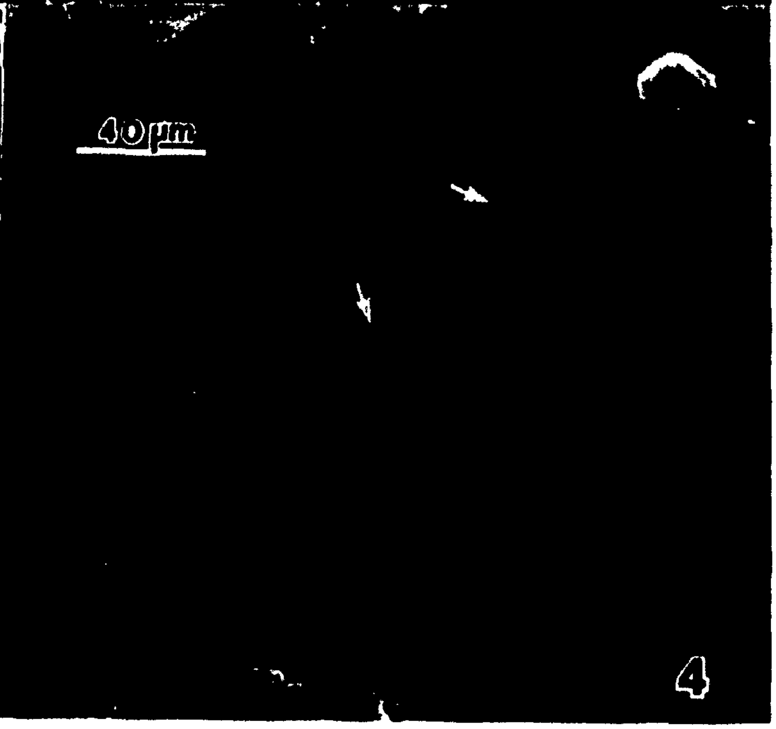

40 µm
4

Figure 5 (continued)

5-Colonization of a green leaf by Alternaria alternata, at 60 h (x 180).

6-A. alternata hyphae in contact with germinating ascospores, at 60 h and 36 h respectively (x 1000).

7-A. alternata conidium (A) and hyphae in contact with germinating ascospores, at 60 h and 36 h respectively (x 1250).

8-Trichoderma viride on a senescent leaf surrounding an ascospore (arrow), at 72 h and 48 h respectively (x 850).


5
6

DISCUSSION

Germ tube inhibition tests on various surfaces have been widely used for screening biocontrol agents. They offer a fairly good compromise between the inhibition zone measurements in petri dishes and direct testing for disease control on plants. Reduction in germ tube length and mycelial growth of the pathogen has often been related to a reduction in the amount of disease on the plant. This has been shown for several pathogens, such as *Drechslera dictyoides* (Austin *et al*., 1977), *Alternaria alternata* (Flavel and Spurr, 1977), *Alternaria zinniae* (Van den Heuvel, 1969), *Alternaria porri* (Fokkema and Lorbeer, 1974), *Botrytis cinerea* (Blakeman and Fraser, 1971) and *Septoria nodorum* (Fokkema and Van der Meulen, 1976). The data presented here appear to indicate that this is also true for *S. sclerotiorum*. Since growth of *S. sclerotiorum* on a nutrient base is usually necessary for infection to occur (Abawi *et al*., 1975; Newton and Sequeira, 1972; Purdy, 1958), inhibition of growth prior to penetration is likely to prevent infection.

Most of the isolates tested caused at least some degree of germ tube inhibition, indicating that *S. sclerotiorum* is sensitive to the presence of a wide range of different fungi. Hartill (1975) attributed the inhibition of *S. sclerotiorum* ascospores on tobacco leaves to the presence of phylloplane fungi stimulated by the presence of pollen. It is not possible, however, to estimate how much inhibition will be required before a reduction of disease incidence can occur. The isolates of *Epicoccum purpurascens* and *Trichoderma viride* were about as effective in reducing germ tube growth as were the isolates of

Cladosporium cladosporioides and red yeast "A". However, when tested for the control of drop, their efficacy was very different. E. purpurascens and T. viride reduced disease incidence significantly while C. cladosporioides and the red yeast had seemingly no effect. Andrews et al. (1983) found discrepencies between results obtained with various in vitro and in vivo methods. Such differences would be expected, since results obtained with in vitro methods are used only as estimates of antagonism. The method used here for scoring infections did not permit a comparison of infected plants with respect to the number of infections, nor to their degree of damage. Because Sclerotinia lesions spread rapidly, it would have been difficult to make such an assessment, especially with badly infected plants. There was, however, an apparent reduction in the degree of damage and number of infection points with the red yeast and C. cladosporioides isolates. If sprayed at higher rates or given a longer incubation period, they may have produced a significant reduction in disease incidence.

Antagonists were screened on healthy green leaf discs because they were more uniform than older leaves. However, a lettuce plant has many dead and senescing leaves, with possibly some wounds or dead leaf tips on some leaves. Thus, the leaf discs could not exactly reproduce the conditions an antagonist and the pathogen would encounter on an intact plant. This factor may also have contributed to discrepancies between in vitro and in vivo tests. The use of artificially killed or injured leaf discs might have been an alternative. However, the purpose of these inhibition tests is to provide a ranking of microorganisms so that further work could be done on several of them. Thus, it should not be expected that the results of these tests would perfectly agree with subsequent in vivo tests.

The success of Alternaria alternata, T. viride and E. purpurascens seems to be due to their ability to rapidly colonize the senescent foliage. This is the usual entry point for S. sclerotiorum (Sitepu and Wallace, 1984), and colonization of these regions by antagonists may reduce the possibilities for infection. The colonization of senescent leaves and dead leaf tips by antagonists was apparent after a few days, by virtue of sporulation observable with the naked eye. Newhook (1957) demonstrated the importance of saprophytic fungi in protecting tomato fruits against Botrytis cinerea by colonizing the dead flower parts. Wood (1951) and Newhook (1951) showed that colonization by saprophytes of wounds and frost injuries on lettuce leaves inhibited B. cinerea. The importance of dead organic matter in lettuce drop infection and the protection given when it is colonized by an antagonist was emphasised by Sitepu and Wallace (1984) in their biocontrol experiment. It remains to be shown whether these selected antagonists can be effective under field situations. These fungi represent a minor part of the microflora of lettuce leaves (Section I), and therefore their populations would have to be artificially increased. Unlike the Fusarium "A" isolate, which was pathogenic on lettuce, these three fungi did not seem to have any negative effect on lettuce growth.

E. purpurascens and A. alternata are common as phylloplane fungi. They show their greatest level of activity when leaves become senescent (Dickinson, 1967; Flannigan and Campbell, 1977; Godfrey, 1974; Lindsey and Pugh, 1976). Alternaria and Epicoccum can grow at low relative humidity levels and resist dessication for long periods of time without damage (Dickinson and Bottomley, 1980; Park, 1982). Their large spores with nutrient reserves permit them to grow under low-nutrient situations (Breeze and Dix, 1981; Dickinson and Bottomley, 1980). SEM observations confirmed their ability to extensively colonize green healthy leaves although growth was more significant on senescent leaves. These are assets for their use in the field as biocontrol agents. Spurr (1977) already has used a non-pathogenic Alternaria isolate as an antagonist on tobacco.

Trichoderma viride, although occasionally found on leaves, cannot be considered a true phylloplane fungus. Probably for nutritional reasons, it does not appear to be able to grow on green, healthy leaves. Trichoderma spp. have however been used successfully to control diseases on aerial plant surfaces (Tronsmo and Dennis, 1977; Tronsmo and Ystaas, 1980; Dubos et al., 1978). Since S. sclerotiorum infections usually occur on senescent plant parts, it can probably be an effective biocontrol agent for this pathogen. The inoculum concentration of 7 x 10^7 spores/ml used here is comparable to the ones used in these field experiments.

It seems that both A. alternata and T. viride interact directly with ascospores. SEM observations indicated that A. alternata deliberately grew towards groups of ascospores and possibly could either parasitize them directly or use nutrients leaking from them. Other workers have reported that various Trichoderma spp. were seen parasitizing other fungi (Dennis and Webster, 1971c; Tronsmo and Raa, 1977). Thus it likely that the isolate of T. viride used in this study is able to attack ascospores. E. purpurascens did not appear to have any direct, physical interaction with S. sclerotiorum. Nevertheless, it was very effective in inhibiting ascospores when it had grown abundantly on senescent leaves. These leaves were usually dripping with a pink-orange liquid presumably secreted by the fungus. It is likely that this pigmented material or some other inhibitory compound(s) was(were) secreted at high enough levels to prevent ascospore germination.

Ascospores on lettuce leaves appear to penetrate by producing small rudimentary appressoria-like structures. Some resemble closely the "sheath" produced on soybean leaves, as shown by Sutton and Deverall (1984). Previous reports have indicated that S. sclerotiorum develops well-defined, appressoria-like structures with various degrees of complexity (Abawi et al., 1975; Sutton and Deverall, 1983; Tariq and Jeffries, 1984). However, in all these studies, a nutritional base was supplied. It would be expected that in the low-nutrient situations which arise on intact leaves , the growth of the pathogen prior to penetration would be rather restricted and penetration structures reduced to a minimum. Infection through stomates seems to be rare and probably occurred by chance.

SUGGESTIONS FOR FURTHER RESEARCH

Now that a number of promising antagonistic fungi have been selected, the next thing to do would be to test them in the field for the control of lettuce drop and other diseases of lettuce. They could also be tested for the control of S. sclerotiorum on more major crops such as beans, rapeseed and carrots. Time of application, formulation of the biocontrol agents, and tolerance to agricultural chemicals should be the main points to consider at this moment.

More knowledge should be acquired on the biology of S. sclerotiorum in Québec. So far, very little is known with respect to the sources of inoculum in vegetable fields. Identification of these sources is a prime factor for the success of any control stategies. On a more fundamental aspect, the process of ascospore penetration and infection could be better documented at the histological and biochemical levels. In particular, the reduced ability of ascospores to infect healthy tissues could be better explained.

A microflora study similar to the one made here could be repeated, but this time concentrating on only the few taxa that occur most often, to determine if they are developing as pesticide-tolerant populations in treated plots. Sampling of the microflora could be done with media supplemented with various concentrations of the pesticide studied.

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APPENDIX I :Raw population data 1983

C: carbaryl
M: maneb
U: untreated control

filamentous fungi: numbers of colonies isolated per plot

treatment	blk	plot	11/07/83	18/07/83	25/07/83	2/08/83
C	1	1	22	26	48	65
C	2	8	28	32	25	58
C	3	15	38	43	61	79
C	4	4	22	20	.	53
C	5	11	30	15	31	85
C	6	18	41	39	57	63
M	1	13	63	46	20	87
M	2	2	27	13	16	64
M	3	9	16	7	33	43
M	4	16	40	17	46	40
M	5	5	25	22	21	38
M	6	12	22	11	41	37
U	1	7	52	53	39	57
U	2	14	46	39	34	78
U	3	3	11	33	51	62
U	4	10	20	20	74	49
U	5	17	41	21	27	54
U	6	6	23	24	38	32

bacteria : numbers of colonies isolated per plot

treatment	blk	plot	11/07/83	18/07/83	25/07/83	2/08/83
C	1	1	66	260	330	328
C	2	8	92	46	141	276
C	3	15	242	544	274	379
C	4	4	45	443	33	270
C	5	11	39	8	174	346
C	6	18	876	32	345	222
M	1	13	291	226	206	369
M	2	2	39	503	173	456
M	3	9	456	143	68	295
M	4	16	193	113	221	248
M	5	5	255	385	81	352
M	6	12	559	462	279	242
U	1	7	217	103	83	341
U	2	14	197	88	140	277
U	3	3	.	95	212	307
U	4	10	49	627	464	109
U	5	17	213	69	56	415
U	6	6	.	109	145	300

APPENDIX II: Raw population data 1984

C: carbaryl
M: maneb
U: untreated control
MA: multiple applications of maneb

filamentous fungi: numbers of colonies isolated per plot

treatment	blk	plot	3/07/84	12/07/84	17/07/84	25/07/84
C	1	1	5	10	125	49
C	2	8	29	70	183	16
C	3	15	22	16	45	89
C	4	22	31	25	15	44
C	5	5	35	40	138	11
C	6	12	15	149	80	35
MA	1	19	9	12	10	2
MA	2	2	28	27	11	2
MA	3	9	29	103	24	2
MA	4	16	15	45	20	2
MA	5	23	4	35	6	4
MA	6	6	16	50	8	4
M	1	13	17	10	35	26
M	2	20	10	15	15	15
M	3	3	28	33	83	37
M	4	10	21	82	73	10
M	5	17	18	28	17	16
M	6	24	17	30	92	28
U	1	7	31	42	89	34
U	2	14	10	20	31	14
U	3	21	3	16	43	27
U	4	4	26	42	44	20
U	5	11	6	120	78	6
U	6	18	23	28	8	6

bacteria : numbers of colonies isolated per plot

treatment	blk	plot	3/07/84	12/07/84	17/07/84	25/07/84
C	1	1	140	88	1605	767
C	2	8	129	194	2977	159
C	3	15	94	51	304	965
C	4	22	58	36	294	45
C	5	5	209	72	214	88
C	6	12	141	353	211	100
MA	1	19	60	62	1632	115
MA	2	2	255	99	81	35
MA	3	9	86	286	2146	8
MA	4	16	90	91	.	.
MA	5	23	23	263	1068	46
MA	6	6	75	233	54	171
M	1	13	151	67	2092	63
M	2	20	33	74	713	297
M	3	3	165	307	313	194
M	4	10	136	196	103	42
M	5	17	78	45	2790	215
M	6	24	110	29	160	626
U	1	7	158	274	214	176
U	2	14	58	460	553	2005
U	3	21	21	213	234	96
U	4	4	111	150	311	72
U	5	11	47	345	39	58
U	6	18	133	67	1571	63

APPENDIX III

Analysis of variance of the microbial population data.

Dependent variable: Log of no. of colonies isolated per plot (LOGD) on four dates

B: blocks(6)
CH: treatments
- U: untreated control
- C: carbaryl
- M: maneb (sing~~le~~-application)
- MA: maneb (multiple applications)

CHDATE: treatment-date combinatio[illegible]
CH*DATE: interaction between treatments and dates

A-Fungal populations in 1983

General linear models procedure

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE
MODEL	16	2.01721258	0.12607579	3.88	0.0001	0.534901
ERROR	54	1.75397721	0.03248106			
CORRECT. TOTAL	70	3.77118979				

SKEWNESS	C.V.	ROOT MSE	LOGD MEAN
-0.0919689	11.7569	0.18022502	1.53292639

SOURCE	DF	TYPE III SS	F VALUE	PR > F
B	5	0.19086974	1.18	0.3333
CH	2	0.26359452	4.06	0.0228
DATE	3	1.38930792	14.26	0.0001
CHDATE	11	1.82320144	5.10	0.0001
CH*DATE	6	0.17400165	0.89	0.5068

```
DUNCAN'S MULTIPLE RANGE TESTS

ALPHA=0.05   DF=54   MSE=0324811

GROUPING      MEAN*     N      DATE

    A        1.7458    18        4
     B       1.5555    17        3
   C B       1.4597    18        1
   C         1.3720    18        2

                             CHDATE

    A        1.8212     6       C4
    A        1.7280     6       U4
    A  B     1.6882     6       M4
  C A  B     1.6223     5       C3
  C A  B     1.6184     6       U3
  C    B     1.4729     6       U2
  C    B     1.4669     6       C1
  C    B     1.4629     6       M1
  C    B     1.4493     6       U1
  C          1.4370     6       C2
  C          1.4369     6       M3
   D         1.2060     6       M2

* log-transformed data
```

B-Fungal populations in 1984

General linear models procedure

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE
MODEL	20	10.43231908	0.52161595	4.96	0.0001	0.569342
ERROR	75	7.89113643	0.10521515			
CORRECT. TOTAL	95	18.32345551				

SKEWNESS	C.V.	ROOT MSE	LOGD MEAN
-0.383154	24.3038	0.32436885	1.33464436

SOURCE	DF	TYPE III SS	F VALUE	PR > F
B	5	0.27146785	0.52	0.7654
CH	3	3.24154079	10.27	0.0001
DATE	3	3.66814202	11.62	0.0001
CHDATE	16	10.16085123	6.44	0.0001
CH*DATE	9	3.25116842	3.43	0.0014

```
DUNCAN'S MULTIPLE RANGE TESTS

ALPHA=0.05   DF=54   MSE=0324811

GROUPING           MEAN        N      DATE

    A             1.5352      24        3
    A             1.5189      24        2
     B            1.1904      24        1
     B            1.0941      24        4

                                     CHDATE

    A             1.8719       6       C3
    A B           1.6161       6       M3
  C A B           1.5855       6       U3
D C A B           1.5699       6       MA2
D C A B           1.5463       6       U2
D C A B           1.5371       6       C2
D C A B E         1.5121       6       C4
D C   B E F       1.4221       6       M2
D C   B E F       1.3018       6       M4
D C   B E F       1.2859       6       C1
D C   B E F       1.2476       6       M1
D C     E F       1.1611       6       U4
D       E F       1.1410       6       MA1
        E F       1.0872       6       U1
          F       1.0673       6       MA3
      G           0.4014       6       MA4
```

C-Bacterial populations in 1983

```
                    General linear models procedure
---------------------------------------------------------------------------
SOURCE      DF   SUM OF SQUARES   MEAN SQUARE   F VALUE   PR > F   R-SQUARE

MODEL       16     2.72361411      0.17022588     1.23    0.2758   0.271157

ERROR       53     7.32080352      0.13812837

CORRECT.    69    10.04441763
TOTAL

                  SKEWNESS       C.V.      ROOT MSE     LOGD MEAN

                 -0.0709493    16.4587    0.37165625   2.25811868
---------------------------------------------------------------------------
SOURCE      DF    TYPE III SS       F VALUE       PR > F

B            5    0.69187592         1.00         0.4265

CH           2    0.41730570         1.51         0.2301

DATE         3    1.07367947         2.59         0.0614

CHDATE      11    2.00028464         1.32         0.2412

CH*DATE      6    0.49963537         0.60         0.7268
---------------------------------------------------------------------------
```

```
DUNCAN'S MULTIPLE RANGE TESTS

ALPHA=0.05   DF=53   MSE=0.138128

GROUPING     MEAN      N     DATE

    A       2.4704    18      4
     B      2.1973    16      1
     B      2.1918    18      3
     B      2.1662    18      2
```

D-Bacterial populations in 1984

General linear models procedure

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE
MODEL	20	9.70831959	0.48541598	2.44	0.0031	0.400552
ERROR	73	14.52901174	0.19902756			
CORRECT. TOTAL	93	24.23733133				

SKEWNESS	C.V.	ROOT MSE	LOGD MEAN
0.154179	20.3356	0.44612505	2.19381811

SOURCE	DF	TYPE III SS	F VALUE	PR > F
B	5	1.25802745	1.26	0.2882
CH	3	0.44030705	0.74	0.5363
DATE	3	6.11544015	10.24	0.0001
CHDATE	15	8.48878079	2.84	0.0016
CH*DATE	9	2.01100351	1.12	0.3580

```
DUNCAN'S MULTIPLE RANGE TESTS

ALPHA=0.05   DF=73   MSE=0.199028

GROUPING          MEAN      N     DATE

     A           2.6327     23      3
      B          2.1021     24      2
      B          2.1015     23      4
      B          1.9534     24      1

                                 CHDATE

  A              2.7220      6      M3
  A              2.7142      6      C3
  A  B           2.6428      5      MA3
  A  B  C        2.4537      6      U3
  A  B  C        2.3281      6      U2
  A  B  C  D     2.2781      6      C4
  A  B  C  D     2.2187      6      M4
  A  B  C  D     2.1651      6      MA2
  A  B  C  D     2.1583      6      U4
     B  C  D     2.0771      6      C1
        C  D     1.9970      6      M1
        C  D     1.9835      6      C2
        C  D     1.9317      6      M2
        C  D     1.8850      6      MA1
        C  D     1.8543      6      U1
           D     1.6807      6      MA4
```

APPENDIX IV: Biochemical tests performed on the bacteria

O/F: Oxidation/fermentation of glucose
- O oxidation
- F fermentation
- \- no activity

PIG: Pigmentation
- \- no pigmentation
- Y yellow
- O orange

MOT: Motility

OXI: Oxidase

ONP: ONPG hydrolysis

ADH: Arginine dihydrolase

LDC: Lysine decarboxylase

ODC: Ornithine decarboxylase

CIT: Citrate utilization

H_2S: H_2S production

URE: Urease

TDA: Tryptophan deaminase

IND: Indole production

VP: Voges-Proskauer

GEL: Gelatin hydrolysis

GLU: Glucose

MAN: Mannitol

INO: Inositol

SOR: Sorbitol

RHA: Rhamnose

SAC: Saccharose

MEL: Melibiose

AMY: Amygdalin

ARA: Arabinose

NO_2: Nitrate reductase

N_2: N_2 gas

CAT: Catalase

Isolate	O/F	PIG	MOT	OXI	ONP	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP
002A2	OF	-	+	+	+	+	-	-	+	-	-	-	-	-
002A3	OF	-	-	+	-	+	-	-	+	-	+	-	-	-
008B1	OF	Y	-	-	+	-	-	-	+	-	-	-	-	-
036C1	OF	-	-	-	+	-	-	-	+	-	+	-	+	-
036C2	OF	-	-	-	-	-	-	-	+	-	+	+	-	-
036C3	OF	-	-	+	+	-	-	-	+	-	-	-	-	-
072A1	—	-	+	+	+	-	-	-	+	-	-	-	-	+
106C1	O	-	-	+	+	-	-	-	+	-	+	-	-	-
106C2	—	Y	-	-	-	-	-	-	+	-	+	+	-	-
115A1	O	-	-	+	+	-	-	-	+	-	-	-	-	-
117C1	—	O	-	-	-	-	-	-	+	-	-	-	-	-

Isolate	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	NO_2	N_2	CAT
002A2	+	+	-	-	-	+	-	+	-	+	+	+	+
002A3	+	+	-	-	-	-	-	+	-	+	+	+	+
008B1	+	+	+	-	-	+	+	+	+	+	-	-	+
036C1	-	+	-	+	-	+	-	+	-	+	-	-	+
036C2	+	+	-	-	-	-	-	-	-	-	-	-	+
036C3	-	+	-	-	+	-	+	+	+	+	-	-	+
072A1	+	-	-	-	-	-	-	-	-	-	+	+	+
106C1	+	+	+	+	+	-	+	+	+	+	+	+	+
106C2	+	-	-	-	-	-	-	-	-	-	+	+	+
115A1	+	-	-	-	-	-	-	-	-	-	-	-	+
117C1	+	+	-	-	-	-	-	-	-	+	+	+	+

Isolate	O/F	PIG.	MOT	OXI	ONP	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP
123B1	OF	Y	-	-	+	-	-	-	-	-	-	-	-	-
134A1	O	-	+	+	-	-	-	-	+	-	-	+	-	-
134A2	—	-	-	+	-	-	-	-	+	-	+	+	-	-
134C1	—	-	+	+	-	-	-	-	-	-	-	-	-	-
146C1	O	-	-	+	-	-	-	-	+	-	+	-	-	
156C1	O	-	-	+	+	-	-	+	+	-	+	-	-	-
167C1	—	-	-	-	+	-	-	-	+	-	-	-	-	-
172C1	O	-	+	+	+	-	-	-	+	-	-	-	-	-
173C1	—	-	-	+	-	-	-	-	+	-	-	-	-	
173C2	—	-	-	-	-	-	-	-	+	-	-	-	-	-
174B1	—	-	-	+	-	-	-	-	+	-	+	+	-	-

Isolate	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	NO_2	N_2	CAT
123B1	+	+	-	+	-	-	+	+	-	+	-	-	+
134A1	-	-	-	-	-	-	-	-	-	-	-	-	+
134A2	+	-	-	-	-	-	-	-	-	-	+	+	+
134C1	-	-	-	-	-	-	-	-	-	-	-	-	-
146C1	-	+	-	-	-	+	-	+	-	+	-	-	+
156C1	+	+	-	-	-	+	-	+	-	+	-	-	+
167C1	+	-	-	-	-	-	+	-	-	-	+	+	+
172C1	+	+	-	-	-	+	+	+	-	+	-	-	+
173C1	-	-	-	-	-	-	-	-	-	-	-	-	+
173C2	-	-	-	-	-	-	-	-	-	-	-	-	+
174B1	+	-	-	-	-	-	-	-	-	-	+	+	+

Isolate	O/F	PIG	MOT	OXI	ONP	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP
177B1	--	-	-	+	+	-	-	-	+	-	-	-	-	-
181A1	0	-	-	-	+	-	-	-	+	-	-	-	-	-
181A2	0	-	-	+	+	-	-	-	+	-	-	-	-	-
185B1	--	-	-	+	+	+	-	-	+	-	-	-	-	
185B2	OF	-	-	+	+	-	-	-	+	-	-	-	-	
199C1	--	-	-	-	+	-	-	-	+	-	+	-	-	-
204B1	--	-	-	+	+	-	-	-	+	-	-	+	-	-
218B1	--	Y	+	+	+	-	+	-	+	-	-	-	-	-
219A1	OF	Y	-	-	+	-	-	-	+	-	-	-	-	-
222C1	--	-	-	-	+	+	-	-	+	-	-	-	-	-
224A1	--	-	-	+	-	-	-	-	+	-	-	+	-	-

Isolate	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	NO_2	N_2	CAT
177B1	+	-	-	-	-	+	-	-	-	+	-	+	+
181A1	+	+	-	-	-	+	+	+	-	+	+	+	+
181A2	+	+	-	-	-	+	-	+	-	+	-	-	+
185B1	+	-	-	-	-	-	-	-	+	-			+
185B2	+	-	-	-	-	-	-	-	+	-	-	-	+
199C1	+	+	+	-	-	-	-	+	-	+	-	-	+
204B1	-	-	-	-	-	-	-	-	-	-			+
218B1	+	-	-	-	-	-	-	-	-	-	+	+	
219A1	+	+	-	-	-	-	-	-	-	-	-	-	+
222C1	+	-	-	-	-	-	-	-	-	-	-	-	+
224A1	-	-	-	-	-	-	-	-	-	-	-	-	+

Isolate	O/F	PIG	MOT	OXI	ONP	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP
240A1	--	-	+	+	+	-	+	-	+	-	-	-	-	-
240B1	--	-	-	+	+	-	+	-	+	-	-	-	-	-
245A1	--	-	-	-	+	-	-	-	+	-	-	-	-	-
247B1	O	-	-	+	+	+	-	-	+	-	-	-	-	-
253B1	OF	-	+	-	-	-	-	-	-	-	-	-	-	-
253B2	O	-	+	+	-	+	-	-	+	-	-	-	-	-
256C1	O	-	-	+	+	+	-	-	+	-	-	-	-	-
259A1	OF	-	+	+	+	+	-	-	+	-	-	-	-	-
260A1	O	-	-	+	+	-	-	-	+	-	-	-	-	-
261A1	O	Y	-	+	+	-	-	-	-	-	-	-	-	-
264C1	OF	-	-	-	-	-	-	-	+	-	-	+	-	-

Isolate	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	NO_2	N_2	CAT
240A1	+	-	-	+	-	-	-	-	-	-	+	+	+
240B1	+	-	-	-	-	-	-	-	-	-	+	+	+
245A1	+	-	-	-	-	-	-	-	-	-	+	+	+
247B1	+	-	-	-	-	-	-	-	-	-	-	-	+
253B1	+	-	-	-	-	-	-	-	-	-	-	-	-
253B2	+	+	-	-	-	-	-	+	-	+	-	-	+
256C1	+	-	-	-	-	-	+	+	-	+	-	-	+
259A1	+	-	-	-	-	-	+	-	+	-	+	+	+
260A1	+	+	+	+	+	+	+	+	+	+	+	+	+
261A1	+	+	-	-	-	-	+	+	+	+	+	+	+
264C1	+	-	-	-		-	-	-	-	-	+	+	+

Isolate	O/F	PIG	MOT	OXI	ONP	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP
271A1	OF	-	+	+	+	-	+	+	+	-	-	-	-	-
273C1	--	Y	-	+	+	-	-	-	-	-	+	-	-	-
273C2	O	Y	+	-	+	-	-	-	+	-	-	-	-	-
280A1	O	-	-	-	-	+	-	-	+	-	-	-	-	-
281B1	O	Y	-	-	+	+	-	-	-	-	-	-	-	-
289C1	--	-	+	+	-	+	-	-	+	-	-	-	-	-
289C2	--	-	-	+	+	+	-	-	+	-	-	-	-	-
297C1	OF	-	+	+	+	-	+	-	+	-	-	-	+	-
300A1	OF	-	+	+	+	-	-	-	+	-	-	-	+	+
306B1	--	-	-	+	+	+	-	-	+	-	-	-	+	+
306B2	O	-	-	+	+	+	-	-	+	-	-	-	-	-

Isolate	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	NO_2	N_2	CAT
271A1	+	+	-	+	-	-	-	+	+	+	+	+	+
273C1	-	-	-	-	-	-	-	-	-	-	+	+	+
273C2	+	+	-	-	-	-	-	-	-	+	+	+	+
280A1	+	+	-	-	-	-	-	+	-	+	-	-	+
281B1	+	+	+	-	-	-	+	-	+	-	-	-	+
289C1	+	+	-	-	-	-	-	+	-	+	-	-	+
289C2	+	+	+	-	-	-	+	+	+	+	-	-	+
297C1	+	+	+	+	+	+	-	+	+	+	+	+	+
300A1	+	+	+	+	+	+	-	+	+	+	+	+	+
306B1	+	+	-	-	-	-	-	+	-	+	+	+	+
306B2	+	+	-	-	-	-	-	+	-	+	-	-	+

Isolate	O/F	PIG	MOT	OXI	ONP	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP
316B1	0	-	-	-	-	+	-	-	+	-	-	-	-	-
316B2	--	Y		+	-	+	-	-	+	-	-	-	+	-
316B3	0		-	-	-	+	-	-	+	+	-	-	-	-
318A1	--	-	-	+	-	-	+	-	+	-	-	-	-	-
323B1	--	Y	-	+	+	-	+	-	+	-	-	-	-	-
323B2	--	-	-	+	-	-	-	-	-	-	-	-	-	-
323C1	--	-	+	+	+	-	-	-	+	-	-	-	-	-
324B1	0	-	+	+	-	+	-	-	+	-	+	-	-	-
328B1	--	0	-	+	-	-	-	-	+	-	-	+	-	-
328B3	--	-	-	-	+	-	-	-	-	-	-	-	-	-
329C1	0	-	+	+	-	+	-	-	+	-	-	-	-	-

Isolate	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	NO_2	N_2	CAT
316B1	-	+	-	-	-	-	-	+	-	+	+	+	+
316B2	+	+	-	-	-	-	-	+	-	+	-	-	+
316B3	-	+	+	-	+	-	-	+	+	+	+	+	+
318A1		+	-	-	-	-	-	-	-	-	+	+	+
323B1	+	+	-	-	-	-	-	-	-	-	+	+	+
323B2	+	-	-	-	-	-	-	-	-	-	-	-	+
323C1	+	-	-	-	-	-	-	-	-	-	+	+	+
324B1	+	+	-	-	-	+	-	+	-	+	+	+	+
328B1	+	-	-	-	-	-	-	-	-	-	+	+	+
328B3	-	-	-	-	-	-	-	-	-	-	+	+	+
329C1	+	+	+	-	+	+	-	+	-	+	+	+	+

Isolate	O/F	PIG	MOT	OXI	ONP	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP
330A1	--	-	+	+	-	-	-	-	+	-	+	-	-	-
331B1		-	-	-	+	+	-	-	+	-	-	-	-	-
332C2	--	0	-	+	+	-	-	-	-	-	-	-	+	-
333B1	--	-	-	-	+	-	-	-	+	-	+	-	-	-
345A1	--	-	-	-	+	-	-	-	+	-	-		-	-
352A1	OF	-	-	-	+	-	-	-	-	-	-	-	+	-
354B1	--	-	-	+	+	-	-	-	+	-	+	+	-	-
358A1	--	-	+	+	-	-	-	+	+	-	-	-	-	-
349C1	OF	-		-	+	-	-	-	-	-	-	-	+	-
349C2	OF	-	-	-	+	-	-	-	-	-	+	-	-	-
362A2	--	-	-	+	+	-	-	-	+	-	-	+	-	-

Isolate	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	NO_2	N_2	CAT
330A1	+	-	-	-	-	-	-	-	-	-	+	+	+
331B1	+	+	-	-	-	-	-	+	+	+	+	+	+
332C2	+	-	-	-	-	-	-	-	-	-	-	-	+
333B1	+	-	-	-	-	-	-	-	-	-	+	+	+
345A1	+	-	-	-	-	-	-	-	-	-	+	+	+
352A1	-	+	+	+	-	+	+	+	-	+	+		+
354B1	+	-	-	-	-	-	-	-	-	-	+	+	+
358A1	-	-	-	-	-	-	-	-	-	-	-	-	+
349C1	-	+	+	+	-	+	+	+	-	+	+	+	+
349C2	-	+	+	-	-	-	+	-	-	-	+	+	+
362A2	+	-	-	-	-	-	+	-	-	-	+	+	+

Isolate	O/F	PIG	MOT	OXI	ONP	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP
364A1	--	-	-	-	+	-	-	-	-	-	-	-	-	-
367B1	--	-	-	-	+	-	-	-	+	-	+	+	-	-
380B1		-	-	-	+	-	-	-	+	-	-	-	-	-

Isolate	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	NO_2	N_2	CAT
364A1	-	+	-	-	-	-	+	-	-	-	-	-	+
367B1	+	-	-	-	-	-	-	-	-	-	-	-	+
380B1	+	+	-	+	-	-	+	+	-	+	+	+	+

APPENDIX V

Measurements of <u>Sclerotinia</u> <u>sclerotiorum</u> germ tube lengths (in mm): batch 1

isol. no.	fungus
A8	<u>Trichoderma</u> <u>viride</u>
RY24	red yeast B
RY28	red yeast A
cont.	control-no antagonist

Obs.	cont.	A8	RY28	RY24	Obs.	cont.	A8	RY28	RY24
1	15	11	12	5	36	11	3	20	6
2	15	20	4	3	37	6	3	7	10
3	30	22	10	6	38	9	5	7	12
4	15	5	4	3	39	7	4	5	10
5	28	6	8	2	40	5	9	7	5
6	18	12	7	8	41	10	5	7	16
7	13	4	11	8	42	10	7	6	7
8	8	4	8	9	43	12	11	9	4
9	8	5	5	11	44	14	3	5	3
10	24	8	4	11	45	17	8	18	4
11	4	4	6	11	46	15	5	12	6
12	10	12	7	8	47	7	5	18	7
13	13	3	7	9	48	10	7	4	10
14	8	10	8	13	49	11	3	5	6
15	6	10	5	7	50	34	7	8	15
16	7	15	4	11	51	6	5	5	13
17	13	9	13	11	52	10	13	5	26
18	24	2	3	3	53	14	4	4	3
19	11	5	7	18	54	11	4	4	7
20	11	7	7	9	55	4	10	5	8
21	24	5	6	16	56	8	3	4	10
22	5	13	3	8	57	6	10	7	10
23	10	5	4	10	58	7	5	10	8
24	20	5	11	10	59	13	7	6	9
25	7	4	8	9	60	9	9	6	13
26	4	13	10	6	61	5	10	4	8
27	16	8	5	6	62	9	3	7	19
28	9	3	8	6	63	8	7	7	11
29	8	9	9	7	64	4	4	11	15
30	17	5	13	6	65	10	10	9	10
31	14	6	9	14	66	9	16	11	5
32	9	6	10	5	67	7	16	5	9
33	32	3	2	7	68	10	4	8	4
34	10	4	7	4	69	15	5	5	13
35	11	6	7	7	70	6	7	7	11

(continued)

Obs.	cont.	A8	RY28	RY24
71	7	9	5	10
72	8	5	3	8
73	8	3	4	11
74	10	10	10	5
75	8	6	11	6
76	5	7	7	5
77	6	6	11	16
78	8	6	7	12
79	7	6	8	11
80	17	6	5	10
81	3	5	5	7
82	6	7	6	11
83	8	9	5	9
84	5	7	17	9
85	8	5	4	7
86	4	7	6	6
87	7	6	4	6
88	10	4	5	12
89	3	4	7	6
90	6	7	8	21
91	10	6	5	14
92	9	4	7	7
93	6	8	5	6
94	7	8	8	10
95	11	5	4	6
96	11	14	5	4
97	20	14	8	14
98	11	12	4	9
99	11	4	6	10
100	14	4	8	7

Statistical analysis of batch 1: Student's t test

	cont.	A8	RY28	RY24
sums of X (totals)	1080	711	728	895
sums of X^2	15350	6472	6246	9689
mean	10.80	7.11	7.28	8.95
% inhib.		34.2	32.6	17.1
S^2	37.23	14.31	9.56	16.96
F		2.6017^{**}	3.8943^{**}	2.1952^{**}
Homogeneity of variance		no	no	no
S(Xc-Xt)		0.7179	0.6840	0.7361
t		5.1400^{**}	5.1462^{**}	2.5132^{**}
Effective df		165	147	174
Significant inhibition		yes	yes	yes

APPENDIX VI

Measurements of Sclerotinia sclerotiorum germ tube length (in mm): batch 2

isol. no.	fungus
A9	Myrothecium verrucaria
A16	Cladosporium cladosporioides
Y3	white yeast B
Y7	white yeast A
cont.	control-no antagonist

Obs.	cont.	A16	Y7	Y3	A9
1	7	3	4	10	7
2	7	5	4	2	7
3	4	3	2	18	8
4	3	3	6	6	13
5	8	3	3	5	6
6	5	13	15	7	4
7	4	4	3	6	3
8	7	4	7	4	15
9	7	3	3	10	6
10	15	5	3	3	5
11	4	3	5	4	9
12	13	4	4	5	8
13	13	2	3	6	6
14	13	5	3	2	5
15	4	4	4	7	5
16	8	3	8	9	6
17	9	3	3	3	5
18	4	3	3	2	2
19	3	3	3	14	3
20	18	3	2	3	2
21	2	3	4	6	4
22	5	4	4	16	12
23	6	2	5	10	12
24	4	3	5	5	4
25	5	4	5	5	26
26	2	4	11	3	4
27	5	3	4	4	4
28	3	3	4	7	4
29	3	5	10	3	26
30	11	4	3	13	5
31	5	3	3	12	11
32	5	5	2	2	4
33	4	4	2	5	4
34	8	5	2	3	13
35	4	6	5	2	8

Obs.	cont.	A16	Y7	Y3	A9
36	12	4	3	3	4
37	10	3	6	6	6
38	5	3	3	2	3
39	3	4	4	3	3
40	6	3	5	3	3
41	9	4	5	10	6
42	7	2	4	5	7
43	9	4	3	10	11
44	5	3	3	5	12
45	5	3	3	10	7
46	11	3	2	3	3
47	4	3	2	3	8
48	11	3	2	9	7
49	4	3	3	8	6
50	4	5	4	12	8
51	6	3	3	2	6
52	5	5	4	5	6
53	13	5	4	5	14
54	8	3	3	5	5
55	7	3	5	10	5
56	7	2	5	24	3
57	11	2	5	9	5
58	5	4	4	6	14
59	10	3	4	5	5
60	9	3	7	12	9
61	5	4	3	4	5
62	4	4	3	7	3
63	4	4	4	8	3
64	4	3	4	5	13
65	9	3	3	5	4
66	4	3	3	2	23
67	6	7	6	14	10
68	5	4	5	6	15
69	4	4	4	9	20
70	4	3	3	8	8
71	4	4	3	6	2
72	4	10	4	10	5
73	3	4	9	14	3
74	10	2	4	11	9
75	5	3	4	4	11
76	6	4	3	13	6
77	3	6	6	8	3
78	8	5	3	2	10
79	6	4	7	7	7
80	9	3	2	10	7
81	12	7	4	4	10
82	3	5	4	5	7
83	9	3	7	4	15
84	4	5	2	5	5
85	3	5	3	28	19
86	3	5	6	9	4

Obs.	cont.	A16	Y7	Y3	A9
87	2	3	2	3	7
88	5	2	3	3	10
89	4	3	2	4	5
90	3	4	4	5	7
91	4	7	4	3	6
92	7	3	3	5	11
93	6	3	3	2	9
94	3	4	4	4	8
95	7	3	5	15	3
96	8	4	3	4	9
97	6	2	5	4	4
98	4	4	9	5	4
99	4	4	7	8	7
100	10	4	8	6	17

Statistical analysis of batch 2: Student's t test

	cont.	A16	Y7	Y3	A9
sums of X (totals)	628	384	424	676	766
sums of X^2	4956	1712	2238	6630	8262
mean	6.28	3.84	4.24	6.76	7.66
% inhib.		38.85	32.48	-7.64	-21.97
S^2	10.224	2.398	4.447	20.810	24.186
F		4.2636**	2.2991**	2.0354**	2.3656**
Homogeneity of variance		no	no	no	no
S(Xc-Xt)		0.3553	0.3830	0.5571	0.5866
t		6.8674**	5.3264**	0.8616	2.3525**
Effective df		143	171	177	170
Significant inhibition		yes	yes	--	--
Significant stimulation		--	—	no	yes

APPENDIX VII

Measurements of _Sclerotinia_ _sclerotiorum_ germ tube length (in mm): batch 3

isol. no.	fungus
A3	_Trichoderma_ sp.
A5	_Alternaria_ _alternata_
A7	_Fusarium_ B
M131	_Fusarium_ A
M146	_Epicoccum_ _purpurascens_
Y11	_Aureobasidium_ _pullulans_
cont.	control-no antagonist

no. of	treatment (isolate)						
observ.	cont.	A7	A5	A3	M146	M131	Y11
1	9	14	13	6	6	6	12
2	8	18	6	11	12	7	20
3	17	13	7	9	8	5	12
4	7	11	9	9	9	7	10
5	9	9	7	12	11	8	10
6	18	21	3	19	16	11	15
7	10	3	12	15	11	6	4
8	21	5	7	14	11	8	9
9	4	12	6	6	8	12	9
10	9	4	4	8	10	9	10
11	8	5	7	10	6	21	8
12	13	9	7	12	29	7	8
13	13	6	4	9	9	6	5
14	8	14	5	5	5	8	9
15	4	7	3	7	12	6	14
16	40	6	6	17	8	11	16
17	13	5	14	9	10	7	13
18	22	6	5	21	8	7	8
19	32	9	13	13	11	9	11
20	29	10	6	7	11	6	12
21	8	10	2	6	4	4	5
22	7	31	4	10	13	6	7
23	28	8	4	9	4	5	6
24	4	6	3	17	10	8	10
25	11	5	6	4	4	9	9
26	8	9	4	5	11	4	8
27	7	5	2	7	11	4	12
28	22	10	9	30	7	15	8
29	5	11	4	25	7	5	14
30	42	6	4	17	6	11	10
31	48	4	10	19	13	8	4
32	30	4	14	9	12	9	6
33	13	16	4	21	10	4	20
34	7	5	4	6	9	5	10

no. of		treatment (isolate)					
observ.	cont.	A7	A5	A3	M146	M131	Y11
35	15	5	6	9	11	9	10
36	4	6	4	7	10	6	13
37	8	9	5	20	13	6	25
38	7	8	4	7	10	5	14
39	11	5	2	8	13	7	9
40	16	6	5	9	4	7	6
41	17	14	7	6	8	4	7
42	17	11	3	18	9	7	11
43	15	11	5	5	7	13	4
44	8	5	7	10	9	8	14
45	35	18	3	11	17	5	7
46	21	14	5	10	25	5	10
47	15	18	2	17	7	8	6
48	17	11	5	5	4	7	6
49	8	28	5	22	13	11	10
50	16	8	2	12	18	10	6
51	18	18	8	10	6	7	12
52	6	8	3	10	8	5	13
53	19	13	6	8	6	5	12
54	7	13	5	5	7	7	15
55	21	9	5	9	8	5	8
56	17	19	4	16	5	6	8
57	9	18	3	6	9	7	22
58	14	4	11	11	8	6	6
59	12	15	20	8	5	8	6
60	22	13	4	13	6	9	14
61	12	9	3	5	4	8	15
62	9	14	5	12	5	4	10
63	14	7	4	10	10	2	8
64	5	4	4	8	9	5	6
65	6	10	8	10	8	8	7
66	12	16	4	6	7	10	5
67	14	9	6	4	5	6	8
68	9	6	3	7	3	3	9
69	11	6	6	16	7	6	11
70	19	9	4	7	7	5	6
71	9	7	10	13	9	7	12
72	12	8	7	10	8	8	9
73	8	19	3	5	6	9	5
74	10	9	8	14	7	5	4
75	10	24	4	19	4	4	10
76	7	8	3	9	5	10	11
77	9	17	2	3	3	7	13
78	21	11	5	18	4	10	6
79	5	13	3	8	6	12	6
80	7	19	5	6	5	13	20
81	4	7	5	6	3	14	10
82	18	18	7	9	7	5	5
83	15	14	7	6	26	9	7
84	6	8	3	8	4	5	7

no. of	treatment (isolate)						
observ.	cont.	A7	A5	A3	M146	M131	Y11
85	12	11	5	19	5	7	5
86	4	14	4	18	5	6	7
87	8	12	3	7	7	8	5
88	18	6	5	10	6	6	7
89	14	4	4	10	10	11	28
90	15	19	3	9	21	10	6
91	8	21	3	7	5	9	5
92	9	13	8	5	3	10	5
93	28	10	9	25	9	11	16
94	12	6	3	27	3	8	11
95	19	12	2	8	4	12	16
96	15	11	2	9	5	4	9
97	24	9	4	9	27	7	22
98	10	10	7	9	4	11	9
99	27	3	9	26	3	7	10
100	13	19	4	6	5	8	12

Statistical analysis of batch 3: Student's t test

	cont.	A7	A5	A3	M146	M131	Y11
sums of X (totals)	1397	1076	551	1099	862	757	1002
sums of X^2	26793	14634	3975	15373	9988	6557	12198
mean	13.97	10.76	5.51	10.99	8.62	7.57	10.02
% inhib.		22.98	60.56	21.33	38.30	45.81	28.27
S^2	73.504	30.87	9.48	33.28	25.83	8.35	21.80
F		2.3810**	7.7495**	2.2084**	2.8452**	8.8039**	3.3720**
Homogeneity of variance		no	no	no	no	no	no
S(Xc-Xt)		1.0216	0.9110	1.0334	0.9967	0.9047	0.9762
t		3.1421**	9.2865**	2.8837**	5.3677**	7.0742**	4.0463**
Effective df		170	124	173	113	121	153
Significant inhibition		yes	yes	yes	yes	yes	yes