ANTAGONISTIC INTERACTIONS INVOLVING TRICHODERMA SPECIES

A thesis presented for the degree

of

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in the University of Stirling

by

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ABSTRACT

An investigation has been made of antagonistic interactions involving Trichoderma species.

The problem was studied from two aspects; (1) the effect of fungistasis on spore germination and (2) the production of non-volatile and volatile antibiotics.

Sensitivity to soil fungistasis was measured on four parameters of germination (final percentage germination, rate of germination, latent period of germination, and germ tube growth rate) for each of the 20 Trichoderma isolates. Sensitivity varied between the isolates and for any one soil fungistasis did not act equally on the four parameters. There was, however, no apparent consistency for any one parameter to be more affected than another when the results for the different isolates are compared. Positive correlations were however shown to exist between the percentage reduction in the latent period and both the percentage reduction in the final percentage germination and the percentage reduction in the germ tube growth rate. The latent period in the control was directly correlated with the percentage reduction in the final percentage germination.

A growth index under fungistatic conditions has been calculated. This has been called the Theoretical Colonization Index and reflects the ability of a spore

population to grow under antagonistic conditions in soil. There is a direct correlation between the percentage reduction in the theoretical colonization index and the percentage reduction in performance of each of the other four parameters.

Variation in the sporulation media made little significant difference to the sensitivity of T. viride (48) spores to soil fungistasis although considerable variation resulted in the theoretical colonization indices.

Bioassay tests with a wide range of fungi indicated that all of the Trichoderma isolates produced non-volatile antibiotics in liquid culture. A large number of biologically active metabolites were produced including a peptide antibiotic similar to the antibiotics alamethicine and suzukacillin.

Work with T. viride (48) indicated that the quality and quantity of the active metabolites produced varied with the quality of nutrients in the culture medium and the maturity of the culture.

All of the isolates produced volatile antibiotics active against germination of Gliocladium roseum conidia. Volatile antibiotic production by T. viride (48) growing on different C-sources was found to vary with respect to C-source and time; maximum biological activity was found to occur at the same time as production of a 'coconut' smell.

The nature of these antagonistic interactions is discussed in relation to the ecology of Trichoderma in soil.

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Thanks are also due to my colleagues at Stirling with whom I have had much stimulatory discussion.

I would also like to express my gratitude to Fran, my wife, for her patience and encouragement and Mrs. J. Weber for the typing.

Finally, the financial support of the Science Research Council is gratefully acknowledged.

The work presented in this thesis is the result of my own investigations and has neither been accepted nor is being submitted for any other degrees.

C. P. Mitchell ..... Candidate

Neville J. Dix ..... Supervisor

28th September 1973 Date

We dance round in a ring and suppose,
but the secret sits in the middle and knows

ROBERT FROST

At first it seemed a little speck,
And then it seemed a mist;
It moved and moved, and took at last
A certain shape, I wist.

SAMUEL TAYLOR COLERIDGE

CONTENTS

INTRODUCTION

Antagonism between fungi involves a variety of interactions; competition for nutrients, predation by other fungi and antibiosis.

One of the difficulties involved in an investigation of antagonism between fungi and fungal ecology in general is interpretation of the definitions used by other workers for "competition" and "antagonism". Competition appears to have at least two meanings in common usage though efforts have been made to try and standardize the definitions (Clark, 1965; Park, 1960, 1967). One of the definitions is 'rivalry for a limiting factor in the environment' which corresponds to that used for competition as one of the three factors involved in antagonism. The second, more broadly based definition, encompasses both the closely defined competition of the first definition but also includes antibiosis thus making it equatable with antagonism. The broad definition of competition was the one used by Garrett (1950) when he first put forward his ideas concerning "Competitive Saprophytic Ability" and will be used in this thesis.

The term Competitive Saprophytic Ability was proposed by Garrett in 1950 and later (1956) defined as "the summation of physiological characteristics that make for success in competitive colonization of dead organic substrates". Garrett (1950) compiled a list of the more obvious physiological and biochemical attributes that could contribute to competitive saprophytic ability of one kind or another which he slightly modified and updated in 1970:

(1) rapid germination of fungal propagules and speedy growth of young hyphae when stimulated by soluble nutrients diffusing from a substrate,

(2) appropriate enzyme equipment for decomposition of the more resistant carbon constituents of plant tissues such as lignin and cellulose,

(3) production of fungistatic and bacteriostatic growth-products including antibiotics,

(4) tolerance of fungistatic substances produced by other soil microorganisms, i.e. tolerance of the general soil antagonism (including soil fungistasis).

In the light of subsequent research it was realised that the term competitive saprophytic ability was only applicable when referring to particular circumstances. Whether the fungus has a high or a low competitive saprophytic ability will be determined to a large extent by the substrate being colonized and by the competing organisms. It therefore became obvious that the substrate being colonized should be stated explicitly i.e. Nannizzia gypsea has a high competitive saprophytic ability on substrates composed of keratin but is unable to compete on the great majority of substrates (Griffin, 1972).

The degree of success attained by any one fungal species in competitive saprophytic colonization of a substrate as well as being determined by its competitive saprophytic ability for that substrate is also determined by its inoculum potential and environmental conditions. "Inoculum potential"

is defined as 'the energy of growth of a fungus available for colonization of a substrate at the surface of that substrate to be colonized' (Garrett, 1956).

Inoculum potential, as a determinant in the process of root infection (for which the term was first developed) is the force that opposes resistance of the host; only if the inoculum potential of the fungus is sufficient to overcome resistance of the host does a successful, progressive infection ensue. A substrate, on the other hand is inert and offers no active resistance to colonization; the resistance to be overcome is constituted by the competition of other organisms. For this reason, the propagule-population (per unit area of substrate surface) component of inoculum potential appears to be relatively more important in competitive saprophytic ability than it is in host infection. A high propagule-population of a particular fungal species at the surface of a substrate gives the species an advantage position which can more than compensate for a low degree of competitive saprophytic ability.

The mechanism of saprophytic competition between fungi has been summarized by Garrett (1970) in the following statement "the share of a substrate obtained by a particular fungal species will be determined partly by its intrinsic competitive saprophytic ability and partly by the balance between its inoculum potential and that of its competitors". This finely balanced interaction can be

drastically turned by slight changes in environmental conditions in favour of one of the interacting organisms. An idea of the complexity of the potential interactions involved can be gleaned from the following diagram (fig.1). The diagram lists the major attributes for competitive saprophytic ability on the left side and on the right the factors modifying the inoculum potential of the spore population and the possible interactions between the various components are indicated. A slight change in any one of these factors will be reflected in modifications of other factors which could influence the success of that population competing for a substrate. The relative significance of the various factors influencing the outcome of attempted colonization of a substrate by a mixed population of organisms is uncertain. This is in the main due to the fact that the experimental technique used to investigate a problem determines to a considerable extent the answers obtained. Wastie (1961), using a modified Cambridge method investigated the competitive saprophytic ability of 14 root-infecting fungi, confined his investigation to considerations of the two characteristics of a fungus which then seemed most likely to determine its success on the agar plate:

(1) growth rate in pure culture on the virgin nutrient agar and (2) tolerance of fungistatic microbial growth products expressed by the ratio growth rate on staled agar/growth rate on virgin agar. He was unable to show any significant correlation between the colonization ability and tolerance to antibiotics but there was a significant correlation between

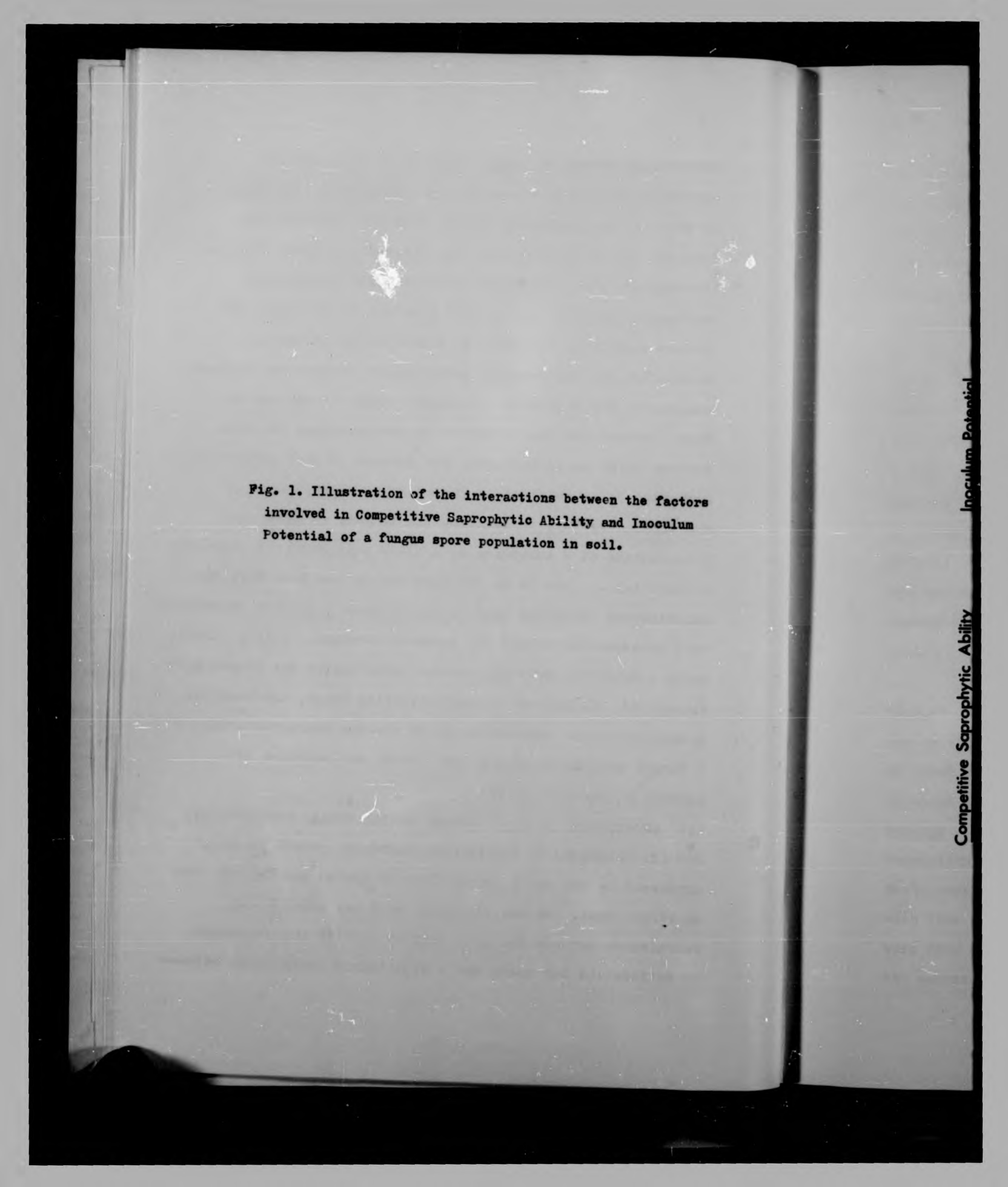

Fig. 1. Illustration of the interactions between the factors involved in Competitive Saprophytic Ability and Inoculum Potential of a fungus spore population in soil.

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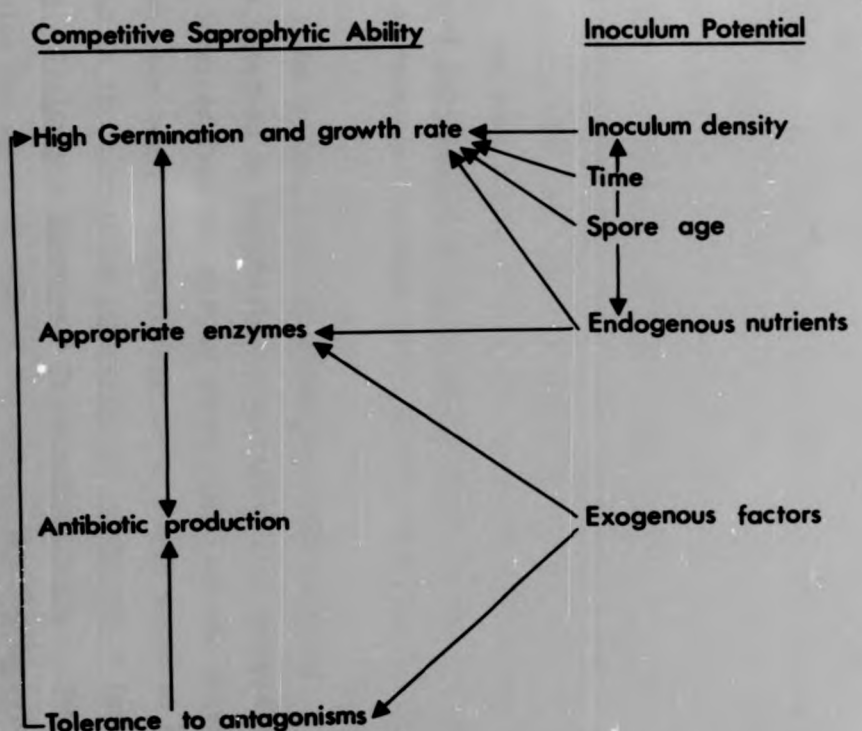

colonization ability and hyphal growth rate in pure culture. These results suggest that the outcome of the struggle between the inoculant fungus and other soil fungi is decided before any fungistatic growth products could accumulate. Similar results were obtained when colonization of autoclaved soil from a mixed inoculum was assessed by noting the presence of fungi in sampling tubes, filled with potato dextrose agar, placed in the soil (Lindsay, 1965). The conclusions concerning saprophytic colonization of virgin nutrient agar do not apply to colonization of the same agar when it has become staled by fungal growth. Dwivedi & Garrett (1968) found that the species spectrum of fungi colonizing staled agar changed progressively with the time and degree of staling due to fungal growth; the most highly staled agar was only colonized by species of Penicillium and Trichoderma which showed a high degree of tolerance to fungistatic growth products of other fungi.

An insight into the number and complexity of factors involved in saprophytic colonization and successions can be obtained from the work of Gibbs (1967) on the colonization of fresh pine logs. At least four factors were shown to be involved in the succession Leptographium lundbergii → Fomes annosus → Peniophora gigantea → Trichoderma viride. The succession was shown to reflect decreasing ability to tolerate toxic substances present in the substrate itself which declined with time and increasing colonization. The initial colonizers were only able to utilize simple sugars and those further on in the succession were also able to utilize the more complex

carbohydrates lignin and cellulose. The order of the succession was also shown to be the order of increasing tolerance to the antibiotics produced by other members of the succession.

From a consideration of these examples it would be foolhardy to argue for the general supremacy of any one factor in influencing competitive saprophytic colonization, for the predominant factor is likely to vary with the circumstances of each case.

Whilst maintaining cognizance of this fact the present work was undertaken to investigate antagonistic interactions in soil with particular reference to the genus Trichoderma.

The genus Trichoderma is a common component of the soil mycoflora, being isolated from a wide range of habitats. The genus was first described by Persoon (1794) to accommodate four species of fungi isolated from rotting wood. Later the genetical connection between Trichoderma viride and the ascomycete Hypocrea rufa was reported by the Tulasne brothers (1865) and Brefeld & vanTavel (1891). In 1902 Oudemans & Koning reported the first occurrence of a species of Trichoderma from the soil. They obtained, in culture, an isolate which they described as T. koningii Oud., differentiated from T. viride by having ellipsoidal as opposed to globose conidia.

The increased study of soil fungi in the early 20th Century led to the isolation of large numbers of Trichoderma isolates from varying habitats. As species

delimitation was obscure there was no agreed naming of certain isolates. Bisby (1939) investigated the variability of isolates of Trichoderma and concluded that in the absence of any reliable distinguishing characters the genus was mono-typic, consisting of one variable species T. viride Pers. ex Fries.

Many mycologists adopted Bisby's view resulting in the name being indiscriminately applied to any green-spored isolates of Trichoderma. Dingley (1957) reported that nine species of Hypocrea from New Zealand produced conidial states referable to the genus Trichoderma thus indicating that the genus could not be mono-typic but that it is composed of closely related species. Studies by Webster (1964), Rifai & Webster (1966a, 1966b), Webster & Rifai (1968) on the perfect and imperfect states of Hypocrea species showed that their Trichoderma states could be distinguished.

Rifai (1964, 1969) established certain characters for classifying the different isolates into species enabling him to make a taxonomic revision of the genus. He adopted the 'species group' concept rather than using a narrowly defined species as the natural variation could make differences between species obscure. Rifai (1969) recognises nine species groups within the genus which are grouped into four large groups (complexes) of rather similar species groups. Hammill(1970) has since reported the occurrence of another species of Trichoderma isolated from soil. The importance of Rifai's work lies in the fact that if mycologists adhere to it then more accurate observations may be made of the ecology of the genus than has been the case in the past.

Unfortunately Soviet mycologists and mycologists of other nationalities still only accept two distinct species; T. koningii Oud. and T. lignorum (Tode) Harz. (Bilai, 1963).

Many workers have reported the isolation of species of Trichoderma from soils of various types in many different countries, but in general the descriptions of the soil types have been vague (except Okado, 1938; Rishbeth, 1950, 1951 and Aytoun, 1953) and for the reasons given above the identity of some of the isolates is in doubt.

Previous studies have shown that sites characterized by excessive moisture and acid soils appear to favour Trichoderma although Dwivedi (1966) has found the genus common in well drained and alkaline soils in India. Apinis (1967) has shown however that Trichoderma species differed in their affinity for moisture which may account for apparent discrepancies of this type in the literature. Danielson (1971), using Rifai's classification found that the largest populations of Trichoderma occurred in F (fermentation) and H (humus) forest floor horizons or under conditions of excessive soil moisture. Using a broad geographic and climatic scale he showed that T. viride and T. polysporum are largely restricted to cool temperate regions; whereas, T. harzianum was largely characteristic of warm climates. T. koningii and T. hamatum were the most widely distributed species groups and were found in all the climatic regions investigated. Under conditions of excessive moisture, strains of T. hamatum and T. pseudokoningii were the dominant forms.

T. polysporum (assuming synonymy with T. album and T. sporulosum) is commonly found in the litter layer of forests (Blandsberg, 1969; Christensen, 1969; Christensen & Whittingham, 1965; Hayes, 1965; Kendrick, 1963; and Macauley & Thrower, 1966) and it has also been isolated from pine wood soil (Ellis, 1940). Boardman (1968) in a study of heather rhizosphere fungi thought that T. album had affinities in habitat and physiology to Penicillium spp. and Dooley (1968) in a similar study considered that T. viride was a true rhizosphere fungus. Work on the succession of fungi on decaying plant remains in the soil has established that in general Trichoderma species are considered to be primary colonizers (Caldwell, 1963; Corbell & Levy, 1963; Dix, 1964; Hering, 1967; Macauley & Thrower, 1966; Merrill & French, 1966; and Tubaki & Yokoyama, 1971).

Much of this information is now only of general value because of the new classification scheme. Even with adherence to this scheme problems will still arise in interpretation of the results of other workers because of the physiological variations in isolates of the same species group.

Many studies with Trichoderma have been concerned with the antagonistic properties of the genus. This area of research was instigated, according to many reviewers, by observations made by Weindling (1932) on the parasitic habit of T. lignorum (Tode) Harz. The earlier work of Vuillemin (1887) on observations of parasitism by T. viride on a Mucor species appears to have been overlooked by most reviewers.

Two forms of antagonism have been observed in culture, especially under acidic conditions; direct parasitism in which Trichoderma hyphae grow around and in some cases eventually penetrate the hyphae of susceptible fungi, and antibiosis where death or inhibition of the other fungus occurs with no physical contact effected (Aytoun,,1953; Dennis, 1970; Dennis & Webster, 1971c; Hashioka et al, 1961; Komatsu & Hashioka, 1964; Weindling, 1932, 1934).

The parasitic action or exploitation of one fungus by another is usually termed mycoparasitism (Barnett, 1964; Boosalis, 1964). Mycoparasitism by a Trichoderma spp was first reported by Weindling (1932) who observed Trichoderma hyphae coiling around hyphae of other fungi notably Rhizoctonia solani. Webster & Lomas (1964) showed that Weindling's strains were not Trichoderma species but belonged to the genus Gliocladium. Other workers, however reported hyphal interaction by isolates considered to be true Trichoderma species with a large number of fungi; coiling (Aytoun, 1953; Chi, 1960; Dennis, 1970; Dennis & Webster, 1971c; Komatsu, 1968) and penetration (Dennis, 1970; Dennis & Webster, 1971c; Durrell, 1966; Slagg & Fellows, 1947).

Of the 80 isolates of Trichoderma tested for hyphal interaction with 6 test fungi by Dennis (1970; Dennis & Webster, 1971c) only 10 did not show coiling around the host hyphae. Some variations in coiling behaviour was observed between isolates of the same species groups, though to a lesser extent than variation in antibiotic production (Dennis, 1970; Dennis & Webster, 1971a,b,c). Isolates of

T. harzianum which showed no apparent ability to produce antibiotics were capable of coiling around other hyphae. Hyphal penetration appeared to be restricted to penetration of Pythium ultimum by non-antibiotic producing isolates of the T. harzianum and T. viride species groups. Further Hashioka & Fukita (1969) found, in an ultrastructural study of mycoparasitism of Trichoderma, Gliocladium and Acremonium to phytopathogenic fungi that "Trichoderma invaded into, coiled around and cut off the host hyphae". No disorganisation of the host cells was observed although an infection papilla similar to a callus or callosity in plant mycosis was found inside the affected host cell wall. Dennis & Webster (1971c) observed vacuolation and disruption of host hyphae in some cases, this was thought to be due to the antibiotic producing capabilities of these isolates; cyclic polypeptide antibiotics similar to alamethicine (produced by Trichoderma) have been shown to produce a similar reaction in bacteria (Newton, 1965).

Griffin (1972) thought that mycoparasitism in the soil was unlikely to be a factor of great ecological importance. Mycoparasitism in itself may not be of great ecological importance but the coiling of Trichoderma around host hyphae is of importance in that it allows any antibiotics produced by the Trichoderma to be present in high concentrations in the immediate environment of the other fungus. The report of Roth (1969) of active mycoparasitism by T. viride on the causal organism of stem rot disease of sugarcane indicates that in some cases mycoparasitism may be of ecological importance.

Trichoderma isolates have been shown to produce a range of non-volatile and also volatile antibiotics active against a range of fungi (Dennis, 1970; Dennis & Webster, 1971a, b).

The ecological significance of these observations have not been fully investigated. Certain aspects of the antagonistic properties of *Trichoderma* have therefore been studied in relation to general antagonisms in soil. The problem was approached from two aspects; (1) the action of the soil environment (general soil antagonism i.e. soil fungistasis) on the growth of different *Trichoderma* species groups and (2) the effect, on selected test fungi, of the external metabolites produced by the *Trichoderma* isolates in order that the overall competitive ability in the soil and the significance of the factors involved in the ecology of the genus may be assessed with respect to the substrate.

MATERIALS AND METHODS

FUNGI

Trichoderma isolates

The following Trichoderma isolates were used in the present study:-

T. hamatum (Ben.) Bain (2 isolates), T. harzianum Rifai (3 isolates), T. koningii Oud. (1 isolate), T. longibrachiatum Rifai (1 isolate), T. polysporum (Link ex Pers.) Rifai (1 isolate), T. pseudokoningii Rifai (1 isolate), T. saturnispora Hammill (1 isolate), T. viride Pers. ex S. F. Gray (10 isolates); full details of their origins are given in Appendix 1.

Test fungi

The following test fungi were used:-

Gliocladium roseum Bainier (2 isolates), G. virens Miller, Giddens & Foster, Cladosporium herbarum Link ex Fr., Trichocladium opacum (Corda) Hughes, Stemphylium dendriticum Sousa da Camara, Fusarium solani (Martius) Saccardo, F. culmorum (W. G. Smith) Saccardo, Botrytis allii Munn, B. cinerea Pers. ex Fr., Penicillium expansum Link ex F. S. Gray, P. funiculosum Thom, P. janthinellum Biourge P. nigricans Bain ex Thom, P. spinulosum Thom; full details of their origins are given in Appendix 2.

Maintenance of Cultures

Pure cultures of all the fungi are maintained on P.D.A. in Petri dishes. Stock cultures were maintained on P.D.A. slopes in MacCartney bottles under sterile mineral oil.

MEDIA

Full details of formulation of all the media and stains used are given in Appendix 3. Carbon sources were added to basal Weindlings medium at the rate of 1000 ppm $C\ell^{-1}$ and N-sources at 300 ppm $N\ell^{-1}$. The C- and N-sources were sterilized in aqueous solution by millipore filtration (0.22 μ pore size) and then added to sterile Weindlings medium.

TECHNIQUES

pH Determination

"B.D.H." narrow range pH papers were used throughout.

Mycelial Dry Weight Determinations

The mycelial mat was dried in a vacuum dessicator ($CaCl_2$) at room temperature overnight and then weighed.

Colony Growth

Colony diameters were measured on the back of the Petri dish using a half meter rule.

Slide Washing

The slides used in all bioassay procedures were soaked in Decon overnight to remove any grease and then thoroughly rinsed in five changes of distilled water and alcohol sterilized.

Preparation of Spore Suspension

Spore suspensions for use in the experiments were collected from cultures growing on P.D.A., after an appropriate incubation time, by washing off with sterile distilled water, concentrated and washed twice by centrifugation at 500 rpm for 5 min and then made up to the required

concentrations; for the small spores e.g. Penicillium spp a concentration of 45 x 10^5 spores cm^{-3} was used and for the larger spores e.g. Stemphylium dendriticum a concentration of 25 x 10^4 spores cm^{-3} was used.

Assay for Sensitivity to Fungistasis

The sensitivity of the spores to fungistasis was measured using the method developed by Dix (1967, 1972). Sifted air-dried soil (1.5 kgm) of pH 4.6 from a mixed deciduous woodland was made up to 60% W.H.C. in a plastic seed tray and the surface smoothed. Strips of sterile Whatman No.1 filter paper 3 x 2 cm were moistened with sterile distilled water and placed on the soil surface, making sure that there was even contact with the soil. A peptone agar block 600μ thick was placed on the surface of each of the pieces of paper. A drop (0.2 cm^3) of a spore suspension (45 x 10^5 spores cm^{-3}) was placed on the surface of each block. Controls were similarly prepared blocks placed on sterile microscope slides on the surface of the same soil, the tray was sealed with a sheet of glass and incubated at 25°C.

At set time intervals 3 agar blocks and the control slide were removed from the soil, stained with lactophenol and cotton blue, a coverslip placed on top and examined microscopically. At least 200 spores were randomly counted on each agar block and the number germinated expressed as a percentage. Measurements were made of 30 germ tubes chosen randomly for each treatment time. A map measurer was used to measure (in arbitrary units) the length of germ tubes

drawn with the aid of a projection microscope at X400 magnification.

Conductivity Measurements

Conductivity measurements of electrolytes released from the spores were made using a conductivity meter, Model MC-1 Mk.5, Electronic Switchgear, London, Limited. The experiments were carried out with the apparatus and materials equilibriated at 25°C in a constant temperature room. Spores, brushed from the surface of the culture were suspended in 15 cm^3 de-ionised water (conductivity 2 μmho cm^{-1}) in the electrode cell. The cell was shaken between readings on a wrist action shaker to minimise the effects of settlement of the spores. Measurements of the conductivity of the solution were taken immediately on addition of the spores to the de-ionised water. This reading was taken as the initial (zero reading) to which all subsequent readings were referred to when comparisons are made of increase in conductivity. Subsequent readings were taken at 1, 2 minutes, at further 2 minute intervals to 10 minutes thereafter readings were taken at 10 minute intervals to the completion of the experiment after 60 minutes. Each experiment was repeated thrice for each spore population. The spore concentration in the electrode cell was determined at the completion of each experiment and the conductivity expressed as standard conductivity for 10^7 spores cm^{-3}. These results were then expressed as percentage increase in conductivity with respect to the initial reading.

Preparation of Culture Filtrates

Two 3 mm diameter blocks were cut from the edge of an actively growing culture on P.D.A. with a No. 3 cork borer and used to inoculate 200 cm^3 Weindlings medium in a litre conical flask. Triplicate flasks were inoculated for each isolate and incubated on a rotary incubator at 150 rpm at 25°C for 6 days. The culture liquid was then filtered through 3 layers of Whatman No.1 filter paper using a Buchner funnel and flask to separate the mycelium.

The 200 cm^3 of culture filtrate was then concentrated in a vacuum rotary evaporator at 40°C to 50 cm^3 (see Appendix 4). The concentrated filtrate was then sterilized by filtration through a Hemmings filter under centrifugation.

Bioassay

Peptone agar blocks (1 mm thick, 3 mm diameter) were placed on sterile microscope slides in a damp chamber. An 0.2 cm^3 aliquot of the concentrated culture filtrate was placed on the upper surface of the block and allowed to dry. Three blocks were used for each treatment, the controls consisted of similarly treated blocks but with an 0.2 cm^3 aliquot of sterile Weindlings medium rather than the culture filtrate added. When the surface of the blocks was dry a drop of a spore suspension of the relevant test fungus was added at the appropriate concentration and then incubated at 25°C.

After the requisite incubation period the slides were removed from the damp chamber, stained with lactophenol and cotton blue, a cover glass placed on top and examined

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After the requisite incubation period the slides were removed from the damp chamber, stained with lactophenol and cotton blue, a cover glass placed on top and examined

microscopically. A total of 200 spores were randomly counted for each block of each treatment and the percentage germination calculated. The differences between the percentages in treatment and in the control were tested for significance by Student's 't' test using arc-sin transformed data.

The lengths of 30 randomly chosen germ tubes were measured with a map measurer in arbitrary units from drawings made with a projection microscope at X400 magnification.

Investigation of Chemical Nature of Culture Filtrates

Source of Antibiotics

Gliotoxin Viridin Suzukacillin	Dr. C. Dennis, ARC Food Research Institute, Norwich.
Trichodermin	Dr. W. O. Godtfredsen, Leo Pharmaceuticals Ballerup, Denmark.
Alamethicine	Dr. G. B. Whitfield, The UpJohn Company, Kalamazoo, Michigan 49001, U.S.A.

Preparation of Chloroform Extract

Four lots of 200 cm^3 culture filtrate were prepared for each *Trichoderma* isolate and pooled. The 800 cm^3 of culture filtrate was concentrated to 100 cm^3 on a rotary evaporator at 40°C. The concentrated culture filtrate was then extracted twice with 100 cm^3 chloroform (AR). The extractions were carried out by shaking the chloroform with the culture filtrate in a separating funnel. The heavier chloroform fraction was allowed to separate out and run off from the aqueous layer. A precipitate formed at the interface of the chloroform and aqueous layers. The pooled chloroform extracts were dried in a rotary evaporator at ambient temperature (approx. 20°C).

Preparation of Ethanolic Extract

After the concentrated culture filtrate had been extracted with chloroform (as above) a white precipitate formed at the interface between the chloroform and aqueous layers. The precipitate was separated from the aqueous fraction by filtration through 3 layers of Whatman No.1 filter paper. Acetone (AR, 100 cm^3) was added to the precipitate and the acetone-soluble fraction of this precipitate was separated by filtration through three layers of Whatman No.1 filter paper. The acetone-soluble fraction was then reduced to dryness in a rotary evaporator at ambient temperature (approx. 20°C), the dry material being redissolved in 1 cm^3 of ethanol (AR). Further purification was not carried out.

CHROMATOGRAPHY

All the chromatography was carried out using Camag thin layer chromatography (T.L.C.) plates. The full details of the techniques and the solvent systems used are given in Appendix 5.

SPECTROPHOTOMETRY

Absorbance of light in wavelength 200-340 nm was carried out using a Unicam SP 1800 UV spectrophotometer using quartz cells, an 0.25 cm slit width was used throughout.

Absorbance of light in the Infra-red region (4000-650 nm) was measured in CCl_4 solution on a Perkin-Elmer 720 Infra-red spectrophotometer.

CHAPTER ONE

THE EFFECT OF SOIL FUNGISTASIS ON THE DIFFERENT SPECIES GROUPS OF TRICHODERMA

"Soil fungistasis" or "the general soil antagonism" is a term used to describe the phenomenon whereby (a) viable fungal propagules not under the influence of endogenous or constitutive dormancy do not germinate in soil in conditions of temperature and moisture favourable for germination, or (b) growth of fungal hyphae is depressed or terminated by conditions of the soil environment other than temperature and moisture (Watson & Ford, 1972).

Among the first to recognize the inhibition of fungus spores in soil were Simmonds et al (1950), who extracted, with water, the viable conidia of Helminthosporium sativum from field soil. They showed that the recovered conidia would germinate on clean moist filter paper but not in the presence of soil, and thought that the inhibition was a kind of antibiosis caused by bacteria. Hessayon (1953) observed inhibition of Trichothecium roseum conidia when placed directly on soil surfaces and Chinn (1953) recorded the failure of spores of several species to germinate when embedded in water agar on glass slides placed in the soil. Dobbs & Hinson (1953) extended these observations and framed a more generalised concept, that of a "widespread fungistasis in soils". This work aroused interest in the nature of the inhibition and on its probable survival value to the fungi.

Soil fungistasis is characterized by (a) a widespread occurrence in natural soils, (b) a non-specific inhibition affecting almost all species tested, (c) co-existence with microbial activity, and (d) annulment by nutrients.

The phenomenon has been recorded from many different soil types; in tropical soils (Dwivedi & Dwivedi, 1971; Griffiths, 1966a, b; Jackson, 1958; Mishra, 1966; Mishra & Kananjia, 1972, 1973), temperate (Chinn, 1967; Dobbs & Griffiths, 1962; Dobbs *et al*, 1960; King & Coley-Smith, 1969; Vaartaja & Agnihotri, 1967) and sub-arctic areas (Cooke, 1967). The level of fungistasis differs amongst different soil types (Dobbs *et al*, 1960; Lingappa & Lockwood, 1961). Payen (1962) found a peat soil not to be fungistatic towards *Fusarium culmorum* macroconidia whereas Chinn (1967) found a peat soil to be the most fungistatic of those tested towards *Cochliobolus sativus* spores and Jackson (1958) recorded a spectrum of inhibition which was very similar in 6 of 7 widely different soils. Fungistasis has been found to be more pronounced in surface than in sub-soil layers of any one soil profile (Chacko & Lockwood, 1966; Dobbs, 1963; Griffiths, 1966a, Jackson, 1960; Mishra & Kanajia, 1973) however, Schüepp & Frei (1969), working with *Trichoderma koningii*, found no correlation between fungistasis and soil depth.

The general consensus is that fungistasis is due to microbial activity. This opinion is based on the results of a large number of experiments in which microbial activity in the soil was reduced or eliminated by several methods, the reduction being concommitant with a reduction in the fungistatic level of the soil. Dobbs (1971), however, has distinguished between microbial fungistasis which he described as "heat and sugar sensitive", and "residual fungistasis" which is "heat and sugar resistant". Residual fungistasis is

abiological and was first reported by Dobbs & Byewater (1959) and has since been reported from a number of soils (Dobbs, 1971; Dobbs & Gash, 1965; Griffiths, 1966a).

Several theories have been advanced to explain the mechanism of fungistasis due to microbial activity. Prior to the work of Dobbs & Hinson (1953) it had been generally assumed that non germination of spores in soil was a result of lack of nutrients, oxygen or excess of carbon dioxide. Later, however, the emphasis was placed on the interpretation that inhibitory substances produced by microorganisms in the soil were responsible. Thereafter an extensive survey was carried out by several workers to try and isolate an organism or a compound to which the role of a "fungistatic principle" could be applied. The search, based on the assumption that only one mechanism was involved, was unsuccessful and resulted in a much confused situation in the literature.

Park (1960, 1961) drew a parallel between the inhibition caused by metabolites produced by fungi in staled cultures and fungistasis suggesting that they may be the major limiting factor of fungal spore germination and mycelial development in soil. Recent work (Robinson & Garrett, 1969; Robinson & Park, 1966) shows that volatile inhibitors are involved in the phenomenon of staling, and these results parallel those found on the soil (Balis & Kouyeas, 1968; Hora & Baker, 1970; Hora et al, 1971; Kouyeas & Balis, 1968) with respect to fungistatic factors. The presence of sporostatic compounds in the soil and their implication in fungistasis has been reported by Garrett (1972).

There has been much discussion in the literature of the inhibitor theory. Brian (1960) concluded that the occurrence of antibiotics in the soil mass, as distinct from their presence in proximity to soil organic matter, was unlikely owing to the absorptive properties of clay particles and the rapidity with which antibiotics introduced into the soil were broken down by microbial action. It has been shown (Pinck, Holton & Allison, 1961; Pinck, Soulides & Allison, 1961; Soulides, Pinck & Allison, 1961, 1962; Soulides, 1965, 1969) that streptomycin adsorbed onto the kaolin fraction of a soil remained undegraded for 28 days during which time, changes in the microbial activity showed that it was slowly released. Jackson (1965) considered that adsorbed antibiotics might be released following a pH change in the soil, produced by local microbial activity. The dismissal of the inhibitor theory with the argument that any inhibitory compounds produced in the soil will be inactivated in one way or another may be premature when consideration is given to the results of Garrett (1972) and Hora & Baker (1971) who, using more refined techniques than have hitherto been used, were able to detect inhibitory compounds in soil.

The inhibitor hypothesis postulates the diffusion of metabolites from microorganisms to the fungal spore. An alternative hypothesis, put forward by Lingappa & Lockwood (1961, 1964) postulates the diffusion of nutrients away from the spore to adjacent metabolizing cells, predominately bacterial. This hypothesis has lost favour due to the difficulty of reconciling the prolonged activity of fungistasis

with the limited amounts of nutrients present in spores. But it is possible that this microbial activity at the spore surface may result in a metabolic change within the spore (Lockwood, 1964). An example of this relationship has been demonstrated for chlamydospores of Thielaviopsis basicola (Hawthorne & Tsao, 1969; Tsao & Hawthorne, 1970); inhibition of chlamydospore germination in soil was thought to be a result of microbial activity at the spore surface which had an adverse effect on the permeability of the chlamydospore wall.

Ko & Lockwood (1967), investigated the possibility that the spore or its environment was deficient in nutrients required for germination. They found that for 18 fungi tested, the ability of spores to germinate on soil was correlated with ability to germinate in the absence of exogenous nutrients. Fungistasis was therefore considered to be explicable solely in terms of nutrient availability, and the inhibitor theory superceded.

Steiner & Lockwood (1969) showed that for 17 fungi, sensitivity to fungistasis was directly correlated with the germination time (time for 50% of the population to germinate) on sterilized soil and inversely correlated with spore size, volume and surface area although the Fusarium spp tested were exceptions to this generalization. They interpreted these results in the light of the 'nutrient' hypothesis. They thought that as the time of germination and spore size were related to sensitivity to fungistasis this indicated that sensitivity to fungistasis was wholly related to the

availability of nutrients; i.e. the germination time will determine the amount of nutrients that may be leached from the spore and the spore size will determine the amount of endogenous nutrient reserves left to that spore for use in germination, the longer the germination time the less will be the remaining nutrient reserves.

The problem of the mechanism of fungistasis is still open though Jackson (1965) and Knight (1970) are of the opinion that it is a result of competition in the broad sense of the word, i.e. it is a result of both competition in the narrow sense and antibiosis (or antagonism).

Sensitivity to fungistasis is usually measured by taking the percentage germination of a spore population after a pre-determined incubation period in contact with the soil and comparing it with the percentage germination in the absence of soil, similarly when assessing the effect of fungistasis on hyphal or germ tube growth.

The sensitivity of mycelial growth to fungistasis has been shown to be less than that of the spores by Old (1965), Hessayon (1953), Steiner & Lockwood (1969) whereas spores were found to be less sensitive to fungistasis than germ tubes by Cooke & Satchuanthananthavale (1968), Dix (1972) and Stover (1958). Hora & Baker (1972) found that with Helminthosporium sativum and H. victoriae there was a 30-50% reduction in conidial germination and a concommitant reduction in germ tube length.

Hsu & Lockwood (1971) investigated the responses of fungal hyphae to soil fungistasis. Hyphae of 20 different fungi were incubated for 4 hours on cellophane placed on soil amended with different amounts of alfalfa (Medicago sativa)

extract. Linear growth, expressed as percent of growth occurring on alfalfa extract agar, was plotted against log concentration of alfalfa extract. The area under the curve, taken as a percentage of the total area, was termed the "relative growth index". A high relative growth index indicated a low sensitivity to fungistasis. They found that relative growth indices were directly correlated with hyphal growth on alfalfa extract agar and with hyphal diameter i.e. thicker, faster growing hyphae are less sensitive to fungistasis than are thinner, slower growing hyphae. They also found an inverse relationship between fungistasis sensitivity indices of spores and relative growth indices of hyphae. The relative growth index was inversely correlated with the germination time of spores on sterilized soil confirming the results of Steiner & Lockwood (1969).

The effect of soil fungistasis on several parameters of germination of 11 species of Penicillium was thoroughly examined by Dix (1972). By measuring percentage germination and germ tube length at fixed time intervals up to 40 hours he was able to define 3 parameters of germination; (1) final percentage germination, (2) latent period of germination (time for 30% of the population to germinate), and (3) germ tube growth rate which when expressed as a percentage reduction with respect to their respective controls enabled him to make comparisons between the species. He found that for any one species soil fungistasis did not reduce the percentage performance equally in all three parameters of germination. There was a positive correlation between the percentage reduction in the latent period and the percentage reduction

in the final percentage germination, and an inverse relationship between percentage reduction in the germ tube growth and the final percentage germination. It was suggested that the effect of soil fungistasis on the growth rate of germ tubes may be the most significant ecologically, but it was stressed that a complete assessment of the ecological effects of soil fungistasis on a fungus can only be based on measurements of the effect on all three parameters of germination.

The ecological status of the different species groups of the genus Trichoderma in soil, as mediated by fungistasis, was investigated with respect to the tenet of Dix (1972).

Each of the Trichoderma isolates was tested for sensitivity to fungistasis using the method described in the Materials and Methods. T. polysporum was not tested for sensitivity to fungistasis as a satisfactory level of germination was never obtained, even in the controls.

In order to ascertain the effect of fungistasis upon the germination of the different Trichoderma species groups the following four parameters of germination were measured;

(1) the final percentage germination,
(2) the rate of germination overall (i.e. the rate of germination to the final percentage germination),
(3) the latent period (the time taken for 25% of the spore population to have germinated),
(4) the germ tube growth rate.

Fig. 1.1. The effect of fungistasis on percentage germination of T. hamatum (22) spores (logistic curve).

●——● Control, o----o Experiment.

age germination

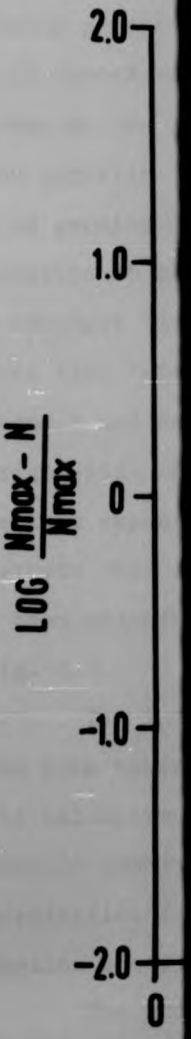
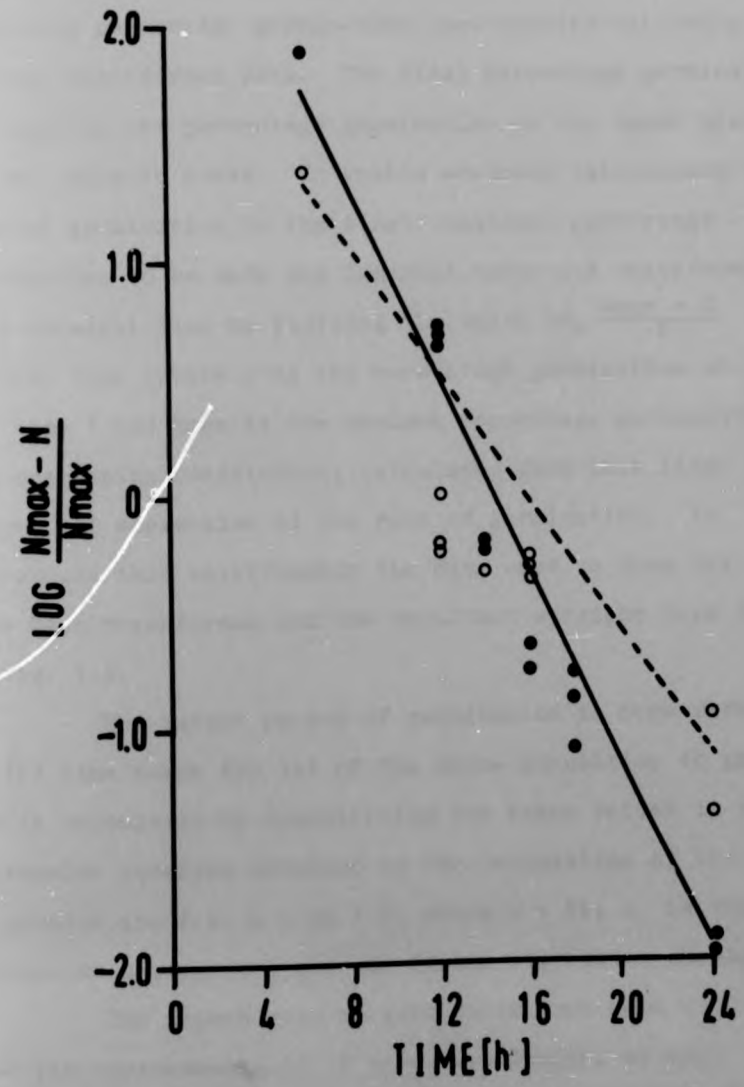


Fig. 1.2. The effect of fungistasis on percentage germination of T. hamatum (22) spores (transformed data).

●—● Control, O- - - -O Experiment.

e germination



The percentage germination for each of the replicates at each time interval were plotted against time and a logistic curve was found to be the best fit for the data, Fig. 1.1 shows the graph of percentage germination against time for T. hamatum (22) spores. All analyses involving percentage germination were carried out using arc-sin transformed data. The final percentage germination is taken as the percentage germination on the upper plateau of the logistic curve. To enable accurate calculation of the rate of germination to the final (maximum) percentage germination to be made the logistic curve was transformed to a straight line by plotting the value $\log \frac{Nmax - N}{N}$ against time (where N is the percentage germination at any time t and Nmax is the maximum percentage germination). The regression coefficient, calculated from this line, is a convenient expression of the rate of germination. To illustrate this relationship the data used to draw fig. 1.1 have been transformed and the resultant straight line shown in fig. 1.2.

The latent period of germination is here defined as the time taken for 25% of the spore population to germinate and is calculated by substituting the known values in the regression equation obtained in the calculation of the rate of germination i.e. $y = ax + b$, where $y = 25$, a is the regression coefficient and b is the regression constant.

The growth rate of germ tubes have been calculated from the measurements of 30 germ tube lengths at each time interval. The regression coefficient of the straight line obtained by plotting the average common logarithmic values for

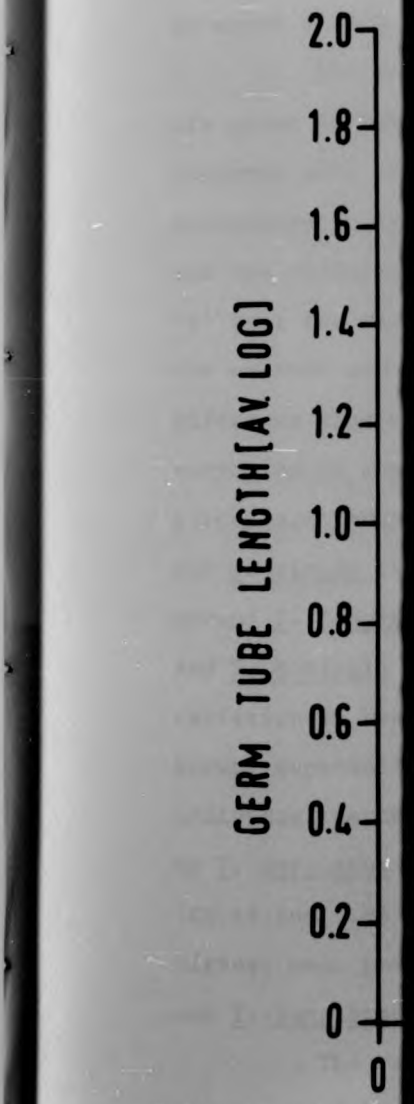
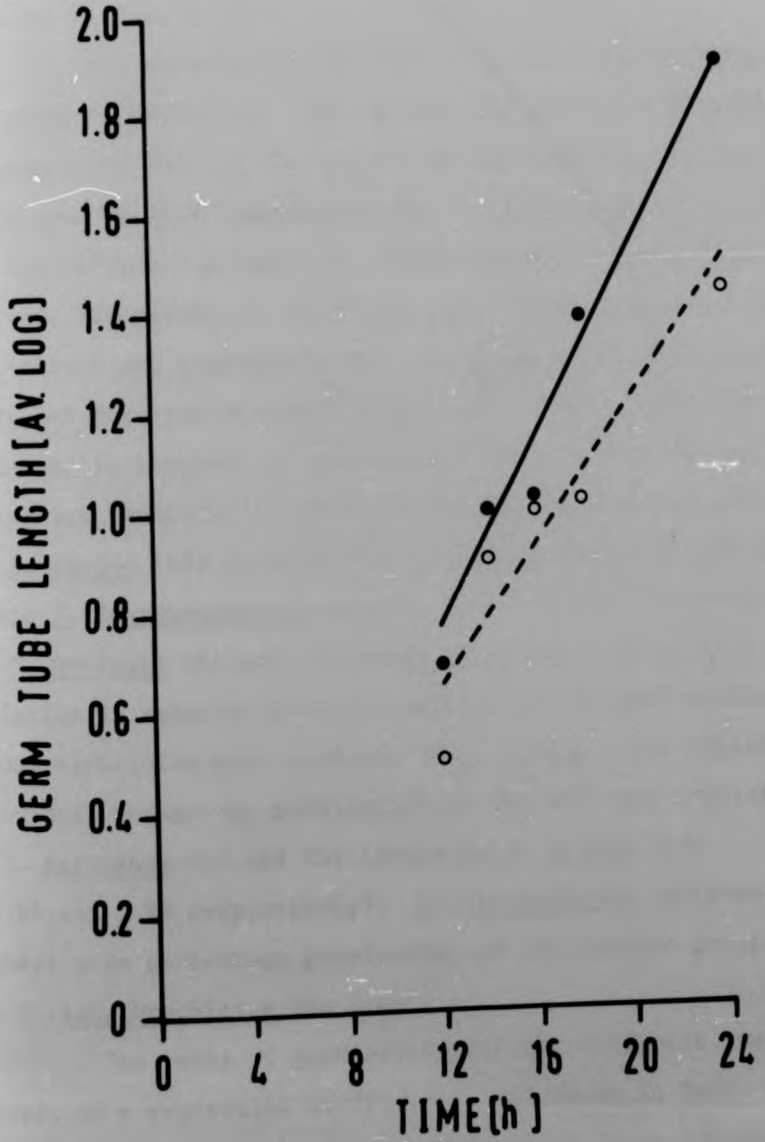


Fig. 1.3. The effect of fungistasis on germ tube growth of T. hamatum (22) spores.

●——● Control, O---O Experiment.

growth of


GERM TUBE LENGTH (AV. LOG)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
4
8
12
16
20
24
TIME (h)

each of the 30 germ tube lengths at each time interval against time is a convenient measure of germ tube growth rate (a graph of germ tube length against time for T. hamatum (22) spores is shown in fig. 1.3).

The results for the final percentage germination are given in Table 1.1. The percentage reduction in performance compared with that of the control of the same isolate has been calculated so that comparisons may be made between the species and the different parameters. When subjected to Student's 't' test the values of the final percentage germination for the control and experiment were all found to be significantly different from one another (Table 1.1). There was a wide variation in response to fungistasis as indicated by the percentage reduction in final percentage germination from 8.0% for T. viride (49) to 84.6% for T. viride (54). Of the species groups T. longibrachiatum was the least affected by fungistasis and T. koningii the most although there was a great deal of variation in response amongst isolates of the same species group, especially with isolates of T. viride. The highest individual percentage germination on the soil was achieved by T. harzianum (1) and the lowest by T. viride (53) (66.6% and 7.8% respectively). T. saturnispora achieved the highest mean percentage germination of the species groups and T. longibrachiatum the least.

The rates of germination for the different species groups, as a regression coefficient, are shown in Table 1.2. The regression equations for the calculated lines are given in Appendix 6. In order to ascertain if the regression lines, and hence the rates, calculated for the control and experiment were significantly different from one another they were

TABLE 1.1 Effect of Soil Fungistasis on Final Percentage Germination of the different Trichoderma species groups.

	Final Percentage Germination			
	Control	Experiment	% Reduction	
T. hamatum				
(15)	74.0	39.6	46.5	***
(22)	79.8	59.5	25.4	***
T. harzianum				
(1)	80.3	66.6	17.0	***
(129)	58.6	25.4	56.8	***
(20)	81.2	59.9	26.2	***
T. koningii	77.8	36.6	52.9	***
T. longibrachiatum	47.1	36.5	22.7	***
T. pseudokoningii	85.4	43.3	49.3	**
T. saturnispora	74.0	53.6	27.6	**
T. viride				
(2)	50.8	35.2	30.7	***
(14)	81.5	21.7	73.4	**
(28)	45.9	25.2	45.1	*
(48)	80.9	48.1	40.6	***
(49)	67.7	62.3	8.0	*
(50)	80.9	66.3	18.1	**
(51)	78.0	41.9	46.3	***
(52)	83.0	24.6	70.4	***
(53)	50.7	7.8	84.6	***
(54)	72.2	51.0	29.4	

* Significant $P < 0.05$)
** " $P < 0.01$,) Student's 't' test (see text)
*** " $P > 0.001$)

TABLE 1.2 Effect of Soil Fungistasis on the Germination Rate of the different Trichoderma species groups.

	Germination rate (as regression coefficient)			
	Control	Experiment	% Reduction	
T. hamatum				
(15)	-0.1817	-0.1443	20.6	NS
(22)	-0.2167	-0.1701	21.5	NS
T. harzianum				
(1)	-0.2390	-0.2215	7.3	NS
(129)	-0.1139	-0.1109	2.6	NS
(20)	-0.2167	-0.1864	14.0	NS
T. koningii	-0.1270	-0.1229	3.2	NS
T. longibrachiatum	-0.0950	-0.1416	0	NS
T. pseudokoningii	-0.1507	-0.0817	45.8	***
T. saturnispora	-0.1786	-0.1537	13.9	NS
T. viride				
(2)	-0.1418	-0.1344	5.2	NS
(14)	-0.1423	-0.0675	52.6	**
(28)	-0.1367	-0.0986	27.9	***
(43)	-0.1968	-0.1463	25.7	NS
(49)	-0.1541	-0.1703	0	NS
(50)	-0.2519	-0.1442	42.8	*
(51)	-0.1447	-0.1437	0.7	NS
(52)	-0.1457	-0.0852	42.9	***
(53)	-0.1383	-0.0453	67.3	***
(54)	-0.1386	-0.0986	28.9	NS

* Significant P < 0.05
** " P < 0.01
*** " P < 0.001
NS Not significant P > 0.05

Variance (F) ratio (see text)

subjected to a variance (F) ratio test. This test examines the variance of the data about the two calculated regression lines and the value of the ratio of the two variances will indicate whether the two regression lines belong to the same normal population distribution or whether, in fact, they come from two significantly different populations. In all cases, except that of T. longibrachiatum and T. viride (50) there was a reduction in the germination rate but this reduction was only significant for 6 of the fungi tested (T. pseudokoningii, T. viride (14), T. viride (28), T. viride (50), T. viride (52), T. viride (53), see Table 1.2). The isolate with the fastest germination rate on soil was T. harzianum (1) which was also relatively insensitive to fungistasis (7.3% reduction), the slowest was T. viride (53) which was also the most sensitive of the isolates tested (67.3% reduction).

The results of the latent period calculations and percentage reduction compared with controls are given in Table 1.3. The percentage reduction is calculated by using reciprocals i.e. $\frac{\frac{1}{C} - \frac{1}{X}}{\frac{1}{C}} \times 100\%$. In two cases, T. harzianum (1) and T. viride (50) the time to reach 25% germination was less in the experiment than in the control whereas T. harzianum (129) and T. viride (53) failed to reach 25% germination in the experiment. There was again a wide variation in sensitivity to soil fungistasis, from 4.4% reduction for T. hamatum (22) to 60.4% for T. viride (14). In addition to those that did not reach 25% germination, T. viride (14) took the longest and T. harzianum (1) the shortest time to reach 25% germination in contact with the soil (35.1 and 9.2 hours respectively).

TABLE 1.3 Effect of Soil Fungistasis on the Latent Period of Germination of the different Trichoderma species groups.

	Latent Period (h)		
	Control	Experiment	% Reduction
T. hamatum			
(15)	12.6	24.9	49.4
(22)	13.0	13.6	4.4
T. harzianum			
(1)	10.8	9.2	0
(129)	16.6	-	100
(20)	12.6	20.1	37.3
T. koningii	13.6	21.8	37.6
T. longibrachiatum	16.3	19.0	14.2
T. pseudokoningii	14.1	19.8	28.8
T. saturnispora	11.4	12.3	7.3
T. viride			
(2)	15.3	23.5	34.9
(14)	13.9	35.1	60.4
(28)	19.2	33.6	42.8
(48)	11.3	13.1	13.8
(49)	11.5	12.8	10.2
(50)	11.0	9.6	0
(51)	14.6	18.7	21.9
(52)	15.6	33.1	52.9
(53)	17.6	-	100
(54)	13.4	16.3	17.8

They were also respectively the most and the least sensitive to fungistasis (60.4% and 0 reduction in latent period).

The germ tube growth rates, as regression coefficients, for the different Trichoderma isolates are shown in Table 1.4. The regression equations of the calculated regression lines are given in Appendix 7. The variance (F) ratio was again used to test for significant differences between the regression lines. There was no reduction in performance by fungistasis of four of the fungi tested (T. koningii, T. viride (2), T. viride (28), T. viride (54)), the germ tube growth rate of T. viride (53) was reduced to the greatest extent (76.9%). Only 6 of the fungi tested (T. hamatum (15), T. harzianum (129), T. longibrachiatum, T. pseudokoningii, T. viride (49), T. viride (53)) had germ tube growth rates on soil significantly different from those in the control. On the soil T. viride (50) showed the fastest and T. longibrachiatum the slowest germ tube growth rate, the growth rate of the latter being significantly different from that of its control ($F = 12.4$ $P < 0.05$).

The most interesting aspect of these results is that, for any one isolate, soil fungistasis did not act equally on the four different germination parameters measured. There was, however, no apparent consistency for any one parameter to be more affected than another when the results for the different isolates are compared. There were, however, a number of relationships between the effects of fungistasis on the different parameters. The percentage reduction in the

TABLE 1.4 Effect of Soil Fungistasis on the Germ Tube Growth Rate of the different Trichoderma species groups.

	Germ tube growth rate (as regression coefficient)			
	Control	Experiment	% Reduction	
T. hamatum				
(15)	0.1222	0.0673	44.9	*
(22)	0.0970	0.0705	27.3	NS
T. harzianum				
(1)	0.0938	0.0804	14.3	NS
(129)	0.0433	0.0140	67.7	**
(20)	0.1133	0.0792	30.1	NS
T. koningii	0.0036	0.0102	0	NS
T. longibrachiatum	0.0224	0.0076	66.1	*
T. pseudokoningii	0.0742	0.0294	60.4	*
T. saturnispora	0.0581	0.0467	19.6	NS
T. viride				
(2)	0.7101	0.7281	0	NS
(14)	0.0828	0.0610	26.3	NS
(28)	0.0711	0.0964	0	NS
(48)	0.1093	0.0642	41.3	NS
(49)	0.1069	0.0569	46.8	*
(50)	0.1338	0.1092	18.4	NS
(51)	0.0849	0.0669	21.2	NS
(52)	0.0612	0.0317	48.2	NS
(53)	0.0718	0.0166	76.9	*
(54)	0.0463	0.0501	0	NS

* Significant P < 0.05)
** " P < 0.01) Variance (F) ratio (see text)
NS Not significant P > 0.05)

latent period was directly correlated with both the percentage reduction in the final percentage germination and the percentage reduction in germ tube growth rate ($r = 0.8882$ $P < 0.001$ and $r = 0.5430$ $P < 0.05$ respectively). Dix (1972) found a direct correlation between the percentage reduction in the latent period and the percentage reduction in the final percentage germination for 11 species of Penicillium. But he found no statistically significant correlation between percentage reduction in the latent period and percentage reduction in germ tube growth rate.

There was no significant correlation between the percentage reduction in germ tube growth rate and the percentage reduction in the final percentage germination which is at variance with the results of Dix (1972) and Hsu & Lockwood (1971) who both found inverse relationships between these two parameters.

The latent period of germination in the control was directly correlated with the percentage reduction in the final percentage germination ($r = 0.9055$ $P < 0.001$) although it was not correlated with any of the other parameters. A similar result was obtained by Steiner & Lockwood (1969), this then is further evidence in favour of the theory that the germination time of a fungus is related to its sensitivity to fungistasis. Analysis of the results of Dix (1972) show that there was a direct correlation between the latent period in the control and both percentage reduction in final percentage germination and the percentage reduction in germ tube growth rate ($r = 0.8244$ $P < 0.001$ and $r = 0.7613$ $P < 0.05$ respectively).

From these experiments fungistasis appears to act principally by delaying the time of germination and reducing the potential germination ability of the spore population.

The effect of time on the sensitivity of the spore to fungistasis can be interpreted by either of the two current hypotheses for the fungistatic mechanism. The length of time in the soil that a spore spends before it germinates determines its sensitivity to fungistasis; this concommitant increase in sensitivity with time could be attributed to increased time being available for inhibitory compounds to pass into the spore or to allow the development, on the spore surface, bacteria producing quantities of antibiotics or for a nutrient debt to be set up by leakage of nutrients from the spore. In only a few cases are the actual rates of either germination or germ tube growth significantly affected suggesting that perhaps the fungistatic effect may be the result of an exertion of exogenous dormancy on the spore, by the environment, which the spore, in order to germinate, has to overcome.

Soil fungistasis has an inhibitory effect upon the germination of spores in the soil; the sensitivity of the spores, as reflected in the response of the four germination parameters, to fungistasis varies within the same isolate, between isolates of the same species group and also between species groups. When considering the ecological implications of soil fungistasis it is important to maintain cognizance of the fact that although the fungus spore population will have parameters of germination which will differ in their response to fungistasis it is the overall growth of the organism in soil that is of the utmost importance in consideration of the survival and propagation of that fungus.

The ability of a spore population in the soil to

germinate, grow and colonize a substrate has been epitomized by Garrett (1970) in the statement "the share of a substrate obtained by any particular fungal species will be determined partly by its intrinsic competitive saprophytic ability and partly by the balance between its inoculum potential and that of competing species".

The present results can be interpreted in accordance with this statement. The effect of the antagonistic environment (soil fungistasis) on the growth of standard populations of spores on a defined substrate (peptone agar) in contact with the soil surface has been measured in these experiments. Three of the attributes (i.e. germination and growth rate, enzyme production and tolerance of antagonism) that make for a high competitive saprophytic ability have therefore been measured; antibiotic production will be considered in a later chapter.

The interaction of these factors may be expressed as an overall growth index which can be calculated by taking the product of the percentage germination at any time and the mean germ tube length of that population at the same time. This index I have termed the "Theoretical Colonization Index" which can be considered to reflect the ability of that spore population to grow and colonize a substrate in soil.

The theoretical colonization index makes it possible for predictions to be made of the ability of different spore populations to germinate, in the presence of a simple nutrient source and competing organisms present in the soil, and colonize that substrate. Attempts to measure a similar aspect of colonization have, in the past, been restricted to the consideration of the effect of tolerance of hyphal

growth to antibiotics (Rao, 1959), growth on agar plates staled after inoculation with soil (Wastie, 1961) or to fungistasis (Hsu & Lockwood, 1971).

The theoretical colonization indices after 24 hours incubation are shown in Table 1.5. For comparative purposes the theoretical colonization indices have also been expressed as percentage reduction compared with the overall growth of that population in the control.

There is variation in both sensitivities (percentage reduction) and indices between the different isolates and between species groups. Individually, T. viride (53) with an index of only 45.2 shows the least ability to colonize the agar block where T. harzianum (1), with an index of 4092.4 has the highest overall growth under antagonistic conditions. Comparing the different species groups T. koningii is the most sensitive to fungistasis with 81.5% reduction in the theoretical colonization index, it has also the smallest mean theoretical colonization index of the species groups (201.1). The T. hamatum species group is the least sensitive (55.1% reduction) but the T. harzianum species group has the highest mean theoretical colonization index (1823.7).

The percentage reduction in the theoretical colonization index is related to all of the other germination parameters measured. There is a direct correlation between the percentage reduction in the theoretical colonization index and percentage reduction in the final percentage germination, percentage reduction in the germination rate, percentage reduction in latent period, the latent period in the control

TABLE 1.5 Effect of Soil Fungistasis on the Theoretical Colonization Index of the different Trichoderma species groups.

	Theoretical Colonization Index		
	Control	Experiment	% Reduction
T. hamatum			
(15)	6011.3	524.8	91.3
(22)	6278.4	1626.5	74.1
T. harzianum			
(1)	8184.6	4092.4	50.0
(129)	1030.1	123.6	88.0
(20)	4723.1	1255.1	73.4
T. koningii	1089.2	201.1	81.5
T. longibrachiatum	765.0	250.8	67.2
T. pseudokoningii	9498.6	422.8	95.6
T. saturnispora	2775.0	1175.4	57.6
T. viride			
(2)	540.3	230.6	57.3
(14)	5890.6	367.2	93.8
(28)	2632.8	215.4	91.8
(48)	8125.7	1686.6	79.2
(49)	6442.6	1515.6	76.5
(50)	2862.6	1151.0	59.8
(51)	5021.8	471.9	90.6
(52)	5839.6	273.4	95.3
(53)	4495.2	45.2	99.0
(54)	4983.8	2188.3	50.0

and percentage reduction in the germ tube growth rate ($r = 0.7759$ $P < 0.001$; $r = 0.5168$ $P < 0.05$; $r = 0.6586$ $P < 0.01$; $r = 0.5157$ $P < 0.05$; and $r = 0.4774$ $P < 0.05$ respectively). It is possible to ascertain the relative importance of each of the parameters in determining the theoretical colonization index of the fungus by placing the correlation coefficients in order of magnitude.

The percentage reduction in the final percentage germination (with a correlation coefficient of 0.7759) is the largest component of the percentage reduction in theoretical colonization index after 24 hours incubation. The percentage reduction in the latent period also has a profound effect ($r = 0.6586$); this is to be expected as there was a strong correlation between the percentage reduction in the latent period and percentage reduction in the final percentage germination. The percentage reduction in the germ tube growth rate is the smallest component. It is possible therefore that the percentage reduction of the final percentage germination by fungistasis may, in some cases, be the main deciding factor in the outcome of competition for a substrate in the soil. That the percentage reduction in the final percentage germination is the major component of the percentage reduction in the theoretical colonization index indicates, and is in support of Garrett's (1970) belief, that the outcome of the struggle for competitive success may be decided early.

Dix (1972) posed the question "wherein within the field of fungal ecology does the signifance of soil fungistasis

lie?" and he discussed the effects of fungistasis on the various parameters of germination that he had measured and tried to assess the ecological significance of depression of any one parameter more than another in competitive situations. It can be concluded from his discussion that the significance of fungistasis will vary in accordance with the particular environmental conditions under investigation. In the final analysis, however, it is not the effect of fungistasis on any one parameter that is of prime importance (although it is still important) but the effect of soil fungistasis on the overall growth of the fungus. This, as has been shown, is easily assessed by use of the theoretical colonization index. By using this index (and also the other parameters of germination) one is able to build up a complete picture of the effect of fungistasis on the dynamics of the colonization ability of a spore population, for not only can the effect on the overall growth be measured but also the degree to which fungistasis affects the different parameters and the relative importance of these in the overall growth and competitive ability of the spore population can be assessed.

CHAPTER TWO

THE INFLUENCE OF C-SOURCE IN THE SPORULATION MEDIUM ON THE SENSITIVITY OF TRICHODERMA VIRIDE (48) SPORES TO FUNGISTASIS

A characteristic feature of soil fungistasis is its annulment by nutrients, but there are conflicting reports concerning the effectiveness of specific nutrients.

Dobbs & Hinson (1953) and Dobbs *et al* (1960) showed that the addition of glucose to soil reversed fungistasis but other workers have found glucose to be ineffective (Chinn & Ledingham, 1957; Jackson, 1958). Jackson (1960) compared the effectiveness of different sugars in agar discs and found the order of effectiveness in reducing fungistasis to be mono- di- tri-saccharides whereas Ematty & Green (1967) using conidia of *Trichoderma viride* found no apparent differences in the effectiveness of a similar range of sugars.

Ko & Lockwood (1967) postulated that the failure of spores to germinate in soil is a result of nutrient depletion of either the spore or the environment in the immediate vicinity of the spore. They found that for 18 fungi tested, the ability of spores to germinate on soil was correlated with ability to germinate in distilled water i.e. in the absence of exogenous nutrients. But four of the fungi that they tested which were nutrient-independent did not germinate on soil. They demonstrated that this inhibition was due to nutrient loss when the spores were leached, supported on millipore filters, with glass distilled water or 0.01M phosphate buffer and showing that leached spores did

not germinate but when placed in a nutrient solution did germinate. Fungistasis, it was concluded, was a result of nutrient depletion caused by leaching in the soil. They claim, that the validity of this leaching system as a model for fungistasis is indicated by the correlation between the pattern of spore germination occurring in the leaching system and on natural soil. But, as has been pointed out by Watson & Ford (1972), the fact that two mechanisms give the same result does not logically show that they are the same. This is borne out to some extent by the failure of this leaching system to duplicate the effect of fungistasis on conidia of Glomerella cingulata one of the four nutrient independent fungi. That nutrients are leached from nutrient independent spores has been demonstrated by Knight (1970) and Bristow & Lockwood (1972). Knight (1970) found that 11.4% of the endogenous reserves of Cochliobolus sativus were leached from ^{14}C-labelled spores over a 32-hour period but this loss of reserves had no injurious effect upon the subsequent germination performance in soil. Loss of ^{14}C-labelled material was greater during dialysis than during incubation over a soil suspension, although the latter treatment resulted in a stronger inhibition of germination. It was therefore suggested that in addition to the nutrient depletion effect inhibitory compounds may also be involved in soil fungistasis.

The effect of fungistasis on nutrient dependent spores was investigated by Yoder & Lockwood (1973). Using conidia of Penicillium frequentans and a combination of natural soil and leaching conditions, with ^{14}C-glucose labelled spores they found that the germination process

consisted of two stages; (1) an irreversible water dependent, nutrient independent initiation of germination stage and (2) production of germ tubes which was dependent upon exogenous nutrient sources. The finding that initiation of germination is water dependent and nutrient independent is in contrast with the results of other workers (Cox & Sisler, 1951; Ekundayo & Carlile, 1964; Marchant & White, 1966; Martín & Nicolás, 1970; Yanagita, 1957) who found that all stages of germination had a requirement for exogenous nutrients. Yoder & Lockwood (1973) found that about half of the labelled materials lost from the ^{14}C-glucose labelled spores occurred as CO_2 during respiration and half in exudates through the spore walls. The exudates were not identified but presumed to be amino acids and carbon compounds similar to those found by other workers (Daly *et al*, 1967; Jones & Snow, 1965; Ko & Lockwood, 1967; Powel & Strange, 1953). They considered that the sensitivity of *Penicillium frequentans* conidia to fungistasis could be explained solely in terms of nutrient deprivation. It is possible therefore that the nutrient status of both spore and substrate may affect the sensitivity of that spore population to fungistasis.

The following investigation was carried out to examine the effect that the carbon source in the sporulation medium has on the subsequent response of *Trichoderma viride* (48) to soil fungistasis. Cultures of *Trichoderma viride* (48) were grown on media with different carbon sources, after incubation the spores were harvested and tested for sensitivity to fungistasis as described in Materials and Methods.

The sporulation media consisted of modified Weindlings medium without glucose (see Appendix 3). The different carbon sources, in the form of simple sugars (glucose, sucrose, fructose, lactose, xylose and mannitol) were sterilized by Millipore filtration (0.22μ pore size) and added to autoclaved modified Weindlings medium at the rate of 1000ppm C l^{-1}.

Five replicate Petri dishes were inoculated using 3mm diameter blocks cut from the edge of an actively growing culture of T. viride 48 growing on PDA with a No.3 corkborer. The blocks were placed singly onto the centre of a Petri dish containing 20 cm^3 of the relevant medium and incubated for 21 days at 25°C.

The same parameters of germination (final percentage germination, rate of germination, latent period and germ tube growth rate) were measured by the methods described in Chapter One.

The results for the effect of soil fungistasis on the final percentage germination are given in Table 2.1. There was

TABLE 2.1 Effect of Soil Fungistasis on the Final Percentage Germination of T. viride (48) spores produced on different sporulation media.

	Final Percentage Germination			
	Control	Experiment	% Reduction	
Glucose	75.4	58.0	23.7	***
Sucrose	90.0	70.6	21.6	***
Fructose	78.8	76.6	2.8	NS
Lactose	70.4	66.8	5.1	NS
Xylose	79.3	57.9	27.0	***
Mannitol	80.1	57.1	29.9	***

*** Significant $P < 0.001$) Student's 't' test
NS Not significant $P > 0.05$) (see text)

a relatively small variation in the percentage reduction in the final percentage germination of the different spore populations. When subjected to Student's 't' test the values for the final percentage germination for the control and experiment for all the spore populations were all (except fructose and lactose) significantly depressed by fungistasis; the mannitol spore population was the most sensitive (29.9% reduction).

The germination rates of the different spore populations, as regression coefficients, are given in Table 2.2.

TABLE 2.2 Effect of Soil Fungistasis on the Germination Rate of T. viride (48) spores produced on different sporulation media.

Sporulation medium C-source	Germination Rate (as regression coefficient)			
	Control	Experiment	% Reduction	
Glucose	-0.2558	-0.2509	1.9	NS
Sucrose	-0.2912	-0.2333	15.8	NS
Fructose	-0.3050	-0.3071	0	NS
Lactose	-0.3091	-0.2691	12.9	NS
Xylose	-0.2590	-0.1832	29.3	*
Mannitol	-0.2628	-0.2065	21.4	NS

* Significant $P < 0.05$) Variance (F) ratio
NS Not significant $P > 0.05$) (see text)

The variance (F) ratio was again used to test for significant differences between the regression lines. The full equations for the regression lines are given in Appendix 8. The germination rate of all the spore populations (except fructose)

was reduced, with respect to the control, by soil fungistasis but this reduction was only significant for the xylose spore population (29.3% reduction $F = 6.38$, $P < 0.05$).

The values for the latent period are given in Table 2.3. In only three cases (fructose, xylose and mannitol)

TABLE 2.3 Effect of Soil Fungistasis on the Latent Period of Germination of T. viride (48) spores produced on different sporulation media.

Sporulation medium C-source	Latent period (hours) Control	Experiment	% Reduction
Glucose	13.1	12.8	-
Sucrose	11.0	10.5	-
Fructose	12.0	12.1	0.84
Lactose	12.1	11.9	-
Xylose	11.1	11.7	5.1
Mannitol	10.8	11.4	5.3

was there any reduction in the latent period in response to fungistasis, and even then the highest percentage reduction was only 5.3% for the mannitol produced spore population. It is probable that such a low percentage reduction is not significant although by the nature of its calculation it is not possible to carry out any statistical test on it.

The germ tube growth rates, as regression coefficients, for the different spore populations are given in Table 2.4 and the full equations for the regression lines in Appendix 9. The variance (F) ratio was again used to test for significant differences between the regression lines.

TABLE 2.4 Effect of Soil Fungistasis on the Germ Tube Growth Rate of T. viride (48) spores produced on different sporulation media.

	Germ tube growth rate (as regression coefficient)			
	Control	Experiment	% Reduction	
Glucose	0.1309	0.1087	17.0	NS
Sucrose	0.1333	0.1265	5.1	NS
Fructose	0.1293	0.1379	0	NS
Lactose	0.1705	0.1413	17.1	NS
Xylose	0.1524	0.0946	37.9	*
Mannitol	0.1477	0.0966	34.6	**

* Significant P < 0.05
** " P < 0.01
NS Not significant P > 0.05
) Variance (F) ratio (see text)

The germ tube growth rate was only significantly depressed by fungistasis in those populations produced on mannitol and xylose (34.6% reduction F = 18.04, P < 0.01 and 37.9% reduction F = 10.96, P < 0.05 respectively).

There were no significant relationships between any of the parameters with respect to sensitivity to soil fungistasis.

Variation of the C-source present in the sporulation medium does not have any significant effect in determining the sensitivity of the resultant spores to fungistasis. If, however, one considers the value of the theoretical colonization index of the different populations, the values of which are given in Table 2.5, it is evident that in this respect the nature of the sporulation medium is important. The glucose population has the least competitive ability on

TABLE 2.4 Effect of Soil Fungistasis on the Germ Tube Growth Rate of T. viride (48) spores produced on different sporulation media.

	Germ tube growth rate (as regression coefficient)			
	Control	Experiment	% Reduction	
Glucose	0.1309	0.1087	17.0	NS
Sucrose	0.1333	0.1265	5.1	NS
Fructose	0.1293	0.1379	0	NS
Lactose	0.1705	0.1413	17.1	NS
Xylose	0.1524	0.0946	37.9	*
Mannitol	0.1477	0.0966	34.6	**

* Significant $P < 0.05$
** " $P < 0.01$
NS Not significant $P > 0.05$
) Variance (F) ratio (see text)

The germ tube growth rate was only significantly depressed by fungistasis in those populations produced on mannitol and xylose (34.6% reduction $F = 18.04$, $P < 0.01$ and 37.9% reduction $F = 10.96$, $P < 0.05$ respectively).

There were no significant relationships between any of the parameters with respect to sensitivity to soil fungistasis.

Variation of the C-source present in the sporulation medium does not have any significant effect in determining the sensitivity of the resultant spores to fungistasis. If, however, one considers the value of the theoretical colonization index of the different populations, the values of which are given in Table 2.5, it is evident that in this respect the nature of the sporulation medium is important. The glucose population has the least competitive ability on

the soil and the sucrose produced population, although it is relatively sensitive to fungistasis (62.0% reduction), has the highest competitive ability; 6X greater than that of the glucose produced spores.

TABLE 2.5 Effect of Fungistasis on the Theoretical Colonization Index of T. viride (48) spores produced on different sporulation media.

Sporulation medium C-source	Theoretical Colonization Index at 18 hrs.		
	Control	Experiment	% Reduction
Glucose	1382.8	388.8	71.88
Sucrose	6167.7	2346.4	61.96
Fructose	3033.0	2048.6	32.46
Lactose	3732.4	2150.3	42.39
Xylose	4342.0	1217.6	71.96
Mannitol	4357.0	1067.8	75.49

How quickly the population grows in soil may be estimated by the rate of increase of the theoretical colonization index with time. This can be determined by taking the regression coefficient obtained from a plot of log theoretical colonization index against time. The effect of fungistasis on the rate of increase in the theoretical colonization index is given in Table 2.6 and the full regression equations in Appendix 10. The variance (F) ratio was again used to test for significant differences between the regression lines. Fungistasis significantly depressed the rate of increase in theoretical colonization index of

three of the spore populations; mannitol, xylose and lactose (40.9%, 40.8%, 25.1% reduction respectively) whereas the fructose population appears to be insensitive to fungistasis.

TABLE 2.6 Effect of Fungistasis on rate of increase of Theoretical Colonization Index of T. viride (48) spores produced on different sporulation media.

Sporulation medium C-source	Rate of increase of Theoretical Colonization Index (as regression coefficient)			
	Control	Experiment	% Reduction	
Glucose	0.2537	0.2300	9.34	NS
Sucrose	0.2378	0.1753	26.28	NS
Fructose	0.2775	0.2886	0	NS
Lactose	0.3540	0.2652	25.08	*
Xylose	0.2354	0.1394	40.78	***
Mannitol	0.2524	0.1492	40.89	*

* Significant $P < 0.05$
*** " $P < 0.001$
NS Not significant $P > 0.05$
) Variance (F) ratio (see text)

There was a direct correlation between the percentage reduction in the rate of increase in theoretical colonization index and percentage reduction in germ tube growth rate ($r = 0.8768$, $P < 0.01$). This indicates that the sensitivity of the germ tube growth rate to fungistasis may be a crucial factor in the continued growth of the fungus in soil. Although the fact that there is a direct correlation between the percentage reduction in theoretical colonization index and the percentage reduction in the final percentage

germination ($r = 0.9761$, $P < 0.001$) does indicate that the proportion of the spore population to germinate is also important.

Yoder & Lockwood (1973) found in their experiment in which they leached ^{14}C-glucose labelled spores of Penicillium frequentans in a model system that there was leakage of substances away from the spores. This leakage, they thought, may be responsible for some of the fungistatic effect. Therefore it was decided to investigate whether leakage of electrolytes from spores produced on the different sporulation media was related to their sensitivity to fungistasis on any of the germination parameters.

Conductivity measurements of electrolytes released from the spores were made using the technique described in the Materials and Methods.

The increase in conductivity with time for the different spore populations is shown in Fig.2.1 and the figures for the percentage increase in conductivity to equilibrium given in Table 2.7. There was a wide variation in the increase in conductivity of the de-ionised water with the spores in suspension, the fructose produced spores reached equilibrium within 30 minutes the others taking about 50 minutes. This gives an indication of the time taken for the spore membranes to adjust to the new environment. A wide variation was also recorded for the percentage increase in conductivity at equilibrium which is a measure of the amount of electrolytes lost; the fructose produced spores only increasing the conductivity by 9.8% whereas the xylose produced spores increased it by 45.0%.

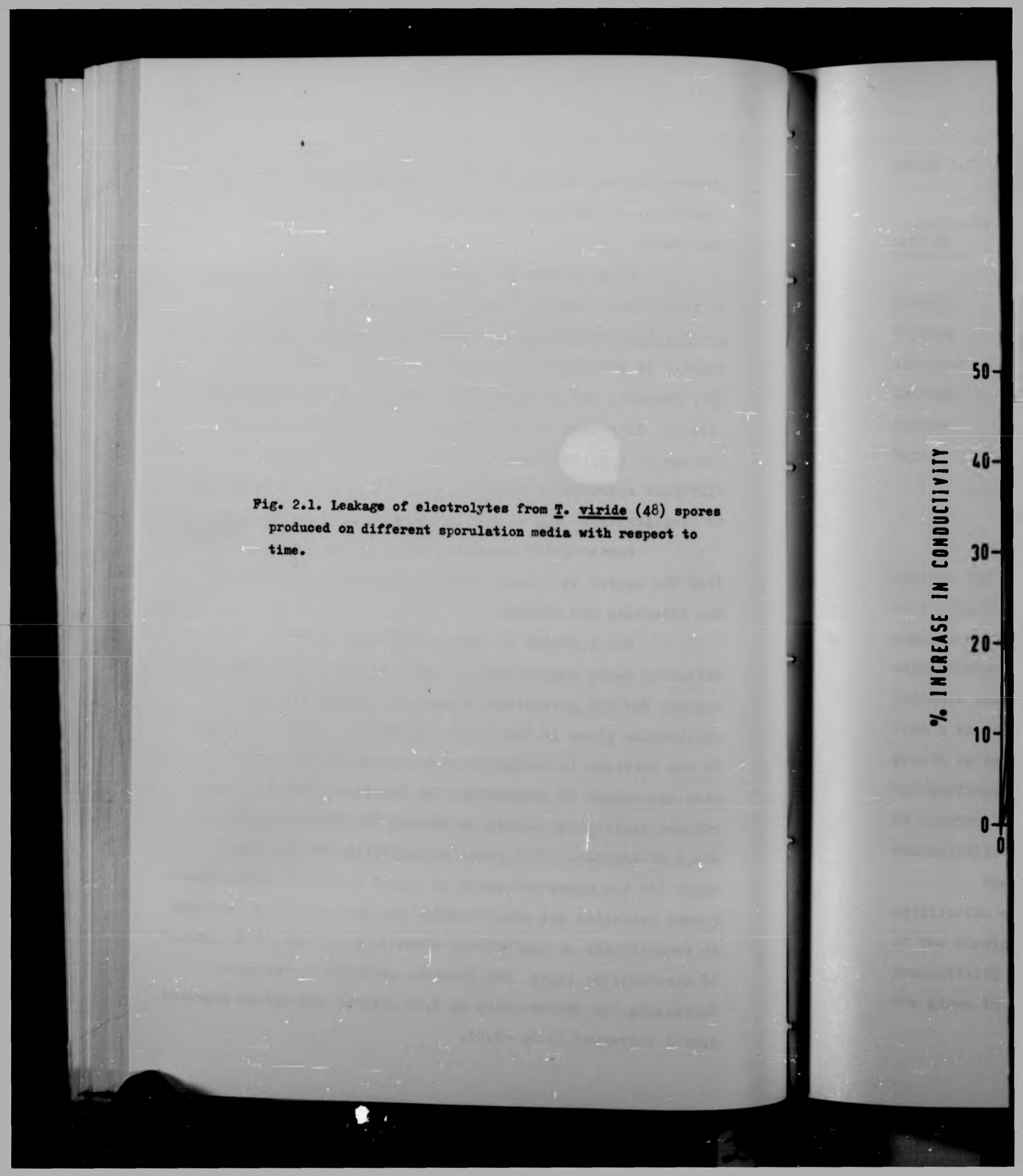


Fig. 2.1. Leakage of electrolytes from T. viride (48) spores produced on different sporulation media with respect to time.

ride (48) spores
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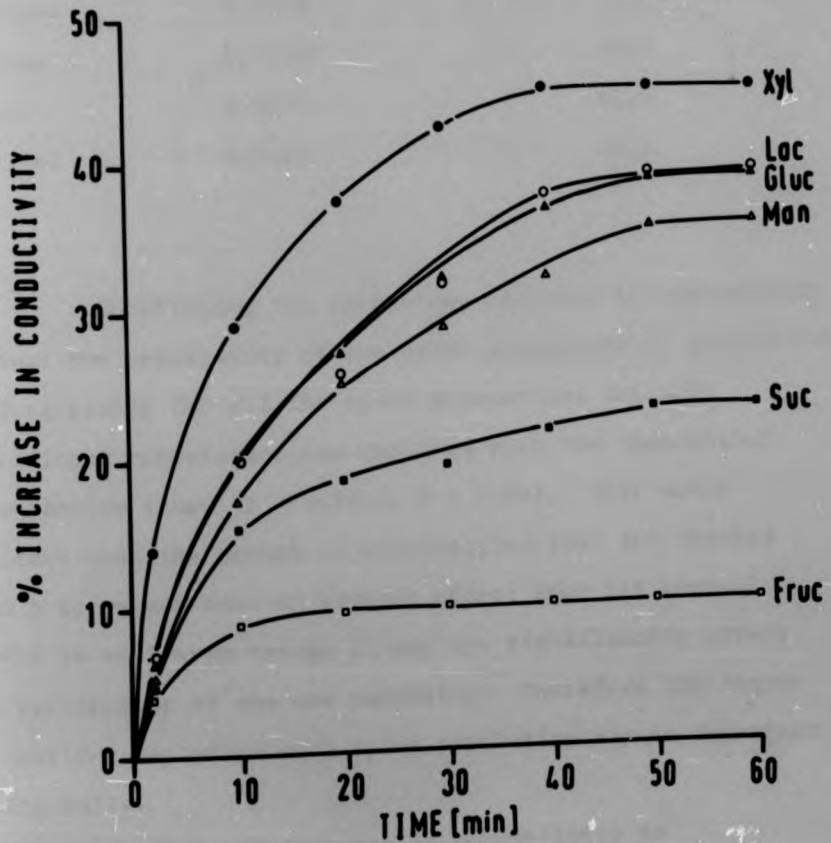

TABLE 2.7 Rates of increase in Conductivity of suspension, in de-ionised water, of T. viride (48) spores produced on different sporulation media.

Sporulation medium C-source	Rates of increase in conductivity to equilibrium (regression coefficient)	% increase in conductivity to equilibrium
Glucose	0.6695	38.6
Sucrose	0.6390	23.0
Fructose	0.4128	9.8
Lactose	0.7338	38.8
Xylose	0.3721	45.0
Mannitol	0.7653	35.5

Correlating the percentage increase in conductivity against the sensitivity of the other parameters of germination to fungistasis for all the spore populations the only significant correlation was obtained with the theoretical colonization index ($r = 0.7813$, $P < 0.05$). This would indicate that the amount of electrolytes that are leached from a spore may have an adverse effect upon its overall growth in soil even though it may not significantly affect the performance of any one parameter. Therefore the degree of nutrient depletion of a spore population may be important ecologically.

The rate of increase in conductivity to equilibrium was calculated as the regression coefficient of the straight line produced by plotting the log increase in conductivity against log time. The regression coefficients are given in Table 2.7 and the full regression equations in

Appendix II. There was no significant correlation between the rate of increase in conductivity to equilibrium and the percentage increase in conductivity at equilibrium nor with the sensitivity of any of the germination parameters measured. This would indicate that it is the amount of leakage that occurs that may influence the sensitivity of the spores to fungistasis and not the rate at which leakage occurs.

CHAPTER THREE

PRODUCTION OF NON-VOLATILE ANTIBIOTICS

The first suggestion that a Trichoderma might produce substances inhibitory to other fungi was the observation by Falck (1931) that wood infested by Trichoderma lignorum was resistant to decay by Coniophora cerebella and Merulius lacrymans. Later Weindling (1934) reported that culture filtrates of T. lignorum were toxic to Rhizoctonia solani and other fungi even at high dilution. This subsequently led to an intensive study into the nature of these metabolites.

The 'lethal principle' isolated in crystalline form by Weindling & Emerson (1936) was thought to be produced by a strain of T. lignorum. But in 1937 Weindling reported that the fungus was in fact Gliocladium fimbriatum and consequently the antibiotic named gliotoxin (Weindling, 1941). Subsequently Brian (1944), Brian & Hemming (1945) and Brian & McGowan (1945) acting on Bisby's paper (1939) and advice, described the production of gliotoxin and viridin by isolates of what they called Trichoderma viride, but it appears that actually they were working with Gliocladium virens Miller, Giddens & Foster (Webster & Lomas, 1964). This information therefore casts an element of doubt on the identity of the 'Trichoderma' isolates used by other workers. However, as Webster & Lomas (1964) pointed out, G. virens is not common in soil whereas Trichoderma is relatively common (especially in acid soils). It is therefore probable that most reports do, in fact, relate to Trichoderma.

Jeffreys, et al (1953) found that some Trichoderma isolates from an acid heath soil (therefore likely Trichoderma)

did in fact have antibiotic activity against Botrytis allii conidial germination. Rishbeth (1950) found that the culture filtrates of 10 East Anglian forest soil Trichoderma isolates all inhibited the germination of Fomes annosus conidia. Gibbs (1967), using an agar-layer technique found that some isolates of Trichoderma spp, identified according to Rifai's classification, showed antibiotic activity against Fomes annosus. From these results it seems reasonable to suppose that some, though not all, Trichoderma isolates produce substances (antibiotics) active against other fungi under suitable conditions but that gliotoxin and viridin have not been definitely identified as being produced by Trichoderma species.

The production and identification of antibiotics produced by Trichoderma isolates has been and still is under investigation.

Antibiotics which have been identified as being produced by true Trichoderma isolates include trichodermin, a sesquiterpene (Adams & Hanson, 1972; Godtfredsen & Vangedal, 1965), suzukacillin (Ooka et al 1966, 1972) and alamethicine (Meyer & Reusser, 1967; Reusser, 1967) both peptide antibiotics with antibacterial and antifungal activity; and an unsaturated monobasic acid with similar properties, antibiotic U-21,963 'Dermadine' (Pyke & Dietz, 1966; Meyer, 1966).

An extensive survey of the production of non-volatile antibiotics by isolates from Rifai's different species groups was undertaken by Dennis (1970; Dennis & Webster, 1971a). Dennis elected to use the agar plate method devised by

Heatley (1947) for antibiotic assay with bacteria and adapted by Gibbs (1967) for use with filamentous fungi. Dennis considered that this method was more comparable with the natural environment of a colonizing fungus and also provided a more convenient quantitative technique than did conidial germination assays.

Dennis found that many isolates produced non-volatile antibiotics active against a wide range of fungi. The ability to produce such antibiotics varied between isolates of the same species group as well as between isolates of different species groups. The susceptibility of the test fungi used to these antibiotics varied widely confirming the work of Gibbs (1967) and Mughogho (1968). Dennis also investigated the chemical nature of the culture filtrates of a selection of isolates and it was found that although gliotoxin and viridin were not produced other antibiotics, including trichodermin and the peptide antibiotics alamethicine and suzukacillin were produced. Production of antibiotic U-21,963 was not tested for as a pure sample was unobtainable due to its instability.

An unstable mycelial localized antifungal antibiotic has been reported from an isolate of Trichoderma koningii (Dayal, et al, 1971). A cyclic peptide mycotoxin, Trichotoxin A, has been isolated from the mycelium of Trichoderma viride isolated from corn infected with southern leaf blight (Hou, et al, 1972) and a total of 11 metabolites have been isolated and characterized from a soil isolate of Trichoderma pseudokoningii by Pakistani workers (Kamal, et al, 1971).

The present study was undertaken to investigate the nature of the antibiotics produced by the 20 Trichoderma isolates and also to assess the activity of Trichoderma culture filtrates against 15 fungi, isolated mainly from soil, in relation to possible antagonistic activity of Trichoderma during the colonization of substrates in competition with other fungi.

Culture filtrates of each of the 20 Trichoderma isolates were produced as described in the Materials and Methods. The initial chemical investigation of the culture filtrates followed the methods described by Dennis (1970; Dennis & Webster, 1971a). Chloroform and ethanolic extracts of the culture filtrates were prepared (see Materials and Methods).

A chloroform extract was also prepared of uninoculated Weindlings medium; virtually no chloroform-soluble substances were found to be present, indicating that the chloroform-soluble substances extracted from the culture filtrates were fungal metabolites. The dried chloroform extracts of the culture filtrates were all resinous in nature.

The dried chloroform extract was redissolved in 1 cm^3 chloroform and tested for biological activity. The assay procedure was essentially the same as that described in the Materials and Methods with the exception that a drop of the chloroform extract was placed on the agar block and the excess chloroform allowed to evaporate off. Gliocladium roseum (1) conidia were used in all the assay procedures and incubated for 18h. The results are given in Table 3.1. The chloroform extracts of half of the isolates were completely inhibitory, the others showed variation in their activity toward the two germination parameters.

TABLE 3.1 Biological Activity of Chloroform and Ethanol Extracts of Culture Filtrates of the different *Trichoderma* species groups against *Gliocladium roseum* (1) conidia (as % reduction).

	Chloroform Extract		Ethanol Extract	
	Percentage Germination	Germ tube length	Percentage Germination	Germ tube length
T. hamatum				
(15)	61.8 ***	41.9 ***	48.6 ***	30.3***
(22)	100	100	48.2 ***	36.3 ***
T. harzianum				
(1)	100	100	56.1 ***	38.8 ***
(129)	86.0 ***	42.9 ***	45.3 **	40.9 ***
(20)	100	100	31.6 ***	33.2 ***
T. koningii	100	100	50.9 ***	29.5 ***
T. longibrachiatum	45.6 ***	40.2 ***	39.5 ***	54.6 ***
T. polysporum	69.0 ***	56.1 ***	9.2 *	38.5 ***
T. pseudokoningii	100	100	35.1 ***	61.8 ***
T. saturnispora	100	100	100	100
T. viride				
(2)	36.9 **	44.7 ***	8.6 **	35.9 ***
(14)	44.9 ***	56.0 ***	22.2 ***	45.1 ***
(28)	36.0 **	47.2 ***	47.4 ***	54.7 ***
(48)	100	100	66.0 ***	59.0 ***
(49)	100	100	100	100
(50)	100	100	29.9 **	44.8 ***
(51)	100	100	28.8 ***	56.2 ***
(52)	47.7 **	50.9 ***	28.7 **	52.4 ***
(53)	26.2 **	41.9 ***	15.9 *	45.2 ***
(54)	22.4 *	50.8 ***	20.2 ***	52.0 ***

* Significant $P < 0.05$)
** " $P < 0.01$) Student's 't' test
*** " $P < 0.001$) (see text)

Chloroform Control 60.9% Germination, 7.4 germ tube length

Ethanol Control 62.2% Germination, 8.6 germ tube length

Each of the extracts was tested to see if any of the known Trichoderma antibiotics were present.

A 10µl aliquot of each extract was spotted onto a Camag thin layer chromatography (T.L.C.) plate with a 'microcap' micropipette, pure samples of gliotoxin and viridin were run as markers. The plates were run in a chloroform:acetone (95:5, v/v) solvent system for 2-3h and, after allowing the solvents to evaporate off, developed by spraying with silver nitrate reagent or phloroglucinol-hydrochloric acid reagent. The former was used for the detection of gliotoxin, which gives a brownish-black spot and the latter gives an orange-red colour with viridin. T.L.C. plates were also run in a second solvent system, ether:ethyl acetate (9:1, v/v), for 20 min and the spots located by absorbance under a UV lamp (254nm) and by halogenation with iodine, the spots were developed by spraying with 1% starch solution to yield a blue colour (Waldi, 1965). In this solvent system viridin and gliotoxin had R_F values of 33 and 37.5 respectively.

A chloroform extract of Gliocladium virens (211), an isolate known to produce both gliotoxin and viridin, was prepared and also run in both solvent systems.

Using this method it was possible to detect very small quantities (5µg) of both gliotoxin and viridin. It was not possible, however, to detect either of the two antibiotics in any of the chloroform extracts from any of the Trichoderma isolates. Both antibiotics were detected in the chloroform extract of the G. virens (211) culture filtrate.

Neither gliotoxin nor viridin was detected in any of the Trichoderma culture filtrates tested by Dennis (1970; Dennis & Webster, 1971a) but gliotoxin was reported to be present in chloroform soluble extracts of Trichoderma culture filtrates tested by Khasanov (1962).

Two other chloroform-soluble antibiotics, Trichodermin and antibiotic U-21,963 'Dermadine' have been reported to be produced by Trichoderma isolates which are considered to be true Trichoderma isolates (Dennis, 1970; Dennis & Webster, 1971a).

A pure sample of trichodermin was chromatogrammed on T.L.C. plates with the chloroform soluble extracts of the Trichoderma isolates. Three solvent systems were used; chloroform:acetone (95:5, v/v), ethyl acetate:cyclohexane (50:50, v/v) and ether:ethyl acetate (9:1, v/v). Trichodermin was detected by spraying with concentrated sulphuric acid and then heating the plates to 100°C for 10-15 min. It was by this method possible to detect very small quantities (1µg) of trichodermin.

Trichodermin was not present in any of the Trichoderma culture filtrates. No tests were carried out for the production of U-21,963 as it was not possible to obtain a sample due to its instability.

From this survey it is clear that the activity of the chloroform-soluble extracts is not due to the presence of any of the known Trichoderma chloroform-soluble antibiotics. A similar conclusion was reached by Dennis (1970; Dennis & Webster, 1971a) after a similar survey.

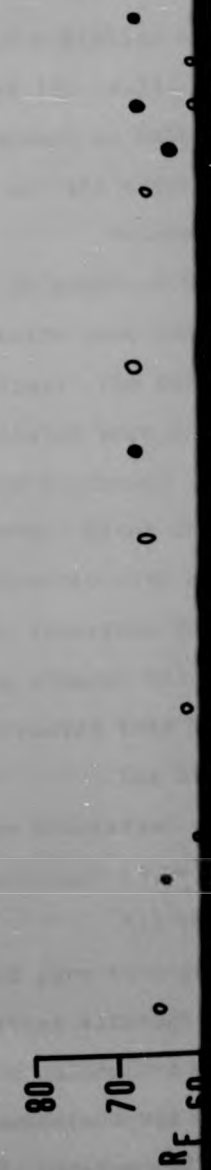

Fig. 3.1. Chromatograms of chloroform extracts of Trichoderma culture filtrates run on T.L.C. plates in the ether: ethyl acetate solvent system (see text).

- O UV absorbance
- ● Iodine

...cts of Trichoderma
... the ether:

Using the ether:ethyl acetate solvent system it was possible to detect several chloroform-soluble compounds present in the culture filtrates. The compounds present in the different extracts varied but in some cases compounds with a similar R_F value were present in different extracts (see fig. 3.1). It was not possible to pin-point any one compound as being responsible for the biological activity of all the extracts.

Polypeptide antibiotics have been reported to be produced by Trichoderma isolates and these earlier results were confirmed by Dennis (1970; Dennis & Webster, 1971a). The culture filtrates of the different Trichoderma isolates were examined to ascertain if these antibiotics were produced. The cultural conditions were those described above. After the concentrated culture filtrates had been extracted with chloroform a white precipitate formed at the interface between the chloroform and aqueous layers, the ethanol soluble component of this precipitate was extracted (see Materials and Methods).

The biological activity of the ethanol extracts was bioassayed using Gliocladium roseum (1) conidia as described above and the results are given in Table 3.1.

All the extracts reduced the percentage germination and germ tube growth of the G. roseum (1) conidia to some extent although the ethanol extracts were not as active as the chloroform extracts. Complete inhibition of both parameters was only produced by two of the extracts (T. hamatum (22) and T. saturnispora). The ethanol extract of T. viride (2) was the least active against percentage germination and T. koningii against germ tube growth.

The ethanol extracts were examined by chromatography to find out if either of the two known Trichoderma peptide antibiotics (alamethicine and suzukacillin) were produced by the Trichoderma isolates. Ethanolic solutions of pure samples of alamethicine, suzukacillin and extracts from the Trichoderma extracts were chromatogrammed on T.L.C. plates in two solvent systems; ethanol:water (70:30) and n-butanol:acetic acid:water (60:20:20). The plates were then removed from the solvents, dried with an industrial drier, and developed by halogenation with iodine and then spraying with 1% starch (Waldi, 1965).

In both solvent systems, alamethicine and suzukacillin had similar R_F values. With the ethanol:water solvent system all the Trichoderma ethanol extracts contained a compound that ran to the same R_F as alamethicine and suzukacillin, in all cases a single spot was observed although some streaking did occur. With the n-butanol:acetic acid:water solvent system in some cases an additional substance was detected which had a higher R_F value than the two peptide antibiotics (see fig. 3.2). This spot never reached the intensity as that which corresponded with the pure antibiotic. A similar result was found by Dennis (1970; Dennis & Webster, 1971a). Further analysis of the amino acid composition of the antibiotics was not carried out.

From these results it can be concluded that all the Trichoderma isolates tested produce peptide antibiotic(s) similar to either alamethicine or suzukacillin. Although no quantitative measurements of the substances were made, it was

Fig. 3.2. Chromatograms of ethanol extracts of Trichoderma culture filtrates run on T.L.C. plates in the n-butanol: acetic acid: water solvent system (see text).

of Trichoderma
the n-butanol:
t).

noticed that the intensity of the spots was greatest in those extracts with a high biological activity indicating that it may be a question of how much rather than which antibiotics are produced that determines the activity of the culture filtrate.

Culture filtrates of the different Trichoderma isolates were prepared and their biological activity bioassayed (see Materials and Methods). Spores of each of the Trichoderma isolates were tested for self-inhibition by its own culture filtrate. The culture filtrates were also bioassayed for activity against conidia of all the 15 test fungi listed in Materials and Methods. The effect of the culture filtrates on two parameters of germination; percentage germination and germ tube length after an optimum incubation time were measured. The incubation time was determined for each fungus individually; 18h was the usual incubation time but for the faster germinating spores (e.g. Stemphylium dendriticum) a 12h incubation period was used.

The different Trichoderma isolates varied in sensitivity to their own culture filtrates (see Table 3.2 and Appendix 12), T. viride (51) had the greatest reduction in percentage germination and T. viride (52) had its germ tube growth reduced the most. The theoretical colonization index was reduced the most for T. viride (52) and least for T. viride (53) (65.5% and 4.5% reduction respectively). It is evident that in conditions in which metabolites can accumulate the germination and germ tube growth of spores of some Trichoderma isolates could be seriously retarded,

TABLE 3.2 Sensitivity of the different *Trichoderma* spp to their own culture filtrates.

	Percentage Reduction		
	Percentage Germination	Germ Tube Length	Theoretical Colonization Index
T. hamatum			
(15)	24.5 **	12.6 NS	34.0
(22)	12.3 *	1.9 NS	25.4
T. harzianum			
(1)	5.7 NS	10.9 NS	16.0
(129)	7.5 NS	3.7 NS	10.9
(20)	19.2 **	3.1 NS	21.7
T. koningii	28.0 **	26.4 **	47.1
T. longibrachiatum	8.0 **	39.7 ***	44.5
T. polysporum	NOT TESTED		
T. pseudokoningii	14.7 **	25.6 **	36.5
T. saturnispora	20.0 *	35.6 ***	40.0
T. viride			
(2)	16.1 **	16.0 *	29.5
(14)	3.1 NS	12.7 NS	15.4
(28)	31.1 *	36.7 ***	56.4
(48)	0.1 NS	13.3 NS	13.4
(49)	35.4 *	34.8 ***	57.9
(50)	40.8 ***	20.2 *	52.7
(51)	53.4 ***	38.1 *	71.2
(52)	31.8 **	49.5 ***	65.5
(53)	19.2 **	0	4.5
(54)	22.5	18.6 NS	36.9

* Significant $P < 0.05$
** " $P < 0.01$
*** " $P < 0.001$
NS Not significant $P > 0.05$

Student's 't' test (see text)

whether this would be an advantage or a disadvantage to the spore population would depend on the situation. Tolerance of its own metabolites would be of advantage if the environmental conditions were otherwise favourable to growth but if the environmental conditions were unfavourable then no advantage would ensue.

The percentage reduction in performance of the percentage germination, germ tube growth and the theoretical colonization index of the 15 test fungi produced by the Trichoderma culture filtrates are given in Appendices 13-27. For comparative purposes the results have been expressed as a percentage reduction in performance with respect to that of the control.

There is considerable variation within the species groups as well as between the different species groups as regards the production of inhibitory external metabolites by the Trichoderma isolates. These results confirm the findings of previous workers (Danielson, 1971; Dennis, 1970; Dennis & Webster, 1971a, b; Gutter, 1957; Hashioka & Komatsu, 1964; Komatsu & Hashioka, 1964; and Moubasher, 1963) that strains differ physiologically although they may be morphologically very similar.

There is also a wide variation in the sensitivity of the individual test fungi to the different culture filtrates; the different culture filtrates affecting the two germination parameters of any one test fungus to varying degrees i.e. in some cases the percentage germination may be reduced more than the germ tube growth and vice versa.

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The results can be examined from two aspects; (1) the general activity of the culture filtrates and (2) the sensitivity of the test fungi to these filtrates.

The overall activity of a culture filtrate in inhibiting the germination and germ tube growth of the test fungi can be determined by summing the percentage reduction in performances of any one parameter for all the test fungi and calculating the mean i.e.

$$\Sigma \frac{\% \text{ reduction}}{15}$$

This value I have termed the Activity Index and can be determined for any culture filtrate for any of the parameters measured. A culture filtrate with a high activity index indicates that it has a high biological activity; the activity indices of the Trichoderma culture filtrates for the different parameters of germination are given in Table 3.3.

There is variation between the activity indices of the Trichoderma culture filtrates against the same parameter; T. viride (48) has the highest activity against the percentage germination of the test fungi and T. polysporum the least (activity indices of 28.9 and 12.9 respectively). Germ tube growth of the test fungi was inhibited to the greatest extent by T. hamatum (15) and least by T. polysporum (activity indices of 42.9 and 22.9 respectively). In all cases the germ tube growth of the test fungi was the most sensitive to the activity of the culture filtrates; the mean activity of the culture filtrates against the percentage germination of the

TABLE 3.3 Activity Indices of Trichoderma Culture Filtrates on Germination of 15 test fungi.

	Percentage Germination	Germ Tube Length	Theoretical Colonization Index
T. hamatum			
(15)	26.2	42.9	58.8
(22)	19.4	24.5	37.9
T. harzianum			
(1)	27.0	37.7	53.6
(129)	16.0	27.3	37.6
(20)	27.8	41.8	55.4
T. koningii	21.0	36.4	49.9
T. longibrachiatum	17.5	28.5	40.9
T. polysporum	12.9	22.9	32.6
T. pseudokoningii	25.3	35.8	50.1
T. saturnispora	20.3	34.6	45.3
T. viride			
(2)	17.7	25.0	37.7
(14)	19.0	33.8	46.3
(28)	20.2	29.9	42.9
(48)	28.9	39.6	58.6
(49)	19.8	32.1	45.4
(50)	19.1	27.1	40.2
(51)	23.7	32.6	50.4
(52)	19.6	29.0	43.4
(53)	21.5	30.3	42.7
(54)	23.6	34.5	49.6

* Delete

* Significant P < 0.05
** " P < 0.01
*** " P < 0.001
NS Not significant P > 0.05

Student's 't' test (see text)

test fungi was 36.4% less than that against germ tube growth. This indicates that there is a possibility that in a competitive situation the germination of the competing spore populations will be inhibited to some extent but that the activity of the inhibitory external metabolites will be greater against the hyphal extension of the competing fungi i.e. production of antibiotics by Trichoderma spp may be most ecologically significant at the later stage of germination as hyphal extension begins.

As was discussed earlier (Chapter One) it is not the effect that inhibitory compounds have on any one parameter of germination that is of overall importance to the fungus but the effect they exert on the overall growth of that fungus which is of prime importance i.e. the effect on the theoretical colonization index.

An activity index can also be calculated for the theoretical colonization index; the activity indices for this parameter are given in Table 3.3. The variation in effect of the culture filtrates on the theoretical colonization index is not as great as that against the other parameters. T. hamatum (15) is the most effective in reducing the theoretical colonization index of the test fungi although T. viride (48) is almost as effective (activity indices of 58.8 and 58.6 respectively). The T. polysporum culture filtrate was again the least active (activity index 32.6).

To compare the inhibitory effects of the external metabolites of the different species groups the mean species group activity indices have been calculated and are given in Table 3.4. T. pseudokoningii is the most effective species

TABLE 3.4 Activity Indices of the Trichoderma species groups.

Species group	No. of Isolates	Percentage Germination	Germ tube length	Theoretical Colonization Index
T. hamatum	2	22.8	33.7	48.4
T. harzianum	3	23.6	35.6	48.9
T. koningii	1	21.0	36.4	49.9
T. longibrachiatum	1	17.5	28.5	40.9
T. polysporum	1	12.9	22.9	32.6
T. pseudokoningii	1	25.3	35.8	50.1
T. saturnispora	1	20.3	34.6	45.3
T. viride	10	21.3	31.4	45.7

group against the percentage germination and the theoretical colonization indices of the test fungi. This species group was found to be a strong producer of inhibitory metabolites by Dennis (1970; Dennis & Webster, 1971a). The T. polysporum culture filtrate was consistently the least effective in reducing the performance of all the germination parameters measured. All the other species groups (except T. polysporum) have activity indices against the theoretical colonization index of the test fungi in excess of 40 (with only a range of 9.2 between them) indicating that there is not a substantial difference between them.

The sensitivity of the test fungi to the culture filtrates can be assessed in a similar manner to that used to assess the activities of the culture filtrates; the value of $\Sigma \frac{\%\ \text{reduction}}{20}$ for any one parameter will give an index of the sensitivity of individual test fungi to the activity of the culture filtrates of all 20 Trichoderma isolates. This value I have called the sensitivity index; the sensitivity indices of the test fungi are given in Table 3.5.

The germination of Botrytis cinerea is inhibited to the greatest extent by the culture filtrates and the germination of Penicillium nigricans the least (sensitivity indices of 35.6 and 5.0 respectively). The germination of the Penicillium spp tested was relatively insensitive, compared to that of the other fungi tested, to the activity of the Trichoderma culture filtrates.

The germ tube growth of Trichocladium opacum was the most sensitive and that of Botrytis cinerea the least sensitive to the activity of the culture filtrates

TABLE 3.5 Sensitivity Indices of the Test Fungi to the Trichoderma culture filtrates.

	Percentage Germination	Germ tube length	Theoretical Colonization Index
Gliocladium roseum (1)	25.6	43.6	60.0
Gliocladium roseum (2)	22.9	28.8	44.2
Gliocladium virens	21.1	35.7	49.2
Cladosporium herbarum	28.8	12.2	33.4
Trichocladium opacum	16.4	48.9	43.4
Stemphylium dendriticum	31.8	43.2	59.2
Fusarium solani	32.0	40.3	57.2
F. culmorum	19.3	37.0	46.6
Botrytis allii	28.0	33.7	51.4
B. cinerea	35.6	22.5	50.0
Penicillium nigricans	5.0	33.4	34.7
P. janthinell um	25.1	23.4	41.3
P. expansum	10.9	48.4	35.0
P. spinulos um	8.6	36.5	41.4
P. finuculos um	10.4	35.2	42.4

(sensitivity indices of 48.9 and 22.5 respectively). In most cases the percentage germination of the spores was less sensitive to the action of the culture filtrates than the germ tube growth; there were two exceptions, Cladosporium herbarum and Botrytis cinerea in which the germ tube growth was the least sensitive to the action of the culture filtrates.

The sensitivity of the theoretical colonization index of the test fungi to the activity of the culture filtrates will give an indication of their ability to compete for a substrate in the presence of the Trichoderma isolates. Gliocladium roseum (1) is the most and Cladosporium herbarum the least sensitive of the test fungi (sensitivity indices of 60 and 33.4 respectively) indicating that the latter will be able to compete more effectively with Trichoderma than will the former. The low sensitivity of the C. herbarum theoretical colonization index to the action of the culture filtrates is probably a result of the fact that the germ tube growth of this fungus is relatively insensitive which will compensate for the high sensitivity of the percentage germination. Again the Penicillium spp showed relatively little sensitivity to the culture filtrates, their tolerance to antagonism is borne out in nature by their ability to compete effectively with other fungi for the colonization of substrates in soil; P. nigricans was the least sensitive of the Penicillium spp tested.

It is interesting to note that the two isolates of Gliocladium roseum (both from soil) had different sensitivity

indices to the culture filtrates i.e. this is another example of the phenomenon reported earlier in which different isolates of the same fungus exhibit different physiological responses.

CHAPTER FOUR

THE EFFECT OF NUTRIENT SOURCE ON THE PRODUCTION OF NON-VOLATILE ANTIBIOTICS BY TRICHODERMA VIRIDE (48)

In the previous chapter it was shown that culture filtrates of a number of Trichoderma isolates from different species groups were biologically active against conidia of a range of test fungi and that the chemical nature of these culture filtrates varied.

It was suggested by Dennis (1970) that the nutrient source, particularly the nitrogen source, may affect the production of antibiotics (particularly polypeptide antibiotics) by Trichoderma isolates.

The effect of amino acid in the culture medium on the production of the peptide antibiotic suzukacillin by a Trichoderma species was investigated by Ooka & Takeda (1972). Suzukacillin formation was found to be increased by the addition of γ-amino butyric acid, L-asparagine, L-arginine, glycine and L-serine. It was thought that the amino acids L-asparagine, γ-amino butyric acid and arginine may act by being amino donors or may accelerate the enzyme system of suzukacillin biosynthesis. Glycine was found by radioisotopic experiments to be directly incorporated into suzukacillin and it is thought that serine may act in a similar manner. L-valine, L-methionine, L-cysteine, L-tyrosine and L-tryptophan all suppressed suzukacillin production without affecting the mycelial growth indicating that the biosynthesis of suzukacillin is independent of that of the mycelium.

The production of biologically active metabolites by T. viride (48) with respect to nutrient source was

investigated to see if relationships similar to those found by Ooka & Takeda (1972) existed between nutrient source and production of biologically active metabolites by this fungus.

Weindlings medium was used in all these experiments; carbon sources, replacing the glucose in Weindlings medium were added at the rate of 1000 ppm $C\ell^{-1}$; nitrogen sources, replacing the ammonium tartrate in Weindlings medium were added at the rate of 300 ppm $N\ell^{-1}$.

The carbon and nitrogen sources were sterilized by millipore filtration (0.22μ pore size) and added to sterile basal Weindlings medium.

Culture filtrates were prepared for each of the media as described in Materials and Methods. The mycelial dry weight of the Trichoderma was determined after 6 days incubation.

The culture filtrates were bioassayed for biological activity using Gliocladium roseum (1) conidia (see Materials and Methods, an 18h incubation period was used) and controls consisted of the relevant sterile medium.

There was a wide variation in the yield of the Trichoderma with the different C-sources; most growth occurred with glucose as the C-source and the yields for the other C-sources have been expressed as a percentage of the glucose yield (see Table 4.1). Mannose, galactose, maltose, xylose and ribose all support the growth of T. viride (48) well. A poor yield was obtained with sorbose, sorbitol, rhamnose and lactose as C-source, similar results for an isolate of T. viride were obtained by Danielson (1971) although he found

TABLE 4.1 Effect of C-source on growth of T. viride (48) and the effect of resultant culture filtrate on Theoretical Colonization Index of Gliocladium roseum (1) conidia

Substrate	T. viride (48) Yield as Percent of Yield on Glucose	Theoretical Colonization Index	
		Control	Experiment
Monosaccharides			
Glucose		447.0	124.1
Fructose	28.7	1038.3	198.3
Galactose	82.8	1011.1	243.6
Mannose	83.5	1054.2	173.2
Ribose	77.0	755.0	406.3
Xylose	78.0	906.1	123.9
Rhamnose	2.3	724.2	565.0
Mannitol	78.3	772.9	163.3
Sorbitol	22.8	1025.3	313.6
Sorbose	13.1	1114.7	288.4
Disaccharides			
Sucrose	28.6	558.5	105.9
Lactose	8.1	952.4	432.7
Maltose	82.5	457.7	202.5
Trisaccharides			
Raffinose	23.9	1106.9	365.7
Polysaccharides			
Starch	70.9	891.3	162.7
Glucose Yield (mg)	1452.0		

greater yields with lactose and sucrose as sole C-source than was the case in these experiments; sucrose was not a very good C-source, producing only 28.6% of the yield obtained with glucose.

There is a variation in the biological activity of the culture filtrates produced on different C-sources against the germination and germ tube growth of the G. roseum conidia. The results are given in Appendix 28. The mannose culture filtrate had the greatest and the rhamnose culture filtrate the least activity against the percentage germination (62.3% and 9.6% reduction respectively). The germ tube growth of G. roseum conidia was reduced most by the galactose culture filtrate and least by the rhamnose culture filtrate (59.3% and 1.0% respectively). The germ tube growth of G. roseum was slightly more sensitive to the action of the Trichoderma culture filtrate than was the percentage germination (mean percentage reduction 46.2 and 44.5 respectively).

The theoretical colonization indices of G. roseum in both control and experiment are given in Table 4.1. The theoretical colonization index in the experiment is a result of the inherent ability of G. roseum to grow on that substrate as indicated in the control and the quantitative and qualitative ability of the Trichoderma to produce antibiotics on that substrate. It will therefore give an indication of the ability of G. roseum to compete with the Trichoderma on that substrate; a high theoretical colonization index indicating a high competitive ability. Gliocladium roseum had the highest theoretical colonization index with the rhamnose

culture filtrate and the lowest with the sucrose culture filtrate (565 and 105.9 respectively). Theoretically this means that G. roseum will be better able to compete with the Trichoderma for colonization of a rhamnose based substrate than one containing sucrose. In the natural environment it is probable that T. viride (48) will be better able to compete than is indicated in these experiments because it has other antagonistic attributes which may affect the development of the G. roseum spores.

There was an inverse relationship between the yield of the Trichoderma growing on the different C-source media and the theoretical colonization index of G. roseum growing on the resultant culture filtrates ($r = -0.5775$ $P < 0.05$) i.e. the greater the growth of the Trichoderma the less the growth of G. roseum indicating a direct relationship between antibiotic production by this isolate of T. viride or that the greater growth of the Trichoderma will result in a greater depletion of the nutrients available to such a level that it will restrict the growth of G. roseum this, however, is unlikely as the medium had a very high initial nutrient status; the effect of low pH in restricting growth can also be discounted (see Appendix 29).

The yield of T. viride (48) mycelium growing on the different N-sources have been expressed as a percentage of the yield obtained on ammonium tartrate (i.e. normal Weindlings medium) and the figures given in Table 4.2.

Most growth occurred with peptone and the least with NO_3-N with yields of 134.3% and 10.0% respectively of that with ammonium tartrate. Ammonium tartrate was the best of the ammonium-N sources tested. Amino-N was found to be

TABLE 4.2 Effect of N-source on growth of T. viride (48) and effect of resultant culture filtrate on Theoretical Colonization Index of Gliocladium roseum (1) conidia

Substrate	T. viride (48) Yield as Percent of Yield on Amm. tartrate	Theoretical Colonization Index	
		Control	Experiment
Ammonium tartrate		447.0	124.1
Ammonium sulphate	21.0	936.7	356.4
Ammonium chloride	30.6	942.8	273.3
Sodium nitrate	10.1	841.8	295.5
γ-amino butyric acid	90.7	712.9	397.4
Glycine	80.2	609.2	482.0
Glutamic acid	74.3	645.0	216.3
Cysteine	27.7	738.7	333.7
Phenyl-alanine	53.5	686.9	395.8
Peptone	134.3	975.3	495.0
Ammonium tartrate yield (mg)	1452.0		

the best N-source (besides peptone) for growth of the Trichoderma, this is probably because the amino acids will also act as C-sources. γ-amino butyric acid gave the highest yield of the amino acids tested, glycine, glutamic acid and phenylalanine were all relatively good nutrient sources but the yield with cysteine was small compared to that with the other amino acids.

The biological activity of the culture filtrates was assessed using G. roseum (1) conidia. The results are given in Appendix 30.

There was a wide variation in the biological activity of the culture filtrates produced on the different N-sources. The ammonium tartrate culture filtrate was the most active against the percentage germination of G. roseum conidia and peptone and phenylalanine the least active (54% and 15.6% reduction respectively). G. roseum germ tube growth was inhibited to the greatest extent by the glutamic acid culture filtrates and least by the glycine culture filtrates (54.9% and 5.0% reduction respectively). The culture filtrates produced with ammonium-N showed greater biological activity than the amino-N culture filtrates against both the percentage germination and germ tube growth of G. roseum (37.5% and 20.2%; 40.1% and 32.1% reduction respectively). G. roseum had the highest theoretical colonization index (see Table 4.2) on the peptone culture filtrate and the smallest on the ammonium tartrate culture filtrate (495.0 and 124.1 respectively) indicating that in a competitive situation peptone would be a better N-source for G. roseum and ammonium tartrate a better N-source for the Trichoderma. Of the amino-N culture filtrates the

G. roseum theoretical colonization index was highest with the glycine culture filtrate and smallest with the glutamic acid culture filtrate (theoretical colonization indices of 482.0 and 216.3 respectively).

The theoretical colonization index of G. roseum was greater with amino-N sources than with ammonium-N sources (means of 365 and 251 respectively) indicating either that the Trichoderma culture filtrate was less biologically active with an amino-N source than with the ammonium-N source or that the amino-N source is better for the growth of G. roseum than the ammonium-N source. From consideration of the overall growth of G. roseum conidia under non-antagonistic conditions (in control) it can be seen that they grow better on the ammonium-N source than on the amino-N source suggesting therefore that the former may be correct.

There was no significant relationship between the amount of growth of the Trichoderma on the different N-sources and the theoretical colonization index of G. roseum in the resultant culture filtrates.

The ability of the test fungus to grow in the culture filtrate with different nutrient sources is not only a function of its sensitivity to the antibiotics produced by the Trichoderma under those conditions but also of its own ability to utilize that substrate. Therefore the nature of the substrate to be colonized can have a profound effect on the outcome of competition i.e. if the Trichoderma was able to produce a large quantity of antibiotics and if G. roseum

were unable to readily utilize that substrate then the *Trichoderma* would probably succeed in the competition. If on the other hand the *Trichoderma* were able to produce a similar amount of antibiotic activity on another substrate and the *Gliocladium* was able to grow strongly on that substrate then the outcome may be completely different. This is further evidence in support of the assertion made by Garrett (1970) that the competitive saprophytic ability of a fungus is substrate specific.

It was not known whether the variation in biological activity of the *Trichoderma* culture filtrates produced on the different nutrient sources was due to qualitative or quantitative differences in the external metabolites produced. Consequently a chromatographic survey was conducted to examine the nature and biological activity of the components of the chloroform and ethanol-soluble extracts of the different culture filtrates.

Chloroform and ethanolic extracts were prepared from the culture filtrates produced with the 6 C- and 6 N-sources and tested for biological activity against *G. roseum* (1) conidia; the results are given in Table 4.3. All of the chloroform extracts, except that of the cysteine culture filtrate, produced total inhibition of germination. There was quite a large variation in the activity of the ethanol extracts; complete inhibition was produced with only one phenylalanine, extract. The sucrose ethanol extract showed the least activity against the percentage germination of *G. roseum* and the lactose ethanol extract the least activity against the germ tube growth.

TABLE 4.3 The effect of the Chloroform and Ethanolic Extracts of Trichoderma culture filtrates on Gliocladium roseum (1) conidia

	Percentage Reduction			
	Chloroform Extract		Ethanol Extract	
	Percentage Germination	Germ tube length	Percentage Germination	Germ tube length
Glucose	100	100	66.0***	59.0***
Sucrose	100	100	71.0***	60.5***
Fructose	100	100	73.4***	66.8***
Lactose	100	100	63.0***	41.0***
Xylose	100	100	80.3***	68.2***
Mannitol	100	100	78.0***	51.4***
$NaNO_3$	100	100	61.3***	67.2***
γ amino-butyric acid	100	100	47.1***	64.8***
Glutamic acid	100	100	66.1***	68.9***
Glycine	100	100	42.7***	56.3***
Cysteine	59.1***	54.5***	72.6***	64.8***
Phenyl-alanine	100	100	100	100

*** Significant at $P < 0.001$ - Student's 't' test (see text)

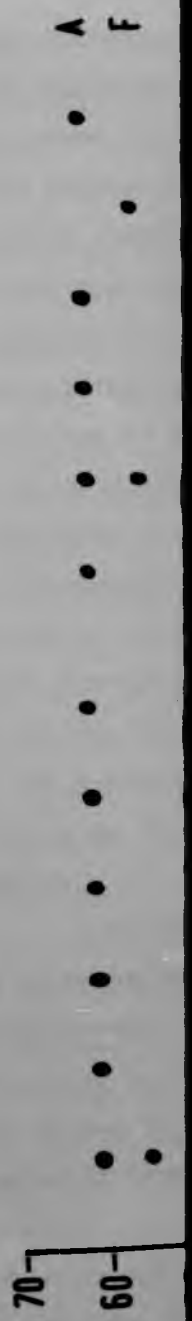

Fig. 4.1. Chromatograms of chloroform extracts of T. viride (48) culture filtrates produced on different nutrient sources (ether: ethyl acetate solvent system).

Glu	Glucose	$NaNO_3$	Sodium nitrate
Suc	Sucrose	ABA	γ amino butyric acid
Fruc	Fructose	Gly	Glycine
Lac	Lactose	GA	Glutamic acid
Xyl	Xylose	Cyst	Cysteine
Man	Mannitol	PhAl	Phenylalanine

...cts of T. viride
...rent nutrient
...stem).

Sodium nitrate
γ amino butyric acid
Glycine
Glutamic acid
Cysteine
Phenylalanine

Aliquots (10 µl) of the ethanol extracts of the culture filtrates were spotted onto a T.L.C. plate and run in the ethanol:water solvent system and the plates developed as described previously. No qualitative differences were evident between the extracts, a spot which ran to a R_F similar to that of alamethicine and suzukacillin was detected in all extracts, the intensity of the spot varied between extracts and appeared to be related to the activity of the extract indicating that variations in biological activity of the extracts may be a result of differences in concentration of the antibiotics produced on the different nutrient sources. Phenylalanine would therefore appear to be the best of the nutrient sources tested for the production of peptide antibiotics. The ethanol soluble extracts of the N-source culture filtrates had a higher activity against G. roseum than did that of the C-source culture filtrates indicating that the presence of a N-source on the medium has a greater influence on the production of peptide antibiotics than do C-sources.

Aliquots (10 µl) of the chloroform soluble extracts were chromatogrammed on T.L.C. plates in the ether:ethyl acetate solvent system and the spots located as described previously. A diagram of the resultant chromatograms is given in fig. 4.1. A total of 7 different substances could be detected from the different extracts and there is an obvious qualitative difference between the different culture filtrates produced on the different nutrient sources. The spots have been assigned a letter to aid identification and they are indicated on the side of the chromatogram (see fig. 4.1). There is a difference in the substances produced in the

different culture filtrates e.g. spot A is present in all but the cysteine culture filtrate whereas spot F is only produced in 3 (glucose, γ amino butyric acid, glutamic acid) of the culture filtrates.

In order to investigate whether there were quantitative as well as qualitative differences between the substances present in the different culture filtrates, the extracts were subjected to preparative T.L.C. An 0.01 cm^3 aliquot of each extract was placed on the base line of the T.L.C. plate in a streak 3 cm long and then chromatogrammed in the ether:ethyl acetate solvent system. The areas of the plates where the spots were known to occur were scrapped from the T.L.C. plate and the soluble material present eluted from the silica gel by extracting twice with 5 cm^3 methanol (AR); the silica gel was removed by centrifugation at 3000 rpm for 5 min. The two eluates of each spot were then pooled and concentrated to half the volume on the rotary vacuum evaporator at room temperature (approximately 20°C). The UV absorption spectrum of each of the eluates was obtained by use of a Unicam SP18000 UV spectrophotometer. The eluates were scanned over the range 200-350nm automatically with methanol (AR) in the reference cell. From the UV absorption spectra it was possible to determine the wavelength at which maximum absorbance (λ max) occurred which is a characteristic property of the substance. The amount of absorbance at the λ max is directly related to the quantity of material present (Beer-Lambert Law) enabling the production of each of the compounds to be monitored. The UV absorption values for each of the eluates from the different nutrient source extracts are given in

Appendix 31. By use of the UV absorption spectra it was possible to discern that two different substances ran to the same R_F as spot A. These two substances will henceforth be referred to as A_1 and A_2; A_1 has a λ max of 240nm and A_2 one of 300nm.

From perusal of these results it is obvious that there are both qualitative as well as quantitative differences between the chloroform solubles in the different culture filtrates.

Each of the eluates has been bioassayed for activity against G. roseum (1) conidia and the results given in Table 4.4. All the eluates (except C and F) are active against the percentage germination and all are active against germ tube growth; the latter parameter is the most sensitive to the activity of the eluates. Substance A_1 is the most active of the two substances with the same R_F; A_1 has more activity against percentage germination than against germ tube growth whereas with A_2 the greatest activity is against germ tube growth.

Eluates C and F are not very active by themselves although they are present in the chloroform soluble fraction of most of the culture filtrates. It is possible that these two substances may not be important in an antagonistic context.

No one eluate was present in all the extracts although either A_1 or A_2 occurred in all extracts except that of the cysteine culture filtrate. It is not possible to pin-point any one eluate as being responsible for the biological activity of any one culture filtrate, although the low activity of the cysteine culture filtrate chloroform

TABLE 4.4 Effect of Chloroform-soluble components of *T. viride* (48) culture filtrates on Germination of *Gliocladium roseum* (1) conidia

Chloroform soluble components				% Reduction	
Glutate	R_F	λ max	Absorbance	Percentage Germination	Germ tube length
A_1	61.0	240	0.430	60.1***	49.4***
A_2	61.0	300	0.945	7.5*	35.9***
B	45.0	260	0.350	7.8**	50.8***
C	20	255	0.230	0	10.7 NS
D	12.5	255	0.230	11.9**	39.6***
E	8	270	0.280	1.1 NS	19.3*
F	55	260	0.350	0	44.7***
G	35	280	0.280	5.7*	34.9***

* Significant $P < 0.05$
** " $P < 0.01$
*** " $P < 0.001$
NS Not significant $P > 0.05$
) Student's 't' test (see text)

Control % Germination = 71.6 Germ tube length = 12.7

extract may be due to the fact that only relatively small quantities of chloroform solubles with biological activity are produced. The overall activity of the culture filtrate is maintained however by the production of a peptide antibiotic in sufficient quantity to give a high biological activity.

As there were variations in the biological activity of the culture filtrates it was thought that an investigation into the relationship between the growth of the Trichoderma and the biological activity of the culture filtrate with respect to time, pH and C-source would yield interesting results.

Culture filtrates for each of the C-sources were prepared at 24h intervals for 6 days, the dry weight of the Trichoderma, the pH of the culture filtrate and its biological activity were determined. The yields of the Trichoderma on the C-sources for each time interval are given in Table 4.5.

TABLE 4.5 Yield (mg) of T. viride (48) grown on different carbon sources at 24h intervals

Time (days)	Glucose	Sucrose	Fructose	Lactose	Xylose	Mannitol
1	6.1	6.1	55.8	30.7	95.3	85.4
2	238.5	151.9	270.5	83.8	197.6	135.7
3	742.3	343.3	281.4	106.9	574.6	316.4
4	1105.0	268.3	309.7	104.1	703.0	637.7
5	692.8	414.5	418.4	106.5	999.1	745.7
6	1452.0	271.7	417.3	117.7	1132.5	1136.5

There is a wide variation in the yields of the Trichoderma grown on the different C-sources for each of the 24h intervals, but for each there was an increase in yield with time. The yield with sucrose as the C-source showed some variation for the different time intervals but there was no significant variation from the overall increase in growth with time. Growth was better with a monosaccharide than a disaccharide C-source; after 6 days a higher yield was obtained with glucose than with lactose (1452 mg and 117.7 mg respectively).

The change in pH of the culture filtrates with time are shown in Table 4.6, in most cases (glucose, sucrose, xylose and mannitol) there is an increase in acidity with time, a pH of 3.0 being reached by day 6. An increase in acidity with time is noted for the fructose and lactose culture filtrates but the increase is not as rapid, after 6 days the pH was 5.0.

TABLE 4.6 The change in pH of T. viride (48) culture filtrate with time when grown on different C-sources

Time (days)	Glucose	Sucrose	Fructose	Lactose	Xylose	Mannitol
0	6.4	6.4	6.4	6.4	6.4	6.4
1	5.5	6.0	6.0	6.0	5.5	5.5
2	4.5	5.0	6.0	6.0	5.0	5.0
3	4.0	4.5	5.5	5.5	4.5	4.5
4	3.5	4.0	5.5	5.5	4.0	4.0
5	3.0	3.5	5.0	5.4	3.5	3.5
6	3.0	3.0	5.0	5.0	3.0	3.0

There is a wide variation in the yields of the Trichoderma grown on the different C-sources for each of the 24h intervals, but for each there was an increase in yield with time. The yield with sucrose as the C-source showed some variation for the different time intervals but there was no significant variation from the overall increase in growth with time. Growth was better with a monosaccharide than a disaccharide C-source; after 6 days a higher yield was obtained with glucose than with lactose (1452 mg and 117.7 mg respectively).

The change in pH of the culture filtrates with time are shown in Table 4.6, in most cases (glucose, sucrose, xylose and mannitol) there is an increase in acidity with time, a pH of 3.0 being reached by day 6. An increase in acidity with time is noted for the fructose and lactose culture filtrates but the increase is not as rapid, after 6 days the pH was 5.0.

TABLE 4.6 The change in pH of T. viride (48) culture filtrate with time when grown on different C-sources

Time (days)	Glucose	Sucrose	Fructose	Lactose	Xylose	Mannitol
0	6.4	6.4	6.4	6.4	6.4	6.4
1	5.5	6.0	6.0	6.0	5.5	5.5
2	4.5	5.0	6.0	6.0	5.0	5.0
3	4.0	4.5	5.5	5.5	4.5	4.5
4	3.5	4.0	5.5	5.5	4.0	4.0
5	3.0	3.5	5.0	5.4	3.5	3.5
6	3.0	3.0	5.0	5.0	3.0	3.0

The effect of time of incubation on the biological activity, as a percentage reduction, of the culture filtrates is shown in fig. 4.2; the values for percentage germination and germ tube length are given in Appendix 32. There is an increase in biological activity of the culture filtrates with time although there are fluctuations about this trend i.e. with the fructose culture filtrate there is a slight drop in activity between days 2 and 3 before increasing again.

There are obvious variations between the effects of the different culture filtrates on the two germination parameters measured of Gliocladium roseum conidia with time. The biological activity of the sucrose culture filtrate is almost the same against both parameters over the 6 day period whereas with the xylose culture filtrate activity against germ tube growth is higher initially than against the percentage germination but this activity falls, the activity against the percentage germination increases and finally after 6 days the activities against both parameters are about equal.

The effects of the culture filtrates on the theoretical colonization index of G. roseum with respect to time are shown in fig. 4.3. The theoretical colonization index of G. roseum falls with respect to the age of the culture filtrate although the rate and amount of the decrease varies between the different C-sources. The initial theoretical colonization index represents the overall growth of G. roseum under non-antagonistic conditions (in control) and can be seen to vary for the different C-sources. Growth is greatest on the fructose culture filtrate and least with glucose, growth on sucrose was not very good either although the other C-sources all provided good growth.

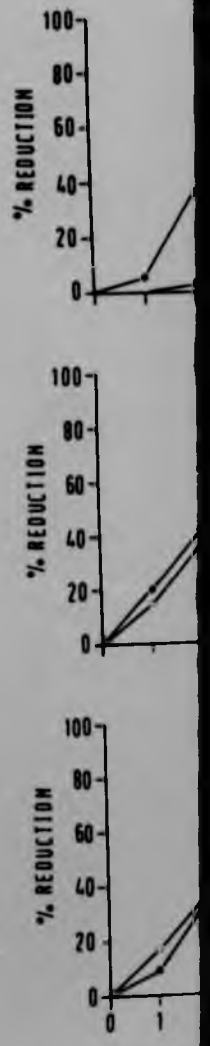

Fig. 4.2. Biological activity of T. viride (48) culture filtrates produced on different C-sources against Gliocladium roseum (1) conidia.

●——● % germination, O——O germ tube length

Controls	% germination	Germ tube length
Glucose	51.0	8.8
Sucrose	65.5	8.5
Fructose	80.9	12.8
Lactose	77.9	12.2
Xylose	78.3	11.6
Mannitol	73.6	10.5

(48) culture
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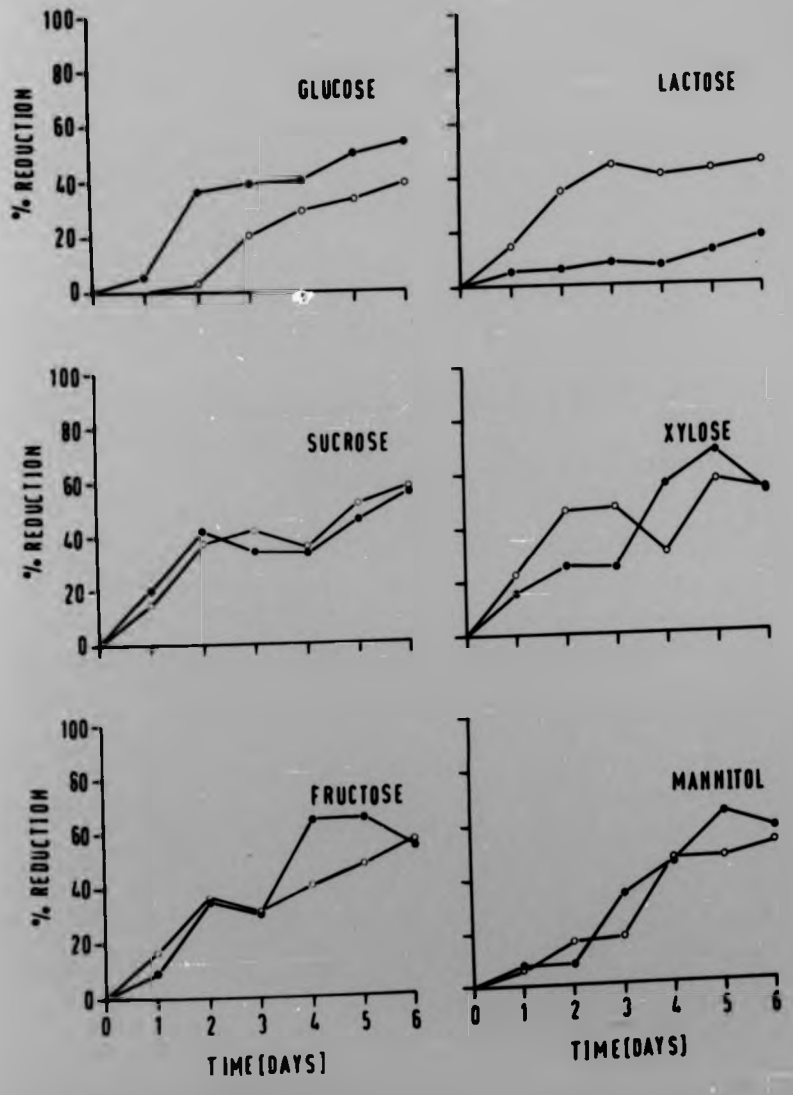


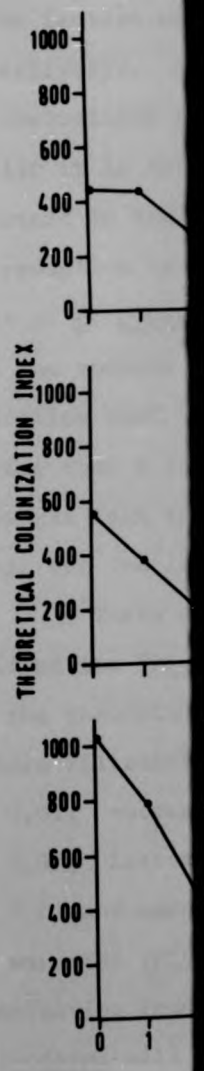
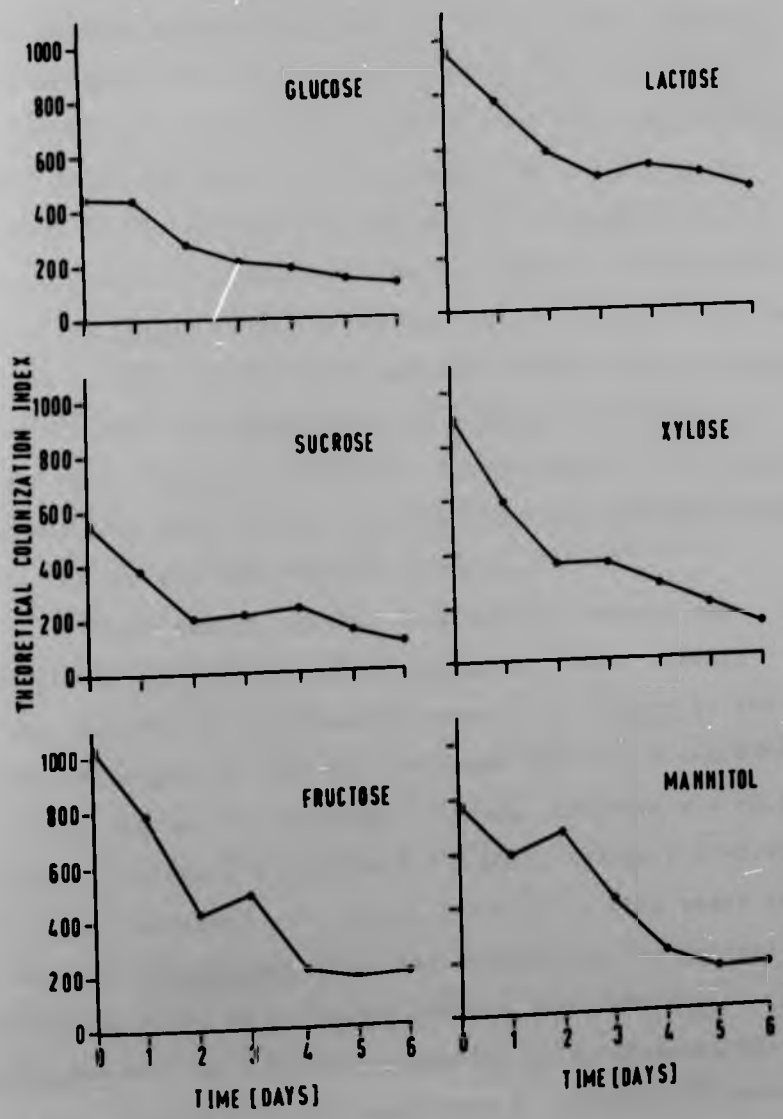


Fig 4.3. Theoretical colonization index of Gliocladium roseum (1) conidia in T. viride (48) culture filtrates produced on different C-sources.

Gliocladium
ure filtrates



The theoretical colonization index for the 6 day culture filtrate was reduced to the greatest extent with respect to the initial value by the xylose and least by the lactose culture filtrate (86.3% and 54.6% reduction respectively). All of the other culture filtrates reduced the theoretical colonization index by over 70%. As discussed earlier it is the theoretical colonization index that is important to the survival and growth of the fungus and not the reduction in performance. The theoretical colonization index of G. roseum in the 6 day old culture filtrate was least with the sucrose and greatest with the lactose culture filtrate indicating that the Trichoderma is a better antagonist on a sucrose than a lactose C-source. The theoretical colonization index was less than 200 for all the 6 day old culture filtrates except for the lactose culture filtrate.

There was an inverse relationship between the yield of the Trichoderma at the different time intervals and the theoretical colonization index of G. roseum in the culture filtrates for all the C-sources (glucose $r = -0.8948$ $P < 0.01$; sucrose $r = -0.9098$ $P < 0.01$; fructose $r = -0.9461$ $P < 0.01$; lactose $r = -0.9906$ $P < 0.001$; xylose $r = -0.9214$ $P < 0.01$ and mannitol $r = -0.9121$ $P < 0.01$). This means that the more the Trichoderma grows the smaller the theoretical colonization index of G. roseum becomes therefore the Trichoderma will be a better antagonist on a substrate that it is able to grow well on, conversely G. roseum will be better able to compete with the Trichoderma on a substrate that it is able to utilize more efficiently than the Trichoderma.

The biological activity of the culture filtrates has been shown to increase with time but it is not known whether this increase in activity is related to qualitative or quantitative (or a combination of both) changes in the external metabolites produced by T. viride (48) with time on the different C-sources. The following experiments were conducted to investigate this relationship.

Chloroform and ethanol extracts were prepared for each of the culture filtrates and each time interval and their biological activity ascertained by bioassay with Gliocladium roseum conidia. The results, as a percentage reduction, are given in fig. 4.4. Greatest activity was shown by the chloroform soluble extracts, in most cases 100% of inhibition of germination was achieved by day 2 although this level of inhibition was not reached by the xylose and lactose culture filtrates chloroform extract until day 3.

There was an increase in activity of the ethanol extract with time, slight variations in activity between the extracts at different times were observed. The activities of the day 6 ethanol extracts of the culture filtrates were similar.

The chloroform and ethanol soluble extracts of the culture filtrates were then examined by T.L.C. using the methods described earlier.

The chloroform solubles were eluted from the T.L.C. plate and their absorbance at λ max determined, the results are given in Appendix 33. One of the more important relationships to be noted is that between eluates A_1, A_2, time and pH change.

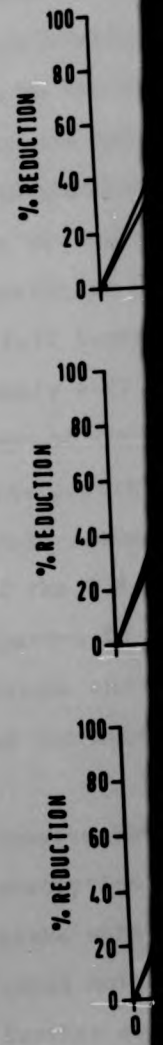


Fig. 4.4. Biological activity of chloroform and ethanol extracts of T. viride (48) culture filtrates produced on different C-sources against Gliocladium roseum (1)

●——● % germination chloroform extract
○——○ germ tube length " "
▲——▲ % germination ethanol extract
△——△ germ tube length " "

Controls	% germination	Germ tube length
Chloroform	71.9	12.3
Ethanol	72.8	15.6

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roseum (1)

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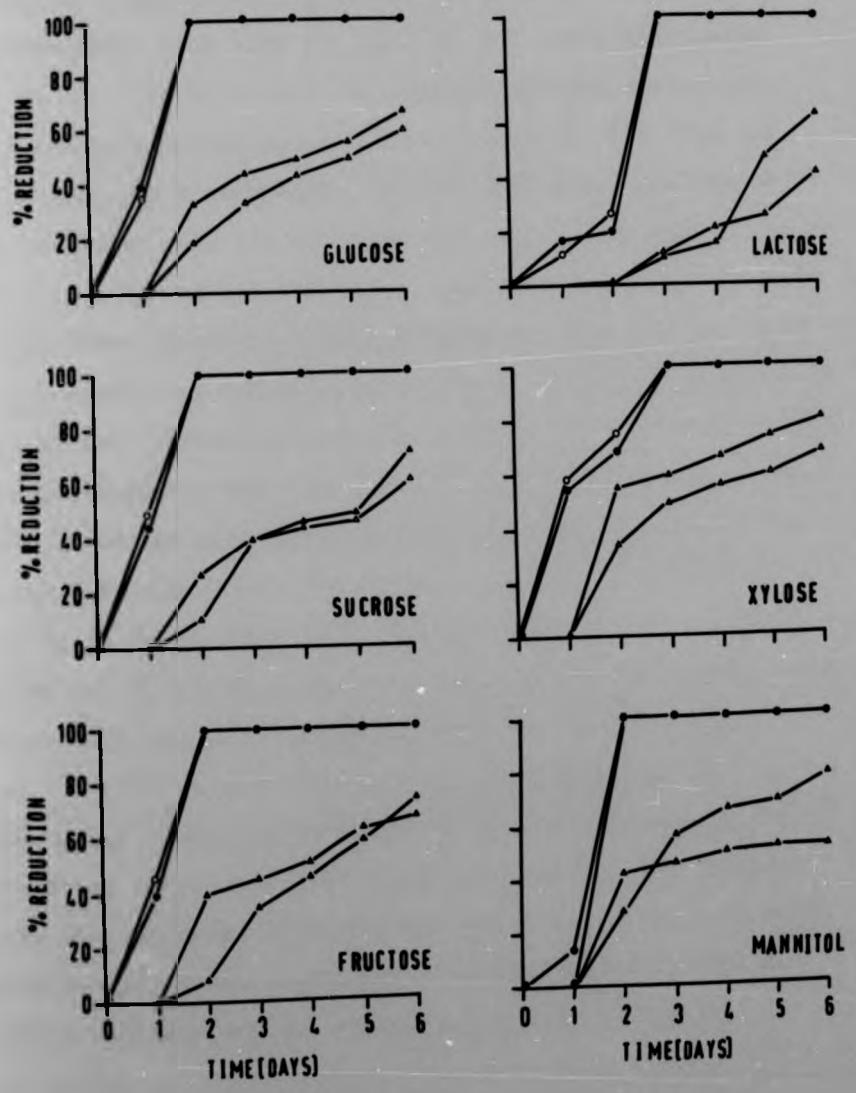


Substance A_1 is produced initially in high quantities at pH 5.5-4.0. The concentration of A_1 falls with time and also increasing acidity; by pH 4.0 A_1 cannot be detected but A_2 is now present and increases in concentration with time and fall in pH. Both substances run to the same R_F in all the solvent systems tested and both are halogenated with iodine indicating that they are both unsaturated compounds. The UV absorption spectra of the two substances are given in fig. 4.5. Sufficient quantities of the two substances were not available to carry out a full investigation into their chemical identity but there was barely sufficient material to obtain an infra-red spectrum of each of the substances (see fig. 4.6) in CCl_4. No conclusive interpretation of the I.R. spectra could be made due to the small amount of material available but from interpretation of the I.R. spectra in conjunction with the UV absorption spectra it is possible that substance A_1 may be an aromatic ketone and that with time and increasing acidity conjugation of the molecule occurred giving rise to substance A_2.

In one case (sucrose day 3) it was possible to observe the transition between the two substances in the UV absorption spectrum of the eluate of spot A; two distinct peaks were observed from the one eluate (see fig. 4.7) which could not be further resolved by chromatography. This is further evidence for the relationship between the two substances.

Of the two substances A_1 is more biologically active than is A_2 indicating that, at least in the initial stages of growth of the Trichoderma when only small quantities

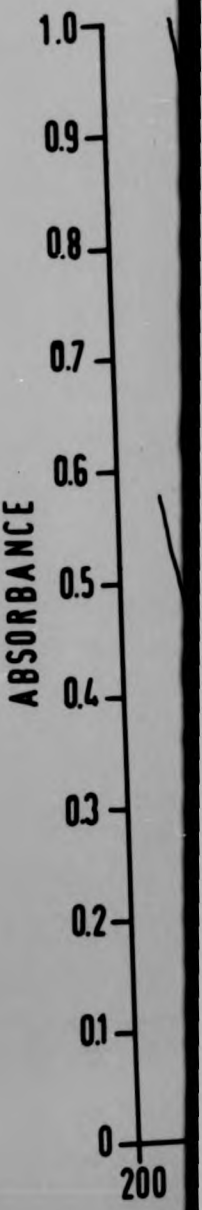


Fig 4.5. UV absorption spectra of eluates A_1 and A_2

and A_2

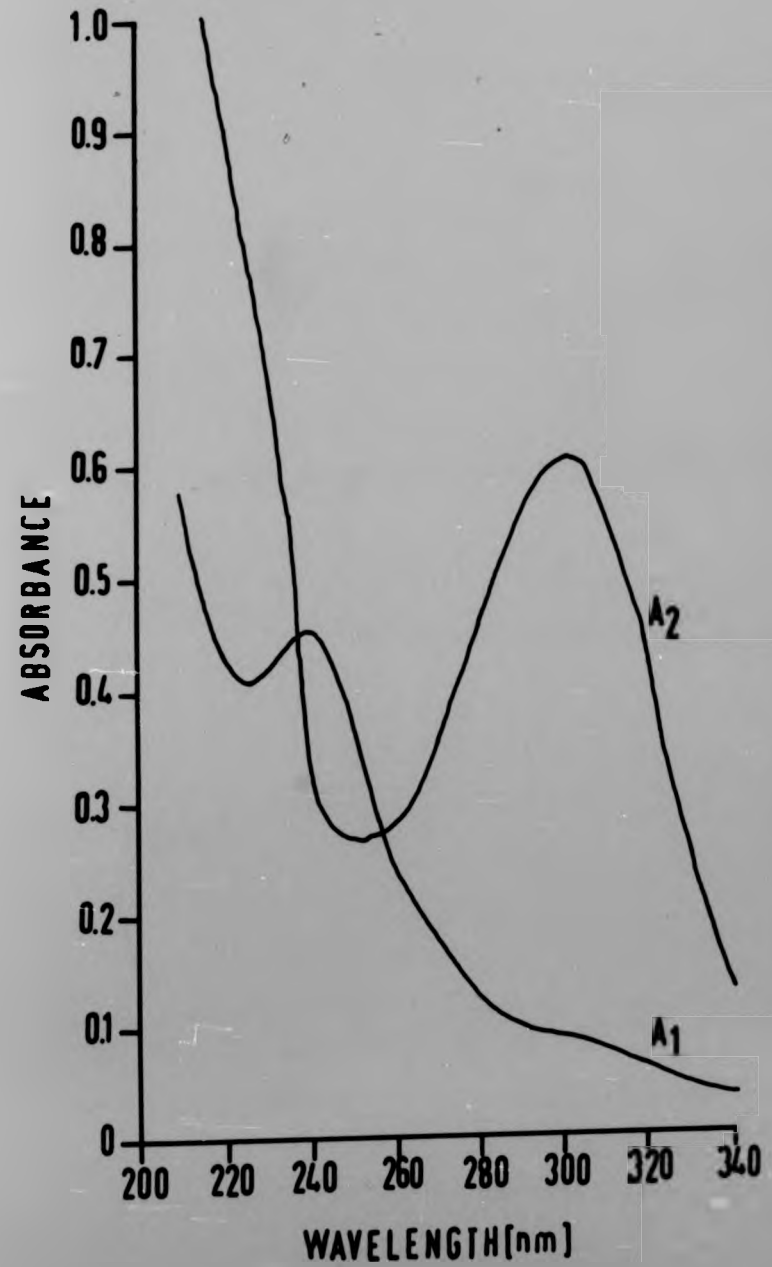
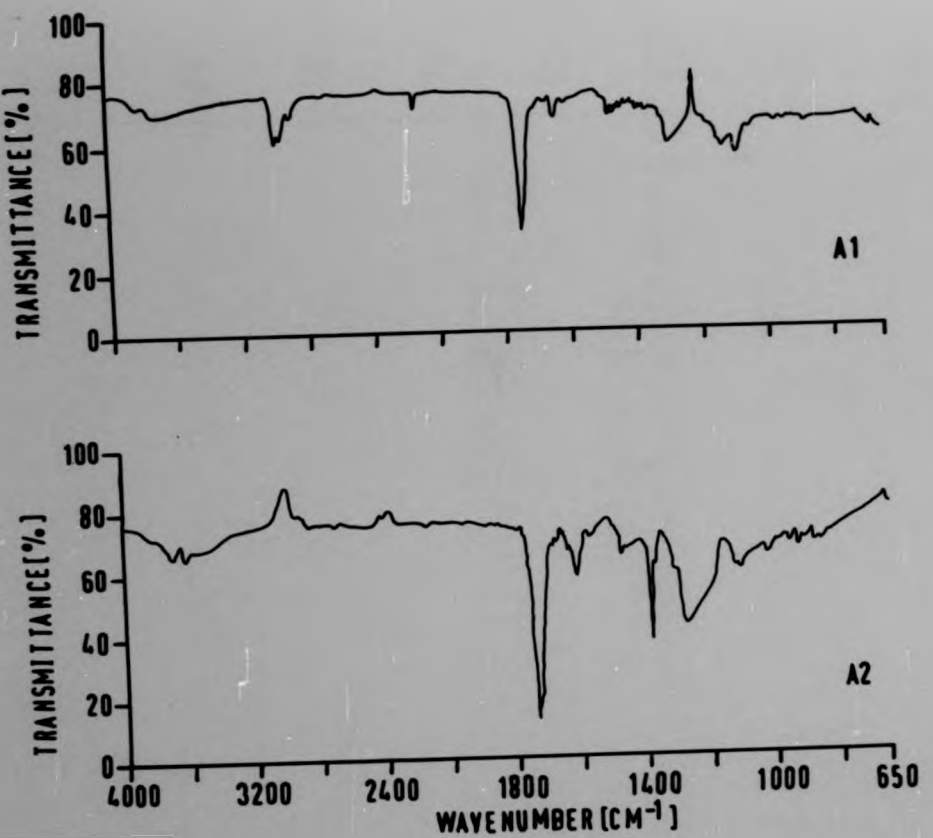

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE
A2
A1
200
220
240
260
280
300
320
340
WAVELENGTH[nm]

Fig. 4.6. IR spectra of eluates A_1 and A_2


TRANSMITTANCE [%]
100
80
60
40
20
0
A1
TRANSMITTANCE [%]
100
80
60
40
20
0
A2
4000
3200
2400
1800
1400
1000
650
WAVE NUMBER [CM^{-1}]

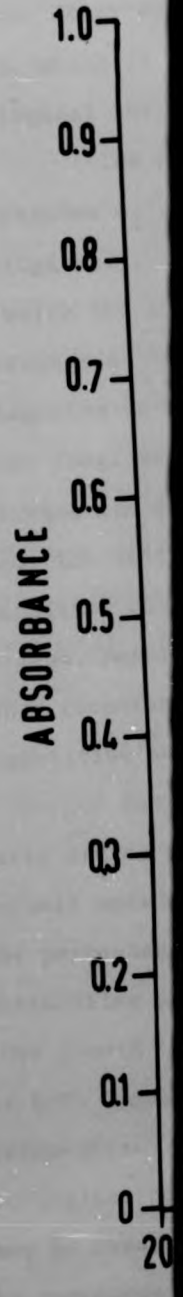
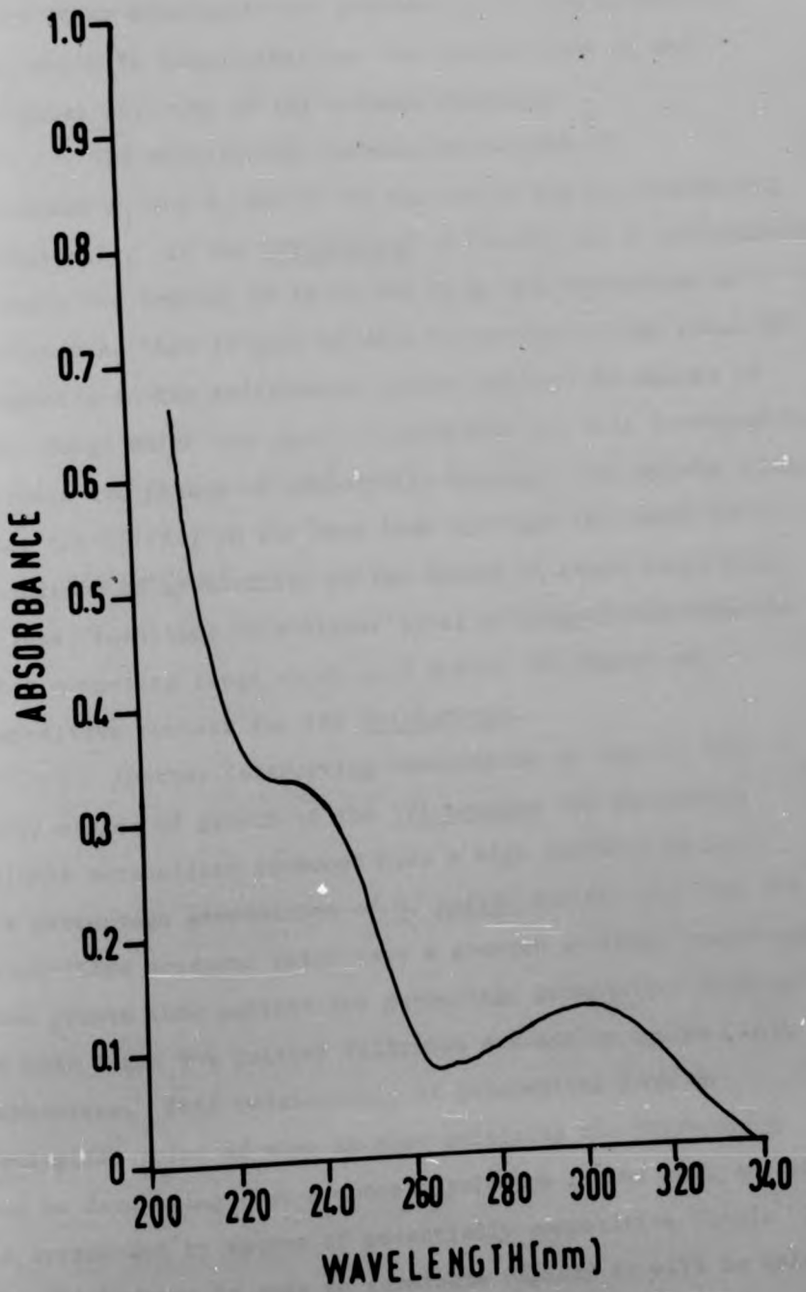


Fig 4.7. UV absorption spectrum of eluate A from Sucrose day three culture filtrate.

rom Sucrose


1.0
0.9
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0
ABSORBANCE
200
220
240
260
280
300
320
340
WAVELENGTH(nm)

of the other substances are present, it is the production of A_1 which is responsible for the greater part of the biological activity of the culture filtrate.

The relationship between production of substances A_1 and A_2 and pH of the medium may be interesting ecologically. If the Trichoderma is growing in an environment in which the initial pH is in the range for production of substance A_1 then it will be able to produce a high level of antagonism in the environment active against the spores of other fungi which are about to germinate and will consequently increase its chance of competitive success. If, on the other hand, the initial pH was less than 4.5 then the level of inhibition of germination of the spores of other fungi will be less, resulting in a higher level of competition from the other competing fungi which will reduce the chance of competitive success for the Trichoderma.

Another interesting observation is that in the early stages of growth of the Trichoderma the chloroform soluble metabolites produced have a high activity against the percentage germination of G. roseum conidia and that the metabolites produced later have a greater activity toward germ tube growth than against the percentage germination although at both times the culture filtrates are active against both parameters. This relationship is interesting from an ecological point of view in that initially the Trichoderma may be developing from a spore population in the soil and will be surrounded by spores of potentially competitive fungi. If the Trichoderma is able to germinate rapidly it will be able

to produce external metabolites which will retard the germination of the other spores and also inhibit the growth of other fungi. As the Trichoderma grows the other fungi, which have overcome the effects of the metabolites, will have germinated and started to grow, the Trichoderma will by this time be producing metabolites which will be inhibitory to the hyphal growth of the other fungi present enabling it to compete on two levels, inhibition of germination of the other fungi and of the developing hyphae.

The production of the peptide antibiotics present in the ethanol extracts could not be monitored accurately but the spots on the chromatograms could be scored according to the intensity of colouration (as judged by eye) after developing the chromatograms with 1% starch (see Table 4.7).

TABLE 4.7 Production of Peptide Antibiotics by T. viride (48) on different C-Sources

Substrate	Time (Days)					
	1	2	3	4	5	6
Glucose			+	++	+++	++++
Sucrose			+	++	++	+++
Fructose			+	++	++	++++
Lactose			+	++	++	+++
Xylose			+	++	+++	++++
Mannitol			+	+	++	+++

\+ presence of peptide antibiotic

Number of crosses indicating the intensity of the spot on the chromatogram.

Peptide antibiotics were not detected until the extract of the third day's culture filtrate and thereafter the spots increased in intensity with time until completion of sampling. There appeared to be a direct relationship between the amount of antibiotic present (intensity of spot) and the biological activity of the extract indicating that accumulation of this antibiotic in the environment would result in decrease in the ability of G. roseum conidia to germinate and grow in that environment provided that the antibiotic is not adsorbed onto clay particles or inactivated by some other mechanism.

The production of the peptide antibiotics does not appear to be as important as that of the chloroform soluble biologically active metabolites under laboratory test conditions but in the natural environment the position may be reversed. It would be interesting to carry out the above series of experiments using natural substrates in soil to ascertain to what extent the environment of the soil modifies non-volatile antibiotic production by Trichoderma isolates and whether any ecological advantage is conferred on a strong antibiotic producer.

CHAPTER FIVE

PRODUCTION OF VOLATILE ANTIBIOTICS

Little work prior to 1970 appears to have been carried out on the production of volatile metabolites with antibiotic properties by the genus Trichoderma. Russian workers, Bilai (1956, 1963), Dymonych (1960) and Khasanov (1962) have reported volatile metabolites of Trichoderma species to be antagonistic to fungi and bacteria.

Dennis (1970; Dennis & Webster, 1971b) tested for production antibiotics by several Trichoderma isolates. The volatile metabolites produced by some isolates were found to be inhibitory to the growth of some of the test fungi used, although the activity was not as great as that exhibited by the non-volatile antibiotics. It was noted that the most active cultures also possessed a characteristic 'coconut' smell. An attempt was made by them to try and ascertain the chemical nature of the metabolites produced by the two most active isolates of T. viride. Acetaldehyde was detected as a component of the volatile products and was tentatively proposed as being responsible for the inhibitory properties of the volatiles, although the possibility that other compounds, not detected by their technique, may be present was not discounted. They did not consider that primary metabolites such as carbon dioxide and ammonia were responsible for the inhibition as the pattern of inhibition was different from that to be expected if these metabolites were implicated.

A contrary opinion was put forward by Hutchinson & Cowan (1972) who maintained that the inhibitory effects of the volatile products of an isolate of T. harzianum could be attributed solely to the amounts of carbon dioxide and ethanol produced. They investigated the chemical nature of the volatile metabolites by use of Gas liquid chromatography (G.L.C.) and the only metabolites that they could detect in more than trivial amounts were carbon dioxide and ethanol. The volatile metabolites were found to inhibit the growth and sporulation of Aspergillus niger and of Pestalotia rhododendri, and the colouration and rate of elongation of seedlings of Lactuca sativa var sativa but no bacteriostatic activity was detected.

The production of a 'coconut' smell by some isolates of Trichoderma has been recorded by several workers (Bilai, 1956; Bisby, 1939; Dennis, 1970; Dennis & Webster, 1971b; Rifai, 1964, 1969). The volatile metabolite that is responsible for this smell has been isolated and characterized and found to be 6-pentyl-α-pyrone (Collins & Halim, 1972), a compound which has also been isolated from peach essence (Sevenants & Jennings, 1971). It was noted that the origin of the compound in the fungus was primarily the spore rather than the mycelium and hence presumably its association with the sporulation process.

The present investigation was undertaken to survey the biological activity of volatile metabolites produced by each of the twenty Trichoderma isolates against the germination and germ tube growth of Gliocladium roseum (1) conidia in relation to antagonistic activity in soil and to investigate any effects that the C-source may have on their production.

Fig. 5.1. Bioassay p
antibiotics.

Fig. 5.1. Bioassay procedure for Trichoderma volatile antibiotics.

Each of the Trichoderma isolates was grown on the modified Weindlings medium for 6 days and then the volatile metabolites that had accumulated above the culture in the Petri dish were assayed for biological activity with G. roseum (1) conidia. Triplicate plates were inoculated for each isolate by placing a 3mm diameter agar block cut from the edge of an actively growing colony in the centre of each Petri dish and then incubated at 25°C for 6 days.

The bioassay procedure was a slightly modified form of that used for bioassay of the non-volatile antibiotics. A sterile microscope slide was inserted into the Petri dish containing the Trichoderma and raised above the culture by two 3mm diameter sterile glass rods and then the peptone agar block placed on the slide (see fig. 5.1). A drop of G. roseum conidial suspension was then placed on each of the peptone agar blocks, the Petri dish lid replaced and incubated at 25°C for 18h before removal of the slide from the Petri dish and the percentage germination and germ tube length determined. The experiment was set up in triplicate for each isolate, the control was incubated in a similar manner but over sterile agar.

The percentage reduction in performances of the percentage germination and germ tube growth of G. roseum conidia in the presence of the Trichoderma volatile metabolites are given in Table 5.1. All the Trichoderma isolates reduced the germination level slightly with little variation between isolates. The percentage germination was significantly reduced by the volatile antibiotics of only five isolates; the three T. harzianum isolates and T. viride (48) and T. viride (49). The volatile metabolites produced by T. viride (53) appear to have the least activity whereas those of T. harzianum (129)

TABLE 5.1 Effect of *Trichoderma* volatile metabolites on Germination of *Gliocladium roseum* (1) conidia

	% Reduction		Theoretical Colonization Index	
	Percentage Germination	Germ Tube Length	Experiment	% Reduction
T. hamatum				
(15)	19.7 NS	21.3 NS	372.0	36.4
(22)	8.9 NS	17.6*	437.4	25.2
T. harzianum				
(1)	27.4*	20.1*	340.2	41.8
(129)	59.9**	51.2***	113.7	80.6
(20)	17.8*	41.7***	280.8	52.0
T. koningii	11.1 NS	7.3 NS	484.1	17.2
T. longibrachiatum	22.2 NS	32.4**	306.0	47.7
T. polysporum	22.5 NS	16.0 NS	381.7	34.7
T. pseudokoningii	11.1 NS	20.4 NS	412.0	29.6
T. saturnispora	16.5 NS	31.8**	333.3	43.0
T. viride				
(2)	23.8 NS	31.3*	304.3	48.0
(14)	18.2 NS	2.5 NS	469.3	19.8
(28)	23.2 NS	32.1**	307.1	47.5
(48)	27.9*	42.7***	241.9	58.6
(49)	35.8*	40.1**	226.9	61.2
(50)	20.9 NS	30.4*	320.6	45.2
(51)	21.3 NS	9.0 NS	419.5	28.3
(52)	25.0 NS	25.1*	329.8	43.6
(53)	6.2 NS	0	548.4	6.2
(54)	22.3 NS	22.1 NS	355.5	39.2

* Significant $P < 0.05$
** " $P < 0.01$
*** " $P < 0.001$
NS Not significant $P > 0.05$
) Student's 't' test (see text)

Control 82.2% germination, 11.9 germ tube length

are the most active against germination of G. roseum conidia (6.2% and 59.9% reduction respectively); the volatile metabolites of most of the Trichoderma isolates reduced the germination level by about 20%.

The level of activity of the volatile metabolites against germ tube growth is higher than that against germination; most of the volatile metabolites of the Trichoderma isolates reducing germ tube growth by between 25 and 30%. The volatile metabolites of T. harzianum (129) are the most active and those of T. viride (53) had no effect on germ tube growth at all.

As has been discussed earlier it is the overall effect on the growth of the spore population that is of the greatest ecological importance, the theoretical colonization indices of G. roseum in the presence of the Trichoderma volatile metabolites are given in Table 5.1, the percentage reduction of the theoretical colonization index with respect to that of the control is also included.

The volatile metabolites of all the Trichoderma isolates reduced the theoretical colonization index of G. roseum although there was variation between isolates in the extent of the reduction. T. harzianum (129) was by far the most active producer of volatile metabolites inhibitory to G. roseum and T. viride (53) the least active (80.6% and 6.2% reduction respectively). The volatile metabolites of only four (T. harzianum (129), T. harzianum (20), T. viride (48), T. viride (49)) of the Trichoderma isolates were able to reduce the theoretical colonization index by greater than 50% this is

in contrast with the higher inhibitory levels of the non-volatile antibiotics. The T. harzianum species group was on the whole the most active producer of volatile antibiotics and the T. pseudokoningii species group the least active. The volatile metabolites of the T. viride species group had quite a high level of activity but there was a wide variation in the activity of the volatile metabolites produced by some of the isolates; T. viride (49) was the most active and T. viride (53) the least (61.2% and 6.2% reduction respectively) but 6 of the 10 T. viride isolates produced volatile metabolites capable of reducing the theoretical colonization index of G. roseum by over 40%.

The 'coconut' smell could be detected in all the active isolates and was most intense when the cultures were beginning to sporulate.

The effect of the C-source in the culture medium on the production of volatile antibiotics by T. viride (48) with respect to time was investigated. Essentially the same methods were used as for the non-volatiles, C-sources at the rate of 1000 ppm C ℓ^{-1} were added to the modified Weindlings medium (see Appendix 3) and the volatile metabolites which accumulated every 24h for up to 6 days were assayed for biological activity with G. roseum (1) conidia. The radial growth of the Trichoderma on the C-sources was monitored by measuring the colony diameter at the beginning of each bioassay procedure, the time at which sporulation initiation was visible to the naked eye was also recorded.

The effects of the volatile metabolites produced by

T. viride (48) growing on the different C-sources on the percentage germination and germ tube growth of G. roseum conidia are shown in fig. 5.2. There was a variation in the amount of biological activity against both germination parameters, in all cases activity was greatest against germ tube growth than germination. The volatile metabolites appeared to be most active (both parameters) from the glucose culture and least active with the lactose culture. There was variation too in the time of peak activity; glucose and sucrose culture volatile metabolites were most active at day 3, fructose at day 4, lactose and mannitol at day 5 and the xylose culture showed a steady increase in activity with time, the day 6 culture having the greatest activity.

A similar relationship existed for the theoretical colonization index (see fig. 5.3). In three cases (glucose, sucrose and fructose) there was a decrease in theoretical colonization index with time until after about day 4 when the activity of the volatile metabolites decreased. The theoretical colonization index of G. roseum over the xylose and mannitol cultures decreased steadily with time though the activity of the volatile metabolites levelled out at about day 5. The lactose culture had two peaks of activity at day 3 and 5 but then the activity decreased.

The growth (colony diameter) of the Trichoderma culture and the time of sporulation (S) have been included in fig. 5.3 so that the relationship between growth, sporulation and biological activity of the volatile metabolites can be more clearly seen. On all the media the colony diameter equalled that

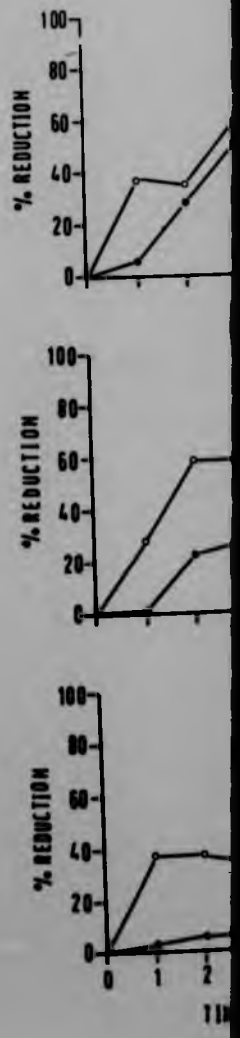


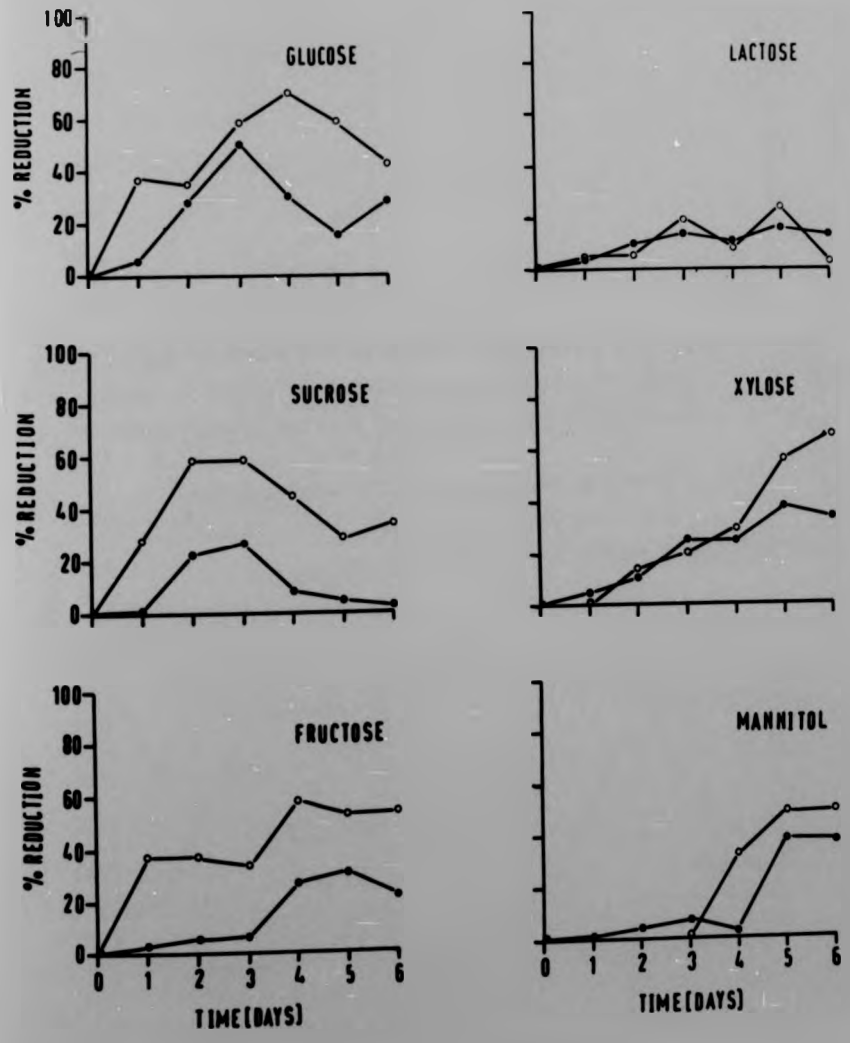
Fig. 5.2. Biological activity of T. viride (48) volatile metabolites produced whilst growing on different C-sources against Gliocladium roseum (1)

●——● % germination, O——O Germ tube length

Control 82.3 % germination
11.9 germ tube length

e (48) volatile
different
)

Germ tube length



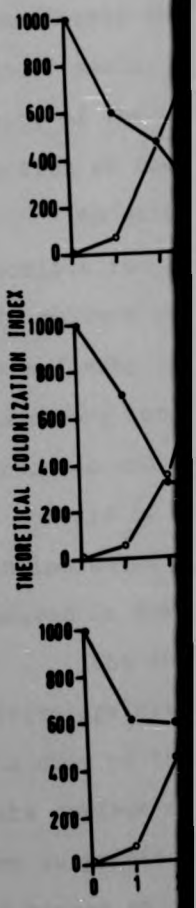


Fig. 5.3. Theoretical colonization index of Gliocladium roseum (1) conidia germinating in volatile metabolites produced by T. viride (48) growing on different C-sources

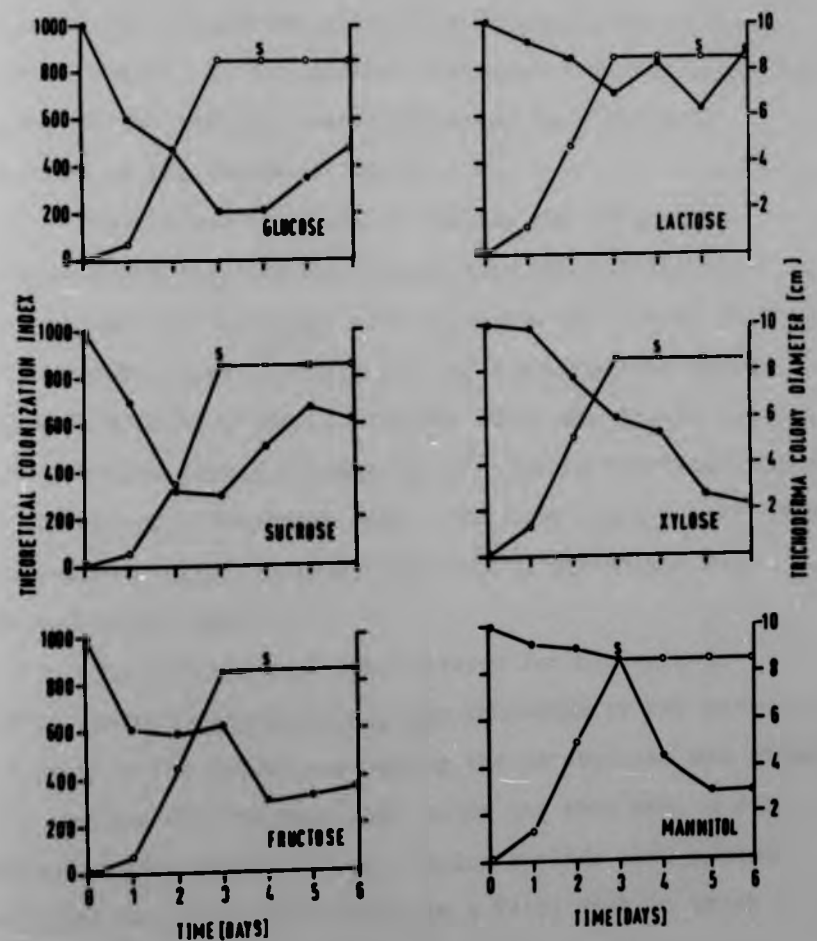
●——● Theoretical colonization index

○——○ Colony diameter

S = initiation of sporulation

of Gliocladium
ile metabolites
ifferent C-sources

ex



of the Petri dish by day 3 and sporulation occurred either at this time (sucrose and mannitol) or 24h later (glucose, fructose and xylose) the lactose culture not sporulating until day 5. In all cases the maximum activity of the volatile metabolites was recorded at the time of sporulation initiation or immediately thereafter coinciding with production of the 'coconut' smell. It is possible therefore that the biological activity of the volatile metabolites may be related to production of the 'coconut' smell.

An attempt was made to isolate the metabolite responsible for the 'coconut' smell from the air above a liquid culture of T. viride (48) (i.e. 200 cm^3 liquid in a 1 litre flask; approximately 800 cm^3 vapour phase) which had a strong coconut smell. The air above the liquid culture was removed and passed through 50 cm^3 sterile distilled water with the aid of a diaphragm pump. The water took up the odour which indicated that at least some of the metabolite had dissolved in the water.

The solution was then assayed for biological activity against Gliocladium roseum (1) conidia by two methods; (1) a drop of the liquid containing the metabolites was placed on the surface of a peptone agar block and when dry, a drop of spore suspension added, (2) by placing a slide with peptone agar blocks on its upper surface in a Petri dish in which a watch-glass containing 3 cm^3 of the liquid containing the metabolites had been placed, a drop of spore suspension was then added to the surface of the agar blocks. The results are given in Table 5.2. No inhibitory activity was recorded by the first bioassay technique although there was stimulation

TABLE 5.2 Effect of an aqueous solution of *T. viride* (48) volatile metabolites on *Gliocladium roseum* (1) conidia

Bioassay Method		Percentage Germination	Germ Tube Length	% Reduction Percentage Germination	% Reduction Germ Tube Length
1	Control	62.9	6.4		
				0.3 NS	Stimulation
	Experiment	62.4	10.3		
2	Control	73.4	9.8		
				11.9**	8.9 NS
	Experiment	64.7	8.9		

** Significant P < 0.01 } Student's 't' test
NS Not significant P > 0.05 } (see text)

of germ tube growth. In the second method nearly 12% reduction in the percentage germination was recorded but only 9% reduction in germ tube growth. These results indicate that probably only a small quantity of the active volatile component(s) was dissolved in the water or that it was partially inactivated. The different results obtained with the two bioassay methods indicate that the volatile component is only active in the vapour phase. The presence of other dissolved volatile metabolites in the water cannot be discounted and in fact the activity observed may be due to their presence and not that of 6-pentyl-α-pyrone. Even so it does seem likely that the 'coconut' smell is associated with the maximum inhibitory activity of the culture and with sporulation.

Sporulation of T. viride in spaces within soil has been observed (Jackson, 1971 personal communication) so it is possible that the volatile antibiotics may be ecologically important in the soil atmosphere, although Hora & Baker (1972) found that the addition of Trichoderma isolates to soil did not significantly increase the volatile fungistatic activity of that soil. The possibility does still remain however that localized volatile antibiotic activity may be important in the colonization of substrates in soil.

DISCUSSION

The work reported here concerned measurements of the effects of antibiotic production by Trichoderma species and soil fungistasis on the ecological status of Trichoderma species with particular emphasis on the colonization, by this genus, of substrates in soil.

The ability of a fungus to colonize a substrate situated in the soil has been termed the competitive saprophytic ability (Garrett, 1970) and has been resolved into four major components:-

(1) rapid germination and hyphal growth,

(2) ability to produce appropriate enzymes,

(3) production of antibiotics,

(4) tolerance of antagonisms.

It was generally considered that the production of antibiotics by the fungus was the most important of these factors in determining the competitive saprophytic ability of a fungus. Park (1960), however, has convincingly argued that it is the tolerance of the fungal propagules to antagonisms that is the main factor in deciding the growth of the fungus in soil, in that if growth were totally inhibited then none of the other factors cited by Garrett would be able to exert any effect.

Therefore before any consideration may be given to the ecological significance of the antagonistic properties of the genus Trichoderma it has first to be established that the isolates under consideration are capable of growing in soil.

The response of the Trichoderma isolates to the general soil antagonism (i.e. soil fungistasis) was therefore measured.

There was a wide variation in the sensitivity of the different Trichoderma isolates, as measured by the response of several germination parameters, to soil fungistasis. Although measuring the sensitivity of specific germination parameters to soil fungistasis is very useful this gives no indication of the amount of growth of the fungus under antagonistic conditions. This aspect can best be investigated by considerations of the theoretical colonization index. The theoretical colonization index is a measure of the overall growth of the fungus under antagonistic conditions and can be readily calculated from data obtained in the assessment of sensitivity of germination to fungistasis. This index encompasses three of the four factors involved in Garrett's concept of competitive saprophytic ability (i.e. germination and growth rates, enzyme production and tolerance of antagonism); it does not include antibiotic production. Although there was a large variation in the theoretical colonization index of the Trichoderma isolates, in most cases substantial growth was made under antagonistic conditions.

One of the advantages of this index is that it is possible, by correlation analysis, to assess the relative importance of each of the germination parameters in determining the subsequent growth of the fungus in soil i.e. the importance of the final percentage germination in governing the theoretical colonization index. The critical periods in spore germination with respect to the ecology of a fungus can therefore be highlighted. Knowledge of these factors will enable more

critical experiments than have been possible in the past to be developed so that a close study may be made of the role of antagonism at various stages of fungal development.

It has therefore been established that some of the Trichoderma isolates may be relatively sensitive to the effects of fungistasis but that they all were capable of actively growing in soil; in some cases large amounts of growth would be expected.

It was pointed out by Garrett (1970) that the competitive saprophytic ability of a fungus will be modified by the substrate being colonized by the nature of the ability of the colonizing and competing fungi to utilize that substrate. It was shown (Chapter Two) that the nature of the sporulation medium has relatively little effect upon the sensitivity of the resultant spore population to fungistasis as measured by the percentage reduction of each of the germination parameters with respect to their controls; but that the theoretical colonization index was able to indicate that the nature of the sporulation medium does have a profound effect on the overall growth of the fungus. The theoretical colonization index showed that there was a large difference in the overall growth of the different spore populations under antagonistic conditions, such that the theoretical colonization index of the sucrose spore population was 6 times greater than that of the glucose population. In terms of growth and colonization of a substrate in soil this substantial difference may be quite crucial in the survival of the fungus in soil. It would be interesting to test this hypothesis directly in soil to ascertain

whether in fact in the antagonistic environment of the soil the superior growth of the sucrose spore population over that of the glucose population occurs and whether this will result in a greater incidence of colonization of the substrate by the sucrose population than the glucose population.

As the Trichoderma isolates have been shown to be capable of growth in soil it is therefore valid that consideration should now be given to their antibiotic production (the remaining component of competitive saprophytic ability) and its relationship to colonization of substrates in soil.

All of the Trichoderma isolates tested produced non-volatile antibiotics which were active against a wide range of fungi. A peptide antibiotic similar to alamethicine or suzukacillin was shown to be produced by most of the isolates but none of the other known or suspected Trichoderma antibiotics (e.g. gliotoxin, viridin, and trichodermin) were found to be produced by any of the isolates examined.

An interesting aspect of the activity of the Trichoderma culture filtrates against the test fungi was that they were more active against germ tube growth than against percentage germination. This would indicate that their greatest ecological importance lies in inhibiting the growth of hyphae of other fungi trying to colonize the same substrate. In this context it is interesting to note the widespread ability of Trichoderma species to coil around hyphae of other fungi reported by Dennis (1970; Dennis & Webster, 1971c). This ability will ensure that the antibiotics produced will be in high concentration in the immediate vicinity of the 'host' fungus.

The time course experiment brought to light another interesting relationship. In the early stages of growth of the Trichoderma the culture filtrate was more active against percentage germination of Gliocladium roseum spores although there was some inhibition of germ tube growth. As time progressed the culture filtrate became more inhibitory towards germ tube growth whilst the activity towards percentage germination was maintained. It is immediately apparent that this relationship will confer an advantage on the Trichoderma in a competitive situation. When the Trichoderma spore population is stimulated to germinate by diffusion of nutrients from a substrate other potential competitors will be likewise stimulated. The Trichoderma, by virtue of its high germination and growth rate will be able to rapidly produce antibiotics which will initially retard the germination of the competing fungi and as the Trichoderma continues its growth it will then be able to inhibit hyphal growth of the competitors and will thus ensure itself a high competitive saprophytic ability. It is the summation of these factors that has resulted in the genus, as whole, being referred to as primary colonizers. There will be obvious differences in the ability of different Trichoderma isolates to compete under different conditions, this will be a reflection, in many cases of their ability to utilize the substrate and also their tolerance to antibiotics produced by other, competing, fungi.

In this respect the results of the investigation of the production of non-volatile antibiotics on different nutrient sources are of interest. There was variation in the nature and quantity of the antibiotics produced on the different

nutrient sources and also in the biological activity of the culture filtrates towards Gliocladium roseum conidia. The variation in the nature and quantity of the antibiotics produced will probably be a reflection of the ability and manner in which the different nutrients are metabolized. To elucidate this aspect further a more detailed biochemical investigation needs to be carried out. Supplying the fungus with radioactively-labelled C- and N-sources and then following their metabolism and production of metabolites by a tracer technique is the obvious answer to this problem. Although the approach may appear simple the actual interpretation of the biochemical pathways involved may prove to be very complex. It would be interesting if this sort of experiment could be carried out using naturally occurring substances so that a less artificial situation could be studied than has been in this work.

The variation in the biological activity of the culture filtrates from the different nutrient sources towards Gliocladium roseum can be resolved into two components which will be specific for the nutrient in the medium; the amount of antibiotics produced by the Trichoderma, and secondly the inherent ability of the Gliocladium to utilize the nutrients. The Gliocladium may be able to produce a large amount of growth on a particular medium such that in spite of the inhibitory effect of antibiotic production by Trichoderma a relatively large amount of mycelium is produced. The ability of the Trichoderma to produce antibiotics on the different media will also vary. It is possible from the results of this experiment to draw some conclusions concerning the interaction between Trichoderma, Gliocladium and substrate. The substrate will

by the nature of its chemical composition influence the ability of the Trichoderma to germinate in an antagonistic environment and to grow on that substrate and produce antibiotics inimical to the Gliocladium. The substrate will also act in a similar manner upon the Gliocladium and will also determine its response to the Trichoderma antibiotics. The substrate can therefore be seen as being the deciding influence in the outcome of competition between two competing fungi. The competitive saprophytic ability of a fungus will therefore be determined by the nature of the substrate involved.

All the Trichoderma isolates tested also produced volatile antibiotics but they were not as active towards germination of Gliocladium roseum conidia as the non-volatile antibiotics. The chemical nature of the substrate was again found to be influential in the biological activity of the volatile antibiotics produced by T. viride (48) with respect to time. Greatest biological activity was observed at initiation of sporulation of the Trichoderma culture which corresponded to the greatest production of the 'coconut' smell. It is therefore probable that the compound responsible for this smell (6-pentyl-α-pyrone), or other volatile metabolites associated with its production, is likely to be involved in the biological activity of the volatile metabolites. Trichoderma viride has been observed to sporulate in soil (Jackson, personal communication) so that it is possible that the volatile metabolites may be biologically active in soil and exert an antagonistic effect that may be advantageous to the Trichoderma.

Inherent in an investigation of fungal ecology is the problem of whether antagonistic interactions between fungi observed under laboratory conditions occur in soil. There has been much debate as to whether antibiotics are produced in soil and if they are whether their production will be of any advantage to the producer.

The presence in soil of antibiotics produced by organisms inoculated into that soil has been demonstrated by many workers (see Brian, 1949, 1957). Most of such work has been done with sterilized soil with or without organic amendments; the amount of antibiotic produced was greater in supplemented than in non-supplemented soil. This indicates that the presence of organic material, mainly carbon sources may be necessary for antibiotic production in soil. In this context it must be remembered that natural soil is composed of a complex of discontinuous microhabitats which may differ widely one from another in any of the factors which determine microbial behaviour within them.

It has been unequivocally demonstrated by Wright (1952) that gliotoxin could be produced in amounts detectable by paper chromatography in unsterilized but heavily supplemented soil. Wright (1954, 1956a, 1956b), also demonstrated production of gliotoxin in pieces of straw and on the surface of seeds which had been inoculated with Trichoderma viride and buried in soil. It has been subsequently established (Dennis, 1970) that the fungus used was in fact Gliocladium virens. It cannot be doubted in these instances, that concentrations of antibiotics sufficient

to influence the pattern of microbial colonization of these substrates must occur. Similar concentrations are also likely to occur in smaller pieces of organic matter in which the quantities of antibiotics produced are too small for detection by conventional means. Jackson (1965), advocates that the development of microtechniques, such as used by Stevenson (1956) and Rangaswarmi & Ethiraj (1962), will result in greater understanding of antibiosis in microhabitats.

In natural conditions, therefore one can envisage that antagonism between soil fungi is not something that takes place evenly throughout the soil but is concentrated in space in the immediate vicinity of available organic substrates. Antagonism is likely to be of greatest ecological significance at the surface of viable seeds or in the rhizosphere and rhizoplane of roots where there are large numbers of fungi (both saprophytes and parasites); their numbers probably being determined by the substances diffusing from the root or seed tissues.

Assuming that antibiotics are produced in soil which seems highly probable there remains the problem of whether they will be biologically active in the soil. In this context the adsorption of antibiotics, biological and chemical breakdown, and the stability of antibiotics at different pH levels are of importance (Jeffreys, 1952). Adsorption of antibiotics on clay colloids is more usually confined to actinomycete antibiotics and some of the bacterial polypeptide antibiotics. Neutral or acidic antibiotics are not adsorbed to the same extent. The accumulation and activity

of antibiotics in soil away from microhabitats of active metabolism has therefore not generally been regarded as plausible.

Although polypeptide antibiotics are often adsorbed onto clay colloids, the polypeptide antibiotics produced by Trichoderma spp have been found to contain different amino acids from those contained in bacterial polypeptide antibiotics (Dennis, 1970; Dennis & Webster, 1971a) and thus may not be as strongly adsorbed. Alamethicine has been shown to titrate as an acid (Meyer & Reusser, 1967) and therefore would not be strongly adsorbed. Other polypeptide antibiotics that are produced by Trichoderma appear to contain the same amino acids as alamethicine and will therefore presumably have similar chemical properties. Even if the antibiotics were either adsorbed or chemically broken down their biological activity may be ensured by the coiling habit of Trichoderma hyphae around other fungal mycelia. This will ensure that the concentration of the antibiotics at the surface of the antagonised fungus will be maintained at a high level.

The metabolites produced by fungi (staling compounds) are thought by Park (1960) to be responsible for the general soil fungistasis. Park's theory was supported by conclusions reached by Griffin (1962). Griffin found that autoclaved soil reinfected with a variety of microorganisms developed fungistatic properties, but that there was no correlation between ability to produce a fungistatic effect in soil and production of an antibiotic effect in culture. Griffin

postulated that normal soil fungistasis may be partly a result of the general saprophytic activities of the soil microflora and partly that of toxic metabolites other than specific antibiotics. Park and co-workers (Robinson, Park & Garrett, 1968; Robinson & Park, 1966; Robinson & Garrett, 1969; Garrett & Robinson, 1969) have found that acetaldehyde and other volatile metabolites are detectable in soil samples and put forward the hypothesis that they may be present in such high concentrations in microhabitats as to be responsible for fungistasis. Further Garrett (1972) has reported that the sporostatic substances acetaldehyde, n-propanol and nonanoic acid were present in soil and that it is possible that they could contribute to soil fungistasis.

One of these sporostatic compounds, acetaldehyde, has been shown to be produced by two isolates of Trichoderma viride (Dennis & Webster, 1971a). Hora & Baker (1972) have shown that when 6 isolates of Trichoderma spp were added individually to sterilized soil and incubated for 7 - 10 days there was a reduction in spore germination of 4 test fungi on agar blocks placed above the soil. Thus these isolates were capable of producing a volatile fungistatic factor in soil.

It therefore seems likely that antibiotics, both volatile and non-volatile, account at least in part for the antagonisms between Trichoderma isolates and other fungi in the natural environment. Such antibiotic production and hence antagonism is likely to occur in microhabitats and that the nutrient status of the substrate will have a profound effect on the outcome of antagonistic interactions with other fungi on that substrate. It is possible, also, that these

antibiotics may contribute to the general fungistatic level of soil.

The antagonistic properties of fungi in soil has focussed the attention of plant pathologists on to their possible use as agents of biological control. This is particularly true of members of the genus Trichoderma. Biological control usually involves the introduction of the Trichoderma into an environment which is relatively permanent and stable and where it is not already present and therefore unlikely to survive. If, however, at least one factor of the environment is changed, the chances of successful introduction may be improved. This may be achieved by sterilization of the soil but this produces drastic changes, and the introduction of biological control agents after such treatment may not be successful because of the unstable conditions created. Trichoderma isolates however have been found to be highly resistant to a wide range of fumigants and herbicides (Bliss, 1951; Hameed & Foy, 1971; Hodges, 1960; Moje et al 1957; Moubasher, 1963; Mughogho, 1968; Richardson, 1954; Singh, 1970; Warcup, 1951; Wilkinson & Lucas, 1969) which means that they can rapidly recolonize fumigated soil and be able, possibly, to exert an antagonistic effect which may be used to combat the incidence of fungal, particularly root, diseases. Garrett (1958) pointed out that the extent of biological control obtained would depend on the inoculum potential of the Trichoderma that developed in the fumigated soil. Mughogho (1968) has shown that the inoculum potential of Trichoderma spp in soil may be high in fumigated soils but that their activity may be inhibited by the presence of other antagonistic microorganisms. Mughogho (1968) suggests that the

failure of dominant populations of Trichoderma in fumigated soils in his experiments to limit the growth of Rhizoctonia solani and Armillaria mellea was due to the fact that these dominant populations must have been made up largely by strains of Trichoderma species groups that were not effective antagonists. He found that members of the T. viride species group were the most effective antagonists and that biological control would be possible if a fumigant which promoted the growth of T. viride, at the expense of other fungi, were developed. From the present work, however, the T. hamatum, T. harzianum, T. koningii, and T. pseudokoningii species groups were all better antagonists than the T. viride species group which would indicate therefore that a large scale screening of Trichoderma isolates would be necessary in order to select the best antagonist for the particular conditions involved.

Trichoderma spp have been shown to be antagonistic to a wide range of root infecting fungi (Boosalis, 1956; Cambell, 1956; Fedorinchik, 1961, 1964; Joffe, 1966; Lai & Bruehl, 1968; Mathur, 1965; Negrutskii & Sychev, 1969; Pudelko, 1970; Rodriguez-Kabana & Curl, 1968; Sanford, 1959; Wood & Tveit, 1955). These investigators have demonstrated control or at least reduction of fungal root diseases in sterile or amended soil by inoculation with Trichoderma spp. Unfortunately when extended to unsterile soil attempts to control the same disease were either unsuccessful or inconclusive. Successful control of Rhizoctonia solani by Trichoderma in unsterile soil was observed by Ogura & Akai (1965) but only when a very high Trichoderma population was

maintained. Control of Sclerotium rolfsii by T. harzianum (Wells et al, 1972); control of Armillaria mellea attack of citrus roots (Ohr & Munnecke, 1972) and charcoal rot disease of Abelmoschus esculantus (Rao & Mukerji, 1972) by Trichoderma viride have been reported.

Biological control of phytopathogenic fungi has been affected by Russian workers by increasing the population of antagonistic Trichoderma spp by directly increasing the inoculum density of the fungus by using biological preparations known as Trichodermin -1, -2, -3. These biological preparations are cultures of antagonistic Trichoderma isolates maintained on an organic substrate. Trichodermin-3, which is in widespread use in Russia is obtained by growing Trichoderma isolates on peat. The preparation is added directly to the soil prior to planting or sowing, the peat also acts as an organic fertilizer. Trichodermin-1 and -2 are grown on cereal grains, chaff and cut straw. Using such preparations several workers (Fedorinchik, 1961, 1964; Nugrutskii & Sychev, 1969) have reported control or reduction in the incidence of diseases of crop plants caused by phytopathogenic fungi under field conditions. Fedorinchik & Buga (1971) have reported a further method in which wheat seeds are pelleted with either spores of T. lignorum or with a carrier (peat) of a definite titre of spores. Using this method they achieved suppression of Helminthosporium sativum pathogenicity under field conditions.

The use of T. viride to control storage rot of hardwood timber has on the other hand met with more success (Hunt et al, 1971; Shields, 1968; Shields & Atwell, 1963). This is probably because there are fewer problems in maintaining

a large Trichoderma population on timber compared with soil.

For biological control, using Trichoderma spp, to be an economically viable proposition it would be necessary to develop a means of establishing a large and active Trichoderma population in a soil from which it may not have been isolated from. It is unlikely that this will be possible until a great deal more is known about interactions between microorganisms in soil. Whether, in the final analysis, biological control using antagonistic organisms is likely to be economically viable will, I think, depend to a large extent on the nature of the disease and the ease with which it can be controlled. Even so it is a project that would benefit from an economic analysis.

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Personal communication

JACKSON, R.M. (1971). Department of Biological Sciences, University of Surrey, Guildford, Surrey.

APPENDIX

APPENDIX 1

Sources of *Trichoderma* isolates

T. hamatum

(15) Dr. C. Dennis, ARC Food Research Institute, Norwich.

(22) Dr. N.J. Dix, University of Stirling, Scotland.

T. harzianum

(1) Dr. C. Dennis

(129) Dr. C. Dennis

(20) Dr. N.J. Dix

T. koningii (4) Dr. C. Dennis

T. longibrachiatum (W.B.C. 4576) Dr. C. Dennis

T. polysporum (C306) Dr. C. Dennis

T. pseudokoningii (P.H.B.T.-H1) Dr. C. Dennis

T. saturnispora (IMI 146852) Commonwealth Mycological Institute, Kew, England.

T. viride

(2) Dr. C. Dennis

(14) Dr. C. Dennis

(28) Dr. N.J. Dix

(48) Soil, Hermitage Wood, Stirling, Scotland

(49) " " " "

(50) Rotting wood, Hermitage Wood, Stirling

(51) Soil, Hermitage Wood, Stirling

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APPENDIX 2

Sources of Test Fungi

Gliocladium roseum (1)	Dr. N.J. Dix, University of Stirling, Scotland.
Gliocladium roseum (2)	Soil, Hermitage Wood, Stirling, Scotland.
Gliocladium virens (211)	Dr. C. Dennis, ARC Food Research Institute, Norwich.
Cladosporium herbarum	Dr. N.J. Dix
Trichocladium opacum	Dr. N.J. Dix
Stemphylium dendriticum	Dr. N.J. Dix
Fusarium solani	Dr. N.J. Dix
Fusarium culmorum	Dr. N.J. Dix
Botrytis allii	Dr. J.W. Mansfield, University of Stirling, Scotland.
Botrytis cinerea	Dr. J.W. Mansfield
Penicillium expansum	Dr. J.W. Mansfield
Penicillium funiculosum	Dr. N.J. Dix
Penicillium janthinellum	Dr. N.J. Dix
Penicillium nigricans	Dr. N.J. Dix
Penicillium spinulosum	Dr. N.J. Dix

APPENDIX 3

Media and Stains

Weindlings Medium

D-Glucose	25.0g
Ammonium tartrate $(CHOHCOONH_4)_2$	2.0g
Potassium dihydrogen phosphate (KH_2PO_4)	2.0g
Magnesium sulphate $(MgSO_4.7H_2O)$	1.0g
Ferrous sulphate $(FeSO_4)$	0.01g
Distilled water	1.0 l

Autoclaved 15 min at 103.4 kNm^{-2}

Modified Weindlings Medium

Normal Weindlings medium	
Oxoid Agar No 3	20.0g
Distilled water	1.0 l

Peptone Agar

Mycological peptone	10.0g
Oxoid Agar No 3	20.0g
Distilled water	1.0 l

Potato Dextrose Agar (P.D.A.)

Potato extract (Oxoid)	4.0g
D+Glucose	20.0g
Oxoid Agar No 1	15.0g
Distilled water	1.0 l

Carbon sources - added at the rate of 1000ppm C l^{-1} to basal Weindlings medium

Monosaccharides	D-Glucose	D+Xylose
	D-Fructose	L+Rhamnose
	D+Galactose	Mannitol
	D+Mannose	D-Sorbitol
	D+Ribose	L-Sorbose
Disaccharides	Sucrose	
	Lactose	
	Maltose	
Trisaccharide	Raffinose	
Polysaccharide	Starch	

Nitrogen sources - added at the rate of 300ppm N l^{-1} to basal Weindlings medium

Ammonium tartrate	Glycine

APPENDIX 3

Media and Stains

Weindlings Medium

D-Glucose	25.0g
Ammonium tartrate $(CHOHCOONH_4)_2$	2.0g
Potassium dihydrogen phosphate (KH_2PO_4)	2.0g
Magnesium sulphate $(MgSO_4 \cdot 7H_2O)$	1.0g
Ferrous sulphate $(FeSO_4)$	0.01g
Distilled water	1.0 l

Autoclaved 15 min at 103.4 kNm^{-2}

Modified Weindlings Medium

Normal Weindlings medium	
Oxoid Agar No 3	20.0g
Distilled water	1.0 l

Peptone Agar

Mycological peptone	10.0g
Oxoid Agar No 3	20.0g
Distilled water	1.0 l

Potato Dextrose Agar (P.D.A.)

Potato extract (Oxoid)	4.0g
D+Glucose	20.0g
Oxoid Agar No 1	15.0g
Distilled water	1.0 l

Carbon sources - added at the rate of 1000ppm C l^{-1} to basal Weindlings medium

Monosaccharides	D-Glucose	D+Xylose
	D-Fructose	L+Rhamnose
	D+Galactose	Mannitol
	D+Mannose	D-Sorbitol
	D+Ribose	L-Sorbose
Disaccharides	Sucrose	
	Lactose	
	Maltose	
Trisaccharide	Raffinose	
Polysaccharide	Starch	

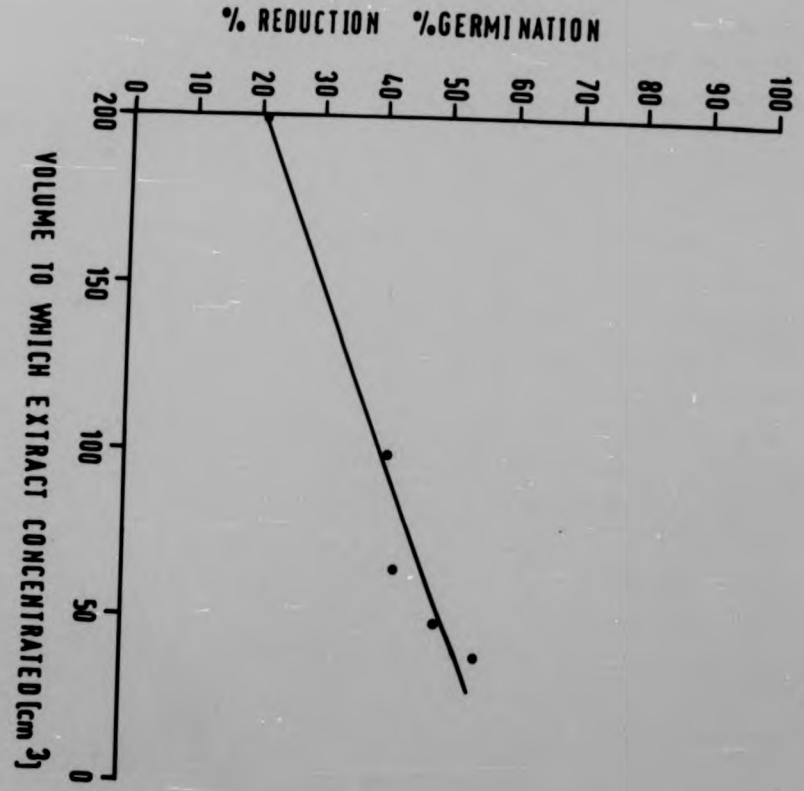
Nitrogen sources - added at the rate of 300ppm N l^{-1} to basal Weindlings medium

Ammonium tartrate	Glycine

Ammonium sulphate	L-Glutamic acid
Ammonium chloride	γ-Amino butyric acid
Sodium nitrate	L-Phenylalanine
Peptone	L-Cysteine

Lactophenol and Cotton Blue Stain

Phenol (pure crystals)	20.0g
Lactic acid (S.G. 1.21)	20.0g
Glycine	40.0g
Distilled water	20.0g
Cotton Blue	0.5g



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APPENDIX 4

Determination of Concentration of *Trichoderma* culture filtrates for use in Bioassay technique

The experiment was conducted to determine the volume to which the *Trichoderma viride* (48) culture filtrate had to be concentrated to give 50 % reduction in percentage germination of *Gliocladium roseum* (1) conidia.

The culture filtrate (200 cm^3) was concentrated to 40 cm^3 with the rotary vacuum evaporator at 40°C and dilutions prepared to give concentrations equivalent to concentrating the culture filtrate to 100, 66.6, and 50 cm^3. The dilutions were then tested for biological activity against the percentage germination of *Gliocladium roseum* conidia after 18h incubation (see Materials and Methods).

The results are given in the accompanying graph of percentage reduction in percentage germination against the volume to which the culture filtrate was concentrated.

The volume to which the culture filtrate had to be concentrated to give 50 % reduction in percentage germination of *G. roseum* conidia was approximately 50 cm^3.

The culture filtrates were all concentrated to this volume before being assayed for biological activity.

APPENDIX 5

Chromatography

T.L.C. Plates

Camag 20 x 20 cm pre-coated T.L.C. plates (0.25 cm thick) with a UV indicator were used throughout.

Micropipettes

The material to be chromatogrammed was applied to the T.L.C. plate using a Drummond 'Microcap' disposable micropipette dispensing either 1 ul or 5 ul as required.

Chromatography Solvents

For Chloroform Soluble Extracts

1) Chloroform : Acetone; 95 : 5, v/v
   Running time 2 - 3 h
2) Ether : Ethyl acetate; 9 : 1, v/v
   Running time 20 min
3) Ethyl acetate : Cyclohexane; 50 : 50, v/v
   Running time 30 min

For Ethanol Soluble Extracts

1) Ethanol : Water; 70 : 30, v/v
   Running time 2 - 3 h
2) n-Butanol : Acetic acid : Water; 60 : 20 : 20, v/v

Chromatography Sprays

Silver nitrate reagent

Silver nitrate, saturated solution in water	1 vol
Acetone	20 vol

Phloroglucinol - hydrochloric acid reagent

Phloroglucinol, 0.7 % in acetone	9 vol
Hydrochloric acid, 40 % in water	1 vol

Starch

Soluble starch, 1 % in water

Sulphuric acid

Conc Sulphuric acid sprayed on T.L.C. plate, developed by heating to 100°C for 10 - 15 min.

APPENDIX 6

Regression equations for straight lines obtained from plot of log $\frac{N_{max} - N}{N}$ against time (see text)

Control

T. hamatum	
(15)	y = -0.1817x + 2.5201, r = -0.8933 P<0.001
(22)	y = -0.2167x + 3.0432, r = -0.9656 P<0.001
T. harzianum	
(1)	y = -0.2390x + 2.8112, r = -0.9097 P<0.01
(129)	y = -0.1139x + 1.9776, r = -0.8374 P<0.001
(20)	y = -0.2167x + 3.0296, r = -0.9683 P<0.001
T. koningii	y = -0.1270x + 1.9611, r = -0.8653 P<0.001
T. longibrachiatum	y = -0.0950x + 1.4138, r = -0.7857 P<0.001
T. pseudokoningii	y = -0.1507x + 2.3940, r = -0.9492 P<0.001
T. saturnispora	y = -0.1786x + 2.2206, r = -0.9045 P<0.001
T. viride	
(2)	y = -0.1418x + 2.0072, r = -0.9083 P<0.001
(14)	y = -0.1423x + 2.2375, r = -0.9233 P<0.001
(28)	y = -0.1367x + 2.3845, r = -0.9703 P<0.001
(48)	y = -0.1968x + 2.4521, r = -0.9344 P<0.001
(49)	y = -0.1541x + 1.9012, r = -0.7540 P<0.001
(50)	y = -0.2519x + 3.0067, r = -0.9711 P<0.001
(51)	y = -0.1447x + 2.3417, r = -0.9259 P<0.001
(52)	y = -0.1457x + 2.5309, r = -0.9789 P<0.001
(53)	y = -0.1383x + 2.3093, r = -0.9443 P<0.001
(54)	y = -0.1386x + 2.0227, r = -0.9004 P<0.001

Experiment

T. hamatum	
(15)	y = -0.1443x + 1.9819, r = -0.9117 P<0.001
(22)	y = -0.1701x + 2.3477, r = -0.9153 P<0.001
T. harzianum	
(1)	y = -0.2215x + 2.1338, r = -0.8309 P<0.05
(129)	y = -0.1109x + 2.0664, r = -0.9462 P<0.001
(20)	y = -0.1864x + 2.7336, r = -0.9845 P<0.001
T. koningii	y = -0.1229x + 1.9296, r = -0.9000 P<0.001
T. longibrachiatum	y = -0.1416x + 2.0309, r = -0.9533 P<0.001
T. pseudokoningii	y = -0.0817x + 1.5261, r = -0.7756 P<0.001
T. saturnispora	y = -0.1537x + 1.8660, r = -0.8360 P<0.001
T. viride	
(2)	y = -0.1344x + 2.4014, r = -0.9473 P<0.001
(14)	y = -0.0675x + 1.7011, r = -0.8279 P<0.001
(28)	y = -0.0986x + 2.4995, r = -0.9141 P<0.001
(48)	y = -0.1463x + 1.7417, r = -0.8347 P<0.001
(49)	y = -0.1703x + 2.2012, r = -0.9111 P<0.001
(50)	y = -0.1442x + 1.5536, r = -0.7051 P<0.001
(51)	y = -0.1437x + 2.3256, r = -0.9438 P<0.001
(52)	y = -0.0832x + 1.8775, r = -0.9414 P<0.001
(53)	y = -0.0453x + 0.9184, r = -0.6980 P<0.01
(54)	y = -0.0986x + 1.5151, r = -0.8270 P<0.001

APPENDIX 7

Regression equations for straight line obtained from a plot of log germ tube length against time (see text).

Control

T. hamatum	
(15)	$y = 0.1222x - 0.9300$, $r = 0.9789$ $P<0.001$
(22)	$y = 0.0970x - 0.3977$, $r = 0.9924$ $P<0.001$
T. harzianum	
(1)	$y = 0.0938x - 0.1053$, $r = 0.9344$ $P<0.01$
(129)	$y = 0.0433x + 0.1256$, $r = 0.9722$ $P<0.01$
(20)	$y = 0.1133x - 0.7513$, $r = 0.9542$ $P<0.001$
T. koningii	$y = 0.0036x + 1.0865$, $r = 0.9556$ $P<0.01$
T. longibrachiatum	$y = 0.0224x + 0.6149$, $r = 0.9679$ $P<0.01$
T. pseudokoningii	$y = 0.0742x + 0.1453$, $r = 0.9177$ $P<0.05$
T. saturnispora	$y = 0.0581x + 0.2491$, $r = 0.9730$ $P<0.001$
T. viride	
(2)	$y = 0.0251x + 0.4136$, $r = 0.9775$ $P<0.01$
(14)	$y = 0.0828x - 0.1981$, $r = 0.9374$ $P<0.05$
(28)	$y = 0.0711x - 0.1029$, $r = 0.9712$ $P<0.01$
(48)	$y = 0.1093x - 0.4538$, $r = 0.9649$ $P<0.001$
(49)	$y = 0.1069x - 0.3999$, $r = 0.9530$ $P<0.001$
(50)	$y = 0.1338x - 0.6626$, $r = 0.9582$ $P<0.001$
(51)	$y = 0.0849x - 0.2952$, $r = 0.9505$ $P<0.05$
(52)	$y = 0.0612x + 0.3078$, $r = 0.8818$ $P<0.05$
(53)	$y = 0.0718x + 0.0991$, $r = 0.8952$ $P<0.05$
(54)	$y = 0.0463x + 0.8135$, $r = 0.8827$ $P<0.05$

Experiment

T. hamatum	
(15)	$y = 0.0673x - 0.3205$, $r = 0.9086$ $P<0.01$
(22)	$y = 0.0705x - 0.1991$, $r = 0.9342$ $P<0.05$
T. harzianum	
(1)	$y = 0.0804x - 0.0477$, $r = 0.9602$ $P<0.01$
(129)	$y = 0.0140x + 0.4223$, $r = 0.9652$ $P<0.01$
(20)	$y = 0.0792x - 0.3772$, $r = 0.8916$ $P<0.01$
T. koningii	$y = 0.0102x + 0.5470$, $r = 0.9550$ $P<0.01$
T. longibrachiatum	$y = 0.0076x + 0.6902$, $r = 0.9972$ $P<0.001$
T. pseudokoningii	$y = 0.0294x + 0.2851$, $r = 0.9644$ $P<0.01$
T. saturnispora	$y = 0.0467x + 0.3128$, $r = 0.9239$ $P<0.01$
T. viride	
(2)	$y = 0.0281x + 0.2017$, $r = 0.9700$ $P<0.01$
(14)	$y = 0.0610x - 0.2851$, $r = 0.9958$ $P<0.001$
(28)	$y = 0.0964x - 1.4011$, $r = 0.9702$ $P<0.05$
(48)	$y = 0.0642x + 0.1456$, $r = 0.8895$ $P<0.01$
(49)	$y = 0.0569x + 0.0897$, $r = 0.9687$ $P<0.001$
(50)	$y = 0.1092x - 0.3811$, $r = 0.9731$ $P<0.001$
(51)	$y = 0.0669x - 0.4701$, $r = 0.9947$ $P<0.001$
(52)	$y = 0.0317x + 0.2739$, $r = 0.9964$ $P<0.001$
(53)	$y = 0.0166x + 0.2739$, $r = 0.9387$ $P<0.05$
(54)	$y = 0.0501x + 0.4236$, $r = 0.9170$ $P<0.05$

APPENDIX 8

Regression equations for straight line obtained from a plot of log $\frac{N_{max} - N}{T}$ against time for the germination of *T. viride* (48) spores produced on different sporulation media.

Control

C-source in sporulation medium	
Glucose	$y = -0.2258x + 3.2587$, $r = -0.9740$ $P<0.001$
Sucrose	$y = -0.2912x + 3.5010$, $r = -0.9915$ $P<0.001$
Fructose	$y = -0.3050x + 3.8973$, $r = -0.9595$ $P<0.001$
Lactose	$y = -0.3091x + 3.9258$, $r = -0.9737$ $P<0.001$
Xylose	$y = -0.2590x + 3.1093$, $r = -0.9729$ $P<0.001$
Mannitol	$y = -0.2628x + 3.0816$, $r = -0.9595$ $P<0.001$

Experiment

Glucose	$y = -0.2509x + 3.3149$, $r = -0.9680$ $P<0.001$
Sucrose	$y = -0.2414x + 2.6820$, $r = -0.8970$ $P<0.001$
Fructose	$y = -0.3071x + 3.9039$, $r = -0.9795$ $P<0.001$
Lactose	$y = -0.2691x + 3.3264$, $r = -0.9733$ $P<0.001$
Xylose	$y = -0.1832x + 2.1697$, $r = -0.8579$ $P<0.001$
Mannitol	$y = -0.2065x + 2.3466$, $r = -0.9074$ $P<0.01$

APPENDIX 9

Regression equations of straight lines obtained fron a plot of log germ tube length against time for spores of T. viride (48) produced on different sporulation media.

Control

C-source in sporulation medium	
Glucose	y = 0.1309x - 1.0310, r = 0.9868 P<0.001
Sucrose	y = 0.1333x - 0.4676, r = 0.9874 P<0.001
Fructose	y = 0.1293x - 1.0295, r = 0.9328 P<0.001
Lactose	y = 0.1705x - 1.4044, r = 0.9648 P<0.001
Xylose	y = 0.1524x - 1.0786, r = 0.9797 P<0.001
Mannitol	y = 0.1477x - 0.9893, r = 0.9941 P<0.001

Experiment

Glucose	y = 0.1087x - 0.8878, r = 0.9610 P<0.001
Sucrose	y = 0.1265x - 0.5910, r = 0.9695 P<0.001
Fructose	y = 0.1379x - 1.0726, r = 0.9794 P<0.001
Lactose	y = 0.1413x - 1.0862, r = 0.9843 P<0.001
Xylose	y = 0.0946x - 0.4244, r = 0.9865 P<0.001
Mannitol	y = 0.0966x - 0.4307, r = 0.9835 P<0.01

APPENDIX 10

Regression equations of straight lines obtained from a plot of log theoretical colonization index against time for spores of *T. viride* (48) produced on different sporulation media.

Control

C-source in sporulation medium	
Glucose	$y = 0.2537x - 1.3347$, $r = 0.9902$ $P<0.001$
Sucrose	$y = 0.2378x - 0.2501$, $r = 0.9751$ $P<0.001$
Fructose	$y = 0.2775x - 1.5884$, $r = 0.9959$ $P<0.001$
Lactose	$y = 0.3540x - 2.4960$, $r = 0.9891$ $P<0.001$
Xylose	$y = 0.2354x - 0.5663$, $r = 0.9929$ $P<0.001$
Mannitol	$y = 0.2524x - 0.7342$, $r = 0.9885$ $P<0.001$

Experiment

Glucose	$y = 0.2300x - 1.2214$, $r = 0.9448$ $P<0.001$
Sucrose	$y = 0.1753x - 0.4357$, $r = 0.9571$ $P<0.01$
Fructose	$y = 0.2886x - 1.6681$, $r = 0.9818$ $P<0.001$
Lactose	$y = 0.2652x - 1.2554$, $r = 0.9888$ $P<0.001$
Xylose	$y = 0.1394x + 0.5645$, $r = 0.9974$ $P<0.001$
Mannitol	$y = 0.1492x + 0.4725$, $r = 0.9517$ $P<0.01$

APPENDIX 11

Regression equations of straight line obtained from a plot of log increase in conductivity in de-ionised water against time for spores of T. viride (48) produced on different sporulation media.

C-source in sporulation medium	
Glucose	y = 0.6695x - 0.8572, r = 0.9669 P<0.001
Sucrose	y = 0.6390x - 0.5504, r = 0.9251 P<0.001
Fructose	y = 0.4128x - 0.1207, r = 0.9587 P<0.001
Lactose	y = 0.7338x - 0.7321, r = 0.9890 P<0.001
Xylose	y = 0.3869x - 0.4122, r = 0.9760 P<0.001
Mannitol	y = 0.7653x - 0.8640, r = 0.9605 P<0.001

APPENDIX 12

Germination of *Trichoderma* isolates in Weindlings Medium (i.e. in controls)

	Percentage Germination	Germ Tube Length
T. hamatum		
(15)	43.9	11.0
(22)	69.1	13.0
T. harzianum		
(1)	71.8	15.0
(129)	71.3	17.7
(20)	43.4	9.5
T. koningii	55.5	24.6
T. longibrachiatum	81.2	7.7
T. pseudokoningii	65.7	8.3
T. saturnispora	65.7	10.1
T. viride		
(2)	72.7	5.4
(14)	66.2	12.1
(28)	50.6	21.9
(48)	83.0	5.5
(49)	56.2	12.0
(50)	83.8	6.1
(51)	67.1	16.0
(52)	54.1	15.4
(53)	41.7	12.9
(54)	69.7	7.3

APPENDIX 13

Effect of *Trichoderma* culture filtrates on Germination of *Gliocladium roseum* (1) conidia (as % reduction)

	Percentage Germination	Germ Tube Length	Theoretical Colonization Index
T. hamatum			
(15)	45.3***	56.1***	80.0
(22)	25.6**	41.4***	56.4
T. harzianum			
(1)	34.4***	56.0***	71.2
(129)	8.8 NS	30.0***	36.2
(20)	27.0**	58.5***	69.7
T. koningii	37.3***	50.4***	68.9
T. longibrachiatum	23.1**	28.7***	45.2
T. polysporum	10.9 NS	30.2***	37.8
T. pseudokoningii	20.1*	50.0***	60.1
T. saturnispora	16.4 NS	33.5***	44.1
T. viride			
(2)	31.6***	44.8***	62.2
(14)	21.1*	46.0***	57.5
(28)	24.7***	39.8***	54.7
(48)	39.0***	50.2***	69.6
(49)	23.8***	39.3***	53.7
(50)	31.0***	43.3***	60.9
(51)	21.8***	47.7***	59.1
(52)	18.1**	40.0***	50.8
(53)	26.1**	37.8***	54.0
(54)	26.8***	49.2***	62.8

* Significant P<0.05)
** " P<0.01) Student's 't' test (see text)
*** " P<0.001)
NS Not significant P>0.05)

Control 51.9% germination
13.4 germ tube length

APPENDIX 14

Effect of *Trichoderma* culture filtrates on Germination of *Gliocladium roseum* (2) conidia (as % reduction)

	Percentage Germination	Germ Tube Length	Theoretical Colonization Index
T. hamatum			
(15)	20.0*	45.4***	56.3
(22)	20.5*	0	6.9
T. harzianum			
(1)	22.3*	40.2***	53.5
(129)	20.0*	34.5***	47.6
(20)	43.3**	32.9***	62.0
T. koningii	35.3**	44.2***	63.9
T. longibrachiatum	21.2*	10.7 NS	29.7
T. polysporum	19.3*	11.6 NS	28.6
T. pseudokoningii	24.4*	29.4***	46.6
T. saturnispora	13.5 NS	33.3***	42.3
T. viride			
(2)	20.7*	26.1***	41.4
(14)	22.8*	41.6***	54.9
(28)	18.0*	29.1***	41.9
(48)	40.2*	35.6***	61.5
(49)	20.9*	17.4**	34.6
(50)	14.5 NS	22.9***	34.1
(51)	13.5 NS	30.3***	39.8
(52)	16.8*	31.2***	42.7
(53)	22.0*	33.1***	47.8
(54)	29.1*	26.3***	47.7

* Significant P<0.05)
** " P<0.01) Student's 't' test
*** " P<0.001) (see text)
NS Not significant P>0.05)

Control 52.8 % germination
10.6 germ tube length

APPENDIX 15

Effect of *Trichoderma* culture filtrates on Germination of *Gliocladium virens* (211) conidia (as % reduction)

	Percentage Germination	Germ Tube Length	Theoretical Colonization Index
T. hamatum			
(15)	20.3***	61.7***	69.5
(22)	11.5*	34.4**	42.0
T. harzianum			
(1)	31.8**	36.1***	56.4
(129)	30.5**	24.1**	47.3
(20)	33.7**	24.8 NS	50.7
T. koningii	29.3**	44.8***	61.0
T. longibrachiatum	23.0**	40.9***	54.5
T. polysporum	24.0**	6.3 NS	28.8
T. pseudokoningii	30.2**	17.6*	42.4
T. saturnispora	18.9**	38.3**	50.0
T. viride			
(2)	18.8*	30.4***	43.4
(14)	30.0**	45.7***	62.0
(28)	20.5***	43.8***	55.3
(48)	15.6 NS	39.0***	48.5
(49)	9.6 NS	28.8*	35.6
(50)	8.8 NS	24.3 NS	30.9
(51)	25.5**	38.2***	54.0
(52)	11.1*	35.6**	45.1
(53)	11.8 NS	54.9***	43.2
(54)	16.7***	45.0***	62.4

* Significant P<0.05)
** " P<0.01) Student's 't' test
*** " P<0.001) (see text)
NS Not significant P>0.05)

Control 70.1 % germination
31.7 germ tube length

APPENDIX 16

Effect of *Trichoderma* culture filtrates on Germination of *Cladosporium herbarum* conidia (as % reduction)

	Percentage Germination	Germ Tube Length	Theoretical Colonisation Index
T. hamatum			
(15)	39.8***	19.9 NS	40.4
(22)	22.5*	0 NS	17.8
T. harzianum			
(1)	43.3***	17.9 NS	53.5
(129)	29.4**	0 NS	23.0
(20)	41.7***	23.3*	55.3
T. koningii	30.6***	12.2 NS	39.1
T. longibrachiatum	18.1**	0 NS	12.7
T. polysporum	25.1***	3.8 NS	27.9
T. pseudokoningii	75.2***	17.8 NS	40.9
T. saturnispora	35.8***	17.3 NS	46.9
T. viride			
(2)	10.8**	0 NS	4.6
(14)	19.8**	23.2*	38.5
(28)	12.8**	10.2 NS	21.6
(48)	38.6***	2.5 NS	40.1
(49)	20.7**	31.0**	45.3
(50)	16.5**	12.3 NS	26.8
(51)	33.3***	25.9*	50.6
(52)	25.1***	7.5 NS	30.8
(53)	13.9***	10.4 NS	22.8
(54)	22.2***	8.4 NS	28.7

* Significant P<0.05)
** " P<0.01) Student's 't' test
*** " P<0.001) (see text)
NS Not significant P>0.05)

Control 63.6 % germination
11.2 germ tube length

APPENDIX 17

Effect of *Trichoderma* culture filtrates on Germination of *Trichocladium opacum* conidia (as % reduction)

	Percentage Germination	Germ Tube Length	Theoretical Colonization Index
T. hamatum			
(15)	23.2***	46.2***	58.6
(22)	20.8***	28.9**	43.7
T. harzianum			
(1)	12.6*	40.6***	48.1
(129)	9.5*	22.4*	29.8
(20)	8.5 NS	31.3***	37.1
T. koningii	14.2*	23.2**	34.2
T. longibrachiatum	17.8**	20.1*	34.4
T. polysporum	16.0**	38.6***	48.4
T. pseudokoningii	15.9*	45.9***	54.5
T. saturnispora	20.0***	25.2**	40.1
T. viride			
(2)	6.8 NS	18.8 NS	24.4
(14)	13.9**	37.0***	45.7
(28)	14.4**	31.9**	41.7
(48)	18.6*	43.4***	53.9
(49)	17.8**	31.0**	43.2
(50)	20.7**	34.4***	48.0
(51)	20.8***	29.8**	44.4
(52)	14.7**	33.8**	43.5
(53)	24.5**	32.2***	48.8
(54)	17.0**	35.1***	46.1

* Significant P<0.05)
** " P<0.01) Student's 't' test
*** " P<0.001) (see text)
NS Not significant P>0.05)

Control 78.3 % germination
76.7 germ tube length

APPENDIX 18

Effect of *Trichoderma* culture filtrates on Germination of *Stemphylium dendriticum* conidia (as % reduction)

	Percentage Germination	Germ Tube Length	Theoretical Colonization Index
T. hamatum			
(15)	27.5*	60.1***	79.0
(22)	23.6 NS	35.9*	51.1
T. harzianum			
(1)	47.0**	62.1***	79.9
(129)	17.9*	30.8**	43.2
(20)	42.8**	60.7***	77.5
T. koningii	19.6*	49.7***	59.5
T. longibrachiatum	28.5 NS	25.5 NS	46.7
T. polysporum	12.6 NS	46.1***	52.9
T. pseudokoningii	55.1***	56.0***	80.2
T. saturnispora	17.9 NS	35.5*	47.0
T. viride			
(2)	21.3 NS	40.6***	53.3
(14)	22.9*	42.2***	55.5
(28)	31.1 NS	42.1*	60.1
(48)	27.6 NS	55.6***	67.9
(49)	23.1 NS	36.7*	51.4
(50)	22.7 NS	17.4 NS	36.2
(51)	40.4*	37.7*	62.9
(52)	34.6*	26.5 NS	51.9
(53)	23.2 NS	47.2**	59.4
(54)	29.6 NS	56.3***	69.3

* Significant P<0.05)
** " P<0.01) Student's 't' test
*** " P<0.001) (see text)
NS Not significant P>0.05)

Control 66.9 % germination
49.6 germ tube length

APPENDIX 19

Effect of *Trichoderma* culture filtrates on Germination of *Fusarium solani* macroconidia (as % reduction)

	Percentage Germination	Germ Tube Length	Theoretical Colonization Index
T. hamatum			
(15)	36.8***	49.8***	68.3
(22)	27.5***	37.0***	54.3
T. harzianum			
(1)	64.9***	64.6***	87.6
(129)	23.0**	42.7***	37.7
T. koningii	30.2***	38.0***	56.8
T. longibrachiatum	28.2***	23.4**	45.0
T. polysporum	2.6**	20.0*	22.1
T. pseudokoningii	28.9**	37.5***	55.6
T. saturnispora	20.1***	27.6***	42.2
T. viride			
(2)	29.2***	10.9 NS	36.9
(14)	27.4***	42.9***	58.5
(28)	27.8***	41.7***	58.0
(48)	40.1***	54.0***	72.4
(49)	19.7***	34.6***	47.5
(50)	29.5***	41.3***	58.6
(51)	34.9***	51.5***	68.5
(52)	29.2***	30.3***	50.7
(53)	33.6***	45.8***	64.0
(54)	56.0***	49.6***	77.8

* Significant P<0.05)
** " P<0.01) Student's 't' test
*** " P<0.001) (see text)
NS Not significant P>0.05)

Control 71.1 % germination
9.5 germ tube length

APPENDIX 20

Effect of *Trichoderma* culture filtrates on Germination of *Fusarium culmorum* macroconidia (as % reduction)

	Percentage Germination	Germ Tube Length	Theoretical Colonization Index
T. hamatum			
(15)	27.5**	41.7***	52.9
(22)	21.3***	5.3 NS	16.9
T. harzianum			
(1)	18.9**	44.3***	54.8
(129)	19.4**	32.6**	45.7
(20)	10.3*	37.8***	44.2
T. koningii	15.2**	47.4***	55.3
T. longibrachiatum	22.5***	39.6***	47.9
T. polysporum	2.5 NS	36.9***	38.4
T. pseudokoningii	16.6*	46.0***	54.9
T. saturnispora	22.3***	7.6 NS	20.0
T. viride			
(2)	29.2***	51.1***	65.4
(14)	15.0*	57.8***	64.2
(28)	32.1***	37.6***	52.8
(48)	23.6**	32.8***	42.8
(49)	23.8**	42.0***	50.8
(50)	20.8***	0.9 NS	12.6
(51)	18.9**	45.5***	55.8
(52)	25.0***	58.1***	68.6
(53)	13.3*	40.8***	48.7
(54)	6.8 NS	34.9**	39.3

* Significant P<0.05)
** " P<0.01) Student's 't' test
*** " P<0.001) (see text)
NS Not significant P>0.05)

Control 71.9 % germination
29.0 germ tube length

APPENDIX 21

Effect of *Trichoderma* culture filtrates on Germination of *Botrytis allii* conidia (as % reduction)

	Percentage Germination	Germ Tube Length	Theoretical Colonization Index
T. hamatum			
(15)	26.7***	49.1***	62.7
(22)	9.3*	27.9**	34.6
T. harzianum			
(1)	18.7***	32.5***	45.1
(129)	22.7***	26.1***	42.9
(20)	31.8***	24.2**	48.3
T. koningii	22.6***	38.2***	52.1
T. longibrachiatum	0 NS	26.5*	26.1
T. polysporum	8.3***	21.1**	27.6
T. pseudokoningii	8.8*	26.2**	32.8
T. saturnispora	25.0**	42.8***	57.1
T. viride			
(2)	17.6**	34.2***	45.7
(14)	24.4***	31.6***	48.1
(28)	38.0***	38.2***	61.7
(48)	68.9***	46.3***	83.3
(49)	28.0***	29.9**	50.5
(50)	39.2**	36.4***	61.3
(51)	40.5***	19.6 NS	52.1
(52)	25.4***	30.4**	48.1
(53)	51.7**	45.4***	73.6
(54)	51.3***	47.9***	74.6

* Significant $P<0.05$)
** " $P<0.01$) Student's 't' test
*** " $P<0.001$) (see text)
NS Not significant $P>0.05$)

Control 73.4 % germination
10.2 germ tube length

APPENDIX 22

Effect of *Trichoderma* culture filtrates on Germination of *Botrytis cinerea* conidia (as % reduction)

	Percentage Germination	Germ Tube Length	Theoretical Colonization Index
T. hamatum			
(15)	36.3**	49.3***	67.6
(22)	46.6**	0 NS	45.0
T. harzianum			
(1)	27.5*	16.5 NS	39.4
(129)	29.5*	18.3 NS	42.4
(20)	33.5**	22.4*	48.4
T. koningii	34.9**	14.5 NS	44.3
T. longibrachiatum	39.8**	17.9 NS	50.5
T. polysporum	36.7**	28.7*	54.9
T. pseudokoningii	36.9**	31.2**	56.6
T. saturnispora	33.8*	32.3**	55.2
T. viride			
(2)	31.1*	6.5 NS	38.2
(14)	26.8*	5.1 NS	30.6
(28)	36.0**	18.2 NS	47.7
(48)	59.6**	36.2**	74.2
(49)	20.5 NS	36.8***	49.8
(50)	34.5**	12.5 NS	42.7
(51)	35.6**	11.0 NS	42.7
(52)	36.5**	12.6 NS	44.5
(53)	41.5**	32.2**	60.3
(54)	34.9*	47.0***	65.5

* Significant P<0.05)
** " P<0.01) Student's 't' test
*** " P<0.001) (see text)
NS Not significant P>0.05)

Control 32.2 % germination
20.8 germ tube length

APPENDIX 23

Effect of *Trichoderma* culture filtrates on Germination of *Penicillium nigricans* conidia (as % reduction)

	Percentage Germination	Germ Tube Length	Theoretical Colonization Index
T. hamatum			
(15)	10.7 NS	32.4***	39.6
(22)	8.6 NS	29.4**	35.4
T. harzianum			
(1)	13.1**	45.1***	52.2
(129)	8.2*	36.4***	41.6
(20)	7.5*	54.2***	57.6
T. koningii	7.2*	54.4***	57.7
T. longibrachiatum	0 NS	45.1***	29.2
T. polysporum	5.5 NS	32.8***	36.5
T. pseudokoningii	6.3 NS	42.7***	46.3
T. saturnispora	0 NS	39.3***	33.2
T. viride			
(2)	11.5**	30.3***	38.4
(14)	3.2 NS	36.4***	38.4
(28)	0 NS	25.9**	22.1
(48)	0 NS	33.5***	33.5
(49)	3.7 NS	27.6**	30.3
(50)	4.8 NS	24.2*	27.8
(51)	0 NS	33.5***	31.2
(52)	0 NS	44.3***	45.0
(53)	2.2 NS	15.6 NS	17.4
(54)	8.1 NS	2.3 NS	10.2

* Significant P<0.05)
** " P<0.01) Student's 't' test
*** " P<0.001) (see text)
NS Not significant P>0.05)

Control 69.2 % germination
13.9 germ tube length

APPENDIX 24

Effect of *Trichoderma* culture filtrates on Germination of *Penicillium janthinellum* conidia (as % reduction)

	Percentage Germination	Germ Tube Length	Theoretical Colonization Index
T. hamatum			
(15)	26.8***	44.6***	59.4
(22)	23.1***	25.2***	42.5
T. harzianum			
(1)	28.2**	10.6 NS	35.8
(129)	5.6*	25.6***	29.8
(20)	47.2***	46.0***	71.5
T. koningii	16.3***	19.4*	32.5
T. longibrachiatum	16.3***	33.9***	44.7
T. polysporum	8.1*	0 NS	2.6
T. pseudokoningii	36.8***	26.0***	53.6
T. saturnispora	21.7*	58.5***	51.8
T. viride			
(2)	19.4***	27.4***	41.4
(14)	27.8***	26.5***	46.6
(28)	23.3**	4.6 NS	27.0
(48)	24.5**	32.3***	48.9
(49)	47.8***	20.8**	58.6
(50)	8.2*	10.1 NS	17.4
(51)	31.1***	20.0**	45.6
(52)	34.7**	21.2**	48.5
(53)	33.1***	13.3 NS	42.0
(54)	20.4*	7.0 NS	25.9

* Significant $P<0.05$)
** " $P<0.01$) Student's 't' test (see text)
*** " $P<0.001$)
NS Not significant $P>0.05$)

Control 39.4 % germination
21.2 germ tube length

APPENDIX 25

Effect of *Trichoderma* culture filtrates on Germination of *Penicillium expansum* conidia (as % reduction)

	Percentage Germination	Germ Tube Length	Theoretical Colonization Index
T. hamatum			
(15)	10.7*	48.9***	54.4
(22)	12.3**	13.3 NS	24.0
T. harzianum			
(1)	7.3 NS	26.5***	32.1
(129)	2.4 NS	30.1***	31.9
(20)	5.9 NS	38.2***	41.9
T. koningii	10.4 NS	36.1***	42.8
T. longibrachiatum	12.7 NS	48.3***	54.9
T. polysporum	6.1 NS	22.0**	26.9
T. pseudokoningii	4.8 NS	46.4***	49.1
T. saturnispora	16.9***	40.4***	50.4
T. viride			
(2)	5.9 NS	29.8***	34.0
(14)	10.4 NS	26.5***	34.3
(28)	11.5 NS	0 NS	6.9
(48)	15.1***	24.7*	36.1
(49)	15.5***	17.0 NS	29.9
(50)	16.9**	40.5***	50.5
(51)	16.4*	0 NS	0
(52)	6.4 NS	12.8 NS	18.4
(53)	21.3***	41.5***	53.9
(54)	9.9 NS	25.9*	33.2

*	Significant	P<0.05)	
**	"	P<0.01)	Student's 't' test
***	"	P<0.001)	(see text)
NS	Not significant	P>0.05)	

Control 81.4 % germination
28.5 germ tube length.

APPENDIX 26

Effect of *Trichoderma* culture filtrates on Germination of *Penicillium spinulosum* conidia (as % reduction)

	Percentage Germination	Germ Tube Length	Theoretical Colonization Index
T. hamatum			
(15)	8.3*	35.8***	41.1
(22)	8.0*	35.2***	40.4
T. harzianum			
(1)	8.2 NS	24.9***	31.1
(129)	8.0 NS	33.4***	38.7
(20)	9.0 NS	67.0***	69.9
T. koningii	5.6 NS	48.0***	50.9
T. longibrachiatum	6.2*	34.0***	38.1
T. polysporum	8.8 NS	15.4*	22.8
T. pseudokoningii	5.5 NS	29.0***	32.9
T. saturnispora	10.8**	48.1***	53.7
T. viride			
(2)	5.0 NS	13.3 NS	17.6
(14)	8.5 NS	14.8 NS	22.1
(28)	6.5**	40.7***	44.6
(48)	11.2**	55.6***	60.5
(49)	10.8***	53.1***	58.1
(50)	8.2**	36.6***	41.8
(51)	14.9**	65.5***	67.6
(52)	9.0***	29.0***	35.4
(53)	3.9*	0 NS	1.3
(54)	15.7**	51.7***	59.3

* Significant P<0.05)
** " P<0.01) Student's 't' test
*** " P<0.001) (see text)
NS Not significant P>0.05)

Control 78.3 % germination
22.1 germ tube length

APPENDIX 27

Effect of *Trichoderma* culture filtrates on Germination of *Penicillium funiculosum* conidia (as % reduction)

	Percentage Germination	Germ Tube Length	Theoretical Colonization Index
T. hamatum			
(15)	13.8*	44.3***	52.0
(22)	9.5 NS	53.3***	57.7
T. harzianum			
(1)	26.3***	48.4***	63.5
(129)	4.9 NS	22.6*	26.4
(20)	25.7**	42.8***	46.1
T. koningii	5.7 NS	25.8**	30.1
T. longibrachiatum	9.7 NS	49.6***	54.5
T. polysporum	7.3 NS	27.8**	33.1
T. pseudokoningii	14.4*	35.9***	45.2
T. saturnispora	12.0*	38.8***	46.2
T. viride			
(2)	7.0 NS	11.7 NS	17.8
(14)	10.8 NS	29.6**	37.2
(28)	5.9 NS	44.3***	47.6
(48)	11.1*	52.1***	85.7
(49)	11.0*	34.9***	42.0
(50)	9.9*	48.9***	54.0
(51)	7.0 NS	13.2***	37.8
(52)	7.4 NS	21.6*	27.4
(53)	0 NS	4.4 NS	2.7
(54)	9.4*	34.3***	40.5

* Significant P<0.05)
** " P<0.01) Student's 't' test (see text)
*** " P<0.001)
NS Not significant P>0.05)

Control 74.3 % germination
19.5 germ tube length

APPENDIX 28

Effect of C-source on Biological activity of *T. viride* (48) culture filtrates against *Gliocladium roseum* (1) conidia.

C-source	Control		% Reduction	
	Percentage Germination	Germ Tube Length	Percentage Germination	Germ Tube Length
Glucose	51.0	8.8	54.0***	39.7***
Fructose	80.9	12.8	55.3***	57.3***
Galactose	73.8	13.7	41.1**	59.3***
Mannose	75.3	14.0	62.3***	56.4***
Ribose	71.9	10.5	54.9**	29.5*
Xylose	78.3	11.6	52.4*	53.4***
Rhamnose	71.0	10.2	9.6*	1.0 NS
Mannitol	73.6	10.5	56.8***	51.1***
Sorbitol	74.3	13.8	45.9**	43.2***
Sorbose	71.0	15.7	38.4***	58.1***
Sucrose	65.5	8.5	55.5***	57.4***
Lactose	77.9	12.2	17.4***	45.0***
Maltose	56.5	8.1	33.7*	33.3***
Raffinose	75.3	14.7	33.4***	50.2***
Starch	71.3	12.5	56.9***	57.8***

* Significant P<0.05
** " P<0.01
*** " P<0.001
NS Not significant P>0.05

Student's 't' test (see text)

APPENDIX 29

Effect of pH on Germination of Gliocladium roseum (1) conidia

The pH of sterile Weindlings medium was adjusted to pH 6.4, 5.0, 4.5, 4.0, 3.0 with hydrochloric acid and then the germination of Gliocladium roseum (1) conidia bioassayed as described in Materials and Methods.

Results

pH	Percentage Germination	Germ Tube Length
6.4	62.9	6.4
5.0	67.0	9.7
4.5	64.8	9.5
4.0	65.7	9.8
3.0	66.5	7.9

The pH of the medium, in the range tested, did not have any significant effect on either the percentage germination or germ tube growth of the germinating G. roseum (1) conidia.

APPENDIX 30

Effect of N-source on Biological activity of T. viride (48) culture filtrates against Gliocladium roseum (1) conidia.

N-source	Control		% Reduction	
	Percentage Germination	Germ Tube Length	Percentage Germination	Germ Tube Length
Ammonium tartrate	51.0	8.8	54.0***	39.7***
Ammonium sulphate	65.5	14.3	39.5***	37.1**
Ammonium chloride	63.7	14.8	48.9**	43.4**
Sodium nitrate	80.7	12.4	35.8***	53.9***
γ-amino butyric acid	65.4	10.9	20.9**	29.2**
Glycine	65.5	9.3	16.8*	5.0 NS
Glutamic acid	55.6	11.6	20.4**	57.9***
Cysteine	64.8	11.4	27.5*	37.1***
Phenylalanine	63.6	10.8	15.6*	31.4***
Peptone	65.9	14.8	15.6 NS	40.1***

* Significant P<0.05)
** " P<0.01) Student's 't' test
*** " P<0.001) (see text)
NS Not significant P>0.05)

APPENDIX 31

The Absorption at max of each of the eluates of the Chloroform extract taken after 6 days incubation for each nutrient source.

Eluate	A_1	A_2	B	C	D	E	F	G
max	240	300	260	255	255	270	260	280
R_F	61	61	45	20	12.5	8	55	35
Glucose	-	0.95	0.35	0.23	0.23	0.28	0.35	0.12
Sucrose	-	0.73	0.41	0.12	0.08	0.14	-	-
Fructose	0.53	0.68	0.16	-	0.09	0.05	-	0.05
Lactose	1.17	-	-	-	0.14	0.64	-	0.31
Xylose	-	0.54	0.15	0.1	0.12	0.24	-	0.18
Mannitol	-	0.12	0.29	0.11	0.11	0.37	-	0.08
Sodium nitrate	0.92	-	-	0.16	0.12	0.36	-	0.13
Yamino butryic acid	0.68	0.29	-	0.16	-	0.24	0.12	0.10
Glutamic acid	-	0.21	0.14	0.06	0.08	0.38	-	-
Cysteine	-	-	-	0.13	-	-	0.33	0.13
Phenylalanine	0.97	-	0.57	-	0.19	-	-	-

APPENDIX 32

Germination of *Gliocladium roseum* (1) conidia on different C-sources.

	Percentage Germination	Germ Tube Length
Glucose	51.0	8.8
Sucrose	65.5	8.5
Fructose	80.9	12.8
Lactose	77.9	12.2
Xylose	78.3	11.6
Mannitol	73.6	10.5

APPENDIX 33

Variation in Absorption of Chloroform soluble eluates from the C-source culture filtrates with respect to time

Time(days)	Eluate A_1	A_2	B	C	D	E	F	G
Glucose								
1	-	-	-	-	-	-	-	-
2	0.60	-	-	-	0.01	0.03	-	-
3	0.43	-	-	-	0.01	0.06	-	-
4	-	0.17	0.06	0.03	0.07	0.09	-	-
5	-	0.31	0.10	0.08	0.07	0.17	-	-
6	-	0.95	0.35	0.23	0.23	0.28	0.35	0.12
Sucrose								
1	-	-	-	-	-	-	-	-
2	0.57	-	0.03	-	-	-	-	-
3	0.13	0.28	0.10	-	0.03	0.07	-	-
4	-	0.44	0.29	0.07	0.04	0.08	-	-
5	-	0.56	0.39	0.08	0.06	0.12	-	-
6	-	0.73	0.41	0.12	0.08	0.14	-	-
Fructose								
1	-	-	-	-	-	-	-	-
2	1.11	-	-	-	-	0.03	-	0.03
3	1.94	-	-	-	0.05	0.05	-	0.03
4	0.40	0.15	0.15	-	0.10	0.06	-	0.04
5	0.46	0.46	0.25	-	0.06	0.08	-	0.04
6	0.53	0.68	0.16	-	0.09	0.05	-	0.05
Lactose								
1	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-
3	-	-	-	-	-	0.04	-	-
4	-	-	-	-	-	0.14	-	-
5	-	-	-	-	-	0.19	-	0.07
6	1.17	-	-	-	0.14	0.64	-	0.31
Xylose								
1	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-
3	0.51	-	0.05	-	-	0.05	-	-
4	-	0.43	0.07	0.09	-	0.13	-	0.10
5	-	0.52	0.13	0.10	0.12	0.15	-	0.15
6	-	0.54	0.15	0.10	0.12	0.24	-	0.18
Mannitol								
1	-	-	-	-	-	-	-	-
2	0.90	-	-	-	-	0.11	-	-
3	0.44	-	-	-	-	0.17	-	-
4	-	0.34	0.17	-	0.15	0.46	-	0.05
5	-	1.18	0.22	0.16	0.42	0.59	-	0.11
6	-	1.20	0.29	0.11	0.11	0.37	-	0.08