INTERACTIONS BETWEEN *TRICHODERMA* SPP. AND SOIL-BORNE PLANT PATHOGENS; IMPLICATIONS FOR BIOLOGICAL CONTROL

by

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ABSTRACT

The research was designed to evaluate the commercially marketed biological control agent Binab T., which contains *Trichoderma* spp., as an alternative to fungicides for the control of soil borne pathogens under greenhouse conditions.

A dual inoculation plate method was used to screen a range of fungi for their susceptibility to antagonism *in vitro* on a nutrient-poor medium (soil extract agar) and nutrient-rich medium (malt extract agar). Various interactions were observed of which the killing of the Oomycete damping-off fungi *Pythium ultimum* and *Pythium debaryanum* was the most striking. On soil extract agar the interaction was characterized by progressive cytoplasmic vacuolation, lysis and a zone of clearing which developed after hyphal contact between the two organisms had been established. Similarly on nutrient-rich media *Trichoderma* acted as an aggressive antagonist competing with and overgrowing a range of fungi; *Pythium multisporum*, *Pythium undulatum*, *Phytophthora citricola*, *Phytophthora cactorum*, *Rhizoctonia solani*, *Corticium praticola* and the wilt pathogens *Verticillium albo-atrum* and *Fusarium oxysporum* f.sp. *lycopersici*.

Subsequent greenhouse trials were employed to assess the extent of antagonism against *P.ultimum* and *P.debaryanum* in soil and the potential of the commercial formulation for control of the damping-off disease. Vermiculite soaked with vegetable juice was the most suitable growth medium and it also provided a convenient and efficient means of delivery. In this way *Trichoderma* inoculum was employed both as a prophylactic, pre-pathogen inoculation treatment and as a simultaneous-inoculation treatment against the pathogens, with varying degrees of success; but in no instance was a commercially acceptable level

of disease control achieved. Use of this Trichoderma carrier-growth system was also ineffective against F.oxysporum f.sp. lycopersici in sterile soil.

Crude filtrates from Trichoderma cultures grown in soil extract liquid medium and from cultures grown on heat-killed Pythium mycelium or from dual cultures with Pythium, failed to reproduce the distinctive interaction with Pythium observed in paired culture tests. Agar plate tests using cellophane and dialysis membranes demonstrated that the Trichoderma produced an unidentified antibiotic. Trichoderma also produced a cellulase system which degraded both Whatmans CM11 cellulose and Avicel, but the hyphal lysis of Oomycetes on low-nutrient status agar could not be attributed to either of these metabolites nor to a physical interaction. Whatever the underlying mechanism the extent of hyphal lysis is clearly insufficient to cause appreciable suppression of Pythium inoculum in soil.

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ABBREVIATIONS

ai	active ingredient
°C	degree Centigrade
cfu	colony forming unit
cm	centimetre
CMC	carboxymethylcellulose
con	control
Cp	Corticium praticola
cv	cultivar
EDTA	Ethylenediaminetetra-acetic acid
Fig	Figure
Fol	Fusarium oxysporum f.sp. lycopersici
f.sp.	forma specialis
g	gram
hr	hour
l	litre
MEA	malt extract agar (2%(w/v))
µm	micron
µg	microgram
µl	microlitre
mg	milligram
ml	millilitre
no	number
P.cact	Phytophthora cactorum
P.c	Phytophthora citricola
PCNB	pentachloronitrobenzene
%	percentage
p	probability level
P.d	Pythium debaryanum
PI	Prophylactic, pre-inoculation treatment
P.m	Pythium multisporum
P.u	Pythium ultimum
P.und	Pythium undulatum
Rs	Rhizoctonia solani
SCH	sucrose casein hydrolysate
SE	standard error of mean
SEA	soil extract agar
sp./spp.	species singular and plural

Th	*Trichoderma harzianum*
Tp	*Trichoderma polysporum*
TRC	Binab *Trichoderma*
TRC + *Pd*	Binab *Trichoderma* used simultaneously with *Pythium debaryanum* to inoculate compost
TRC + *Pu*	Binab *Trichoderma* used simultanesously with *Pythium ultimum* to inoculate compost
TRC PI *Pd*	Binab *Trichoderma* incorporated into compost seven days prior to inoculation with *Pythium debaryanum*
TRC PI *Pu*	Binab *Trichoderma* incorporated into compost seven days prior to inoculation with *Pythium ultimum*
TRC PI	Binab *Trichoderma* incorporated into compost but omitting the pathogen inoculation step after the seven day time period
UV	ultra violet light
V_8	V_8 - vegetable juice
Vaa	*Verticillium albo-atrum*
Verm	uninoculated vermiculite soaked with V_8 vegetable juice incorporated into compost
vs	versus
v/v	volume/volume
wp	wettable powder
wt	weight
w/v	weight/volume

1. LITERATURE REVIEW

1.1 INTRODUCTION

In a balanced stable soil ecosystem populations of soil microorganisms are in dynamic equilibrium. Within this biologically buffered system, populations of each microorganism fluctuate within defined limits and, stimulatory and inhibitory interactions between organisms achieve this biological balance. Thus, in these undisturbed ecosystems disease epidemics are rare and in most plant communities it is likely that biological control operates to a degree but remains unrecognized.

Man has attempted to utilize these inhibitory interactions by selecting microbial competitors or natural enemies to effect control of specific plant pathogens. However, the overall concept of biological control has been used in a broader sense to encompass methods such as plant breeding to improve resistance. For example, Baker and Cook (1974) suggested that biological control is "the reduction of inoculum density or disease-producing activities of a pathogen or parasite in its active or dormant state, by one or more organisms, accomplished naturally or through manipulation of the environment, host or antagonist, or by mass introduction of one or more antagonists". Alternatively, narrow definitions have been proposed (Garrett, 1970), and Deacon (1983) defined biological control of both plants and pests as "the practice in which, or process whereby, the undesirable effects of an organism are reduced through the agency of another organism that is not the host plant, the pest or pathogen, or man".

The genus Trichoderma (Persoon) is considered by many authors to contain a number of potential biological control agents. In general, Trichoderma spp. are aggressive competitors with high growtn rates, and are nutritional opportunists able to utilize a range

of substrates, produce antibiotics, and some are mycoparasites. T.harzianum (Rifai), a widely studied species aggregate, has been used successfully as a soil treatment under field conditions. Wells et al. (1972) were among the first to report successful field control of tomato stem rot caused by Sclerotium rolfsii with preparations of T.harzianum and success against this pathogen on peanuts was also obtained in the field by Backman and Rodriguez-Kabana (1975) and by Chet et al. (1979). Similarly isolates of T.harzianum have been successfully used by Elad et al. (1981 b) to control Rhizoctonia solani in strawberry fields and by Lewis and Papavizas (1980) to control cucumber fruit rot caused by the same pathogen. Difficulties with classification and identification of Trichoderma isolates have been encountered and in discussions here the scheme outlined by Rifai (1969) is used where possible; hence references to species are to species aggregates as defined by that author.

Much attention has been focused on biological control of seedling diseases using seed, root and soil treatments. Success for example against Pythium spp. have been gained with Trichoderma spp. (Wright, 1956; Fajola and Alasoadura, 1975), Pencillium frequetans and T.viride (Liu and Vaughan, 1965), Corticium sp. (Hoch and Abawi, 1979) Chaetomium globosum (Harman et al., 1978) and Pythium oligandrum (Al-Hamdani et al., 1983).

The reduction of adverse effects of an organism via biological control clearly has certain potential benefits over more conventional chemical control methods. The high toxicity of many chemicals, environmental pollution combined with the ecological disruption often caused, and its inadequacy for long term control, have been further incentive to develop biocontrol methods operative within present day agricultural practices.

1.2 MECHANISMS OF ANTAGONISM BETWEEN MICROORGANISMS

Antagonism, the adverse effect of one organism on another, may broadly be divided into (a) antibiosis, the inhibition of one organism by a metabolite of another, (b) competition for example for nutrients, oxygen or space or some other commodity whose supply is limited but is required by the pathogen (c) hyperparasitism, mycoparasitism and predation (Baker and Cook, 1974; Baker, 1980; Cook, 1981). Dennis and Webster (1971 a, b, c) studied the antagonistic nature of members of the genus Trichoderma in vitro under three sections, the production of non-volatile antibiotics, volatile antibiotics and hyphal interactions. They found that many of the isolates from different species of Trichoderma which they tested produced non-volatile antibiotics and coiled around hyphae of test fungi whilst only some isolates produced volatile inhibitory metabolites. Barnett and Binder (1973) suggested that species of Trichoderma capable of hyperparasitizing plant pathogens would provide highly efficient antagonists, whilst Deacon (1983) suggested that coiling, often interpreted as a sign of mycoparasitism, may simply enhance the effects of antibiotics. However in many instances when a significant reduction in disease does occur subsequent to Trichoderma application, the mechanism via which biological control is achieved is unknown. It is possible that an interplay of mechanisms occurs with antibiosis, mycoparasitism and competition for example acting simultaneously or progressively at different stages.

As early as 1934 Weindling reported the parasitism of T.lignorum (Tode) Harz on S.rolfsii and R.solani. More recently T.hamatum (Bon.) Bain isolated from soils naturally suppressive to R.solani induced suppressiveness when conidia were added to a clay loam soil which was subsequently assayed with radish. Reductions in disease also occurred with Pythium spp. on peas and S.rolfsii on beans (Chet and Baker, 1981). Application

of this antagonist as a seed treatment of radish and pea was also effective in controlling disease induced by R.solani and Pythium spp. respectively (Harman et al., 1980). Chet and Baker (1981) found that although no inhibition zones indicative of antibiotic production in vitro were observed this antagonist produced fungal cell wall-degrading enzymes in vitro and this ability was implicated in its success as a biological control agent. In further studies T.hamatum was observed coiling round, penetrating and growing within mycelium of R.solani and Pythium ultimum and the formation of appressorial-like structures were noted (Chet et al., 1981). Elad et al. (1983) corroborated these reports by demonstrating mycoparasitism of S.rolfsii and R.solani by T.hamatum and an isolate of T.harzianum using scanning electron microscopy.

In experiments where Trichoderma spp. have been successful as biocontrol organisms, hyphal interactions such as coiling or penetration have often been observed in laboratory screens. The mycoparasitism observed in vitro is often implicated as a mechanism of control. However Ayers and Adams (1981) concluded that out of the more than 30 species of fungi and bacteria cited as antagonists of Sclerotinia spp. only four including isolates of T.viride Pers. ex Fr. were destuctive towards Sclerotinia spp. in soil.

The isolate of T.harzianum used as a biocontrol agent of S.rolfsii on lupin, tomato and peanut by Wells et al. (1972) was reported to inhibit the growth of and kill the plant pathogen in culture; whereas Backman and Rodriguez-Kabana (1975) and Grinstein et al. (1979) did not establish a mode of action for the successful control of this pathogen on peanut. Hadar et al. (1979) reported that T.harzianum which they found an effective biocontrol agent of R.solani on bean, tomato and eggplant produced cell wall lytic enzymes and they observed hyphal interactions in vitro. Elad et al. (1980) demonstrated that an isolate of

T.harzianum, isolated from naturally infested soil, was able to lyse mycelia of both S.rolfsii and R.solani and invaded mycelium of S.rolfsii under growth conditions unfavourable to the pathogen.

In general tests in vitro offer only circumstantial evidence as to the mechanism(s) of disease suppression in vivo and verification of for example parasitism under natural conditions is highly desirable. Further Atkinson et al. (1975) concluded that the genotype of the host plays a major role in governing the characteristics and composition of microbial populations in the rhizosphere with plant exudates directly affecting the soil microorganisms. Thus the absence of the host in laboratory screens imposes considerable constraints on predicting interactions in vivo.

1.3 STRATEGIES FOR DISEASE CONTROL

Cook (1981) divided the mechanisms of biological control into three major strategies (1) the reduction of pathogen inoculum by antagonistic microorganisms (2) the protection of plant surfaces against infection and (3) the management of physiological incompatibility between host and pathogen.

The destruction of pathogen propagules while in the dormant state by the action of hyperparasites or predators may effectively reduce the inoculum potential below a threshold level able to cause disease. Papavizas and Lumsden (1980) suggested that a reduction in the inoculum density of the pathogen would create an environment more favourable to plant growth in which more effective use could be made of small amounts of fungicide, resistant or tolerant cultivars, and cultural control techniques. Oospores of Pythium, Phytophthora and downy mildew fungi are prone to hyperparasitism by a range of organisms and 80-90% are reported to be parasitized naturally (Cook, 1981). Sneh et al. (1979)

investigating the parasitism of oospores of Phytophthora megasperma var. sojae, P.cactorum, Pythium sp. and Aphanomyces euteiches in soil found that a number of organisms including Oomycetes, Chytridiomycetes, Hyphomycetes, Actinomycetes aand bacteria were able to invade and destroy oospores. The mycoparasite Sporidesmium sclerotivorum has been successfully used in the field to reduce the numbers of sclerotia of Sclerotinia minor, cause of lettuce drop (Adams and Ayers, 1982). Its ability to establish in field plots, destroy sclerotia produced on the infected lettuce plants and give significant reductions in disease incidence demonstrate its potential as a biocontrol agent. Huang (1977) found that Coniothyrium minitans parasitized and killed the sclerotia that developed on diseased roots of Sclerotinia-infected plants in a sunflower field naturally infested with both fungi. The mycoparasite parasitized sclerotia within the root and stem causing a reduction in the number of penetrating viable sclerotia. Henis and Papavizas (1983) proposed that a strain of T.harzianum was only able to degrade sclerotia of S.rolfsii in the presence of nutrients made available when sclerotia were subjected to treatments such as drying and heating. T.harzianum and the pathogen competed for this same food base for degradation and germination respectively.

Taber and Pettit (1981) and Shokes and Taber (1983) reported a reduction in sporulation by Cercosporidium personatum when leaf spots on peanuts became colonized by Hansfordia sp., whilst in the absence of this hyperparasite a mass of secondary inoculum was produced. Similarly Corke and Hunter (1979) found that inoculation of pruning wounds on apple trees with Bacillus subtilis, an antagonistic organism capable of remaining within apple leaf scar tissue throughout the winter and spring (Swinburne, 1973), and with Cladosporium cladosporioides isolated from non-sporing Nectria cankers, prevented inoculum formation by the wound pathogen Nectria galligena. Use of these microbial

inoculants resulted in 96% fewer conidia being released in rain over the following years.

Displacement by antagonistic saprophytes of pathogens which increase their inoculum in host material may be an effective means of reducing disease incidence since "possession is nine points of the law for microorganisms in relation to host tissue" (Baker and Cook, 1974). An organism which colonizes the host tissue is able to deplete the nutrients rendering them unavailable for other organisms. An example of this principle is usedfor biological control of Fusarium roseum 'Culmorum'. If wheat stubble is allowed to stand into the wet season in the Pacific Northwest region of the U.S.A. the stubble becomes colonized by common airborne saprophytes which pre-empts invasion and colonization by the pathogen. In the absence of this prior colonization by saprophytic fungi, F.roseum rapidly colonizes clean straw when ploughed under so increasing its population in the soil (Cook, 1970; Baker and Cook, 1974). Lin and Cook (1979) found that complete displacement of a pathogen may not be required for disease suppression. In the absence of a food base Fusarium roseum 'Avenaceum' is only weakly pathogenic to lentils. The presence of competitive saprophytes including T.viride and two species of Mucor in the soil inoculum effectively weakened F.roseum which was unable to compete with these fast growing fungi for the food base required to incite disease.

Sublethal chemical or physical treatments may facilitate displacement of a pathogen by an antagonist by weakening or inhibiting growth of the former. Bliss (1951) found that the native fungal antagonists especially Trichoderma spp. survived and increased in carbon disulphide-treated Citrus orchard soils and controlled Armillaria mellea which was weakened by the fumigant. Similarly Ohr et al. (1973) found that sublethal fumigations with methyl bromide reduced A.mellea populations. This decline was primarily

due to Trichoderma which was found to be twice as resistant in vitro to methyl bromide as was Armillaria.

Cook (1981) included the influence of viruses or virus like organisms causing diminished virulence and vigour in this category of biological control. Hypovirulence of the destructive chestnut blight fungus Endothia parasitica is transmissible and linked with the presence of a double stranded RNA mycovirus. In France the use of hypovirulent strains of Endothia appears to be successfully suppressing the disease (Deacon, 1983). In order for biological control to operate a hypovirulent strain introduced into a canker must be of the same compatibility group as the virulent strain already present. Anastomosis between hyphae of a compatible pairing results in transfer of hypovirulence to the virulent strain and the canker subsequently heals.

The second major approach to biological control involves the protection of plant surfaces against infection. The introduction of microbial antagonists to plant surfaces can 'protect' the host by providing a biological barrier against the pathogen. In Britain Peniophora gigantea is commercially manufactured and used as a stump inoculant against Heterobasidion annosum (formerly Fomes annosus). Sachets of spores in a sucrose solution are added to water and the suspension is brushed over the stump surface of freshly felled pines. Stump protection by the antagonist prevents infection from airborne spores and is comparable in efficacy with chemical treatment. It has the advantage that the inoculum of the antagonist increases by sporophore production six to twelve months after inoculation and can grow down into the stump tissue preventing the pathogen from entering the roots through which H.annosum can grow to infect nearby trees (Deacon, 1983). Two other commercial successes, the use of Agrobacterium radiobacter var. radiobacter strain 84 on fruit and ornamental plants to control crown gall caused by A.tumefasciens and ectomycorrhizae on pine to protect pine roots from Phytophthora cinnamomi, also fall into this category (Cook, 1981).

Trichoderma spp. have been reported to protect wounds and on plum *T.viride* spores applied to fresh pruning wounds 48 hours prior to the arrival of the pathogen effectively protected wounds from *Chondostereum* (= *Stereum*) *purpureum* causing silver leaf disease (Grosclaude, 1970). In contrast Corke (1974) used *Trichoderma* inoculum as a curative treatment and observed reductions in silver leaf symptoms over a two year period. Summer inoculations of wounds in red maple with *T.harzianum* delayed or prevented invasion of *Hymenomycetes* for at least two years, and after 31 months only 14% of the antagonist-treated wounds yielded Hymenomycetes compared with 50% in wounded-only controls. Despite this success Pottle *et al*. (1977) concluded that *T.harzianum* was not a pioneer colonist of fresh wounds in red maple and with time it probably lost its dominance in wood, allowing Hymenomycetes to invade.

Bacterial and fungal antagonists are often applied to seeds to protect them against root pathogens (Chang and Kommedahl, 1968; Kommedahl and Mews, 1975). Kerr (1972) applied the antagonistic strain of *A.radiobacter* to peach seeds to protect them from crown gall in soil infested with *A.tumefasciens*. Mitchell and Hurwitz (1965) reported that inoculation of tomato seeds with rhizosphere bacteria (*Arthrobacter* sp.) suppressed damping-off caused by *P.debaryanum* and Howell and Stipanovic (1979, 1980) reported that a strain of *Pseudomonas fluorescens* isolated from the rhizosphere of cotton seedlings, protected cotton against damping-off in soils infested with *R.solani* and *P.ultimum*. Seed treatments are usually more effective in controlling pre-emergence damping-off than post-emergence damping-off (Wright, 1956, Liu and Vaughan, 1965; Windels and Kommedahl, 1978). Oshima (1966) (in Kommedahl and Windels, 1981) used *T.lignorum* to protect tobacco seedlings from infection by *S.rolfsii*. A powder of antagonist spores containing soil and rice bran was applied to seedlings. The resulting reduction in disease incidence compared with the controls was greatest

when the treatment was used at transplanting and again one month later.

The management of physiological incompatibility between host and pathogen was proposed by Cook (1981) as a third major strategy for disease control. In his view this included plant breeding and more novel approaches such as induced resistance in which avirulent or non-pathogenic strains of pathogens are used to induce a resistance response in the host. Prior inoculation with avirulent or non-pathogenic organisms is known to stimulate host resistance in vascular wilt pathogens (Matta and Garibaldi, 1977; Baker, 1981).

1.4 METHODS OF INTRODUCING AND ENHANCING THE ACTIVITY OF ANTAGONISTS IN SOIL

The introduction of an alien microorganism into the soil environment may in theory be possible if the organism is better adapted to the particular ecological niche than the established residents or if it is capable of altering the environment to its advantage. Alternatively mass introduction can be used temporarily or permanently to swamp the residents (Baker, 1980). In reality introduction and establishment of an antagonist at high population levels in a stable ecosystem may be extremely difficult. The microbial balance must be shifted in favour of the antagonist to enable the organism to establish itself and proliferate. Natural soils appear to be resistent to alteration by the introduction of large numbers of antagonists and Garrett (1955,1956) pointed out that attempts to establish high populations of an inoculant organism, if previously isolated from soil generally fails because the population is a reflection of the habitat and any introduction in the absence of a change in the habitat would be transient. This point was illustrated with reference to the work of Weindling and Fawcett (1936) who found that only when soil was acidified

to pH 4.0 were conditions suitable for the establishment and activity of *T.lignorum* at a sufficiently high population level to protect *Citrus* seedlings from *Rhizoctonia* damping-off. Heavy inoculations in the absence of acidification were ineffective.

Shock treatments used as techniques for facilitating the introduction or enhancing the activity of resident microbial antagonists have included steam sterilization, fumigation and fungicide treatment. Munnecke *et al*. (1976) working on *Citrus* roots infected with *A.mellea* found that sublethal heat treatments of bare or balled roots produced results similar to those observed by Bliss (1951) using carbon disulphide described earlier. Resident species of *Trichoderma* resistant to the temperatures used parasitized the weakened pathogen and the treatment reduced the survival of *A.mellea*. Soil treated by steam at 100°C for 30 minutes has provided a suitable medium for effective biocontrol of *R.solani* and *P.ultimum*. Broadbent *et al*. (1971) introduced species of *Bacillus* or *Streptomyces* into treated soil and reduced damping-off caused by *P.ultimum* and *R.solani*. However they also reported that in some cases introduced microorganisms increased disease incidence. Olsen and Baker (1968) found that *Bacillus subtilis* and various Actinomycetes antagonistic to *R.solani* flourished in soil treated with aerated steam, so reducing the survival of the pathogen. In both cases the *Bacillus* isolates demonstrated a high degree of specificity for particular strains of *R.solani* and Olsen and Baker (1968) found that *B.subtilis* strains also exhibited specificity for certain soils. Clearly a situation such as this would complicate and hamper the use of a microorganism in biological control.

Moubasher (1963), reported that recolonization of carbon disulphide-treated soil by *Trichoderma* spp. occurred faster than by other organisms. Mughogho (1968) found that treatment of soil with various chemical fumigants promoted the development of dominant populations

of Trichoderma which in extreme cases could reach 100% of the recolonizing fungus flora. Trichoderma was the dominant fungal recolonizer of both acid and alkaline soils treated with allyl alcohol and several species groups including T.harzianum were included in these dominant populations. The rapid growth of Trichoderma in sterilized soils was also noted by Ferriss (1984), who found that the fungal populations of soils previously autoclaved and aerated for 28 days predominantly comprised Trichoderma spp.

Langerak (1977) found that treatments of Narcissus bulbs with thiram, organomerculials, formalin or the antibiotic pimaricin protected the roots and bulb from the bulb rot pathogen Fusarium oxysporum f.sp. narcissi by stimulating antagonistic Trichoderma and Pencillium spp. which colonized the surface of newly developed roots. Stankova-Opocenska and Dekker (1970) reported that seed treatments with the systemic fungicide 6-azauracil protected cucumber seedlings from Pythium débaryanum although this pathogen was somewhat insensitive to the fungicide in vitro. They proposed that suppression of the pathogen resulted from an increase in rhizosphere bacteria possibly caused by a change in the root exudate composition induced by the fungicide treatment. Similarly an unidentified bacterium antagonistic to species of Rhizoctonia, Pythium, Phytophthora, Fusarium and Trichoderma was favoured by low levels of hexachlorophene and may have been responsible for the reduction of cotton diseases observed (Pinckard 1970).

Wells et al. (1972) successfully controlled S.rolfsii, the cause of tomato stem rot, under field conditions using T.harzianum grown on autoclaved ryegrass and soil. They proposed that successful biological control depends on the provision of a readily usable food base to ensure vigorous growth of the antagonist. This approach of using a nutrient substratum for growing and delivering Trichoderma has been adopted by many research workers and a variety of application methods

have been developed. Mollases-enriched diatomaceous earth granules were successfully employed by Backman and Rodriguez-Kabana (1975) whilst wheat bran was employed by Henis et al. (1978), Hadar et al. (1979) and Elad et al. (1981). A mixture of wheat bran and sawdust was used by Elad et al. (1980), sand and cornmeal by Lewis and Papavizas (1980) and recently Sivan et al. (1984) reported that a wheat-bran plus peat mix was the most efficient substrate tested for growth of an isolate of T.harzianum.

Thus this technique of augmenting soils with large populations of an antagonist has proved successful and is frequently used in preference to applications of the organism alone. Spores introduced into natural soils in the absence of a nutrient source are prone to the effects of fungistasis (Lockwood, 1977). A supply of a suitable organic food base may overcome the effects of fungistasis enabling large populations of the antagonist to be introduced, temporarily inundating the infection court and increasing the likelihood of achieving successful biological control.

Thus the approach used in early experimental work in which antagonists were introduced directly into soils met with limited success (Weindling and Fawcett, 1936; Wood and Tveit, 1955), with many attempts at introducing alien antibiotic-producing organisms failing to control root diseases. However some success was reported when antagonists were introduced into sterilized soils (Garrett, 1956). The removal of resident competitive microorganisms combined with the presence of abundant nutrients often associated with sterile soil probably accounts for these successes.

Papavizas and Lewis (1981) proposed that the choice of growth medium may directly affect the efficacy of the antagonist used for soil augmentation. A strain of T.harzianum was a more effective biocontrol agent when grown on cornmeal than when grown on alternative

nutrients. The presence of rich nutrient sources promotes antibiosis (Baker and Cook, 1974) with antibiotic production often being most marked in the vicinity of organic substrates (Wright 1955, 1956; Baker 1968). Elad et al. (1982) detected high lytic activities in wheat bran cultures of T.harzianum and in sterile soil amended with wheat bran inoculum compared with soil inoculated with T.harzianum in the absence of the food base. Papavizas and Lewis (1981) found that a newly discovered antagonist (Stilbum sp.) when grown on carriers enriched with mollases V_8 - vegetable juice, cornmeal or bran was effecive against R.solani-incited damping-off of radish but not when grown on spent coffee grounds, or castor bean pomace. Further, they described a new method for its commercial use for growth and delivery of T.harzianum. Perlite treated with four% methyl cellulose as an adhesive and enriched with five% mollases was coated with a preparation of T.harzianum grown on low quality oat seed which had been air dried and ground to pass through a ten mesh screen. The efficacy of one strain of Trichoderma sp. was improved when grown on this medium, producing a significantly greater suppression of damping-off by R.solani when compared with alternative substrates.

1.5 INTEGRATION WITH CHEMICAL AND CULTURAL CONTROL METHODS

Results of experiments using biological control agents in combination with chemicals suggest that the resulting synergistic control responses may allow the fungicide to be applied in reduced quantities. Henis et al. (1978) integrated the use of pentachloronitrobenzene (PCNB) at four µg ai/g soil and T.harzianum against damping-off of radish induced by R.solani. They found that the presence of the fungicide together with the biocontrol agent had a synergistic effect on the decrease of the pathogen propagules and an additive effect on disease control. Similarly Hadar

et al. (1979) found that low concentrations of PCNB which proved ineffective alone, improved control of R.solani when applied together with wheat bran cultures of T.harzianum, giving more effective control of damping-off of eggplant seedlings than T.harzianum alone. In a similar study (Chet et al. (1979) found that by integrating low dosages of PCNB with T.harzianum a synergistic interaction between both treatments resulted in greater control of damping-off of bean seedlings by S.rolfsii than the sum of the two treatments used individually. They concluded that this form of integrated control minimized pollution of the soil and reduced interference in the biological balance.

Fusarium roseum 'Culmorum' may be reduced if wheat stubble becomes colonized by common saprophytes as discussed earlier. Furthermore, the pathogen is inhibited by bacteria in moist soils but causes severe foot rot under dry conditions. In Washington state an integrated programme to conserve water and so decrease losses from this disease has been developed. Techniques such as dust mulching and chisel ploughing to increase water infiltration are employed, and varieties of high water efficiency are grown. By late seeding and the use of minimal amounts of fertilizer plants are kept small in the autumn and spring to reduce water consumption and delay water stress (Baker,1981). Similarly Lewis and Papavizas (1980) obtained encouraging results by integrating cultural methods with biocontrol to suppress fruit rot of cucumber by R.solani. Corticum sp. and a strain of T.harzianum grown on sand-cornmeal medium when raked into soil reduced disease and were as effective as the fungicide captafol but not as effective as chlorothalonil in plots that had been disked (5-7 cm deep). In ploughed plots (20-25 cm deep) in which pathogen inoculum was removed from its optimum position in the upper few centimeters of soil, disease reduction was as effective as that given by either fungicide. Moreover the use of T.harzianum or captafol, with mechanical ploughing resulted in a synergistic reduction in cucumber fruit rot.

1.6 INDUCTION OF NEW BIOTYPES OF TRICHODERMA

Improvements in the yield and quality of enzymes synthesized by Trichoderma spp. in industrial microbiology have been achieved via genetic improvements. Mutation and selection have been used to obtain strains of T.reesii Simmons that produce more enzymes of the cellulase complex than the wild type. This concept has been used to obtain new biotypes of T.harzianum induced by ultraviolet irradiation (Papavizas et al. 1982), and exposure to high concentrations of fungicides.

Emphasis has been placed on inducing biotypes resistant to fungicides, in particular benomyl, and which possess greater biological control properties (Papavizas and Lewis, 1981). Papavizas et al. (1982) irradiated conidia of a T.harzianum wild strain with UV-light for 100 minutes. Surviving colonies were isolated and grown on vegetable juice agar and conidia from these were allowed to germinate, subjected to a second irradiation, and the process repeated a third and final time. Induced biotypes of T.harzianum obtained in this way tolerated high concentrations of benomyl (100-500 µg/ai/ml). They also differed considerably from the wild strain in growth habit and appearance, survival ability in soil and in fungitoxic metabolite production (against Sclerotium cepivorum). Some of UV-induced biotypes were more effective than the wild strain in suppressing P.ultimum-incited damping-off of peas when used as a seed treatment and they suppressed damping-off of cotton and radish induced by R.solani and white rot of onion caused by S.cepivorum when used to augment soil. For soil inoculations aqueous suspensions of conidia and dried sand-cornmeal wheat bran preparations containing a modified gliotoxin fermentation medium were both used. The saprophytic colonization of table beet in soil by R.solani was suppressed more effectively by five of the UV-induced biotypes tolerant to benomyl than by the wild strain. The authors concluded that the new biotypes resulted

from mutation rather than selection alone (Papavizas *et al*. 1982).

Isolates of *T.harzianum* tolerant to the fungicides chlorothalonil, procymidone, iprodione and vinclozolin were obtained by Abd-El Moity *et al*. (1982) by exposing four wild strains to the fungicides. However prolonged and repeated exposure of mycelia and conidia to benomyl did not produce tolerant isolates. In contrast with the results of Papavizas *et al*. (1982) tolerance was not always a stable characteristic and it was suggested that in many cases tolerance may be due to non-genetic adaptation (training), and in order to preserve tolerance cultures may need to be maintained on media amended with the appropriate fungicide. A combination of iprodione and an iprodione-tolerant isolate of *T.harzianum* gave significantly greater control of onion white rot than did the chemical alone. This combination and the iprodione-tolerant isolate alone gave the best disease control under field conditions.

Papavizas and Lewis (1983) induced ten new biotypes tolerant to methyl benzimidazole carbamate fungicides namely benomyl, thiabendazole and thiophanate-methyl (up to 100 µg/ai/ml). When conidia of these UV-induced biotypes were used as seed treatments several were more efficient at suppressing *P.ultimum*-incited damping-off of peas than the wild strain. Similarly two of the new biotypes also gave significantly better control of damping-off and blight of beans caused by *S.rolfsii* than the wild strain and one biotype was reported to be an effective biocontrol agent of *Fusarium* wilt of chrysanthemum (Locke *et al*. 1982).

Thus long exposures to UV-irradiation and to various fungicides is a feasible means of inducing new stable biotypes of *Trichoderma* spp. Results suggest that some of these new biotypes may possess superior biocontrol properties and may be compatible with chemical fungicides for use in an integrated control programme.

1.7 SUPPRESSIVE SOILS

Naturally occurring pathogen-suppressive soils have been recognized in which the pathogen either is unable to establish or alternatively establishes but causes no disease. Also recognized are soils where the pathogen establishes but disease declines with monoculture (Baker and Cook, 1974). Suppressiveness is often lost after treating soils with aerated steam or with fumigants indicating that the absence of disease is biological in nature. Multiple antagonists are often implicated as are abiotic factors such as pH, soil type and moisture but in the majority of cases the precise mechanism of control remains unclear. Studies of naturally suppressive soils may elucidate underlying mechanisms by determining the organisms and environmental factors involved in what are frequently considered to be examples of natural biological control (Baker and Cook, 1974; Baker, 1980; Hornby, 1983).

Fusarium-suppressive soils have been reported and studied by many authors and include examples of "resistant" soils (Hornby, 1983) in which the pathogen fails to establish or establishes but produces little or no disease. Soils suppressive to *Fusarium* wilt of pea caused by *F.oxysporum* f.sp. *pisi* and Panama disease of bananas caused by *F.oxysporum* f.sp. *cubense* were identified in the 1930's in Wisconsin and Central America respectively (Baker and Cook, 1974). Studies on Panama disease found that in short life sandy soils, bananas dies three to four years after planting and developed high pathogen populations. This contrasted with long-life clay soils which developed only low pathogen populations and in which banana plants remained healthy for up to twenty years. This was attributed to the presence of montmorillonite clays in long life soils which were absent in short life soils, the proportion of which was directly related to suppression (Baker, 1981). It was found that these clays stimulated bacterial activity primarily by maintaining the pH at a level

suitable for sustained metabolism such that growth of the pathogen was checked (Stotzky and Rem, 1966, 1967).

Fusarium oxysporum consists of a saprophytic non-pathogenic population with about fifty specialized pathogenic clones (formae speciales). These non-pathogenic clo es have been implicated as competing with the pathogenic ones on or within the root. Cook (1981) suggested that these may induce resistance in the hosts. The evidence suggests that a soil suppressive to one forma specialis of *F.oxysporum* will also be suppressive to other formae speciales (Smith and Snyder, 1972). This indicates that formae speciales have similar characteristics that affect their interaction with the suppressive microbiota. However supression does not extend to the saprophytic *F.oxysporum* or *F.roseum* or *F.solani* (Cook, 1981; Baker, 1981).

Muskmelons have been grown in the Chateaurenard région of France with little *Fusarium* wilt disease (*F.oxysporum* f.sp. *melonis*) even though the disease is often very severe in two nearby regions (Schroth and Hancock, 1982). Alabouvette *et al*. (1979) found that higher populations of non-pathogenic clones of *F.oxysporum* were present in suppressive soils and following heat treatment addition of clones of *F.oxysporum* either native to or alien to the soil restored suppressiveness. Their results pointed to the involvement of other microorganisms perhaps working synergistically with the non-pathogenic clones. Smith (1977), found that the germination of chlamydospores and growth of hyphae of *F.oxysporum* f.sp. *vasinfectum* and *F.oxysporum* f.sp. *tracheiphilum* were less in suppressive soils. Large populations of *Arthrobacter* sp. developed more rapidly in suppressive soils than in conducive soils and were associated with germlings of *F.oxysporum* f.spp. *vasinfectum* and *tracheiphilum*, and increasing in numbers as the germ tubes grew. However inhibition or lysis of *Fusarium* mycelium was reported to be slight

on agar plates whereas Bacillus sp. and Actinomycetes caused extensive lysis. In contrast Mitchell and Hurwitz (1965) observed extensive lysis of F.oxysporum f.sp. lycopersici in dual culture with a strain of Arthrobacter isolated from tomato rhizosphere. The rapid multiplication of bacteria and lysis of the fungus that often occurs in Fusarium-suppressive soils may be due to a depletion of nutrients and oxygen by bacterial activity in the vicinity of the germ tubes and the presence of deleterious metabolites (Hornby, 1983).

Scher and Baker (1980, 1982) found a fluorescent Pseudomonas sp. isolated from Fusarium wilt-suppressive soil in the Salinas Valley in California which induced suppressiveness to the flax with pathogen (F.oxysporum f.sp. lini). Further, this species identified as P.putida produced siderophores which are high affinity iron chelators and found that competition for iron was responsible for the suppressiveness since the pathogen required iron for germ tube elongation. In studies investigating the survival of P.putida populations Dupler and Baker (1984) concluded that the ability to induce suppressiveness to Fusarium wilts was dependent on their root-colonizing ability.

Cook (1981) summarised the differences often observed between Fusarium wilt suppressive and conducive soils:- (1) A rapid decline in pathogen population occurs in suppressive soils compared with conducive ones; (2) Several times more inoculum for infection is required per unit mass of soil than in conducive soils; (3) In the presence of nutrients the percentage of chlamydospore germination is lower and the growth rate of germ tubes is less suppressive than in conducive soils.

Phytophthora cinnamomi although present in an avocado grove in a rain forest area in Queensland, Australia, has caused little root rot for forty to

fifty years. Suppressive soil becomes fully conducive when inoculated with the pathogen following treatment with steam at 100°C for 30 minutes but remains suppressive if treated at 60°C for 30 minutes (Broadbent and Baker, 1975; Baker (1981). Thermo - tolerant spore-forming bacteria or Actinomycete antagonists are thought to be members of a multiple antagonist microflora. The suppression is believed to be a function of the undisturbed rain-forest soils and may be temporarily lost after waterlogging or flooding, probably as a consequence of alterations to the microbial balance (Broadbent et al., 1971). High levels of organic matter and calcium, with the ammonium nitrogen and calcium tied up in the organic cycle are characteristic of these Phytophthora-suppressive soils, with the pH near neutrality and a large and diverse microbiota being present (Hornby, 1983). Large quantities of poultry manure, composted plant material and lime or dolomite used by Australian avocado growers has given satisfactory control in avocado groves (Baker, 1980, 1981; Cook, 1981). Malajczuk et al. (1983) found that soil extracts from red earth forest soil suppressive to P.cinnamomi stimulated sporangial production and greater hyphal lysis by microorganisms compared with extracts from yellow and red podsol conducive to disease development. Although sporangial production was stimulated in red earth many of the sporangia were non-viable confirming reports by Nesbitt et al. (1977); that reduced viability of sporangia inspite of the increase in numbers can lead to inoculum reduction in suppressive red earth soils.

Pegg (1977) (In Baker, 1981) described the use of sulphur to reduce the pH below 5.4 for the control of Phytophthora root rot of pineapple. Antagonistic and nitrifying bacteria were inhibited and nitrogen which remained in the ammonium form was somewhat inhibitory to Phytophthora. T.viride favoured by these acid conditions apparently controlled the disease and it was proposed that the antibiotics gliotoxin and viridin stable only at low pH may be involved.

The extensively studied decline in disease caused by the take-all fungus *Gauemannomyces graminis* var. *tritici* occurs after continuous wheat cultivation. Disease reaches a maximum after two to three years and then declines to a tolerable level. Multiple antagonists are apparently involved and the live virulent pathogen is required for suppressive microflora to develop (Baker and Cook, 1974). Antagonistic antibiotic-producing pseudomonads which colonize roots may be involved (Cook and Rovira, 1976; Deacon, 1983) since high populations were found in association with wheat roots grown in suppressive soils (Weller and Cook, 1981). Other proposed mechanisms for a take-all decline include antagonism in host residues and loss of virulence (Deacon, 1983; Hornby, 1983).

Similarly the potato common scab pathogen (*Streptomyces scabies*) declines after eight years of monoculture in California and Washington soils (Baker, 1981). Suppressiveness is lost when soil is autoclaved but suppressiveness can be transferred. Scab was reduced when 10% suppressive soil and 1% alfalfa meal were incorporated into conducive soil (Menzies, 1959).

Certain bean fields in the Columbia River Basin are suppressive to *Fusarium solani* f.sp. *phaseoli* which causes root rot of bean. Hyphal growth is extensive in these soils but chlamydospores are small and fewer in number than in conducive soils and are formed slowly. In contrast large chlamydospores were formed rapidly in conducive soils but hyphal growth was limited. Autoclaving eliminated suppression but suppressiveness could not be transferred to other soils (Burke, 1965).

Henis *et al.* (1979) used successive crops of radish to induce suppressiveness to *R.solani*-incited seedling blight. A fresh crop of host seeds was planted every seven days in trays of pathogen-infested soil and by the fifth crop virtually no blight occurred.

Liu and Baker (1980) concluded that this was due to *Trichoderma* spp. They found that the numbers of propagules of *Trichoderma* spp. increased as suppressiveness increased and that the inoculum density of *R.solani* was inversely proportional to these *Trichoderma* spp. populations. Suppressiveness could be induced when conidia of *Trichoderma* were added to conducive soil at the same density as found in suppressive soils. Suppressiveness developed more rapidly in acid soils which are known to favour *Trichoderma* spp. and the latter were re-isolated from mycelial mats of the pathogen incubated in suppressive soils. Successive plantings using alternative hosts which did not lead to increases in the *Trichoderma* population failed to induce suppressiveness.

Since many of the mechanisms operating in soil are presumptive these experiments which, by analogy with Koch's postulates provide strong evidence that *Trichoderma* spp. are responsible for suppression, are an important development. However Deacon (1983) pointed out that hyphae of introduced *R.solani* are likely to autolyse in the absence of a suitable substrate and *Trichoderma* may just colonize the hyphal remains accounting for its presence on buried hyphal mats of the pathogen.

In both natural and induced suppressive soils microbial antagonism appears to play a central role and is likely to be correlated with certain physical and chemical properties, and abiotic factors such as soil type and the presence of clay mineral fractions. In soils which demonstrate a long sustained suppressiveness the situation must be complex and it seems unlikely that a few individual properties or organisms determine suppressiveness. More likely populations of a number of microorganisms are involved being favoured by abiotic factors which interact to promote and maintain a more durable form of disease suppression.

Thus Trichoderma spp. have been implicated in several fore-mentioned control processes including the example of induced suppressiveness to R.solani by continual cropping. However inspite of extensive research the practical commercial success of Trichoderma spp. in crop protection is limited. Trogoff and Ricard (1976) reported the successful control of dry bubble disease of mushroom by Trichoderma spp. and in France a Trichoderma preparation in a nutrient base has been registered for the control of this pathogen (Verticillium malthousei). Similarly Trichoderma spp. have been effective in ameliorating symptoms of the silver leaf pathogen (Chondostereum (=Stereum) purpureum) in plum and perry pear trees (Grosclaude et al., 1973; Corke, 1974) and a Trichoderma preparation has received clearance from the registration authorities in Britain for pilot scale tests.

The rationale behind this project was to evaluate this same Trichoderma preparation as a biocontrol agent against a range of diseases with a view to its eventual commercial use for the control of soil pathogens under greenhouse conditions. This preparation (Binab T, Bioinnovation AB, Sigtuna, Sweden) is a composite of two species aggregates of T.harzianum and T.polysporum (Rifai) and henceforth is referred to as TRC.

2. INTERACTIONS BETWEEN TRC AND SOIL FUNGI ON AGAR MEDIA

2.1 INTRODUCTION

The antagonistic ability of microorganisms isolated from soil is often assessed using agar plate tests. The dual inoculation plate test described by Dennis and Webster (1971 c) has been widely used to estimate antagonism against specific target organisms in pure culture. This technique has proved useful for screening large numbers of candidate organisms for antagonism (Broadbent et al., 1971; Wells and Bell, 1979; Sivasithamparam and Parker, 1980; Bell et al., 1982) and has selected effective Trichoderma antagonists from soil which have subsequently proved to be successful biocontrol agents in greenhouse or field experiments (Hadar et al., 1979; Elad et al., 1980).

Thus the dual culture method was used to investigate the antagonistic activity of the Binab TRC and assess the susceptibility of various fungi to antagonism in vitro.

2.2 MATERIALS AND METHODS

2.2.1 Fungi

The Binab T.Trichoderma preparation (TRC) was supplied as a wettable powder (wp) formulation by Stokes Bomford Chemicals Limited. The commercial preparation was stored at 4°C and fresh TRC cultures were obtained from this powder at three monthly intervals.

The following fungi were obtained from the culture collection held at Imperial College:-

Pythium ultimum Trow, Pythium debaryanum Hesse, Pythium undulatum Peterson, Pythium multisporum Poitras, Rhizoctonia solani Kuhn, Corticium praticola Kotila., Fusarium oxysporum f.sp. lycopersici (Sacc.) Synd.

and Hans. Race O (Gabe, 1975), Verticillium albo-atrum Reinke and Berth., Phytophthora citricola Sawada, Phytophthora cactorum (Lebert and Cohn) Schroter.

The constituents of the Binab T. Trichoderma preparation namely Trichoderma harzianum Rifai and Trichoderma polysporum Rifai were kindly supplied by Dr. P. Morris, Imperial College, University of London.

All stock cultures were maintained under sterile liquid paraffin on 2%(w/v) malt extract agar slopes. These cultures were stored at 4°C and were subcultured at three monthly intervals. For all experimental procedures actively growing colonies were maintained on 20.0 ml of either malt extract (2%(w/v)) or soil extract agar per Petri dish and incubated at 20°C.

2.2.2 Nutrient Media

All chemicals used were Analar grade or the highest purity commercially available. The following media were prepared and were sterilized by autoclaving at 121°C for 20 minutes:

Malt Extract Agar 2%(w/v) (MEA)

Malt Extract (Oxoid)	20.0 g
Agar	20.0 g
Distilled water	1000 ml

V_8 - Agar

V_8 vegetable juice (Campbells Soups Ltd)	200 ml
Agar	20.0 g
Distilled water	800 ml

Soil Extract Agar (SEA)

Glucose	1.0 g
K_2HPO_4	0.5 g
* Soil Extract	100 ml
Bacto-Difco Agar	20.0 g
Distilled water	900 ml

* Preparation: Soil extract was prepared by autoclaving 1000g of garden soil in 1000ml of tap water for 30 minutes at 121°C. After cooling the soil suspension was filtered through double layers of muslin to remove large soil particles. 0.5g of calcium carbonate was added to the suspension and filtration was continued through double layers of filter paper in a Buchner funnel attached to a water vacuum pump. The extract was filtered through increasing numbers of filter paper until a clear liquid was obtained. The extract was bottled in 50.0ml aliquots and sterilized at 121°C for 20 minutes. The extract was stored at 4°C in the cold room until required. The pH of the extract was between 6.0 and 6.5.

2.2.3 Dual Inoculation Plate Tests

Screening *in vitro* using the Binab *Trichoderma* (*TRC*) preparation relied on dual culture tests as used by Dennis and Webster (1971(c)), for assessment of hyphal interactions.

Disposable Petri dishes (9.0cm) containing 20.0ml of medium were used for all *in vitro* plate tests. A 5.0mm disk of the test organism cut from the margin of an actively growing culture with a sterile 5.0mm diameter corkborer, was positioned diametrically opossite a 5.0mm disk of *TRC* obtained in the same manner. Disks were placed at the periphery of the plate. Controls of each organism alone were included to assess unimpeded growth rates.

All plates were incubated at 20°C and were observed daily. Growth was measured in mm using calipers (Camlab, Cambridge) and was recorded as the mean of two measurements taken at right angles across the unaffected and healthy looking fungal colony viewed from the undersurface of the plate. Paired interactions and controls were routinely replicated three times and the means for each organism expressed with its standard error.

The maximum possible growth was 86.60mm. Dual inoculation tests were repeated at least three times for each test organism.

2.2.4 Transects

Squares of agar, approximately $5.0mm^2$ were cut from the agar surface of dual cultures at 1.0cm intervals along a diameter transecting the plate. Each square was transferred to a fresh agar plate, usually MEA and incubated at 20°C. Plates were viewed daily and growth indicated that the organism was viable and had grown to the position on the plate sampled. In cases where TRC dominated the plate by overgrowing the pathogen colony transects also indicated the subsequent viability of that second organism.

RESULTS

2.3 Dual Inoculation Plate Tests using Pythium ultimum and Pythium debaryanum as the test organisms

2.3.1 Malt Extract Agar (MEA) (2% w/v)

Growth of P.ultimum and P.debaryanum on rich nutrient agar such as 2% MEA was rapid with complete colonization normally occurring within three days of inoculation on control plates (Figure 1). In dual culture with TRC growth of Pythium spp. was inhibited and on approaching the antagonist the shape of the Pythium colony often altered giving a straighter and flatter outline to the growing edge.

P.ultimum and P.debaryanum both characteristically produce dense white mycelium on this medium with a mass of aerial hyphae giving a 'fluffy' appearance. Contact and overlap of the two colony edges occurred by the third day of incubation and progressive growth

of TRC over the Pythium colony caused hyphae of the latter to collapse. This may have initially been due to the physical pressure exerted by the TRC hyphae as it grew over the target mycelium, however flattened sometimes water-soaked like areas of Pythium were often observed well ahead of the TRC growing margin (Plate 1).

Pythium ceased growth after contact had been established and at this stage the growth rate of TRC was also occasionally reduced compared with controls. However TRC growth proceeded and dense sporulation usually in the typical zonal ring formation occurred throughout the region previously colonized by Pythium mycelium. Sporulation frequently occurred at the boundary between TRC and Pythium.

Occasionally a portion of the Pythium colony beyond the water-soaked and flat mycelium survived remaining 'fluffy' for a short period but was confined to a small peripheral region of the plate surrounding the inoculum disk. However, the continued extension of TRC over the agar soon led to total colonization of the plate usually seven to ten days after inoculation (Figures 1 and 3), leaving no visible traces of surviving pathogen mycelium.

Growth from samples of agar taken along a transect that joined the two original inoculum disks confirmed that TRC was the dominant organism and Pythium if detected was restricted to the vicinity of the initial inoculum disk.

2.3.2 V_8 - Agar

The interaction between TRC and P.ultimum and P. debaryanum was essentially the same as that occurring on malt extract agar. Upon contact with TRC, Pythium ceased growing and TRC continued its progress across the plate growing over the Pythium colony. Complete colonization

Figure 1

Growth of TRC and *P.ultimum* in single and dual culture on MEA

●———● *P.ultimum* in dual culture

●- - -● *P.ultimum* control grown in axenic culture

○———○ TRC in dual culture

○- - -○ TRC control grown in axenic culture

Bars denote the standard error of the mean

Figure 1

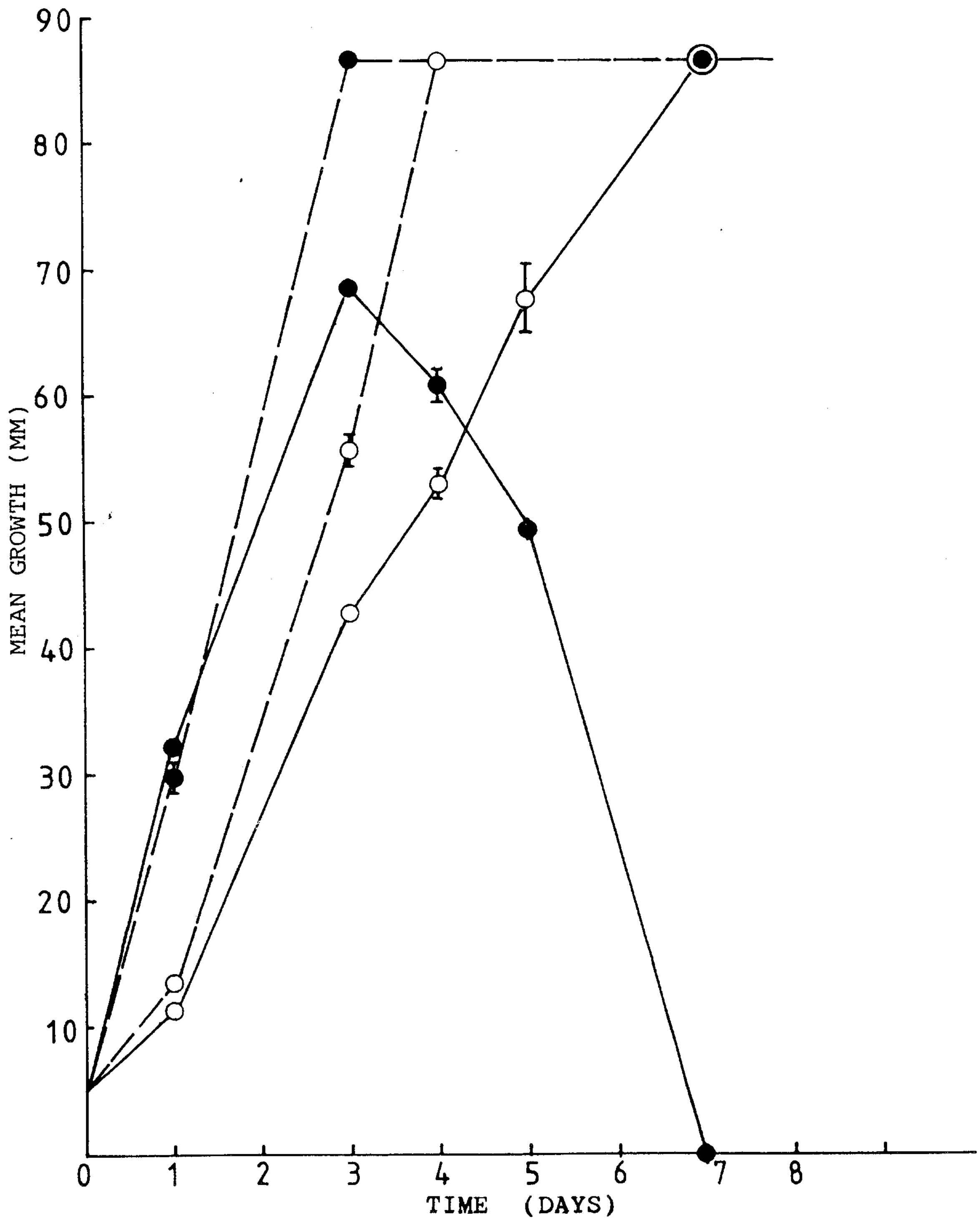


Figure 2

<u>Growth of TRC and P.ultimum in single and dual culture on SEA</u>

●——● *P.ultimum* in dual culture

●— —● *P.ultimum* control grown in axenic culture

○——○ *TRC* in dual culture

○— —○ *TRC* control grown in axenic culture

Bars denote the standard error of the mean

Figure 2

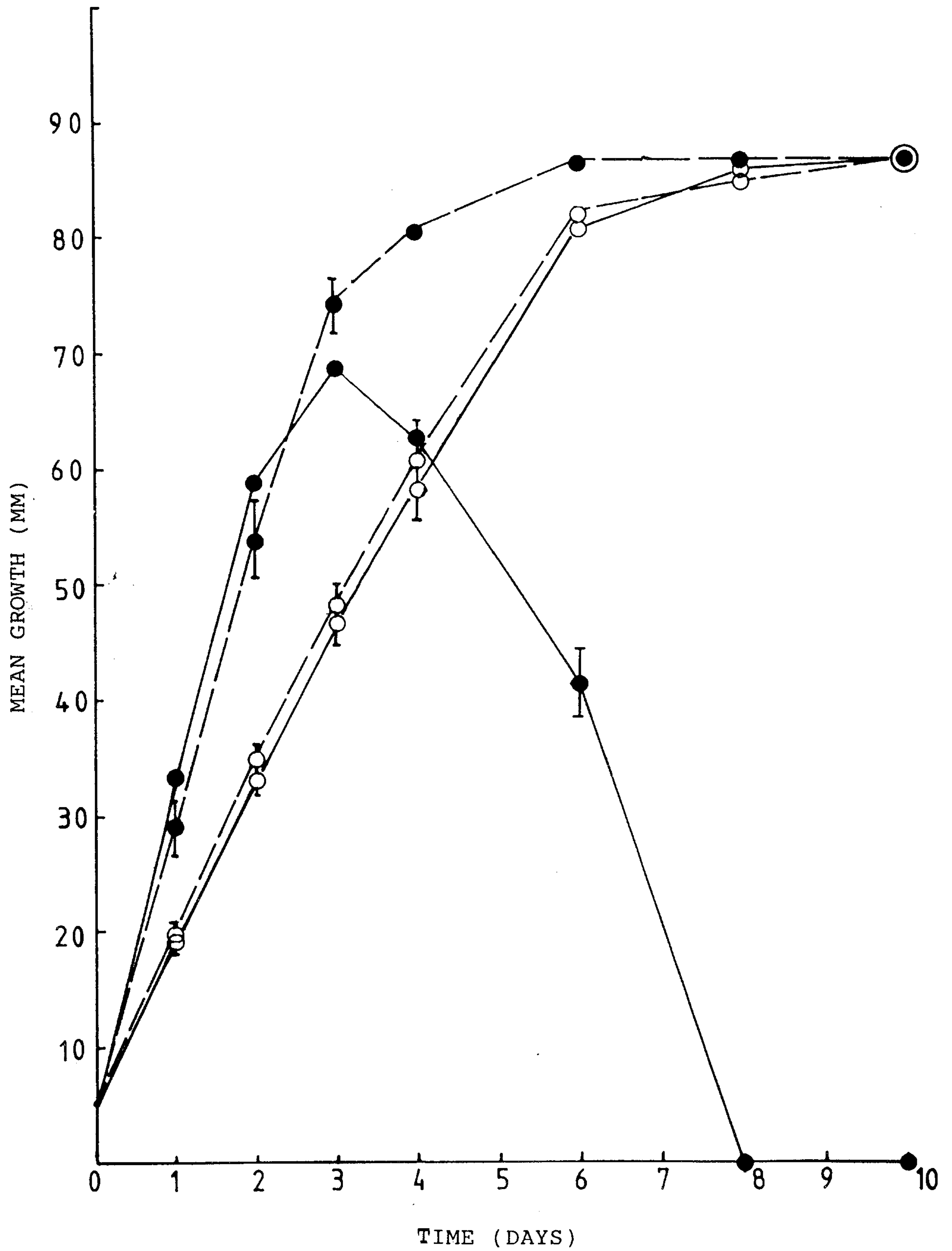


Figure 3

<u>Growth of TRC and P.debaryanum in single and dual culture on MEA</u>

●——● *P.debaryanum* in dual culture
●- - -● *P.debaryanum* control grown in axenic culture
○——○ *TRC* in dual culture
○- - -○ *TRC* control grown in axenic culture
Bars denote the standard error of the mean

Figure 3

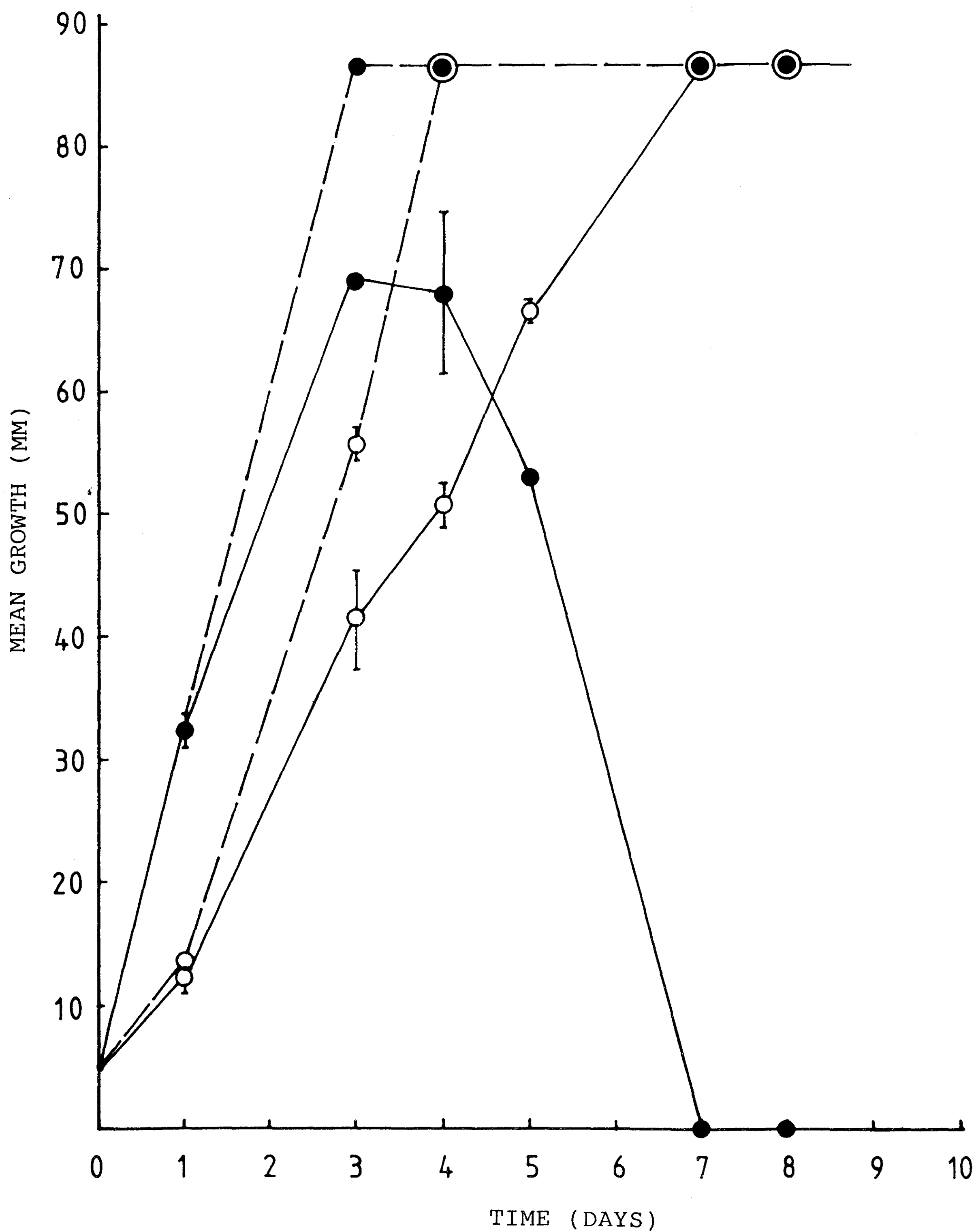
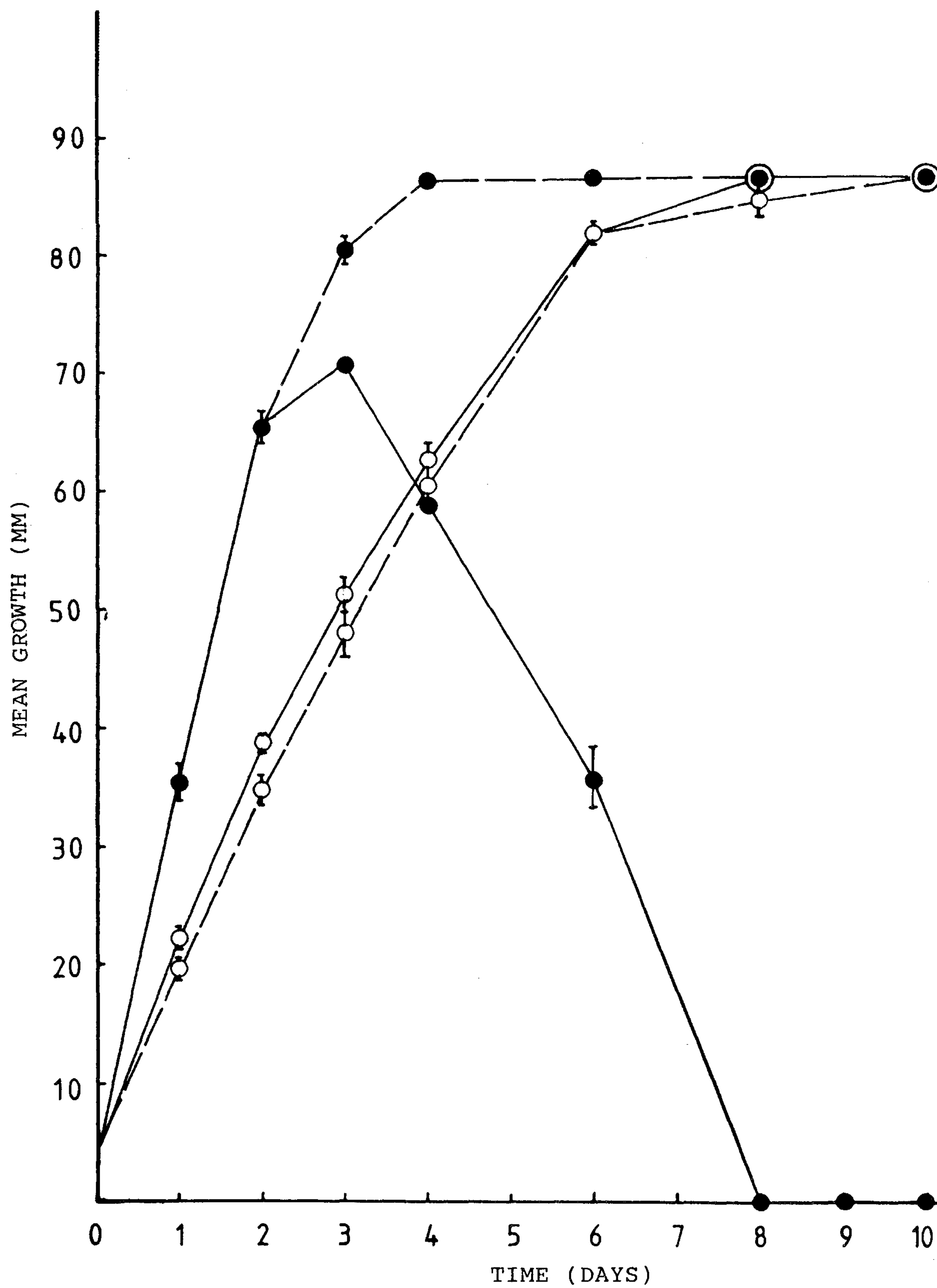


Figure 4

Growth of TRC and P.debaryanum in single and dual culture on SEA

●——● P.debaryanum in dual culture
●- - -● P.debaryanum control grown in axenic culture
○——○ TRC in dual culture
○- - -○ TRC control grown in axenic culture
Bars denote the standard error of the mean

Figure 4



by TRC with dense sporulation generally occurred by seven to ten days after inoculation Pythium was not re-isolated from transect samples taken from a position on the agar surface distal to the original inoculum disk.

Microscopic examination of hyphae was extremely difficult on both MEA and V_8 agar. The dense growth of both organisms and the profuse sporulation of TRC on these rich media prevented detailed examination of Pythium hyphae. However disintegration and disorganization of the cytoplasm in Pythium hyphae was occasionally observed in the region of overlap, similar to the observations of Howell (1982) in tests with Gliocladium virens.

2.3.3 Soil Extract Agar (SEA)

SEA was used for dual inoculation plate tests using both P.ultimum and P.debaryanum as the test pathogens. This medium is probably more appropriate for screening potential interactions for use in soil than some of the richer nutrient media used previously.

Both Oomycetes reacted in a highly distinctive fashion to the presence of TRC on this medium. Prior to contact, growth of both fungi was normal, but once within close proximity (1-3mm) to the TRC colony, the Pythium spp. developed a flattening and thinning of the mycelium. This initially affected the region of the colony directly opposing and closest to TRC and implicated the activity of antibiotic-like metabolites, but since the latter is often associated with rich media, it may be of more limited importance on SEA. Pythium hyphae in this region closest to the TRC colony edge soon ceased extension growth, although regions of the colony further removed from the TRC remained healthy and continued growing (Figure 5) until also confronted by the outer margins of the TRC colony.

Once TRC grew into the margin of the Pythium colony a zone of clearing developed, 2-3mm in width beyond the region of overlap directly in front of the TRC leading edge. This zone which usually developed three to five days after inoculation was often widest at the centre of the Petri dish and was readily visible to the naked eye. Formation of this zone of clearing in the manner described accompanied the growth of TRC as it progressed deeper into the established Pythium colony (Figure 5). Microscopically it was characterized by a granulation of cytoplasm leading to total lysis of the hyphal contents. Septa were observed in this characteristically coenocytic fungus, a condition often associated with old hyphae (Wheeler, 1978), but which can also develop in response to stress conditions (M. J. Carlile, personal communication), but any barrier they may have constituted perhaps separating damaged areas in contact with TRC from the unchallenged portion of the Pythium colony, was only temporary. The final stages were marked by the hyphal walls becoming less distinct, but there was no evidence even in old colonies for widespread wall degradation.

The continued growth of TRC across the Pythium colony resulted in the production of further clear interaction zones and rings of spores. Thus, TRC rapidly established itself as the dominant organism colonizing the entire agar surface (Figures 2 and 4). Samples of agar taken along transects usually failed to yield P.ultimum and P.debaryanum upon sub-culturing; although Pythium was on occasions re-isolated from the vicinity of the original inoculum disk.

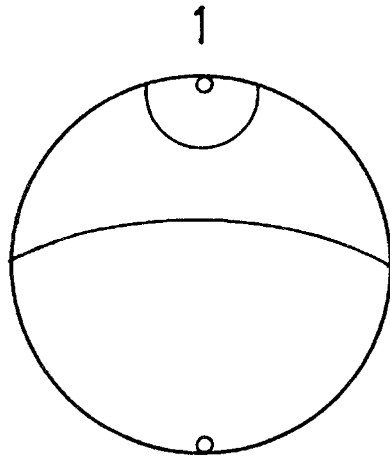
The characteristic zone of clearing formed with TRC was investigated in greater detail with the component species of the Binab preparation, T.polysporum and T.harzianum individually against both P.ultimum and P.debaryanum of the three Trichoderma cultures, T.polysporum grew the slowest yet produced the most distinctive interaction with both Pythium spp. A very

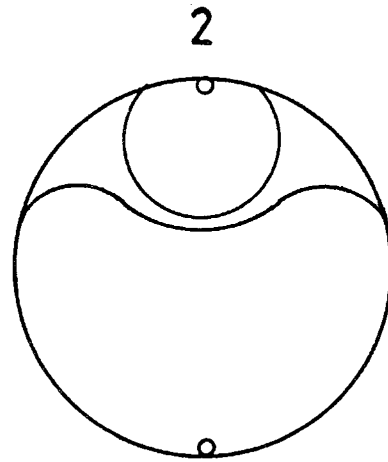
Figure 5

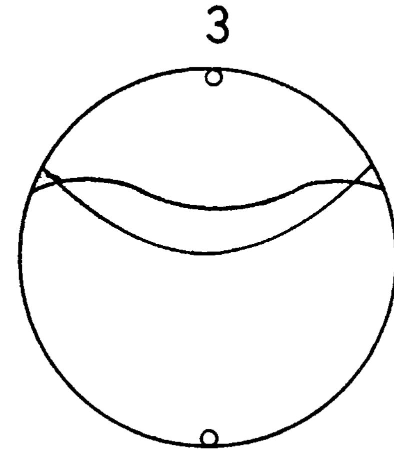
Interactions between TRC and Pythium spp. on Soil Extract Agar (SEA)

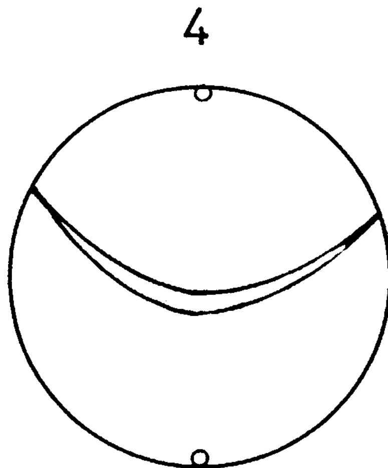
Interactions between TRC (top) P.ultimum and P.debaryanum (bottom) on SEA. 1. Growth of both organisms prior to contact 2. Inhibition of the test organism in a region of the colony directly opposing TRC colony. 3. Overlap of the organisms, the edge of the test organisms becomes faint and indistinct. 4. First formation of the characteristic interaction zone henceforth termed a clear zone, immediately in front of the TRC growing edge. The clear zone is most distinct in the central region. 5. TRC continues to grow producing another clear zone of lysis. 6. Complete cover of the agar surface with sporulation rings of TRC

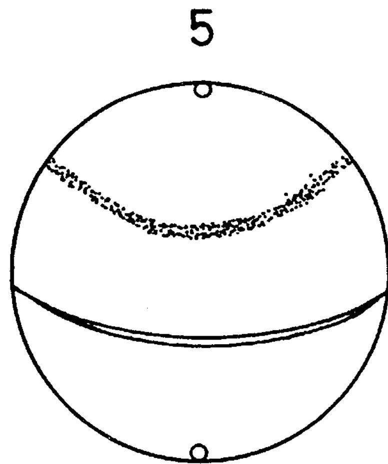
Figure 5

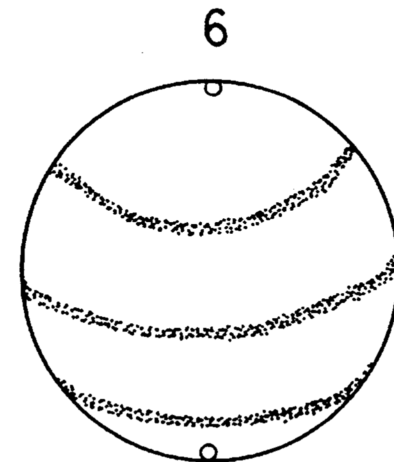












pronounced and marked zone of clearing developed between T.polysporum and P.ultimum and P.debaryanum five days after inoculation (Plate 2). This zone was wider (up to 4.0mm) than that observed with TRC and a yellow-brown discolouration was often associatd with lysis in the area of overlap at a position corresponding approximately to the original edge of the Pythium colony. However, T.polysporum did not grow progressively across the plate in the characteristic manner associated with colonization by TRC. The slower growth rate of T.polysporum resulted in a colony of smaller size than TRC and allowed Pythium to colonize a greater area, in some cases more than 85% of the agar surface, prior to encountering hyphae of the opposing organism. Growth of T.polysporum was therefore reduced to a greater extent than Pythium in comparison with controls (Tables 1 and 2).

Following contact, growth of T.polysporum initially continued unaltered but soon extended into a region where hyphae of the two organisms intermingled and in which the growing edge of T.polysporum could not be clearly distinguished. Thus despite the obvious antagonism of T.polysporum against Pythium spp. observed initially, the course of events subsequent to the clear zone formation could not be readily discerned, but the growth rate of T.polysporum through the Pythium-colonized region of the agar plate appeared to decline. Moreover unlike TRC and T.harzianum, T.polysporum failed to produce further clear zones subsequent to day five and both T.polysporum and Pythium could be re-isolated from the region of visible intermingling which occupied approximately half the plate by day 16. Extended incubation resulted in the development of characteristic white clumps of T.polysporum spores at the periphery of some plates, which confirmed that inspite of its irregular and slow growth, T.polysporum was capable of continued growth in the presence of viable Pythium, reaching the edge of the plate most distal to its inoculum disk 21-28 days after inoculation. In regions of mutually

Table 1

The effect of TRC, *T.harzianum* and *T.polysporum* on growth of *P.ultimum* growth in dual culture on SEA.

	% Inhibition					
DAYS	TRC	Pu	Th	Pu	Tp	Pu
1	0	3.9	11.2	0	9.3	5.6
2	6.1	12.4	9.4	12.4	0	8.4
3	6.9	5.4	22.9	4.8	0	4.0
4	8.0	8.0	23.8	11.9	0	11.6
7	5.2	25.7	28.9	20.4	5.2	23.2
8	4.1	48.7	26.4	22.6	14.5	22.3
9	0.1	77.2	17.2	26.4	8.5	24.9
10	0	100	5.1	34.6	9.7	26.9
11	0	100	3.5	55.0	22.1	26.9
12	0	100	2.9	72.4	24.3	26.9
14	0	100	1.8	84.0	30.4	26.9
15	0	100	0	84.4	32.1	26.9
16	0	100	0	100	33.2	26.9

% inhibition compared with the controls in which each organism was grown in axenic culture TRC Binab *Trichoderma*; Th *T.harzianum*; Tp *T.polysporum*; Pu *Pythium ultimum*

Table 2

The effect of TRC, T.harzianum and T.polysporum on growth of P.debaryanum in dual culture on SEA

	% Inhibition					
DAYS	TRC	Pd	Th	Pd	Tp	Pd
1	0	2.1	3.7	0	10.7	0.1
2	0	0	0	0.3	0	5.5
3	0	14.1	1.5	10.1	0	12.5
4	0	22.1	22.2	12.7	0	11.7
7	0	54.8	6.5	25.9	22.7	15.4
8	0	91.4	7.7	28.5	28.7	16.3
9	0	100	5.0	41.7	38.9	16.3
10	0	100	5.4	49.2	40.5	14.9
11	0	100	3.0	63.0	47.4	16.4
12	0	100	0.8	87.2	46.4	16.4
14	0	100	0	100	46.2	16.4
15	0	100	0	100	50.5	16.4
16	0	100	0	100	50.0	16.4

% Inhibition compared with controls in which each organism was grown in axenic culture TRC Binab Trichoderma; Th T.harzianum; Tp T.polysporum; Pd Pythium debaryanum

intermingling hyphae in dual culture tests, the margins of the individual colonies cannot be easily distinguished especially in the presence of aerial hyphae, and thus measurements of the irregular growth of T.polysporum may have underestimated the true extent of its presence within the Pythium colony.

In contrast the events with T.harzianum were very similar to those seen in paired culture tests using TRC. In each case defined zones of clearing first developed in dual cultures on the fifth day of incubation and colonization of the plate by T.harzianum was evident from the presence of sporulation rings across the plate. Complete cover of the Pythium colonies and agar surface by T.harzianum took 14-16 days whilst equivalent growth by TRC took 10 days (Tables 1 and 2). The growth rate of T.harzianum was retarded by the presence of P.ultimum and to a lesser extent P.debaryanum, being inhibited prior to and immediately after initial contact. The growth of TRC was not affected in this way by P.debaryanum whilst P.ultimum had a marginally debilitating effect. In contrast with results on rich media, generally little inhibition of TRC was observed in paired culture tests on SEA and on occasions growth appeared to be stimulated. It is possible that nutrients released from lysed Pythium hyphae enabled TRC to progress rapidly across the plate establishing itself as the dominant organism.

SUMMARY

2.3.4 TRC was an aggressive antagonist of both P.ultimum and P.debaryanum on MEA, V_8 and SEA. The interactions on MEA and V_8 clearly demonstrated the intense competitive nature of the Binab TRC against Pythium. However, the results were broadly similar to the reactions sen on MEA with other test organisms (Sections 2.5 to 2.6) with TRC competing vigorously for the available resources, inhibiting growth of the other fungi and rapidly overgrowing them. Nevertheless, dual culture tests with these Pythium

Plate 1

TRC and *P. ultimum* in paired culture on MEA

Overgrowth of the *P.ultimum* colony by TRC (upper). Progressive TRC colonization is accompanied by abundant sporulation above the target pathogen. The *Pythium* mycelium collapses producing flat, sometimes water-soaked regions, which contrast with the healthy, characteristic 'fluffy' mycelium (white regions)

Plate 2

T.polysporum and *P.ultimum* in paired culture on SEA

Growth of *P.ultimum* (lower) ceases on contact with *T.polysporum* (upper); dieback of the *Pythium* mycelium ensues, producing a marked interaction zone between the two colonies. Clear zones in this case were observed on two successive days only

Plate 1

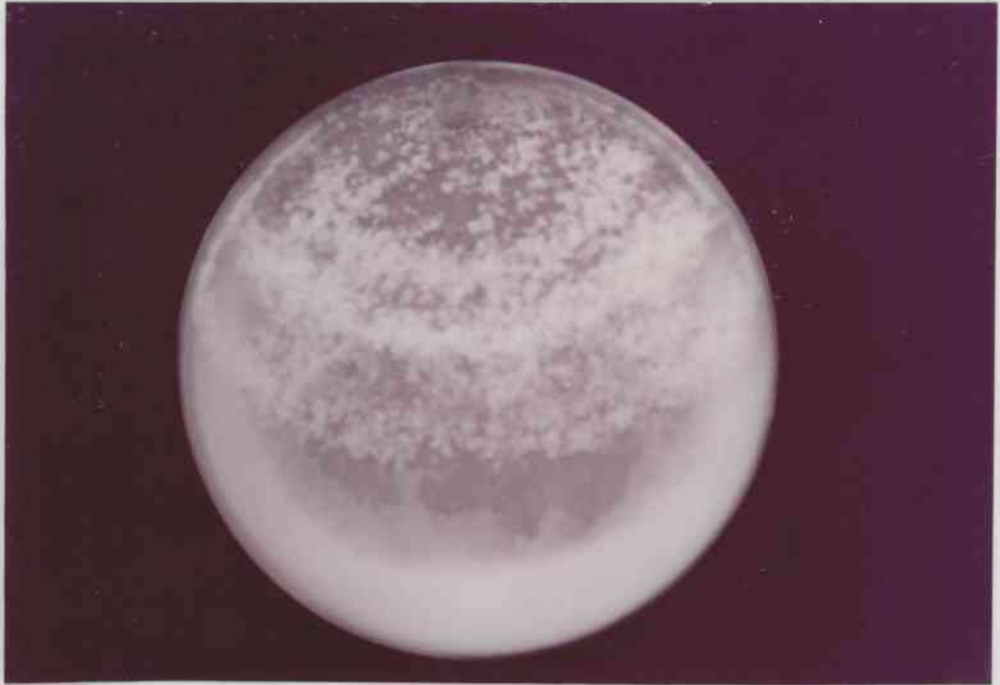


Plate 2

spp. produce more striking and dramatic results than obtained with other candidate organisms. This may in part be due to the growth habit of Pythium spp. on rich media, but the cessation of its rapid growth, together with an apparent dieback, implicated a more active form of antagonism than simple overgrowth.

The zone of clearing and lysis that developed on SEA developed ahead of the TRC colony, within the Pythium colony, occurring in general on 2-4 successive days in parallel with the growth of TRC. In comparison with the activities of T.harzianum and T.polysporum, TRC was the most competitive becoming the dominant organism within 10 days in paired culture tests with Pythium spp. T.harzianum closely mimicked the behaviour of TRC in dual culture whilst T.polysporum produced a marked zone of clearing, but appeared to lack the competitive antagonistic capacity of the other two cultures in vitro. Although total inhibition of Pythium growth did occur, T.polysporum did not appear adversely to affect the overall viability of the Pythium to the degree observed with T.harzianum and TRC.

Inhibition of TRC was sometimes observed on MEA and V_8 agar after initial contact with the pathogen. The slower growth rate may have been due to a depletion of nutrients although this seems unlikely on media of rich nutrient status, more probably this was due to the physical presence of the pathogen which hindered the progress of TRC. Conversely little inhibition of TRC was observed in paired culture tests on SEA.

2.4 Interactions between TRC and other Oomycetes

Zones of clearing and lysis developed within colonies of Pythium multisporum and Pythium undulatum when these were used in paired culture tests with TRC on SEA. Both species and in particular P.multisporum grew more slowly than the Pythium spp. used previously,

Table 3

Percentage inhibition of TRC and Pythium multisporum growth in dual culture on MEA and SEA

	% Inhibition			
	MEA	TRC vs P.multisporum		SEA
DAYS	TRC	P.multisporum	TRC	P.multisporum
1	9.8	0	3.0	0
2	13.7	0	0	0
3	17.5	0	0	0
4	17.2	0.3	0	0
7	12.9	18.2	0	27.9
8	0	100	0	46.1
9	0	100	0	95.1
11-18	0	100	0	100

Table 4

Percentage inhibition of TRC and Pythium undulatum growth in dual culture on MEA and SEA

	% Inhibition			
	MEA	TRC vs P. undulatum		SEA
DAYS	TRC	P.undulatum	TRC	P.undulatum
1	16.9	0	0	0
2	15.7	0	0	0
3	17.0	7.8	0	0
4	16.5	26.0	0	1.4
7	18.8	36.8	0	43.1
8	0	100	0	59.1
9	0	100	0	100
11-18	0	100	0	100

% Inhibition compared with controls in which each organism was grown in axenic culture TRC Binab Trichoderma

but the sequence of events on both MEA and SEA media was broadly similar to that already described (section 2.3.1 to 2.3.3). Due to this slower growth, inhibition occurred at a later stage and clear zones were most distinct in dual cultures on SEA seven days after inoculation with P.multisporum and P.undulatum. These zones were not always as marked as previously observed with P.ultimum, but confirmed that the growth rate of the test Oomycete spp. largely determined when development of this zone occurred, since overlap of the colony edges preceded clear zone formation.

Greater inhibition of TRC growth occurred on MEA compared with SEA (Tables 3 and 4) and in some paired culture tests employing the latter medium, the growth of the antagonist appeared to be stimulated compared with controls. On MEA the growth rate was slightly reduced for the first seven days whilst Pythium spp. were either unaffected or were stimulated, giving greater mean growth values than the controls until the colony margin reached that of the TRC colony. Slight checks in TRC growth were also noted in these tests on both media immediately before and after contact with these test fungi. A transient increase in growth rate of P.multisporum and P.undulatum was also observed on both media prior to irreversible inhibition which occurred once contact with TRC had been made. TRC domination of P.multisporum and P.undulatum on MEA occurred by the eighth day of incubation and was evident 9-12 days after inoculation on SEA. Neither Pythium spp. could be recovered from transects and TRC had clearly become the dominant organism.

Domination by TRC also occurred in tests against Phytophthora citricola and Phytophthora cactorum on MEA and SEA (Tables 5 and 6) and likewise resulted in inhibition of both test organisms on contact. The sequence of events was very similar to results observed with Pythium spp. except that no distinct clear zones

Table 5

Percentage inhibition of TRC and Phytophthora citricola grown in dual culture on MEA and SEA

	% Inhibition TRC vs T.citricola			
	MEA		SEA	
DAYS	TRC	P.citricola	TRC	P.citricola
1	2.4	7.5	32.3	11.9
2	22.2	8.0	16.6	15.9
3	3.8	3.9	6.5	19.0
4	6.2	13.8	16.6	13.9
5	6.0	46.3	9.9	24.5
6	3.6	88.2	0.4	24.0
7	0	100	9.6	35.4
8	0	100	3.0	47.6
9	0	100	0	63.6
10	0	100	0	100

Table 6

Percentage inhibition of TRC and Phytophthora cactorum grown in dual culture on MEA and SEA

	% Inhibition TRC vs P.cactorum			
	MEA		SEA	
DAYS	TRC	P.cactorum	TRC	P.cactorum
1	0	0	26.3	0
2	6.3	5.9	18.5	0
3	0	9.2	1.6	1.6
4	0	11.3	10.8	0
5	2.0	30.4	4.7	0.8
6	0	72.7	0	6.4
7	0	100	0.6	21.1
8	0	100	0	38.1
9	0	100	0	54.9
10	0	100	0	100

% Inhibition compared with controls in which each organism was grown in axenic culture TRC Binab Trichoderma

developed on SEA. As seen with other test fungi TRC growth continued after contact with these slow growing fungi, and progressed over the test colony which became fainter with time colonizing the entire agar surface such that only TRC could be re-isolated.

2.5 Interactions between TRC and Corticium praticola and Rhizoctonia solani

Growth of both Corticium praticola and Rhizoctonia solani was inhibited by the Binab TRC (Tables 7 and 8). On SEA hyphae of these test organisms progressed across the plate until meeting mycelium of the opposing TRC colony, in a position approximately half-way across the agar surface. After contact growth of both the pathogen and antagonist was inhibited, but this was only a slight and temporary effect on TRC which recovered to grow over the completely inhibited C.praticola and R.solani colonies, successfully colonizing the entire agar surface 11-14 days after inoculation. With time mycelium of the pathogens being sparser on this nutrient-poor medium became fainter in appearance and transects generally failed to re-isolate the test fungi.

On MEA the two opposing colonies met approximately three to six days after inoculation. In general the edges of the two colonies overlapped and produced a region of denser appearance two to three mm in width. This region of thicker growth visible at the periphery of the pathogen colony, often contained more aerial hyphae, which may have been formed by the pathogen in response to the opposing TRC hyphae which were also present. Similar observations were noted by Sivasithamparam and Parker (1980) who found that a region of dense growth sometimes occurred with the take all fungus Gauemannomyces graminis var. tritici in the region of contact in paired culture tests with Aspergillus spp., by Wells and Bell (1974) in reactions between Sclerotium rolfsii and Trichoderma spp. and by Butler (1957) with a mycoparasitic isolate of R.solani.

Table 7

Percentage inhibition of TRC and Corticium praticola growing in dual culture on MEA and SEA

% Inhibition

TRC vs C.praticola

MEA			SEA		
DAYS	TRC	C.praticola	DAYS	TRC	C.praticola
1	0	7.5	1	0	0
3	2.6	5.7	2	1.1	0
6	25.9	22.3	3	8.3	0
8	13.0	44.0	4	0	6.4
10	9.8	49.8	5	3.0	14.0
12	1.9	79.2	7	7.6	25.1
14	0	100	9	0	56.3
			11	0	100
			14	0	100

Table 8

Percentage inhibition of TRC and Rhizoctonia solani growing in dual culture on MEA and SEA

% Inhibition

TRC vs R.solani

MEA			SEA		
DAYS	TRC	R.solani	DAYS	TRC	R.solani
1	8.1	0	1	0	0
3	0	8.7	2	1.7	0
6	24.8	24.2	3	0	0
8	15.2	38.3	4	3.6	0
10	12.0	38.0	5	6.3	6.6
12	4.3	66.8	7	10.2	19.8
14	0	100	9	2.7	37.7
			11	1.1	82.6
			14	0	100

% Inhibition compared with controls in which each organism was grown in axenic culture TRC Binab Trichoderma

Plate 3

TRC and *C.praticola* in paired culture on MEA

Growth of *C.praticola* (lower) is inhibited whilst TRC (upper) overgrows the colony, eventually producing dense regions of TRC spores above the target pathogen. The margin of the *C.praticola* colony is defined and often contains more aerial hyphae

Growth of both TRC and the test organism in the region of contact ceased and extension occurred at the sides of each colony, until contact had also been established along the entire front. A distinct boundary separated the two colonies at this stage with the region of mutually intermingling hyphae forming on overlapping interaction zones (Plate 3a).

Occasionally, growth of both colonies was checked for one to two days, but usually TRC growth continued slowly but was mainly restricted to the edge of the Petri dish. In general TRC re-established itself after this brief check and in time grew directly over the Corticium or Rhizoctonia colony.

The antagonist invariably sporulated profusely at the boundary of the pathogen colony and spores were subsequently observed above the entire area of the test organism colony. Neither C.praticola nor R.solani could be recovered from transect samples plated onto fresh agar. It is likely that this is in part due to the large numbers of Trichoderma conidia present which could have obscured any wholly mycelial organism.

2.6 Interactions between TRC and Verticillium albo-atrum and Fusarium oxysporum f.sp. lycopersici

Verticillium albo-atrum being one of the slower growing test organisms screened presented little opposition to the competitive and aggressive TRC. In SEA growth of V.albo-atrum was inhibited by the advancing TRC mycelium and complete colonization of the plate and the test colony by TRC occurred 11 days after inoculation (Table 9). Although the outline of the V.albo-atrum colony was still visible, as observed with other fungi, this became fainter with time indicatng a loss in viability, and was soon obscured completely by the TRC. Similarly on MEA plates TRC rapidly became the dominant organism.

Table 9

The percentage inhibition of TRC and *Verticillium albo-atrum* grown in dual culture on MEA and SEA

	% Inhibition TRC vs Vaa			
	MEA		SEA	
DAYS	TRC	Vaa	TRC	Vaa
1	0	7.8	0	5.1
2	1.4	4.2	0	0
3	4.1	2.3	0	0
4	2.9	0	0	0
7	8.9	29.5	0	12.4
8	8.7	41.6	1.5	14.9
9	4.4	72.3	1.7	21.7
11	0	100	6.5	60.8
15	0	100	0	100
18	0	100	0	100

Table 10

The percentage inhibition of TRC and *Fusarium oxysporum f.sp. lycopersici* grown in dual culture on MEA and SEA

	% Inhibition TRC vs Fol			
	MEA		SEA	
DAYS	TRC	Fol	TRC	Fol
1	0	7.8	0	0
2	7.7	4.2	0	2.1
3	7.3	0	0	3.0
4	5.7	0	2.8	1.6
7	12.7	19.9	1.6	3.1
8	15.6	27.8	3.1	10.9
9	11.5	67.2	5.8	17.2
11	11.5	72.0	10.6	18.3
15	0	100	10.6	31.1
18	0	100	10.6	33.9

% Inhibition compared with controls in which each organism was grown in axenic culture TRC Binab *Trichoderma*; Vaa *Verticillium albo-atrum*; Fol *Fusarium oxysporum f.sp. lycopersici*

On reaching the Verticillium colony TRC continued growing often sporing profusely on the small test colony itself (Plate 4). A slight yellow discolouration was frequently observed on the edge of the Verticillium colony implying that lysis and death resulted subsequent to TRC colonization. On neither media could mycelium of the test fungus be recovered from samples of agar taken along transects.

Fusarium oxysporum f.sp. lycopersici was more resistant to TRC domination in vitro (Table 10). On SEA contact between mycelium of the two opposing organisms resulted in mutual inhibition. This was maintained for a period of 14 days and neither organism appeared to progress during this period. On MEA Fusarium growth was inhibited but a distinct boundary similar to that observed with C.praticola and R.solani was sometimes formed. The extent to which and rapidity with which TRC colonized the plate largely depended on the size of the opposing Fusarium colony (section 2.7). In these tests the F.oxysporum f.sp. lycopersici colony was small and the TRC growth prior to contact rapid. However, on meeting the Fusarium colony TRC did not advance directly over the test colony but extended peripherally surrounding the Fusarium colony first. Occasionally, conidia of TRC were observed confined to the margin edge of the test colony and growth of TRC appeared to be temporarily checked by the presence of the opposing Fusarium (Plate 5). However, under the conditions of this test, total colonization of the Fusarium colony eventually resulted with further growth of TRC progressing over the colony accompanied by the production of large numbers of spores which swamped the test colony. Fusarium mycelium could not be re-isolated from paired cultures on MEA whilst successful re-isolation of Fusarium and TRC on SEA confirmed that both organisms remained viable but both were inhibited.

Plate 4

TRC and *V.albo-atrum* in paired culture on MEA

TRC (upper) colonizes the entire agar surface and conidia completely obscure the small *Verticillium* colony (lower)

Plate 5

TRC and *F.oxysporum* f.sp. *lycopersici* in paired culture on MEA

Growth of TRC (upper) is checked on meeting the *Fusarium* colony (lower), but TRC growth proceeds over the pathogen leading to total colonization, thus producing a reaction as seen in plate 4. Characteristic rings of TRC conidia are evident.

Plate 4

Plate 5

2.7 Paired culture tests using staggered inoculation on 2% (w/v) MEA using TRC, T.harzianum and T.polysporum against F.oxysporum f.sp. lycopersici and V.albo-atrum

Disks of Fusarium and Verticillium mycelium were used to inoculate the surface of MEA plates 7-21 days prior to Trichoderma inoculation in paired culture tests. This staggered inoculation allowed unimpeded pathogen growth hence producing colonies of greater size than with simultaneous inoculation (section 2.6), before being challenged by the opposing Trichoderma mycelium.

Growth of Fusarium alone for 7 or 14 days resulted in colonies that covered approximately 50% and 75% of the agar surface respectively, preceding introduction of TRC, T.harzianum or T.polysporum. Subsequently measurements of each colony were taken over a 14 day period. They revealed inhibition of the Trichoderma colonies compared with controls, which was particularly acute with the greater Fusarium growth (75% cover) (Table 11b). This highlights the artifacts that may be generated in culture tests which utilize organisms with different growth rates.

In both cases, visual observations and the results of agar sampling taken along transects across the plate confirmed that subsequent to contact, mingling of the colony edges occurred with both Fusarium and TRC being successfully re-isolated from this region. This interaction generally produced an easily distinguished and sometimes discoloured region which clearly delimited the colonies. With 50% Fusarium colonization, growth of the latter appeared to be inhibited subsequent to contact (Table 11a) although occasionally mycelium of this pathogen could be recovered 0.5-1.0cm beyond this interaction suggesting that growth may have continued albeit at a much reduced rate. Similarly growth of Trichoderma was checked on contact (Table 11a), but TRC and to a lesser extent T.harzianum appeared to continue extension, but in contrast with results in simultaneous inoculation tests, growth directly over the Fusarium

Table 11

<u>Percentage inhibition of Trichoderma and Fusarium oxysporum f.sp. lycopersici growing in dual culture on MEA using a staggered inoculation</u>

(a) 7 day interval

(b) 14 day interval

Table 11a

7 days Fol growth prior to <u>Trichoderma</u> inoculation

	% Inhibition					
	TRC vs Fol		Th vs Fol		Tp vs Fol	
DAYS	TRC	Fol	Th	Fol	Tp	Fol
1	8.3	0	3.3	4.0	10.7	4.0
2	7.7	0	2.0	1.4	9.9	1.8
3	18.4	2.8	8.3	4.6	6.5	4.2
6	34.1	13.9	32.3	15.2	20.4	11.0
8	42.5	17.8	37.1	20.1	32.4	15.7
12	42.3	18.6	37.0	20.6	43.6	14.6
14	39.3	19.6	38.8	21.3	42.7	15.4

Table 11b

14 days Fol growth prior to <u>Trichoderma</u> inoculation

	% Inhibition					
	TRC vs Fol		Th vs Fol		Tp vs Fol	
DAYS	TRC	Fol	Th	Fol	Tp	Fol
1	0	0	0	3.3	0	0
2	14.5	0.7	46.1	1.2	23.4	0
5	52.0	6.5	49.2	7.6	47.4	3.4
6	59.8	5.9	59.0	6.3	43.6	4.3
7	57.7	8.3	64.2	8.7	61.2	4.6
8	59.8	9.0	65.8	8.8	62.0	4.3
9	57.3	11.1	64.6	10.9	62.0	6.5
14	58.6	11.1	64.8	10.9	75.3	6.8

% Inhibition compared with controls in which each organism was grown in axenic culture where Fol <u>Fusarium oxysporum</u> f.sp. <u>lycopersici</u>; TRC Binab <u>Trichoderma</u>; Th <u>Trichoderma harzianum</u>; Tp <u>Trichoderma polysporum</u>

colony was rarely seen. Subsequent to the 14-day period scattered regions of spores were noted on the test colony which allowed successful re-isolation of TRC from a distal position furthest away from the original inoculum disk. This also occurred in tests with T.harzianum but conversely in tests using T.polysporum the latter could not be re-isolated. Thus, with extended incubation TRC and T.harzianum probably proceeded around the periphery of the agar plate or grew under and through the Fusarium mycelium.

With 75% Fusarium prior colonization, growth of all three Trichoderma colonies was severely restricted for the duration of the 14-day observation period, compared with the controls (Table 11b). After contact had been established the growth rate of Fusarium was reduced and results of transect samples suggested that T.polysporum growth ceased and there was only limited further extension of T.harzianum. TRC growth continued after the 14-day period and as observed with the 50% Fusarium cover, resulted in re-isolation of both TRC and Fusarium from the region of the plate colonized by Fusarium.

Due to the slower growth rate of V.albo-atrum 26 days prior growth was necessary to provide approximately 50% cover of the agar surface prior to Trichoderma introduction. Results were broadly similar to the reactions observed with Fusarium with TRC once again appearing to more aggressive than its constituents. A distinct boundary occurred in the region of contact and on occasions this was preceded by a region of sparser Verticillium growth which developed as the two colonies approached each other. Growth of both organisms was inhibited on meeting (Table 12) and Verticillium could not be re-isolated from this region. Further incubation eventually resulted in total colonization by TRC and to a lesser extent T.harzianum. TRC spored profusely and T.harzianum more sparsely above Verticillium colonies. On plates supporting growth of both Verticillium and

Table 12

<u>Percentage inhibition of Trichoderma and Verticillium albo-atrum growing in dual culture on MEA using a staggered inoculation 26 day interval</u>

26 days Vaa growth prior to *Trichoderma* inoculation

	% Inhibition					
	TRC vs Vaa		Th vs Vaa		Tp vs Vaa	
DAYS	TRC	Vaa	Th	Vaa	Tp	Vaa
1	11.1	2.2	18.0	2.9	10.7	4.4
2	14.5	3.2	20.7	3.1	7.9	3.5
3	18.4	3.2	20.0	4.0	2.3	2.2
6	31.8	6.3	35.4	7.2	8.4	3.7
8	33.6	9.1	37.1	8.5	20.4	7.2
12	20.1	14.5	22.9	15.0	33.1	11.0
14	21.9	14.7	20.9	15.3	30.0	11.9

% Inhibition compared with controls in which each organism was grown in axenic culture where *Vaa* *Verticillium albo-atrum*; TRC Binab *Trichoderma*; Th *Trichoderma harzianum*; Tp *Trichoderma polysporum*

T.polysporum mutual inhibition occurred with re-isolation of both organisms being possible from the distinct interaction zone that developed on contact.

Thus, in general Verticillium appeared to be more susceptible to antagonism in vitro by TRC and T.harzianum than Fusarium, however clearly the age of the Verticillium mycelium may have largely pre-disposed the colony to antagonism. Furthermore, these results demonstrate one of the major drawbacks of using simultaneous-inoculation paired culture tests which are likely to underestimate the resistance of candidate test cultures to antagonism, especially if growth is slow in vitro. In the standard test the organism occupying the greatest area of agar possesses the greater spatial advantage and an established colony has the opportuniity to sequester nutrients unimpeded before introduction of a second organism.

2.8 Tests using other Trichoderma cultures against Pythium ultimum on SEA

Ten other Trichoderma isolates were used in dual inoculation plate tests with P.ultimum. The species aggregates as defined by Rifai (1969) to which these isolates belonged were not determined, but numbers 8, 9, 21 and 27 were supplied as isolates of T.harzianum and isolated 2 and ZN59 as isolates of T.viride. All the isolates apart from T.reesei were reported to have demonstrated antagonism against various test organisms in agar plate tests (J. Nicklin, P. Woodgate-Jones and J. Flood, personal communication) while T.reesei was found to degrade cellulose in vitro (Section 3.7).

Zones of clearing were observed in paired culture tests with isolates 2 and 21. However the zones were not as well defined as observed with the Binab TRC. The following types of interactions similar to those distinguished by Porter (1924) were observed on SEA and were placed in the following four categories:-

Table 13

The interaction between Trichoderma isolates with Pythium ultimum on SEA

	Trichoderma Isolates	The presence of absence of clear zones
a	8	-
a	9	-
a	21	+
a	27	-
b	R.176	-
b	R.198	-
b	R.199	-
c	ZN59	-
c	Isolate 2	+
d	T.reesei	-
e	Binab TRC:-	++
d	T.harzianum)*	++
d	T.polysporum)	+++

- denotes the absence of clear zones in dual culture tests with P.ultimum on SEA

+ denotes the presence of clear zones in dual culture tests with P.ultimum on SEA on a scale of + to +++ where +++ represents the most pronounced clear zone

* T.harzianum and T.polysporum are the constituents of the Binab TRC preparation, the most marked clear zones developed in paired culture tests with T.polysporum

a. T.harzianum from Dr. J. Nicklin, Birbeck College, University of London; b. Trichoderma spp. isolates from Dr. J. Flood, University of Bristol; c. T.viride isolates from Dr. P. Woodgate-Jones, Long Ashton Research Station; d. T.reesei from Dr. P. Morris, Imperial College, University of London, T.harzianum and T.polysporum were originally obtained from Dr. J. Ricard Bio-Innovation AB Binab, Sigtuna, Sweden; e. Binab TRC from Stokes Bomford Chemicals Ltd.

1. Mutual intermingling of the two organisms after contact demonstrated by:- isolates 8, R.176, R.199, T.reesei.

2. Mutual inhibition with clear separation between the two organisms demonstrated by:- isolate 27.

3. Inhibition of P.ultimum on contact, with the Trichoderma colony continuing to grow over the test organism at a reduced or unchanged rate demonstrated by:- isolates 9, ZN59, R.198.

4. After meeting clear zones developed ahead of the Trichoderma colony edge, resulting in dieback and death of Pythium mycelium as the antagonist continued to progress over the agar demonstrated by:- Binab TRC and isolates 2 and 21.

Thus the formation of clear lysis zones was a relatively specific interaction confined to three Trichoderma cultures (Table 13). Its occurrence did not correlate with other known attributes of the isolates such as trichodermin production (ZN59, P. Woodgate-Jones, personal communication), hyphal coiling (R. 176, R. 198, R. 199, J. Flood, personal communication) nor did it occur with the cellulase producing T.reesei isolate (P. Morris, personal communication; section 3.7).

2.9 DISCUSSION

The antagonism demonstrated by the Binab TRC in vitro indicated that this isolate may be a potentially useful organism for biological control. Two levels of antagonism were observed, the first directed against the Pythium spp. exemplified in particular by P.ultimum and P.debaryanum took a distinctive form resulting in dieback and disintegration of the test colony (section 2.3.4), the second involved aggressive and effective

competition by TRC and was observed in interactions with the remaining test organisms. TRC progressively colonized the test colony by growing over its mycelium but although re-isolation of the pathogen was not often possible, no dieback of the mycelium was observed. The outline of the colony became fainter with time but the mycelium remained visible. On SEA the reaction between TRC and *F.oxysporum f.sp. lycopersici* resulted in mutual inhibition indicating that this organism was perhaps the most resistant to TRC antagonism *in vitro*.

Although the dual inoculation method of screening *in vitro* was employed extensively it suffers from a number of inadequacies. Results must be interpreted with caution and certainly cannot predict the efficacy of a candidate antagonist under greenhouse or field conditions (Kommedahl and Windels, 1978). Clearly agar media presents on environment very different from natural soil conditions and studies which use two organisms in isolation and excludes interactions with the soil-inhabiting microflora and the host, is itself artificial. In generaly agar tests favour the saprophytic antagonist rather than the pathogen (Baker and Cook, 1974) since the former is frequently able to utilize a greater range of nutrients and is free from the effects of other highly competitive microorganisms co-inhabiting the soil environment. However, such tests may elucidate potential mechanisms or antagonistic properties via which disease suppression can be effected. Antibiosis often occurs on agar (Baker and Cook, 1974) and media such as MEA which contains a level of nutrients that is likely to exceed and differ from those normally occurring in nonammended soil, may preferentially promote this form of antagonism. Antibiotic production sometimes resulting in marked inhibition (Johnson and Curl, 1972; Baker and Cook, 1974) is often favoured by rich media and the emphasis that is sometimes placed on this mechanism of biocontrol may in part be due to the choice of agar used in tests.

Despite the drawbacks, microorganisms obtained from soil have been screened using paired cultures and certain Trichoderma isolates found to be capable of lysing or coiling around hyphae of the pathogen have subsequently been used successfully in greenhouse and field trials (Hadar etal., 1979; Elad et al., 1980; Chet and Baker, 1981). Nevertheless, in the absence of its demonstration under natural conditions, mycoparasitism like antibiosis may prove to be a phenomenon largely confined to or selected for by laboratory tests. Moreover successful suppression of a pathogen in vivo is likely to operate through a complexity of interactions involving other organisms and incorporating a combination of mechanisms such as competition, antibiosis and mycoparasitism. Although there is a tendency perhaps to overestimate the importance of a single mechanism when observed in dual culture tests in vitro, such methods are an effective tool for screening large numbers of candidate organisms for antagonism, although in the process it is likely that potentially useful organisms may be over-looked. Papavizas and Lewis (1983) confirmed this when they found that none of the Trichoderma biotypes used to suppress damping-off of peas caused by P.ultimum had demonstrated antibiosis exhibited as zones of inhibition on potato dextrose agar. Moreover Chet and Baker (1981) found that T.hamatum isolated from Rhizoctonia solani suppressive soil produced cell wall degrading enzymes in vitro, yet no inhibition zones indicative of antibiosis were observed on the synthetic agar medium.

The reaction observed in these tests on nutrient-poor SEA between TRC and Pythium spp. involved the development of a definite interaction zone epitomized by the formation of a dead and lysed region termed here a zone of clearing. No such interaction was observed with other test organisms and this interaction was confined to members of the genus Pythium (Table 13). However in contrast with the results of Dennis and Webster (1971 c) in no instance was hyphal penetration or coiling around Pythium hyphae observed. The zone

of lysis and clearing developed ahead of the TRC leading edge and the region of overlap suggesting that the interaction was induced either by contact of at least some hyphae or simply required the presence of hyphae of both organisms in the same portion of agar for lysis to occur. The results differ from the hyphal interference that occurs between Coprinus heptemerus and Ascobolus crenulatus which affects only those cells of the latter in contact with the antagonist and is not influenced by the composition of the growth medium (Ikediugwu and Webster, 1970). However a similar loss in opacity together with granulation and vacuolation of the cytoplasm were noted. On MEA hyphae of the Pythium test organism appeared to overlap the TRC colony edge, lying above the TRC mycelium when viewed from the upper surface of the plate. The resulting dieback and apparent collapse and disintegration of the Pythium mycelium was accompanied by the progressive growth of TRC which proceeded over the pathogen colony. Similar results were obtained by Howell (1982), using Gliocladium virens and P.ultimum in dual culture tests on potato dextrose and on agar which incorporated cotton seed filtrate and soil extract. Hyphal penetration was not observed but coagulation and disintegration of cytoplasm in the pathogen hyphae, attributed to the effects of antibiotics, was observed in the areas of hyphal contact.

Although interactions on MEA are liable to involve antibiosis, tests conducted on SEA revealed that clear zones were not formed by all the antibiotic or toxic metabolite-producing strains of Trichoderma tested (section 3.4.4). The areas of sparser Verticillium mycelium noted on MEA in the areas of the colony opposed by TRC implicate the action of antibiotic-like diffusable metabolites, whilst the greater resistance to antagonism and colonization exhibited by Fusarium may be related to its ability to resist or overcome the effects of such TRC products (section 3.4.3). The isolate of F.oxysporum tested by Dennis and Webster (1971 a) was also found to be largely unaffected by non-volatile

antibiotics produced by various Trichoderma isolates and it was the most resistant of the test organisms used. They also confirmed that greater antibiotic production occurred on rich media and used MEA in preference to water agar. Thus, although the changes in colony shape observed in this study especially with Pythium spp. on both MEA and SEA could be due to the action of metabolites produced by the TRC antagonist, on SEA being low in nutrients, competition may play a more important role in the underlying mechanism.

Sivasithamparam and Parker (1980) pointed out the importance of using media similar in nutrient status to the natural environment and thus used both nutrient poor and rich media to screen 132 isolates of wheat rhizosphere fungi against the take-all fungus G.graminis var. tritici. Of the various types of reactions observed a region of dense growth (similar to the response observed in tests with C.praticola and R.solani) developed on plates of both nutrient rich and nutrient-poor media in which it was concluded that neither organism was inhibited. They strongly implicated antibiosis and did not classify or discuss overgrowth as a form of antagonism but found that the type of interaction that occurred between two organisms was not always consistent with different media. Further, they observed lysis within the pathogen colony in a position removed from the test organism deep into the older portion of the take-all mycelium. This type of reaction occurred on a nutrient-poor medium and was attributed to the action of antibiotics but fresh hyphae were often seen to re-colonize this area.

In this study only two other isolates besides TRC were found to produce clear zones with P.ultimum (Table 13). The component species of the Binab preparation T.harzianum and T.polysporum both produced clear zones. However, these constituents within TRC did not act synergistically in this respect since T.polysporum, the isolate which was least competitive, produced more marked zones

of clearing than TRC. The reactions with T.polysporum suggests that the mechanism leading to colonization of the agar plates and domination of the test organism cannot solely rely on the formation of these lysis zones. Overgrowth, classified as an aggressive and competitive response often did not occur with T.polysporum although such an attribute would indeed augment antagonistic activities in vivo.

Bell et al. (1982) used paired culture tests on a V_8 based medium to screen 77 isolates of Trichoderma against several pathogens. The degree of antagonism observed was scored into five ratings with overgrowth and complete cover of the agar representing the most severe form of antagonism. Thus according to this scale TRC acted as a highly aggressive antagonist against all the test organisms used on both media with the exception of Fusarium. However, this method of scoring antagonism also used by Bell and Wells (1977) may give very different results if the pathogen is allowed to colonize a greater area of the plate prior to being challenged by TRC (section 2.7). Although colonization of Verticillium and to a lesser extent Fusarium did eventually occur after an extended incubation, the overall impression was one of reduced antagonism and clearly use of a short observation period may result in different conclusions. Bell et al. (1982) found that only a very small percentage of the Trichoderma isolates were highly antagonistic towards the two test species of Pythium used (two percent were antagonistic against P.aphanider-matum and nine percent with P. myriotylum). In contrast when the results using isolates of four anastomosis groups of Rhizoctonia solani were averaged, 85 percent of the isolates of Trichoderma were antagonistic. For the purposes of this study R.solani and C.praticola were treated as two different organisms although in reality they are probably both isolates of R.solani (Thanetophorus cucumeris (Frank) Donk) that demonstrated differences in growth and overall appearance on the agar media used. However their behaviour

in dual culture tests with TRC were similar with TRC generally covering two-thirds of the agar rapidly, eventually covering and overgrowing both of these fungal colonies.

Competition viewed in these agar tests may be related to sporulation, since T.polysporum was a slow end relatively sparse sporing culture in comparison with T.harzianum which produced an abundance of conidia. TRC often sporulated at the boundaries with contiguous colonies a phenomenon noted by Ellison et al. (1981) in agar tests using two or more inoculations of isolates of T.viride (which usually only produced spores at the periphery of the colony in Petri dishes) on the same plate. Checks in TRC growth observed on contact with a test organism may have been due to a shift from extension growth to sporulation. Thus in the environment of a paired culture agar plate, greater sporulation may have been induced by the presence of another organism that would not occur normally in nature.

Frequently only TRC was re-isolated from samples of agar taken along the transects. This may also reflect the intrinsic bias of the technique towards recovery of heavily sporulating fungi. In general the test fungi were all of poor or non-spore producing capacity at least under the culture conditions employed. More useful information may have been obtained if agar blocks from beneath the surface could have been isolated from the test colony excluding the surface-growing TRC mycelium and spores. The limited success achieved with re-isolations of the test organisms from the peripheral regions surrounding the original inoculum disk, suggests that reduced TRC sporulation sometimes associated with these areas may have aided recovery of the test pathogen. However growth of the test pathogen from the sampled agar blocks used may have easily been obscured or even inhibited on the fresh agar plates by the presence of TRC. Similarly Bell et al. (1982) transferred the original inoculum disks of Pythium spp. from paired

culture plates tests with Trichoderma isolates, onto a selective Pythium medium and if growth did not occur after four days it was assumed that death had occurred.

Interactions on both MEA and SEA in which only TRC could be re-isolated suggest that this was the dominant organism. Growth was inhibited and the viability of the test fungi was reduced by TRC growth over and probably through the target colony, with the production of large numbers of spores; re-isolation of the test fungus in the presence of this highly aggressive antagonist was generally not possible.

3. TESTS TO INVESTIGATE THE MECHANISM OF CLEAR ZONE PRODUCTION

3.1 INTRODUCTION

The clear zone which developed in dual culture tests between TRC and Pythium spp. on SEA clearly indicated that an interaction between the organisms occurred to the detriment of the Pythium sp., however the underlying mechanism remained unknown. In this section the interaction was examined in greater detail and various explanations proposed and tested. One possibility which implicated depletion and/or competition for essential growth factors was investigated by incorporating additional supplements of soil extract or glucose into the agar medium.

The production and effects of inhibitory metabolites produced by TRC were studied using a method similar to that described by Mughogho (1968) and Dennis and Webster (1971 a) for testing the antibiotic activity of various isolates of Trichoderma spp. Both cellophane and dialysis membranes were used on MEA and SEA and P.ultimum was employed initially as the test organism. Cellophane enables metabolites of varying molecular weights to pass through to the agar surface whilst dialysis membrane selectively allows passage of only low molecular weight metabolites. Cellophane and dialysis membranes were used in further tests to separate the opposing TRC and Pythium colonies in paired culture tests and also to investigate whether physical contact was a prerequisite for clear zone formation.

Tests were made to reproduce with cell-free extract the antagonistic interaction of TRC with Pythium spp. Culture filtrates were examined for the presence of any active component. They were harvested from cultures of TRC and Pythium grown individually and simultaneously, together with filtrates of TRC grown

on autoclaved Pythium mycelium. The celluloytic ability of TRC was also investigated with a view to its possible involvement in the characteristic interaction on SEA.

3.2 MATERIALS AND METHODS

3.2.1 Preparation of cellophane and dialysis membrane

Circles of cellophane (British Rayophane, Pu600) and dialysis membrane (Medicell International) 9.0cm in diameter were boiled in an approximately 0.5%(w/v) solution of EDTA for 10-15 minutes to deplasticize and clean the membranes prior to use. The membrane circles were thoroughly rinsed in several changes of distilled water to remove all traces of EDTA, and finally they were sterilized by autoclaving in distilled water at 121°C for 20 minutes.

When cool the membranes were aseptically layered singly over the surface of 20.0ml of agar in Petri dishes. Using a sterile spatula each membrane was smoothed over the agar surface to remove air bubbles and so produce an even surface. For ease of removal the membranes were slightly larger than the area of the agar surface and so formed an overlap at the edges.

3.2.2 Tests to investigate the effects of TRC metabolites

Disks of TRC, 5.0mm in diameter were cut from actively growing colonies growing on 20.0ml of 2% MEA. One disk was used to inoculate centrally each membrane-covered agar plate. Plates were incubated at 20°C for 2-3 days, after which the membrane and adhering TRC colony was removed, and a 5.0mm diameter disk of the test fungus growing on 2% MEA was placed directly on the agar surface in a central position corresponding to that previously occupied by the TRC

disk. The plates were replaced in the 20°C incubator and were examined daily.

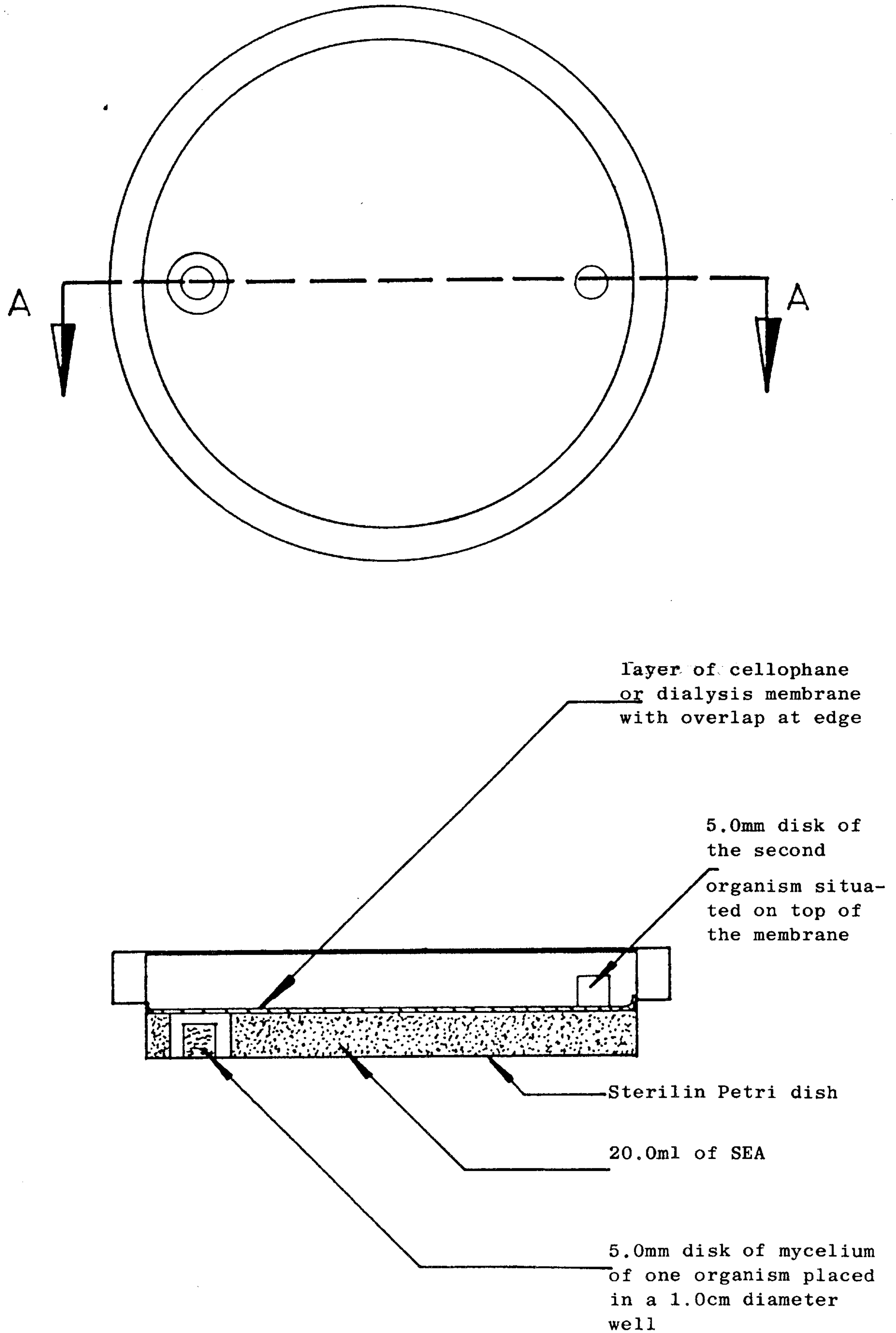
Controls consisted of plates covered with membranes which had not been inoculated with TRC and were thus inoculated only with the test fungus after the 2-3 days delay. Alternatively, controls using the test organism instead of TRC were used to inoculate the membranes. Subsequent removal of the membrane was followed by inoculation of the agar surface by the same test fungus. Growth was assessed periodically as described earlier (section 2.2.3), and unless otherwise stated all experiments were replicated five times.

3.2.3 Dual culture tests using cellophane and dialysis membranes

The cellophane and dialysis membranes prepared as described previously, were sterilized at 121°C for 20 minutes. A well was cut in a plate of SEA using a sterile 1.0cm diameter corkborer at a distance of 2.0cm from the edge of the plate. In this well a 5.0mm disk of mycelium which had been cut from an actively growing culture of the test organism was placed. A single circle of either cellophane or dialysis membrane was next layered over the agar surface and smoothed to give an even cover. A 5.0mm disk of the second organism was placed diametrically opposite the well on the surface of the membrane 2.0cm from the edge of the dish (figure 6). The plates were incubated at 20°C with five fold replication.

Controls comprised each of the three organisms grown individually on plates using the same organism both above and below the membrane layer. Additionally controls were included using the standard plate test arrangement but omitted a separating barrier.

Figure 6



Transect samples across the agar surface at 1.0cm intervals were taken 14 days after inoculation. Portions of the membrane taken at approximately 2.0-3.0cm intervals were also plated onto 2% MEA. The experiment was repeated three times.

3.2.4.1 Culture filtrates

The soil extract medium used for paired culture tests was employed as a liquid growth medium and was prepared as in section 2.2.2, omitting the agar constituent: 10.0ml of liquid soil extract medium was dispensed into McCartney bottles and sterilized by autoclaving at 121°C for 20 minutes. The bottles were cooled in a sterile air environment and were inoculated with a 5.0mm disk of mycelium taken from an actively growing culture. The bottles were incubated on angled stands (25° from the horizontal) at 20°C. Growth of TRC, *P.ultimum* and *P.debaryanum* was sparser than in richer liquid media and sporulation of TRC was reduced.

When sufficient growth had occurred (usually 10-14 days for TRC) the bottles were removed and the culture fluids harvested by filtration under reduced pressure through sterile filters of various composition with a final pore size of a 0.45µm or less.All the apparatus including the filter holder had been sterilized prior to use and precautions were taken to maintain the sterility of the filtrates. These were kept cool throughout the proecdure and were either used immediately or were stored in 2.0ml aliquots at -21°C until required.

3.2.4.2 Freeze drying of culture filtrates

Filtrates for freeze drying were dispensed in 50.0ml aliquots in 500ml flasks, shell frozen and lyophilized to dryness. The powder was re-constituted in 5.0ml of sterile distilled water, filter sterilized and where necessary stored in aliquots of 1.0ml at -21°C.

3.2.5 Assay methods

Various methods to assay the biological activity of culture filtrates were assessed and filter paper disks including antibiotic disks (Whatmans AA disks) impregnated with culture filtrate were used. As an alternative wells of various sizes cut in the agar were filled with culture filtrate. Different methods of introducing the test organism were also tested. Seeding molten agar with *Pythium* mycelium gave unreliable results and surface inoculation was selected as a more appropriate method.

The well method of assaying culture filtrates was found to be the most suitable. 1.0cm diameter wells were generally used and these were filled with 200-250µl of culture filtrate. Three wells were used on each plate, arranged in a line 3.5cm from the edge of Petri dish. A 5.0mm disk of the test organism was inoculated at the periphery of the agar plate in a position distal from the wells. Growth was monitored before and after contact with the filtrate-containing wells. A circular arrangement with central inoculation was occasionally used but the former was selected for routine screening.

SEA (20ml per plate) was employed for all the tests. *P.ultimum* was selected as the primary test organism and growth was assessed as described in section 2.2.3. All plates were incubated at 20°C and treatments were replicated three times unless otherwise stated. All tests were repeated a minimum of three times.

3.2.6 Modified cellulose medium (Bravery, 1968)

KH_2PO_4	1.0 g
KCl	0.5 g
$MgSO_4.7H_2O$	0.2 g
$CaCl_2$	0.1 g
Agar	20.0 g
* Cellulose	10.0 g
$(NH)_4SO_4$	0.54 g
Thiamine HCl	0.001 g
Distilled water	to 1 litre

* Cellulose powder was ball milled as a 4%(w/v) suspension for 72 hours. Whatmans CM11 cellulose and Avicel (P101, Honeywell and Stein Ltd.) were both used.

The modified cellulose medium which omitted alternative carbon sources (DL-asparagine, yeast extract and hemicellulose) was used in preference to the cellulose agar described by Eggins and Pugh (1962). Cellulolytic activity could be detected after a shorter incubation period with the former (within 7 days compared with approximately 21 days with the latter). Molten modified cellulose agar (10.0ml) was poured into sterile, cool test tubes. The agar was inoculated with 1.0cm diameter disks of mycelium and incubated at 20°C. Zones of activity were easily observed in tubes of cellulose agar with defined depths of clearing developing soon after inoculation and thus these were found to be more suitable than agar plates.

RESULTS

3.3 Further studies on the Interaction occurring between TRC and Pythium on SEA

3.3.1 The effect of Soil Extract Concentration

The soil extract prepared as described earlier was incorporated into the agar base (section 2.2.2) to give the following soil extract concentrations by volume:- 5%, 10%, 20%, 40% and 80%. The range included the concentration routinely used (ie 10%(v/v)) and was designed to investigate the effects of soil extract concentration on subsequent interactions in paired culture tests(section 2.2.3) between TRC and P.ultimum.

The characteristic zones of clearing developed with all five concentrations of soil extract used. Complete cover of the agar plate by TRC occurred within the 12-day period. Clear zones developed when the mean diameter of the TRC colony were between 50 and 60mm.

Contact between the two colonies was rapid with the two highest concentrations used and thus zones developed by the third day of incubation (Figure 7d and 7e). With 5% and 10%(v/v) soil extract concentrations a marked clear zone was observed on the fifth and fourth day of incubation respectively (Figure 7a and 7b). Although clear zones were first observed on the 20%(v/v) plates by day five these were not as distinct as observed with the other concentrations until the sixth day.

The increased soil extract concentrations did not appear to influence the characteristic interaction that occurred between TRC and P.ultimum (section 2.3.3).

Figure 7

The effect of soil extract concentration on TRC and *P.ultimum* growth in dual culture
(a) 5%(v/v), (b) 10%(v/v), (c) 20(v/v), (d) 40%(v/v), (e) 80%(v/v)

▲——▲ *P.ultimum* in dual culture;

▲– – –▲ *P.ultimum* control (growth in axenic culture);

△——△ TRC in dual culture;

△– – –△ TRC control (growth in axenic culture);

denotes the development of clear zones;

growth is expressed as mean colony diameters;

bars denote the standard error of the mean.

Figure 7a

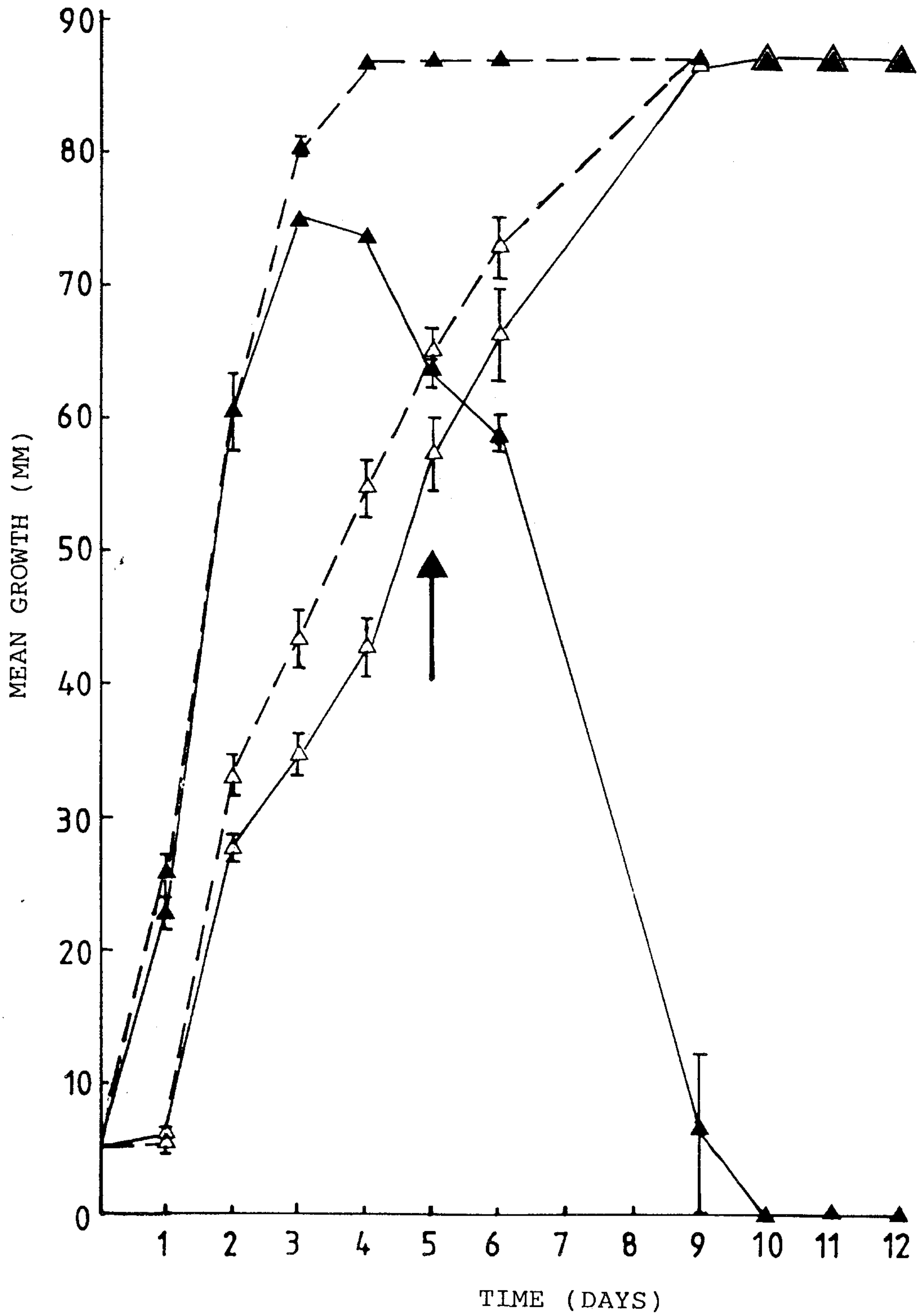


Figure 7b

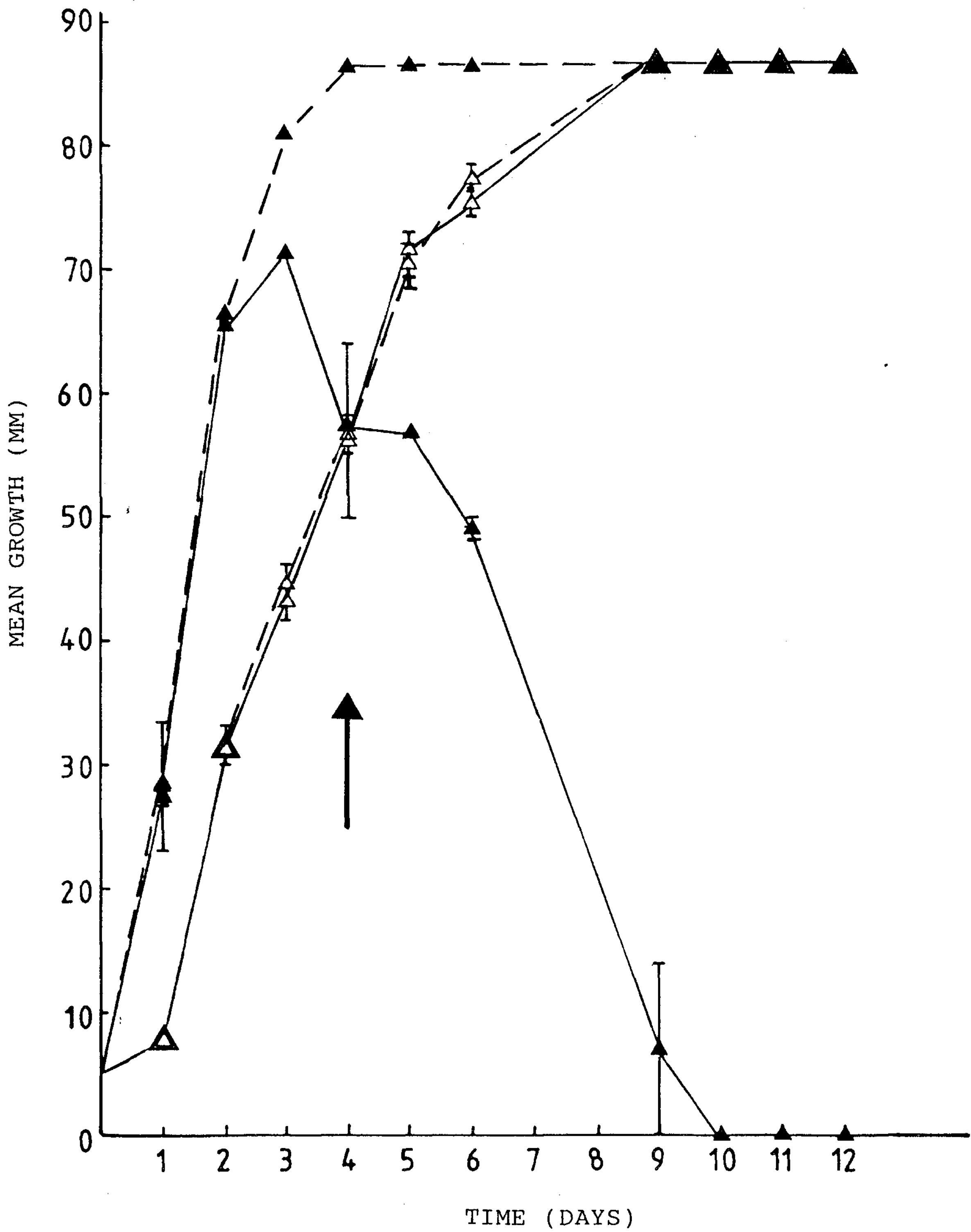


Figure 7c

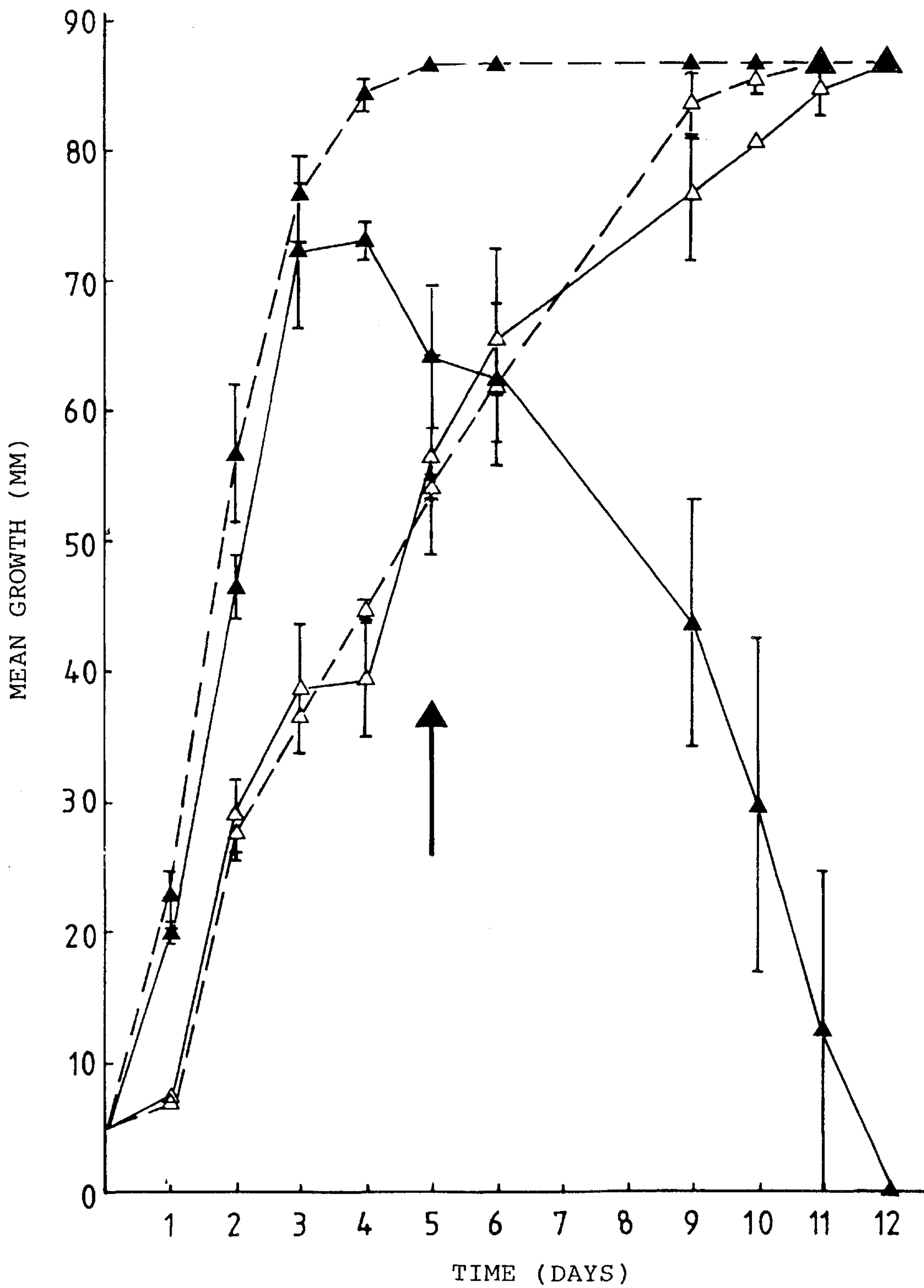


Figure 7d

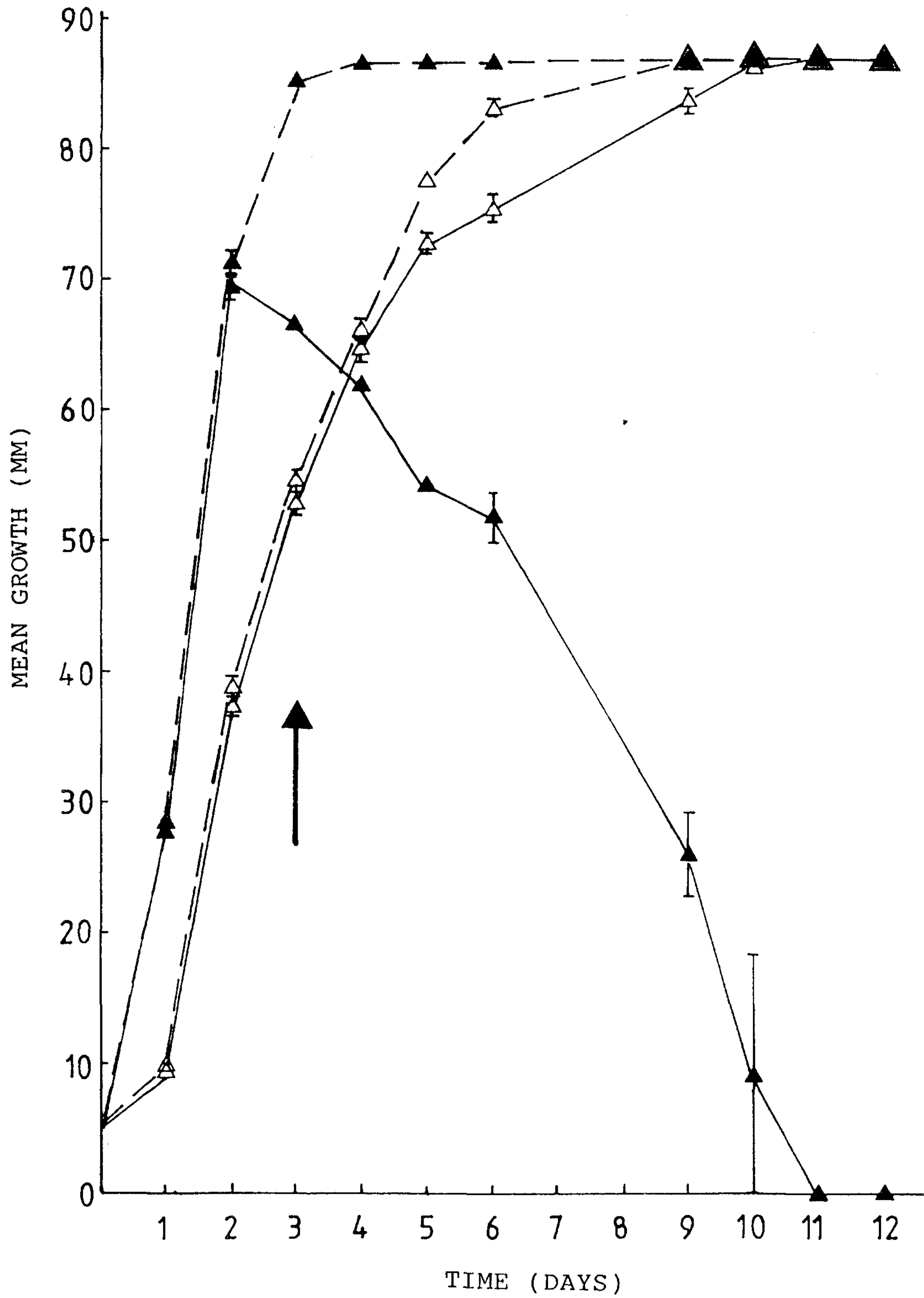
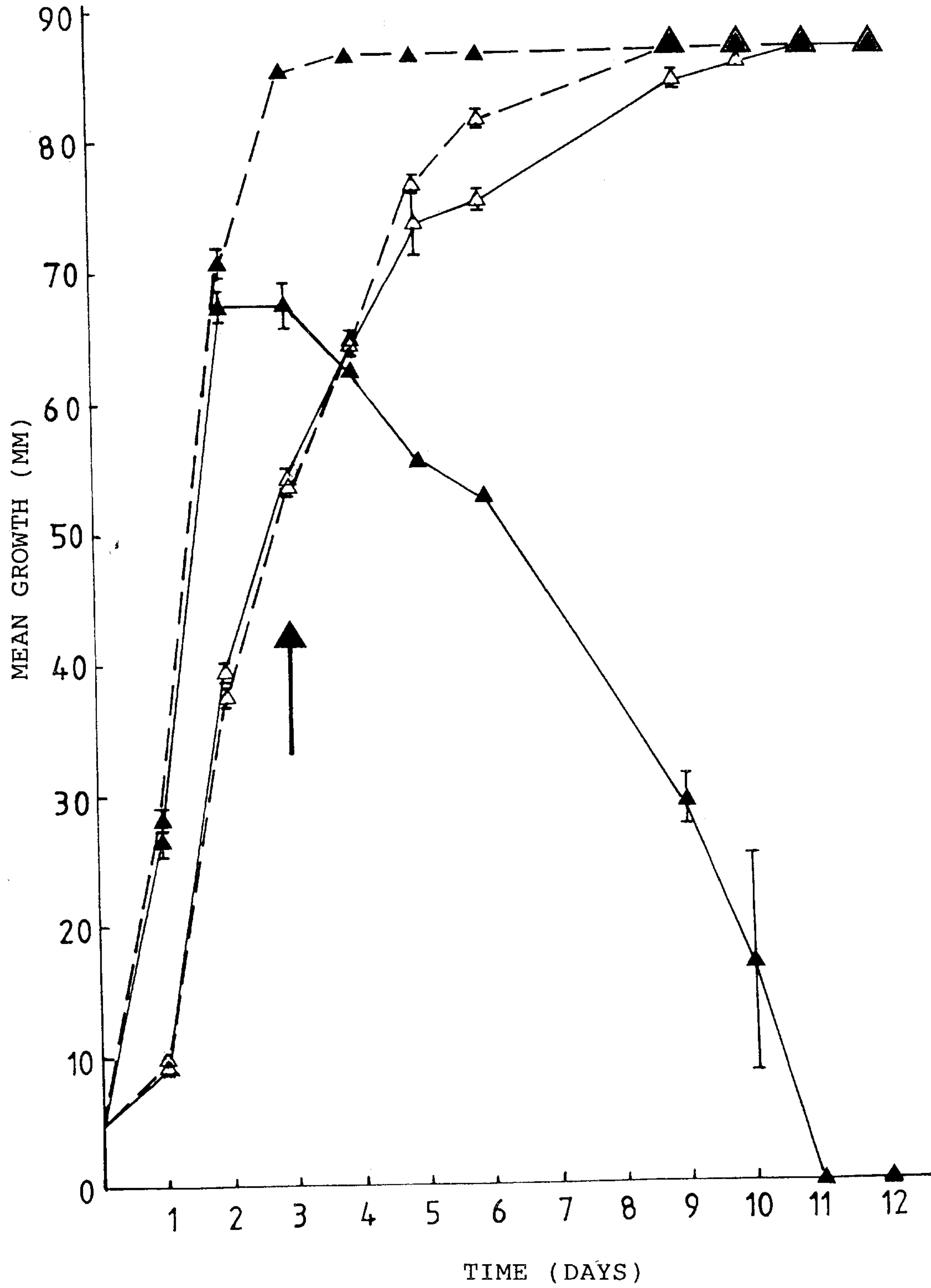


Figure 7e



If death and lysis of hyphae of *P.ultimum* is due to depletion of nutrients, additional soil extract did not counteract this.

3.3.2 The effect of Glucose Concentration

The amount of glucose incorporated into SEA was varied from the usual 1.0g up to 100.0g of glucose/l of agar. A range of five concentrations, 0.1%(w/v), 1%(w/v), 2%(w/v), 5%(w/v) and 10%(w/v) (the equivalent molarities being between 5.6mM and 555mM) were used in paired culture tests between TRC and *P.ultimum* as described in section 2.2.3.

Zones of clearing developed with all five concentrations of glucose tested. *P.ultimum* growth was halted after contact and subsequent overgrowth by TRC occurred after the fourth day of incubation with the first three concentrations and after five days with the two highest concentrations (Figures 8a to 8c). The decline in *P.ultimum* surface area observed was due to the gradual encroachment by TRC accompanied by lysis of the *Pythium* mycelium. However in the case of the two higher glucose concentrations, clear zones formed at a later stage (day seven) and in this experiment growth was slower with the 10%(w/v), concentration. Glucose at 1%(w/v) appeared to support optimum growth of the TRC controls and complete colonization by TRC in dual culture occurred by day ten. Similarly complete colonization of control plates by *P.ultimum* occurred within the first five days with both 0.1%(w/v) and 1%(w/v) of glucose. Thus although glucose-dependent variations in growth rates were observed, in all five cases interactions between TRC and *P.ultimum* resulted in the development of clear zones, (Figures 8a to 8e).

Figure 8

The effect of glucose concentration on growth of TRC and *P.ultimum* in dual culture
(a) 0.1%(w/v, (b) 1%(w/v), (c) 2%(w/v),
(d) 5%(w/v) and (e)10%(w/v)

▲——▲ *P.ultimum* in dual culture;

▲- - -▲ *P.ultimum* control (growth in axenic culture;

△——△ TRC in dual culture;

△- - -△ TRC in control (growth in axenic culture);

↑ denotes the development of clear zone;

growth is expressed as the mean colony diameter;

bars denote the standard error of the mean.

Figure 8a

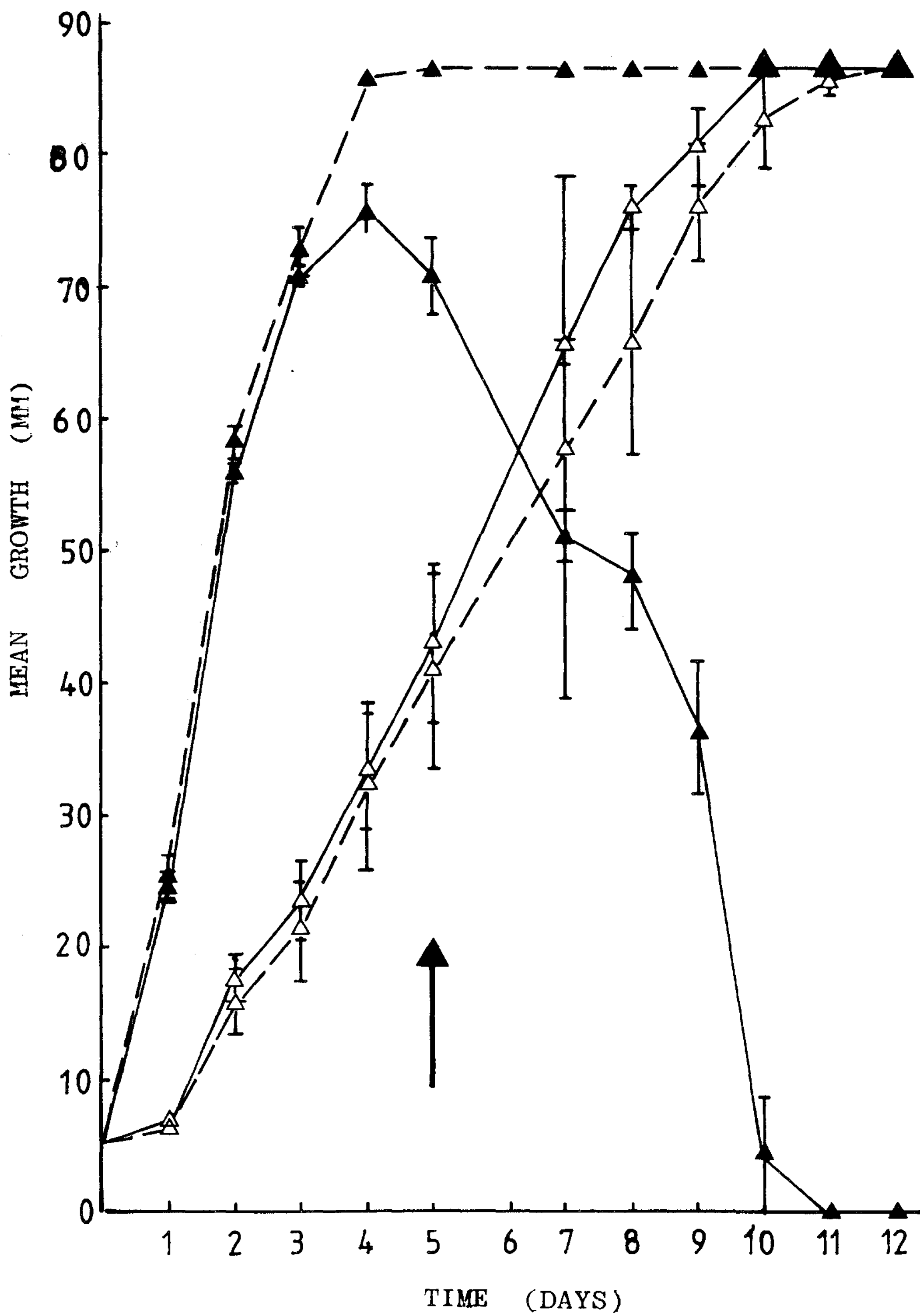


Figure 8b

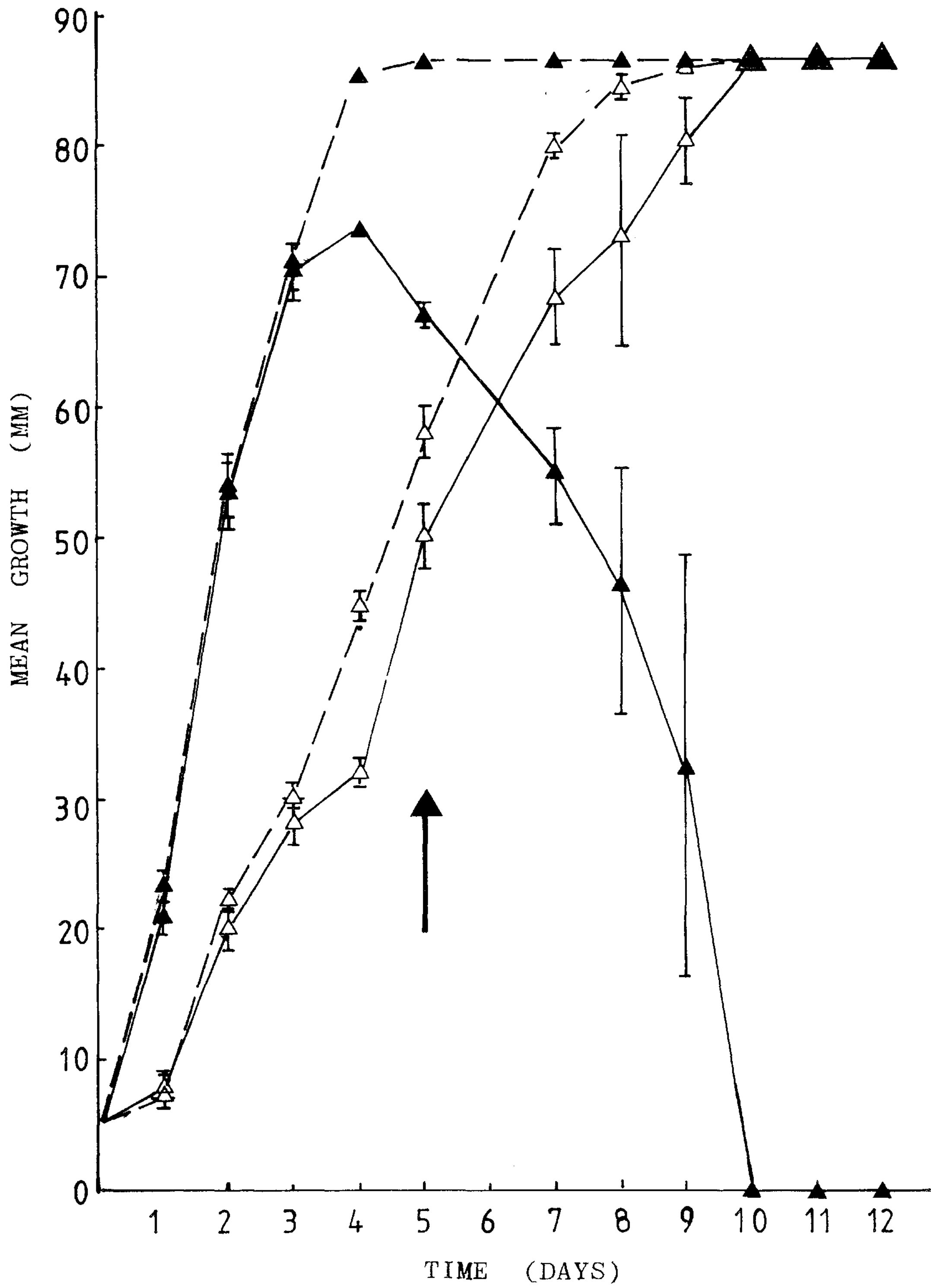


Figure 8c

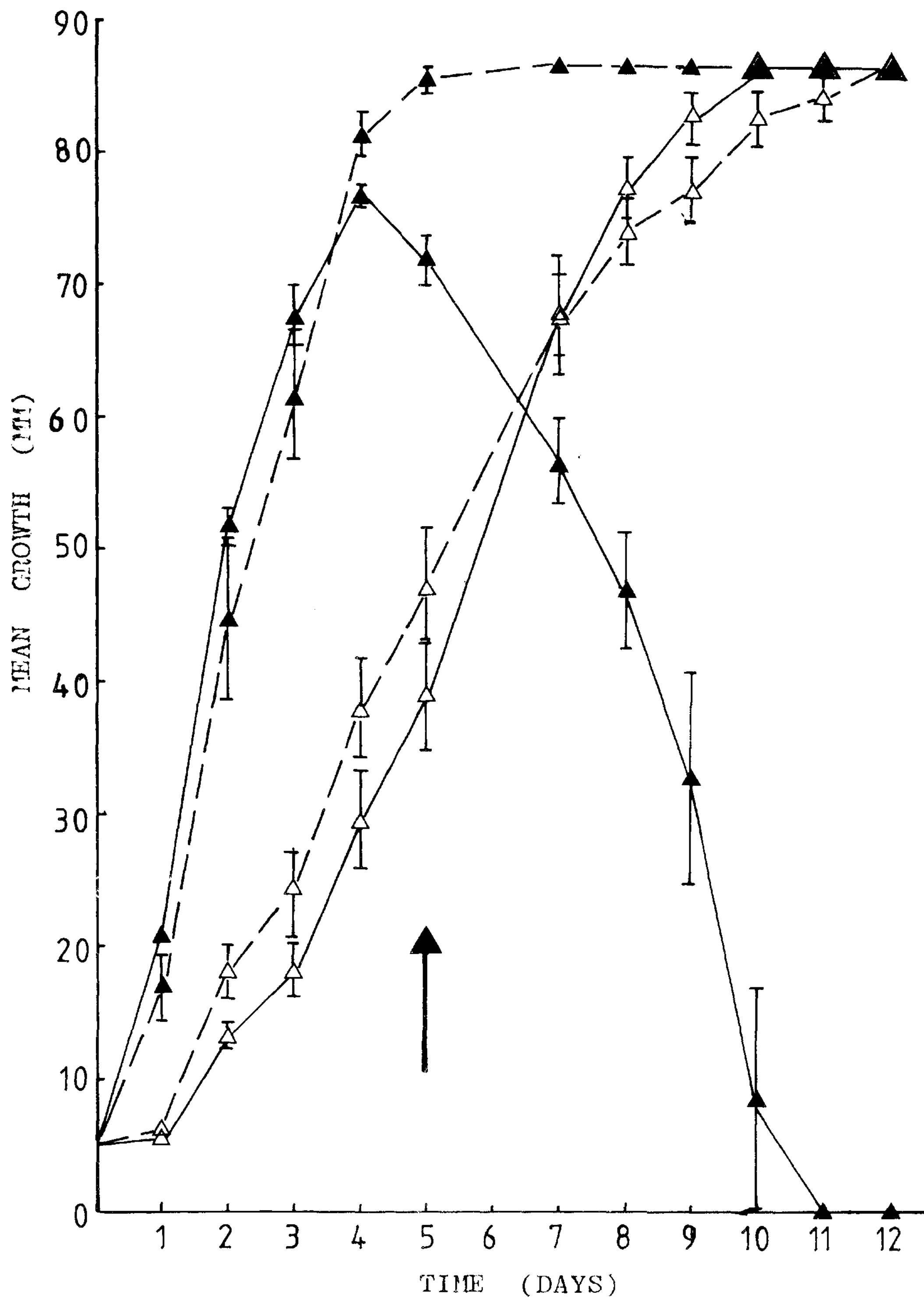


Figure 8d

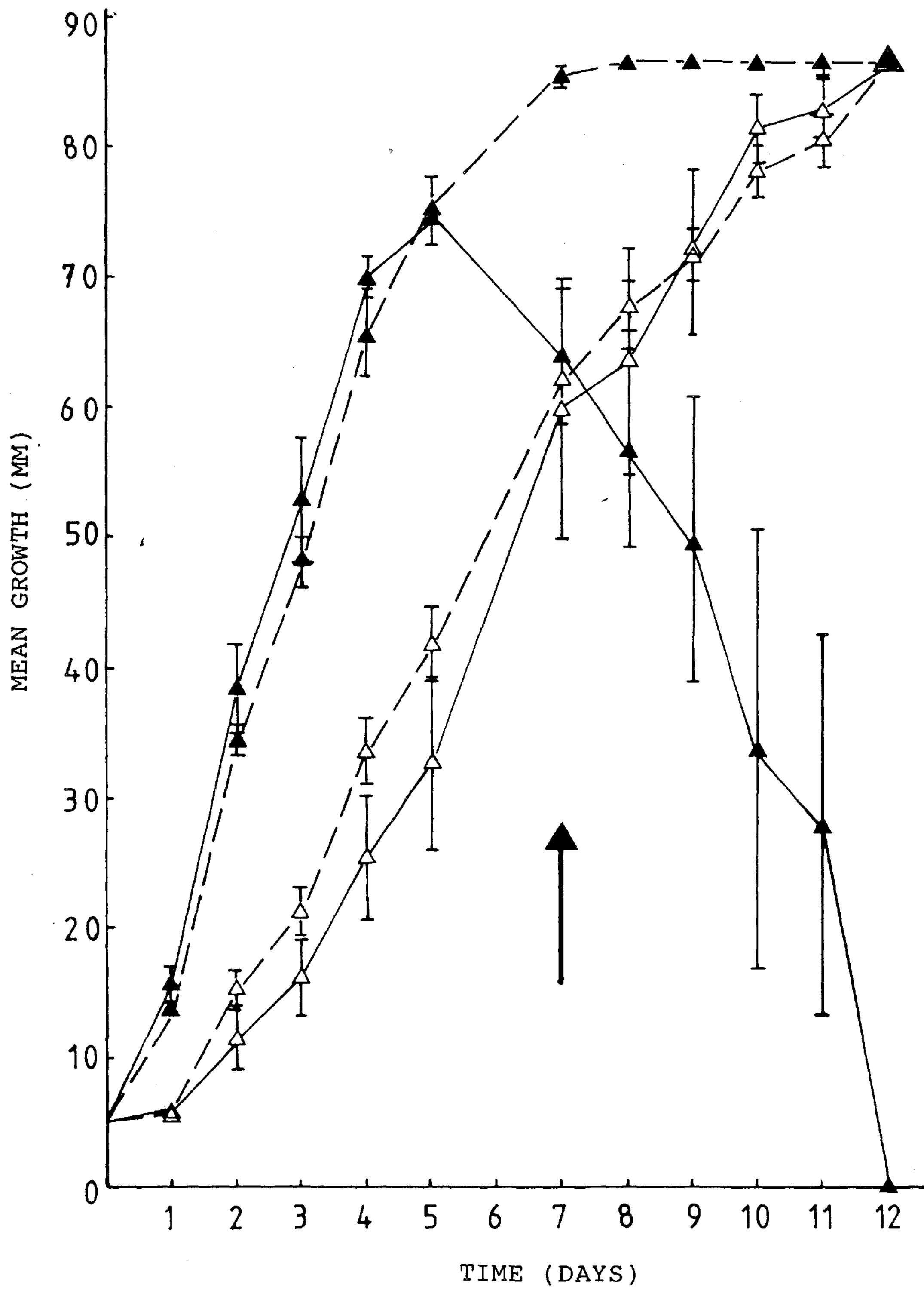
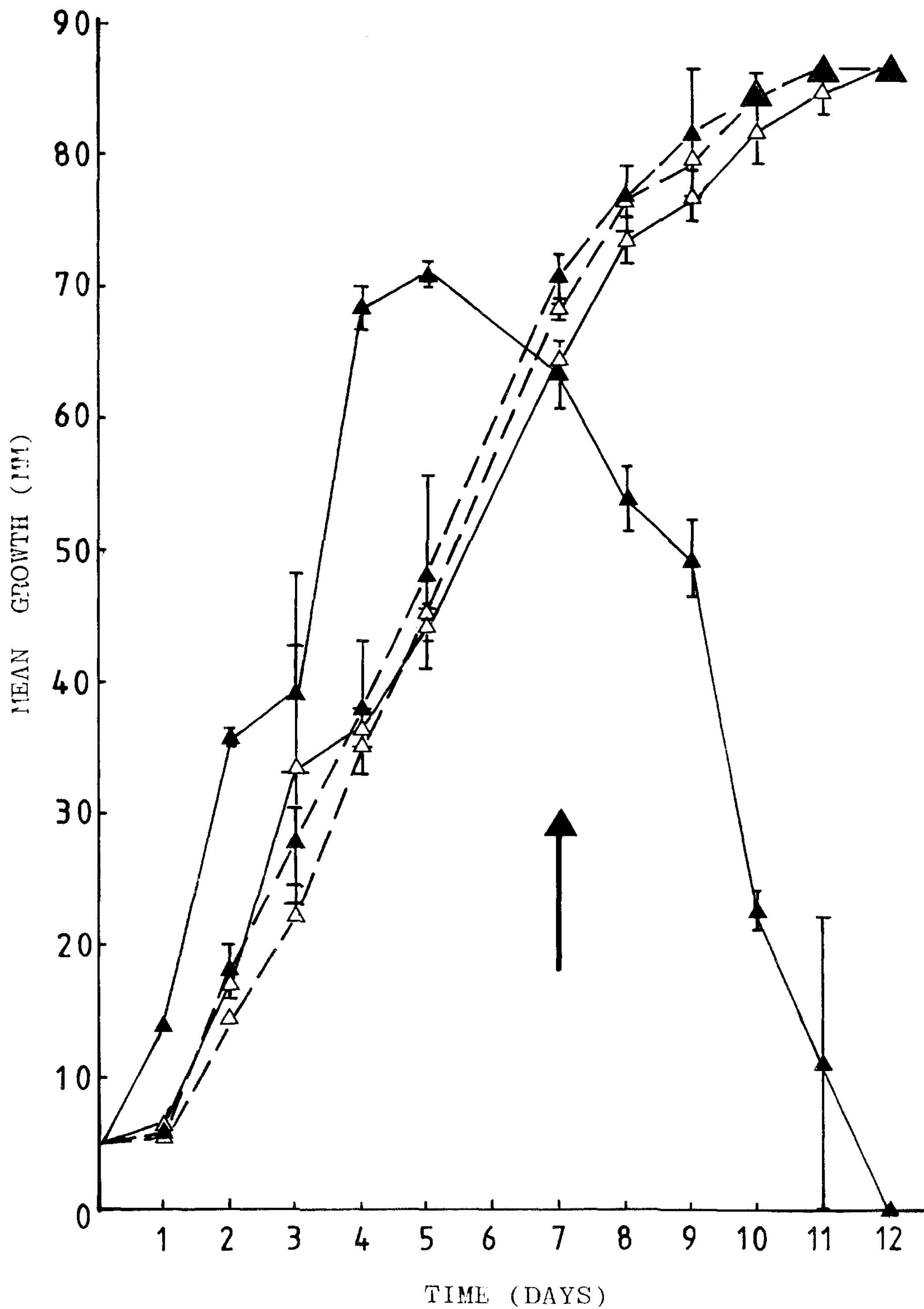


Figure 8e



3.4 EXPERIMENTS TO INVESTIGATE THE EFFECTS OF PRIOR TRC GROWTH ON CELLOPHANE AND DIALYSIS MEMBRANES

3.4.1 MEA (2%(w/v)) and SEA

Replacement culture experiments indicated that different reactions occurred on the two nutrient media employed. On MEA growth of P.ultimum was completely inhibited in tests using both cellophane and dialysis membranes (Tables 14a and 14b). On SEA growth of P.ultimum was relatively unaffected although slight reductions in growth rate were on occasions noted on plates previously treated with TRC. However similar effects were also obtained with the P.ultimum-inoculated controls. This probably reflects the depletion of nutrients that would naturally occur within the zone of agar previously occupied by P.ultimum or TRC.

The inhibition observed on MEA was durable with no growth occurring over a 14-day period. Transfer of the inoculum disk to fresh MEA did not result in growth indicating death rather than stasis of Pythium. The similar inhibition obtained with dialysis membranes (Table 14a) implied that inhibition was not enzymic in nature and so implicated the production of a low molecular weight component. The inhibition specifically associated with TRC and was not observed with the P.ultimum inoculated controls.

3.4.2 MEA (5%(w/v))

This test investigated the response of P.ultimum to prior colonization of rich nutrient media by TRC cellophane and dialysis were used as before on 5%(w/v) MEA. SEA and 2%(w/v) MEA were used for comparisons and uninoculated controls were used for all three treatments which were each replicated five times.

Table 14a

The effect of TRC grown on cellophane and dialysis membranes on subsequent growth of P.ultimum using MEA

Pu colony diameter on MEA

	CELLOPHANE MEMBRANE		
DAY	PRIOR INOCULATION with TRC	UNINOCULATED CONTROL	PRIOR INOCULATION with Pu
1	NIL	26.20 ± 0.90	35.03 ± 0.89
2	NIL	67.95 ± 0.96	82.78 ± 1.49
3	NIL	86.60 ± 0	86.60 ± 0
4	NIL	86.60 ± 0	86.60 ± 0

	DIALYSIS MEMBRANE		
DAY	PRIOR INOCULATION with TRC	UNINOCULATED CONTROL	PRIOR INOCULATION with Pu
1	NIL	27.98 ± 0.54	33.44 ± 0.26
2	NIL	73.04 ± 0.22	72.10 ± 1.50
3	NIL	86.60 ± 0	86.60 ± 0
4	NIL	86.60 ± 0	86.60 ± 0

Pu: P.ultimum

Figures are the mean colony diameter (mm) ± S.E.

Arrangements were replicated five times

Table 14b

The effect of TRC grown on cellophane and dialysis membranes on subsequent growth of P.ultimum using SEA

PU colony diameter on SEA

	CELLOPHANE MEMBRANE		
DAY	PRIOR INOCULATION with TRC	UNINOCULATED CONTROL	PRIOR INOCULATION with Pu
1	28.33 ± 0.63	27.92 ± 0.79	22.13 ± 1.10
2	63.99 ± 1.22	66.24 ± 1.22	49.34 ± 2.95
3	86.60 ± 0	86.60 ± 0	79.09 ± 4.73
4	86.60 ± 0	86.60 ± 0	86.60 ± 0

	DIALYSIS MEMBRANE		
DAY	PRIOR INOCULATION with TRC	UNINOCULATED CONTROL	PRIOR INOCULATION with Pu
1	24.11 ± 1.56	30.03 ± 0.58	24.87 ± 0.38
2	60.48 ± 3.68	66.86 ± 0.81	60.02 ± 1.37
3	86.60 ± 0	86.60 ± 0	86.60 ± 0
4	86.60 ± 0	86.60 ± 0	86.60 ± 0

Pu: P.ultimum

Figures are the mean colony diameter (mm) ± S.E.

Arrangements were replicated five times

Total inhibition of subsequent P.ultimum growth resulted from both cellophane and dialysis membrane treatments on 5% and 2% MEA. No breakdown of this inhibition was noted over the 12-day observation period. Inhibition as a result of prior TRC growth was confined to media of rich nutrient status and did not occur on the low nutrient status SEA. The complete inhibition that occurred on richly supplemented 5% MEA confirmed that nutrient depletion was unlikely to account for the inhibition observed on 2% MEA.

3.4.3 Range of Activity

TRC was used against a range of test organisms using dialysis membrane to separate the TRC colony from the agar surface. Experiments used MEA as before and were repeated at least three times. Growth of the following Oomycetes was completely inhibited:- P.ultimum, P.multisporum, P.undulatum and Phytophthora citricola (Table 15). P.debaryanum was less susceptible than the other Oomycetes tested but growth was nevertheless severely retarded.

Of the other fungi tested Rhizoctonia solani was very susceptible with growth often being totally inhibited. Growth of Corticium praticola and Verticillium albo-atrum was also inhibited whilst Fusarium oxysporum f.sp lycopersici appeared to be least affected by TRC metabolites (Table 15). This agrees with the results of Dennis and Webster (1971(a)) who studied the effects of Trichoderma non-volatile antibiotics and reported that the pathogen F.oxysporum was the most resistant of the test organisms used.

Inoculum disks from plates showing 100% inhibition were removed and plated onto fresh MEA and fresh inoculum disks of the test organism were used to re-inoculate those plates demonstrating inhibition. With the exception of C.praticola no growth resulted from any inoculum

Table 15

Percentage Inhibition of growth compared with controls on 2% MEA previously inoculated with TRC on dialysis membrane

	% Inhibition								
DAY	Pm	P.und	Pu	Pd	Pc	CP	Rs	Fol	Vaa
1	100	100	100	100	100	100	100	10.9	100
2	100	100	100	100	100	100	100	10.7	100
3	100	100*	100*	100*	100	100	100	22.6	59.6
4	100	100	100	96.1	100	98.1*	100	14.7	62.1
7	100*	100	100	68.5	100	84.1	100	8.9	67.3
8	100	100	100	30.5	100	76.0	100*	8.3	64.4
10	100	100	100	3.3	100*	68.9	100	6.9	61.6
11	100	100	100	0	100	68.8	100	1.9*	52.8
12	100	100	100	0	100	66.7	100	0.12	48.9

Pm : *Pythium multisporum*;
P.und: *Pythium undulatum*;
Pu : *Pythium ultimum*;
Pd : *Pythium debaryanum*;
Pc : *Phytophthora citricola*;
Cp : *Corticium praticola*;
Rs : *Rhizoctonia solani*;
Fol : *Fusarium oxysporum f.sp. lycopersici*;
Vaa : *Verticillium albo-atrum.*

*denotes when growth in controls reached the edge of the Petri dish completely covering the agar surface. In the case of Vaa complete cover took more than 21 days. Control plates were covered with dialysis membrane and inoculated with the respective test organism instead of TRC, membranes were removed and the agar surface re-inoculated with the same test organism.

Table 16

Second Inoculation of plates demonstrating Inhibition

% Inhibition

DAYS	Pm	P.und	Pu	Pc	Cp*	Rs
2	24.9	0	25.2	34.7	69.4	57.8
3	30.0	14.3	0	22.5	70.8	64.4
4	24.1	25.2	0	55.4	56.7	56.2
5	13.4	0	0	39.0	41.1	48.1
6	0	0	0	24.3	35.6	25.7
9	0	0	0	4.6	0	0

% Inhibition compared with growth of the test organism in control plates which had been previously inoculated with the test organism instead of TRC. Results are the mean of three replicates

Pm : *Pythium multisporum*

P.und : *Pythium undulatum*;

Pu : *Pythium ultimum*;

Pc : *Phytophthora citricola*;

Cp : *Corticium praticola*;

Rs : *Rhizoctonia solani*

*Results of two replicates only since only two plates showed inhibition.

disk when transferred to fresh agar: growth from one disk of C.praticola was observed. When plates demonstrating inhibition were re-inoculated with their respective test organism, some growth always resulted and inhibition was weaker than in the first assay (Table 16). Inhibition was most severe with C.praticola and R.solani whilst P.ultimum overcame any inhibitory effects by day three. On one plate total inhibition of R.solani extended to the second inoculation with no growth occurring from the fresh inoculum disk. Inhibition of the test organisms decreased as they extended beyond the region of agar previously covered by the TRC colony on the membrane.

3.4.4 Tests using six Trichoderma isolates against P.ultimum

The Binab TRC was used in a comparative test on MEA with five other Trichoderma isolates (section 2.8). Each was grown on dialysis membrane following the routine procedure and their effects on subsequent P.ultimum growth was noted over a ten-day period.

P.ultimum was totally inhibited by four of the Trichoderma isolates tested (Table 17) namely R.198, Isolate 2, ZN59 and the Binab TRC. Greater inhibition was observed within the first four days with isolate R.199 than R.176 but complete cover of the agar surface by P.ultimum occurred in both cases within the ten-day period.

Thus the inhibition of P.ultimum that resulted after inoculation with the Binab isolate was non-specific. Inhibition also occurred with a range of Trichoderma isolates which included a known Trichodermin antibiotic producer namely ZN59. This further suggested that inhibition was due to a low molecular weight antibiotic substance.

Table 17

The effect of Trichoderma spp. grown on dialysis membrane on subsequent P.ultimum growth on MEA

% Inhibition of P.ultimum

TRICHODERMA ISOLATES

DAYS	TRC	R.176	R.198	R.199	ISOLATE 2	ZN59
1	100	15.9	100	100	100	100
2	100	18.0	100	90.3	100	100
4	100	0	100	31.9	100	100
6	100	0	100	0	100	100
8	100	0	100	0	100	100
10	100	0	100	0	100	100

Complete cover of the agar surface by P.ultimum control cultures occurred on day 4. Each treatment was replicated three times.

3.5 DUAL INOCULATION PLATE TESTS USING CELLOPHANE AND DIALYSIS MEMBRANE

TRC and P.ultimum or P.debaryanum were used in the arrangement described in section 3.2.3, and Figure 6. With TRC positioned above the membrane clear zones were observed in the Pythium mycelium growing beneath both cellophane and dialysis membranes (Tables 18a and 18b). These were distinct by day five and coincided with the development of clear zones on control paired culture plates with no separating membrane barrier between the organisms. In plates with the position reversed, that is with TRC situated below and Pythium above the membrane, observations were more difficult. TRC grew in an irregular manner below the membranes giving a very patchy, uneven appearance to the colony. However a zone similar to that observed when TRC was on the upper surface, was detected in some of the replicates.

Growth measurements taken from days three to seven are given in Tables 18-20. However growth especially of the organism below the membrane could not easily be distinguished subsequent to day seven.

Microscopic examination indicated that the interaction zones consisted of lysed Pythium hyphae, generally separated by septa from unaffected regions of the colony. Sporangia were occasionally noted scattered in the Pythium colony growing beneath the membranes and it is likely that surface moisture introduced on the membranes induced this response.

Neither P.ultimum nor P.debaryanum could be re-isolated from the regions of agar distal to the original inoculation point on plates where they were the sub-membrane organism. TRC although showing an aberrant growth pattern beneath the membrane was successfully re-isolated from samples of agar taken from such colonies. The recovery of both P.ultimum and

Dual Culture Tests with Pythium spp. growing beneath cellophane or dialysis membranes (lower) inoculated against TRC positioned above the membrane layer (upper)

Table 18a

Clear zones were observed on both cellophane and dialysis membrane covered plates by day 5. Zones were more distinct on cellophane covered plates on day 5 and on day 7 with dialysis membrane covered plates in both cases zones were very marked.

Table 18b

Clear zones were visible by day 5 with both cellophane and dialysis membrane covered plates. Zones were more pronounced on cellophane covered plates but were more diffuse in appearance than those observed with Pu. By day 7 zones were still evident with both but were not as defined as seen with *Pu.*

Pu : *Pythium ultimum*;

Pd : *Pythium debaryanum*;

Growth is expressed as the mean colony diameter(mm) ± the standard error of the mean

Table 18a

Pu (lower) vs TRC (upper)
Growth (mm)

DAYS	CELLOPHANE		DIALYSIS	
	Pu	TRC	Pu	TRC
3	64.46±0.38	31.96±1.71	61.59±3.32	27.74±2.09
4	65.15±1.06	42.74±2.30	65.16±1.73	39.03±1.81
5	58.48±2.70	57.18±4.25	63.31±0.90	50.55±1.99
7	46.64±3.75	73.69±2.33	52.76±1.25	67.68±1.83

Table 18b

Pd (lower) vs TRC (upper)
Growth (mm)

DAYS	CELLOPHANE		DIALYSIS	
	Pu	TRC	Pu	TRC
3	67.13±1.53	33.13±1.47	65.28±1.91	31.23±0.34
4	67.91±1.14	45.19±1.48	68.83±0.10	43.38±0.34
5	60.52±1.00	57.90±4.26	63.03±1.53	52.28±1.57
7	50.34±0.61	72.08±0.20	52.30±1.12	67.22±0.41

Table 19

Dual culture tests with TRC growing beneath cellophane or dialysis membrane (lower) inoculated against Pythium spp. positioned above the membrane layer (upper

Table 19a

The growth habit of TRC made accurate measurements of TRC difficult. The colonies were very irregular in shape giving a patchy appearance and contained areas of dense and sparse growth. Clear zones similar to those observed with TRC positioned above the membrane were visible on a few plates on day 5 only.

Table 19b

Clear zones similar to those seen with TRC in the upper position above the membrane were visible by day 5 with some replicates. The area of Pd mycelium lying above TRC had a flat and sparser appearance than areas away from the TRC colony. Growth of TRC was aberrant as described above.

*by day 7 margins of the TRC colonies could not be easily distinguished and measurements therefore probably underestimate the true extent of TRC growth.

Table 20

Dual inoculation controls in the absence of a membrane layer

Clear zones developed by day 5 with both P. ultimum and P.debaryanum

Pu : Pythium ultimum;

Pd : Pythium debaryanum;

Growth is expressed as the mean colony diameter (mm) ± the standard error of the mean

Table 19a

DAYS	TRC (lower) vs Pu (upper) Growth (mm) CELLOPHANE Pu	TRC	DIALYSIS Pu	TRC
3	56.98±0.76	27.03±1.52	59.59±1.77	25.31±0.99
4	61.14±3.06	42.19±1.00	66.31±1.68	38.51±1.12
5	55.58±0.28	66.30±0.45	58.09±0.59	57.76±2.46
7 *	62.48±5.48	66.90±0.43	69.39±1.64	63.38±1.68

Table 19b

DAYS	TRC (lower) vs Pd (upper) Growth (mm) CELLOPHANE Pd	TRC	DIALYSIS Pd	TRC
3	64.00±0.43	23.39±0.55	64.28±1.53	28.33±2.68
4	68.71±0.18	33.75±3.14	66.82±3.24	46.96±7.12
5	63.62±3.94	52.46±2.07	56.78±1.78	62.14±5.36
7 *	68.86±0.78	56.54±3.72	73.43±2.40	63.29±1.94

Table 20

DAY	Growth (mm) TRC vs Pu Pu	TRC	TRC vs Pd Pu	TRC
3	60.28±1.45	26.78±2.54	67.58±1.05	25.08±0.57
4	68.65±1.09	37.27±4.10	72.68±0.72	35.12±0.89
5	65.[illegible]2±1.86	47.68±5.54	65.55±1.31	46.97±2.16
7	53.95±3.40	66.42±5.54	54.83±1.86	69.57±2.21

P.debaryanum from the upper membrane surface indicates that these remained viable when grown as the uppermost organism. TRC was also re-isolated from membranes even when growing as the lower organism probably due to mycelium and spores adhering to the undersurface of the membrane when removed.

In controls using the same organism both above and below the membrane, naked-eye observations suggested that a boundary developed between the two colonies, approximately half-way between the two inoculation sites. However growth rates were unaffected and culture expansion continued both above and below the membrane surface. Pythium mycelium growing below the membrane consisted of sparser and straighter hyphae than that growing on the membrane upper surface and the differences in growth habit therefore could account for this initial demarcation. Microscopic examination of these controls indicated that apart from the differences in growth habit observed P.ultimum, P.debaryanum and TRC grew satisfactorily both above and below the membrane and no adverse effects on viability were detected.

3.6 CULTURE FILTRATE EXPERIMENTS

3.6.1 Tests using non-lyophilized, unconcentrated TRC culture filtrate

Filtrates were harvested from cultures of TRC growing in liquid soil extract media. The cultures were filtered through Whatmans GF/A glass microfibre filtres to remove the hyphal mat and through 0.2µm Whatmans cellulose nitrate filters. Filtrates (50µl) were introduced into each of five wells (five mm in diameter), autoclaved culture filtrates and sterile distilled water were used as controls. Growth of Pythium was unaffected by the presence of TRC culture filtrates and complete cover of the agar surface occurred

within three to four days. No clear zones were observed.

The experiment was repeated using sterile six mm diameter antibiotic disks (Whatmans AA disks) which had been soaked in culture filtrate for two hours. Filtrate (100µl) was also used to impregnate 20mm diameter filter paper disks which were positioned in a circular arrangement. Again neither the growth rate nor growth habit was affected by the culture filtrates compared with that seen in the controls. No zones of lysed or dead Pythium hyphae were observed.

3.6.2 Effect of lyophilized and non-lyophilized culture filtrates from mixed growth of TRC and P.ultimum

The characteristic zone of clearing which developed on SEA plate tests occurred in the presence of both organisms. By analogy the effect of filtrates collected from mixed cultures was investigated by growing TRC in liquid media in the presence of either living or heat-killed Pythium mycelium and compared with filtrates from cultures of TRC and P.ultimum grown singly.

Disks of P.ultimum mycelium five mm in diameter were used to inoculate soil extract liquid medium and the cultures were grown at 20°C for one week. After this period the cultures were killed by autoclaving and were re-inoculated with a five mm disk of TRC. McCartney bottles containing 10 mls of soil extract were also inoculated simultaneously with both P.ultimum and TRC and individually with P.ultimum and TRC only. Fifteen bottles were used for each treatment and an additional set inoculated with P.ultimum only was autoclaved prior to assay and included for comparison.

After ten days incubation, the filtrates were harvested through 0.4 and 0.2 μm cellulose nitrate filters. Aliquots of each treatment (50ml) were lyophilized and reconstituted in sterile distilled water (section 3.2.4.2) to give a ten-fold increase in concentration. Freeze dried and equivalent untreated culture filtrates used in wells (100 μl), on filter paper disks (100μl) or antibiotic disks (100μl) in tests with P.ultimum did not produce any adverse effects on growth rate or habit compared with sterile distilled water controls. Thus lyophilized and non-lyophilized filtrates from P.ultimum cultures which had been autoclaved and re-inoculated with TRC, simultaneously inoculated P.ultimum and TRC cultures and autoclaved P.ultimum cultures failed to demonstrate any deleterious effects on P.ultimum growth either before or after contact.

The test was repeated using plates previously seeded with P.ultimum to test the effects of the treatment filtrates on established four-day old mycelium of the test organism. No detrimental effects were observed subsequent to the introduction of the culture filtrates and no differences between the treatments and the controls were noted.

3.6.3 Use of polycarbonate filters for harvesting culture filtrates

Polycarbonate filters (Nucleopore) provided an alternative non-cellulose based autoclavable membrane. Culture filtrates which had been sterilized through polycarbonate filtrates of 0.40μm and 0.2μm pore size were used in a series of assays with both P.ultimum and P.debaryanum.

TRC was grown with autoclaved cultures of P.ultimum and P.debaryanum as described earlier (section 3.6.2) and the filtrate was subjected to freeze drying as were extracts from pure cultures of TRC, P.ultimum

Table 21

The effect of lyophilized culture filtrates harvested through polycarbonate filtres on growth of P.ultimum

For abbreviations see below

Table 22

The effect of lyophilized culture filtrates harvested through polycarbonate filtres on growth of P.debaryanum

Pu: Pythium ultimum;

Pd: Pythium debaryanum;

TRC: Binab Trichoderma;

SE: soil extract;

Autoclaved Pu/Pd + TRC: seven to ten day old Pythium mycelium killed by autoclaving and followed by re-inoculation with TRC;

Pu/Pd + TRC: simultaneous mixed growth of both Pythium and TRC

Pythium colonized the entire agar surface by day four in all cases.

Results are the mean diameter growth (mm) $\pm$ the standard error. Treatments 3 and 4 when autoclaved produced no deleterious effects on Pythium growth and gave results analogous to treatment 6 in both cases. No adverse effects on Pythium growth were observed with the non-lyophilized equivalent treatments with growth being very similar to the results with the lyophilized filtrates.

Table 21

Lyophilized Treatments	TEST ORGANISM P. ULTIMUM DAYS 1	2	3
1. Pu - Autoclaved + TRC	27.30±2.25	49.64±3.14	71.95±2.69
2. Pu + TRC	25.43±1.93	55.96±0.51	82.13±0.70
3. TRC	34.76±1.73	59.13±3.10	80.09±1.95
4. Pu	23.79±2.70	42.03±5.02	65.45±4.21
5. Treatment(1) Autoclaved	33.43±1.18	56.25±2.17	79.54±0.33
6. Treatment (2) Autoclaved	26.49±0.93	63.08±0.40	75.03±0.24
7. SE liquid medium	29.88±0.84	53.19±0.49	76.82±0.29
8. Sterile distilled water	28.99±1.48	52.34±1.87	74.79±0.93

Table 22

Lyophilized Treatments	TEST ORGANISM P. DEBARYANUM DAYS 1	2	3
1. Pd - Autoclaved + TRC	35.42±0.86	61.37±1.34	80.18±0.54
2. Pd + TRC	27.07±1.36	65.48±0.84	81.64±0.43
3. TRC	34.52±2.87	58.67±4.39	80.08±2.25
4. Pd	35.24±0.72	61.18±0.47	82.24±0.46
5. Treatment (1) Autoclaved	35.40±0.88	60.69±1.93	80.93±1.27
6. Treatment (2) Autoclaved	25.63±1.36	61.83±1.94	79.94±0.10
7. SE liquid medium	35.23±1.31	61.00±1.67	80.75±0.80
8. Sterile distilled water	35.60±2.73	60.70±4.15	79.85±2.34

and P.debaryanum grown in liquid media and culture solutions harvested from simultaneous mixed growth of viable P.ultimum and TRC. The controls included equivalent filtrates which had been autoclaved and uninoculated soil extract medium which had also been lyophilized. Wells (one cm diameter) were each filled with 200µl of liquid, three wells were used on each plate and each treatment was assayed on three plates. Both P.ultimum and P.debaryanum were used as the test organisms and the treatments were used in a minimum of three trials.

No adverse effects on the growth rate or growth habit of the test organisms were observed (Tables 21 and 22) and Pythium mycelium was found to cover the entire agar surface by the fourth day in all cases.

3.6.4 Culture filtrates of T.polysporum

Lyophilized and non-lyophilized culture filtrates of TRC, grown either alone or in combination with viable or heat-killed Pythium mycelium, had failed to produce any deleterious effects on Pythium growth and in no instance had any interaction analogous with that observed in paired culture tests been observed.

Two membered SEA plate tests had also demonstrated that a very distinct zone of interaction also developed between colonies of the constituent species of the Binab preparation, namely T.polysporum and T.harzianum and P.ultimum and P.debaryanum (section 2.3.3). Although not idential in nature to the overall reaction observed with TRC, the initial response was particularly marked in interactions with T.polysporum and a very pronounced clear zone developed soon after contact with the Pythium colonies. Thus following the routine procedure culture filtrates of T.polysporum were assayed for activity against the two Pythium spp. T.polysporum was grown in combination with heat-killed and viable Pythium

mycelium as before and all culture filtrates were sterilized through polycarbonate filters. Both lyophilized and non-lyophilized extracts were tested using the well method (200µl per well) and each treatment was assayed on five plates. Extracts from equivalent treatments with TRC were included for comparison.

In no instance were any deleterious or unusual effects on P.ultimum or P.debaryanum growth observed either prior to or after contact with the wells. In all cases growth was comparable with the equivalent autoclaved treatments and sterile distilled water controls. Similarly no differences were noted between the TRC and T.polysporum treatments and Pythium completely covered the agar surface in all the treatments and controls by the fourth day of incubation.

3.7 CELLULOYTIC ACTIVITY

The Binab TRC was found to produce distinct clear regions in tubes containing modified cellulose agar (section 3.2.6), after six days incubation at 20°C. The depth of clearing was comparable with and frequently greater than that caused by Trichoderma reesei, a known cellulase producer (Theodorou et al., 1980). The Binab TRC degraded both Whatmans CMII cellulose and Avicel. No such degradation was observed with either P.ultimum or P.debaryanum.

Liquid Avicel cellulose medium prepared by omitting the agar constituent was used in further tests. Disks of TRC mycelium (five mm in diameter) were used to inoculate ten ml of sterile liquid medium in McCartney bottles and these were incubated stationary on angled stands for 10-14 days at 20°C. Cultures were harvested by filtration through 0.45µm polyamide filters (Sartorius) which had been sterilized using ethylene oxide (475 mg/dm^3 at 700 Torr) for six hours at 29°C followed by de-gassing overnight prior to

use. The harvested filtrate (200µl) was pipetted into each of three one cm diameter wells cut in SEA. The plates were inoculated with P.ultimum and autoclaved culture filtrate and distilled water were used in controls. Each treatment was replicated five times.

This test was repeated three times and on each occasion P.ultimum grew over the entire agar surface within three to six days. No zones of clearing or lysis were observed but in two of the three trials a thickened band of P.ultimum hyphae developed approximately ten mm away from the wells. The band was approximately four mm wide and consisted predominantly of aerial hyphae. However a similar region was also found near wells containing autoclaved culture filtrate indicating that this response was not caused by the presence of cellulase, and is likely to be a nutritional effect or be due to metabolic products which may have accumulated in the liquid medium. Growth of P.ultimum proceeded from this band to the edge of the plate with no other unusual effects being observed.

Furthermore when T.reesei was used in dual inoculation plate tests against P.ultimum and P.debaryanum on SEA, no zones of clearing or lysis or dead Pythium hyphae were observed. Unlike with TRC, Pythium growth was not affected by the presence of T.reesii (section 2.8) and hyphae of the two organisms intermingled without any apparent ill-effect on either organism.

3.8 DISCUSSION

The formation of zones of inhibition and lysis similar to those under study have often been attributed to the effects of antibiotics (Baker and Cook, 1974; Sivasithamparam and Parker, 1980; Howell, 1982). Using a technique to detect the production of non-volatile antibiotics (Mughogho, 1968; Dennis and Webster, 1971 a ; Tronsmo and Dennis, 1978), TRC was found to produce on antibiotic or antibiotics with a wide spectrum of activity, but its production appeared to be confined to MEA.

The formation of clear zones on SEA did not appear to be influenced by either the glucose or soil extract concentration present in the agar medium, and therefore the importance of nutrient competition in the underlying mechanism could not be ascertained in this experiment. Optimum levels of carbon and nitrogen in the growth medium were not determined although the carbon to nitrogen ratio may be more critical than absolute levels of either component individually. Although composition of the soil extract was not known it was assumed that the nitrogen status was not limiting and that it was available in a form which could be utilized by both organisms. Competition for other growth factors such as minerals, vitamins amino-acids and microelements which are also required for optimum growth may have been operative. Kraft and Erwin (1967, 1968) found that the nutrients in exudates from germinating mung bean seeds affected the virulence of P.aphanidermatum, and different amino acids as nitrogen sources influenced virulence especially when the density of zoospores was low.

The phenomenon described by Ikedi gwu and Webster (1970 a) of hyphal interference by Coprinus heptemerus against Pilobolus crystallinus and Ascobolus crenulatus appears to be a mode of antagonism which

inhibits and kills hyphae. The interaction operates on contact, or at very close distances, and is accompanied by vaculation, granulation and a loss of hydrostatic pressure. The effect is localized with affected cells at the interface with the antagonist losing opacity. All the coprophilous basidiomycetes tested exhibited to different degrees the interference against A.crenulatus (Ikediugwu and Webster, 1970 b). A range of representative fungi including T.viride were susceptible to interference by C.heptemerus but neither T.viride nor any of the other non-basidiomycete fungi, with one exception Stilbella erthrocephala, exhibited hyphal interference against A.crenulatus. Further work by Ikediugwu et al. (1970) also demonstrated symptoms of hyphal interference by Peniophora gigantea against Heterobasidion annosum in paired culture tests. Cells of H.annosum became transparent except at the region of contact where the protoplast was opaque.

The mechanism of hyphal interference appears to involve alterations in membrane integrity. Changes in the plasmalemma cause altered permeability and a wide extra-plasmalemmal zone develops between the plasmalemma and the cell wall, mitochondria swell and electron dense material is deposited in affected cells (Ikediugwu, 1976 a. b). Clearly any similarities between this and the Pythium-TRC interaction requires confirmation using electron microscopy to investigate accompanying ultrastructural changes. Besides the symptoms induced in susceptible cells there are other similarities between this form of antagonism and that of TRC against P.ultimum and P.debaryanum. In both cases the hyphal tip of the antagonist produced the strongest interference response, the interaction could not be reproduced with culture filtrates but did occur through cellophane membrane, and it was independent of the carbon-nitrogen ratio of the medium.

Results in the present study implicate a diffusable low molecular weight metabolite as the agent of mycelial

clearing, but all attempts to detect such a metabolite in filtrates of Trichoderma liquid cultures were unsuccessful. Failure to produce any effects on Pythium growth with culture filtrates may have been due to the accumulation of metabolites (staling substances) in the liquid medium, which suppressed the activity of the clearing factor or at least prevented reactions progressing to completion. Hickey (1953) found that certain 18-carbon unsaturated fatty acids suppressed the activity of the polyene antibiotic ascosin. Moreover, fungal cell surfaces contain enzymes active in extracellular degradation of nutrients rendering them available for absorption (Zalokar, 1965), and thus close association or binding to the hyphal cell wall may prevent the release of active components into culture solutions. However acetone-extracts of cell wall preparations of TRC, and P.ultimum mycelium did not produce any adverse effects on Pythium when incorporated into wells cut in SEA. Similarly macerated portions of the agar from clearing zones in paired cultures did not demonstrate any activity. In neither case was Pythium growth affected either before or after contact: the mycelium appeared healthy and complete cover of the agar surface ensued within four to five days.

The hydrolysis of crystalline cellulose is complex and three different types of cellulolytic activity have been recognized: endoglucanase, exoglucanase and cellobiase. Reese et al. (1950) proposed the Ci-Cx concept in which the component responsible for forming shorter linear chains from native cellulose (possibly by causing breakage of the hydrogen bonds between cellulose chains) was designated the Ci component, and the chains were then hydrolysed by the Cx component. The endoglucanase equates with Cx and exoglucanase is thought by some authors to be synonymous with Ci.

Exoglucanase (B-1, 4-glucan cellobiohydrolase), endoglucanase (B-1, 4-glucan glucanhydrolase) and

B-glucosidase or cellobiase are involved in the cellulase activity of T.viride, and several endoglucanases including a low molecular weight enzyme have been found. Similarly in T.koningii, one exoglucanase and at least four endo-glucanases have been isolated and characterized. It is thought that the three enzymes exoglucanase, endoglucanase and cellobiase act in a synergistic or co-operative manner (Goksoyr and Eriksen, 1980). Many microorganisms produce cell-bound cellulases and degradation occurs following contact with the substrate (Fogarty and Kelley, 1979). Portions of the cellulolytic systems may also bind to cellulose material present in the medium (Cowling and Kelman, 1963; Goksoyr and Eriksen, 1980), possibly the Pythium mycelium. Although results using dialysis membrane in this study suggested that the reaction was not enzymic in nature, TRC is able to degrade cellulose and as such the cellulosic dialysis membrane itself would be prone to digestion, leading in time to an alteration in porosity. The ability to produce cellulose-degrading enzyme provides a possible mechanism of antagonism against Pythium spp. since their cell walls contain cellulose. However solutions from TRC cellulose medium liquid cultures failed to produce zones of clearing in Pythium colonies.

Dick and Hutchinson (1966) recognized the biological activity of volatile fungal metabolites produced by a wide spectrum of fungal cultures, and Dennis and Webster (1971 b) showed that isolates of Trichoderma produced volatile antibiotics effective against a range of test organisms, including P.ultimum, with acetaldehyde being identified as one of the inhibitory metabolites present in the volatile fraction. Fungi are known to produce ethylene (Nickersen, 1948; Young et al., 1951; Ilag and Curtis, 1968) and some basidio-mycetes are reported to produce hydrogen cyanide (Robbins et al., 1950; Lebeau and Hawn, 1963; Ward and Thorn. 1965). In the reaction under study close contact between the two organisms was necessary and no inhibition

of Pythium growth occurred prior to contact. This suggests that the involvement of volatile metabolites is unlikely. Nevertheless, no precautions were taken to collect volatile components and failure to detect activity in culture filtrates especially after lyophilization could be due to this. Hutchinson and Cowan (1972) identified carbon dioxide and ethanol as volatile metabolites from cultures of T.harzianum, and Tamimi and Hutchinson (1973) reported the presence of acetaldehyde, acetone, ethanol carbon dioxide and other metabolites in head space gases above cultures of Trichoderma spp. The degree of inhibition of growth of a range of assay fungi was accounted for largely by differences in the rate of carbon dioxide production.

Low concentrations of any causal agent in culture filtrates might have precluded detection by the assay systems employed. Consequently concentration by lyophilization was used but a ten-fold decrease in solvent volume proved ineffective. Liquid medium has been found to be less conducive to antibiotic production than its equivalent agar counterpart (Hsu and Lockwood, 1969). Fajola and Alasoadura (1975) however, demonstrated the activity of an antifungal antibiotic in liquid cultures of a mycoparasitic isolate of T.harzianum grown in malt extract solution which was effective against Pythium aphanidermatum.

Low level contamination of filtrates by TRC was a continual problem and to combat this three to four filtrations through sterile filters were required prior to use. It is conceivable that failure to demonstrate activity in crude culture filtrates may have been due to adsorption of active constituents during this process. Ko (1983) studying the hormones produced by Phytophthora parasitica, reported that Millipore filters adsorbed hormones which stimulated the opposite mating type to form oospores, but Nucleopore polycarbonate filters were ineffective in this respect and failed to retain the hormones. Moreover hormones extracted

were effective when adsorbed onto small pieces of Millipore filter but not when added directly to agar cultures. It was postulated that the filters prevented inactivation of the hormones by the agar medium. Involvement of a similar type of metabolite in the reaction observed in this study may also have been prone to such effects. Polycarbonate filters being non-cellulosic were used for filtration in preference to cellulose nitrate, but effects exerted by the agar medium particularly in the well method of assay may have prevented detection of activity.

Although no evidence of mycoparasitism of TRC was observed, parasitism can occur without the penetration of the host (Boosalis, 1964), and certain parasites contact their hosts with special hyphal branches which do not penetrate the host cell. Such mycoparasites are thought to glean nutrients by effecting changes in host permeability. Mycoparasitism is influenced by the carbon to nitrogen ration of the agar medium (Barnett, 1963; Berry, 1959; Griffith and Barnett, 1967). Shigo *et al*. (1961) found that *Piptocephalis frequetans* was highly parasitic on *Penicillium frequetans* in liquid media only after high concentrations of microelements were added with manganese being the most effective. The degree of parasitism on agar was correlated with concentration of available nitrogen in the medium, the percentage of soluble nitrogen present in the mycelium of the host, and inversely related to the glucose concentration. It has been proposed that nutrition of the host may influence the production of certain enzymes which may in turn affect the degree of parasitism and influence the supply of amino acids and other nutrients within the host cells (Shigo, 1960).

Papavizas and Lumsden (1980) discussed the phenomena of germination-lysis and mycolysis of dormant propagules. The former occurs in the presence of nutrients with lysis of germlings occurring in the

absence of a host before they produce new resistant propagules. It is thought that competition from populations of saprophytes stimulated by the presence of nutrients causes nutrient-stress in the pathogen and hyphae subsequently lyse. Lysis of R.solani hyphae in soil has been reported (Olsen and Baker, 1968) and lysis by soil bacteria is promoted by a high carbon to nitrogen ratio (Papavizas, 1963). Mycolysis results in loss of protoplasm in fungal structures and enzymic breakdown of cell wells. Bacteria (including Pseudomonas spp.) were observed with Phytophthora cinnaomomi in culture and are thought to be responsible for lysis of sporangia and hyphae (Malajczuk et al., 1977). Siderophores produced by Pseudomonas spp. have been reportd to be inhibitory to several soil fungi (Misaghi et al., 1982) but inhibition could be reversed by the addition of iron. Siderophores act by tightly chelating available iron rendering it unavailable to other organisms. Scher and Baker (1980, 1982) demonstrated the importance of iron in soils suppressive to the Fusarium flax with pathogen and found that suppressiveness could be induced by Pseudomonas putida and synthetic iron chelators. Iron was required for microconidial germ tube elongation and competition for iron was responsible for suppression. Hubbard et al. (1983) reported that T.hamatum were ineffective as a biocontrol agent in soils of low iron content due to the inhibitory pseudomonad colonies and it was concluded that these produced siderphores. The possible involvement of a similar mechanism was investigated in tests in which the synthetic chelating agent Chelex 100 (Biorad Lab) was incorporated into SEA. No adverse effects on either P.ultimum or TRC growth were observed, and in paired culture tests with both organisms clear zones were comparable with controls not containing the chelating agent.

The formation of clear zones in the Pythium/TRC interaction on SEA is reminiscent of hyphal interference described by Ikedi gwu and Webster (1970 a), in

that the defined zone develops ahead of the TRC leading edge. This implies that a diffusable metabolite produced by the youngest portion of the TRC colony is involved. However, the formation of this zone may be a consequence of the non-septate nature of the *Pythium* mycelium, resulting in an extension of the affected zone to beyond that in contact with TRC. Since the clear zone response occurred on SEA but not on MEA, the reaction may be related to the nutrition of both organisms. A low nutritional status may predispose *Pythium* to lysis, a situation compounded by the presence of a second organism contributing to nutrient depletion. Additional stress imposed by lytic enzymes and/or antibiotics would further contribute to the unfavourable environment. A combination of factors such as these seems the most likely explanation for the hyphal clearing effect. Thus the absence of one contributory factor such as nutrient-stress induced by competition would prevent the interaction from being reproduced with culture filtrates. In general inhibitory metabolites produce more marked effects on nutrient-poor media (Baker and Cook, 1974), and use of the thin layer chromatographic method developed by Lazarovitz *et al*. (1982) might in future studies be helpful in identifying specific products of TRC toxic to *Pythium* spp. However the underlying mechanism behind this characteristic interaction remains unclear as does the phenomenon of hyphal interference. The practical success of *P.gigantea* for the control of *H.annosum* which may rely at least in part on hyphal interference provides impetus for further studies.

4. EVALUATION OF TRICHODERMA AS A BIOCONTROL AGENT UNDER GREENHOUSE CONDITIONS

4.1 INTRODUCTION

An approach frequently used in biological control is to introduce a microbial antagonist to a pathogen infection court such as seeds, seedlings, wounded aerial and subterranean plant parts (Baker and Cook, 1974; Mangenot and Diem, 1979; Kommedahl and Windels, 1981). The treatment of seeds and roots and also soil for the control of seedling diseases using spores or mycelia of antagonistic microorganisms has given promising results (Liu and Vaughan, 1965; Hoch and Abawi, 1979; Locke *et al.*, 1979; Harman *et al.*, 1981; Chet and Baker, 1981). To accomplish successful mass introductions of an antagonist into soil Papavizas and Lewis (1981) stressed the importance of utilizing food bases to effect a temporary yet effective swamping of the infection court by the antagonist. This technique has proved effective for biological control of certain soil-borne pathogens (Wells *et al.*, 1972; Ahmed and Tribe, 1977; Moody and Gindrat, 1977; Hadar *et al.*, 1979; Elad *et al.*, 1980).

The experiments in this section were designed to assess the Binab T *Trichoderma* as a potential biocontrol agent under greenhouse conditions. Emphasis was placed *P. ultimum* and *P.debaryanum* as the target pathogens, causes of seed decay and pre-and post-emergence damping-off of many crops, against which *TRC* had demonstrated marked antagonism *in vitro*, and additionally *F.oxysporum* f.sp.*lycopersici* a tomato vascular wilt pathogen.

4.2 MATERIALS AND METHODS

The damping-off pathogens P.ultimum and P.debaryanum

4.2.1 Preparation of Microbial Inoculum

Vermiculite cultures were prepared in two litre Erlenmeyer flasks using 100-150g of the dry vermiculite carrier soaked with 20% (v/v) V8 juice (Campbells V8 - vegetable juice). The liquid was added in a 1:4 (w/v) ratio of dry vermiculite to diluted V8 juice. The medium was sterilized at 121°C for 30 minutes and inoculated when cool with ten disks (1.0cm diameter) cut from four-day old fungal cultures grown on 20ml of 2% (w/v) MEA. The flasks were well shaken and were incubated in the light at room temperature (approximately 20°C). After 5-7 days the flasks were examined and if there was insufficient growth were re-inoculated as before, shaken vigorously and re-incubated until required. The actively growing vermiculite cultures were used on the tenth day of incubation. Prior to use the flasks were shaken to ensure an even distribution of spores and mycelium and approximately 1.0g samples were plated onto 2% MEA to check for viability and contamination.

4.2.2 Incorporation of the fungal cultures into the growth medium

The experimental units used throughout the study were 5" square plastic pots which were sterilized using a 2% (v/v) solution of domestos, thoroughly rinsed and dried prior to use. The vermiculite inoculum (50.0ml) was incorporated into the upper portion of the potting compost in the following manner. Pots were completely filled with Fisons Levingtons potting and seed compost and the top layer (approximately 400-500ml) was removed and placed in a large beaker into which the inoculum was added and the two thoroughly mixed. The seeded layer was then replaced in the pots to give a final concentration of 3-4% (v/v) per pot (approximately 10% (v/v) of the seeded layer of compost). For both TRC only treated- and pathogen only treated- pots, 50.0ml of inoculum were used. In pots containing both

TRC and the pathogen equal volumes of their respective vermiculite inocula were incorporated together as described above. Controls were prepared using uninoculated vermiculite - V8, TRC only inoculum and pots containing compost only. All treatments unless stated otherwise, were replicated five times.

4.2.3 Seed sowing and seedling emergence

Fresh seed stocks were used for each experiment, sealed unopen stocks being stored at 4°C. Seeds were surface sterilized in 10% (v/v) sodium hypochlorite (1.0-1.4% available chlorine) for three to five minutes and were rinsed using several changes of sterile distilled water.

Pea (*Pisum sativum* L.) and cabbage (*Brassica oleracea* L., var. *capitata* L.) were used predominantly although lettuce (*Lactuca sativa* L.), tomato (*Lycopersicon esculentum* Mill.) and dwarf bean (*Phaseolus vulgaris* L.) seeds were also employed. Cabbage seeds, cv. Golden Acre-Progress (Suttons) were sown 20 per pot to a depth of 13mm, while 20 seeds of lettuce cv. Tom Thumb (Suttons) and tomato cv. Moneymaker (Suttons) were sown approximately 6mm deep. Pea seeds, cv. Onward (Suttons) and bean seeds, cv. Red Mexican (University of London, Botany Supply Unit) were each sown 10 per pot to a depth of 50mm. Accurate sowing was achieved using marked, sterilized corkborers to place each seed at the desired depth.

The pots were arranged in a completely randomized or in a randomized block design for each host as required in the misting unit of a greenhouse. This location was used for all the damping-off experiments and contained no supplementary lighting. The majority of experiments were conducted during the months October to April. The heating system was not operated and temperatures in general ranged from approximately 5-20°C. The greenhouse benches were cleaned with a 2-5% (v/v) solution of domestos and were rinsed with water between each experiment.

Pots were regularly watered and experiments which continued into the warmer summer months employed the misting facility to keep the compost moist and minimize the temperature rise during periods of warmer weather.

4.2.4 Use of TRC inoculum as a prophylactic treatment

In experiments where a prophylactic treatment henceforth referred to as a pre-inoculation treatment (PI) treatment was included, TRC was grown as vermiculite - V8 inoculum and was incorporated into compost as described earlier. Pots were watered and were left to stand in the greenhouse for seven days at 15°C ± 5°C. Pathogens were introduced into these pots using the same inoculation procedure as before. A separate TRC treatment was also included using simultaneous inoculation of the pathogen and fresh TRC inoculum. Thus in these experiments two methods of introducing TRC were assessed for each pathogen. TRC pre-inoculated pots which had not been subsequently inoculated with a pathogen were used as a fourth control treatment.

4.2.5 Final fresh weight analysis

After recording the final stand of emerged seedlings, the pots were each emptied and the plants removed together with as much of their root system as possible. The roots were gently washed under running tap water to remove excess soil and were blotted dry between absorbent paper. Plants recovered from each pot were weighed individually and the mean fresh weight for each pot and hence each treatment calculated.

4.2.6 Statistical Treatment of data

Emergence of pathogen only and pathogen plus TRC treated seedlings was expressed as a percentage of the uninoculated vermiculite controls. Final emergence and fresh weight data were subjected to analysis of

variance and the means were compared using Duncan's new multiple range test (Steel and Torrie, 1966).

4.2.7 Trichoderma selective media

4.2.7.1 Elad et al. (1981)

$MgSO_4.7H_2O$	0.20 g
K_2HPO_4	0.90 g
KCl	0.15 g
NH_4NO_3	1.00 g
Glucose	3.00 g
Chloramphenical	0.25 g
* Dexon (60% wp)	0.30 g
** PCNB (75% wp)	0.20 g
Rose Bengal	0.15 g
Agar	20.00 g
Distilled water	1000 ml

* p - dimethylaminobenzenediazo sodium sulphonate

** pentachloronitrobenzene

4.2.7.2 Papavizas (1981)

V_8	200 ml
Glucose	1.00 g
Agar	20.00 g
Distilled water	800 ml

The agar was autoclaved separately in 500ml of distilled water and mixed with the dilute V_8 after autoclaving. The following microbial agents were added µg/ml:

Neomycin sulphate	100
Bacitracin	100
Penicillin G	100
Chloroneb	100
Nystatin	20
Chlorotetracycline HCl	25
Sodium propionate	500
PCNB	100

4.2.7.3 TRC selective media

The TRC selective medium used in this study was a modified version of the Papavizas (1981) medium incorporating Metalaxyl (25% wp) 200 µg/ml (Ciba-Geigy Agrochemicals) in place of chloroneb and triton X-100 2.0ml/l.

4.2.7.4 Peptone-Dextrose-Rose Bengal Agar (Martin,1950)

Agar	20.0 g
KH_2PO_4	1.0 g
$MgSO_4.7H_2O$	0.5 g
Peptone	5.0 g
Dextrose	10.0 g
Rose bengal (1%)	3.3 ml
Distilled water	1000 ml
Streptomycin	30.0 mg

All the constituents except rose bengal and streptomycin were dissolved in water and the mixture heated slowly while stirring until it started to boil. It was removed from the heat and the rose bengal added. After autoclaving the medium was cooled and streptomycin added prior to dispensing.

The vascular wilt pathogen F.oxysporum f.sp. lycopersici

4.2.8 Growth of tomato plants

Surface sterilized tomato seeds (cv. Moneymaker) were germinated in Levingtons compost and seedlings at the first or second true leaf stage were repotted into 5" square pots filled with a 1:1:1 (by volume) mixture of Levingtons compost, John Innes No.2 and sand. Plants were watered at least once weekly with Long Ashton nutrient solution (Hewitt, 1952) and when necessary sprayed with Murphy's systemic insecticide (containing dimethoate) or Bio 'Sprayday' (containing

permethrin) to control glasshouse whitefly (Trialeurodes vaporariorum). From October-March supplementary illumination was provided by mercury vapour lamps on a 16-hour photoperiod.

4.2.9 Preparation of spore suspensions

4.2.9.1 F.oxysporum f.sp. lycopersici

The pathogen was grown in sucrose-casein hydrolysate liquid medium (SCH) of the following composition:-

SCH

Casamino acids (vitamin free, Difco)	4.6 g
KH_2PO_4	1.0 g
$MgSO_4.7H_2O$	0.5 g
Sucrose	15.0 g
Glucose	0.5 g
* Trace Element solution	1.0 ml
Distilled water	1000 ml

*Trace Element solution

$FeSO_4.7H_2O$	20 mg
$ZnSO_4.7H_2O$	100 mg
$Na_2MoO_4.2H_2O$	2.0 mg
$CuSO_4.5H_2O$	2.0 mg
$MnCl_2.4H_2)$	2.0 mg
Distilled water	1000 ml

100ml of SCH were dispensed in 250ml Erlenmeyer flasks and were autoclaved at 121°C for 20 minutes

The cooled, sterilized, liquid medium was inoculated with a 1.0cm diameter agar disk taken from actively growing agar plate cultures and the flasks were incubated at room temperature on an orbital shaker for six days. The cultures, which grew largely by budding resulting in a dense growth of predominantly yeast-like cells, were harvested by low speed centrifugation and washed with distilled water.

The cell concentration was estimated by haemocytometry and adjusted by dilution.

4.2.9.2 TRC

1.0g of the Binab TRC w.p preparation was dispersed in 10ml of sterile distilled water. The suspension was mixed and filtered through four layers of muslin. The resulting spore suspension was diluted if necessary with sterile distilled water.

4.2.9.3 *T.harzianum* and *T.polysporum*

Agar slopes or plate cultures of *T.harzianum* and *T.polysporum* were flooded with sterile distilled water and the surface of the colony gently scraped. The resulting spore suspension was filtered through muslin as above and the concentration determined using a haemocytometer.

4.2.10 Pathogen inoculation of plants

Roots of the wilt susceptible tomato seedlings were wounded by inserting a scalpel blade into the soil on either side of the stem, to a depth of approximately 3.0cm-5.0cm. Unless otherwise stated inoculum consisted of 20.0ml of six day old SCH shake cultures (10^7 to 10^8 cells/ml) of the *Fusarium* wilt pathogen which was poured over the soil surface. Wounding facilitated entry of the pathogens and in a few experiments where re-potting preceded inoculation additional artificial wounding was not necessary.

4.2.11 Evaluation of disease symptoms

The degree of infection exhibited by pathogen inoculated plants was assessed using arbitary scales of yellowing and wilting with the overall appearance of each plant being recorded in detail. Height measurements from the soil surface to the apical bud, the degree

of adventitious rooting and epinasty, the numbers of abscissed leaves and the fresh weight were noted. Yellowing was assessed on a scale of 0 to 100% where 0 = healthy, 20% = yellowing of 1 leaf in the lower half of the stem, 40% = yellowing of >1 leaf in the lower half of the stem, 60% = yellowing of 1 leaf in the upper half of the stem, 80% = yellowing of >1 leaf in the upper half of the stem, 100% = wilting and death.

Wilting of each leaf was assessed on a scale of 0 to 4 and the mean for the plant obtained. 0 = healthy, 1 = slight wilting of 1 or 2 leaflets, 2 = pronounced wilting of the terminal leaflets or moderate wilting extending over the whole leaf, 3 = complete and severe wilting of the leaf, 4 = leaf dead or abscissed.

4.2.12 Preparation of vermiculite media and soil inoculation

Trichoderma vermiculite cultures were prepared as before (section 4.2.1) and sterilized 5" square pots were filled with alternate layers of soil and *Trichoderma* vermiculite inoculum which were thoroughly mixed to give either approximately 20%(v/v) or 50%(v/v) final concentrations of *Trichoderma* vermiculite - V_8 inoculum as required.

4.2.13 Serial dilution of macerated stem segments

Tomato plants after visual assessment were cut at soil level and the basal segment with a fresh weight of 1.0g (approximately 2.0cm length) was removed, surface sterilized for 3-5 minutes in 10%(v/v) sodium hypochlorite and rinsed in three changes of sterile distilled water for three minutes each time. The segment was then macerated in 10.0ml sterile distilled water for 5 minutes using a MSE Atomix mechanical homogeniser of full speed. It was necessary to cut the segment using a sterile razor blade prior to maceration in order to obtain an adequate comminution of the tissue.

The extract was filtered through 4 layers of muslin and 1.0ml of the extract was serially diluted in sterile distilled water to obtain 10^{-1} to 10^{-6} dilutions. 1.0ml of each dilution was plated onto each of three agar plates containing MEA amended with the antibacterial agent chloramphenicol (100 mg/l) (Waller, 1981) or PCNB agar (Nash and Snyder, 1962; Papavizas, 1967) and incubated at 22-25°C for 3-5 days or until colonies were visible and were counted using a Gallenkamp colony counter.

4.3 RESULTS

The damping-off pathogens P.ultimum and P.debaryanum

4.3.1 Selection of host-pathogen combinations in the presence and absence of TRC

The pathogenicity of Pythium ultimum and Pythium dabaryanum was assessed using five hosts (pea, cabbage, lettuce, tomato and bean) in TRC-treated and untreated compost. Three-fold replication and a completely randomized design were used.

Pre-emergence damping-off and to a lesser extent post-emergence damping-off resulted in decreased final stands for four of the five hosts tested. Both pathogens reduced final stands of pea, lettuce and cabbage whilst P.ultimum had a greater effect on tomato emergence than did P.debaryanum (Table 23 & Fig 9). Neither pre- nor post- emergence damping-off of beans was observed during the course of the experiment. In the presence of TRC a significant increase in emergence was observed with pea and cabbage seeds sown in pots treated with P.ultimum and P.debaryanum. Similarly mean lettuce and tomato final stands in P.ultimum + TRC-treatments were significantly greater than in P.ultimum-only inoculated pots (Table 23 c,d). Conversely with tomato significantly lower emergence occurred in P.debaryanum treatments amended with TRC than in the pathogen only treatment.

Table 23

The effect of *Pythium ultimum* and *Pythium debaryanum* on final seedling stands and fresh weight means (g) of pea, cabbage, lettuce, tomato and bean in the presence and absence of TRC

Treatments:- Pu - *Pythium ultimum*, Pu+TRC - *Pythium ultimum* used simultaneously with Binab *Trichoderma* to inoculate compost Pd - *Pythium debaryanum*, Pd+TRC - *Pythium debaryanum* used simultaneously with Binab *Trichoderma* to inoculate compost.

Controls:- Con - uninoculated compost, Verm - uninoculated vermiculite soaked with V_8 - vegetable juice introduced into compost, TRC - Binab *Trichoderma* inoculum incorporated into compost alone (no pathogen inoculum present). 50.0ml of vermiculite inoculum was used in each case, 20 seeds of cabbage, lettuce and tomato and 10 seeds of pea and bean were sown in each pot, fresh weight is given in grams. Results are the means of three replicates. Any two means not underscored by the same line differ significantly. Any two means underscored by the same line do not differ significantly. (P=0.05)

Table 23 (a - e)

a. PEA - EMERGENCE

Pu	Pd	Pd+TRC	Pu+TRC	Verm	Con	TRC
2.33	3.0	7.0	7.67	7.67	8.0	10.0

PEA - FRESH WEIGHT (g)

Con	Pu	TRC	Pd+TRC	Pu+TRC	Pd	Verm
1.38	1.69	1.86	1.94	2.04	2.10	2.24

b. CABBAGE - EMERGENCE

Pd	Pu	TRC	Pd+TRC	Pu+TRC	Con	Verm
11.0	12.33	16.0	16.67	17.0	18.0	18.67

CABBAGE - FRESH WEIGHT (g)

Pu	TRC	Pd	Pu+TRC	Pd+TRC	Verm	Con
0.11	0.15	0.16	0.17	0.18	0.2	0.2

c. LETTUCE - EMERGENCE

Pu	Pd	Pd+TRC	Verm	Pu+TRC	TRC	Con
11.33	14.0	15.0	18.67	19.0	19.67	19.67

LETTUCE - FRESH WEIGHT (g)

Pu	TRC	TRC+Pu	Verm	TRC+Pd	Con	Pd
0.15	0.30	0.32	0.34	0.36	0.40	0.44

d. TOMATO - EMERGENCE

Pu	TRC	Pd+TRC	Pu+TRC	Pd	Con	Verm
14.67	16.0	17.0	18.0	19.33	20	20

TOMATO - FRESH WEIGHT (g)

Pu	TRC	Verm	Pu+TRC	Pd+TRC	Con	Pd
0.16	0.21	0.22	0.23	0.26	0.41	0.42

e. BEAN - EMERGENCE

Con	Pd	Pd+TRC	Verm	Pu	Pu+TRC	TRC
9.0	9.67	9.67	9.67	10	10	10

BEAN - FRESH WEIGHT (g)

Pu	Pu+TRC	TRC	Verm	Con	Pd	Pd+TRC
2.86	3.10	3.32	3.32	3.50	3.63	3.99

Mean final fresh weights gave varied results but the lowest values for cabbage, lettuce, tomato and bean seedlings were obtained with the P.ultimum treatments (Table 23 b,c,d). These were significantly (P=0.05) lower than the P.ultimum + TRC treatment for lettuce only.

Thus from the results of this preliminary screen, pea and cabbage gave promising results and appeared to be suitable candidate hosts for further studies using TRC as a potential biocontrol agent of the damping-off fungi under study.

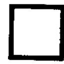
4.3.2 Use of TRC as a seed treatment against P.ultimum and P.debaryanum

The Binab T Trichoderma preparation was used to coat seeds in a preliminary experiment designed to investigate and assess an alternative mode of application. Wright (1956) and Liu and Vaughan (1965) reported successful use of Trichoderma spp. as a seed dressing against Pythium spp. using mustard and beet seeds respectively and Papavizas and Lewis (1983) successfully used biotypes of T.viride with pea seeds. In studies using antagonistic organisms the timing and method of application is critical. The method evaluated here was designed to deliver the potential biocontrol agent into the vicinity of the germinating seed. Use of an antagonist as a seed treatment should confer a spatial advantage on the antagonist and thus provide greater 'protection' against the damping-off fungi (Kommedahl and Windels, 1981).

Pea and cabbage seeds were surface sterilized in 10%(v/v) sodium hypochlorite solution for five minutes. The seeds were rinsed in several changes of sterile distilled water and dried between sterile filter papers. A 3%(w/v) solution of carboxymethyl cellulose (CMC) as used by Tveit and Wood (1955) was prepared in sterile distilled water. To this a 10%(w/v) suspension of the Binab w.p. in sterile distilled water was added. The

Figure 9

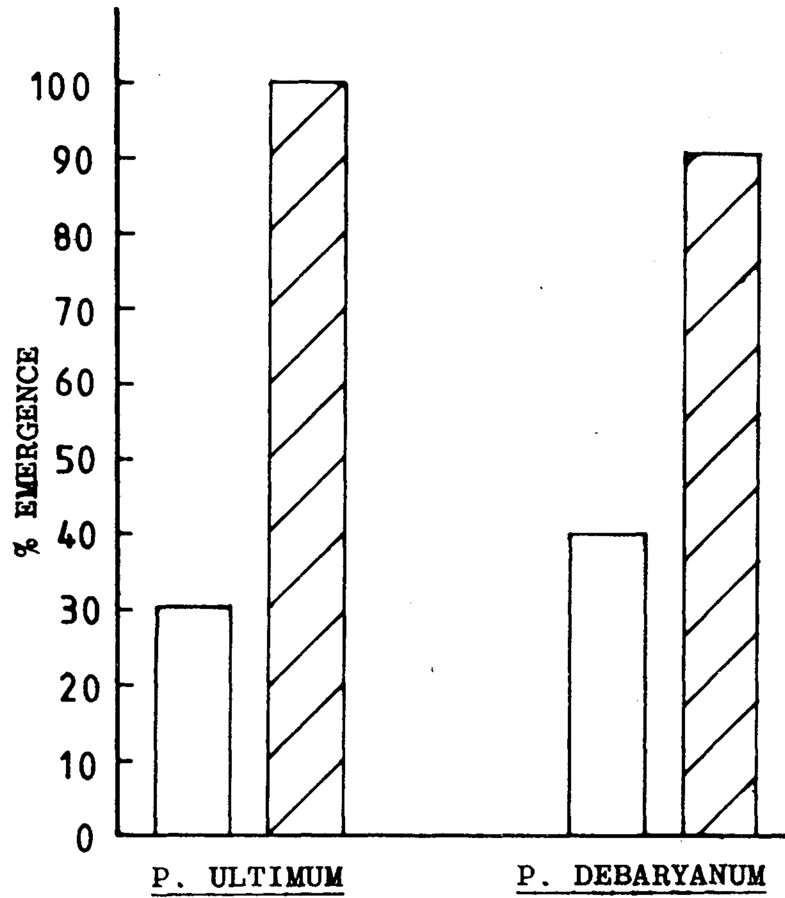
The effect of P.ultimum, P.debaryanum alone and with TRC on final emergence expressed as a percentage of the vermiculite control on (a) pea, (b) cabbage, (c) tomato, (d) lettuce (e) bean.

 Pathogen only treatment

Pathogen + TRC treatment

Figure 9

(a) pea



(b) cabbage

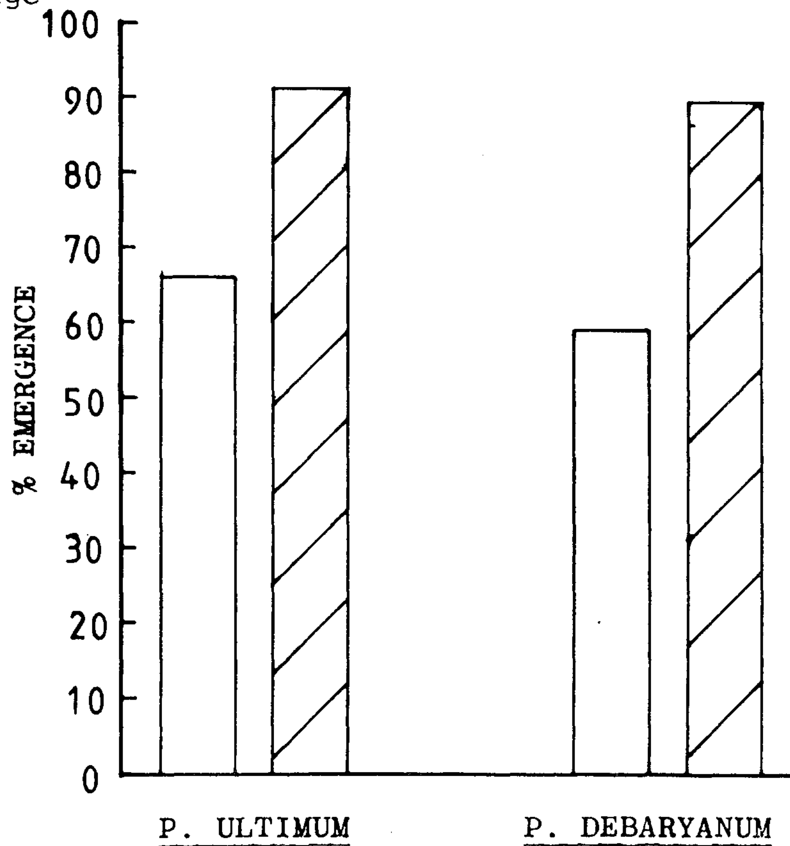
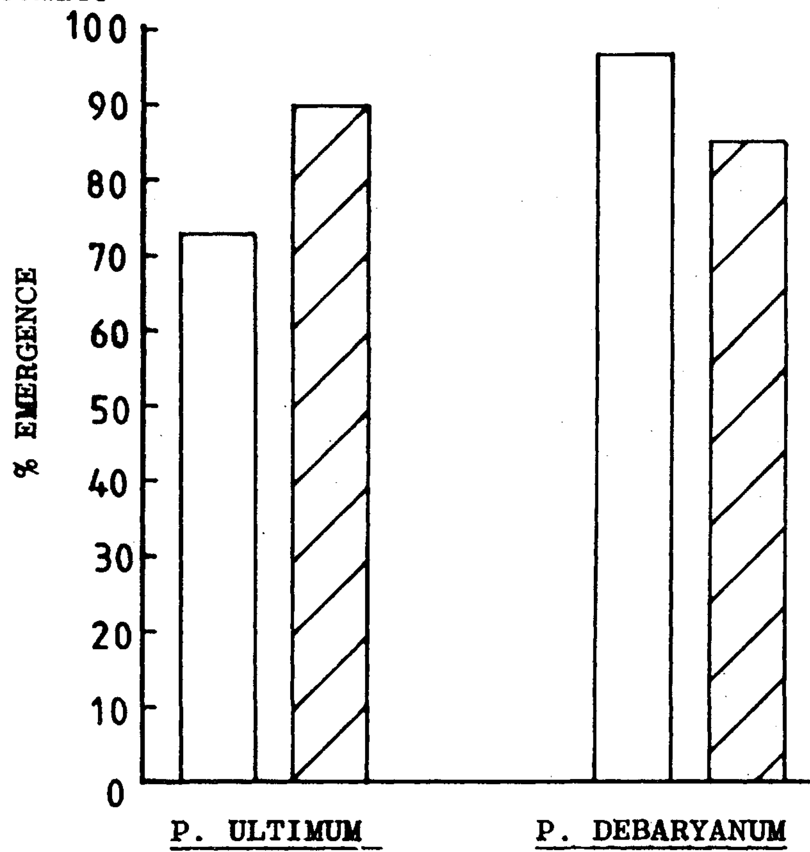


Figure 9

(c) tomato



(d) lettuce

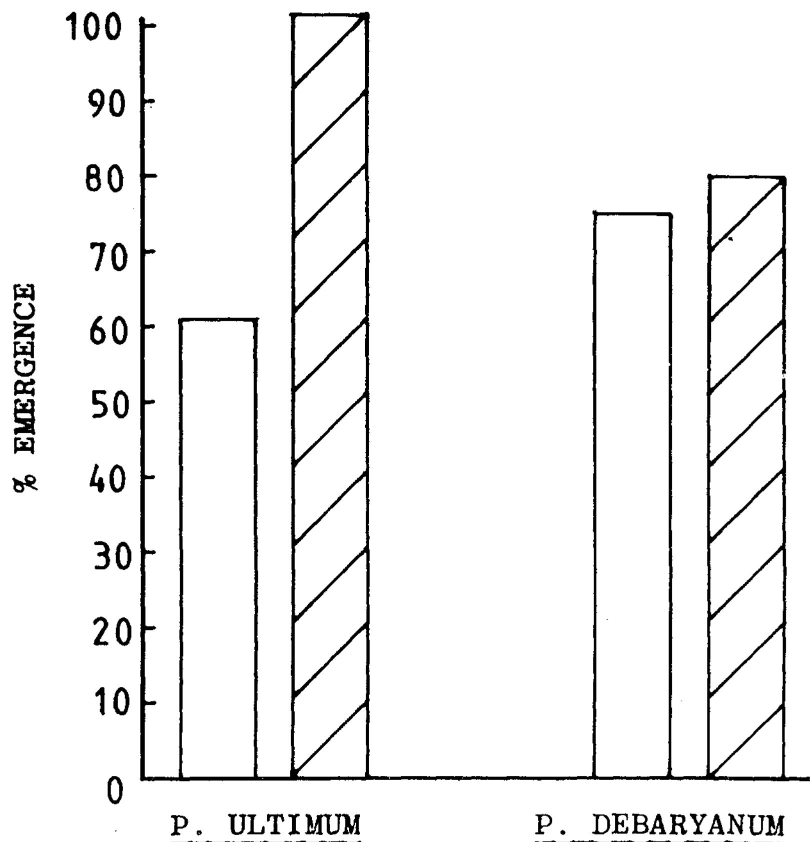
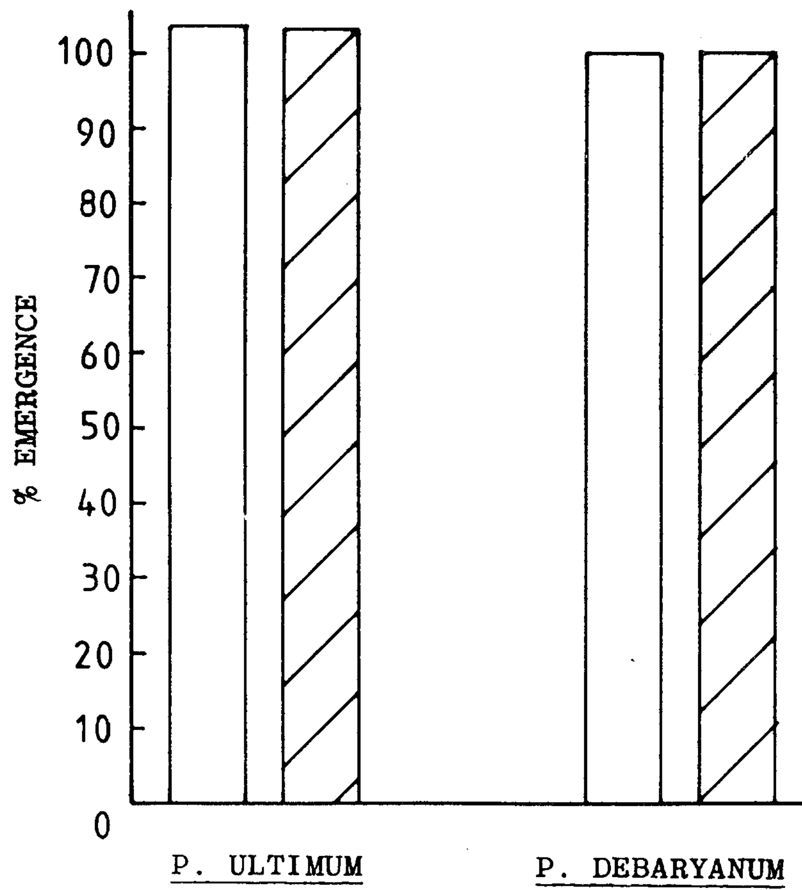


Figure 9
(e) bean



seeds were well coated with the paste of TRC spores and were left to stand in the mixture for one hour, after which they were removed and air-dried overnight on sterile filter papers placed in sterilized plastic containers.

TRC grew profusely from coated seeds plated onto 2% MEA plates suggesting that the spores were largely unaffected by the use of CMC. Germination of the seeds on agar plates indicated that the coating process did not adversely influence viability. Both P.ultimum and P.debaryanum were grown as vermiculite-V_8 cultures and 30.0ml of inoculum was used to infest compost in each pot. Seeds were sown and observed over a 21-day period.

In this experiment pea seeds were found to be highly susceptible to infection by P.ultimum and P.debaryanum with nil emergence in two of the three P.ultimum treatments (Table 24 a). There were no significant differences between the treatment means for either pathogen. The TRC treatment did not significantly improve the mean fresh weight obtained with P.debaryanum-treated seedlings. For both pathogens cabbage seeds coated with CMC only gave significantly greater final emergence than those treated with TRC (Table 24 b). However no differences were found in the corresponding final fresh weight means.

For tomato and lettuce erratic results were obtained (Table 24 c, d) with no clear trends emerging despite some statistically significant differences being obtained. As in the previous experiment bean disease levels were low (results not shown) and as with the other hosts there was no indication of consistent protection by any seed treatment. The mean fresh weight obtained with the TRC treatment of lettuce seeds planted in P.ultimum-infested soil was significantly greater than the results of the other two P.ultimum treatments although high final seedling stands occurred in all

Table 24

The Effect of TRC used as a seed treatment on final seedling stands and fresh weight (g) of hosts grown in P.ultimum and P.debaryanum infested compost

Pu	-	uncoated, surface sterilised seeds sown in compost infested with 30.0ml of vermiculite-V_8 inoculum of Pythium ultimum
Pu-TRC	-	seeds coated with the Binab TRC wettable powder formulation (10% w/v) mixed in carboxymethylcellulose (3% w/v) preparation and sown as above
Pu-CMC	-	seeds coated with carboxymethylcellulose as above omitting the TRC content and sown in Pythium ultimum infested compost as above
Pd	-	uncoated, surface sterilised seeds sown in compost infested with 30.0ml of vermiculite-V_8 inoculum of Pythium debaryanum
Pd-TRC	-	seeds coated with the Binab TRC wettable powder formulation (10% w/v) mixed in carboxymethylcellulose (3% w/v) preparation and sown as above
Pd-CMC	-	seeds coated with carboxymethylcellulose as above omitting the TRC content and sown in Pythium debaryanum infested compost as above
CONTROL	-	uncoated seeds sown in compost containing 30.0.ml of inoculated vermiculite.20 seeds of cabbage, lettuce and tomato and 10 pea seeds were sown in each pot, fresh weight is given in grams; results are the means of three replicates; means not underscored by the same line are significantly different; means underscored by the same line are not significantly different (P=0.05)

Table 24 (a - d)

a. PEA - EMERGENCE

Pu-TRC	Pu	Pu-CMC	Pd-TRC	Pd-CMC	Pd	Control
0	0	1.00	1.00	1.00	2.00	8.67

PEA - FRESH WEIGHT (g)

Pu-TRC	Pu	Pd-CMC	Pd-TRC	Pd	Pu-CMC	Control
0	0	1.06	1.70	2.40	2.83	2.96

b. CABBAGE - EMERGENCE

Pd-TRC	Pu	Pu-TRC	Pd-CMC	Pu-CMC	Pd	Control
8.00	10.67	11.00	11.00	15.33	15.33	18.00

CABBAGE- FRESH WEIGHT (g)

Pd-TRC	Pd-CMC	Pd	Pu-TRC	Pu-CMC	Pu	Control
0.23	0.25	0.33	0.35	0.37	0.38	0.45

c. TOMATO - EMERGENCE

Pd-CMC	Pu-TRC	Pd-TRC	Pu-CMC	Pd	Control	Pu
0.33	2.00	2.33	12.00	12.00	19.67	20.00

TOMATO-FRESH WEIGHT (g)

Pd-CMC	Pd-TRC	Pd	Pu-CMC	Pu-TRC	Pu	Control
0.04	0.07	0.10	0.11	0.20	0.20	0.31

d. LETTUCE - EMERGENCE

Pd	Pd-TRC	Pd-CMC	Pu-CMC	Pu	Pu-TRC	Control
6.00	7.00	9.00	16.00	17.33	18.00	20.00

LETTUCE - FRESH WEIGHT (g)

Pd-TRC	Pd	Pd-CMC	Pu	Pu-CMC	Control	Pu-TRC
0.25	0.36	0.37	0.40	0.45	0.51	0.56

three treatments, the same treatment of tomato was significantly greater than the P.ultimum-CMC treatment only.

In no instance was there a significant increase in emergence of the TRC treatment compared with the untreated seeds planted in pathogen infested compost. Results with the CMC treatments gave varied results with final seedling stands sometimes being significantly greater and sometimes less than the TRC and pathogen treatments. This suggests that CMC may alone influence disease infection and or seed germination more than the presence of TRC. It is likely that this highly viscous 'sticking agent' may itself be utilized as a substrate or nutrient source and may as such cause changes in the microflora surrounding the seed. Harman et al. (1981) found that cellulose amendments in the form of carboxymethylcellulose decreased the numbers of Trichoderma propagules in soil when used to treat radish and pea seeds and a slight increase in disease incidence on radish caused by Rhizoctonia solani was also observed.

Seed treatments with conidia of Trichoderma spp. offers a potentially useful means of delivering the antagonist in large numbers close to the infection court and this technique has been used successfully to supress disease in naturally infested soils (Harman et al., 1981; Papavizas et al., 1982; Windels and Kommedahl, 1982). However the results of this preliminary experiment were less promising than those obtained using TRC as a soil treatment. Thus for the remainder of this study greater emphasis was placed on the use of a standardised soil inoculation procedure for evaluation of TRC as a biocontrol agent of P.ultimum and P.debaryanum under greenhouse conditions.

4.3.3 The effect of TRC on disease incidence in non-sterile compost

The objective of this experiment was to examine in greater detail the effect of TRC on the level of damping-off in non-sterile compost using the two hosts selected namely pea and cabbage. Each treatment was replicated five times and the pots were completely randomized. Emergence was scored over a 21-day period and the mean final seedling stand and fresh weights are summarised in Table 25.

Of the two hosts tested, pea was the more susceptible to both damping-off pathogens with emergence never being more than 45% of the controls. In no instance was a statistically significant increase in emergence noted for this host as a result of the TRC treatments. This was also true for the P.ultimum + TRC cabbage treatment whilst for P.debaryanum on cabbage TRC resulted in a significantly lower degree of emergence compared with the P.debaryanum only treatment. Thus in contrast with the results obtained in section 4.3.1 the TRC treatments did not produce any statistically significant increases in emergence. Similarly, in neither experiment did the presence of TRC give significant increments in the mean fresh weight of emerged pea on cabbage seedlings grown in pathogen inoculated compost.

4.3.4 Effect of TRC on disease incidence in a soil-peat growth medium

Disease incidence was assessed in an alternative soil-based growth medium using John Innes No.2 supplemented with peat mixed in a 2:1(v/v) ratio. Trichoderma spp. are widely distributed naturally occurring soil saprophytes (Danielson and Davey, 1973) and thus TRC may produce optimum growth and colonization in conditions using a true soil-based rather than peat-based 'soil-less' compost as used hitherto. Pots were inoculated as before. Treatments were replicated five times and were completely randomized.

Table 25

The mean final seedling stands and fresh weights (g) of pea and cabbage in TRC treated non-sterile compost infested with the damping-off pathogens

For abbreviations see legend to Table 1

10 pea seeds and 20 cabbage seeds were sown in each pot; fresh weight means are given in grams. Results are the means of five replicates

Means not underscored by the same line are significantly different; means underscored by the same line are not significantly different. (P=0.05)

Table 25

PEA - EMERGENCE

Pu	Pu+TRC	Pd	Pd+TRC	TRC	Verm	Control
0.4	1.2	2.8	3.0	7.0	7.0	7.2

PEA - FRESH WEIGHT (g)

Pu	Pu+TRC	Verm	Control	Pd+TRC	TRC	Pd
1.97	3.72	4.60	5.36	5.62	6.22	7.42

CABBAGE - EMERGENCE

Pu	Pd+TRC	Pu+TRC	Pd	TRC	Verm	Control
13.0	13.0	13.80	16.20	16.60	16.80	19.40

CABBAGE - FRESH WEIGHT (g)

Pu	Control	Pd	Pu+TRC	Pd+TRC	TRC	Verm
1.77	1.93	1.96	2.03	2.65	2.99	3.49

TRC treatment of the soil resulted in no significant increases in emergence for any of the possible host and pathogen combinations (Table 26). Compared with the previous experiment, the P.debaryanum-treatments showed a marginal increase in emergence with pea and a decrease with cabbage, whilst a slightly greater level of emergence was observed with the P.ultimum treatment on cabbage. Therefore the results of this experiment were broadly similar to those obtained previously and there were no obvious treatment effects attributable to the growth medium for either the pathogens or TRC. Fresh weight analysis revealed that no significant differences existed between the treatment means or between these and the controls.

The addition of peat lowered the pH of the soil and this was comparable with that of soil-less compost (pH 5.5-6.0 approximately). Trichoderma spp. are reported preferentially to colonize soils of low pH (Baker and Cook, 1974; Liu and Baker, 1980; Marshall, 1982). Chet and Baker (1981) reported that the efficacy of the antagonist T.hamatum was improved when used in acidified soil indicating that lower pH favours the induced suppressiveness.

John Innes No.2 and Levingtons compost are similar in their inorganic nutritional composition (Fisons Horticultural Products technical information) and in the absence of any significance of one over the other, Levingtons "soil-less" compost was selected for all further studies. Not only is it a medium used widely by nursery men for raising seedlings under glass but it also provides a convenient medium for dispersal of vermiculite-grown fungal inoculum, allows easy removal of the seedling together with their root systems and has the additional advantage of greater between batch uniformity. High moisture content is reported to favour Pythium disease development (Hendrix and Campbell, 1973). The high moisture retention properties of this growth medium is therefore a further useful feature especially when greenhouse experiments are conducted under conditions

Table 26

The effect of TRC on damping-off of pea and cabbage induced by P.ultimum and P.debaryanum in a growth medium containing John Innes No.2 and peat mixed in a 2:1(v/v) ratio

For abbrev iations see legend to Table 1

10 pea seeds and 20 cabbage seeds were sown in each pot; fresh weight is given in grams. Results are the mean of five replicates
Means underscored by the same line are not significantly different (P=0.05)

Table 26

PEA - EMERGENCE

PU	Pu+TRC	Pd+TRC	Pd	TRC	Control	Verm
0.80	1.80	3.60	5.00	6.00	6.40	6.80

PEA - FRESH WEIGHT (g)

TRC+Pu	Pd+TRC	Pu	Pd	TRC	Control	Verm
2.27	2.52	2.95	3.71	3.78	3.89	4.12

CABBAGE - EMERGENCE

Pd	Pu+TRC	Pd+TRC	Pu	Control	Verm	TRC
12.20	12.80	13.80	14.40	16.20	18.00	18.80

CABBAGE - FRESH WEIGHT (g)

Pd+TRC	Pd	Control	TRC	Verm	Pu+TRC	Pu
0.94	0.99	0.99	1.02	1.07	1.15	1.23

of high temperatures during summer.

4.3.5 Use of TRC as a pre-inoculation, prophylactic treatment against the damping-off pathogens

In this experiment TRC was introduced into the upper layers of compost seven days prior to pathogen inoculation. It was considered possible that establishment and growth of TRC in the critical layer of compost could be maximised in the absence of competition from the pathogens during this incubation period. TRC was grown in the usual manner and was incorporated into compost following the routine procedure outlined earlier. The treated pots were kept moist and were incubated in the greenhouse for seven days, after which they were inoculated with either P.ultimum or P.debaryanum and sown with the appropriate hosts (section 4.2.4). At this stage a treatment using simultaneous inoculation of the pathogen and TRC was included for comparison.

Use of TRC in this alternative pre-inoculation mode did not significantly reduce disease incidence with either pea or cabbage (Table 27). No significant differences were found between the emergence results for the six pathogen treatments of pea. The P.ultimum and P.ultimum + TRC treatments of cabbage gave high final seedling stands indicating that a little if any damping-off had occurred, but significantly lower emergence was apparent with the TRC PI-P.ultimum treatment. P.debaryanum on the other hand caused more disease on cabbage with the P.debaryanum + TRC and TRC PI - P.debaryanum treatments giving very similar results. The TRC inoculations did not result in any significant improvement of mean fresh weights of either host grown in pathogen infested compost.

Thus, with the exception of the TRC PI P.debaryanum treatment of cabbage which gave only marginally higher emergence, the pre-inoculation TRC treatments consistently

Table 27

The effect of TRC applied as a prophylactic pre-inoculation (PI) treatment on the final stands and fresh weights of pea and cabbage grown in Pythium infested compost

Treatments:- Pu - Pythium ultimum, Pu+TRC - Pythium ultimum used simultaneously with Binab Trichoderma to inoculate compost TRC PI Pu - a pre-inoculation, prophylactic treatment using the Binab Trichoderma followed by inoculation with Pythium ultimum after a seven-day time lapse. Pd - Pythium debaryanum, Pd+TRC Pythium debaryanum used simultaneously with Binab Trichoderma to inoculate compost, TRC PI Pd - a pre-inoculation, prophylactic treatment using the Binab Trichoderma followed by inoculation with Pythium debaryanum

CONTROLS:- Con - uninoculated compost, Verm - uninoculated vermiculite soaked with vegetable juice introduced into compost, TRC - Binab Trichoderma inoculum incorporated into compost alone (no pathogen inoculum present, TRC PI - treatment of compost with the Binab Trichoderma as described for the pre-inoculation, prophylactic treatment omitting the pathogen inoculation after a seven-day time lapse

50.0ml of vermiculite inoculum was used in each case. 10 pea seeds and 20 cabbage seeds were sown in each pot, fresh weight is given in grams. Results are the means of five replicates.

Means underscored by the same line are not significantly different (P=0.05)

Table 27

PEA - EMERGENCE

Pu	Pu+TRC	TRC PI Pu	TRC PI Pd	PD+TRC	Pd	TRC PI	Con	Verm	TRC
0.2	0.2	0.2	0.4	1.20	1.40	6.00	6.80	7.20	7.40

PEA - FRESH WEIGHT (g)

Pu+TRC	TRC PI Pu	Pu	Pd+TRC	TRC PI Pd	Pd	Verm	TRC	Con	TRC PI
0.04	o.45	0.56	0.86	1.33	2.70	2.75	3.01	3.02	3.06

CABBAGE - EMERGENCE

Pd	TRC PI Pd	Pd+TRC	TRC PI Pu	Pu	Pu+TRC	Verm	Con	TRC PI	TRC
9.6	10.00	10.40	13.00	16.60	18.00	18.00	18.40	19.00	19.20

CABBAGE - FRESH WEIGHT (g)

TRC PI Pu	Pd+TRC	TRC PI Pd	Pd	Pu+TRC	Pu	TRC	TRC PI	Verm	Con
0.36	0.36	0.38	0.39	0.41	0.43	0.45	0.47	0.49	0.53

Figure 10

The effect of TRC used as a pre-inoculation treatment on percentage seedling emergence with time for pea and cabbage

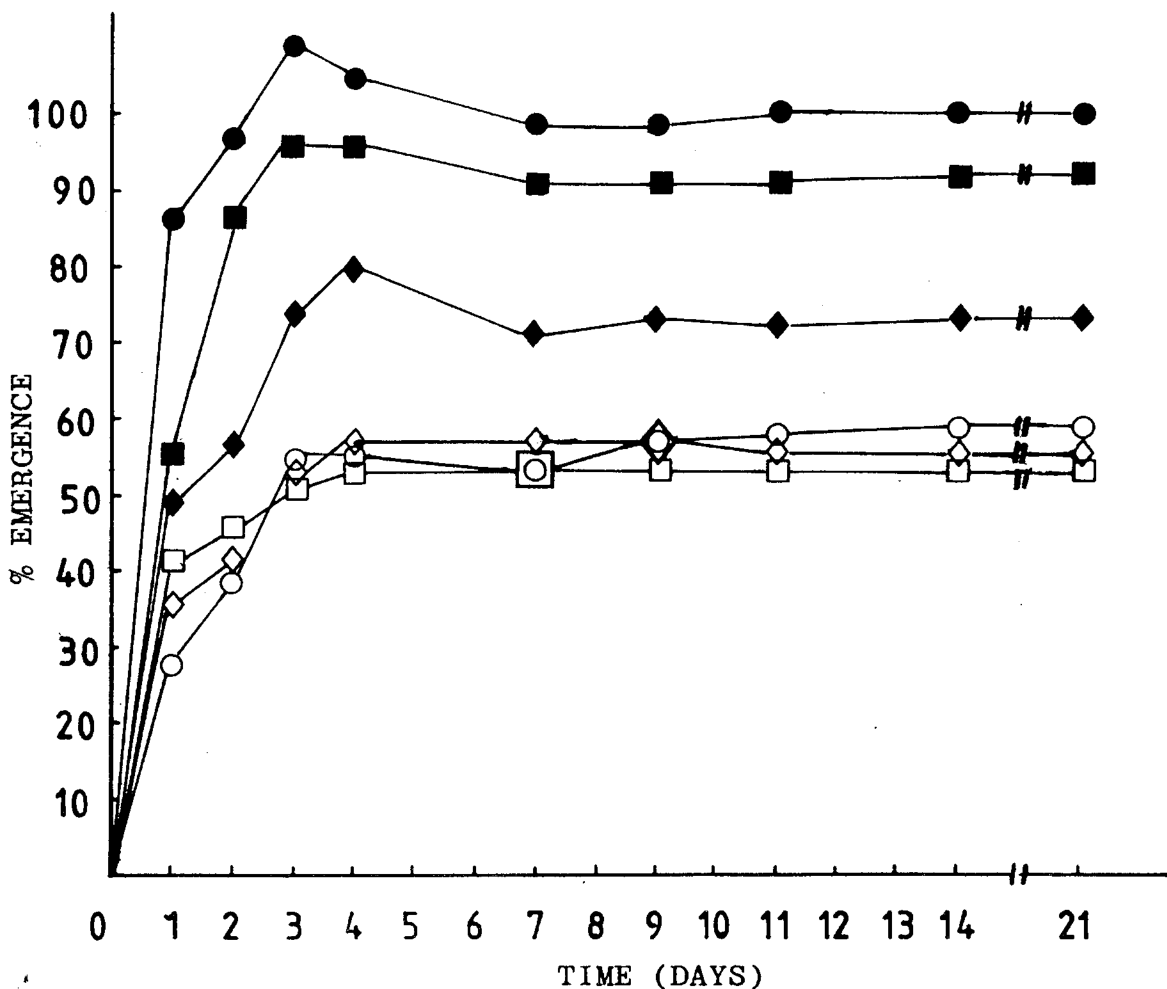
For abbreviations see legend to Table 5

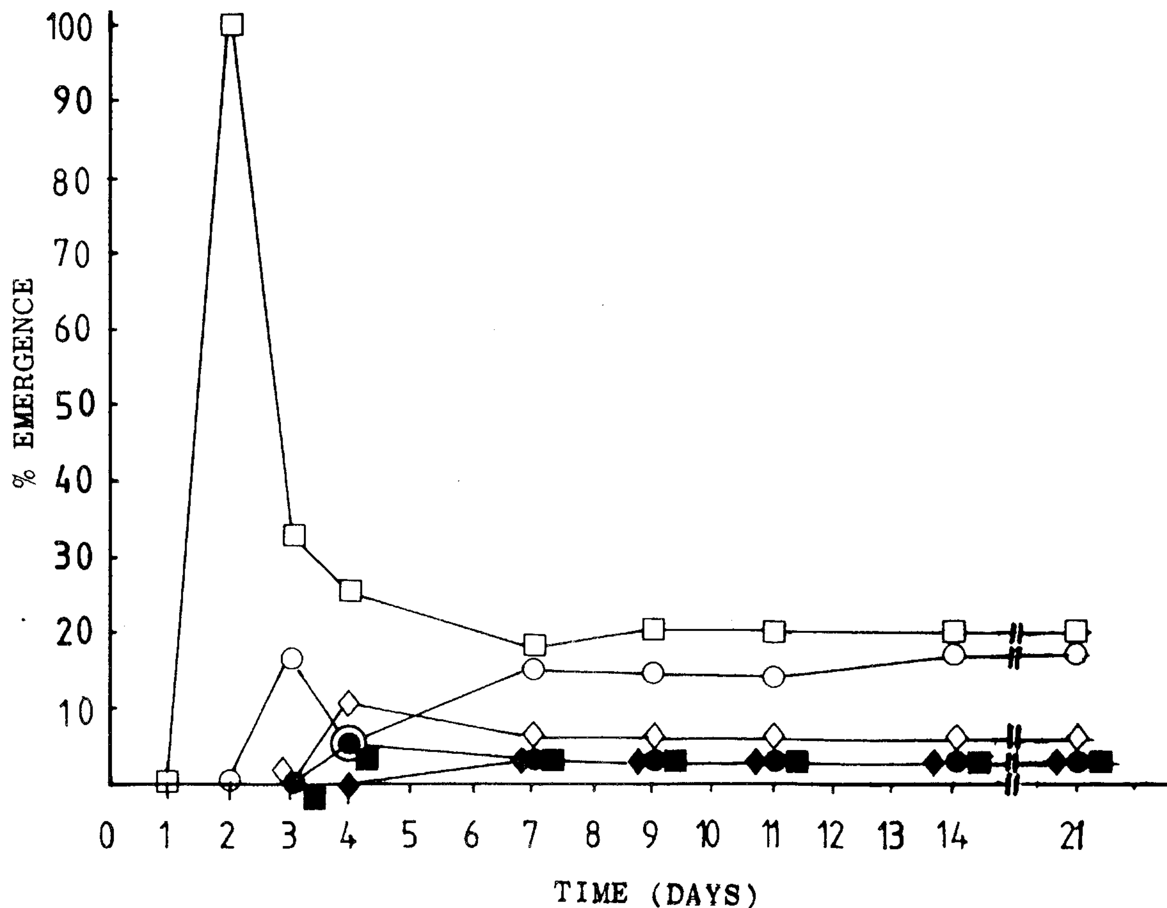
■——■ Pu; ●——● Pu + TRC;

◆——◆ TRC PI Pu; □——□ Pd;

○——○ Pd + TRC; ◇——◇ TRC PI Pd

Percentage emergence is expressed as a percentage of the vermiculite controls and is plotted against days after first emergence


CABBAGE
% EMERGENCE
10
20
30
40
50
60
70
80
90
100
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 21
TIME (DAYS)


PEA
% EMERGENCE
10
20
30
40
50
60
70
80
90
100
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 21
TIME (DAYS)

produced emergence less than or equal to the pathogen only treatments throughout the course of the experiment (Figure 10). Sharp decreases in percentage emergence noted within the first seven days are due to increases in emergence of the vermiculite controls. No significant reductions in disease attributable to TRC were subsequently found as judged by the mean final seedling stands.

Use of a seven day 'pathogen free' incubation is in theory a useful means of establishing a dominant population of TRC to act as a biological barrier against the damping-off pathogens and as such it was employed in all succeeding experiments. However results of this particular experiment suggested that with the pre-inoculation treatment, the ecological balance was not shifted sufficiently in favour of TRC and antagonism and under these experimental conditions no selective advantage over the simultaneous inoculation technique was gained.

Provision of a suitable nutrient food base is considered by many authors (Wells *et al.*, 1972 Backman and Rodriguez-Kabana, 1975; Moody and Gindrat, 1977; Lewis and Papavizas, 1980; Elad *et al.*, 1980; Papavizas and Lewis, 1981) to be an essential pre-requisite for successful introduction Harman *et al.* (1981) using conidia of a *Trichoderma* sp. as a seed treatment also noted that peat introduced on the seed coats promoted the production of *Trichoderma* propagules in soil. Thus delivery of TRC on its vermiculite carrier soaked with V_8 juice into a peat based soil-less compost could potentially enable TRC, other conditions being favourable, to proliferate and increase its biomass.

4.3.6 The interaction between TRC and *P.ultimum* and *P.debaryanum* in autoclaved compost

The introduction of an alien organism into a stable ecosystem may be facilitated by inducing changes in

the environment. Baker (1980) categorised the effects of using chemicals, heat treatment or the addition of organic amendments as "shock" changes. The objective of this experiment was to introduce TRC together with its vermiculite-V_8 carrier and food base into autoclaved compost both prior to pathogen inoculation and in simultaneous inoculation procedure as described in the previous experiment.

Pea seeds grew poorly with nil emergence in all three P.ultimum treatments (Table 28). Emergence was slightly greater in the P.debaryanum + TRC treatment but this did not differ significantly from the results of the other P.debaryanum treatments. Similarly no statistically significant differences existed between the mean final fresh weight values at the 5% probability level.

Although an overall increase in emergence was observed as a result of TRC treatment of cabbage infected with both P.ultimum and P.debaryanum, in no instance was this increase, compared with the pathogen only treatments, statistically significant. Analysis of the fresh weight data revealed that the mean value obtained with the P.ultimum only treatment was significantly lower than that of the P.ultimum + TRC treated plants, but did not differ significantly from the TRC PI - P.ultimum result.

This experiment produced unusual results with considerable variation occurring between replicates of the same treatment. Emerged seedlings were often unhealthy in appearance and final fresh weights were low. Poor emergence was also noted for the controls with the pea host being particularly affected. The condition of the control seedlings strongly suggested that the environment in which they were germinating and growing was in some way deleterious.

Autoclaving (at 121°C for 20 minutes) does

Table 28

Assessment of TRC as a bicontrol agent of P. ultimum and P.debaryanum in autoclaved compost

For abbreviations see legend to Table 5

10 pea seeds and 20 cabbage seeds were sown in each pot, fresh weight is given in grams. Results are the mean of five replicates. Compost was autoclaved at 121°C for 30 minutes.
Means underscored by the same line are not significantly different (P=0.05)

Table 28

PEA - EMERGENCE

Pu	Pu+TRC	TRC PI Pu	TRC PI Pd	Pd	TRC	Pd+TRC	TRC PI	Con	Verm
0	0	0	1.00	1.33	2.33	2.67	2.67	3.67	5.33

PEA - FRESH WEIGHT (g)

Pu	Pu+TRC	TRC PI Pu	Pd	Con	TRC	Verm	TRC PI	TRC PI Pd	Pd+TRC
0	0	0	3.07	3.37	3.64	3.75	4.74	5.06	5.11

CABBAGE - EMERGENCE

Pu	Pu+TRC	Pd	TRC PI Pu	Pd+TRC	TRC PI Pd	Con	TRC	Verm	TRC PI
4.33	4.67	7.33	7.33	9.00	13.33	15.67	16.0	16.67	17.67

CABBAGE - FRESH WEIGHT (g)

Pu	TRC	TRC PI Pd	Con	TRC PI	Verm	Pd	Pd+TRC	TRC PI Pu	Pu+TRC
2.02	2.14	2.23	2.27	2.31	2.74	3.91	3.94	4.83	6.33

not provide the ideal method of changing the environment since this 'shock' treatment is likely to result in an ecological vacuum rather than the selective creation of "niches" (Baker, 1980). However Mughogho (1968) reported that species of Trichoderma and in particular T.harzianum were the dominant fungal recolonizers of fumigated soil treated with allyl alcohol. Nevertheless autoclaving may have led to colonization by other opportunist organisms and this may partially explain the low emergence observed in particular with pea.

With both pathogens marked reductions in emergence resulted implying that they were able to colonize the sterilized compost medium rapidly. Hoch and Abawi (1979) also noted that higher levels of damping-off caused mainly by P.ultimum occurred in autoclaved soils. Species of Pythium are considered to be pioneer colonists (Hendrix and Campbell, 1973) and autoclaving which can eliminate naturally occurring antagonists may therefore exacerbate the disease. The low emergence in control treatments probably resulted from contaminating Pythium or other pathogenic organisms, possibly seed borne. Autoclaving also alters the nutrient balance (Ferriss, 1984) of compost and this may encourage the formation of an unusual microflora in compost. Thus this method of introducing TRC does not produce optimum growth conditions for seedlings and does not lead to significant control of the damping-off pathogens under study.

4.3.7 The Influence of increased volumes of TRC inoculation on disease incidence

The results of preceding experiments had given varied and inconsistent results with the techniques used proving ineffective in promoting significant biological control by TRC. In order to compete effectively with the pathogens it is essential that rapid growth and colonization by TRC occurs directly after inoculation, with the subsequent population maintained at a high

Table 29

Effects of increased volumes of TRC on disease incidence caused by P.ultimum and P.debaryanum in pea and cabbage

For abbreviations see legend to Table 5.

10 pea seeds and 20 cabbage seeds were sown in each pot, fresh weight is given in grams. 100ml of TRC vermiculite-V_8 inoculum and 50ml of P.ultimum or P.debaryanum vermiculite-V_8 inoculum were used. Results are the means of five replicates.
Means underscored by the same line are not significantly different (P=0.05)

Table 29

PEA - EMERGENCE

TRC PI Pu	Pu	Pu+TRC	Pd	TRC PI Pd	Pd+TRC	TRC PI	Con	TRC	Verm
0.20	0.80	0.80	2.80	3.00	3.60	6.60	7.20	7.20	7.80

PEA - FRESH WEIGHT (g)

TRC PI Pu	Pu	Pu+TRC	Pd	TRC PI Pd	Con	Verm	TRC	TRC PI	Pd+TRC
1.08	1.67	2.25	4.49	4.56	4.73	5.22	5.24	5.24	5.76

CABBAGE - EMERGENCE

Pd+TRC	Pd	TRC PI Pd	Pu+TRC	TRC PI Pu	Pu	TRC PI	Con	TRC	Verm
6.20	8.00	8.00	13.00	13.00	13.40	16.60	17.40	17.40	18.00

CABBAGE - FRESH WEIGHT (g)

Pd+TRC	Pd	TRC PI Pd	Pu+TRC	Pu	TRC PI Pu	Verm	TRC PI	TRC	Con
0.50	0.79	0.87	0.91	0.98	0.98	1.08	1.13	1.15	1.23

level for the critical period of plant protection. This experiment attempted to promote this by introducing double the volume of TRC inoculum used previously whilst the volume of pathogen inoculum applied was maintained at a constant 50.0ml.

Pots were inoculated with 100ml of TRC vermiculite - V_8 inoculum which was employed in a seven day pre-inoculation treatment prior to pathogen infestation and in a simultaneous inoculation with the pathogens. The six treatments and four controls were each replicated five times and in this and all succeeding experiments the pots were arranged in a randomized block design.

Incorporation of TRC and the pathogen in a 2:1(v/v) ratio failed to reduce disease significantly as measured by the final seedling stands with either pea or cabbage (Table 29). During the course of the experiment slight increases in emergence were noted with the TRC pre-inoculation of *P.debaryanum* (TRC PI - *P.debaryanum*) compared with the *P.debaryanum* only treatment with both hosts, but the results obtained with the TRC simultaneous inoculation had opposite effects on *P.debaryanum* in pea and cabbage (Figures 11a and 12a).

The effect of TRC treatments on fresh weights are summarised in Table 29. No significant differences were found between the results of the three *P.ultimum* and three *P.debaryanum* treatments of pea. With cabbage the TRC PI - *P.debaryanum* treatment resulted in a significantly greater mean fresh weight than the simultaneous *P.debaryanum* and TRC inoculation. However neither treatment mean differed statistically from that of the *P.debaryanum* only treatment. No differences in fresh weights were found between the *P.ultimum* treatments on cabbage.

Use of an increased volume of the TRC inoculum did not lead to significant reductions in disease

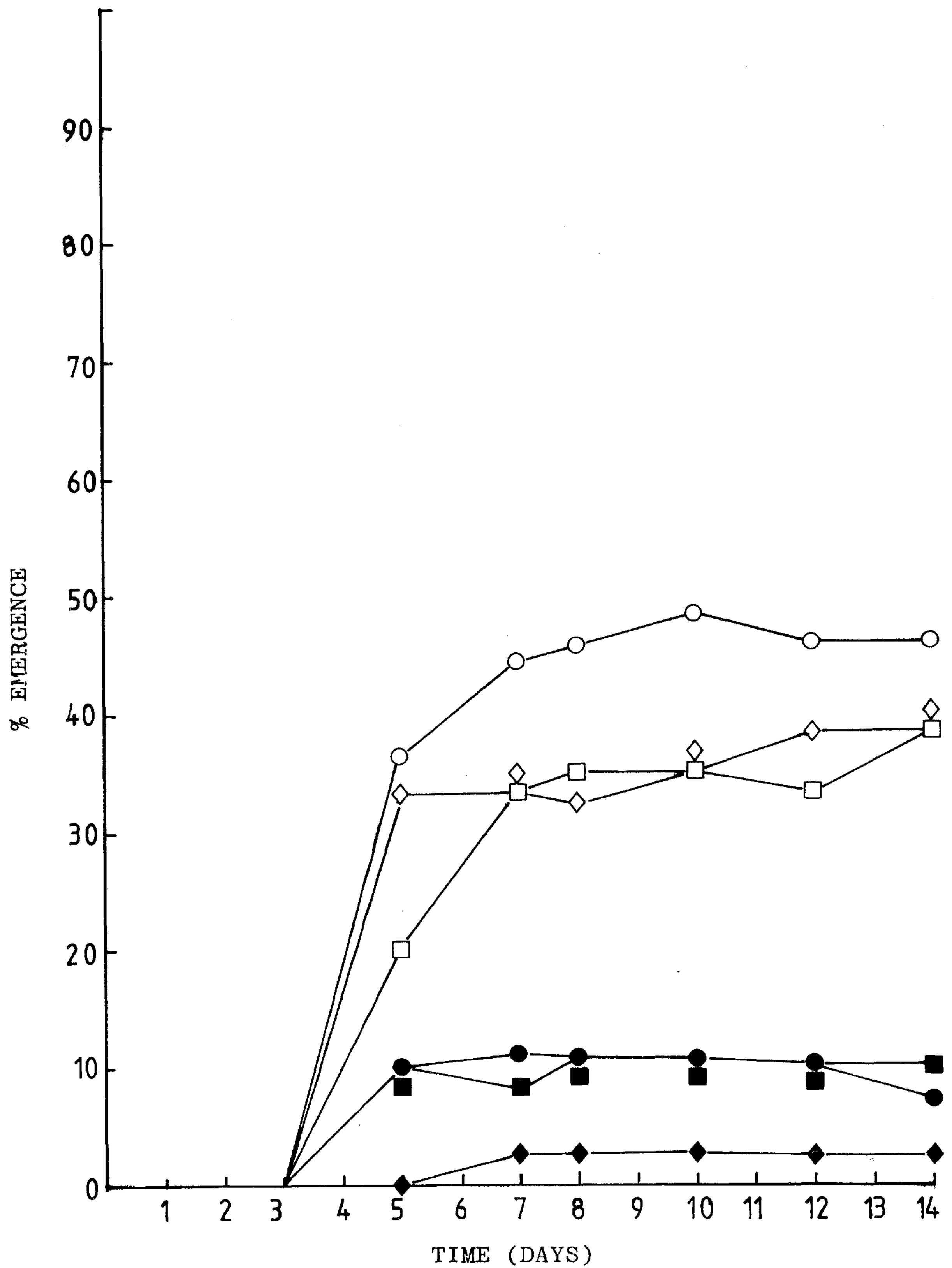
Figure 11

The percentage emergence of pea grown in TRC treated and untreated soil infested with P.ultimum or P.debaryanum for the first fourteen days of emergence in two successive experiments

For abbreviations see legend to Table 5

■——■ Pu; ●——● Pu+TRC; ◆——◆ TRC PI Pu;
□——□ Pd; ○——○ Pd+TRC; ◇——◇ TRC PI Pd;
a - experiment 4.3.7; b - experiment 4.3.8
Emergence calculated as a percentage of the vermiculite control was plotted against time in days after first emergence

Fig. 11a PEA



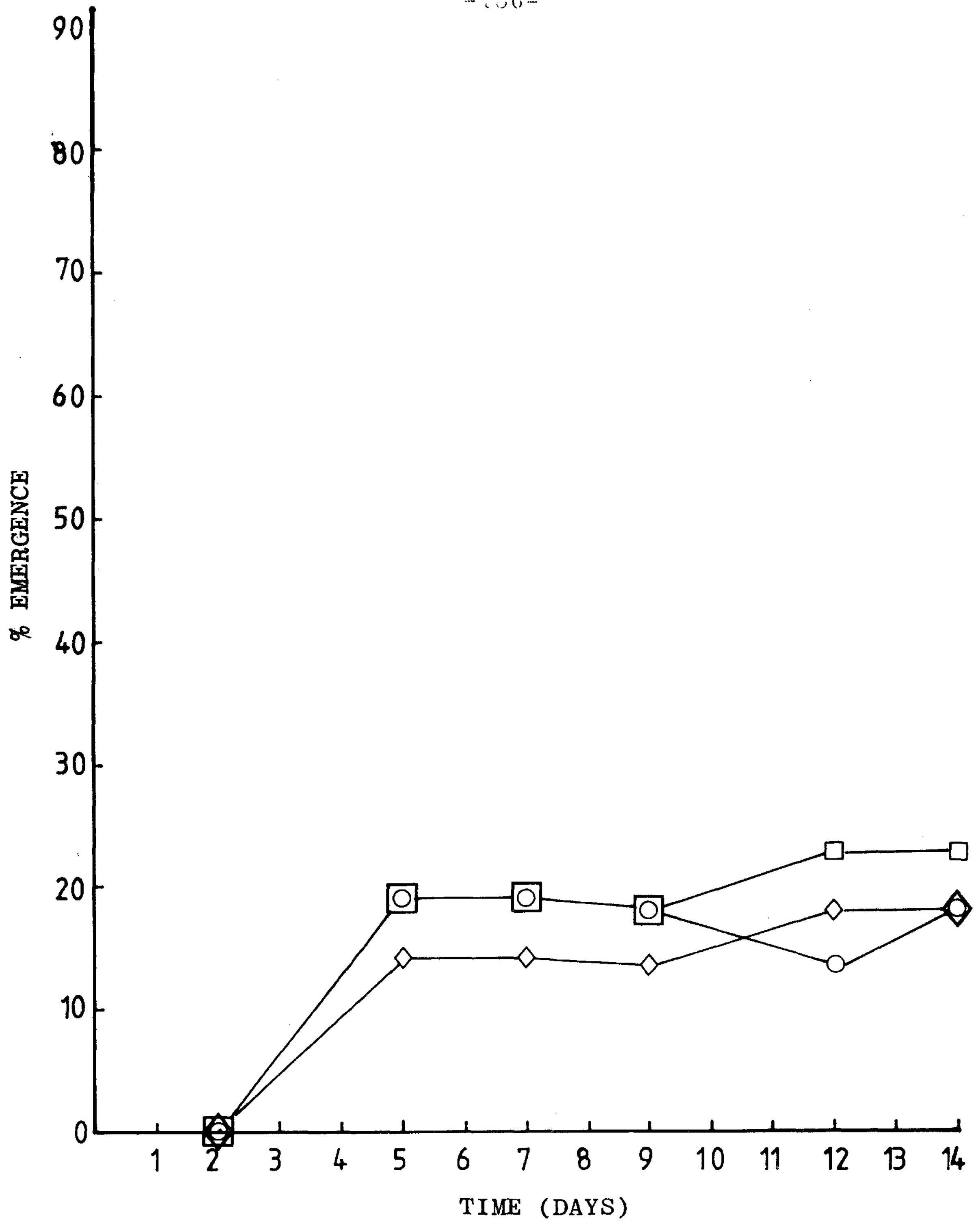


Fig. 11b PEA

incidence or in the final seedlings stands either when incorporated prior to or simultaneously with Pythium spp. Simultaneous inoculation of TRC and the pathogen in particular requires immediate and intense antagonistic activity by the former in order for a significant reduction in disease incidence to result. Increased volumes of an aggressive antagonist introduced into soil may temporarily swamp the infection court and produce a greater impact on disease incidence (Baker and Cook, 1974; Papavizas and Lewis, 1981). A small or relatively static population of TRC will have little if any effect on a rapidly expanding Pythium population stimulated by the presence of host exudates. Increased volumes of TRC inoculum used prior to seed sowing and introduction of the pathogen (ie a pre-inoculation treatment) may produce sufficiently large, well distributed and active populations to compensate for any differences in growth rate. Clearly in this trial both P.ultimum and P.debaryanum were able to overcome any adverse effects caused by TRC and were thus able to initiate infection.

4.3.8 Replant experiment to investigate the longevity of P.ultimum and P.debaryanum in the presence of TRC

A replant experiment was designed to investigate the TRC-pathogen interaction and its effect on subsequent disease levels over an extended time period.

The experiment directly followed the preceding greenhouse trial. After the seedlings had been removed at the end of experiment 4.3.7, the compost was replaced and the pots were re-sown with the appropriate host two to three days later. No additional inoculum was incorporated. Emergence and disease incidence were monitored over a period of 30 days after first emergence was observed.

Both cabbage and pea became markedly diseased,

Table 30

Disease Incidence in pea and cabbage hosts in a replant experiment designed to investigate the interaction between TRC and P.ultimum and P.debaryanum over an extended incubation period

10 pea seeds and 20 cabbage seeds were sown in each pot, fresh weight is given in grams. Pots prepared in section 4.3.7 were replanted with the appropriate host, no additional inoculum was incorporated. Results are the mean of five replicates.
Means underscored by the same line are not significantly different (P=0.05)

Table 30

PEA - EMERGENCE

Pu	Pu+TRC	TRC PI Pu	Pd+TRC	Pd	TRC PI Pd	TRC PI	Con	TRC	Verm
0	0	0	0.80	1.00	1.00	1.80	2.40	4.00	4.20

PEA - FRESH WEIGHT (g)

Pu	Pu+TRC	TRC PI Pu	Con	Verm	TRC PI	TRC PI Pd	TRC	Pd+TRC	Pd
0	0	0	4.47	4.59	4.72	5.66	6.13	10.33	14.46

CABBAGE - EMERGENCE

Pd	TRC PI Pd	Pu+TRC	Pd+TRC	Pu	TRC PI Pu	TRC PI	Con	TRC	Verm
3.00	3.00	3.40	4.40	4.60	5.40	10.60	12.80	13.20	14.00

CABBAGE - FRESH WEIGHT (g)

TRC	Con	Verm	TRC PI	Pu	Pd	Pd+TRC	Pu+TRC	TRC PI Pu	TRC PI Pd
5.15	5.17	5.19	9.56	12.90	19.04	20.35	21.44	22.12	25.46

Figure 12

The percentage emergence of cabbage grown in TRC treated and untreated soil infested with P.ultimum or P.debaryanum for the first fourteen days of emergence in two successive experiments

For abbreviations see legend to Table 5

■—■ Pu; ●—● Pu+TRC; ◆—◆ TRC PI Pu;
□—□ Pd; ○—○ Pd+TRC: ◇—◇ TRC PI Pd;
a - experiment 4.3.7; b - experiment 4.3.8.
Emergence calculated as a percentage of the vermiculite control was plotted against time in days after first emergence. The greatest variation occurred within the first seven days and thus the results for only the first 14 days are given. The initial sharp changes in emergence are primarily due to increases in emergence in the vermiculite controls and to a lesser extent post-emergence damping-off

Fig. 12a CABBAGE

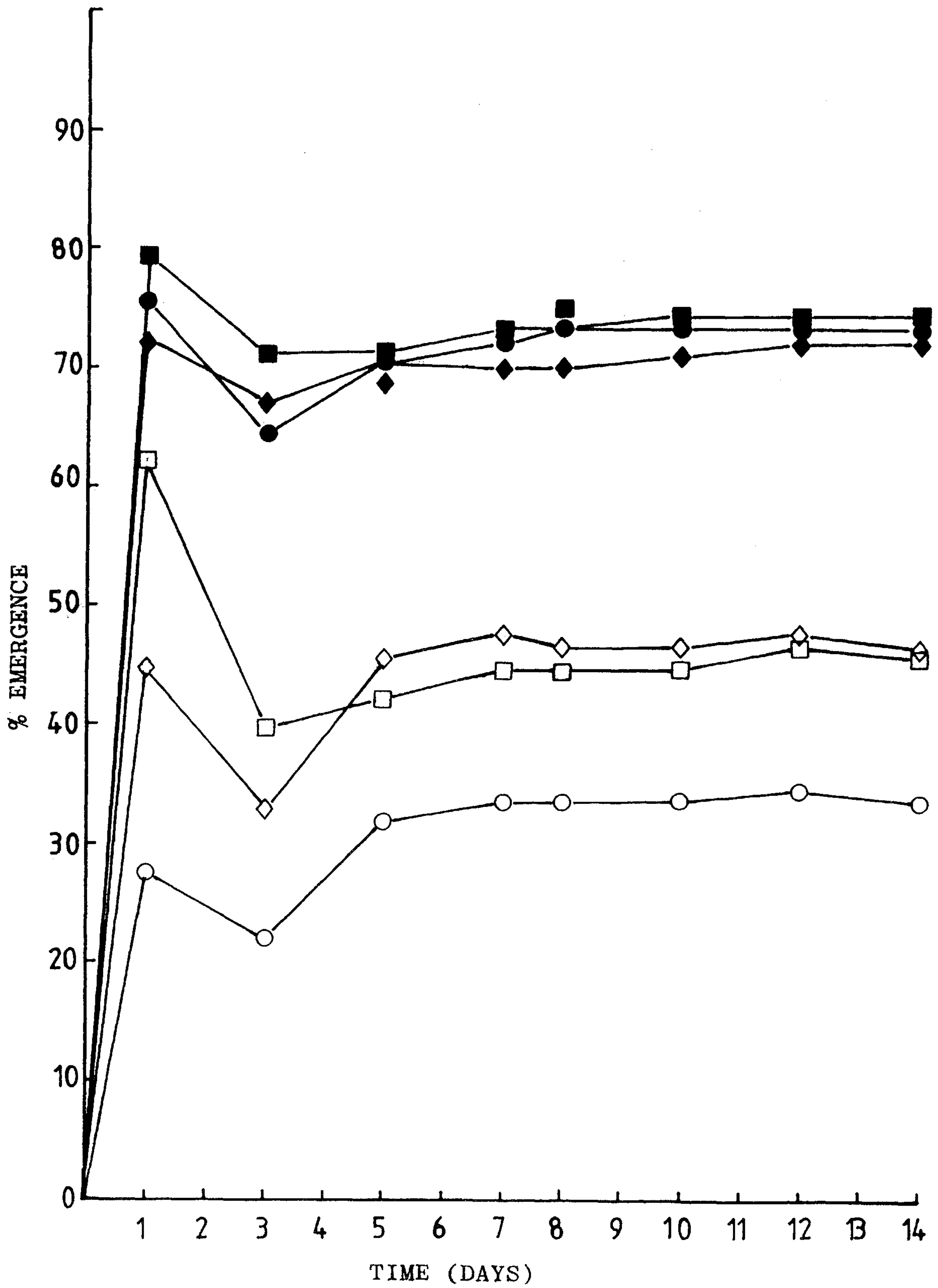
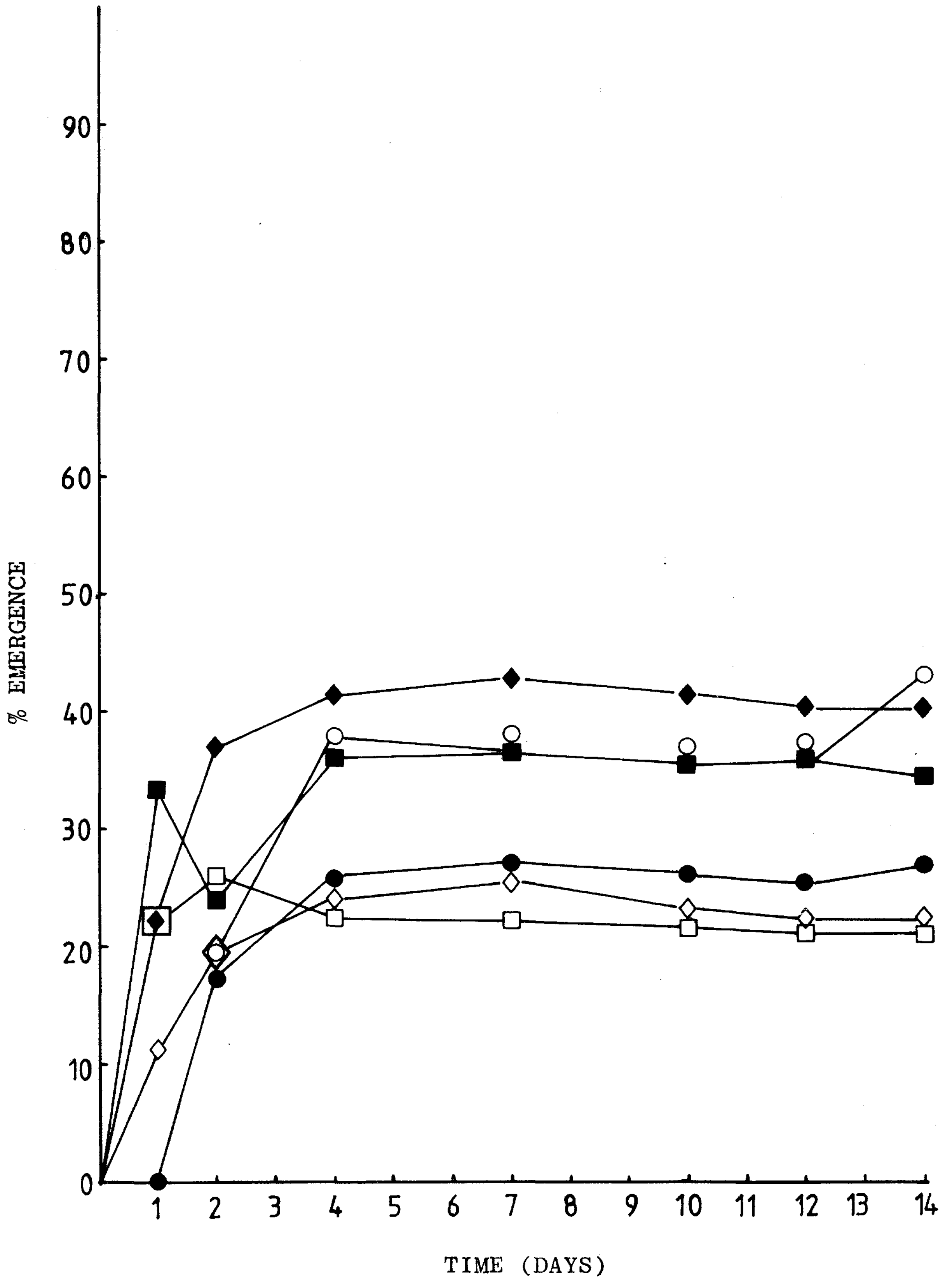


Fig. 12b CABBAGE



there being no significant differences between the pathogen infested treatments with or without TRC. Emergence was low for both the pea and cabbage controls, this being more acute for pea. Although for cabbage emergence was marginally increased in the TRC PI - P.ultimum and P.debaryanum + TRC treatments compared with the pathogen only treatment, no significant reductions in disease incidence were observed. Similarly no significant increases in seedling fresh weight was conferred by the presence of TRC in the pathogen infested treatments.

The anomalous situation whereby large mean fresh weights were obtained for emerged plants growing in pathogen infested compost are high-lighted in this experiment. This was due to a combination of high pre-emergence damping-off resulting in low emergence, favourable post-emergence environmental conditions together with an extended growth period. Thus, seedlings which survived the initial damping-off phase subsequently grew vigorously when free of competition and achieved significant fresh weight gains.

Greater disease was observed in this experiment compared with the preceding one (Tables 29 and 30). Comparisons between the percentage emergence over a fourteen day period confirm that a marked reduction in emergence for both (Figure 11 a and b) cabbage (Figure 12 a and b) occurred in the replant experiment. The period of susceptibility of the hosts is relatively short with older seedlings developing marked resistance. A concomitant decline in the levels of Pythium would therefore be expected through the later stages of cropping in section 4.3.7 and through the brief intercropping period prior to the introduction of fresh host seeds.

In theory this period should provide an ideal time for TRC to proliferate and exert its antagonistic effect on Pythium inoculum. However the poor emergence observed with the controls clearly indicates that conditions

for seed germination were not optimal and suggests that resident or contaminating pathogens may have been favoured by the replant procedure. Thus the high levels of disease observed in this experiment may only be in part due to the presence of artificially introduced Pythium inoculum.

4.3.9 Use of a low nutrient growth medium for TRC, P.ultimum and P.debaryanum

Tests in vitro had demonstrated the existence of two types of interaction between TRC and P.ultimum and P.debaryanum. The first occurred on high nutrient status media and implicated the production of antibiotic-like metabolites. The second interaction characteristically developed on media of low nutrient status, in particular SEA, and resulted in lysis and death of the Oomycete mycelium.

All previous greenhouse experiments had utilized a rich nutrient supplement in growth medium, namely V_8 - vegetable juice. In this experiment the latter was replaced by liquid soil extract which was used to soak the vermiculite carrier and thus provide a medium of low organic nutrient status.

TRC, P.ultimum and P.debaryanum were grown on vermiculite soaked with 10%(v/v) soil extract preparation (section 2.2.2) following the routine procedure (section 4.2.1) and flasks were incubated at room temperature (approximately 20°C) for two weeks. The resulting inoculum was incorporated into compost in the normal manner to give a final 10%(v/v) concentration in the seeded layer. The experimental design included a seven-day prior inoculation of TRC (pre-inoculation (PI) treatment) and a simultaneous inoculation of both TRC and the pathogen as used in earlier experiments.

Severe damping-off of pea seeds occurred resulting

Table 31

The effect of using a low nutrient status vermiculite medium on *Pythium* induced damping-off in the presence of TRC

For abbreviations see legend to Table 5

10 pea seeds and 20 cabbage seeds were sown in each pot, fresh weight is given in grams. *P.ultimum*, *P.debaryanum* and TRC were grown on vermiculite soaked with 10%(v/v) soil extract, 50.0ml of inoculum was used to introduce each organism into compost. Results are the means of five replicates.
Means underscored by the same line are not significantly different (P=0.05)

Table 31

PEA - EMERGENCE

Pu	Pu+TRC	TRC PI Pu	Pd	TRC PI Pd	Pd+TRC	Con	Verm	TRC	TRC PI
0	0	0	0.20	1.00	1.80	3.20	4.00	5.20	5.60

PEA - FRESH WEIGHT (g)

Pu	Pu+TRC	TRC PI Pu	Con	Pd	TRC PI Pd	TRC PI	TRC	Verm	Pd+TRC
0	0	0	4.83	4.98	5.29	5.52	7.06	8.63	10.03

CABBAGE - EMERGENCE

Pu	TRC PI Pu	Pu+TRC	TRC PI Pd	Pd+TRC	Pd	TRC PI	TRC	Verm	Con
4.40	4.60	5.80	6.20	7.40	8.60	10.40	14.40	16.40	17.40

CABBAGE - FRESH WEIGHT (g)

Con	TRC	TRC PI	Verm	TRC PI Pd	TRC PI Pu	Pd	Pd+TRC	Pu+TRC	Pu
4.44	4.67	4.88	5.16	7.52	8.15	8.24	8.37	8.95	15.71

in nil emergence in all P.ultimum treatments (Table 31). In P.debaryanum infested treatments emergence was also low, but for pea was significantly greater with the P.debaryanum + TRC treatment compared with the P.debaryanum only treatment. No significant difference was found between the two TRC treatments of P.debaryanum. Although significantly greater emergence did occur in the controls, the overall vigour of the seedlings indicated that environmental conditions did not favour germination and growth. With the cabbage pathogen treatments, differences between the emergence means were non-significant. In contrast with the pea results, the two TRC cabbage controls were lower than the vermiculite and uninoculated controls with the TRC PI pre-inoculation treatment of cabbage being significantly lower and that of pea significantly higher than both the controls not containing TRC.

Analysis of the fresh weight means revealed that there were no significant differences between the three P.debaryanum treatments of pea or between these and the control means. The anomalous results noted in the preceding experiment pertaining to the fresh weights of emerged plants grown in infested compost also occurred in this trial. A significantly greater fresh weight was obtained for the severely damped-off P.ultimum treatment of cabbage whilst the remaining cabbage treatments and controls were all contained in a separate larger subset with a statistically non-significant range (Table 31).

Although pathogen growth was sparser in the vermiculite-soil extract medium, use of this inoculum did not appear adversely to affect the pathogenicity of either P.ultimum or P.debaryanum. Johnson et al. (1981) found that growth of P.ultimum was partly independent of virulence but P.ultimum growth on media deprived of sucrose and nitrogen caused only minor symptoms on cotton hypocotyls. There is no evidence from this trial of an enhancement of antagonism of TRC by using a low nutrient status growth medium.

4.3.10 The Interaction between TRC and the damping-off fungi in the presence of a straw amendment

Tests *in vitro* demonstrated that the Binab *TRC* produces cellulolytic enzymes (section 3.7) and thus possesses a mechanism by which biological control might operate selectively against Oomycetes. Incorporation into compost of a straw amendment would provide on additional source of cellulose which might augment antagonism by *TRC* if based on cellulase activity.

Autoclaved sterile wheat straws cut into three centimetre lengths were incorporated into non-sterile compost to give a final concentration of approximately 1.5%(w/w). The amended compost was inoculated with 50.0ml per pot of *TRC*, *P.ultimum* or *P.debaryanum* vermiculite-V_8 inoculum as required following the routine procedure. The effect of a straw amendment was also monitored in a pre-inoculation treatment in which *TRC* inoculum was incubated in straw-amended soil for seven days prior to pathogen inoculation.

Both hosts were severely diseased with nil emergence scored for all replicates of the three *P.ultimum* treatments of pea (Table 32). With the exception of the latter, slight improvements in emergence during the course of the experiment were noted for the pathogen + *TRC* treatments, excluding the simultaneous *P.debaryanum* + *TRC* treatment of cabbage (Figure 13). Increases were more pronounced with the *TRC* PI - *P.debaryanum* treatments, and in the case of cabbage the final seedling stand was significantly greater than either the *P.debaryanum* + *TRC* or *P.debaryanum* only treatment (Table 32). However this improved emergence was not raised to the level attained by the four control treatments. The slight improvements in emergence of cabbage also obtained with the *TRC* treatment of *P.ultimum* were not significantly greater than the pathogen only treatment. No significant differences were found between the mean treatment fresh weights for either pea or cabbage (Table 32).

Table 32

Damping-off of pea and cabbage by P.ultimum and P.debaryanum in straw-amended compost containing TRC

For abbreviations see legend to Table 5.

10 pea seeds and 20 cabbage seeds were sown in each pot, fresh weight is given in grams. Autoclaved sterile wheat straw was incorporated into non-sterile compost to give a final concentration of approximately 1.5%(w/w). Results are the mean of five replicates.
Means underscored by the same line are not significantly different (P=0.05)

Table 32

PEA - EMERGENCE

Pu	Pu+TRC	TRC PI Pu	Pd	Pd+TRC	TRC PI Pd	Verm	Con	TRC	TRC PI
0	0	0	0.80	1.00	2.40	6.20	6.20	6.40	6.80

PEA - FRESH WEIGHT (g)

Pu	Pu+TRC	TRC PI Pu	Verm	TRC	Pd+TRC	Con	TRC PI	Pd	TRC PI Pd
0	0	0	6.74	8.36	8.56	9.29	9.56	10.64	12.66

CABBAGE - EMERGENCE

Pu	Pd	Pd+TRC	Pu+TRC	TRC PI Pu	TRC PI Pd	TRC PI	Con	TRC	Verm
2.60	4.80	4.80	5.20	5.60	10.40	14.40	16.20	17.20	19.40

CABBAGE - FRESH WEIGHT (g)

TRC PI Pu	Pu+TRC	TRC	TRC PI Pd	TRC PI	Verm	Pu	Con	Pd+TRC	Pd
1.98	2.33	2.66	3.24	3.71	3.90	4.19	4.54	5.72	6.46

Figure 13

The percentage emergence of pea and cabbage in Pythium infested and TRC treated compost amended with straw over a fourteen-day period

For abbreviations see legend To Table 5

■——■ Pu; ●——● Pu+TRC; ◆——◆ TRC PI Pu;
□——□ Pd; ○——○ Pd+TRC; ◇——◇ TRC PI Pd;

Emergence is expressed as a percentage of the vermiculite control

Fig. 13 PEA

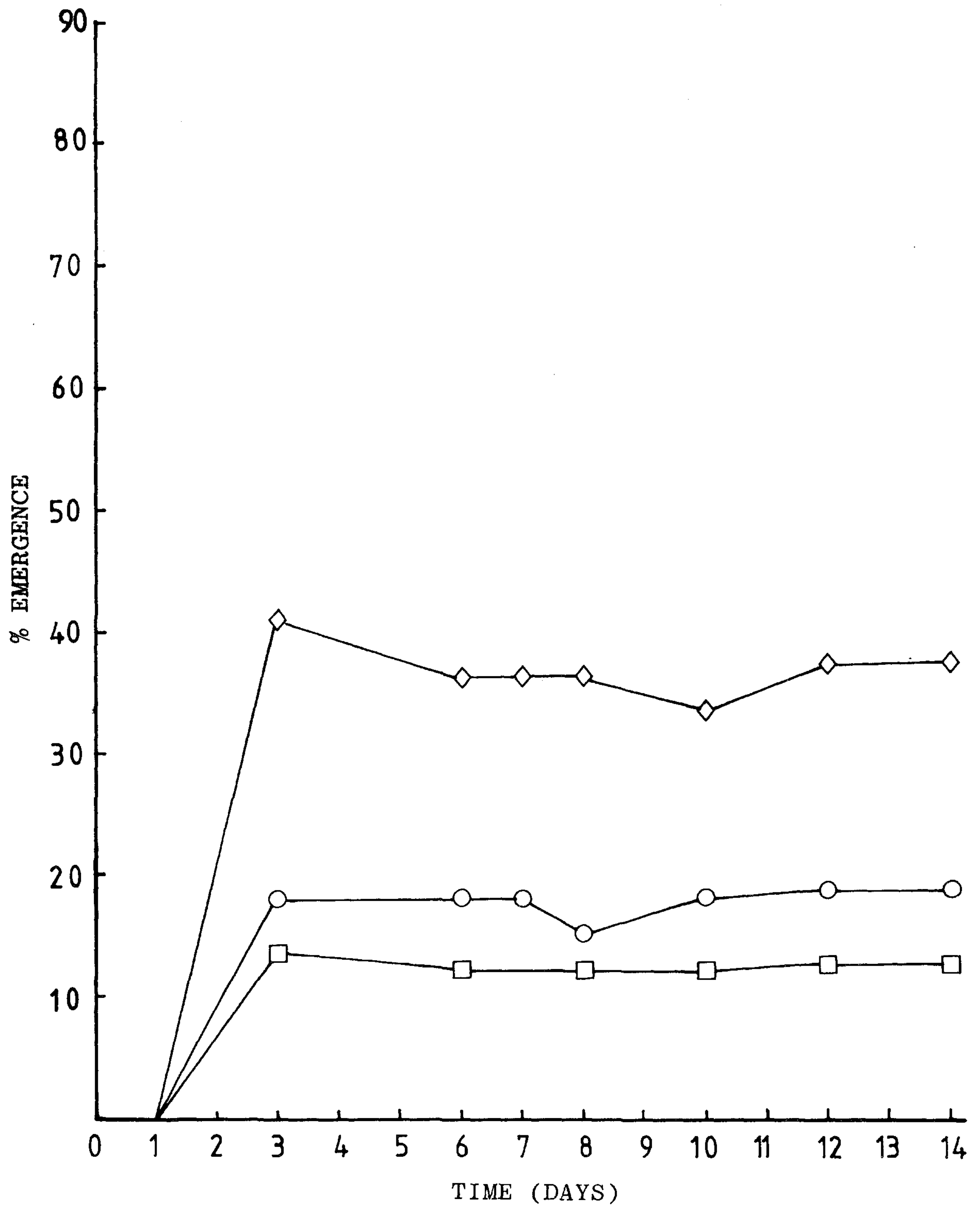
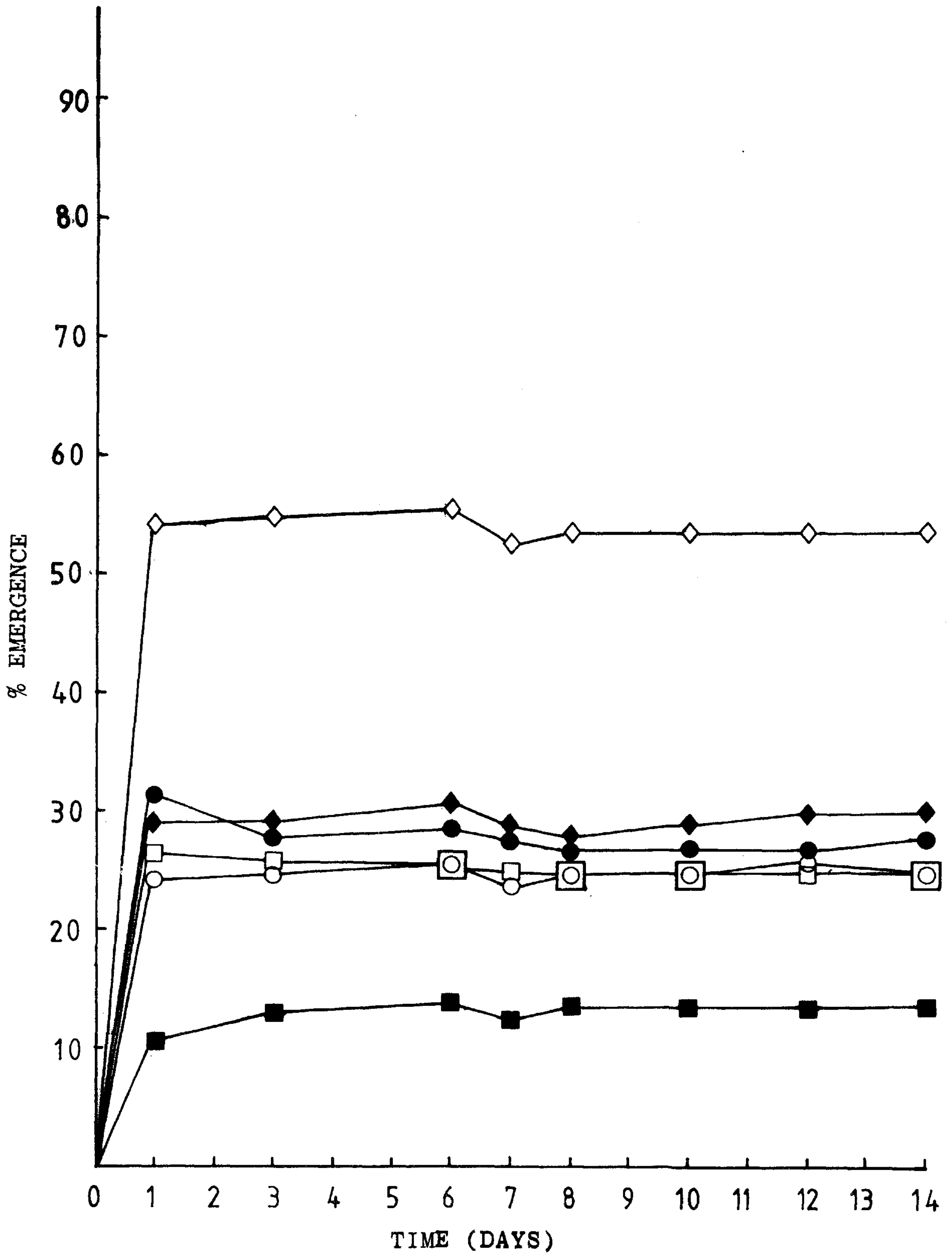


Fig. 13 CABBAGE



Use of a pre-inoculation treatment of TRC combined with a straw amendment in this experiment may favour antibiosis with straw acting as a site of active antibiotic production by TRC (Wright, 1955 ; Baker, 1968) or inducing the production of cellulolytic enzymes. However significant disease reduction was only obtained with one treatment and both forms of TRC inoculation were totally ineffective against *P.ultimum* infections of pea. Species of *Pythium* are not able to compete well for food bases if they are already well colonized (Hendrix and Campbell, 1973) and hence possession of the straw base by TRC prior to pathogen inoculation, possibly augmented by antibiosis may effectively preclude colonization by the pathogens and other resident organisms.

Hancock (1977) reported that increases in *P.ultimum* inocula were closely associated with the introduction into soil of fresh plant residues. With simultaneous inoculation of TRC and the pathogen, competition may ensue with rapid colonization and reproduction on the sterile straw by the pathogen resulting in high levels of disease as observed in this experiment. Nevertheless, Baker and Cook (1974) concluded that pathogens present in cellulosic material are likely to be replaced by highly competitive saprophytes and thus increases in pathogen inoculum could potentially be limited to be superseded by increases in TRC with time.

In the absence of any detailed studies regarding the sequential colonization of straw in compost, the effects of straw decomposition on the pathogens, TRC and the host, the overall result suggests that this treatment favours the pathogen rather than TRC. Although limited success was achieved with cabbage using the TRC pre-inoculation treatment against *P.debaryanum*, clearly the repercussions of integrating a straw amendment are likely to be complex since in no other combination was disease significantly suppressed by TRC.

4.4 QUANTITATIVE STUDIES OF TRICHODERMA POPULATIONS

Quantitative studies of TRC colonization required the use of a sensitive *Trichoderma* selective medium for re-isolation and enumeration of the antagonist. Various media (section 4.2.7) were assessed including the peptone-rose-bengal agar (Martin,1950), a modified version of the latter used by Liu and Baker (1980) which included pentachloro-nitro-benzene (PCNB) at 100μg/ml, a selective medium described by Elad *et al* (1981) and a medium developed by Papavizas (1981). The latter two were specially formulated as selective for *Trichoderma* spp. but each contained essential fungicide components, Dexon and Chloroneb respectively which were not available commercially in the UK. In their absence the media were found to lose their reported specificity and similarly the Martin's media in both the modified and unmodified form did not meet the degree of selectivity required for quantitative assessments of TRC populations.

Mucor, *Rhizopus*, *Penicillium* and *Aspergillus* spp were frequent contaminants and these fast growing fungi frequently obscured or were confused with colonies of *Trichoderma* spp. The media which contained PCNB as a constituent also recovered *Fusarium* spp. and Oomycetes, since PCNB has little effect on these fungi and is often used selectively to recover these organisms (Waller,1981). Thus the PCNB agar (Nash and Snyder,1962) and modified PCNB agar (Papavizas,1967) often used for re-isolation of *Fusarium* spp. from soil, and the Gallic acid (Flowers and Hendrix,1969) and modified Kerr's agar (Hendrix and Kuhlman,1965) recommended for the re-isolation of *Pythium* spp. (Johnson and Curl,1972) with PCNB as a constituent, also supported growth of *Trichoderma* spp. Moreover in experiments where high populations of these pathogens were also present, accurate estimation of *Trichoderma* populations proved extremely difficult highlighting the need for a more sensitive *Trichoderma* medium.

The medium which proved to be the most promising for the re-isolation of TRC was a modified version of that developed by Papavizas (1981) in which metalaxyl (Ridomil, Ciba-Geigy) took the place of the chloroneb (section 4.2.7.3)). Growth of P.ultimum and P.debaryanum in vitro was totally inhibited at all metalaxyl concentrations used to amend V_8 agar, whilst TRC appeared to be unaffected (Table 33).

The medium was also used in a time course experiment in which TRC population levels were investigated using serial dilution technique following inoculation of compost with Pythium and/or TRC vermiculite inoculum sown with pea seeds in the usual manner. In general the population of Trichoderma appeared to remain relatively constant during the time course (Table 34) and was not adversely affected by the presence of the Pythium spp. The addition of Triton X-100 appeared to reduce but did not eliminate the problem of contamination by fast growing mucoraceous fungi. This largely agrees with the findings of Papavizas and Lumsden (1982) who found that the addition of alkaryl polyether alcohol improved the Trichoderma selectivity of media. However the presence of contaminating colonies as obtained in these tests leads to inaccuracies in enumeration and may be directly responsible for much of the variation observed.

Thus, preliminary quantitative analysis of the population and distribution of the Binab antagonist was largely hampered by the failure to devise an entirely suitable medium and the problem was increased by the presence of large populations of the pathogens under study. Papavizas (1981) pointed out that the development of a selective culture media for all Trichoderma spp. might be an impossible feat, and with the medium used here the degree of selectivity for the constituent species of the Binab preparation was not investigated. The major advantage of this medium was that it did not contain PCNB which not only reduces the recovery of c.f.u. belonging to Trichoderma spp. at concentrations as low as 25µgai/ml (Papavizas,1981) but can also re-isolate Fusarium spp. and Oomycete fungi.

Table 33

Percentage inhibition of P.ultimum, P.debaryanum and TRC growth on V_8 agar amended with Metalaxyl

% Inhibition P.ultimum *
Metalaxyl µg/ai/ml

DAY	25	50	100	200	250
1	100	100	100	100	100
2	100	100	100	100	100
3	100	100	100	100	100
4	100	100	100	100	100

% Inhibition P.debaryanum *
Metalaxyl µg/ai/ml

DAY	25	50	100	200	250
1	100	100	100	100	100
2	100	100	100	100	100
3	100	100	100	100	100
4	100	100	100	100	100

% Inhibition TRC *
Metalaxyl µg/ai/ml

DAY	25	50	100	200	250
1	5.9	0	0	0	0.2
2	11.4	2.7	0	10.9	12.4
3	0	0	0	0	0
4	0	0	0	0	0

% Inhibition compared with controls in which organisms was grown on non-amended V_8 agar

* Inoculated as 5mm disks of mycelium

Table 34

Quantative assessment of Trichoderma population in TRC and pathogen infested compost

COLONY FORMING UNITS PER GRAM OF COMPOST (X 10^5)

TIME	TREATMENTS TRC	Pu+TRC	Pd+TRC
0	5.3 ± 0.15	5.4 ± 0.22	4.5 ± 0.25
3 HRS.	4.9 ± 0.16	5.6 ± 1.8	3.9 ± 0.38
6 HRS.	1.4 ± 0.4	1.5 ± 0.18	2.3 ± 0.26
1 DAY	1.6 ± 0.28	0.8 ± 0.8	2.5 ± 0.36
2 DAYS	2.0 ± 0.29	2.5 ± 0.22	2.9 ± 0.40
3 DAYS	2.4 ± 0.25	3.6 ± 0.66	4.2 ± 0.22
6 DAYS	11.0 ± 2.1	4.9 ± 0.52	4.7 ± 0.37
8 DAYS	7.0 ± 0.28	9.2 ± 0.10	7.6 ± 0.09
10 DAYS	13.0 ± 1.7	8.3 ± 0.7	9.8 ± 0.89
14 DAYS	14.0 ± 2.6	26 ± 2.9	4.8 ± 0.28

For abbreviations see legend to Table 5

TRC and Pythium were each incorporated into compost as vermiculite-V_8 inocula (50.0ml) in the routine manner and 10 pea seeds were sown in each pot. Five samples of compost were randomly collected using sterile corkborers and were each suspended in sterile distilled water. A 1.0ml aliquot of a X4000 dilution was plated onto each of three plates containing the Trichoderma selective medium(20.0ml). Plates were incubated in the light at room temperature (approximately 20-25°C) and were viewed daily. Generally colonies were counted after 4-7 days. Numbers of colony forming units are multiples of 10^5. Results are presented with three standard errors

4.5 DISCUSSION

Consistent reductions in disease incidence were not obtained with either method of TRC application. With the exception of the preliminary screen in no instance was a commercially acceptable level of disease control obtained. Significant increases in emergence were only obtained with the TRC treatments in two other experiments, with the TRC PI - P.debaryanum treatment of cabbage in an experiment incorporating straw (section 4.3.10), and to a lesser extent with the P.debaryanum + TRC treatment of pea which utilized a low nutrient status growth medium (section 4.3.9).

The ability of TRC to suppress disease was monitored in three ways: the first scored the numbers of successfully emerged seedlings over a minimum period of 21 days, the other two methods used the mean final seedling stand together with the mean fresh weight in grams which were analysed statistically in multiple range tests. The first assessment method, seedling emergence with time, was expressed as a percentage of the vermiculite control and generally the greatest variation was confined to the first seven days of emergence, with results stabilizing for the remaining period of observation (Figure 10). Sharp declines in percentage emergence noted during this initial period were due either to post-emergence damping-off and/or to increases in emergence of the vermiculite controls. The smaller subsequent decreases indicated that only limited damping-off occurred after this period and agrees with the observations reported by Stephens et al. (1981), who found that the majority of Pythium isolates tested induced little additional damping-off from 9-23 days after seedling emergence. The uniform method of seed sowing aided scoring by ensuring that in the absence of infection, emergence within replicates of the same treatment generally occurred at approximately the same time. Germination and the first emergence of seedlings usually occurred within seven to ten days from the sowing date.

Thus pre-emergence damping-off caused the greatest reductions in emergence although post-emergence damping-off was also observed. Seed vigour and conditions favouring germination are balanced against infection. If germination is rapid, the effects of the pathogen may be avoided or minimized, the emerged seedlings becoming more resistant with age. The presence of an antagonist may alter this balance in favour of seed emergence. If for example the onset of infection is delayed due to suppression of Pythium inoculum or by adverse environmental conditions, the host may evade the pathogen by growing rapidly through the stage of maximum susceptibility.

Initial trials used TRC as a soil and seed treatment. Harman et al. (1980,1981) reported the successful use of the mycoparasite T.hamatum (Bon.) Bain as a seed treatment against R.solani and Pythium spp. and found that the addition of CMC tended to decrease protection by and the establishment of T.hamatum in soil whereas in the presence of peat its population density in soil was increased but the degree of control was not improved. In comparison T.harzianum was unable to protect pea and radish seeds from Pythium spp. and R.solani and colonization of the seed surface was poor. Harman et al. (1980), further suggested that colonization of seed coats may be related to cellulolytic ability with cellulase aiding colonization by providing glucose for the fungus.

Wright (1956) found that antibiotic-producing strains of Trichoderma used as a seed dressing gave the most effective control of Pythium spp. on white mustard, whilst Al-Hamdani et al., (1983) successfully used the mycoparasite Pythium oligandrum with cress seeds in P.ultimum infested soil and sand. However, the Binab TRC employed as a soil treatment giving considerably better results. Windels (1981) using conidia of Pencillium oxalicum to treat pea seeds found hyphal growth and sporulation on the seed coat three days after planting. Similarly

Hubbard et al. (1983) reported that in the absence of vigorous competition by bacteria, T.hamatum was capable of forming a hyphal mat on pea seeds within 24 hours.

The carbohydrate and amino acids of seed and root exudates can stimulate germination of P.ultimum propagules up to 20mm from the host surface (Stanghellini et al.,1983). A relatively small TRC population introduced on the seed may therefore be unable to compete with rapid germination, penetration and infection characteristic of pathogenic Pythium spp. Moreover the stimulatory effect of exudates on the pathogen may override any selective advantage conferred on the antagonist. Stanghellini and Hancock (1971a) found that in soil sporangia of P.ultimum commenced germination by 1.5 hours and reached a maximum 3 hours after nutrients were added, while penetration of peach roots by P.ultimum occurred 5.8 hours after inoculation (Miller et al.,1966). Spencer and Cooper (1967) studying the infection of cotton seedling radicles by Pythium spp. found that mycelial fragments and zoospores took 12 and 2 hours respectively to effect penetration.

Use of TRC as a soil treatment massively augments the growth medium with actively growing, and sporulating cultures of the antagonist. However the apparent level of reduction in disease incidence observed in section 4.3.1 could not be repeated in any of the successive trials. The generally high levels of emergence that resulted infer that conditions for the pathogens and infection were not optimal in this experiment and may have largely accounted for this anomaly (Figure 9).

Pea and cabbage were selected from the results with the soil treatment as hosts for more detailed studies. The former proved to be highly susceptible, with the cultivar consisting of both smooth and wrinkled seeds. Exudates from cultivars of wrinkle-seeded peas are reported to have more sugars than smooth-seeded peas (Flentje and Saksena, 1964b) and the former are more prone to pre-emergence damping-off (Short and Lacy, 1976; Windels

and Kommedahl, 1982). Cabbage gave an overall greater level of emergence in the presence of the pathogens, and it was considered that use of two hosts with apparently different degrees of susceptibility might improve the relevance of the assessment methods.

Tests *in vitro* had clearly demonstrated the antagonistic nature of *TRC* against a range of organisms. Results of laboratory screens are not readily extended to experiments conducted *in vivo* and Kommedahl and Windels (1978) concluded that neither laboratory nor greenhouse trials could alone predict the performance of an antagonist under field conditions. The present greenhouse trials were designed to assess the disease-controlling ability of the Binab *TRC* which had already demonstrated antagonism against *Pythium* spp. in agar plate tests.

Results of dual culture tests had confirmed that *TRC* was an aggressive competitor on rich growth media and thus the choice of a carrier-V_8 nutrient system could potentially promote successful competition by large populations of introduced *TRC*. Furthermore, since antibiotic production invariably occurs in the vicinity of organic substrates such as seed coats and straw (Wright, 1955, 1956 ; Baker 1968), the use of a rich nutrient source might promote antibiotic production. Baker and Cook (1974) concluded that antibiosis in the broadest sense (that is the inhibition of one organism by a metabolite of another) was optimal under nutrient-replete conditions. However they also pointed out that the effects on a target organism would be less in such situations than in a nutrient deficient environment.

Apart from the instances already mentioned, *TRC* when applied as a soil treatment did not successfully control damping-off. This broadly agreed with the findings of Hoch and Abawi (1979) who found that isolates of *T.harzianum* grown on corn leaf meal were ineffective at controlling damping-off of table beet caused principally by *P.ultimum*. A serious disadvantage of using the same

growth medium for both pathogen and antagonist is that selective advantages will be conferred on both organisms in terms of nutrient availability. Failure to obtain disease control may have been due to the increased pathogenicity resulting from the use of a rich growth medium as for example was reported by Johnson et al. (1981) who found that the virulence of P.ultimum increased with high concentrations of sucrose and nitrogen. However, in the present trials severe disease also occurred when an alternative low nutrient status medium was used (section 4.3.9). Kelley (1976) reported that one of the major causes for the failure to obtain disease control of Pytophthora cinnamomi causing damping-off of pine seedlings with T.harzianum was the pathogen's ability to exploit nutrients leached from the T.harzianum-impregnated clay granules. A selective advantage was also gained by Pythium which was stimulated when Trichoderma pellets supplied by Binab were used to inoculate soil (Gindrat et al., 1977). The Pythium presumably colonized the organic nutrients provided by the pellets suggesting that use of a nutrient food base for the antagonist is not feasible in the presence of Pythium spp. Lumsden et al., (1983) studying the effects of composted sewage sludge found an increase in T.harzianum resident in soils following amendment with the organic matter which appeared to stimulate the activity of microorganisms. Damping-off by Pythium spp. was either not affected, increased or decreased depending upon the host and species used, although suppression was enhanced when the time between application and planting was extended.

Results in vitro had suggestd that a form of active antagonism by TRC was favoured by nutrient deficient conditions such as occurred on SEA. When this was used in vivo a marginal increase in disease control was only obtained with the P.debaryanum + TRC treatment of pea, with no other significant improvements or effects on Pythium pathogenicity being observed.

In straw-amended compost (section 4.3.10) modification of the environment in one instance only (TRC PI -

P.debaryanum treatment of cabbage) appeared to favour the antagonist rather than the pathogen to give an increase in emergence. Cellulose can also be degraded by *Pythium* spp. (Taylor and Marsh, 1963; Alexander, 1977) and it is likely that both *TRC* and *Pythium* are able to colonize the sterile straw to gain a selective advantage and increase inoculum density. Prior colonization by *TRC* in a pre-inoculation treatment could in theory be a useful form of antagonism against competitive saprophytes such as *Pythium* spp. The organism able to colonize the straw first may benefit most since an organism already occupying a substrate will retain possession (Bruehl and Lai, 1966; Baker and Cook, 1974). Results suggest that *Pythium* spp. were able to do this, despite the presence of *TRC* in the majority of treatments. The introduction of such an amendment into compost will precipitate a concomitant increase in microbial activity, with a diverse population arising which may itself exert profound effects on both the pathogen, *TRC* and perhaps even the host.

Similarly in the replant experiment (section 4.3.8), it is likely that damped-off seedlings and remnants from the previous experiment provided an exploitable nutrient source giving rise to enhanced pathogen inoculum levels (Hancock, 1981). *Trichoderma* spp. as active soil saprophytes would however also be subject to the same potential benefits. In general *Pythium* spp. are not considered to be vigorous competitors in soil because their saprophytic activities are greatly restricted by antagonistic microflora (Hendrix and Campbell, 1973; Lifshitz and Hancock, 1983). In theory therefore a host-free period should provide an ideal time for *TRC* to proliferate and exert its antagonistic effects on *Pythium* inoculu.

Clearly detailed quantitative studies are required to investigate the fate of *TRC* subsequent to introduction. The lack of quantitative methods has been a constant and major drawback in investigating the survival and ecology of *Trichoderma* spp. (Papavizas, 1981) and only one medium was found to be suitably sensitive and selective for use

with TRC (section 4.4). Results in this preliminary trial suggested that the population of TRC remained relatively constant in all the treatments agreeing with the findings of Papavizas (1981), who found that conidia of T.harzianum when introduced into soil one day before planting did not proliferate in the rhizosphere of pea seedlings. Similarly use of conidia as a seed treatment did not improve survival and T.harzianum failed to establish in the rhizosphere of bean and pea seedlings.

Research into biological control often uses soils naturally infested with the pathogen under study (Papavizas et al., 1982; Hubbard et al., 1983), or uses antagonists originally isolated from naturally disease suppressive soils (Harman et al., 1980, 1981; Chet and Baker, 1981). In this study artificial inoculation had to be relied on as a means of introducing both the pathogen and the antagonist. Thus, no explanation can be given for the low levels of disease that occurred in some experiments (eg: P.ultimum infection of cabbage section 4.3.5. P. dabaryanum infection of tomato section 4.3.1). The importance of relating emergence in TRC treatments to the pathogen only and control treatment is self-evident; the inclusion of numerous controls is necessary to determine the effects of individual components and treatments on emergence. In some experiments (eg: sections 4.3.6 and 4.3.8) the low emergence clearly indicates that other pathogenic organisms and/or adverse environmental conditions resulted in reduced emergence. In experiments where exacerbated disease incidence occurred as a result of the experimental procedure used, it is highly plausible that positive effects of TRC were completely obscured by enhanced pathogenicity and/or increased inoculum density.

Observations in preliminary experiments revealed that germination was frequently rapid in the TRC-control treatments with emergence often being faster than in the vermiculite or compost only controls. This prompted the use of a final fresh weight as an additional criterion for evaluating subtle effects of TRC. Comparison using

emergence data and final seedling stands do not take into account the health and, vigour and growth of the seedlings. P.ultimum can cause poor root development and stunting of emerged seedlings (Hendrix and Campbell, 1973) and the presence of an antagonist has been noted to improve plant growth (Elad et al., 1980).

In practice however, the fresh weight data did not always provide meaningful comparisons. Greater final fresh weights were frequently obtained with pathogen-infected treatments. This often occurred when external environmental conditions favoured subsequent growth of the emerged seedlings. In control pots where greater numbers of seedlings were generally present (in the absence of the pathogens) growth soon became limited by overcrowding. Conversely in pathogen treatments in which limited numbers of plants emerged growth of the survivors was rapid and prolonged in the competition-free environment. Large fresh weight gains were thus often obtained and emergence and fresh weight data for treatments invariably did not agree. However a significant increment in seedling stand was obtained with the P.ultimum + TRC treatment of cabbage in section 4.3.6 compared with the P.ultimum only treatment where emergence was similar for both. In general though, use of the mean final fresh weights is probably only useful for use in shorter term experiments than attempted here.

The wilt pathogen *F.oxysporum* f.sp. *lycopersici*

In these preliminary greenhouse trials the bio-control potential of *T.harzianum* and *T.polysporum* or the commercial mixture (TRC) were evaluated against *F.oxysporum* f.sp. *lycopersici*. The four isolates of *Verticillium albo-atrum* tested mere less pathogenic and the high ambient temperatures that prevailed at the time of the trials favoured *Fusarium* infection but suppressed symptoms of *Verticillium* wilt.

4.6.1 Application of Trichoderma to soil as a spore suspension

The components of the Binab preparation *T.harzianum* and *T.polysporum* were used in combination for initial experiments, since the commercial TRC preparation was in short supply. Using plants at the third to fourth true leaf stage, roots were wounded (section 4.2.10) 20mls and of *T.harzianum* and *T.polysporum* (10^6 spores/ml) were poured over the soil surface together with 20mls of *F.oxysporum* f.sp. *lycopersici* (10^6 cells/ml). Both autoclaved and non-sterile soils were used. Treatments were replicated five times and a completely randomized design was employed.

Disease symptoms rapidly developed in autoclaved soils treated with both the pathogen alone and the pathogen plus *Trichoderma* treatments. Epinasty or drooping of the petioles and vein clearing were first observed with the latter treatment, but both treatments produced marked wilting and yellowing, with ratings of three to four on the wilting index and more than 80% on the yellowing scale two to three weeks after inoculation.

Similarly disease incidence with *Fusarium* plus *Trichoderma*-inoculated host plants grown in non-sterile soil was identical to the *Fusarium*-only treatment. Wilt

symptoms similar to those observed in autoclaved soil took longer to develop, occurring four weeks after inoculation with less yellowing being observed giving a score of 60% in both cases.

Comparisons between the pathogen only and pathogen plus Trichoderma treatments in both non-sterile and sterile soil, showed that in terms of wilting, yellowing, height and overall appearance of the host plants, no obvious treatment differences could be ascribed to Trichoderma.

Further experiments in which the inoculation with T.harzianum and T.polysporum and TRC were repeated, confirmed that in autoclaved soil disease developed rapidly invariably resulting in very marked yellowing, wilting and stunting. This indicated that in the absence of other soil microorganisms Fusarium was able to compete successfully with the introduced Trichoderma population when added simultaneously in this form and, implied that rapid re-colonization of the soil lead to exacerbated levels of disease with severe wilting and death often occurring two weeks after inoculation.

To investigate the distribution of Trichoderma two simple re-isolation procedures were tried similar to methods described by Johnson and Curl (1972). Excess soil was removed from the tomato root system by shaking and the larger soil particles were removed with forceps. Root segments (one to two cm in length) were vigorously shaken in five to ten ml of sterile distilled water for three to five minutes and one ml of the suspension was transferred to agar plates. Cooled (45°C), molten potato dextrose agar (Oxoid) (PDA) or MEA (20.0ml) was added to each plate and the contents were mixed by gently tilting. Growth of both Trichoderma spp. and Fusarium resulted indicating that both these organisms were present in the rhizosphere.

Similarly using root tip segments, particles of soil were removed as before and the root pieces were washed

in twenty changes of sterile distilled water. The roots were blotted dry on sterile filter paper and were transferred to PDA or MEA plates amended with chloramphenicol (100 mg/l). Trichoderma was also identified on these plates confirming it to be closely associated with the root surface.

The presence of Trichoderma in the rhizosphere indicated that despite the likely optimal and strategic position of the organism, it was unable to prevent Fusarium infection in either autoclaved or non-sterile soil.

The increase in disease severity observed in autoclaved soils is likely to reflect the pathogen's capacity for rapid proliferation in treated soil. This was also noted with a crown and root rot isolate of F.oxysporum by Rowe et al. (1977) who observed rapid recolonization of autoclaved soil by airborne microconidia of the pathogen. The results obtained here imply that effective colonization of the autoclaved medium occurred even in the presence of Trichoderma spp.

4.6.2 Application of TRC by root dipping

Tomato plants at the third to fourth true leaf stage were removed from soil and the roots were washed free of soil particles. Roots were placed in a TRC spore suspension containing 10^6 spores/ml for 30 minutes. The plants were re-potted into autoclaved or non-sterile soil and were inoculated with Fusarium in the usual manner.

There was no visual difference between Fusarium infected plants which had been treated with TRC and those which had not. Symptoms developed at approximately the same time in each treatment, the first symptoms being loss of turgor of the leaves and yellowing, classical wilting occurred 10-14 days after inoculation and in no instance did the TRC treatment appear to reduce the severity of symptoms.

4.6.3 Application of TRC by injection

Spore suspensions of TRC (10^6 spores/ml) were injected into the stem of tomato plants grown in non-sterile soil at the third to fourth true leaf stage. Despite the use of a fine hypodermic needle (no.30) the successful introduction of any appreciable volume of fluid was very difficult due to the high back pressure encountered. Injections into the intercellular spaces of leaves and cotyledons resulted in chlorosis and necrosis and successful delivery of small quantities of the spore suspension into the stem invariable caused a wound response. The stems were covered with parafilm or vaseline to prevent invasion by secondary organisms and prevent water loss. Plants were inoculated with Fusarium in the usual manner 24, 48 and 72 hours after injection.

In no instance did TRC introduction cause any appreciable reduction in disease and in all cases the degree of infection was comparable with the control plants injected with sterile distilled water. Failure to obtain disease control was not unexpected as the method of delivery was haphazard and when successful only small quantities of the spore suspension (approximately 0.25 - 0.5ml) could be successfully introduced without causing major wounding.

4.6.4 Soil inoculations using TRC vermiculite-V_8 inoculum

The results of experiments utilising spore suspensions of Trichoderma clearly demonstrated that in this form it was unable to suppress disease in either autoclaved or non-sterile soil. The introduction of "naked" spores into soil has drawbacks, spores being prone to the effects of fungistasis (Lockwood, 1977; Papavizas and Lewis, 1981) and the preceding results suggested that additional methods of shifting the ecological balance in favour of the 'Trichoderma were required. Growth substrates such as wheat bran and autoclaved barley seeds were examined for the production of large quantities of Trichoderma inoculum

but none possessed any substantial advantages over the vermiculite-V_8 vegetable juice medium described earlier (section 4.2.12). TRC vermiculite inoculum was incorporated into autoclaved soil to give a final concentration of 50%(v/v) and was applied simultaneously with *Fusarium* and as a prophylactic treatment prior to pathogen inoculation. In the latter, TRC inoculum was introduced into autoclaved soil into which the seedlings were transplanted and allowed to grow for one, two or four weeks prior to inoculation with *Fusarium*. All treatments were replicated five times and were completely randomized and the seedlings were observed over a four-week period subsequent to pathogen inoculation.

Results obtained in this experiment exhibited much variability. Symptoms of severe infection were prevalent in all treatments. Simultaneous inoculation of *Fusarium* and TRC resulted in rapid infection and severe disease ratings giving rise to death of the plants within two to three weeks of inoculation. Older plants in particular those used for the four-week pre-inoculation treatment had severe disease ratings, with more than 80% yellowing, weakened stems and considerable leaf abscission but death generally did not occur. This probably reflects the greater capacity of older plants to compensate for the effects of infection and frequently new growth was observed subsequent to necrosis and leaf abscission.

In each of the three TRC pre-treatments, plants became severely diseased with a range of symptoms other than wilting and yellowing being observed. TRC did not alleviate disease symptoms when compared with pathogen-only inoculated plants. The results strongly suggested that the large quantities of vermiculite inoculum introduced into autoclaved soil provided a less than optimum growth medium. Such an environment was detrimental to the growth of tomatoes including the uninfected controls, and may in the presence of the pathogen have pre-disposed them to infection.

The experiment was repeated using autoclaved soil into which was incorporated a smaller quantity of the TRC vermiculite based inoculum (20% by volume). Only two time regimes were used, a simultaneous inoculation of *Fusarium* and TRC and a two-week pre-inoculation of TRC prior to pathogen inoculation.

As in the previous experiment characteristic wilt symptoms as described by Walker (1971) frequently did not develop. Once again a range of symptoms were demonstrated by infected plants with considerable variation occurring between replicates of the same treatment. Severely reduced leaf area, epinasty and distortion of the younger leaves, chlorosis and abscission, marked development of adventitious roots, stunting and shortened internodes, poor stem development and necrosis were all evident. Yellowing and wilting scores of 60-80% and 1-2 respectively were usual. A combination of symptoms was often observed on any one plant and these were generally different from symptoms expressed by other plants, not only between treatments but also between replicates of the same treatment. This variation in symptom expression largely precluded the use of a single overall disease rating since the scoring of disease by visual symptoms could not provide a sufficiently sensitive and bias-free estimate of infection.

Expression of visual symptoms in wilt diseases is not always proportional to the extent of infection. The numbers of *Fusarium* cfu obtained from serial dilution of the basal portion of the stem did not correlate with the severity of symptoms observed and *Fusarium* was easily re-isolated from relatively symptomless plants. The range of symptoms observed were unusual and may have been caused by the method and form of TRC inoculation. The within treatment variability precluded accurate recording of results but the overall response was of severe disease development to *Fusarium* inoculation regardless of the presence of TRC.

4.7 DISCUSSION

The application methods employed in these preliminary trials against *Fusarium* were designed to introduce the *Trichoderma* in a manner that facilitated rapid colonization and establishment. Soil drenches using spore suspensions of *T.harzianum* and *T.polysporum* or *TRC* apparently failed to fulfil this requirement and there was no evidence of disease suppression. Nevertheless the results clearly demonstrated the competitive nature of *Fusarium* in soil and revealed that in autoclaved soil enhanced levels of disease compared with that in non-sterile soil always resulted. This presumably occurred as a consequence of the microbiological vacuum created by autoclaving which allowed rapid colonization and proliferation by the pathogen, an ability also noted for tomato crown and root rot isolates of *F.oxysporum* by Rowe *et al.*,(1977) in autoclaved soil and by Marois *et al.*,(1981) and Marois and Mitchell (1981 a,b) in fumigated soil.

In contrast with results obtained here with the root dip treatment with *TRC*, Jordan and Tarr (1978) found that various microorganisms including *T.viride* when used to treat roots of strawberry runners were effective against *Verticillium dahliae* in reducing wilt disease and increasing plant growth. Further, they found that in soils amended with *T.viride* the populations of *V.dahliae* declined. Similarly Dutta (1981) reported that root dips into culture filtrates and culture suspensions of *T.viride* and *Penillium chrysogenum* effectively controlled *V.albo-atrum* and proposed that antibiotics and/or saprophytic growth of the antagonists suppressed growth of the pathogens. Moreover cultures of *Cephalosporium* have been found to mitigate *Fusarium* wilt symptoms on tomato (Smith, 1957; Chisler *et al.*, 1962) whilst Youssef (1960) found that symptoms of *Fusarium* wilt were inhibited in highly susceptible tomato plants treated with cultures or culture filtrates of *P.chrysogenum*. Similarly, Matta (1966) found that by pre-treating tomato roots with *V.dahliae* *F.oxysporum f.sp.*

dianthi and f.sp.callistephi, wilt symptoms were reduced whilst T.viride had very little effect. Greenhouse trials with a UV-induced biotype of Trichoderma suggest that this may be effective for the control of Fusarium wilt of chrysanthemum (Locke et al., 1982).

The injection method tested in this study was not an effective means of introducing TRC and those spores that were successfully introduced were probably delivered into damaged cavities in the pith rather than into vascular tissues. Corke (1974) reported that Trichoderma treatments significantly reduced symptoms caused by the silver leaf pathogen in perry pears as a result of colonization after spores had been introduced into the trunks. Saprophytic bacteria and pathogens of low viruleace are known to exist within the vascular elements of plants, particularly trees, causing minimal injury by their presence (Kreutzer and Baker, 1975). However the vascular system of tomato plants is very different from the woody relatively inert system of trees, and Baker (1981) pointed out that the ability to survive within the vascular system was confined to a small number of very well adapted microorganisms. He further surmised that artificially introduced soil micro-organisms were very unlikely to thrive or suppress vascular pathogens in this habitat.

The lack of success using spore suspensions for root dipping, injections and as soil drenches prompted a shift in emphasis to soil inoculation techniques employing a carrier and food base, which offers an attractive often successful alternative mode of antagonist application (Papavizas and Lewis, 1981). Large populations of TRC were introduced into autoclaved soil and final vermiculite concentrations were high (20% and 50% (v/v)), but, when deployed at these concentrations in sterilized soil growth of the host plants was adversely affected. This was particularly evident at the 50%(v/v) concentration which appeared to affect the growth of un-inoculated plants and the progress of the Fusarium wilt disease.

The detrimental effects of incorporating vermiculite inoculum using both concentrations were unexpected as vermiculite is widely used in horticulture. The presence of large quantities of the carrier may physically alter the soil structure and hence affect aeration or cause water saturation, whilst the high ion exchange capacity of the vermiculite medium can have a marked influence on the nutrient balance of the soil, perhaps even resulting in nutrient deprivation. Additionally autoclaving is known to alter the nutrient balance and thus may also produce adverse effects on plant growth (Dawson et al., 1965). Such effect in the context of this particular syndrome would greatly complicate the evaluation of disease since many of the wilt symptoms can be induced by diverse causes such as waterlogging, soil compaction, prolonged exposure to ethylene and biotic causes such as *Pseudomonas solanacearum*, tomato spotted with virus and the potato cyst nematode (Pegg, 1981). Alteration in nutrient status may produce symptoms akin to wilt symptoms such as foliar chlorosis or interveinal necrosis (Agrios, 1969) and such effects were observed in uninoculated controls treated with vermiculite. Moreover the concentration and the balance of nutrients can play an important role in determining wilt disease levels (Bell and Mace, 1971). Walker and Foster (1946) concluded that high nitrogen and low potassium favoured *Fusarium* wilt of tomatoes while low nitrogen and high potassium retarded it whilst disease was reported to develop more slowly as the concentration of nutrient solution used to water tomato plants was increased (Stoddard and Dimmond, 1948). Calcium and boron concentrations have also been found to affect the level of disease (Eddington and Walker, 1958) and increases in pH via liming of the soil resulted in decreases in the severity of *Fusarium* wilt (Jones and Overman, 1971). Cellulose amendments of soil are reported to increase the severity of *Fusarium* wilt of peas presumably by increasing inoculum density (Guy and Baker, 1977).

Subbao-Rao et al. (1961) investigating the effects of rhizoplane fungi that *T.viride* suppressed the uptake

of inorganic ions (phosphate, sulphate and bicarbonate) but increased the uptake of glucose, whilst Fusarium sp. used to infest the roots of two other cultures suppressed the uptake of all the metabolites. They proposed that infestation of the roots influenced the metabolism of the whole plant and suggested that in the presence of Trichoderma although the amount of glucose taken in was increased, the upward translocation of sugars or their precursors was decreased since the amount of sugars accumulating in the shoot was reduced. Further, Trichoderma infestation of the roots of these same tomato varieties did not affect their ability to convert sugar into amino acids.

Clearly in this study the use of an overall arbitary scale of disease was not sufficiently sensitive to defect minor reductions in symptoms. Unless a more sensitive or quantitative method of disease assessment can be achieved together with conditions which produce a more uniform display of symptoms, the efficacy of TRC can not be evaluated accurately or effectively. Serial dilution of macerates of basal stem segments (1.0g) cut at soil level (section 4.2.13) failed to demonstrate any consistent differences in the number of Fusarium cfu present in TRC or non-TRC treated plants. In general the pathogen infected plants contained approximately 10^5 spores/ml. Moreover it was found that the number of cfu did not reflect the degree of infection exhibited and was not therefore always proportional to the severity of symptoms observed. Fusarium could be re-isolated from relatively symptomless plants.

Fungi able to invade the specialized environment of the host's vascular system largely escape the antagonism of soil microorganisms (Baker, 1981). These greenhouse trials in which the soil was augmented with large populations of Trichoderma were designed to suppress the pathogen inthe period preceding host penetration and infection. With pathogens such as Fusarium which are capable of attacking roots rapidly by multiple infections (Dutta, 1981). This must be a relatively short time period in the life

cycle of the pathogen. In reality biological control may have a greater impact if used in the decline phase of the pathogen following host death in the plant debris and soil environment. However Marois et al., (1981) successfully used a combination of five fungal antagonists, including three isolates of T.harzianum, to reduce the incidence of Fusarium crown rot of tomato in fumigated field soil as determined by the percentage of plants with lesions, and found that pathogen populations declined in soils amended with the antagonists. (Marois and Mitchell (1981 b) reported that the effect was probably due to antagonism by one member of the fungal community (Aspergillus ochraceus) which was found to predominate. Conversely a dominant population of T.harzianum occurred when the incidence of infection was high. They proposed that T.harzianum was not effective or capable of restricting the pathogenic activities of the pathogen.

In autoclaved or fumigated soils recolonization may be discussed in terms of r and K strategies used in other fields of ecology (Odum, 1971; Southwood, 1976). The first recolonizers are r-strategists able to colonize virtual ecological vacuums. They have fast growth rates and are opportunist organisms able to deplete rapidly available resources and recover quickly from low population levels. The early recolonizing community is of low diversity giving rise with time to more diverse stable communities comprising K-strategists which typically are larger and slower growing often having a high competitive ability and are able to utilize the remaining resources over a longer period of time. The r-type organisms are usually unsuccessful in communities of diverse K species and K species are usually found in diverse communities with other K species (Marois and Mitchell, 1981(b)). Control of a r-type pathogen may be possible if r-type organisms are established prior to the pathogen or by establishing K species before or after the pathogen. Bowen (1980) considered that in theory biological control might be successful against K organisms which make large investments in each offspring and generally recover more slowly from reductions in population number.

The results obtained in this study suggest that the *Fusarium* tomato with pathogen is not suitable for primary biological control screens *in vivo*. The vascular wilt pathogen causes a vastly complex physiological and biochemical syndrome (Pegg, 1981) which may manifest itself in a number of ways making quantitative evaluation of disease severity difficult. This coupled with the high degree of variability encountered in the present trials rendered impossible the detection of small differences in disease levels. There was no evidence of any appreciable suppression of disease by *T.harzianum* and *T.polysporum* or *TRC* in either sterile or non-sterile soil and the results suggest that *Fusarium* competes very effectively with *Trichoderma* spp. being capable of rapid increases in inoculum density in autoclaved soil. Moreover, stem rotting and symptoms which may have been caused by secondary organisms invading the autoclaved medium were occasionally observed and further experimental work with *TRC* vermiculite inoculum in non-sterile soil would be desirable. Recolonization of autoclaved soil by opportunist organisms able to exploit and proliferate in a community of low diversity may have included not only pathogenic organisms but also antagonists of *TRC*. Further, there was no evidence of disease control within the host which considering the highly specialized nature of that ecological niche is not a surprising outcome. If *Trichoderma* spp. are to exert any appreciable degree of control of the wilt pathogens it is most likely to occur during the prolonged survival phase between successive susceptible host plants. This aspect of the pathogen life cycle has not been tested and indeed may be amenable to examination using longer term and larger scale experiments than could be attempted here.

5. GENERAL DISCUSSION

Even a well adapted soil saprophyte able to establish itself in competition with a resident microflora may be unable to maintain its population levels in excess of those occurring naturally. This may result inspite of favourable physical or chemical factors such as the provision of a carrier food base provided for its encouragement (Papavizas and Lewis, 1981). Further, the mass introduction technique used in this study is liable to encourage a corresponding increase in the populations of antagonists of the desired control organism. Hubbard et al. (1983) recently found that short, rod shaped fluorescent Pseudomonas spp. were antagonists of Trichoderma causing lysis, and their presence in soil prevented control of Pythium under iron-limiting conditions. A nutrient source such as V_8 juice may alter the status quo, it being a substrate which is suitable for use by a range of organisms. Any such alteration could potentially favour not only antagonists of TRC, but pathogenic organisms also, and reduce the activity of indigenous saprophytic organisms capable of containing Pythium. Organisms better adapted to the changed environment or possessing a spatial advantage, for example true spermosphere or rhizosphere organisms, may multiply and prevent establishment of TRC in areas necessary for active antagonism against plant pathogens.

Pythium spp. are considered to be successful pioneer colonists and Lifshitz and Hancock (1983) concluded that P.ultimum, considered to be the most abundant and widespread pathogenic Pythium sp. in soil (Pieczarka and Abawi, 1978), fulfilled this role by being able to germinate and extend its germ tubes rapidly, hence avoiding competition. Hancock (1981), studying the survival of P.ultumum, concluded that mycoparasites could be successfully used in situations where oospores and sporangia were concentrated. However

in calculations to assess the distribution of the pathogen populations in soil it was concluded that filamentous mycoparasites would need to derive sufficient nutrients from propagules to enable linear growth to occur between propagules. He concluded "considering the metabolic energy and quantities of structural components necessary for linear growth and the efficiency of homing necessary it would be unlikely that this occurs".

Stanghellini *et al.* (1983) studying the distribution of *Pythium aphanidermatum* found that oospore inoculum densities effective for infection must be located within one mm of the host surface. If this is a general pre-requisite for infection under natural conditions, the presence of an antagonist positioned in close proximity to the host surface might successfully reduce disease incidence in *Pythium* infested soil. This emphasizes the importance of selecting antagonists suited to this environment and the choice of a suitable application method which will if possible confer a spatial advantage on the microbial antagonist.

Use of *Trichoderma* as a seed dressing theoretically satisfies these requirements and successes reported by Wright (1956) with *T.hamatum* (Bon.) Bain and with UV-induced biotypes of *Trichoderma* spp. (Papavizas *et al.*, 1982, Papavizas and Lewis, 1982) confirm this. In theory colonization of the seed coat by an active antagonist could pre-empt organisms causing seed damage by virtue of prior occupation of the substrate and exploitation of exudates produced by the germinating seed. Rovira (1965) further pointed out that the microflora associated with the seed coat may not always be transferred to the rhizosphere and Kommedahl and Windels (1981), doubted whether even subsequent recovery of an antagonist introduced on the seed from the rhizosphere was good evidence of its establishment there. Seed treatments with antagonists have on occasions failed to protect roots. (Kerr, 1961; Mitchell and

Hurwitz, 1965; Kommedahl and Windels, 1978) and Papavizas (1981) recovered very few colonies of T.harzianum from rhizosphere soil of roots ten cm away from treated seeds.

Successes with seed applications questions the validity of using large quantities of antagonists especially in soil inoculation techniques. Kuter et al. (1983) reported that suppression of R.solani in container media amended with composted hardwood bark was closely associated with populations of Trichoderma spp. which increased in the rhizosphere of radish in suppressive soils. However further work by Nelson et al. (1983) with an isolate of T.harzianum confirmed that suppression in this case did not depend on the numbers of propagules present and did not correlate with high populations. Thus the emphasis on promoting large antagonist populations in bulk soil may be misdirected, if disease control depends on individual environmental factors operating on a more local scale, to encourage antagonism directly in the infection court. Hadar et al. (1984) found that in a fine sandy loam 10^8 conidia of the Trichoderma spp. tested /gram of soil were required to control Pythium, whilst in other studies using clay loam soils, populations in the region of 10^4 to 10^5 conidia/gram of soil produced the same effect. Ammonia an important mycostatic volatile in alkaline soils may limit the efficacy of T.hamatum as a biocontrol agent, in contrast T.harzianum appears to be insensitive to the gas (Schippers et al. 1982).

The mechanism of antagonism operating in soil is frequently unknown, there being only circumstantial evidence for mycoparasitism or antibiosis as detected with agar plate tests. Nevertheless Trichoderma spp. which demonstrate mycoparasitism and/or antibiosis in dual culture tests, have proved successful biocontrol agents of Pythium spp. (Fajola and Alasoadura, 1975; Chet and Baker, 1981; S.rolfsii (Wells et al., 1972;

Elad et al., 1980) and R.solani (Hadar et al., 1979; Lewis and Papavizas, 1980: Elad et al., 1981). Papavizas and Lewis (1983) proposed that antibiosis in vitro may have a further use as a means of detecting genomic changes in UV-induced biotypes by monitoring the ability of putative mutants to produce inhibitory metabolites on agar.

In the case of the Binab TRC the most likely mechanisms for biological control appeared to be competition and antibiosis since no evidence of penetration or coiling characteristic of mycoparasitism was observed in paired culture tests. Many authors doubt the importance of antibiosis as a mechanism of biological control in soil (Baker, 1968; Papavizas and Lumsden, 1980), and in turn this questions the use of antibiosis in vitro as a means of selecting potential disease controlling agents. Moreover, emphasis placed on mechanisms of antagonism often diverts attention from equally important attributes of an antagonist such as survival, adaptability, sporulation and growth in soil. Kelley (1976) suggested that T.harzianum failed to reduce damping-off of pine seedlings by Phytophthora cinnamoni because it was incapable of effective antagonism beneath the soil surface or under wet conditions. He also found that T.harzianum was unable to reduce damping-off caused by indigenous unidentified pathogens.

Clearly, dual culture tests cannot predict whether an organism will establish itself and proliferate in soil. The approach used by Marois et al. (1981) who selected antagonists that were capable of increasing rapidly in a natural rooting medium (fumigated soil) to occupy the root environment of the host, and that increased the ratio of inoculum density to infection incidence under growth chamber conditions was an appropriate and successful alternative to agar-plate tests. Unfortunately nothing is known of the suitability of such methods for use in non-sterile soil. In contrast with the results of Marois and Mitchell (1981 a b) isolates of T.harzianum

have failed to establish readily in rhizosphere soil. (Papavizas, 1981; Kraft and Papavizas 1983) and preliminary quantitative studies on TRC populations in the present study suggest that the population remained relatively stable. Hadar et al. (1984) noted that higher populations of Trichoderma spp. developed near plants which grew from captan-treated seeds and suggested that this was due to a check in growth of competitive organisms. Papavizas (1981) concluded that the reduced survival of T.harzianum in rhizosphere soil resulted from a lack of sporulation, accounting for the low numbers of T.harzianum cfu recovered when used as a seed treatment and as a soil treatment prior to planting. He proposed that this may explain why Trichoderma spp. appear to require food bases for establishment in soil in order to exert disease control (Harman et al., 1981; Papavizas et al., 1982).

There is evidence that exogenous nutrients supplied together with a Trichoderma antagonist can be preferentially used by Pythium spp. if also present (Kelley, 1976; Gindrat et al., 1977). Flentje and Saksena (1964 a) found that Pythium attacked peas usually between 48 and 96 hours after planting and Fajola and Alasoadura (1975) reported that germination of T.harzianum was slower (82.6% in twelve hours) than P.aphanidermatum which was capable of 100% germination in three hours. Rapid germination of the pathogen in the presence of nutrients may prevent reductions in disease by even an aggressive antagonist.

The type of growth medium appears to influence the biocontrol properties of antagonists (Papavizas and Lewis, 1981; Elad et al., 1982; Lewis and Papavizas, 1983) and assessment of TRC as a biocontrol agent in the presence of alternative nutrient sources may produce more promising results. Further, the antagonistic properties of TRC and other microorganisms could potentially reduce the saprophytic colonization by Pythium spp. and cause a decrease in pathogen inoculum density

when incubated together. The predominant use of mycelium and conidia of Trichoderma spp. for biological control studies has also largely overlooked the possible role of chlamydospores in disease control. Henis et al. (1982) observed that when Trichoderma spp. penetrated sclerotia of S.rolfsii, conidia were produced on the surface and chlamydospores were produced in large numbers within sclerotia.

An important point emerging from the use of UV-induced biotypes is that not all of them are equally effective biocontrol agents of specific pathogens (Papavizas et al., 1982; Papavizas and Lewis, 1983). The relationship between antagonist and pathogen appears to be a very close one with antagonists exhibiting a high degree of selectivity for different species and strains (Olsen and Baker, 1968; Mughogho, 1968; Broadbent et al., 1971; Kommedahl and Windels, 1978). Similarly one isolate of a Trichoderma sp. antagonistic against one pathogen in vitro is often innocuous against another (Dennis and Webster, 1971 a b; Wells et al., 1972; Hadar et al., 1979). Bell et al., (1982) found that individual isolates of Trichoderma antagonistic to one isolate of R.solani belonging to one anastomosis group were not antagonistic to other isolates belonging to the same group or to isolates in other anastomosis groups. Howell and Stipanovic (1979, 1980) found that a strain of Pseudomonas fluorescens isolated from cotton rhizosphere protected cotton seeds against damping-off in soils infested with R.solani and P.ultimum. Two antibiotics pyrrolnitrin effective against R.solani but not P.ultimum, and pyroluteorin effective against P.ultimum but not R.solani, are produced by the same culture and may explain the specificity exhibited for these pathogens. Use of the appropriate antibiotic as a seed treatment gave the same degree of protection against either of the two pathogens as obtained with living cultures. Elad et al. (1982) found that different isolates of T.harzianum produced different levels of hydrolytic enzymes when grown with mycelium of

R.solani, S.rolfsii or P.aphanidermatum in soil. This was correlated with their ability to control the respective pathogens under greenhouse conditions. Bell et al. (1982), proposed that several genes of both antagonist and pathogen were involved in determining the various levels of in vitro antagonism observed. They further proposed that if these and other genetic factors interacted with the environment, the likelihood of finding a single adaptable antagonist of wide "host" range is unlikely.

Lack of success with microbial antagonists may also result from specificity if the "wrong" target pathogens are employed for screening. A large scale empirical approach using many more pathogens and strains of pathogens might be necessary to overcome this. Factors inherent to the antagonist may interact with the environment (soil type, nutrient amendments, moisture, pH and temperature) and the host to limit the use of a potential antagonist further.

In view of this specificity the likelihood of using Trichoderma strains which demonstrate antagonism in wood (Corke, 1978) for successful suppression of pathogens in other environments appears remote. Moreover, the continuous culturing of these strains outside the soil environment may have resulted in the progressive selection of certain characteristics to the detriment of competitive saprophytic ability in soil. Thus although Binab TRC shows promise as a biocontrol agent in both standing and felled timber, its usefulness for the control of soil - borne pathogens is marginal at best.

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