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# EVALUATION OF MICROBIAL EXTRACTS FOR CONTAMINATION CONTROL IN PLANT TISSUE CULTURE SYSTEMS

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# EVALUATION OF MICROBIAL EXTRACTS FOR CONTAMINATION CONTROL IN PLANT TISSUE CULTURE SYSTEMS

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## DECLARATION

The work presented in this thesis was carried out by the candidate himself and due acknowledgement has been made of the assistance received. This work has not been accepted for any other degree and is not concurrently being submitted in candidature for any other award.

## CONTENTS

## ABSTRACT

EVALUATION OF MICROBIAL EXTRACTS FOR CONTAMINATION CONTROL IN PLANT TISSUE CULTURE SYSTEMS

SHAYNE HUSSAIN

Culture filtrates of 13 microbial antagonists exhibited *in vitro* growth inhibition of a range of test contaminations of herbaceous and woody plant tissue culture systems. Filtrates produced by *Bacillus subtilis* and *Trichoderma viride* isolates displayed the greatest broad-range inhibitory activity. Microscopic analysis of antagonized fungal mycelia revealed altered hyphal morphology. Maximum filtrate inhibitory activity was produced when selected antagonists were cultured within a pH range of 5-7 and a temperature range of 20-35°C. Filtrates were thermo-stable at 70°C and could be stored for up to 4 weeks with only a minimal reduction in their inhibitory activity. Bulk-volume production of inhibitory filtrates of a *T. viride* isolate was achieved by optimization of fermentation pH, temperature, and aeration conditions. Plant culture species displayed different responses when grown on media incorporated with microbial filtrates. Microscopy studies at the cellular level revealed reduced cell densities and cellular distortion in plant tissues treated with phytotoxic doses of microbial filtrates. Non-phytotoxic doses of filtrated produced by the *B. subtilis* and *T. viride* isolates produced a reduction in the density of opportunistic contaminations in herbaceous plant tissue cultures when applied as prophylactic treatments. Microbial filtrates proved totally ineffective when employed as post-infection sterilants in contaminated plant cultures. The efficacy of selected microbial filtrates was not comparable to that of conventional antibiotics when assessed for their ability to control contamination levels in herbaceous and woody plant culture systems. Further purification of microbial filtrates for enhanced inhibitory activity is discussed along with the possibility of co-cultivation of micro-organisms with plant tissue cultures as a means of biocontrol of phytopathogens.

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CHAPTER 1

# INTRODUCTION

Plant tissue culture can be defined as the growing of 'sterile' plant cells or tissues separate from the mother plant on artificial media under optimum growth conditions *in vitro*. It is an important technique in the study of plant metabolism, plant genetics, plant morphogenesis and plant physiology, in the genetic transformation of plants, in elimination of plant pathogens, in preservation of important plant species in limited space, and in rapid multiplication of plant tissues *in vitro* ("micropropagation") (George & Sherrington, 1984; Grierson & Covey, 1984).

The *in vitro* culture of plant cells and tissues takes place on a medium containing sucrose, mineral salts and vitamins. This substrate is clearly under continual threat of contamination by bacteria or fungi. Although aseptic conditions are usually implied, many cultures are not or do not stay aseptic *in vitro* and contamination by microorganisms is considered to be the single most important reason for losses during *in vitro* culture of plants (Boxus & Terzi, 1987; Leggatt *et al*, 1988; Leifert *et al*, [in press]). Chronic contamination by microorganisms is thus a major problem faced by all commercial micropropagation companies (Constantine, 1986). Short-term solutions to contamination problems such as the application of antibiotics and chemotherapeutic agents are unlikely to eliminate the risk completely. Such anti-microbial agents are often phytotoxic or may retard plant tissue growth (Dodds & Roberts, 1981; Mathews, 1988) and continued use may lead to dependence on the drug (Falkiner, 1990).

There exists a huge potential for the application of biologically-based control systems to *in vitro* plant culture techniques. If biocontrol agents could be employed to eliminate airborne contaminants and endogenous infections from cell and tissue cultures, then the requirements for asepsis in plant propagation techniques could be reduced. The complete automation or partial mechanization of micropropagation techniques (cost-saving systems which are particularly vulnerable in terms of increased contamination problems) could then be facilitated. Stages of micropropagation such as shoot multiplication which involve high labour costs and repetitive processes are most worthy of mechanisation (see Cassells, 1990).

### 1.1 Contaminants of plant tissue culture and media

The list of organisms described as contaminants in plant tissue cultures and media includes viruses, bacteria, yeasts, fungi, mites and thrips (Blake, 1988; Enjalric *et al*, 1988; Leggatt *et al*, 1988). Contamination with bacteria, fungi, and yeasts are considered the most serious and have been described extensively in the literature (Knauss & Miller, 1978; Trick & Lingens, 1985; Boxus & Terzi, 1988; Leifert *et al*, 1989; Leifert *et al*, [in press]). Contaminant bacteria may exceed the number and weight of any other group of microorganisms on tissue culture media, and their rapidity of growth and ability to utilise different forms of nutrients under widely different conditions is surpassed by no other group. Mites and thrips found in tissue cultures do not usually harm the plant cultures directly but introduce other contaminants into sterile plant cultures (Blake, 1988). Fungi, yeast and many bacterial contaminants produce visible growth in plant culture medium and are usually detected during visual assessment of cultures. However, some contaminants, especially bacteria, do not produce visible growth in the plant or propagation media and these are usually described as being 'latent' or 'endogenous' (Fisse *et al*, 1987; Cassells *et al*, 1988). Some of these contaminations can live

endogenously in plant tissues without causing disease symptoms. The significance of these associations is not yet fully understood. Many latent infections are, however, distinctly pathogenic towards plants (Hayward, 1974). *In vitro* culture media is particularly conducive to the rapid growth of such infections and allows them to multiply and overgrow the explant (Knauss & Miller, 1978; Deprest *et al*, 1980). Contamination by pathogenic latent bacteria can result in reduced multiplication rates of *in vitro* plant cultures and often sudden death of whole plant cultures after many sub-cultures is induced (De Fossard & De Fossard, 1988; Long *et al*, 1988). Latent infections are particularly a problem in woody plant cultures (Young *et al*, 1984) and may only become apparent after a number of passages in culture, resulting in considerable loss of time and resources (Deprest *et al*, 1980; Debergh & Maene, 1981).

A wide variety of bacterial genera and species have been described as latent and/or accidental contaminants in plant tissue and cell cultures. Important bacterial genera repeatedly isolated by different workers from different plant species are *Bacillus*, *Erwinia*, *Pseudomonas*, *Acinetobacter*, *Agrobacterium*, *Corynebacterium*, *Enterobacter*, *Flavobacterium*, *Micrococcus*, *Staphylococcus* and *Xanthomonas* (Knauss, 1976; Cassells, 1986; Boxus & Terzi, 1987; Cornu & Mitchell, 1987; Mathias *et al*, 1987; Podwyszynska & Hempel, 1987; Enjalric *et al*, 1988; Hennerty *et al*, 1988; Long *et al*, 1988; Reuther, 1988). Relative numbers of contaminant bacteria present in plant tissue cultures and media vary considerably depending on factors such as explant origin and method of sterilisation, type of plant species and growth medium, cultural conditions, length of time the plant material has been in culture. Yeasts have been described as contaminants in plant tissue cultures and media (Boxus & Terzi, 1988; Enjalric *et al*, 1988; Leggatt *et al*, 1988). The most frequently isolated yeast species are *Candida* and *Rhototorula* (pink yeasts) species (Leifert *et al*, [in press]). These species

belong to a group of osmophilic yeasts which show a high sugar and salt tolerance (Tibury, 1980) and hence are well adapted to grow in plant culture media. Fungi are repeatedly found in plant tissue cultures and media. Fungi isolated from plant tissue cultures include *Neurospora, Aspergillus, Penicillium, Botrytis, Microsporium, Cladosporium and Philophora* (Enjalric, 1988; Hussain & Lane [in press] ).

### 1.2 Sources of contamination

Fungi, yeasts and bacteria can be introduced into *in vitro* cultures with the plant material if the initial surface sterilisation is inefficient (Darvill & Alberhiem, 1984; Cornu & Mitchell, 1987). High levels of bacteria and fungal sporophytes on the plant tissues taken as explants may reduce the success of the initial sterilisation (Duhem *et al*, 1988; Knauss, 1976). Most laboratories use sodium hypochlorite or various commercial bleaches routinely for surface sterilisation of explants but other chemicals such as mercuric chloride, alcohols, fungicides and antibiotics have been used for surface treatment (see Falkiner, 1990). Concentrations and exposure times for the different chemicals depend greatly on the type and size of the explant. Only the outer tissues of the explant which come in contact with the chemicals are disinfected. A successful treatment is only possible, therefore, if the internal tissues which were not reached by the chemicals are free of contaminants. Sudden appearance of visible growth of contaminants at later *in vitro* stages after many sub-cultures has been attributed to contaminants which were introduced with the initial plant material. The apparent lag period between introduction and appearance of visible growth of the contaminants can be explained by the presence of latent or endogenous bacteria, which need to adapt to the *in vitro* environment before their appearance (Bastiens, 1983; Cassells, 1986; Cornu & Mitchell, 1987; Fisse *et al*, 1987).

equipment contamination

However, this latent population is not the only source of infection. Individual *in vitro* plant cultures must be manipulated, dissected and sub-cultured on many different occasions in micropropagation systems. At each subculture the plant material is subjected to risk from contamination either by faulty handling, by faulty equipment such as unclean filters in laminar flow cabinets, or inadequate sterility within the general transfer area. The introduction of contaminants during the handling of clean plant cultures has been described by various authors (Boxus & Terzi, 1987; Kunnemann & Faaji-Groenen, 1988). Infection with bacteria of the genus *Bacillus* (which form alcohol and heat resistant endospores (Claus & Berkeley, 1986; Sneath *et al*, 1986) due to inefficient sterilisation of instruments used for handling of plants has been reported (Boxus & Terzi, 1987). Spread of these bacteria which are resistant to instrument flaming and able to survive in alcohol for several hours from infected to non-infected plant cultures via the alcohol used to sterilise instruments may then occur. The survival of alcohol resistant contaminants in Dettol and sodium hypochlorite solutions as also been reported (Kunnemann & Faaji-Groenen, 1988). Heat resistant *Bacillus* species have been reported to survive the autoclaving of media. Autoclaving for 20 minutes at 110$^{o}$C was reported to allow the survival of "a more thermostable germ", which could be killed by doubling the autoclave time (Boxus & Terzi, 1987). Inefficient aseptic technique during media preparation and pouring into gamma-irradiated plastic containers subsequent to autoclaving has been demonstrated by sterility testing of the poured media (Leifert et al, 1989).

### 1.3 Effects of contaminants on in vitro plant growth

Bacterial contaminants found on explants are usually plant pathogens, opportunistic plant pathogens or saprophytic bacteria living in the rhizosphere or on aerial plant surfaces, and many produce similar symptoms on *in vivo* and *in vitro* plants (Duhem *et al*, 1988; Enjalric *et al*, 1988; Leifert *et al*, 1989). Many

Many of the non-pathogenic plant inhabiting bacteria found on explants are adapted to utilise dead or stressed plant tissue and the nutrients of the plant medium for their metabolism Krieg & Holt, 1984; Sneath *et al*, 1986) and usually overgrow the explant after transfer onto initiation media.

Many latent bacteria do not grow or persist on the growth medium of Murashige and Skoog (1962) in the absence of plant material (Trick & Lingens, 1985). For most latent bacterial contaminants, competition for the mineral and carbohydrate nutrients in the medium is less likely to be an important reason for reduction in plant growth. Bacterial contaminants have, however, been shown to reduce plant growth and/or kill plants by producing phytotoxic metabolites such as lactic acid (Leifert *et al*, 1989) and bacterial metabolites such as organic acids, antibiotics, and plant growth regulators have been linked to plant growth reductions *in vivo* (Lynch, 1976; Lynch, 1978; Bakker & Schippers, 1987). Yeast contaminants grow well on plant media even in the absence of plant material (Leifert *et al*, [in press]). By reducing the medium pH to below 3, by metabolising much of the carbohydrate in the medium and by producing phytotoxic fermentation products such as ethanol and acetic acid, *Candida* and *Rhototorula* yeasts create an extremely unfavourable environment for plant growth. Yeast contamination usually results in plant death within 1 to 3 sub-cultures after introduction into plant tissue cultures. Like the yeasts, fungal contaminations also thrive on plant tissue culture media and often they may overgrow the explant (Boxus & Terzi, 1987). Observable detrimental effects on plant cultures include necrosis ('browning' and subsequent death of cultured explants), chlorosis of leaves, and reduced growth rates (Leifert, 1990).

## 1.4 Control of contaminants

Microbial contamination of any process has considerable implications. Human safety and financial losses are the main considerations. The preferred method of prevention of contamination is by careful attention to aseptic technique, the use of cabinets with sterile filtered air, and judicious choice of disinfectant such as hypochlorite or alcohol for the decontamination of the plant tissue (Watts & King, 1973; De Fossard & De Fossard, 1988; O'Riordain, 1988). Should these precautions prove insufficient, and financial losses are faced, remedies must be invoked to reduce these contamination problems.

Antibiotic agents have been considered for the prevention, or at least control, of microbial contamination of plant tissue cultures for many years (Katznelson & Sutton, 1951; Morgan & Goodman, 1985). In general, latent endogenous bacterial infections must be eliminated through meristem culture. However, rifampicin has been an effective agent for cultured tissues infected with bacteria (Phillips *et al.*, 1981). Problems are often encountered when employing antibiotics in such systems, and thus their application is not generally recommended. Often antibiotics target a specific pathogen. In plant tissue culture systems , however, a wide range of contaminant organisms may be present which therefore necessitates the use of a combination of antibiotics.

In shoot cultures of woody plants combinations of several antibiotics have proved more effective than any one used singularly. However, such combinations may not always be compatible *in vitro* (Young et al, 1984). Antibiotics may reduce or totally inhibit growth and secondary metabolite production and they tend to select for resistant biotypes (Cullen & Andrews, 1986).

Antibiotic compatibility with plant tissue culture media is also important. A wide range of different plant tissues may be cultured and these are grown on media whose constituents may vary considerably. For clinical purposes

antibiotic sensitivity tests are performed on the simplest possible media which allow growth, to limit the possible interactions between the constituents of the medium and the antibiotic. Some problems are quite specific and well documented, such as the effect of magnesium on the activity of 'gentamicin' towards *Pseudomonas aeruginosa* (Garrod & Waterworth, 1969). The activity of some antibiotics may be significantly affected by pH changes; streptomycin is 500 times as active at pH 8.5 than at pH 5.5 (Garrod *et al*, 1973).

As many antibiotics are toxic to certain plant species or tissues it is necessary to test selected antibiotics and antibiotic cocktails for phytotoxic effects on *in vitro* plant cultures for a given application, and a non-phytotoxic dose selected. Such phytotoxic assessments are presented [Table 1.4.1]. The problems connected with the use of antibiotics together with increasing awareness of possible deleterious effects of fungicides and chemotherapeutic agents on the ecosystem has led to a growing interest in biological control of plant pathogenic microorganisms (Mukerji, 1983).

what about grown resistant new bacteria — due to controlled conditions?

Table 1.4.1 The range and phytotoxicity of antibiotics and chemotherapeutic agents employed in plant tissue culture systems.

| Antibiotic | Target Organism | | | | Microbial Toxicity ($ugml^{-1}$) | Plant Toxicity ($ugml^{-1}$) | Applications ($ugml^{-1}$) |
|---|---|---|---|---|---|---|---|
| | A | B | C | D | | | |
| Amphotericin B | - | - | + | + | 2.5 | >5 | cc, stm |
| Carbenicillin | + | - | - | - | 500 | >1000 | cc, pr, stm |
| Cefotaxime | - | + | - | - | 90 | >100 | cc, pr |
| Chloramphenicol | - | - | - | - | 120 | >10 | 10-35 es |
| Gentamicin sulphate | + | + | - | - | 50 | >100 | cc |
| Nystatin | - | - | + | + | 50 | >40 | cc, stm |
| Rifampicin | + | + | - | - | 15 | >25 | es |
| Kanamycin | + | + | - | - | 100 | >2 | cc, stm |
| Vancomycin.HCl | + | - | - | - | 5 | >100 | cc, es stm |

es, explant sterilization; cc callus culture; pr, plant regeneration; stm, shoot-tip micro-propagation;

A, Gram positive bacteria B, Gram negative bacteria C, yeasts D, moulds

[Reference source: Sigma Plant Cell Culture Catalogue, 1990]

A major challenge in plant pathology is to introduce or develop new disease control strategies, as the more traditional controls become obsolete, and to do so without the greater use of chemicals. Biologically-based control systems offer many advantages to commercial micropropagation and to society in general. Biological control of plant pathogens and contaminants of plant tissue culture and media is the use of one or more biological processes to lower inoculum density of the pathogen/contaminant or reduce its disease producing activities. Desirable attributes of anti-microbial organisms and their metabolic products for use in biocontrol systems would include:

(1) Large capacity for anti-microbial activity *in vitro*

(2) Broad spectrum activity against microbial contaminants and pathogens

(3) Non-toxic to consumers (e.g. humans, farm animals)

(4) Not deleterious to the normal growth and metabolism of plant cultures

(5) Simple biosynthetic pathways to facilitate genetic manipulation

(6) Active in an environment conductive to the contaminant or pathogen and stable in variable adverse conditions

(7) Compatible with plant culture growth media

(8) Compatible with other biocontrol agents and/or integration with antibiotics for an enhanced spectrum of activity

(9) Tolerant to tissue culture manipulations (sub-culturing and environmental modifications)

(10) Systemic in plant tissues

(11) Minimal risk of contaminant resistance

(12) Anti-microbial extracts should be stable and easily sterilised

(13) Not affected by changes in pH of growth media

(14) Inexpensive to produce on a large scale

(15) Low toxicity and low allergenic agents preferred

Antagonistic bacteria and their metabolites are extremely important in the biological control of plant pathogens. Antagonists are microorganisms with potential to interfere with the growth or survival of plant pathogens and thereby contribute to biological control (Mukerji, 1983). Bacteria produce "natural antibiotics" (Baker & Cook, 1974). Various phytosphere bacteria have been shown to produce potent antifungal compounds with a wide spectrum of activity, including *Pseudomonas cepacia, P.fluorescens, Erwinia herbicola, Serratia liquifaciens and Bacillus* species (Becker & Hedges, 1986). Some *Pseudomonas* spp. produce antibiotics effective against other bacteria (Fermor & Lynch, 1988; Sakthivel *et al.*, 1986), and certain *Bacillus* species, especially *B.subtilis*, produce antibiotics with activity against all types of pathogenic microflora (see Broadbent *et al.*, 1971; Schreiber *et al.*, 1988).

The parasitism of one fungus by another ("mycoparasitism") has been recognized for over a century. A great number of fungi have been observed growing on other fungi in nature, and where a nutritional relationship is demonstrated, the antagonist is termed a "mycoparasite" (Baker, 1987). Since parasitism may eventually culminate in the death of host cells, mycoparasitism may be adopted in biological control of plant pathogenic fungi and fungal contaminants of plant tissue culture media to substantially reduce their inoculum densities (Baker & Cook, 1974). Some parasites may increase their biomass exclusively by mycoparasitism, whereas others antagonize hosts only during competition for substrates. Therefore, effective management of host, substrate and pathogen interactions can enhance the activity and biocontrol potential of mycoparasites. A number of mycoparasites have been reported, including the genera *Trichoderma, Woronia, Rozella, Gonatobotrys, Gonatobotryum, Calcarisporium, Piptocephalis, Tieghemiomyces, Syncephalis, Dimargaris* and *Dispira* (Barnett *et al*, 1964). Various authors have reported the application of mycoparasites as biological control agents against phtopathogenic fungi (Sierota, 1976; Abd-El-Moity & Shatla, 1979;

Lynch & Heben, 1985; Labudova & Gogorova, 1988; Vakilli, 1984). In particular, the role of *Trichoderma* as a biocontrol agent has been exploited by many workers (see Mukerji, 1983), though there are no reports of its use as a control agent in plant tissue culture systems. Morris and Lane (1990) noted that specific strains of *Trichoderma* were effective for optimum control of *Botrytis*, particularly *B.fabae*, when grown in dual culture. In particular, pre-inoculation of *B.fabae* with culture filtrates and spore suspensions of *Trichoderma* offered some preventative control.

Co-culture of viable mycoparasites as a means of controlling microbial pathogens in plant tissue culture systems has been reported (Herman, 1987), but competition effects often lead to overgrowth of the plant tissue by the antagonist itself. However, the antimicrobial substances which these antagonists produce provides a feasible alternative (Morris & Lane, 1990). As these antimicrobial substances are produced by organisms that are natural plant symbionts or live in close association with the intact plant, then it is possible that they are less toxic to plant cultures than conventional antibiotics. Exploitation of the antimicrobial activity of bacterial antagonists and mycoparasites and their respective metabolites could facilitate efficient biocontrol protocols in plant cell and tissue culture systems.

### 1.5 Aims of present study

Attempts have been made to develop a biologically-based control system employing the antagonistic properties of microorganisms to eliminate airborne contaminants and endogenous latent infections from plant tissue culture systems. Using such a control system, the requirements for asepsis in plant propagation techniques could be reduced and the complete automation of micropropagation techniques could be facilitated.

The effects of a range of known microbial antagonists (Barnett *et al*, 1964) have been surveyed against a spectrum of common air-borne

contaminants and isolated latent infections of plant tissue cultures. Culture filtrates produced by antagonistic micro-organisms have been examined in terms of their capacity to suppress and inhibit growth of test contaminations, both as a short- and a long-term response (see Schreiber *et al*, 1988; Kope & Fortin, 1989). Extracts produced by antagonists have been screened for any deleterious effects on the growth and development of test callus and organ cultures of a range of herbaceous and woody culture systems at the macro and micro level. Non-phytotoxic doses of extracts have been applied as prophylactic treatments and as sterilising agents so to determine their application as infection control agents. Selected extracts have been improved by manipulation of the culture environment of the antagonist in order to optimize control potential. Further studies involved an assessment of the systems identified in terms of their potential for broad-spectrum use as sterilants and antimicrobial agents in plant tissue culture systems. This related primarily to the control of endophytic organisms and latent infections in herbaceous and woody culture lines - a factor which has been acknowledged to be a major barrier to micropropagation in many instances (Wilkins *et al.*, 1985). The use of biocontrol and chemical sterilisation procedures in integrated approaches to disease elimination in cultured tissues has been evaluated, with a view to possible commercial use.

CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Plant tissue culture studies

### 2.1.1 *Plant materials*

*In vitro* cultures of various plant species were employed throughout this study. These included tobacco (*Nicotiana* spp.) and witch-hazel (*Hamamelis* spp.), both provided by Polytechnic South West stock plant collections, and birch (*Betula* spp.) and rosewood (*Dalbergia* spp.), supplied by Novalal plc., Surrey, England. Prior to explant removal, parent stock plants were carefully screened for evidence of disease or inferior growth and any such material was discarded, thus ensuring that plant cultures were established from healthy, disease-free stock material.

### 2.1.2 *Growth media*

Cultured plant species were grown on the basic mineral medium of Murashige & Skoog (MS) (1962) [Table 2.1.2.1] supplemented with various plant growth regulators and vitamins [Table 2.1.2.2]. The basic media was supplied in powder form by Flow Laboratories, Irvine, Scotland, and was reconstituted using distilled water. Shoot-tip cultures of witch-hazel were grown on a composite medium [Table 2.1.2.3]. Media sterilization was by autoclaving at 121$^{O}$C at 15psi for 15 minutes.

**Table 2.1.2.1** Basic mineral medium of Murashige & Skoog (MS) (1962) for growth of various plant species and witch-hazel (WH) composite media.

| Mineral | Murashige & Skoog (MS) ($mgL^{-1}$) | Witch-hazel ($mgL^{-1}$) |
|---|---|---|
| $CaCl_2.2H_2O$ | 440.000 | 110.000 |
| $FeSO_4.7H_2O$ | 27.800 | - |
| $KH_2PO_4$ | 170.000 | 42.500 |
| $KNO_3$ | 1900.000 | 475.000 |
| $MgSO_4.7H_2O$ | 370.000 | 92.500 |
| $FeNa_2.EDTA$ | 37.300 | 20.000 |
| $NH_4NO_3$ | 1650.000 | 412.500 |
| $CoCL_2.6H_2O$ | 0.025 | 0.025 |
| $CuSO_4.5H_2O$ | 0.025 | 0.025 |
| $H_3BO_3$ | 6.200 | 6.200 |
| KI | 0.830 | 0.830 |
| $MnSO_4.4H_2O$ | 22.300 | 18.000 |
| $Na_2MoO_4.2H_2O$ | 0.025 | 0.250 |
| $ZnSO_4.7H_2O$ | 8.600 | 8.600 |
| Nicotinic acid | 0.500 | - |
| Pyridoxine HCl | 0.100 | - |
| Thiamine HCl | 0.100 | 6.400 |
| Myo-Inositol | 100.000 | 100.000 |
| Glycine | 3.000 | - |
| BAP | - | 2.500 |

[Supplemented with 2% (w/v) sucrose, 1% Difco agar. pH 5.6. BAP: Benzyl amino purine].

Table 2.1.2.2 Supplements to Murashige & Skoog (MS) (1962) media

| Plant Species | Supplements ($mgL^{-1}$) |
|---|---|
| Tobacco (callus) | 2.0 IAA<br>0.2 kinetin |
| Tobacco (root) | 0.2 IAA<br>0.2 kinetin |
| Tobacco (shoot) | 0.02 IAA<br>1.0 kinetin |
| Birch | 0.5 BAP |
| Pineapple | 2.0 BAP |
| Rhododendron | 0.1 BAP |
| Rosewood | 0.1 pantothenic acid<br>0.1 folic acid<br>0.1 biotin<br>2.0 kinetin |

IAA, indole acetic acid; BAP, benzyl amino purine

## 2.1.3 Plant culture and manipulations

Tissue explants were sterilized in 70% ethanol for 1 minute, followed by immersion in 10% sodium hypochlorite (commercial bleach) + Tween 80 wetting agent (2 drops per 100ml) for up to 20 minutes dependant on species. Explants were rinsed three times in distilled water. Stock cultures were grown in 200ml glass jars containing approximately 50mls of agar medium. Four explant tissue sections were incubated on each dish. All jars were sealed with parafilm to reduce the risk of accidental contamination. Cultures were incubated at 21-25$^{o}$C and sub-cultured after 28 days growth. Stock callus and root cultures were maintained in darkness. Shoot cultures were cultured under artificial lighting, provided by parallel 'cool white' fluorescent tubes installed above and below the cultures. The luminous intensity was 2000 Lux.

All tissue transfers and manipulations were conducted within sterile air flow hoods (Slee Ltd., London, England). All instruments used for aseptic work were sterilized by autoclaving at 121$^{O}$C (15 psi) for 15 minutes. Filter-sterilization of solutions was performed using Minisart NP sterile filter units (pore size 0.22um) in conjunction with sterile syringes.

2.1.4 *Infection testing of plant material*

All *in vitro* stock plant cultures were routinely checked for microbial contamination at each sub-culture. Callus and agar samples were plated out on to Nutrient Agar (NA) and Malt Agar (MA) and observed for contaminant growth after incubation at 22$^{O}$C and 30$^{O}$C, respectively, for 2-3 days. If bacterial growth was not visible culture sections were transferred into a 25ml screw-capped bottle containing 10ml of sterility test medium consisting of half-strength Murashige & Skoog medium (1962) without hormones but supplemented with ($gL^{-1}$): Nutrient Broth (No.2 Oxoid), 10; yeast extract (Difco), 10; beef extract (Difco), 3; glucose, 5 and incubated at 30$^{O}$C for 3 weeks, as described by Leifert *et al.* (1989).

## 2.2 Isolation and identification of contaminants

Characterization of contaminations can yield important information about the source of infections, the importance of different sources, and the approach which has to be taken to eliminate contaminations. Certain contaminations are exclusive to the skin or other tissues of humans or other mammals, such as *Staphylococcus epidermis* and *Lactobacillus acidophilus* (Kandler & Weiss, 1986; Kloos & Schleifer, 1986) and thus their presence in plant cultures suggests the introduction of these contaminations via the handling of plant material. A number of bacterial species are more likely to be introduced from the stock plant itself as a direct result of insufficient sterilization procedures. Contaminant species previously found exclusively on

stock plants include *Pseudomonas fluorescens* (Palleroni, 1984), *Agrobacterium radiobacter*, and *Erwinia caratovora* (Lelliot & Dickey, 1984). Leifert *et al.* (1989) stressed the importance of a reliable identification method which can separate the various species of contaminations found on micro-propagated plants as a requirement for the successful production control and prevention or elimination of infections. These sentiments are echoed in this review, though in preliminary studies the broad-spectrum activity of anti-microbial agents towards ubiquitous genera of contaminations is investigated.

### 2.2.1 *Isolation of micro-organisms*

Micro-organisms were isolated from the following plant species grown *in vitro* and obtained from Polytechnic South West stock plant collections, and from commercial micro-propagation laboratories: *Nicotiana, Hamamelis, Rhododendron, Betula, Ananus*, and *Dalbergia*. Micro-organisms were isolated from stock explants and shoot cultures which had been grown *in vitro* for 6 weeks and 5 months, respectively. Infected callus cultures exhibited grey or brown calli and reduced growth rates. Shoot cultures of *Nicotiana, Ananus,* and *Dalbergia* exhibited severe leaf chlorosis. The appearance of latent endogenous bacterial infections in shoot-tip cultures of *Hamamelis* resulted in a rapid decline in the health of the host plant culture and its subsequent death.

Sections of plant cultures and their agar exhibiting disease symptoms were placed into nutrient broth plus 5% w/v sucrose (NBS) and incubated at 27$^{o}$C for 48 h or until obvious microbial growth occurred (Leggatt *et al.* 1988). The micro-organisms were then plated onto nutrient agar with 5% w/v sucrose (NAS) to produce separate colonies which were re-plated until only single types of colonies were evident. Fungal and yeast infections were isolated directly from the surface of contaminated shoot cultures, growth agar, and calli, and inoculated onto malt agar plates with 5% w/v sucrose (MAS). Plates

were incubated at 22$^{O}$C for 48 h and visible micro-organisms were re-plated to give single type lawns.

### 2.2.2 *Characterization of isolates*

Each bacterial and fungal isolate was examined after growth on NAS (27$^{O}$C for 48 h) and on MAS (22$^{O}$C for 48 h), respectively. Bacterial characterization included colony colour and morphology, Gram's stain (Mackie & McCartney, 1956), appearance and motility under phase contrast microscopy, catalase production by addition of hydrogen peroxide (Mac Faddin, 1980), oxidation/fermentation test (Hugh & Leifson, 1953), heat resistance tests (growth at 55$^{O}$C), and gelatin liquefaction. Ability to grow on Pseudomonas agar was used to distinguish fluorescent *Pseudomonas* species. Heat-resistant Gram-positive rods were tentatively identified as *Bacillus* species; Gram-positive, catalase-negative rods were tentatively identified as *Lactobacillus* species; Gram-negative, oxidase-negative rods were tentatively identified as *Erwinia* species (Table 2.2.2.1). Fungal and yeast morphology was examined visually and microscopically.

**Table 2.2.2.1** Identification of bacterial infections.

| Characteristic | *Bacillus* | *Erwinia* | *Pseudomonas* | *Lactobacillus* |
|---|---|---|---|---|
| Colony colour on NA | Beige | Beige | Beige | Beige |
| Gram stain | + | - | - | + |
| Rods | + | + | + | + |
| Motility | + | + | + | + |
| Catalase | + | + | + | - |
| Heat resistance | + | - | - | - |
| Hugh & Leifson | + | + | - | - |
| Oxidase | ND | - | + | - |
| Gelatin liquefaction | + | - | + | - |
| *Pseudomonas* agar | - | - | + | - |

ND, not detected.

### 2.2.3 *Source of contaminations*

Those contaminations isolated from propagation media and from stock explants and shoot cultures which had been grown *in vitro* for 6 weeks and 5 months, respectively, were noted (Table 2.2.3.1).

Table 2.2.3.1 Contaminations isolated from micro-propagated plants and propagation media.

| Plant species | Propagation media | Stock explants | Shoot cultures |
|---|---|---|---|
| *Nicotiana* | *P.chrysogenum*<br>*A.nidulans* | *Erwinia spp.*<br>*P.fluorescens* | *A.niger*<br>*Botrytis spp.* |
| *Hamamelis* | - | - | *Bacillus*<br>*Erwinia*<br>*Pseudomonads* |
| *Betula* | *Botrytis* | - | *Lactobacillus* |
| *Dalbergia* | *Botrytis* | - | *Lactobacillus* |
| *Ananus* | *P.chrysogenum*<br>Pink yeasts | *P.chrysogenum* | - |
| *Rhododendron* | *A.niger* | - | *Bacillus*<br>*P.fluorescens* |

## 2.3 Microbial antagonists

### 2.3.1 *Literature survey of potential antagonists*

An extensive literature survey of potential microbial antagonists was conducted using a DIALOG database system in combination with Polytechnic South West Learning Resource Centre and British Library reference resources. The range of micro-organisms surveyed and considered for screening in these studies is shown (Appendix I). Clearly, due to constraints on material resources and laboratory time it was not possible to screen all of the potential antagonists listed. The criteria upon which selection of these micro-organisms for screening studies was based included their history and potential as biocontrol agents, their availability, and their purchase cost.

**Table 2.3.2.1** The range, source and reported activity of microbial antagonists included in preliminary screening programmes.

| Antagonist | Strain | Source | Anti-fungal activity | Anti-bacterial activity |
|---|---|---|---|---|
| A Bacterial | | | | |
| *Bacillus subtilis* | SR1 | AGC | + | + |
| *Bacillus subtilis* | SR2 | AGC | + | + |
| *Pseudomonas fluorescens* | 95.6 | AGC | + | + |
| *Pseudomonas fluorescens* | 106.6 | AGC | + | + |
| *Citrobacter freundi* | 69.5 | AGC | - | + |
| *Enterobacter aerogenes* | 85.4 | AGC | - | + |
| *Staphylococcus gallinarum* | 215.4 | AGC | - | + |
| *Gram positive Coryneform* | 221.4 | AGC | - | + |
| *Alcalcigenes faecalis* | 106.1 | AGC | - | + |
| B Fungal | | | | |
| *Piptocephalis virginiana* | 70910 | CMI | + | - |
| *Syncephalis nodosa* | 25/1875 | CMI | + | - |
| *Calcarisporium hiemophillum* | 274831 | CMI | + | - |
| *Dispira parvispora* | 101210 | CMI | + | - |
| *Ampelomyces quisqualis* | 272851 | CMI | + | - |
| *Dimargaris verticillata* | 278511 | CMI | + | - |
| *Trichoderma viride* | 24039 | PSW | + | + |
| *Trichoderma viride* | 170657 | PSW | + | + |
| *Trichoderma viride* | 153416 | PSW | + | + |
| *Trichoderma viride* | 45553ii | PSW | + | + |
| *Gliocladium roseum* | 40022 | PSW | + | + |

AGC - Agricultural Genetics Company Limited; PSW - Polytechnic South West;

CMI - Commonwealth Mycological Institute.

*2.3.2 Stock antagonists*

Stock cultures of microbial antagonists exhibiting potential as *in vivo* control agents at the field level (see Barnett et al, 1964; Barnett & Binder, 1973; Becker & Hedges, 1986) were supplied under licence from the Agricultural Genetics Company Limited, Cambridge, and from Polytechnic South West culture collections, Plymouth. Mycoparasitic antagonists were also supplied by the Commonwealth Mycological Institute, Kew, Surrey. The range, source, and reported spectrum of activity of these microbial antagonists is summarised in Table 2.3.2.1. Stock bacterial cultures were inoculated on to nutrient agar slopes and incubated at 30$^{o}$C for 72 hours. Stock fungal cultures were inoculated onto malt agar slopes and incubated at 22$^{o}$C for 72 hours.

*2.3.3 Production of microbial extracts*

Fungal and bacterial antagonists were cultured in malt broth (pH 5.6, 22$^{o}$C) and nutrient broth (pH 6.2, 30$^{o}$C), respectively, for 8 days on a rotary shaker (120rpm). Cultures were centrifuged at 2000rpm (5 min) and filtered twice through Whatman No.1 filter paper to remove as much of the microbial material as possible. Crude filtrates from replicate flasks were pooled, to give 500ml crude filtrate, which was then concentrated by evaporation at 50$^{o}$C to 50% volume.

*2.3.4 Extraction of active preparations*

Active preparations were extracted from the concentrated crude culture filtrates by precipitation with (1) acetone, (2) methanol, and (3) chloroform (see Dennis & Webster, 1971a). Two successive extractions, each with 50ml of organic solvent, were carried out by shaking the solvent with the aqueous fraction in a separating funnel. An emulsion formed which was dispersed by centrifugation at 3000rpm for 5mins. After centrifugation the two layers were carefully poured into a separating funnel. The heavier solvent fraction was allowed to separate out and run off from the aqueous layer. The pooled

extracts were dried in a rotary vacuum evaporator at room temperature. Dried extracts were re-dissolved in a minimum amount of solvent and further concentrated by freeze-drying [Barath *et al.* 1964]. This residue was re-dissolved in 5ml of the organic solvent (80%), filter-sterilized (0.22um), and stored at $4^{O}C$. In addition to partial purification, the extraction procedure ensured that unwanted growth media would not be provided for the target contaminant micro-organisms.

## 2.4 Preliminary screening methods

The usual method of detecting antimicrobial activity in preparations of unknown activity is by means of a simple diffusion test, where filter paper discs saturated with the preparations are placed on agar bioassay plates seeded with test micro-organisms (see Kavanagh, 1972). After incubation of the plates, clear zones of inhibited microbial growth around the disk indicate the presence of anti-microbial activity in the preparation. Caution must be exercised when interpreting the results of such exclusion zone assays. Diffusion of the anti-microbial compound from the disk is determined by the diffusion constant (a function of the molecular weight of the compound), temperature, pH, salt concentration, and concentration of agar in the assay media (Kavanagh, 1963). Inoculum size is also significant, as large inocula reduce inhibition zones to some extent. The ideal inocula is one which gives an even, dense growth without being confluent (Cooper,1972). Thus diffusion assays are relative and standard assay conditions must be specified and suitable controls employed for the assays to have meaning.

2.4.1 *Bioassays for anti-microbial activity*

Microbial lawn bioassays were employed to detect the antimicrobial activity of culture extracts towards test contaminations (Bauer *et al.*, 1966). Suspensions of test contaminants were added to typical molten-agar plant tissue culture media (Murashige & Skoog, 1962) at 42-44$^{O}$C to produce a final concentration of approximately 2000 conidia/ml and 5 000 000 cells/ml for fungal and bacterial isolates, respectively. 10ml of seeded agar was poured into 15cm petri dishes. Sterile bioassay filter paper discs (6mm) were soaked for 2 seconds in microbial extracts and subsequently placed in the centre of bioassay plates. Similar filter paper discs were soaked in solvent and sterile distilled water controls. Fungal and bacterial contaminant bioassay plates were incubated at 22$^{O}$C and 30$^{O}$C, respectively. Clear zones of inhibited microbial growth were measured in 3 replicate plates after 48 h incubation. The diameter of clear zones of inhibited contaminant growth around the discs correspond to the activity of the microbial extracts.

## 2.5 Cultural optimization of extract production

If the anti-microbials produced by the antagonistic microorganisms are to be employed successfully for the control of contaminations of plant tissue culture systems, then it is essential that their activity is maximised. In this study, cultural modifications to the growth of selected antagonists are made, in an attempt to optimize the activity of the culture extracts produced. An assessment of extract stability is also made.

2.5.1 *Cultural optimization and time-course bioassays*

Most micro-organisms which produce anti-microbial metabolites are mesophilic (i.e. the optimum temperature range for growth is within the range 23-37$^{O}$C), (see Porter, 1972). Antibiotics are also known to be produced by certain thermophilic actinomycetes with optima above 50$^{O}$C. The majority of

fungi grow well at room temperature, ordinarily 20-22$^{o}$C, and when incubators are used the temperature is usually set at 23-25$^{o}$C. Some fungi are thermotolerant and grow well above 30$^{o}$C, but most do not (Persson-Huppel, 1963). While pathogenic bacteria frequently require incubation temperatures of 37$^{o}$C, most antibiotic-producing strains are grown at temperatures of 32$^{o}$C or lower (Porter, 1972).

Fungi grow well in acid environments, but they are not restricted to them (Racle, 1965). Most will grow normally within a pH range of 4.5 to 8.0. Many bacteria and actinomycetes, however, will not grow at a pH much below 5.5 to 6.0, and the optimum lies between 6.5 and 7.5. The pH of the growth medium changes during growth depending on factors such as the buffering capacity of the medium and the type of metabolites produced (Danielson & Davey, 1973).

In an attempt to optimize the production of antimicrobial activity in shaker-flask systems, cultural modifications were made to the growth of the microbial antagonists. Selected bacterial and fungal antagonists were cultured in 100ml of liquid nutrient broth and malt broth, respectively. The initial pH of the two types of growth media was buffered over a range pH 2 to pH 9 with 1M NaOH and 1M HCl, before autoclaving. Following sterilization for 10 mins at 121$^{o}$C (15 p.s.i.), replicate flasks were incubated on rotary shakers (120rpm) over a temperature range of 15-30$^{o}$C. Culture filtrates were bioassayed for anti-microbial activity (sections 2.4.1) after 4 and 8 days incubation of the bacterial and fungal antagonists, respectively.

Culture filtrates of antagonists were then cultured under these optimum conditions of pH and temperature for up to 10 days so as to determine the optimum time in culture for maximum antimicrobial activity. Filtrate samples were aseptically removed from shaker flasks every 48 h and bioassayed (section 2.4.1).

2.5.2 *Fermenter scale-up studies*

If only small quantities of anti-microbial metabolites are desired, it is customary to grow cultures in selected liquid media in flasks placed on a reciprocating or rotary shaking apparatus (see section 2.3.2). When larger quantities of the metabolites are required (e.g. for large-scale commercial application) it is necessary to scale-up the fermentation into stirred aerated bottles or tanks. In this case the conditions which have first been established in flasks (see section 2.5.1) may have to be modified to produce maximum activity in larger volumes of media. In general, microorganisms can be grown in the same media minus agar that are employed for culture maintenance, but some modifications to the media composition, and also to culture conditions such as temperature, aeration, and pH, must be made to in order to promote a desirable level of anti-microbial metabolite (see Hutter *et al.*, 1978). Each microbial culture passes through the stages of growth, metabolite formation, and senescence, and the goal of fermentation improvement is to extend the period of biosynthesis (i.e. to achieve and maintain a high level of the desired active component) (see Stanbury & Whitaker, 1984).

Practically all antibiotic producers are highly aerobic organisms and thus aeration must be provided in deep fermentation systems. Air is supplied both by vigorous stirring and by the introduction of air at the bottom of the fermenter. Foaming in tank fermentations may then become a problem, but can be controlled by the addition of anti-foaming agents (e.g. octadecanol in sunflower oil). This addition is carefully controlled as the anti-foam agent may itself be toxic to some organisms, thus causing depressed yields of anti-microbial metabolites (Hutter *et al.*, 1978).

In these studies, attempts were made to optimize the inhibitory effects of filtrates produced by the *T.viride* strain 24039 by modification of cultural conditions. A laboratory scale fermenter was employed to assist optimization of fermentation pH, temperature and aeration, and also to determine that the

bulk-volume production of anti-microbial extracts is achievable. A 2L LH fermenter system, model 502D (Fig.2.5.2.1), was employed for the fermentation of the two microbial antagonists. Temperature, pH, dissolved oxygen content and anti-foam (octodecanol) were all controlled using automated bioreactor control panels. The fermenter vessel, containing 1L of malt broth was sealed and autoclaved at 121$^{O}$C / 15 p.s.i. The sterile vessel was inoculated with a spore suspension from an agar slant of the antagonist (3% v/v). Cultivation temperature, pH and aeration were varied over a series of fermentation runs each lasting 10 days, and samples of the culture filtrate sampled taken and bioassayed (section 2.4) every 48 hours against a susceptible test organism.

2.5.3 *Stability assessments*

Physical characterization of the selected anti-microbial preparations was employed to assess their stability (Schreiber *et al.*, 1988). pH stability was evaluated by buffering extract preparations to different pH values, using 1M NaOH and 1M HCl. Heat stability of extract preparations was assessed by heating samples up to 100$^{O}$C for 30 minute periods, so as to determine the critical temperature above which the filtrate inhibitory activity is reduced. Extract stability in various solvent systems was assessed by shaking 0.1ml of dried extract preparation with 0.2ml of chloroform and methanol. After each of the above treatments, bioassays were conducted (section 2.4.1) to determine the stability of the extract preparations to the various physical conditions. Temporal stability was assessed by storing filtrate samples at 4$^{O}$C and at room temperature for up to 4 months.

## 2.6 Phytotoxicity studies

Agar dilution methods (see Kavanagh, 1972) were employed to evaluate the phytotoxic effects of those extracts exhibiting anti-microbial

activity in preliminary bioassay tests (section 2.4.1). Various doses (0.5-20%v/v) of microbial extracts were incorporated in to culture media [Table 2.1.2.2] to determine their phytotolerant level. Extracts were added filter-sterilized to 20mls of the autoclaved medium in 9cm petri dishes. Explants and shoot-tips of plant materials were surface-sterilized (section 2.1.3) and cultured on the appropriate growth media, and maintained in a growth room at 21-25$^{O}$C. Artificial light was provided by parallel 'cool white' fluorescent tubes installed above and below the cultures. The luminous intensity was 2000 Lux. Tobacco root cultures were maintained in darkness. The influence on shoot proliferation, shoot length, and fresh weight (callus + organs) were noted after 6 weeks. Similarly, the number and length of roots, and fresh weight of tobacco root cultures were noted over the test period. The recovery capacity of those plant tissues exhibiting reduced growth was assessed in subsequent culture over several passages.

### 2.7 Extract activity against contaminated cultures

The previously determined maximum permissive doses which did not interfere with shoot proliferation were tested for their ability to control latent, endogenous infections of *in vitro* plant culture systems. Shoot tips of a range of woody plant cultures susceptible to latent infections (see section 2.1.1) were excised from healthy parent plants. The tissues were surface-sterilized as previously described (section 2.1.3) and then transferred to appropriate culture media (Table 2.1.2.2) inoculated with the maximum permissive dose of individual and combinations of extracts. In addition, cultures of *Nicotiana* were deliberately contaminated by exposure to a typical non-sterile laboratory environment so that the ability of the extracts to control accidental contaminations could be assessed. All cultures were incubated for 28 days as previously described (2.1.1). The appearance and frequency of atmospheric

contaminations and latent infections in each extract-dose trial was observed over the test period.

### 2.8 Control of bacterial growth in short-term tobacco explant cultures

To determine the effectiveness of various antibacterial agents, tobacco stem explant cultures were deliberately contaminated with growing bacterial suspensions of two types, *Bacillus* and *Pseudomonas* species. Actively growing suspensions of the bacteria were obtained by incubation of pure cultures for 24 h in standard tobacco explant culture media (section 2.1.2). Explant cultures were infected by the addition of 10 µl of one of the bacterial suspensions at the beginning of the explant culture period. The density of the bacterial population was estimated after 48 h and expressed on an arbitrary scale of 0-5, where 0 indicates complete absence of bacteria and 5 indicates mass overgrowth. All antimicrobial agents were filter-sterilized prior to inclusion in explant culture media. The antibiotic Rifampicin was dissolved in a small amount of dimethylsulfoxide prior to dilution with water.

### 2.9 Treatment of witch-hazel donor plants with antimicrobials

Witch-hazel stock plant material was sprayed (to run off) on three occasions at weekly intervals with antibiotics, antagonistic extracts, and chemotherapeutic agents. Control plant material was sprayed similarly with distilled water. The treated plant materials were subsequently employed for establishing *in vitro* shoot-tip cultures. The percentage of contaminated cultures (n=10) arising from the treated stock materials was assessed after 4 weeks incubation at 25$^{O}$C. All experiments were repeated once. The antibiotic Rifampicin was dissolved in a small volume of dimethylsulfoxide prior to dilution in water.

## 2.10 Electron microscopy studies

Plant materials examined in the course of the ultrastructural studies were grown under identical conditions to those used for phytotoxicity studies. Electron microscopy studies were employed to assess the effects of the extract preparations on plant cell and tissue ultrastructure. Preliminary attempts at preserving delicate and friable plant tissues for observation by transmission electron microscopy (Phillips EM 300 TEM, at 80Kv) and scanning electron microscopy (Jeol 35C SEM) using conventional fixing and staining procedures proved unsuccessful, resulting in extensive tissue damage and collapse of cellular structures similar to that reported by Boyde & Franc (1981).

Cold-stage scanning electron microscopy (cryo-SEM) enables biological materials to be observed in a near natural state after freezing, or cryo-fixation (Ryan *et al.*, 1988). Chemical fixation and contact with solvents is avoided, levels of specimen hydration are maintained, low melting-point materials are stabilized, volume changes are minimised (Boyde & Maconnachie, 1979), and internal structures can be revealed by freeze-fracture. The technique thus enables biological samples to be examined rapidly and free from artifacts commonly associated with more conventional methods (Sargeant,1988).

### 2.10.1 *Specimen freezing and cryo-mounting*

Specimen tissues were removed from culture media and washed free of agar with distilled water. Specimen sections ($3mm^3$) were mounted onto a cooled specimen holder block using carbon cement, before being rapidly frozen using a plunge cooling device (200mm into a liquid nitrogen slush, $-196^0C$, atmospheric pressure, at approximately $5ms^{-1}$).

2.10.2 *Cryo-scanning electron microscopy*

The frozen specimen was transferred under vacuum to the preparation chamber (CT-1500, Oxford Instruments) of a JEOL 35C scanning electron microscope. Within the preparation chamber the specimen was supported during freeze-fracture and sputter coating by a stage cooled to approximately -180$^{O}$C. The frozen specimen was fractured under vacuum using a cooled scalpel blade. The specimen was transferred to the microscope stage and the fractured face examined at low kV on the microscope monitor. The stage temperature was raised to -80$^{O}$C for ice sublimation and the fractured face observed until sufficient etching had occurred.

A cooled anti-contaminator plate held at -180$^{O}$C trapped sublimated volatiles. The specimen was transferred to the preparation chamber at -180$^{O}$C and sufficient dry Argon admitted to allow sputter coating of the specimen from a gold target (10 minutes duration). The specimen was transferred back to the microscope stage for examination (25kV) and photographic recording.

CHAPTER 3

# *IN VITRO* GROWTH INHIBITION OF CONTAMINATIONS BY CELL-FREE CULTURE FILTRATES OF ANTAGONISTIC MICRO-ORGANISMS

Previous reports (e.g. Dennis & Webster, 1971a; Herman, 1987; Schreiber *et al.*, 1988; Kope & Fortin, 1989) have demonstrated that anti-microbial compounds present in the cell-free culture media of antagonistic micro-organisms are able to suppress the growth of a range of phytopathogens when bioassayed *in vitro*. The aim of these studies was to identify which, if any, of our antagonists produced cell-free liquid culture extracts which exhibited growth inhibition of typical test contaminations isolated from plant tissue culture systems [section 2.2] on *in vitro* bioassay plates. Any such antagonists would then be selected for further screening. Changes in the morphology of fungal contaminations antagonized by culture extracts were also examined in an attempt to evaluate the mode of action of microbial antagonists.

## 3.1 Screening for *in vitro* antagonism

Culture filtrates of microbial antagonists were bioassayed for *in vitro* growth inhibition of a range of fungal and bacterial contaminations isolated from plant tissue culture systems [Tables 3.1.1. and 3.1.2, respectively]. Extracts produced by 13 of the 21 (62%) antagonistic micro-organisms inhibited the growth of the test contaminations with varying degrees of effectiveness. The remaining 8 antagonists did not affect the phytopathogens in any manner. Extracts of the antagonistic isolates produced variable clear zones of inhibited growth on the different phytopathogen lawns.

Table 3.1.1 Growth inhibition of fungal contaminations by culture filtrates of a range of antagonistic micro-organisms[1].

| | Test Contaminant Species | | | |
|---|---|---|---|---|
| **Antagonist** | *Penicillium* | *Aspergillus* | *Botrytis* | Yeasts |
| *B.subtilis SR1* | 2 | 0 | 1 | 0 |
| *B.subtilis SR2* | 3 | 0 | 1 | 0 |
| *P.fluorescens 95.6* | 1 | 0 | 1 | 0 |
| *P.fluorescens 106.6* | 1 | 0 | 1 | 0 |
| *P.virginiana 70910* | 1 | 0 | 0 | 0 |
| *S.nodosa 25/1875* | 0 | 0 | 1 | 0 |
| *G.simplex 245819* | 0 | 0 | 0 | 0 |
| *C.hiemophillum 274831* | 0 | 0 | 0 | 0 |
| *D.parvispora 101210* | 0 | 0 | 0 | 0 |
| *A.quisqualis 272851* | 2 | 1 | 1 | 0 |
| *D.verticilliata 278511* | 0 | 0 | 0 | 0 |
| *T.viride 153416* | 4 | 2 | 3 | 2 |
| *T.viride 24039* | 4 | 2 | 4 | 2 |
| *T.viride 45553ii* | 3 | 1 | 2 | 1 |
| *T.viride 170657* | 3 | 2 | 2 | 1 |
| *G.roseum 40022* | 1 | 1 | 1 | 0 |

[1] Inhibition zones were measured along the diameter of the filter disc to the circumference of the zone of the inhibited growth. Inhibition zones were monitored after 48 h incubation. Values represent the average of 3 replicates. Appropriate acetone and sterile distilled water controls were provided and did not inhibit any test organism.

Key: **0** zero inhibition **1** 0-5mm **2** 6-10mm **3** 11-20mm **4** >20mm

Table 3.1.2 Growth inhibition of bacterial contaminations by culture filtrates of a range of antagonistic micro-organisms[1].

| | Test Contaminant Species | | | |
|---|---|---|---|---|
| **Antagonist** | *Erwinia* | *Bacillus* | *Pseudomonas* | *Lactobacillus* |
| *B.subtilis* SR1 | 0 | 1 | 0 | 1 |
| *B.subtilis* SR2 | 0 | 2 | 1 | 1 |
| *P.fluorescens* 95.6 | 2 | 0 | 2 | 0 |
| *P.fluorescens* 106.6 | 2 | 0 | 1 | 0 |
| *E.aerogenes* 85.4 | 1 | 0 | 1 | 0 |
| *C.freundi* 69.5 | 0 | 0 | 0 | 0 |
| *S.gallinarum* 215.4 | 0 | 0 | 0 | 0 |
| *Coryneform* 221.4 | 0 | 0 | 0 | 0 |
| *A.faecalis* 106.1 | 0 | 0 | 0 | 0 |
| *T.viride* 153416 | 0 | 1 | 0 | 0 |
| *T.viride* 24039 | 0 | 2 | 1 | 0 |
| *T.viride* 170657 | 0 | 1 | 1 | 0 |
| *T.viride* 45553ii | 0 | 1 | 0 | 0 |

[1] Inhibition zones were measured along the diameter of the filter disc to the circumference of the zone of the inhibited growth. Inhibition zones were monitored after 48 h incubation. Values represent the average of 3 replicates. Appropriate acetone and sterile distilled water controls were provided and did not inhibit any test organism.

Key: **0** zero inhibition **1** 0-5mm **2** 6-10mm **3** 11-20mm **4** >20mm

Extracts produced by the *T.viride* isolates exhibited the largest spectrum of activity (both antifungal and antibacterial), inhibiting the growth of 75% of the test contaminations after 48h incubation on microbial lawns. Extracts produced by *T.viride* strain 24039 were most effective producing the largest inhibition zones of the microbial antagonists [Plate 3.1.1]. Extracts of *A.quisqualis* and *G.roseum* both exhibited growth inhibition of the test fungal contaminations only. Their antimicrobial activity was less pronounced than that of the *T.viride* extracts.

Of the bacterial antagonists, extracts produced by the *B.subtilis* isolates displayed the largest spectrum of antimicrobial activity (both antifungal and antibacterial) [Plate 3.1.2]. Extracts of both *P.fluorescens* strains exhibited growth inhibition of 50% of the contaminations surveyed, including both fungal and bacterial phytopathogens. Extracts of *E.aerogenes* produced a small zone of inhibition on lawns of the *Erwinia* and *Pseudomonas* contaminations. None of the extracts from the other bacterial antagonists exhibited contaminant growth inhibition.

Of the test contaminations, *Penicillium* and *Botrytis* species were the most sensitive (both susceptible to growth inhibition by 11 of the antagonists) and the yeast and *Lactobacillus* species the least sensitive (susceptible to only 5 and 2 antagonists, respectively).

### 3.2 Sustained inhibitory activity

Extracts of those antagonists exhibiting growth inhibition on test contaminant lawns after 48 h incubation were selected for further screening (with the exception of extracts of *Syncephalis nodosa*, which only produced a very slight amount of inhibition on only one of the test contaminant lawns, and was thus omitted from further screening). The inhibitory activity of selected extracts was monitored over an extended period, and a second extract dose (0.2ml) applied to bioassay discs at day 8 (Figs. 3.2.1 to 3.2.6).

**Plate 3.1.1.**

Inhibition zones produced by culture filtrates of *T.viride* (strain 24039) on microbial lawn bioassay plates (90mm diameter) of test contaminant species after 48 h incubation.

A *Penicillium* spp.

B *Aspergillus* spp.

C *Botrytis* spp.

D Yeasts

E *Bacillus* spp.

F *Pseudomonas* spp.

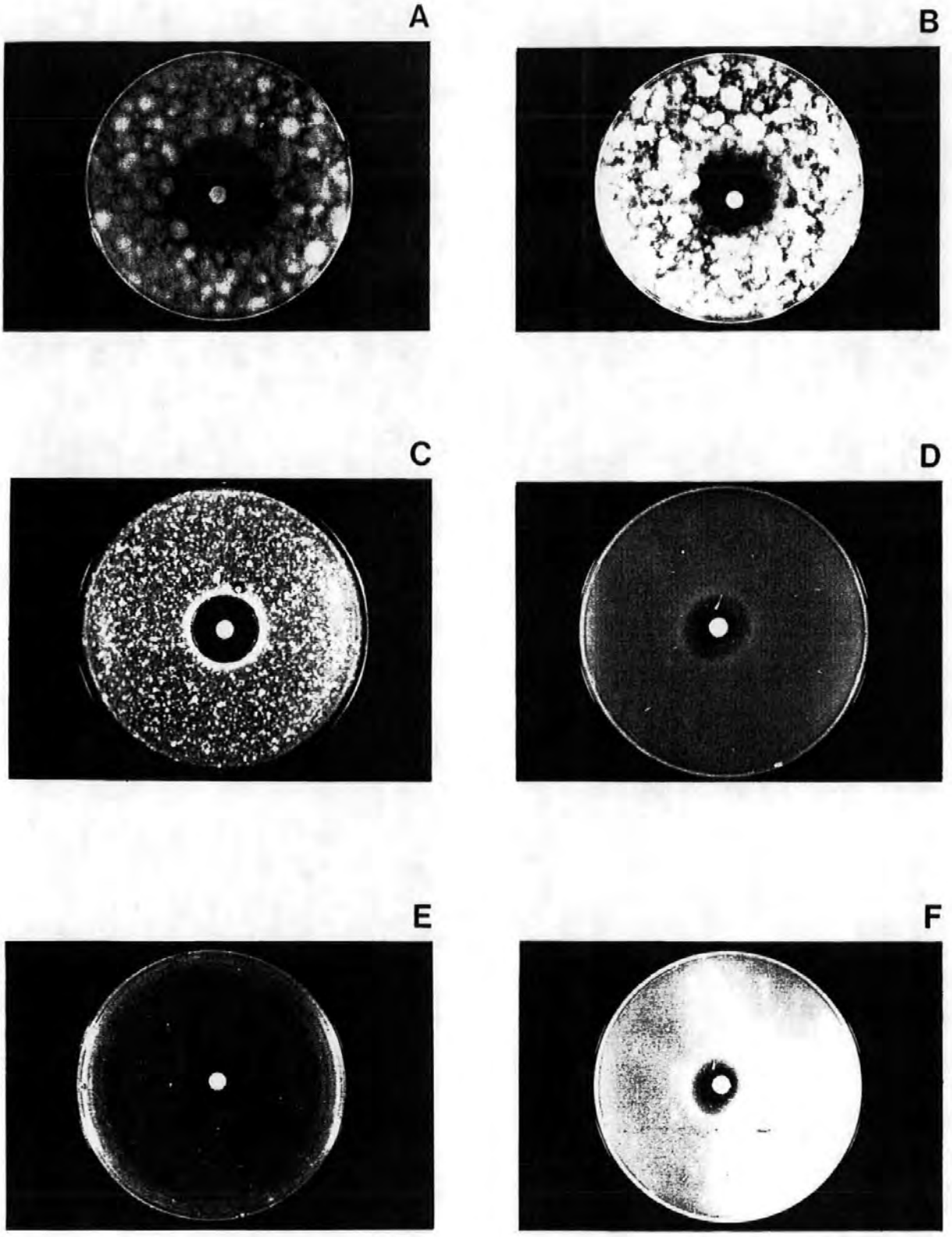

A
B
C
D
E
F

**Plate 3.1.2.**

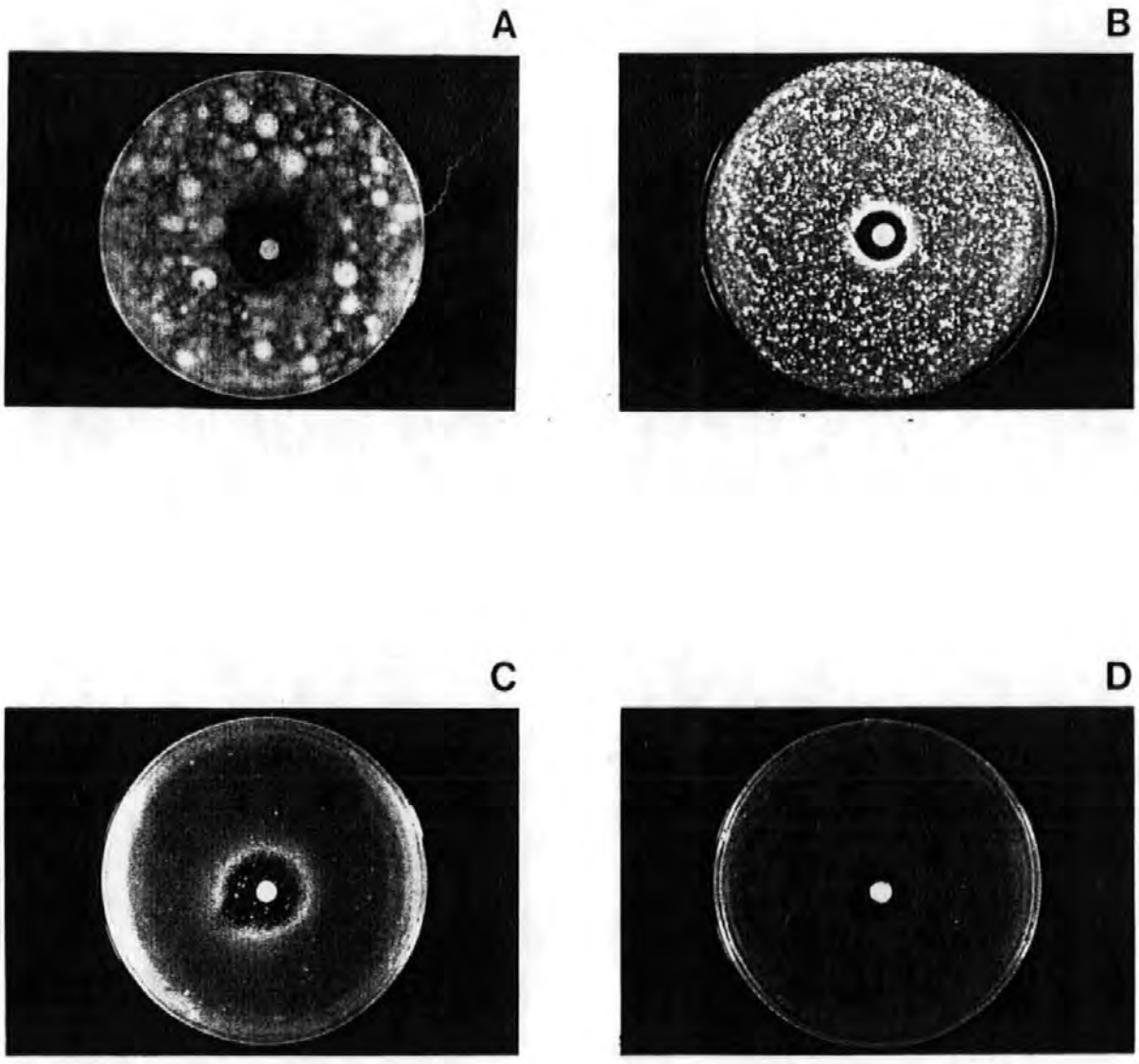
Inhibition zones produced by culture filtrates of *B.subtilis* (strain SR2) on microbial lawn bioassay plates (90mm diameter) of test contaminant species after 48 h incubation.

A *Penicillium spp.*

B *Botrytis spp.*

C *Bacillus spp.*

D *Lactobacillus spp.*


A
B
C
D

The diameter of inhibition zones produced by culture extracts of the *B.subtilis* isolates declined consistently on all test microbial lawns over the initial 7 day incubation period (Fig. 3.2.1). After a secondary extract application growth inhibition was stabilized for approximately 48 h on all lawns. At 14 days incubation, inhibition zones produced by extracts of strains SR1 and SR2 were only evident on lawns of *Penicillium*, and *Penicillium* and *Bacillus*, respectively. All other contaminant lawns overgrew the bioassay discs. The growth inhibition of the *Penicillium* lawns at day 14 was 75% of the inhibition at day 2 (i.e. extracts of the *B.subtilis* isolates exhibited continued growth inhibition of *Penicillium* species for up to 2 weeks when bioassayed *in vitro*).

Growth inhibition of all test microbial lawns decreased rapidly after 6 days incubation on bioassay plates inoculated with extracts from the *P.fluorescens* isolates (Fig. 3.2.1). A secondary application of the extract at day 8 failed to restrict the growth of the microbial lawns. The only test contaminations whose growth was restricted after the 14 day incubation period were the *Erwinia* and *Pseudomonas* species growing on bioassay plates inoculated with extracts from strain 95.6 (2% growth inhibition).

Growth inhibition of contaminant lawns by extracts from the *T.viride* isolates declined steadily over the 14 day incubation period (Fig. 3.2.3a & 3.2.3b). Inhibition was greatest on lawns of the *Penicillium* and *Botrytis* contaminations. The growth inhibition of these lawns at the end of the trial was still 60-80% of the inhibition noted after 48 h incubation, for all of the *T.viride* extracts assayed except those from strain 170657. This latter strain was the least effective of this group of antagonists in terms of sustained growth inhibition. A secondary application of the *T.viride* extracts at day 8 restricted the growth of the microbial lawns for up to 48 h. A tertiary application at day 10 failed to restrict growth of microbial lawns any further, and thereafter growth inhibition continued to decline steadily.

Extracts from the *G.roseum* and *A.quisqualis* isolates displayed a similar pattern of growth inhibition decline (Figs. 3.4 & 3.5). A temporary arrest of contaminant lawn growth was observed after the secondary extract application, followed by a rapid decline in growth inhibition irrespective of subsequent extract applications.

Growth inhibition of *Erwinia* and *Pseudomonas* lawns by extracts from *E.aerogenes* was uninhibited after 96 h and 48 h incubation, respectively (Fig. 3.6), and subsequent extract applications also failed to restrict contaminant growth.

### 3.3 Morphology of antagonized species

Mycelia of the test fungal contaminations were removed from the confronted colony edge at the point of inhibition and placed on slides for microscopic examination. All of the antagonized fungi displayed altered hyphal morphology. The types of morphological changes were of a similar nature irrespective of the extract employed. Five distinct types of change to hyphae of the test fungi were noted. These were a bulging and swelling of hyphae, thickened hyphal cell walls, coiling of hyphae, increased vacuolation, and increased septation (Plates 3.3.1 - 3.3.3). An example of bulging and swelling was seen in the *Aspergillus* spp. antagonized by the cell-free culture filtrates of *T.viride* strain 24039 (Plate 3.3.1a). Swellings were noted along the length of the antagonized hyphae. The *Aspergillus* spp. also exhibited thickened cell walls when antagonized by extracts of *G.roseum* (Plate 3.3.1b). The thickened walls of the antagonized hyphae measured 1.25-1.5um in diameter, whereas in untreated controls hyphae measured 0.5-0.75um in diameter. Bulging of hyphal tips and increased septation were features observed in *Botrytis* spp. antagonized by the cell-free culture extracts of *P.fluorescens* (95.6), and *B.subtilis* (SR2), respectively (Plate 3.3.2). Curling of hyphae was noted in *Penicillium* spp. when antagonized by *B.subtilis* extracts (Plate 3.3.3).

**Plate 3.3.1**

Hyphae of *Aspergillus* spp. antagonized by the cell-free culture filtrates of antagonistic micro-organisms.

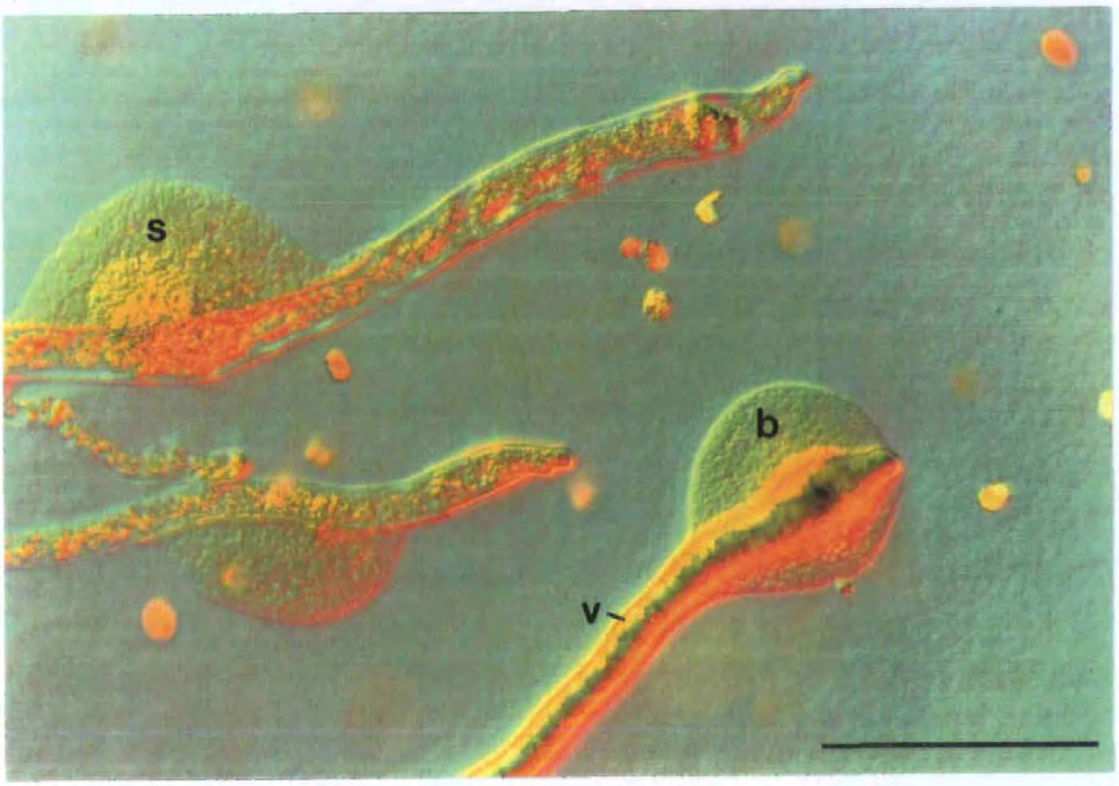
A. Antagonism by filtrates of *T.viride* (strain 24039)

B. Antagonism by filtrates of *G.roseum*.

b - bulging hyphal tips;
c - thickened cell walls;
s - swollen hyphae;
v - increased vacuolation.

[Bar, 45um]

A




B

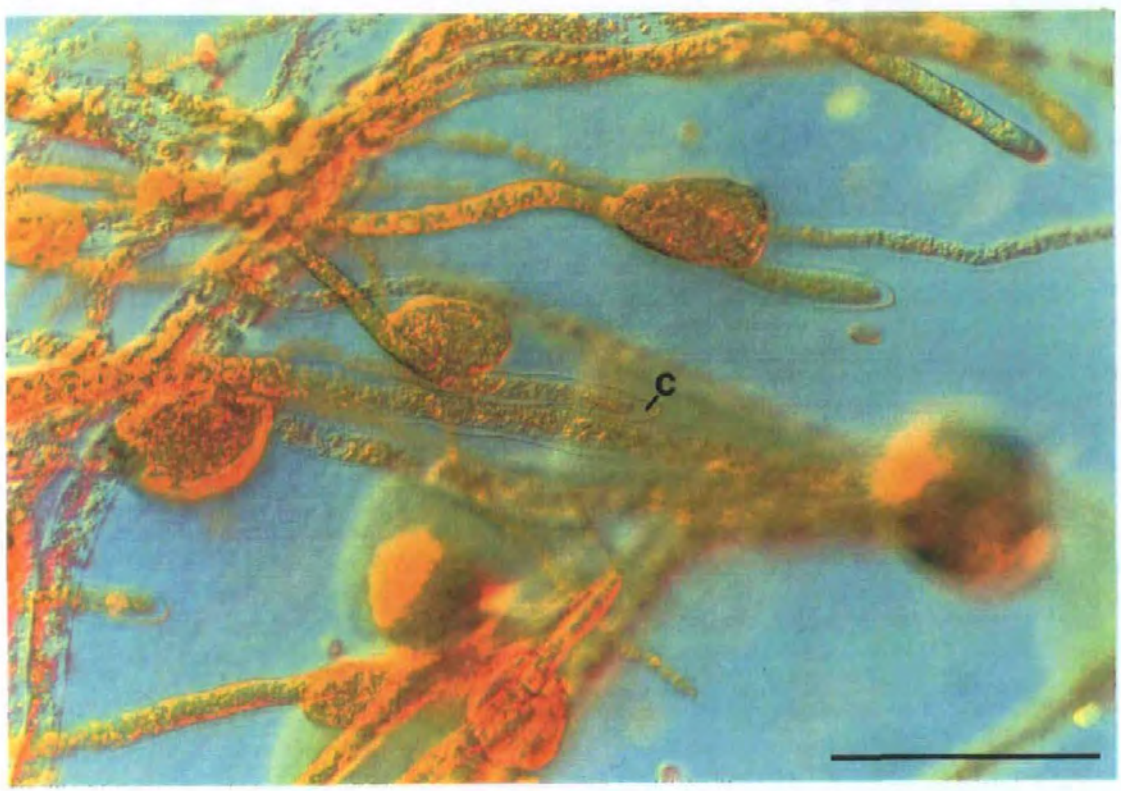


**Plate 3.3.2**

Hyphae of *Botrytis* spp. antagonized by the cell-free culture filtrates of antagonistic micro-organisms.

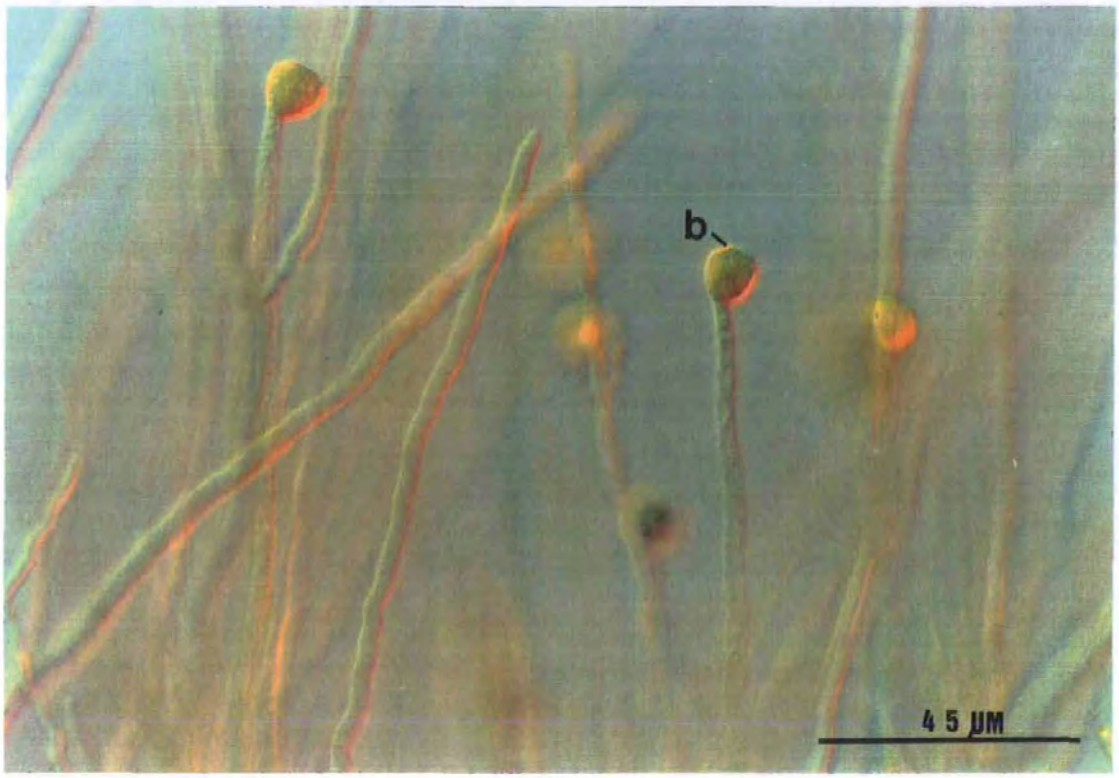
A. Antagonism by filtrates of *P.fluorescens* (strain 95.6).

B. Antagonism by filtrates of *A.quisqualis*.

b - bulging hyphal tips; s - increased septation;

[Bar, 45um]

A




B

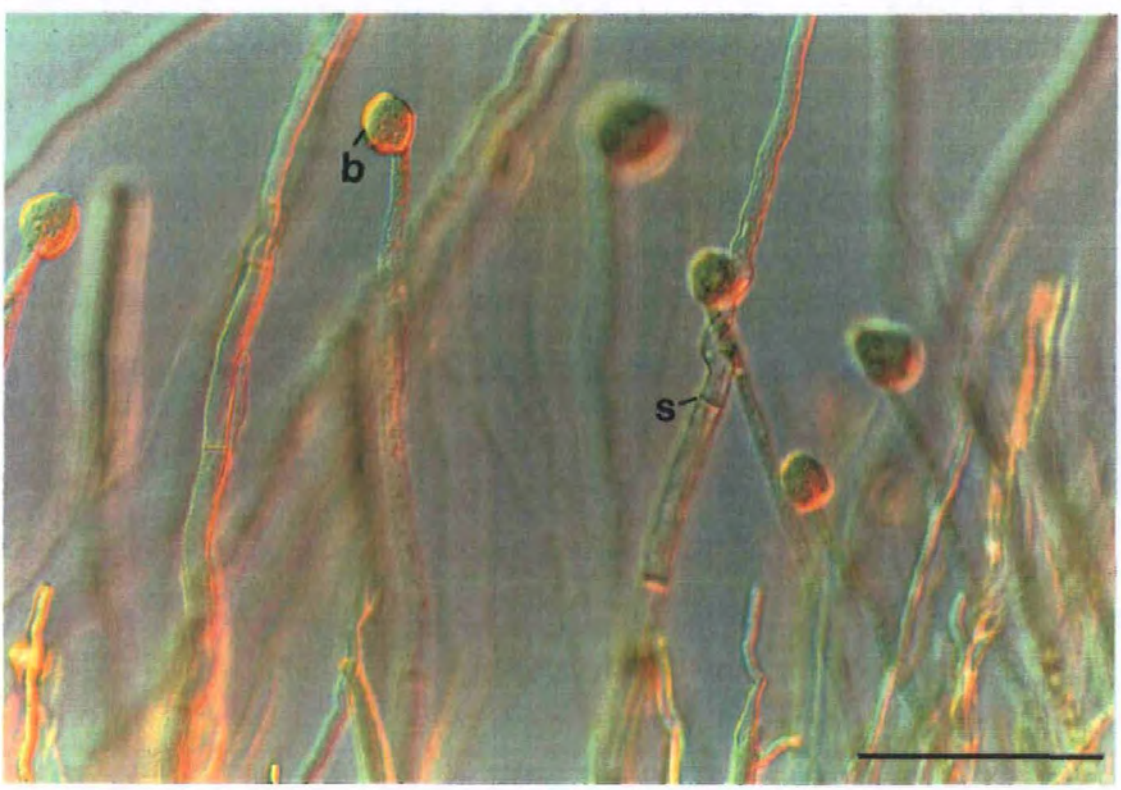
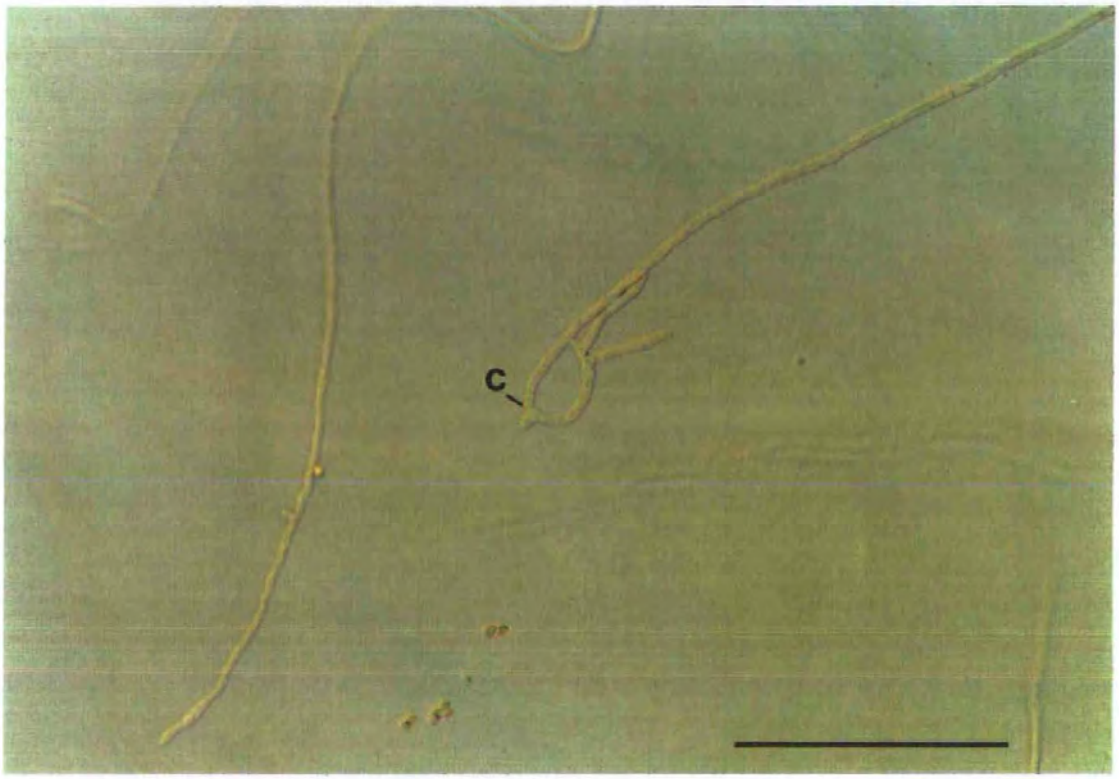


**Plate 3.3.3**

Antagonism of hyphae of *Penicillium* spp. by the cell-free culture filtrates of *B.subtilis* (strain SR2).

c - curling of hyphae.

[Bar, 45um]


C

## Discussion

Antagonistic reactions between micro-organisms are well documented (see review by Mukerji & Garg, 1988), but there are relatively few reports that the extracellular metabolites produced by antagonist micro-organisms can be deleterious to the growth of phytopathogens (see Broadbent *et al.*, 1971; Dennis & Webster, 1971a; Pusey & Wilson, 1984; Morris & Lane, 1990; Schreiber *et al.*, 1988). Kope & Fortin (1989) have reported the presence of anti-microbial compounds in the cell-free culture media of 7 ectomycorrhizal fungi using a variation of the agar plug method of Dennis & Webster (1971a). Growth inhibition of test fungi was noted when they were bioassayed *in vitro* against the cell-free exudate of compounds released from the ectomycorrhizal fungi.

The culture techniques and the methods employed in these studies showed anti-microbial activity to be evident in the cell-free liquid culture extracts of 13 of the micro-organisms screened for *in vitro* antagonism. The extracts restricted the growth of test fungal and bacterial contaminations of plant tissue culture systems, and altered the morphology of some of the phytopathogens.

Extracts produced by the *T.viride* and *B.subtilis* isolates were widely antagonistic towards the range of fungal and bacterial contaminations tested (i.e. these extracts showed low pathogen specificity *in vitro*). Extracts produced by the *P.fluorescens*, *E.aerogenes*, *G.roseum* and *A.quisqualis* isolates also exhibited growth inhibition of test contaminations on microbial lawn bioassay plates, though the extent and range of anti-microbial activity was less pronounced compared with extracts from the *T.viride* and *B.subtilis* isolates. Extracts of these latter two antagonists displayed sustained growth inhibition of *Penicillium* and *Botrytis* spp., and temporarily arrested the growth of all microbial lawns after a secondary extract application.

It was evident from the results obtained in this study that of the mycoparasites, extracts produced by the necrotrophic species (i.e. *Trichoderma*, *Ampelomyces*, and *Gliocladium*) exhibited greater anti-microbial activity than those produced by the biotrophic types (Appendix I). Upon reflection this is perhaps not so surprising, since the necrotrophic mycoparasites are destructive, and having made contact with their host they secrete toxic substances which kill the host cells. In contrast, the biotrophic mycoparasites often co-exist with their host and may not necessarily produce toxic metabolites whilst favourable growth conditions prevail (Barnett & Binder, 1973).

The genus *Trichoderma* has attracted considerable attention as a potential agent for the control of microbial contaminations and plant pathogens. It is ubiquitous, easy to isolate and culture, grows rapidly on many substrates, is rarely pathogenic on higher plants, acts as a mycoparasite, competes well for food and site, produces antibiotics, and has an enzyme system capable of attacking a wide range of plant pathogens. The anti-microbial activity of *Trichoderma* species was first reported by Weindling (1937). More recent studies have shown that many *Trichoderma* isolates are antagonistic ones and produce volatile and non-volatile antibiotics in culture filtrates active against a range of micro-organisms (see Dennis & Webster, 1971a&b; Mukerji & Garg, 1988). Morris & Lane (1990) observed that pre-inoculation of *Botrytis fabae* with the cell-free culture filtrates of specific strains of *T.viride* offered some preventative control of the plant pathogen. Similar growth inhibition of *Botrytis* lawns by extracts of *T.viride* isolates was noted in this study [Figs. 3.2.3a&b].

*Ampelomyces quisqualis* is a common hyperparasite of powdery-mildews and exerts its effect by penetration from cell to cell through the septal pores of the mildew and continues growth during the gradual degradation of the infected cells (Hashioka & Nakai, 1980). There is no evidence of toxin

production by *A.quisqualis* (see Beuther *et al*, 1981). However, experiments conducted in this study contradict this observation, since cell-free extracts of the antagonist did inhibit the growth of lawns of *Penicillium*, *Aspergillus*, and *Botrytis* species.

*Gliocladium* species, including *G.roseum* and *G.virens*, have been shown to be parasitic on a wide variety of plant pathogens including species of *Botrytis, Fusarium, Rhizoctonia*, and *Sclerotia* (Walker & Maude, 1975; Howell, 1982). Species of *Gliocladium* have been shown to produce anti-fungal antibiotics in liquid culture which exhibit strong antibiotic activity against *Fomes annosus, Rhizoctonia solani*, and *Fusarium* and *Mucor* species (Dennis & Webster, 1971a). However, in those experiments the filtrates of *Gliocladium* were often found to be fungistatic as opposed to fungicidal. In this report, extracts produced by *G.roseum* strain 40022 displayed slight antagonism toward species of *Penicillium*, *Aspergillus*, and *Botrytis*.

Several species of the genus *Bacillus* (e.g. *B.penetrans*, *B.subtilis*) are also well known producers of anti-microbial agents (see Goswami & Bose, 1982; Pusey & Wilson, 1984; Becker & Hedges, 1986; Cubeta *et al.*, 1985; Pusey, 1989). Results obtained here for the *B.subtilis* strains SR1 and SR2 [Tables 3.1.1 & 3.1.2] are consistent with those of Schreiber *et al.* (1988) who reported that filter-sterilized crude culture filtrates of a *B.subtilis* isolate (grown on potato dextrose agar for 10 days at $30^{O}$C) produced clear zones of inhibition of up to 40mm and 10mm on lawns of *P.chrysogenum* and *B.subtilis*, respectively, using the Kirby-Bauer method (Bauer *et al.*, 1966). Observations noted in this study on exclusion zone assays using strains SR1 and SR2 are also substantiated by Babasaki and co-workers (Babasaki *et al.*, 1985), who noted that a *B.subtilis* isolate (strain 168) was inhibitory to the growth of lawns of other *Bacillus*, and *Aspergillus* spp. However, contrary to the findings of Babasaki's group, our *B.subtilis* strains did exhibit slight inhibition of *Lactobacillus* spp. An antibiotic culture extract obtained from another strain of

*B.subtilis* (designated strain B-3) has reported activity against a wide range of plant pathogenic fungi (including species of *Penicillium, Botrytis, Fusarium, Pythium, Phytophthora*, and *Ceratocytis*) when assayed by growth exclusion zone tests (see Pusey, 1989).

The fluorescent *Pseudomonads* have received much commercial attention as biocontrol agents (see Cook & Baker, 1983). This group includes the species of *P.fluorescens, P.putida, P.aeruginosa, P.chlororaphus, P.aureofaciens,* and *P.syringae*. These species are known to produce a variety of secondary metabolites with different modes of antibiosis, which display potent, wide spectrum anti-fungal and anti-bacterial activity (see reviews by Broadbent *et al.*, 1971 and Becker & Hedges, 1986). Most of these compounds are nitrogen-containing heterocyclic compounds. Among these are phenazines and indole derivatives (Leisinger & Margraff, 1966). Unusual amino-acids and peptides, including iron-chelating agents such as siderophores (iron-carrying fluorescent pigments) are also produced by antagonistic strains of *P.fluorescens*. Siderophores (e.g. 'pseudobactin') exhibit antibiosis by depriving the pathogen of iron (Becker & Hedges, 1986). *In vitro* antibiosis against mycelial growth and sclerotial germination of *Rhizoctonia solani* by cell-free siderophores of *P.fluorescens* strains has been reported by Savithiry & Gnanamanickam (1987), who suggested that antagonistic strains of *P.fluorescens* might prove valuable for the efficient management of plant pathogens. Sakthivel *et al.* (1986) noted similar *in vitro* antagonism to fungal and bacterial plant pathogens, including species of *Fusarium* and *Rhizoctonia*, and *Pseudomonas* and *Xanthomonas, by* the siderophores of a *P.fluorescens* strain. In this report, filter-sterilized crude filtrates of P.fluorescens strains 95.6 and 106.6 displayed *in vitro* antagonism of species of *Erwinia*, *Pseudomonas*, *Penicillium*, and *Botrytis*.

Extracts produced by the *Enterobacter aerogenes* isolate (85.4) have been shown to possess anti-bacterial activity (Agricultural Genetics Company,

unpublished data), and this claim was verified by experiments conducted in this study (slight growth inhibition of *Erwinia* and *Pseudomonas* was observed).

As previously noted (section 2.4) caution should be exercised when interpreting a putative effect of a crude cell-free extract on microbial contaminations. A reduction in contaminant growth does not necessarily imply an antibiotic reaction, rather it could be an alteration to the physiological environment (e.g. pH) that is affecting microbial growth. However, since the hyphae of the antagonized fungi studied here displayed an altered morphology when exposed to filtrates, it would indicate the presence of anti-microbial compounds in the extracts of the antagonists. Changes in hyphal morphology were associated with a range of inhibition zones. With the *Aspergillus* spp. a zone of inhibition of 0-5mm was recorded when confronted by extracts from *G.roseum* and of 6-10mm when confronted with *T.viride* strain 24039, but in both cases swelling and bulging of hyphal tips were noted. From the size of the inhibition zone and from the degree of altered morphology, a relative range of sensitivity of the contaminants to the anti-microbial extracts could be noted.

Betina *et al.* (1972) and Barathova & Betina (1976) had confronted mycelium with known antibiotics and described the changes to hyphal morphology as branching, budding, curling, and lysis, with extensive branching being the most frequent reaction. Kope & Fortin (1989) also described hyphal branching as the most frequent morphological change observed when fungal mycelium were confronted with the cell-free extracts of ectomycorrhizal fungi. Dennis & Webster (1971a) observed greater branching and vacuolation of hyphae of fungi antagonized by culture filtrates of a *T.viride* isolate.

In this screening programme, swelling and bulging of hyphal tips was the most frequent morphological change observed. The feature of hyphal lysis was not displayed by any of the antagonized fungi. After prolonged incubation

the test fungal contaminations overgrew the bioassay plates. Therefore it is possible that (1) the changes observed are a survival reaction of the antagonized fungi, (2) they are able to regrow when conditions become favourable, and (3) hyphal lysis would be fatal to the survival of the fungus.

This preliminary screening programme yielded 6 groups of antagonists whose extracts were selected for further assessment on their potential for contamination control and/or eradication of *in vitro* plant cell and tissue culture infections. These antagonists included isolates of *T.viride* (4 strains), *B.subtilis* (2 strains), *A.quisqualis*, *G.roseum*, and *E.aerogenes*.

**Figure 3.2.1**

% Growth inhibition of test contaminations by culture extracts of *B.subtilis* isolates.

**A.** *B.subtilis* strain SR1

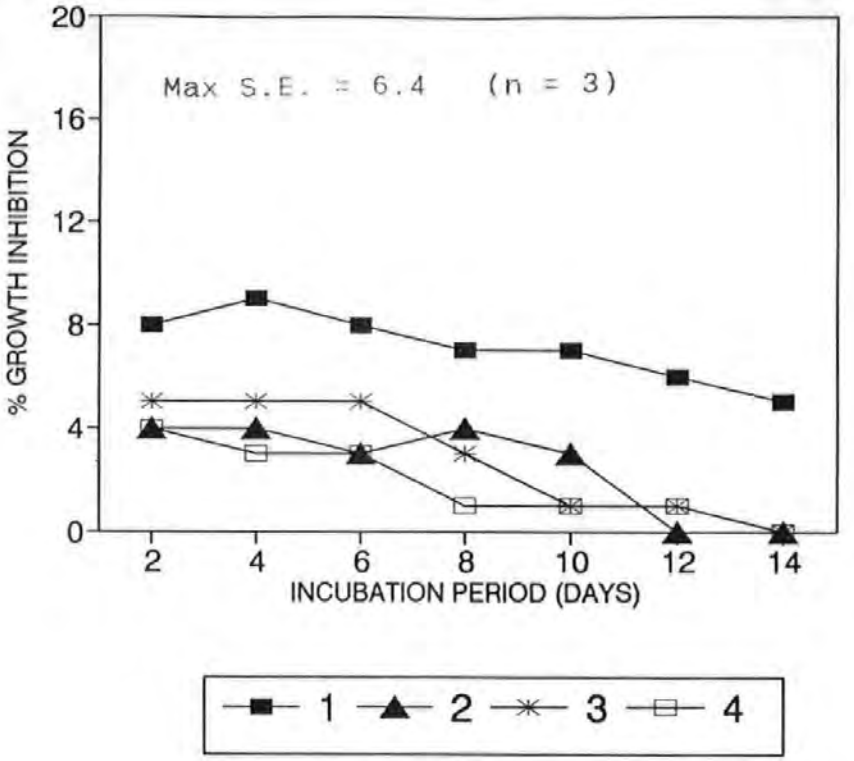
**B.** *B.subtilis* strain SR2

**1** - *Penicillin* spp.

**2** - *Botrytis* spp.

**3** - *Bacillus* spp.

**4** - *Lactobacillus* spp.


Max S.E. = 6.4 (n = 3)
% GROWTH INHIBITION
20
16
12
8
4
0
2
4
6
8
10
12
14
INCUBATION PERIOD (DAYS)
1
2
3
4

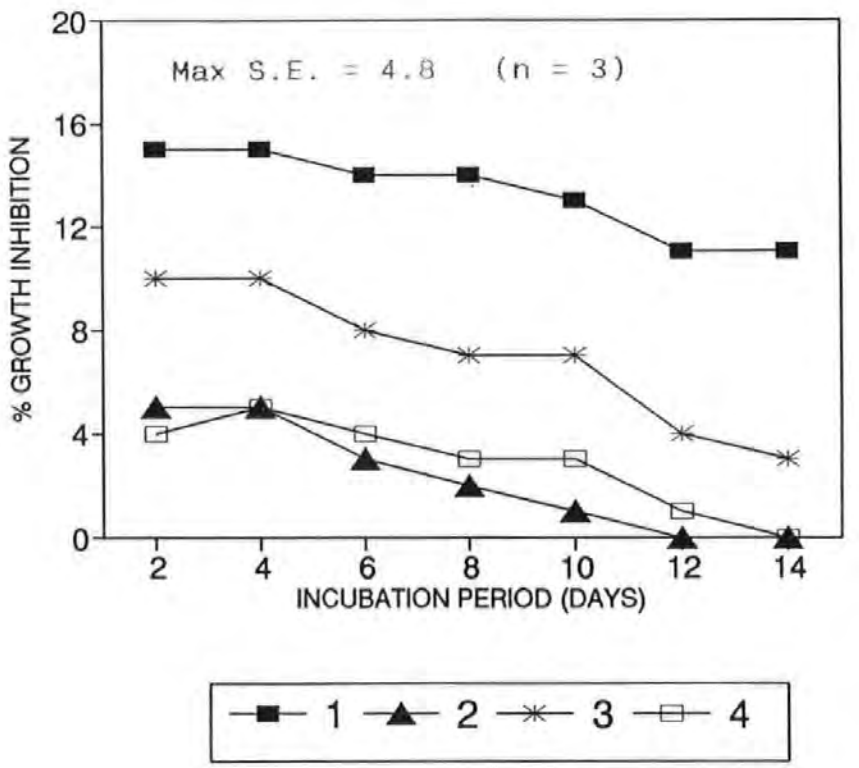

Max S.E. = 4.8 (n = 3)
% GROWTH INHIBITION
20
16
12
8
4
0
2
4
6
8
10
12
14
INCUBATION PERIOD (DAYS)
1
2
3
4

**Figure 3.2.2**

% Growth inhibition of test contaminations by culture extracts of *P.fluorescens* isolates.

**A.** *P.fluorescens* strain 95.6

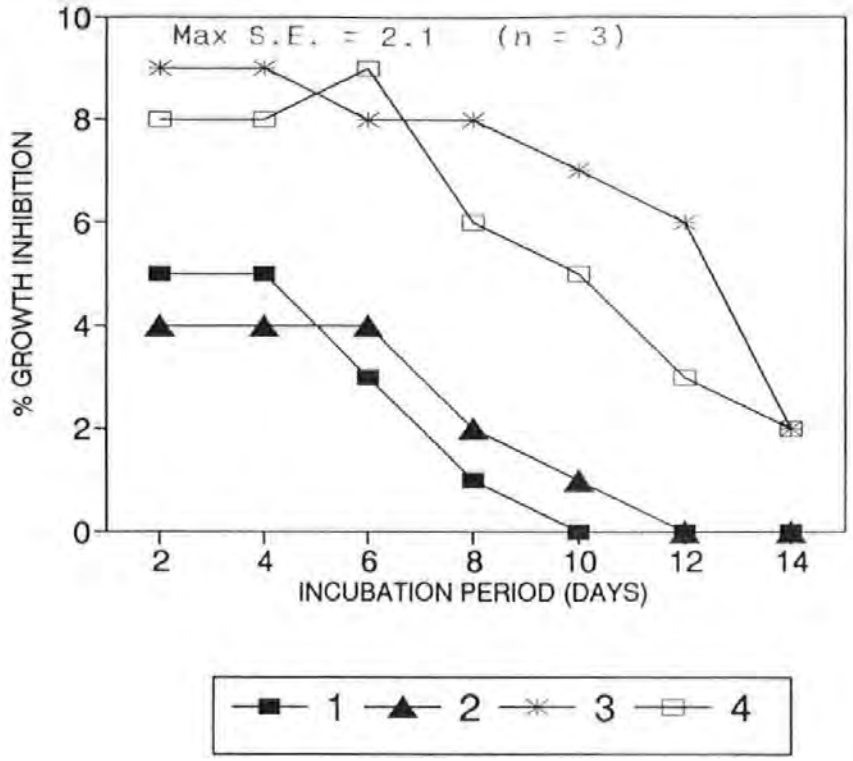
**B.** *P.fluorescens* strain 106.6

**1** - *Penicillin* spp.

**2** - *Botrytis* spp.

**3** - *Erwinia* spp.

**4** - *Pseudomonas* spp.


Max S.E. = 2.1 (n = 3)
% GROWTH INHIBITION
INCUBATION PERIOD (DAYS)
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2
4
6
8
10
2
4
6
8
10
12
14
1
2
3
4

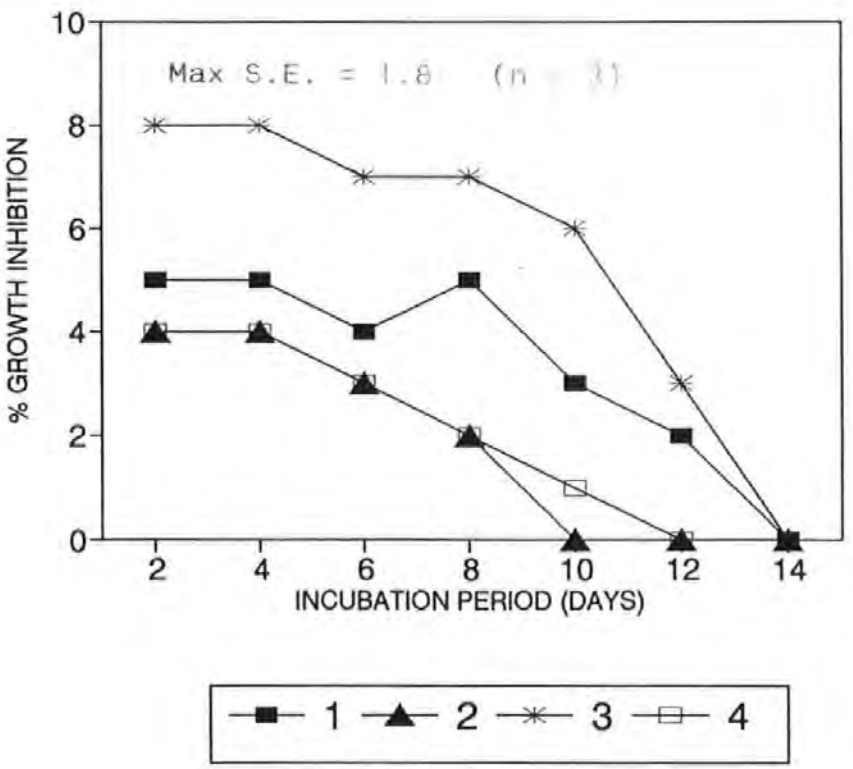

Max S.E. = 1.8 (n = 3)
% GROWTH INHIBITION
INCUBATION PERIOD (DAYS)
0
2
4
6
8
10
2
4
6
8
10
12
14
1
2
3
4

**Figure 3.2.3a**

% Growth inhibition of test contaminations by culture extracts of *T.viride* isolates.

**A.** *T.viride* strain 170657

**B.** *T.viride* strain 24039

**1** - *Penicillin* spp.

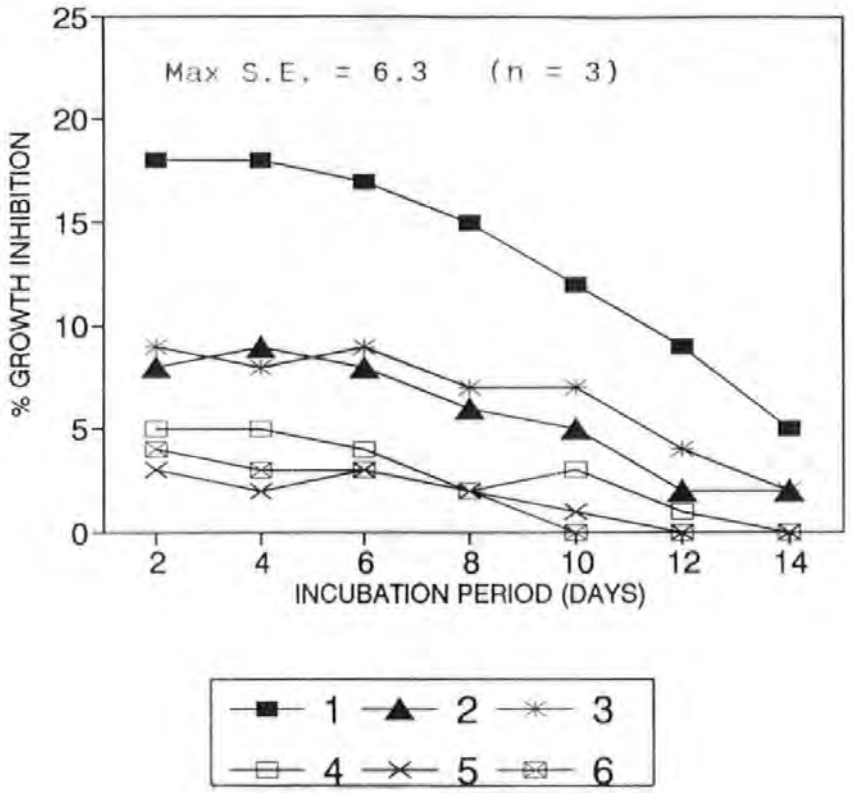
**2** - *Aspergillus* spp.

**3** - *Botrytis* spp.

**4** - Yeasts

**5** - *Bacillus* spp.

**6** - *Pseudomonas* spp.


Max S.E. = 6.3 (n = 3)
% GROWTH INHIBITION
INCUBATION PERIOD (DAYS)
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5
10
15
20
25
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4
6
8
10
12
14
1
2
3
4
5
6

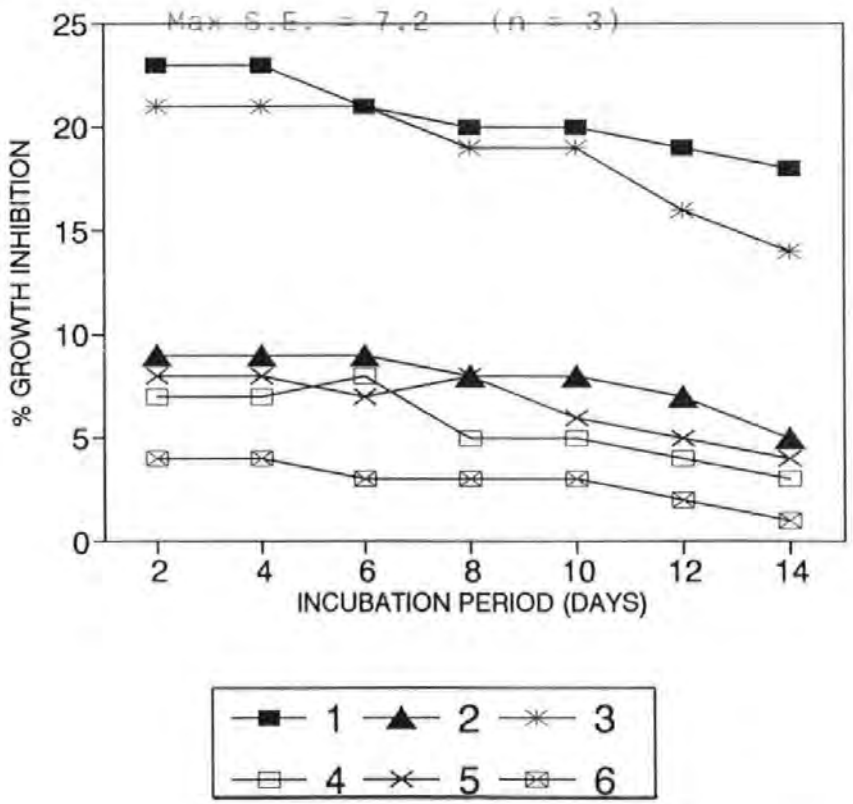

Max S.E. = 7.2 (n = 3)
% GROWTH INHIBITION
INCUBATION PERIOD (DAYS)
0
5
10
15
20
25
2
4
6
8
10
12
14
1
2
3
4
5
6

**Figure 3.2.3b**

% Growth inhibition of test contaminations by culture extracts of *T.viride* isolates.

**A.** *T.viride* strain 153426

**B.** *T.viride* strain 45553ii

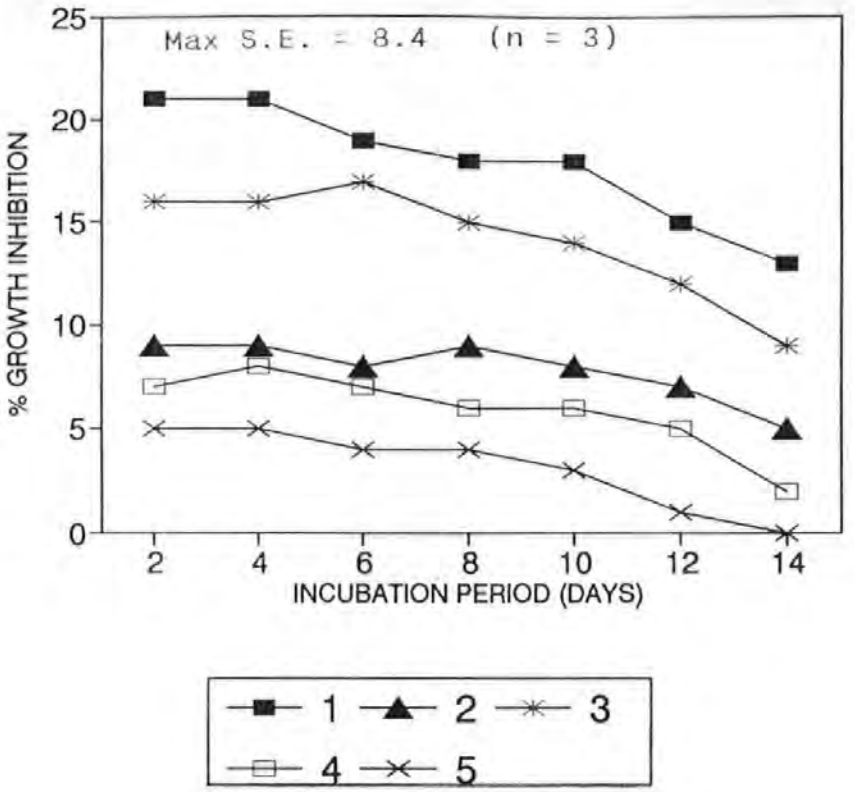
**1** - *Penicillin* spp.

**2** - *Aspergillus* spp.

**3** - *Botrytis* spp.

**4** - Yeasts

**5** - *Bacillus* spp.


Max S.E. = 8.4 (n = 3)
% GROWTH INHIBITION
INCUBATION PERIOD (DAYS)
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5
10
15
20
25
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4
6
8
10
12
14
1
2
3
4
5

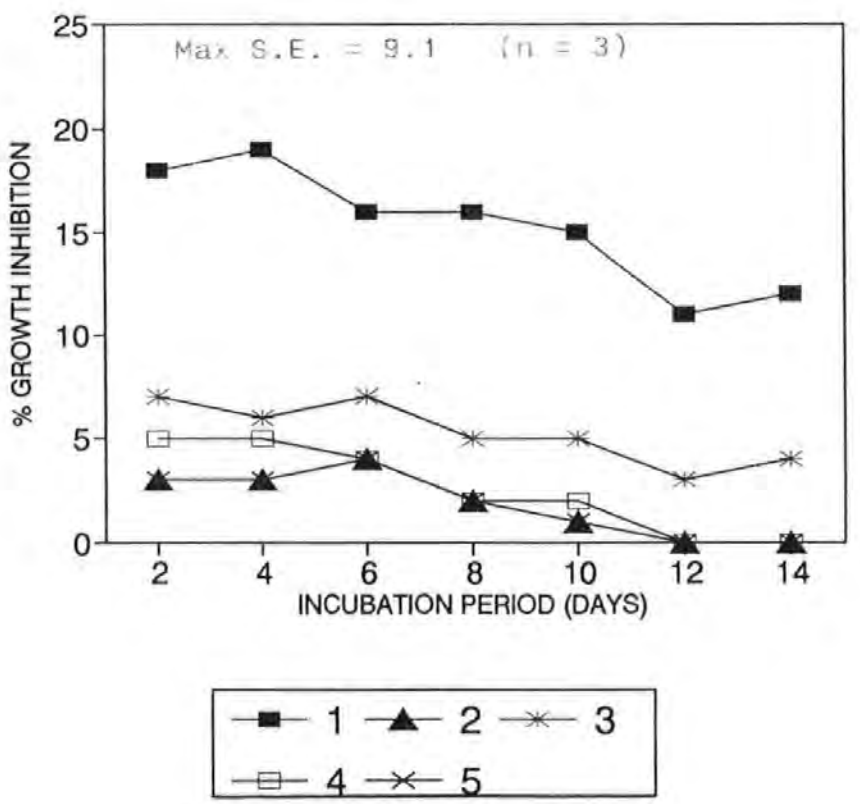

Max S.E. = 9.1 (n = 3)
% GROWTH INHIBITION
INCUBATION PERIOD (DAYS)
0
5
10
15
20
25
2
4
6
8
10
12
14
1
2
3
4
5

**Figure 3.2.4**

% Growth inhibition of test contaminations by culture extracts of *G.roseum*.

**1** - *Penicillin* spp.

**2** - *Aspergillus* spp.

**3** - *Botrytis* spp.

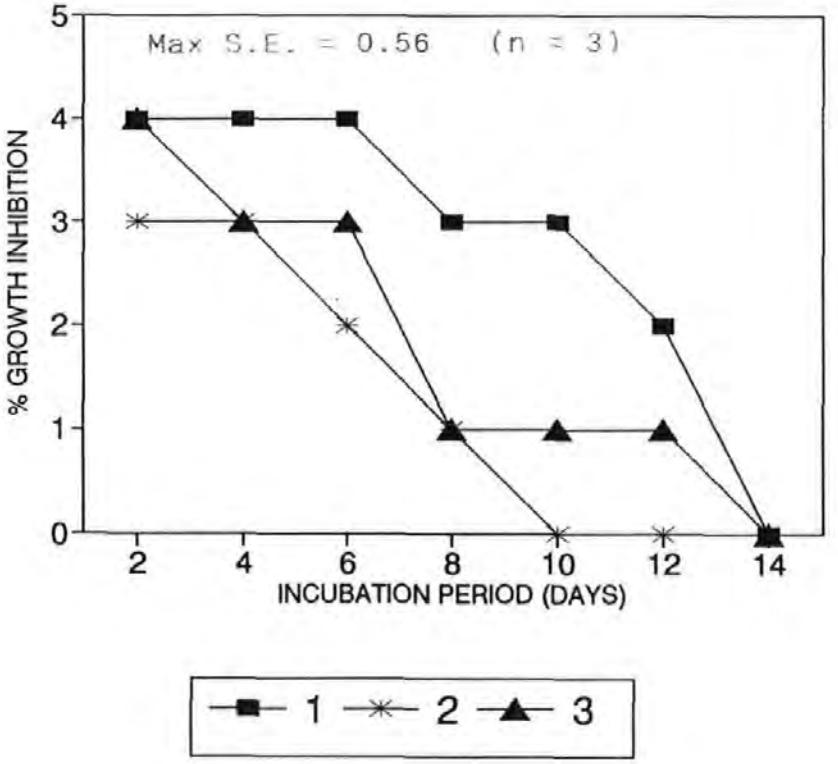

Max S.E. = 0.56 (n = 3)
% GROWTH INHIBITION
0
1
2
3
4
5
2
4
6
8
10
12
14
INCUBATION PERIOD (DAYS)
1
2
3

**Figure 3.2.5**

% Growth inhibition of test contaminations by culture extracts of *A.quisqualis*.

**1** - *Penicillin* spp.

**2** - *Aspergillus* spp.

**3** - *Botrytis* spp.

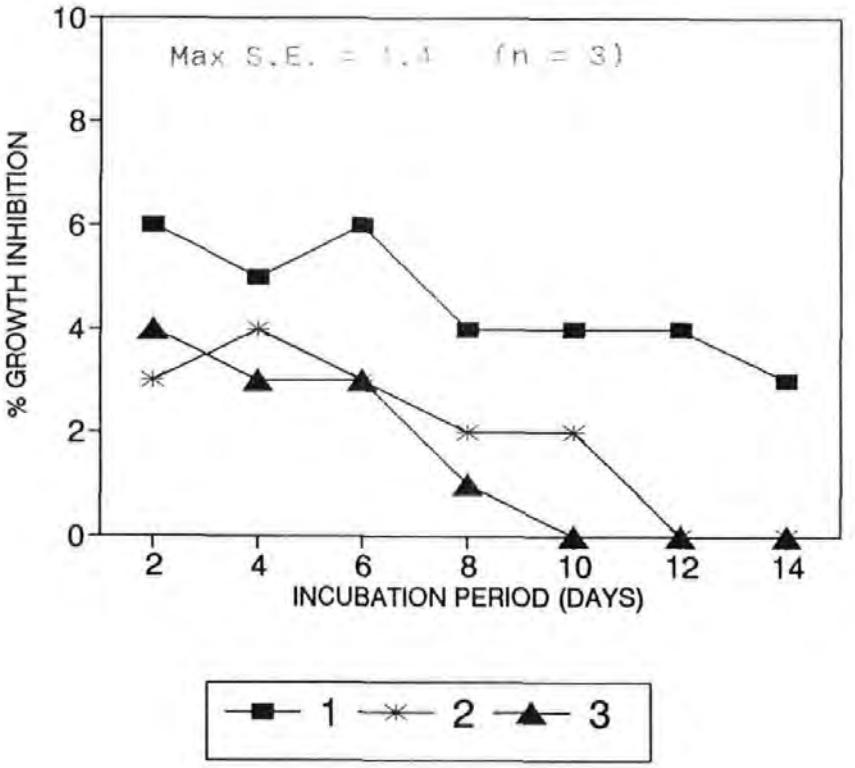
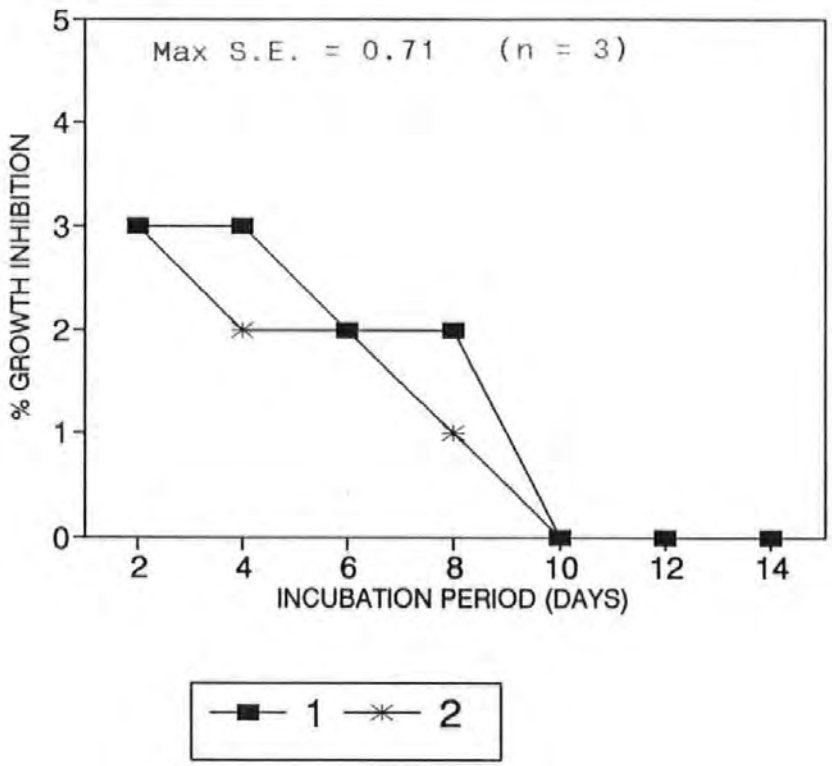

Max S.E. = 1.4 (n = 3)
% GROWTH INHIBITION
10
8
6
4
2
0
2
4
6
8
10
12
14
INCUBATION PERIOD (DAYS)
1
2
3

**Figure 3.2.6**

% Growth inhibition of test contaminations by culture extracts of *E.aerogenes*.

1 - *Erwinia* spp.

2 - *Pseudomonas* spp.


Max S.E. = 0.71 (n = 3)
% GROWTH INHIBITION
0
1
2
3
4
5
2
4
6
8
10
12
14
INCUBATION PERIOD (DAYS)
1
2

CHAPTER 4

# CULTURAL OPTIMIZATION OF INHIBITORY-FILTRATE PRODUCTION FOR MAXIMAL ANTI-MICROBIAL ACTIVITY

The production of anti-microbial activity by antagonists is connected with the accumulation of secondary metabolites (Person-Huppel, 1963). Their antagonistic efficacy may be closely related to pH and temperature conditions during growth (Sierota, 1976). This investigation attempted to determine the pH value and the temperature regime most favourable for the production of maximum anti-microbial activity by culture filtrates of selected antagonists. The bulk-volume production of anti-microbial filtrates of *T.viride* strain 24039 was optimized using a laboratory-scale fermenter system. The stability of filtrates produced under optimum conditions for maximum activity was assessed over a range of pH values, after different heat treatments, in various solvent systems, and after prolonged storage periods.

## 4.1 Cultural optimization and time-course bioassay

Due to time and resource constraints only those antagonistic extracts exhibiting the greatest growth inhibition of test contaminations (see chapter 3) were selected for further screening here. The following microbial antagonists were selected for inclusion in cultural optimization investigations: *T.viride* strain 24039, *B.subtilis* strain SR2, *P.fluorescens* strain 95.6, *E.aerogenes*, *A.quisqualis*, and *G.roseum*. The effect of growing these antagonists under various pH and temperature regimes on the inhibitory activity of their filtrates is presented (Figs. 4.1.1 - 4.1.3).

Growth inhibition of susceptible test organisms was greatest when antagonists were cultured in growth media buffered to between pH 5.0-7.0. The filtrates of antagonists grown at initial pH ranges 2-4 and 8-9 exhibited the least growth inhibition of test contaminations. Control discs (saturated with control initial-

inhibition of test contaminations. Control discs (saturated with control initial-media at the same pH values and non-inoculated with filtrates) did not inhibit the growth of test organisms and were overgrown by the microbial lawns after 72 h incubation. Average changes in the initial pH of the various culture media were noted after growth of the antagonists. The least changes were observed at initial media pH 2.0 and 3.0 (increased to 2.3 and 3.6, respectively), and the greatest at pH 4.0 and 5.0 (increased to pH 5.8 and 6.4, respectively), (Fig.4.1.5). For initial pH values in the range 6.0 - 7.0, the final pH increased to between pH 7.2 and 7.4, and thereafter did not change appreciably. No pH changes were observed in non-inoculated controls.

Only the two extreme incubation temperatures investigated (15 and 35$^{O}$C) proved less favourable for the production of anti-microbial filtrates by the antagonists (Figs. 4.1.1 - 4.1.3). Filtrates of the fungal antagonists displayed greatest growth inhibition of test organisms when they were incubated within the range 20-30$^{O}$C, whilst the bacterial antagonists favoured slightly higher incubation temperatures (25-35$^{O}$C) for the production of filtrates with the greatest inhibitory activity. The optimum range of growth medium pH and incubation temperatures for the production of maximum inhibitory activity by the various antagonists is summarised (Table 4.1.1).

Growth inhibition of test contaminants by filtrates of the bacterial and the fungal isolates was at a maximum when the antagonists were grown for between 4 and 6 days, and 6 and 8 days, respectively (Fig. 4.1.4).

**Table 4.1.1** Optimum range of growth medium pH and incubation temperature for the production of maximal inhibitory activity by microbial antagonists.

| Filtrate | Initial pH | Temperature (°C) |
|---|---|---|
| *T.viride* 24039 | 5-7 | 20-30 |
| *B.subtilis* SR2 | 5-7 | 25-35 |
| *P.fluorescens* 95.6 | 6-7 | 25-35 |
| *E.aerogenes* | 6 | 30-35 |
| *A.quisqualis* | 5-7 | 25-30 |
| *G.roseum* | 6-7 | 20-25 |

## 4.2 Bulk volume production of inhibitory filtrates of *T.viride*

Growth inhibition of susceptible test organisms was exhibited by culture filtrates of *T.viride* strain 24039 grown in a laboratory-scale fermenter system (LH model 502D) (Fig. 4.2.1). Good growth of the *T.viride* culture was observed at the various media pH values and incubation temperatures employed (Fig. 4.2.2). The greatest biomass yields were recorded for the higher temperature and pH value assessed. The inhibitory effect of filtrates was also found to be greatest at the higher pH value of 6 and incubation temperature, 25$^{o}$C. This might suggest that the production of inhibitory activity by antagonists is related to biomass production. The dissolved oxygen content of the fermenter system which produced filtrates with the greatest inhibitory activity was 40% (Fig. 4.2.3). However, above this $DO_2$ level inhibitory activity decreased in proportion to an increase in percentage biomass production. This suggests that inhibitory activity is proportional to biomass production up

to a critical level, above which the production of inhibitory secondary metabolites is reduced at the expense of the increased biomass production. In these investigations on the growth of *T.viride* strain 24039 in a fermenter system, the critical biomass level was determined to be 48.8% (Fig.4.2.3) i.e. at this biomass level the inhibitory activity was at a maximum, above this level of biomass production activity decreased. Time-course bioassays established that the optimum time in culture for maximum inhibitory activity of filtrates of *T.viride* strain 24039 produced under optimum conditions was 6 days (Fig. 4.2.1).

### 4.3 Stability assessments

Culture filtrates of the antagonists exhibited greatest inhibitory activity when buffered within a pH range of 4-8 (Figure 4.3.1). At pH values outside of this range all of the extracts displayed reduced inhibitory activity when bioassayed against susceptible test contaminants. Control bioassay plates (discs saturated with the various pH buffers without inhibitory filtrates) did not inhibit contaminant growth and were overgrown by microbial lawns after 48 h incubation.

The inhibitory activity of all culture filtrates decreased substantially after heat treatment at 70$^{O}$C (Fig. 4.3.2a), and after heat treatment at 90$^{O}$C the activity of all filtrates had declined to zero. The inhibitory activity of extracts from the *P.fluorescens* and *A.quisqualis* began to decline after heat treatment at 60$^{O}$C (Fig. 4.3.2a). The inhibitory activity of all extracts remained stable for up to 15 minutes incubation at 70$^{O}$C, thereafter activity progressively decreased (Fig. 4.3.2b).

Without exception extracts exhibited reduced inhibitory activity after storage at 4$^{O}$C for more than one month (Fig. 4.3.3.a). Activity continued to decline over the four month storage period to less than 20% of the original activity for extracts of *T.viride* strain 24039 and *B.subtilis* strain SR2, and to

Table 4.3.1 Growth inhibition of fungal contaminations by culture filtrates of a range of antagonistic micro-organisms following methanol extraction[1].

| | Test Contaminant Species | | | |
|---|---|---|---|---|
| **Antagonist** | *Penicillium* | *Aspergillus* | *Botrytis* | Yeasts |
| *B.subtilis SR1* | 3 | 0 | 1 | 0 |
| *B.subtilis SR2* | 4 | 0 | 2 | 0 |
| *P.fluorescens 95.6* | 2 | 0 | 2 | 0 |
| *P.fluorescens 106.6* | 2 | 0 | 2 | 0 |
| *P.virginiana 70910* | 0 | 0 | 0 | 0 |
| *S.nodosa 25/1875* | 0 | 0 | 0 | 0 |
| *G.simplex 245819* | 0 | 0 | 0 | 0 |
| *C.hiemophillum 274831* | 0 | 0 | 0 | 0 |
| *D.parvispora 101210* | 0 | 0 | 0 | 0 |
| *A.quisqualis 272851* | 1 | 0 | 0 | 0 |
| *D.verticilliata 278511* | 0 | 0 | 0 | 0 |
| *T.viride 153416* | 3 | 2 | 3 | 2 |
| *T.viride 24039* | 3 | 2 | 3 | 1 |
| *T.viride 45553ii* | 2 | 1 | 1 | 1 |
| *T.viride 170657* | 2 | 1 | 1 | 1 |
| *G.roseum 40022* | 1 | 0 | 1 | 0 |

[1] Inhibition zones were measured along the diameter of the filter disc to the circumference of the zone of the inhibited growth. Inhibition zones were monitored after 48 h incubation. Values represent the average of 3 replicates. Appropriate methanol and sterile distilled water controls were provided and did not inhibit any test organism.

Key: **0** zero inhibition **1** 0-5mm **2** 6-10mm **3** 11-20mm **4** >20mm

Table 4.3.2 Growth inhibition of bacterial contaminations by culture filtrates of a range of antagonistic micro-organisms following methanol extraction[1].

| | Test Contaminant Species | | | |
|---|---|---|---|---|
| **Antagonist** | *Erwinia* | *Bacillus* | *Pseudomonas* | *Lactobacillus* |
| *B.subtilis* SR1 | 0 | 2 | 1 | 1 |
| *B.subtilis* SR2 | 0 | 2 | 2 | 1 |
| *P.fluorescens* 95.6 | 2 | 0 | 2 | 0 |
| *P.fluorescens* 106.6 | 2 | 0 | 2 | 0 |
| *E.aerogenes* 85.4 | 1 | 0 | 1 | 0 |
| *C.freundi* 69.5 | 0 | 0 | 0 | 0 |
| *S.gallinarum* 215.4 | 0 | 0 | 0 | 0 |
| *Coryneform* 221.4 | 0 | 0 | 0 | 0 |
| *A.faecalis* 106.1 | 0 | 0 | 0 | 0 |
| *T.viride* 153416 | 0 | 0 | 0 | 0 |
| *T.viride* 24039 | 0 | 1 | 0 | 0 |
| *T.viride* 170657 | 0 | 1 | 0 | 0 |
| *T.viride* 45553ii | 0 | 1 | 0 | 0 |

[1] Inhibition zones were measured along the diameter of the filter disc to the circumference of the zone of the inhibited growth. Inhibition zones were monitored after 48 h incubation. Values represent the average of 3 replicates. Appropriate methanol and sterile distilled water controls were provided and did not inhibit any test organism.

Key: **0** zero inhibition **1** 0-5mm **2** 6-10mm **3** 11-20mm **4** >20mm

Table 4.3.3 Growth inhibition of fungal contaminations by culture filtrates of a range of antagonistic micro-organisms following extraction in chloroform[1].

| | Test Contaminant Species | | | |
|---|---|---|---|---|
| **Antagonist** | *Penicillium* | *Aspergillus* | *Botrytis* | Yeasts |
| *B.subtilis SR1* | 2 | 0 | 1 | 0 |
| *B.subtilis SR2* | 3 | 0 | 1 | 0 |
| *P.fluorescens 95.6* | 1 | 0 | 1 | 0 |
| *P.fluorescens 106.6* | 1 | 0 | 1 | 0 |
| *P.virginiana 70910* | 1 | 0 | 0 | 0 |
| *S.nodosa 25/1875* | 0 | 0 | 0 | 0 |
| *G.simplex 245819* | 0 | 0 | 0 | 0 |
| *C.hiemophillum 274831* | 0 | 0 | 0 | 0 |
| *D.parvispora 101210* | 0 | 0 | 0 | 0 |
| *A.quisqualis 272851* | 1 | 0 | 0 | 0 |
| *D.verticilliata 278511* | 0 | 0 | 0 | 0 |
| *T.viride 153416* | 3 | 2 | 2 | 2 |
| *T.viride 24039* | 3 | 3 | 2 | 1 |
| *T.viride 45553ii* | 2 | 1 | 1 | 1 |
| *T.viride 170657* | 2 | 2 | 1 | 1 |
| *G.roseum 40022* | 1 | 1 | 1 | 0 |

[1] Inhibition zones were measured along the diameter of the filter disc to the circumference of the zone of the inhibited growth. Inhibition zones were monitored after 48 h incubation. Values represent the average of 3 replicates. Appropriate chloroform and sterile distilled water controls were provided and did not inhibit any test organism.

Key: **0** zero inhibition **1** 0-5mm **2** 6-10mm **3** 11-20mm **4** >20mm

Table 4.3.4 Growth inhibition of bacterial contaminations by culture filtrates of a range of antagonistic micro-organisms following extraction in chloroform[1].

| | Test Contaminant Species | | | |
|---|---|---|---|---|
| **Antagonist** | *Erwinia* | *Bacillus* | *Pseudomonas* | *Lactobacillus* |
| *B.subtilis* SR1 | 0 | 1 | 0 | 0 |
| *B.subtilis* SR2 | 0 | 1 | 0 | 0 |
| *P.fluorescens* 95.6 | 1 | 0 | 1 | 0 |
| *P.fluorescens* 106.6 | 1 | 0 | 1 | 0 |
| *E.aerogenes* 85.4 | 0 | 0 | 0 | 0 |
| *C.freundi* 69.5 | 0 | 0 | 0 | 0 |
| *S.gallinarum* 215.4 | 0 | 0 | 0 | 0 |
| *Coryneform* 221.4 | 0 | 0 | 0 | 0 |
| *A.faecalis* 106.1 | 0 | 0 | 0 | 0 |
| *T.viride* 153416 | 0 | 0 | 0 | 0 |
| *T.viride* 24039 | 0 | 1 | 1 | 0 |
| *T.viride* 170657 | 0 | 1 | 1 | 0 |
| *T.viride* 45553ii | 0 | 0 | 0 | 0 |

[1] Inhibition zones were measured along the diameter of the filter disc to the circumference of the zone of the inhibited growth. Inhibition zones were monitored after 48 h incubation. Values represent the average of 3 replicates. Appropriate chloroform and sterile distilled water controls were provided and did not inhibit any test organism.

Key: **0** zero inhibition **1** 0-5mm **2** 6-10mm **3** 11-20mm **4** >20mm

zero activity for all other extracts assessed. This reduction in inhibitory activity was even more pronounced when the extracts were stored over the same period at room temperature (Fig. 4.3.3b), activity being less than 10% of the original level after 3 months storage for all of the extracts assessed.

Filtrate extraction in methanol enhanced the inhibitory activity of some of the filtrates towards specific contaminant species (Tables 4.3.1 & 4.3.2). The greatest activity increases following methanol extraction were noted, to varying degrees, for filtrates of the *Bacillus* and *Pseudomonas* isolates active against contaminant *Penicillium*, *Botrytis*, *Bacillus*, and *Pseudomonas* species. Conversely, methanol extraction reduced the activity of *T.viride* filtrates towards fungal contaminant species by up to 33%. Contaminant *Pseudomonas* species, previously determined to be sensitive to acetone-extracted *T.viride* filtrates (Table 3.1.2), were not susceptible to inhibition by methanol-extracted *T.viride* filtrates (Table 4.3.2). The inhibitory activity of culture filtrates towards test contaminations was not altered by extracting the filtrates in chloroform (Tables 4.3.3 & 4.3.4).

**Discussion**

In Chapter 3 it was established that certain antagonistic micro-organisms were capable of producing cell-free culture filtrates which exhibited inhibitory activity towards the growth of various contaminants of plant tissue cultures and their media. If these anti-microbials are to successfully employed for the control of such contaminations on a commercial scale, then it is essential that their activity is maximised (see Sierota, 1976; Hutter *et al*, 1978; Falkiner, 1990). Investigations conducted in this study attempted to elucidate the optimal growth medium pH and incubation temperature regimes for the production of maximum anti-microbial activity in filtrates of selected antagonists inhibitory towards the growth of test contaminations.

Experiments reviewed in this study showed that filtrates with the greatest inhibitory activity were produced when antagonists were cultured within an initial media pH range of 5 - 7. Inhibitory activity was observed over the entire pH range assessed, but was least effective at the two extreme pH values tested (pH 2 and pH 9). These observations were consistent for filtrates of all of the antagonists studied, irrespective of their nature, or of that of the test contaminations. These results on the effects of pH are contradictory to those of Sierota (1976) who observed the influence of acidity on the inhibitory effects of filtrates of *T.viride* towards the growth of *Fomes Annosus*. Sierota noted that the inhibitory effect of the filtrates was found only at low pH values (2.3 and 4.3) and that with increasing pH the inhibitory effect was seen to decrease. These differences in the observed inhibitory activity of the *T.viride* filtrates noted by Sierota, compared to those observed in this thesis, might be attributed to the use of different strains of the antagonist, different bioassay media (Sierota employed a glucose carbon source base medium), and / or the use of different susceptible test micro-organisms. However, pH effects noted in this study were consistent with the findings of Dennis & Webster (1971a) who did not observe any major differences in the anti-microbial activity of filtrates produced by various species of *Trichoderma* inhibitory to the growth of a range of susceptible test organisms when the antagonist was grown in initial media pH 4.0 and 6.5. Sierota (1976) associated changes in pH during the growth of the antagonist with changes in the $NH_4^+$ ion concentration brought about by nutrient utilization. This explanation could also account for the pH changes observed in these experiments.

The optimal range of antagonist growth temperatures which produced maximum anti-microbial filtrate activity was 20-30$^{O}$C, and 25-35$^{O}$C for the fungal and bacterial antagonists, respectively. These findings were as expected, since most micro-organisms which produce anti-microbial metabolites are mesophilic (i.e. the optimum temperature range for growth and metabolite production is within the range 22-37$^{O}$C), (see Porter, 1972).

Time-course bioassays established that the optimal time in culture for the production of filtrates with maximum inhibitory activity was 6-8 days and 4-6 days for fungal and bacterial antagonists, respectively. It is possible that as nutrients in the growth medium became depleted and microbial growth rates decelerated, the production of inhibitory secondary metabolites declined. The results of these time-course bioassays are consistent with those noted by commercial laboratories investigating anti-microbial activity of filtrates produced by similar bacterial antagonists (P. Rodgers, pers. comm.).

Fermenter studies established that the bulk-volume production of inhibitory filtrates by the antagonist *T.viride* strain 24039 was achievable when the environmental conditions pH, temperature, and aeration were optimized. These optimal conditions were defined as: a constant media pH 6, 40% dissolved oxygen content (maintained by adjustment of agitation speed), 25$^{O}$C incubation temperature, and a 6 day growth period. Therefore the production of large quantities of the inhibitory filtrates could be facilitated should they be employed on a commercial scale as contamination control agents.

Since it is likely that the environmental conditions in plant tissue culture growth media will change during incubation as nutrients become depleted, plant cultures develop, and metabolic by-products are produced, it is possible that the efficacy of supplementary anti-microbial agents in the medium may vary (see Falkiner, 1990). Therefore it is important to be aware of the scope of activity of anti-microbial agents, and to conduct sensitivity tests in the medium in to which the agent is to be added for prophylaxis (Falkiner, 1988).

It has been shown with conventional antibiotics that pH changes may significantly affect their activity; streptomycin is 500 times as active at pH 8.5 than at pH 5.5, while tetracyclines are more active at an acid pH (see Garrod *et al*, 1973). It has been demonstrated in these experiments that inhibitory culture filtrates of all of the antagonists tested here were most stable with respect to their anti-microbial activity when employed within a culture media pH range of 4-8. Since changes in the pH environment during growth of plant tissue cultures are not normally as extensive as this, it is unlikely that their activity would be greatly affected if employed in such systems.

The inhibitory filtrates were stable with respect to heat treatments when incubated below 70$^{o}$C. Whilst *in vitro* plant cultures are never incubated at such high temperatures, it is important to establish heat stability as often antimicrobials may be added to growth medium after some form of heat sterilization, such as autoclaving or pasteurization (see Falkiner,1990). This would be particularly significant if the anti-microbials were to be employed on a large scale, as then the utilisation of low volume, sterile-membrane filters for ensuring asepsis of the agents would be neither practical nor economically viable.

The inhibitory effect of all of the filtrates decreased after storage for prolonged periods. The greatest inhibitory effects were observed when the filtrates were employed in the first few weeks after production, followed by a dramatic reduction in activity. The inhibitory action of the filtrates may possibly have lessened due to oxidation processes and breakdown of the inhibitory metabolites, as suggested in similar studies by Sierota (1976).

Characterization tests indicated that dried extract preparations were soluble in both absolute methanol and chloroform, as well as acetone. Methanol extraction proved successful in enhancing the activity of some of the filtrates inhibitory towards specific contaminant species. Chloroform extraction did not enhance the inhibitory activity of any of the culture filtates. The optimal solvent-extraction systems which exhibited the greatest inhibitory activity for each of the selected filtrates, were employed in subsequent investigations reported in this study.

In conclusion, the series of experiments reported in this chapter have established: (1) the optimum environmental growth conditions for the production of maximum filtrate inhibitory activity; (2) that the large-scale production of inhibitory filtrates is achievable; and (3) the scope of filtrate activity with respect to various physical parameters. These findings are employed in subsequent investigations contained within this thesis.

**Figure 4.1.1**

Optimization of culture medium pH and incubation temperature for production of maximum inhibitory activity by microbial antagonists grown in shaker-flask systems.

A. *T.viride* strain 24039

B. *G.roseum*

1 - 15$^{O}$C; 2 - 20$^{O}$C; 3 - 25$^{O}$C; 4 - 30$^{O}$C 5 - 35$^{O}$C.

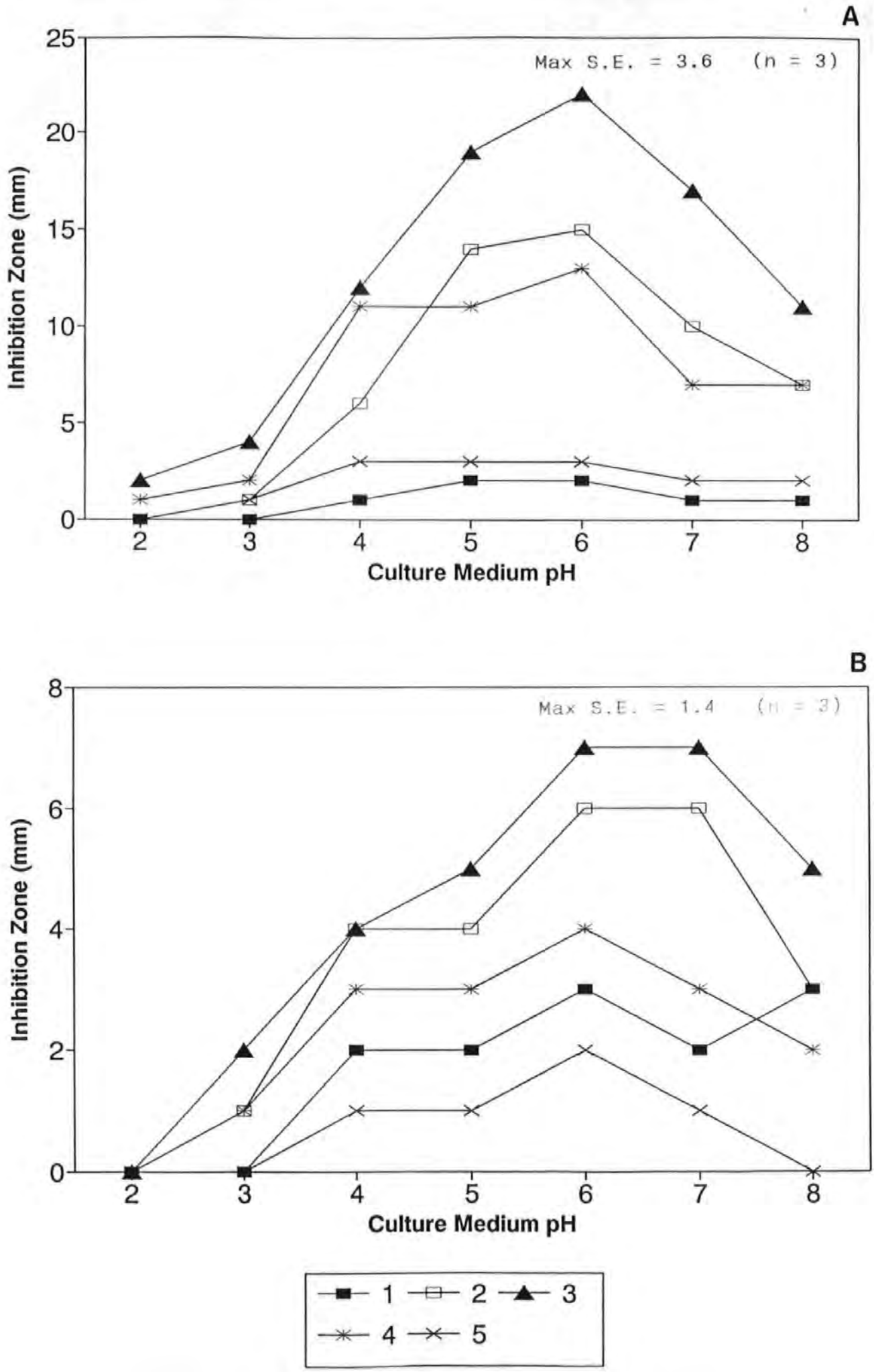

A
Max S.E. = 3.6 (n = 3)
Inhibition Zone (mm)
25
20
15
10
5
0
2
3
4
5
6
7
8
Culture Medium pH
B
Max S.E. = 1.4 (n = 3)
Inhibition Zone (mm)
8
6
4
2
0
2
3
4
5
6
7
8
Culture Medium pH
1
2
3
4
5

**Figure 4.1.2**

Optimization of culture medium pH and incubation temperature for production of maximum inhibitory activity by microbial antagonists grown in shaker-flask systems.

A. *B.subtilis* strain SR2

B. *P.fluorescens*

1 - 15$^{o}$C; 2 - 20$^{o}$C; 3 - 25$^{o}$C; 4 - 30$^{o}$C 5 - 35$^{o}$C.

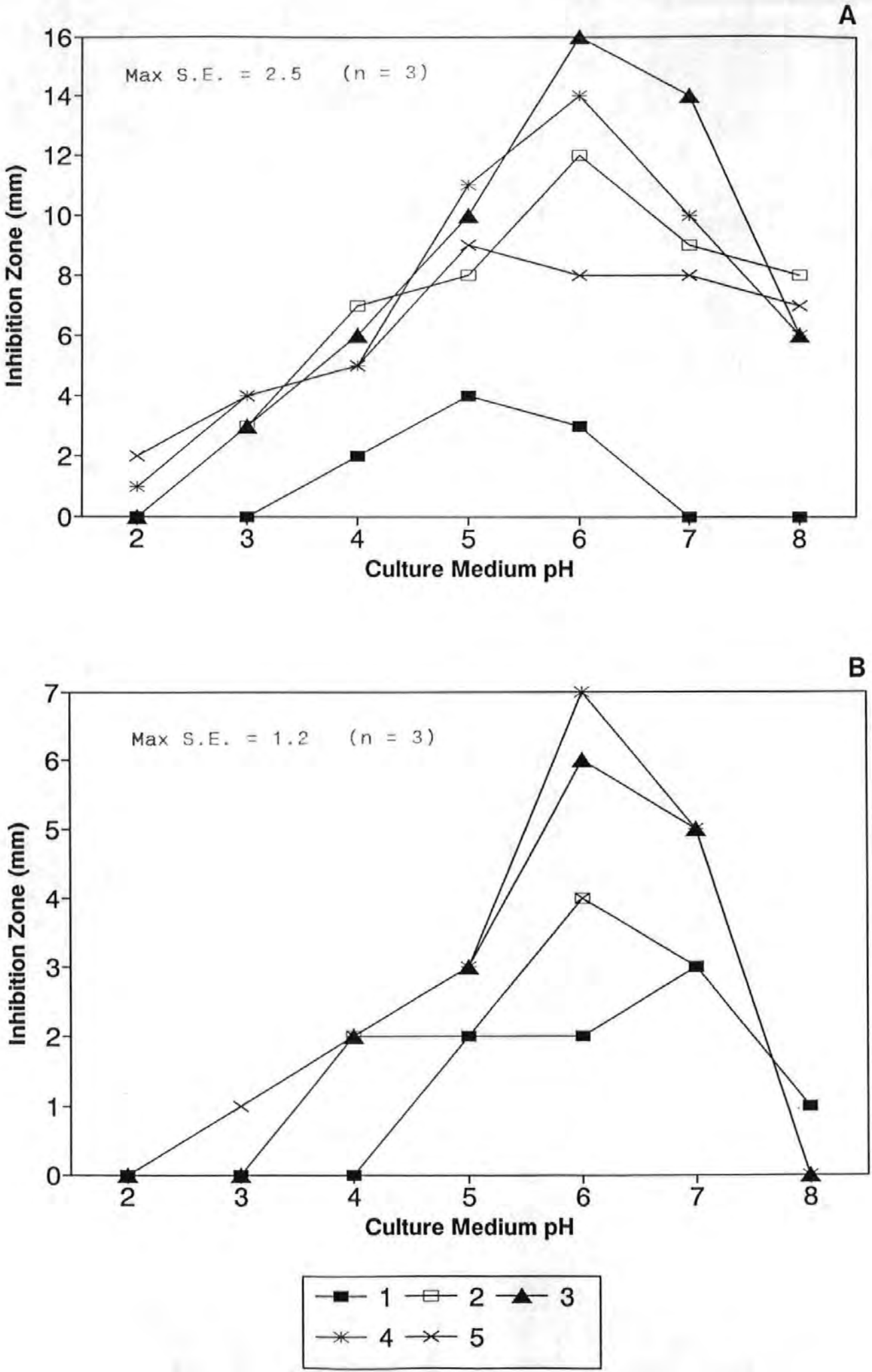

A
Max S.E. = 2.5 (n = 3)
Inhibition Zone (mm)
Culture Medium pH
B
Max S.E. = 1.2 (n = 3)
Inhibition Zone (mm)
Culture Medium pH
1
2
3
4
5

**Figure 4.1.3**

Optimization of culture medium pH and incubation temperature for production of maximum inhibitory activity by microbial antagonists grown in shaker-flask systems.

A. *E.aerogenes*

B. *A.quisqualis*

1 - 15$^{O}$C; 2 - 20$^{O}$C; 3 - 25$^{O}$C; 4 - 30$^{O}$C 5 - 35$^{O}$C.

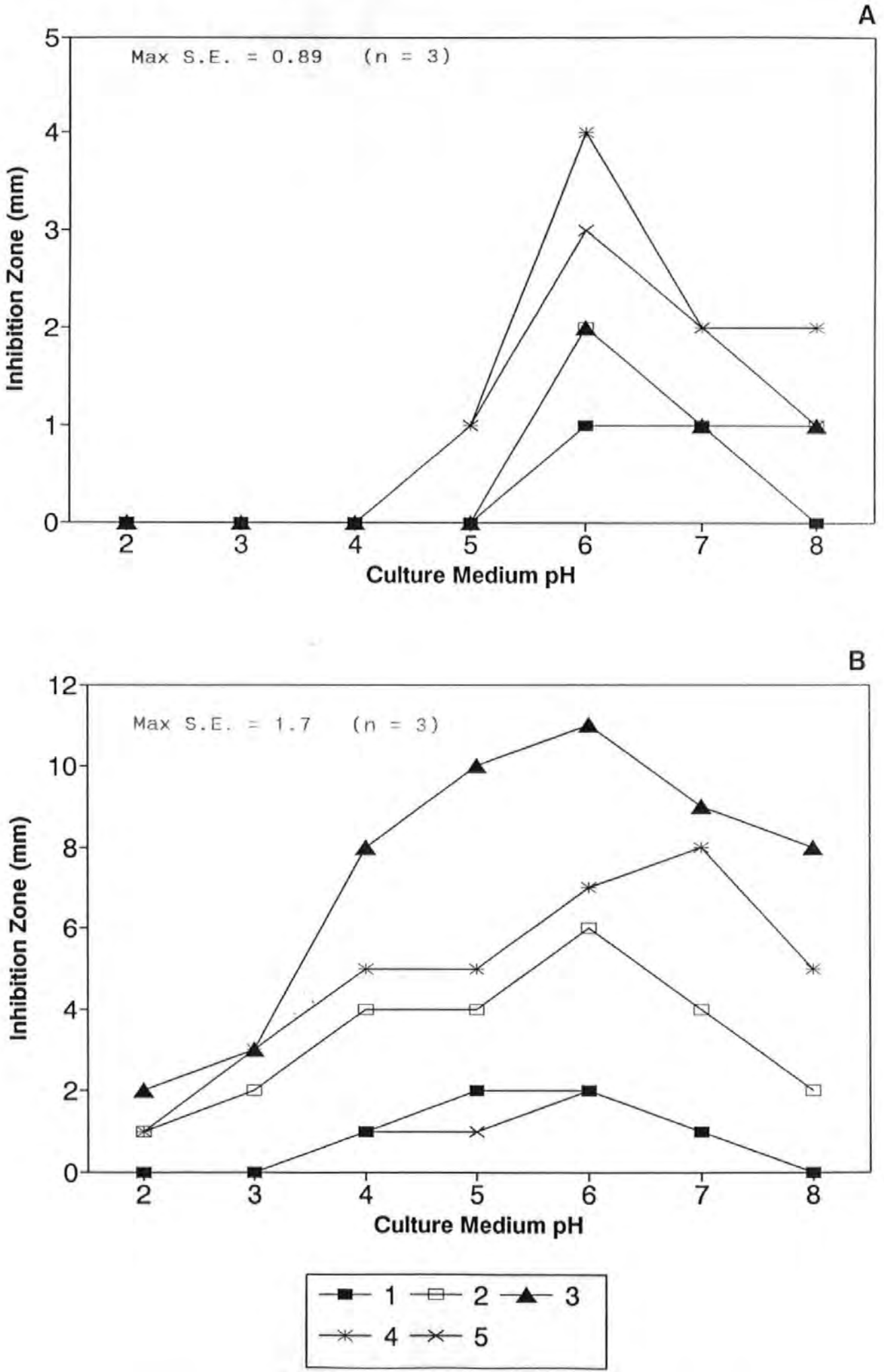

A
Max S.E. = 0.89 (n = 3)
Inhibition Zone (mm)
Culture Medium pH
B
Max S.E. = 1.7 (n = 3)
Inhibition Zone (mm)
Culture Medium pH
1
2
3
4
5

**Figure 4.1.4**

Time-course bioassays of culture filtrates produced by antagonistic micro-organisms.

**A.**

Bacterial antagonists were cultured for up to 10 d in AGC broth at 27$^{O}$C. 48 h filtrate samples were bioassayed for antimicrobial activity towards susceptible test organisms.

A *B.subtilis* SR1

B *B.subtilis* SR2

C *P.fluorescens* 95.6

D *P.fluorescens* 106.6

E *E.aerogenes*

**B.**

Fungal antagonists were cultured for up to 10 d in malt broth at 25$^{O}$C. 48 h filtrate samples were bioassayed for antimicrobial activity towards susceptible test organisms.

A *T.viride* 24039

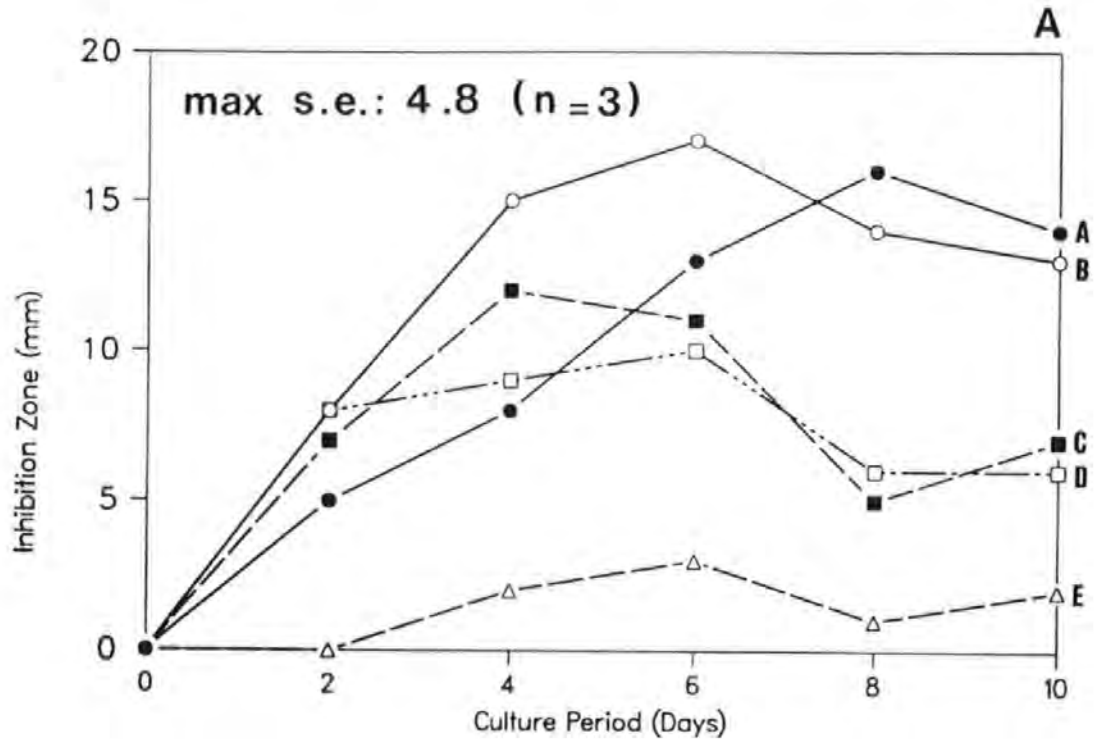
B *T.viride* 153416

C *T.viride* 170657

D *T.viride* 45553ii

E *A.quisqualis*

F *G.roseum*


A
max s.e.: 4.8 (n=3)
Inhibition Zone (mm)
Culture Period (Days)
A
B
C
D
E

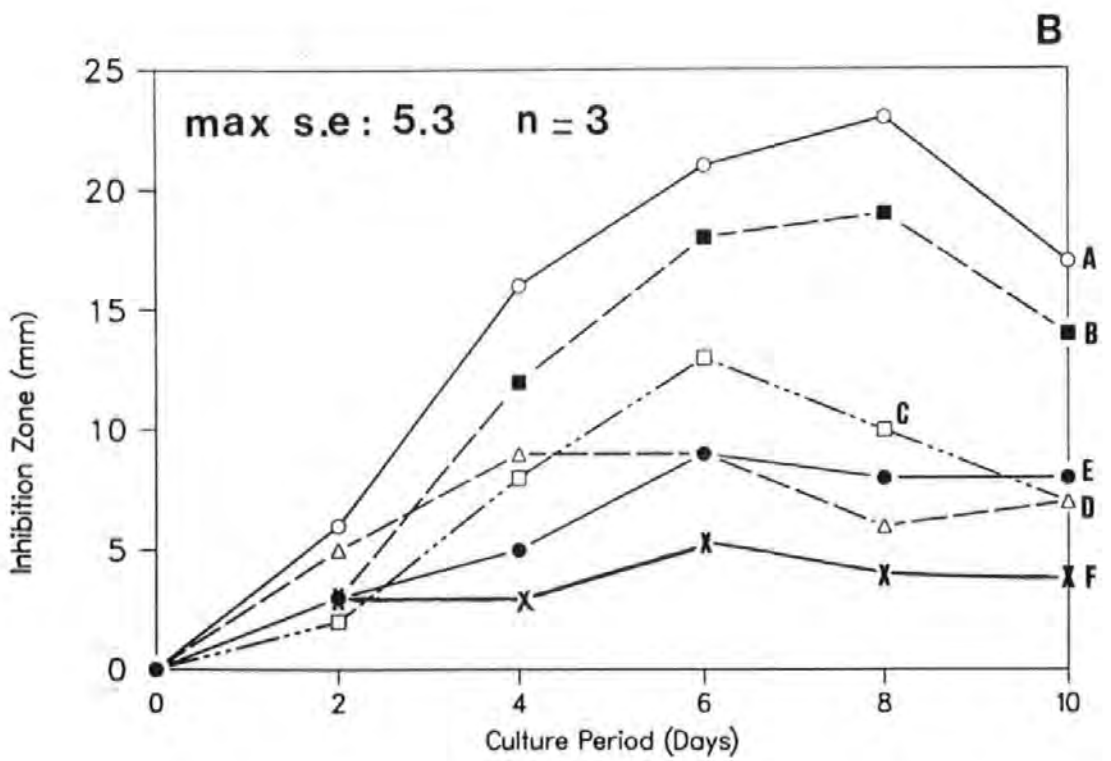

B
max s.e: 5.3 n=3
Inhibition Zone (mm)
Culture Period (Days)
A
B
C
D
E
F

**Figure 4.1.5** Changes in initial pH of growth media.

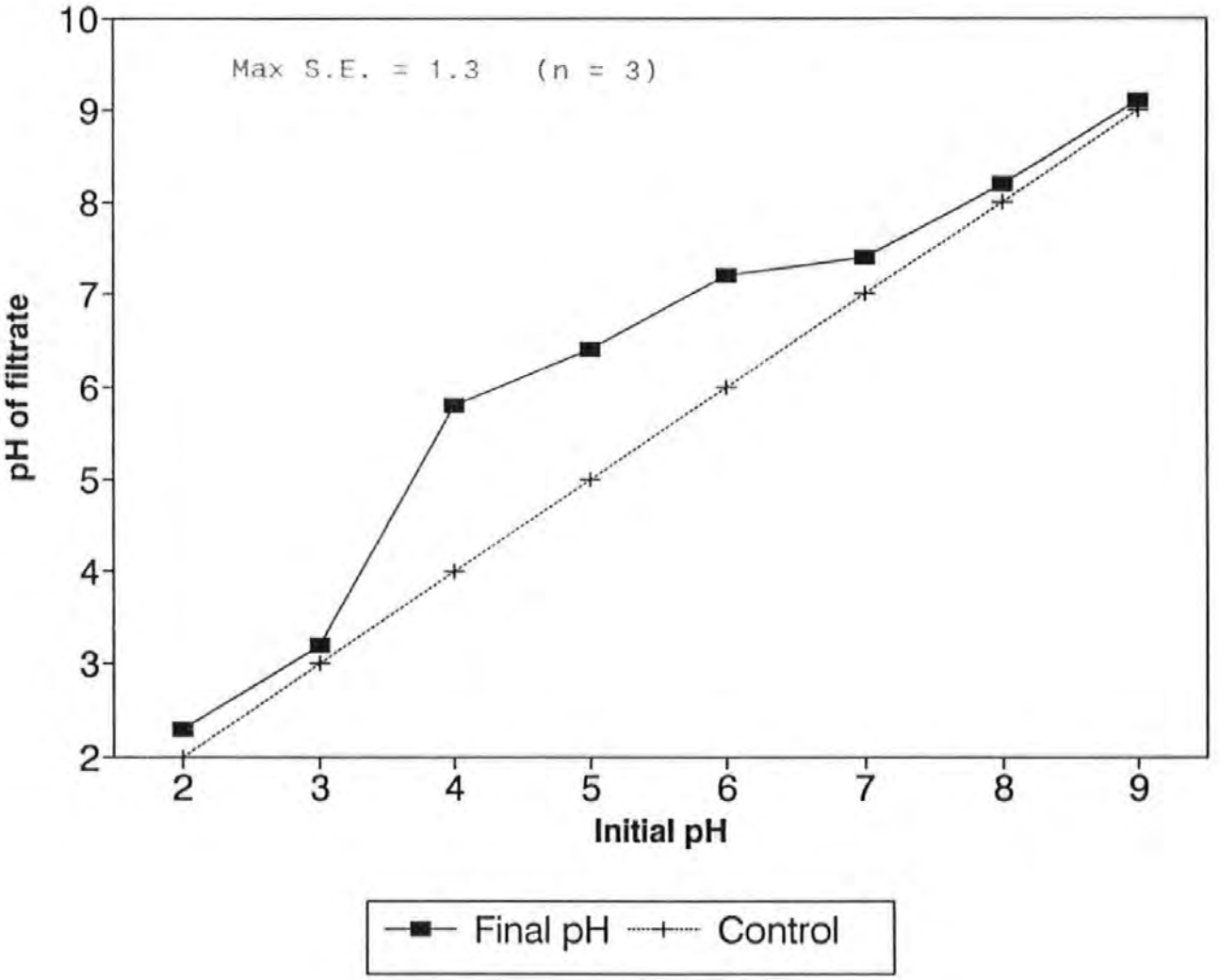


**Figure 4.2.1**

Effect of culture medium pH and incubation temperature on production of maximum inhibitory activity by *T.viride* strain 24039 grown in a laboratory scale fermenter system (LH 502D) for up to 10 days at a constant $DO_2$ of 45%.

1 - 20$^{O}$C, pH5

2 - 20$^{O}$C, pH6

3 - 25$^{O}$C, pH5

4 - 25$^{O}$C, pH6

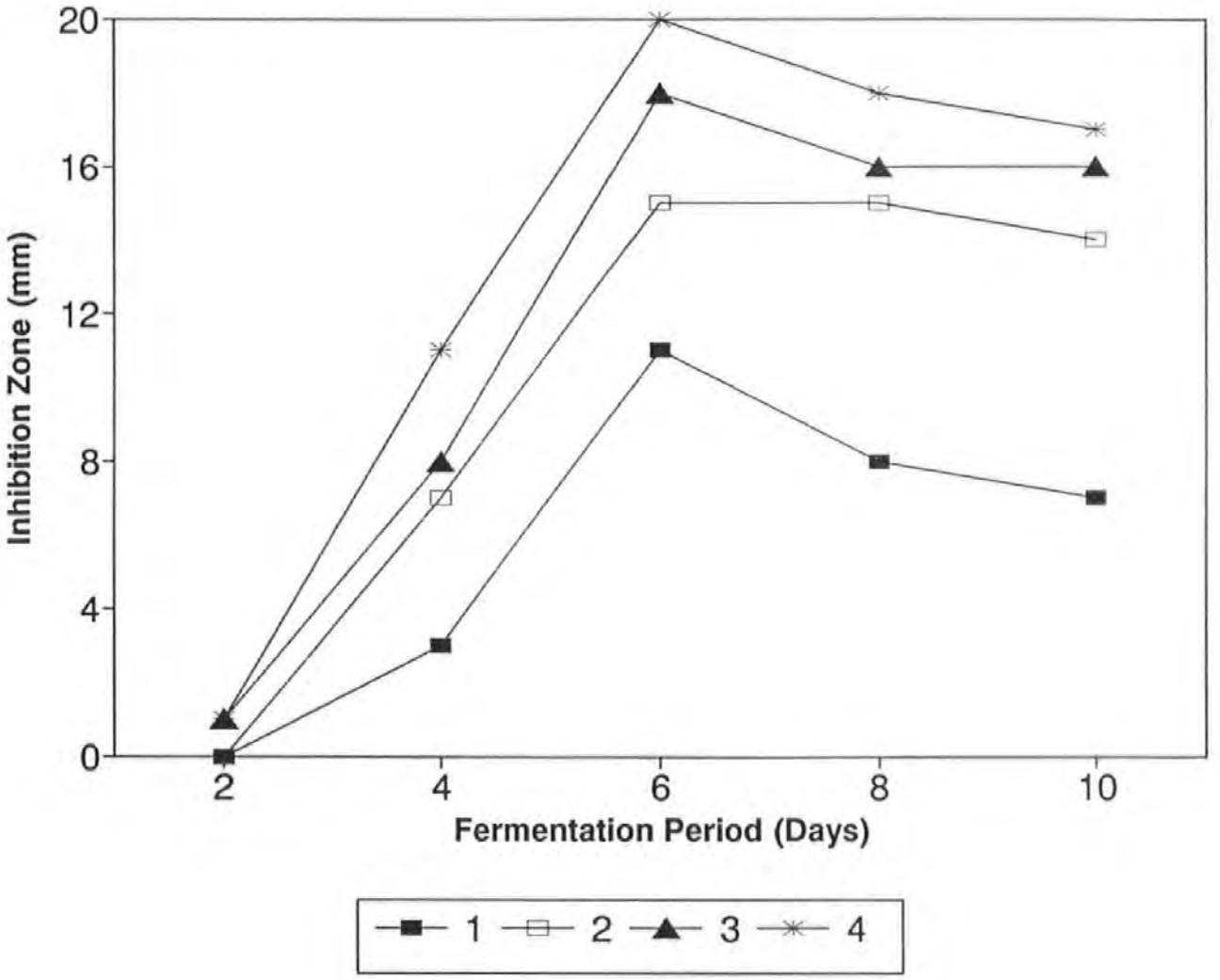

20
16
12
8
4
0
Inhibition Zone (mm)
2
4
6
8
10
Fermentation Period (Days)
1
2
3
4

**Figure 4.2.2**

Growth of *T.viride* strain 24039 in a laboratory scale fermenter system (LH model 502D) at various pH values and incubation temperatures for up to 10 days at a constant $DO_2$ of 45%.

1 - 20°C, pH5

2 - 20°C, pH6

3 - 25°C, pH5

4 - 25°C, pH6

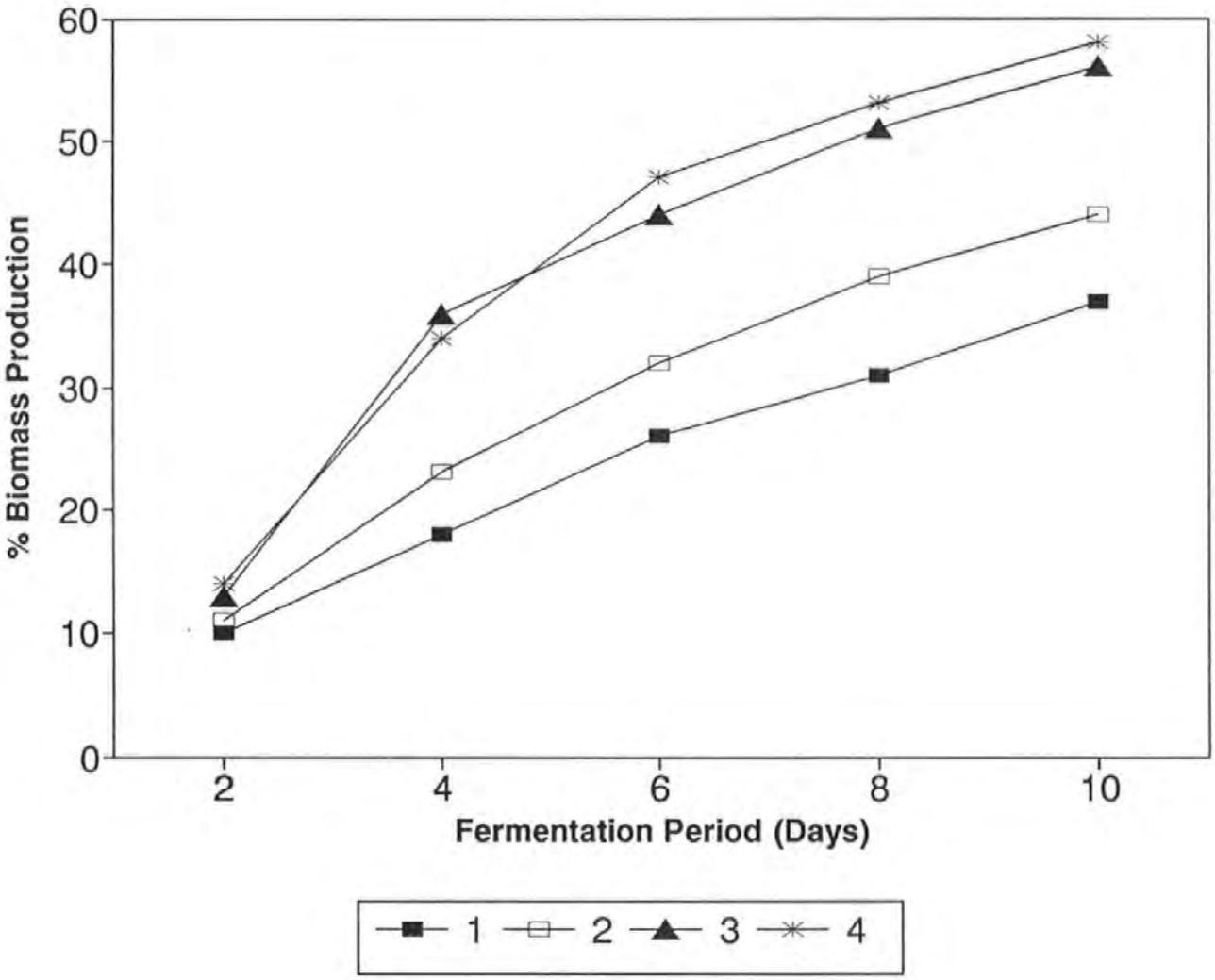

60
50
40
30
20
10
0
% Biomass Production
2
4
6
8
10
Fermentation Period (Days)
1
2
3
4

**Figure 4.2.3**

Effects of dissolved oxygen content on biomass production and anti-microbial activity of *T.viride* strain 24039 cultured in a fermenter system ($25^{O}C$, pH6) for 6 days.

1 - Inhibition zone (mm)

2 - % Biomass production

c - critical % biomass level above which inhibitory activity decreases.

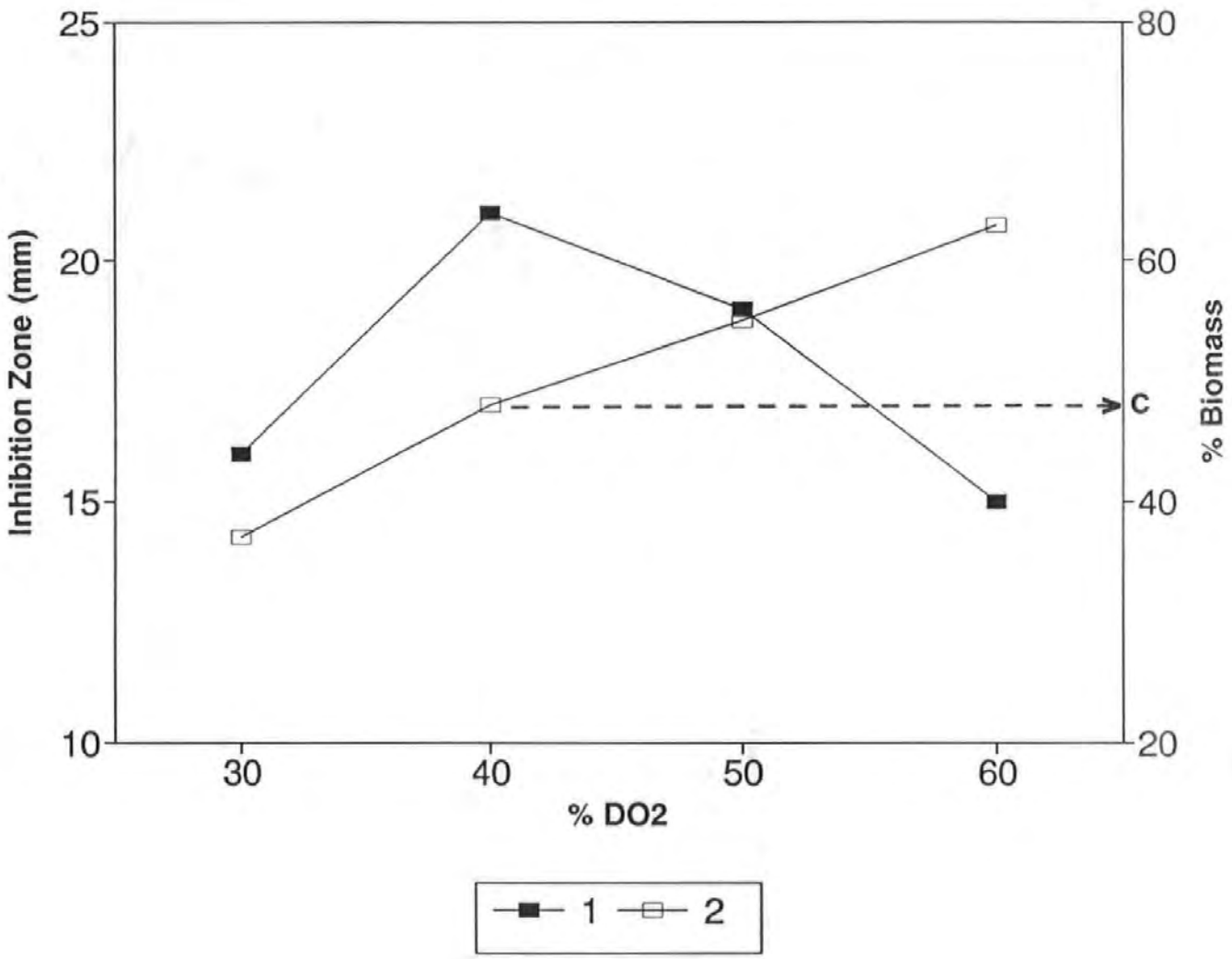

25
20
15
10
80
60
40
20
Inhibition Zone (mm)
% Biomass
C
30
40
50
60
% DO2
1
2

**Figure 4.3.1**

pH stability of cell-free culture filtrates produced by microbial antagonists.

1 - *T.viride* strain 24039

2 - *B.subtilis* strain SR2

3 - *P.fluorescens* strain 95.6

4 - *A.quisqualis*

5 - *G.roseum*

6 - *E.aerogenes*

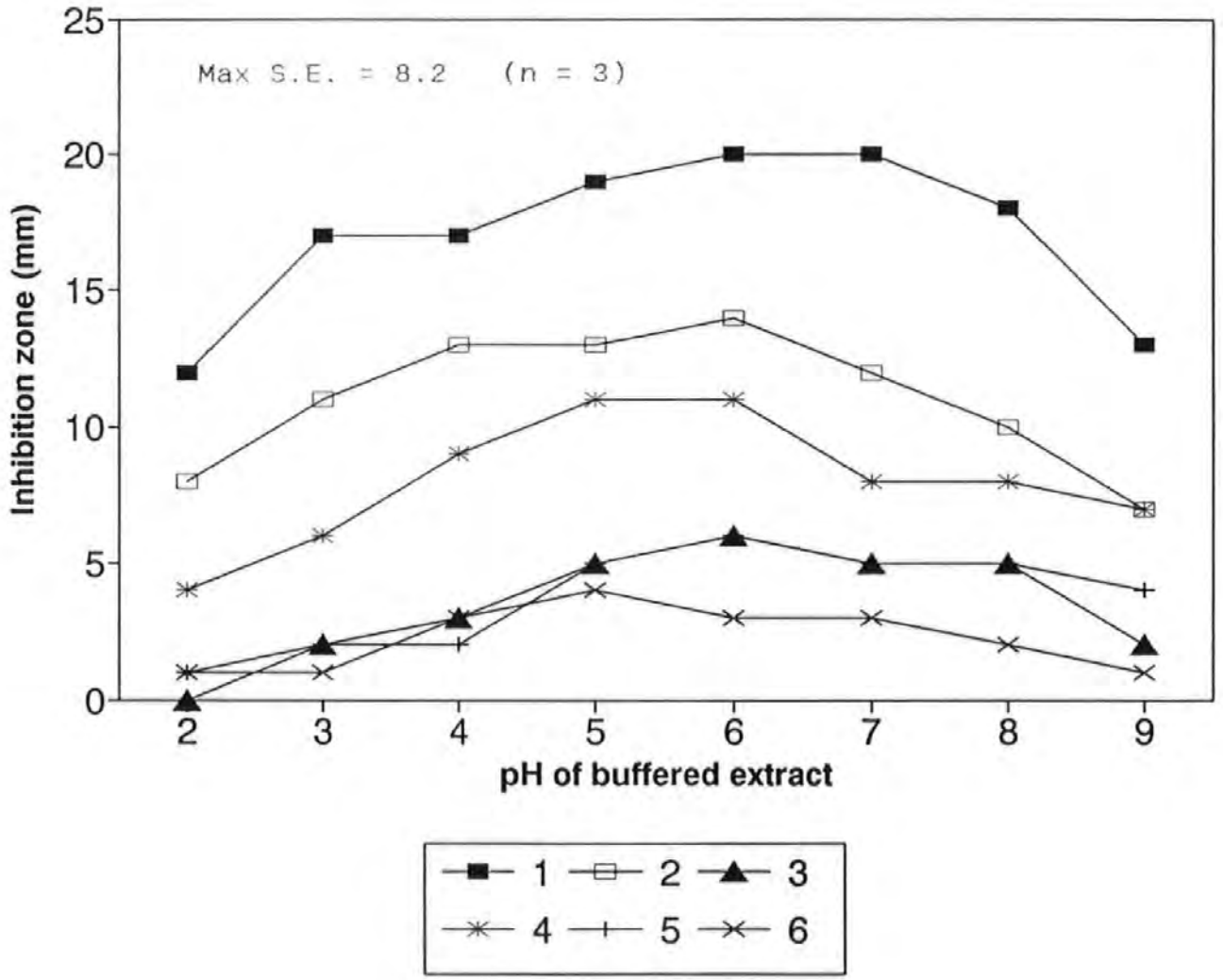

Max S.E. = 8.2 (n = 3)
Inhibition zone (mm)
25
20
15
10
5
0
2
3
4
5
6
7
8
9
pH of buffered extract
1
2
3
4
5
6

**Figure 4.3.2**

Temperature stability of cell-free culture filtrates produced by microbial antagonists.

A. Filtrate samples bioassayed after heating at various temperatures up to 100$^{O}$C for 30 minutes.

B. Filtrate samples bioassayed after heating at 70$^{O}$C for up to 30 minutes.

1 - *T.viride* strain 24039

2 - *B.subtilis* strain SR2

3 - *P.fluorescens* strain 95.6

4 - *A.quisqualis*

5 - *G.roseum*

6 - *E.aerogenes*

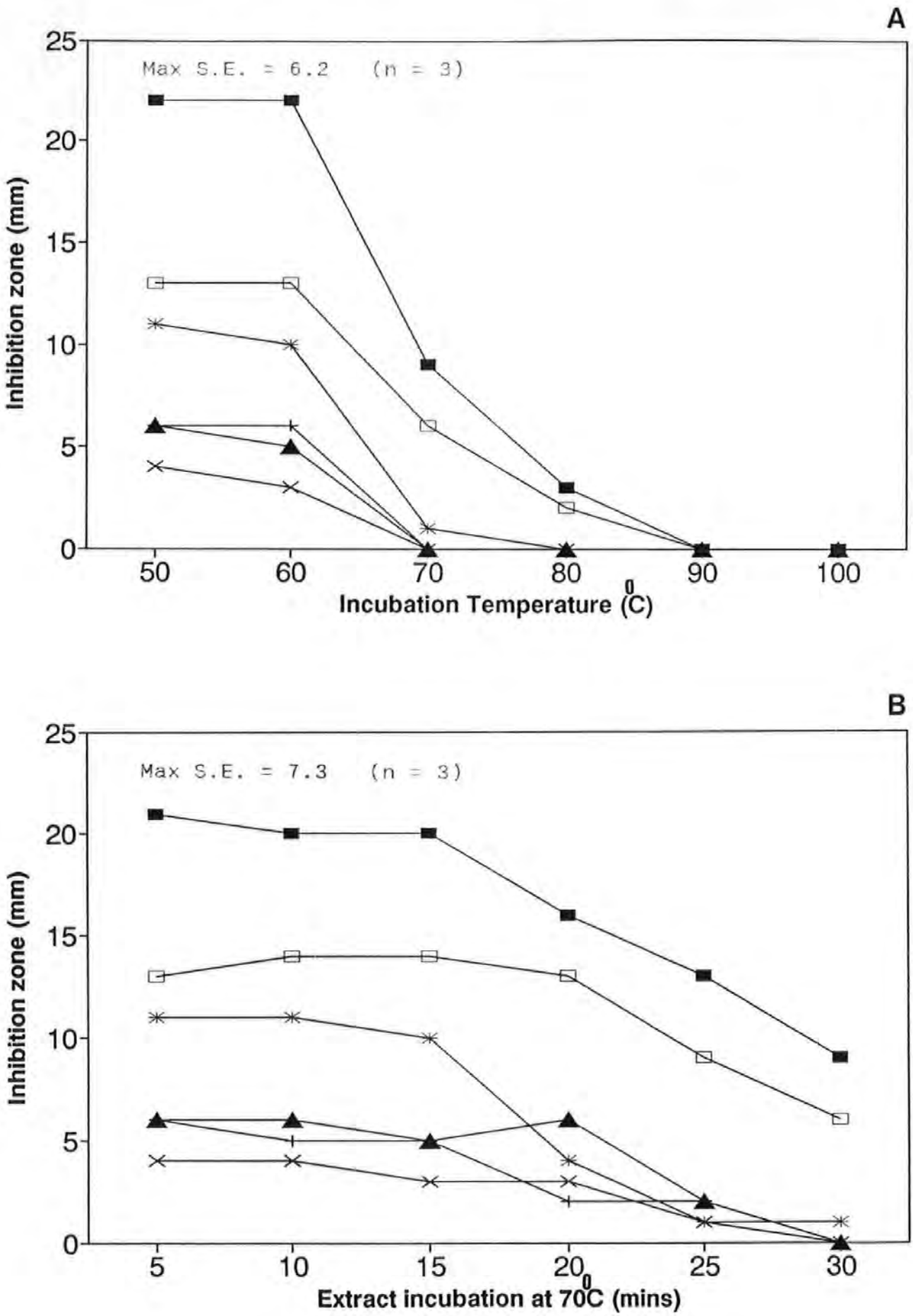

A
Max S.E. = 6.2 (n = 3)
Inhibition zone (mm)
25
20
15
10
5
0
50
60
70
80
90
100
Incubation Temperature (°C)
B
Max S.E. = 7.3 (n = 3)
Inhibition zone (mm)
25
20
15
10
5
0
5
10
15
20
25
30
Extract incubation at 70°C (mins)
1
2
3
4
5
6

**Figure 4.3.3**

Temporal stability of cell-free culture filtrates produced by microbial antagonists.

A. Filtrate storage at $4^{O}C$ for up to 4 months.

B. Filtrate storage at room temperature for up to 4 months.

1 - *T.viride* strain 24039

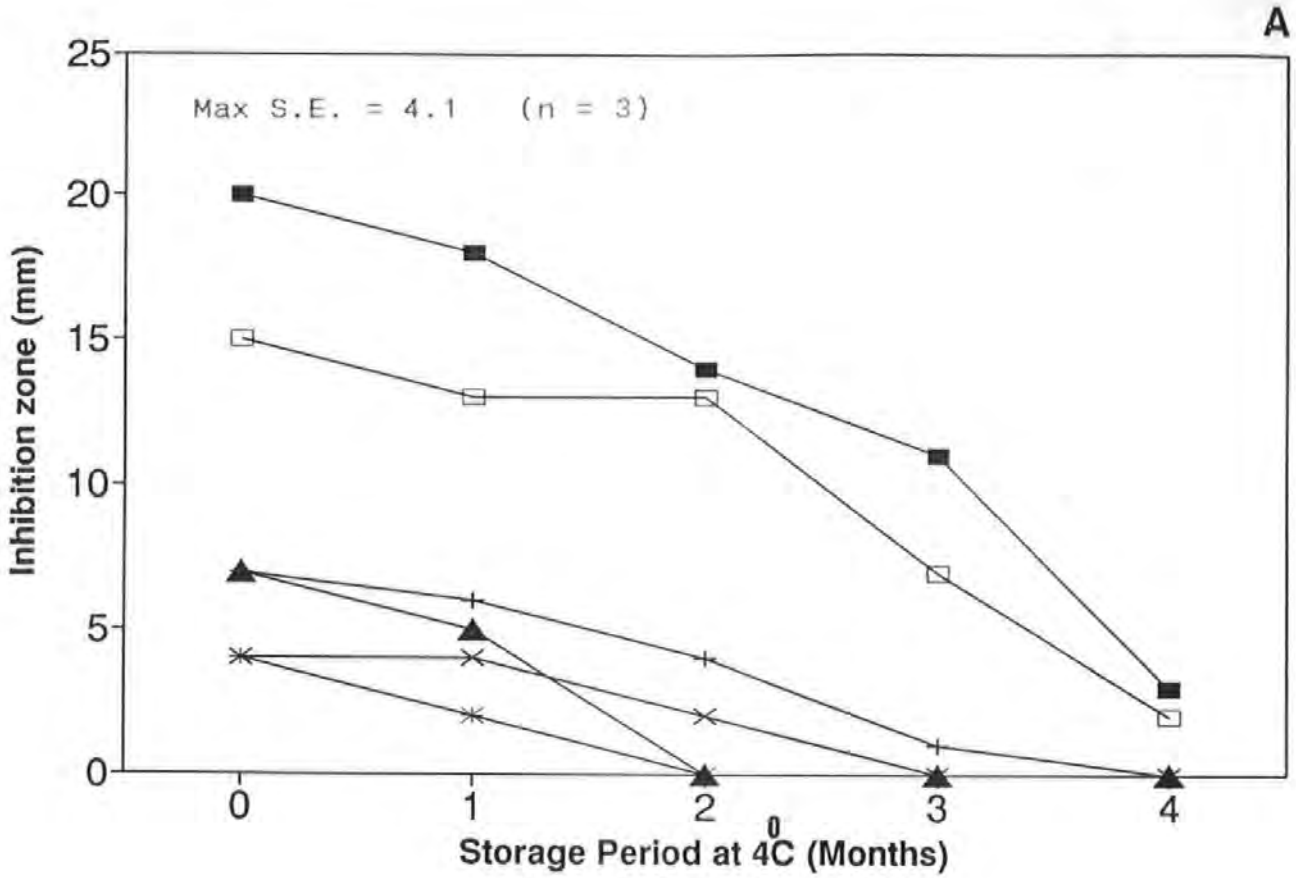
2 - *B.subtilis* strain SR2

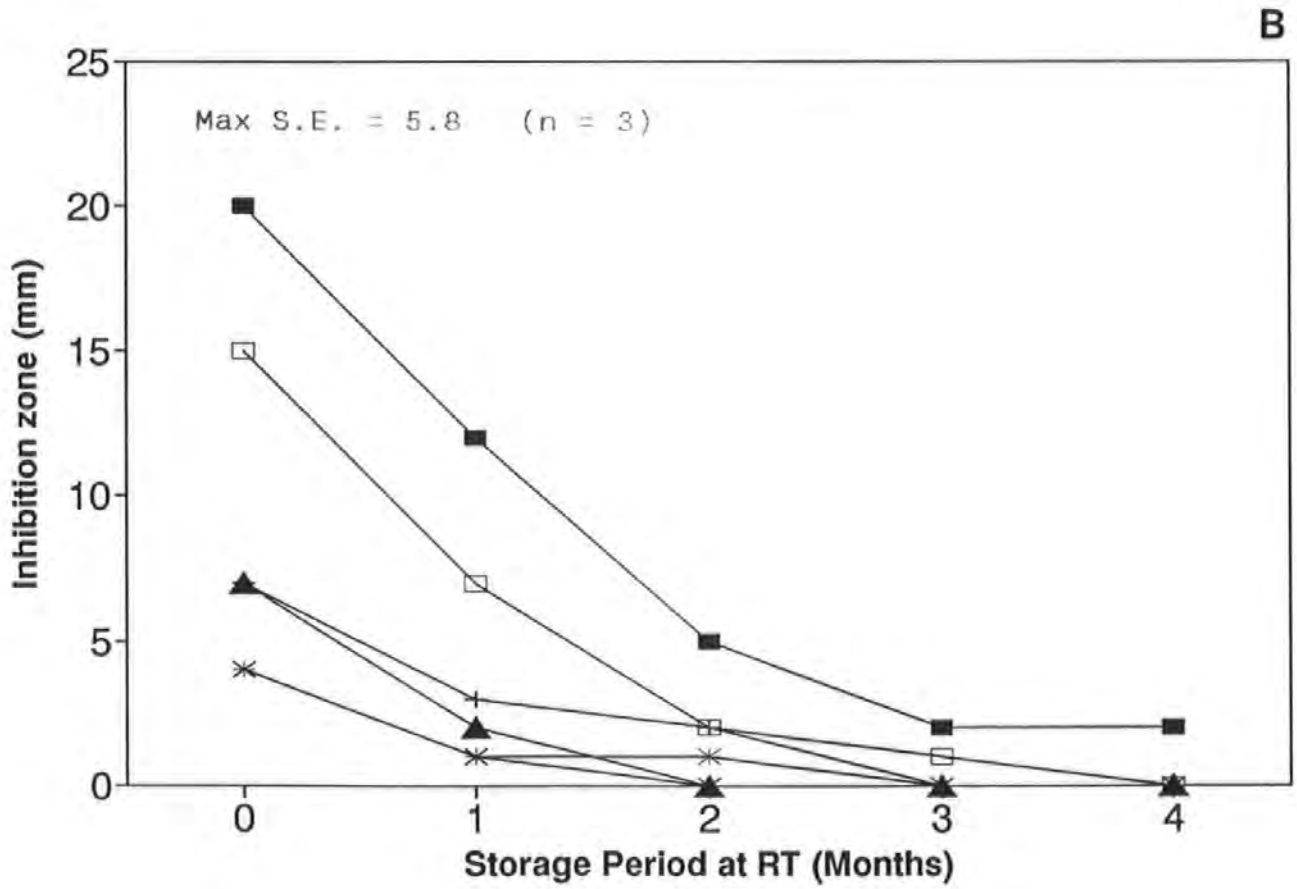
3 - *P.fluorescens* strain 95.6

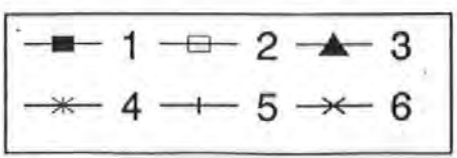
4 - *A.quisqualis*

5 - *G.roseum*

6 - *E.aerogenes*


A
Max S.E. = 4.1 (n = 3)
Inhibition zone (mm)
25
20
15
10
5
0
0
1
2
3
4
Storage Period at 4C (Months)


B
Max S.E. = 5.8 (n = 3)
Inhibition zone (mm)
25
20
15
10
5
0
0
1
2
3
4
Storage Period at RT (Months)


1
2
3
4
5
6

CHAPTER 5

# EVALUATION OF THE PHYTOTOXICITY OF CELL-FREE CULTURE FILTRATES IN PLANT TISSUE CULTURE SYSTEMS

Traditionally antibiotic compounds have been employed as a supplement to culture media to eliminate or reduce accidental contaminations and latent endogenous infections in plant tissue culture systems (see review by Falkiner, 1990). However, their application is not generally recommended, as they are often phytotoxic or may retard plant tissue growth (Dodds & Roberts, 1981; Eichholtz *et al.*, 1982; Pollock *et al.*, 1983) and prolonged exposure increases the risk of resistance and may even progress to dependence on the drug (Falkiner, 1990).

Previous reports have described the antimicrobial activity of cell-free culture filtrates produced by antagonistic micro-organisms (e.g. Broadbent *et al.*, 1971; Dennis & Webster, 1971a; Morris & Lane, 1990; Schreiber *et al.*, 1988; Kope & Fortin, 1989). As such antagonistic micro-organisms are often natural plant symbionts or live in close association with the intact plant, their culture filtrates are less likely to be toxic to plant cells or tissues (Herman, 1987). Therefore it is feasible that such filtrates could be employed in plant tissue culture systems for the prevention of contamination. However, prior to application it is essential that their phytotoxicity is assessed. The purpose of this study was to determine the phytotoxicity of a range of microbial culture filtrates exhibiting anti-microbial activity (see chapter 3) and therefore to evaluate their potential for application as contamination control agents. The recovery capacity of plant species which exhibited reduced growth when cultured in extract-incorporated media was assessed when they were subsequently grown in extract-free media for several growth passages.

## 5.1 *In Vitro* Phytotoxicity Studies

Due to time and resource constraints only representatives of those antagonistic species exhibiting the greatest inhibition of test contaminations in preliminary tests (see chapter 3) were selected for phytotoxicity studies (i.e. *T.viride* strain 24039, *B.subtilis* strain SR2, *P.fluorescens* strain 95.6, and *G.roseum*). The results of these studies are shown in Figs. 5.1.1 - 5.1.5. Plates 5.1.1 and 5.1.2 illustrate the effects of filtrates produced by the *T.viride* strain 24039 isolate (as described in Chapter 3) on shoot, and on root cultures of tobacco, respectively. The maximum permissive doses of the various culture filtrates (i.e. the dosage which does not significantly reduce growth of test plant cultures as determined by the parameters number of shoots or roots [N], length of shoots or roots [L], and fresh weight of cultures [FW]) are summarised in Table 5.1.1.

The plant cultures showed different responses to the various microbial culture filtrates tested (Figs. 5.1.1 - 5.1.5). The maximal permissive doses of the filtrates varied for the different plant species studied (Table 5.1.1). In general, filtrates produced by the *T.viride* and *B.subtilis* isolates were the least phytotoxic towards the various plant cultures (both tolerated up to 7.5% v/v dosage). Filtrates produced by the *P.fluorescens* and *G.roseum* isolates were only tolerated at lower doses (2.5% v/v), with the exception of the tobacco shoot cultures, which continued to develop without any significant effect when grown on media containing up to 5% v/v *P.fluorescens* filtrate. Maximal permissive doses were less varied with respect to the number, length, and fresh weight of shoots or roots of a particular type of culture (i.e. the phytotoxic effect of filtrates towards the 3 different growth parameters studied was of a similar magnitude for any one particular plant species).

Table 5.1.1

Highest permissive doses (%v/v) of various microbial culture filtrates incorporated in to growth media which do not significantly reduce the development of a range of plant tissue cultures grown for 6 weeks.

| | | Highest Permissive Dose (%v/v) | | | |
|---|---|---|---|---|---|
| **Plant Spp.** | **Extract** | **N** | **L** | **FW** | **Maximum Allowed Dose (%v/v)** |
| **Tobacco (Shoot)** | TV | 5.0 | 5.0 | 5.0 | 5.0 |
| | BS | 7.5 | 5.0 | 7.5 | 7.5 |
| | PF | 5.0 | 5.0 | 2.5 | 5.0 |
| | GR | 1.0 | 1.0 | 1.0 | 1.0 |
| **Tobacco (Root)** | TV | 5.0 | 5.0 | 5.0 | 5.0 |
| | BS | 7.5 | 10.0 | 7.5 | 7.5 |
| | PF | 2.5 | 5.0 | 5.0 | 2.5 |
| | GR | 2.5 | 5.0 | 5.0 | 2.5 |
| **Witch-Hazel** | TV | 7.5 | 5.0 | 7.5 | 7.5 |
| | BS | 5.0 | 7.5 | 5.0 | 5.0 |
| | PF | 2.5 | 5.0 | 2.5 | 2.5 |
| | GR | 2.5 | 5.0 | 2.5 | 5.0 |
| **Birch** | TV | 7.5 | 5.0 | 5.0 | 7.5 |
| | BS | 5.0 | 5.0 | 5.0 | 5.0 |
| | PF | 2.5 | 5.0 | 2.5 | 2.5 |
| | GR | 5.0 | 2.5 | 2.5 | 2.5 |
| **Rosewood** | TV | 5.0 | 7.5 | 7.5 | 5.0 |
| | BS | 5.0 | 7.5 | 5.0 | 5.0 |
| | PF | 2.5 | 2.5 | 5.0 | 2.5 |
| | GR | 2.5 | 2.5 | 2.5 | 2.5 |

Number of shoots or roots [N], Length of shoots or roots [L] and fresh weight [FW].

*T.viride* (TV), *B.subtilis* (BS), *P.fluorescens* (PF) and *G.roseum* (GR).

**Plate 5.1.1**

Phytotoxic effects of culture filtrates produced by *T.viride* strain 24039 on shoot proliferation in *Nicotina* cultures.

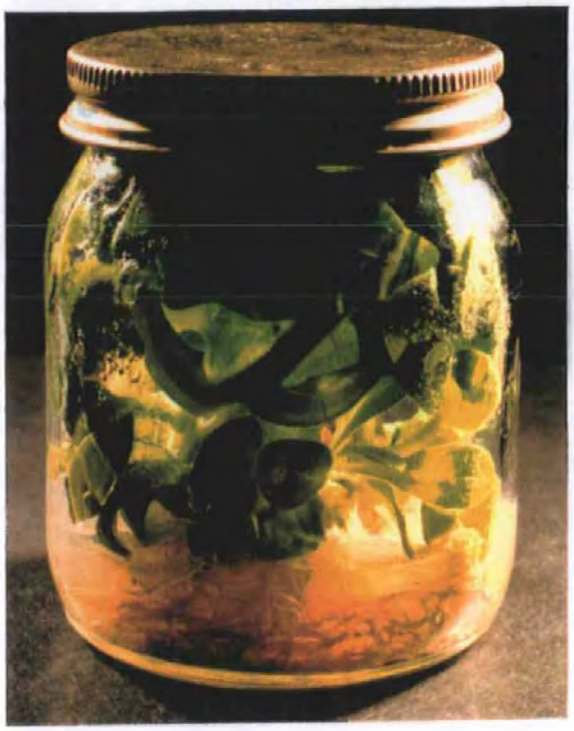
A range of extract doses were incorporated in to tobacco culture growth media upon which *Nicotiana* explants were cultured for 6 weeks at 21-25$^{O}$C.

A Untreated control

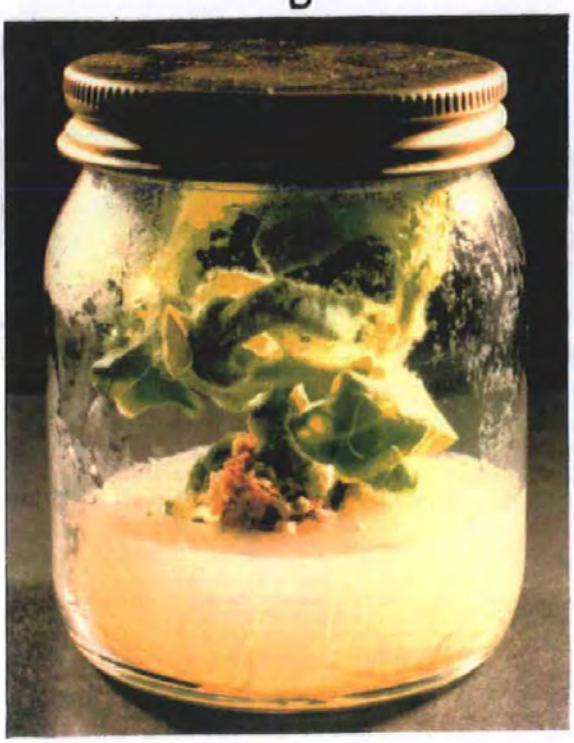
B 7.5 % v/v

C 10 % v/v

A



B



C

**Plate 5.1.2**

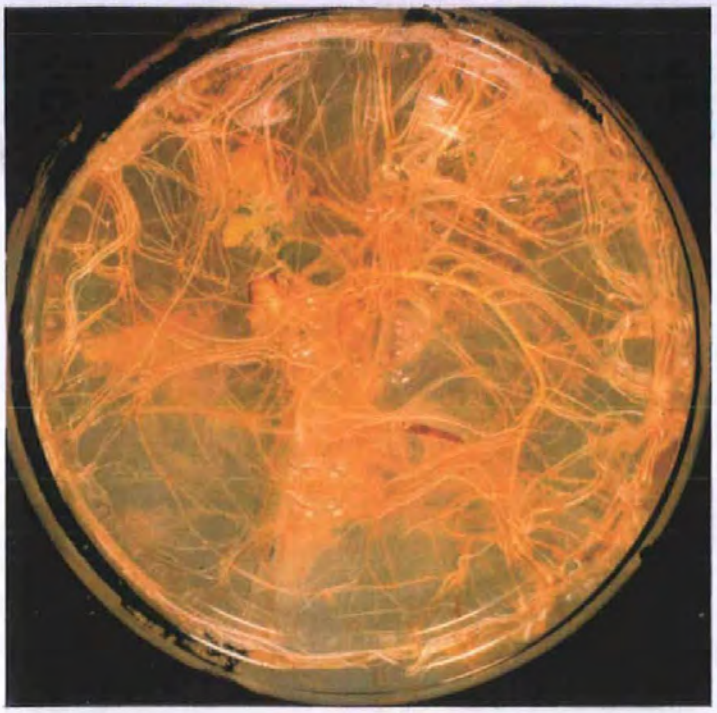
Phytotoxic effects of culture filtrates produced by *T.viride* strain 24039 on root development in *Nicotina* cultures.

A range of extract doses were incorporated in to tobacco culture growth media upon which *Nicotiana* explants were cultured for 6 weeks at 21-25$^{O}$C.

A Untreated control

B 10 % v/v

C 20 % v/v



A

B

C

Of the test plant species, none appeared particularly resistant or particularly susceptible to the various culture filtrates (i.e. maximum allowed doses were all within a similar range of 2.5 -7.5% v/v), with the exception of the tobacco shoot cultures, which could only tolerate levels of *G.roseum* of up to 1% v/v.

## 5.2 Recovery Capacity

Shoot and root proliferation in the various plant cultures under study was observed when the cultures were grown in extract-free media for up to four passages, after previously being grown in media containing extract doses greater than the maximum allowed levels (Figs. 5.2.1 - 5.2.5). These results are summarised in terms of the recovery capacity of the various plant cultures, with respect to the number of shoots produced, in Table 5.2.1.

The plant cultures displayed different recovery responses to the various microbial filtrates tested (Figs. 5.2.1 - 5.2.5). These different recovery capacities varied in terms of the number of growth passages required before the plant cultures exhibited (1) an increase in shoot or root proliferation [$N_O$], and (2) shoot or root proliferation of up to 50% and 100% of that level obtained when plant cultures were grown in media containing the maximum allowed dose of filtrate [$N_{50}$ and $N_{100}$, respectively]), as illustrated in Table 5.2.1.

The tobacco shoot and the tobacco root cultures both displayed a slow recovery in terms of increased proliferation, as defined by the above parameters $N_O$, $N_{50}$, and $N_{100}$. Both the witch-hazel and the rosewood plant cultures exhibited very poor growth recovery, and often a total cessation of culture growth was observed (i.e. death of the plant culture, as exemplified by a high proportion of necrotic tissue, brown calli, and the appearance of chlorotic leaves and stems in such cultures). Contrary to these observations, the birch plant cultures displayed a relatively good recovery capacity, attaining

previously observed levels of shoot proliferation when treated with all the filtrates tested with the exception of those from the *G.roseum* strain.

With reference to the anti-microbial filtrates themselves, those produced by the *B.subtilis* and *G.roseum* isolates were the most deleterious to plant cultures. Without exception, none of the test plant culture species treated with filtrates from these two isolates recovered to pre-treatment proliferation levels (i.e. 100% recovery) after being grown for up to 4 passages in extract-free media. Three of the 5 plant cultures treated with the *G.roseum* filtrates failed to recover whatsoever and death of these cultures eventually occurred. With the exception of the rosewood species, all test plant cultures treated with filtrates from the *T.viride* isolate recovered to pre-treatment proliferation levels after being grown for up to 4 passages in extract-free media. Filtrates from the *P.fluorescens* isolate were the least damaging of all the extracts tested, and for 4 of the 5 plant culture species studied shoot or root proliferation returned to pre-treatment levels after only 2 or 3 growth passages in extract-free media.

Table 5.2.1

Recovery capacity of various plant cultures after being grown in media containing doses of anti-microbial filtrates greater than the previously determined maximum allowed levels.

| Plant Spp. | Extract | Maximum Allowed Level (% v/v) | Test Dose (% v/v) | $N_o$ | $N_{50}$ | $N_{100}$ |
|---|---|---|---|---|---|---|
| **Tobacco (Shoot)** | **TV** | 5.0 | 7.5 | 2 | 0 | 3 |
| | **BS** | 7.5 | 10.0 | 2 | 2 | >4 |
| | **PF** | 5.0 | 7.5 | 2 | 0 | 3 |
| | **GR** | 1.0 | 2.5 | 2 | 2 | >4 |
| **Tobacco (Root)** | **TV** | 5.0 | 7.5 | 1 | 0 | 4 |
| | **BS** | 7.5 | 10.0 | 3 | 0 | >4 |
| | **PF** | 2.5 | 5.0 | 2 | 0 | 3 |
| | **GR** | 2.5 | 5.0 | D | D | D |
| **Witch-Hazel** | **TV** | 7.5 | 10.0 | 2 | 3 | 4 |
| | **BS** | 5.0 | 7.5 | D | D | D |
| | **PF** | 2.5 | 5.0 | 1 | 1 | 2 |
| | **GR** | 5.0 | 7.5 | D | D | D |
| **Birch** | **TV** | 7.5 | 10.0 | 2 | 0 | 3 |
| | **BS** | 5.0 | 7.5 | 3 | 0 | 4 |
| | **PF** | 2.5 | 5.0 | 2 | 0 | 2 |
| | **GR** | 2.5 | 5.0 | 2 | 2 | >4 |
| **Rosewood** | **TV** | 5.0 | 7.5 | 3 | 3 | >4 |
| | **BS** | 5.0 | 7.5 | 2 | 0 | >4 |
| | **PF** | 2.5 | 5.0 | 2 | 0 | 4 |
| | **GR** | 2.5 | 5.0 | D | D | D |

No : Number of growth passages required before plant cultures exhibit an increase in the number of shoots or roots produced.

$N_{50}$ : Number of growth passages required before plant cultures exhibit a level of shoot production of at least 50% of that level obtained when plant cultures were grown in media containing up to the maximum allowed dose of anti-microbial filtrates. Zero indicates that previous growth in excessive extract doses did not reduce the number of shoots produced by more than 50%.

$N_{100}$ : Number of growth passages required before plant cultures exhibit a level of shoot production similar to that level obtained when plant cultures were grown in media containing up to the maximum allowed dose of anti-microbial filtrates .('D' represents zero recovery (i.e. death) of the plant culture).

TV, *T.viride*; BS, *B.subtilis*; PF, *P.fluorescens*; GR, *G.roseum*

## 5.3 Phytotoxic effects at the cellular level

Morphological changes in 28 day old callus cells of *Nicotiana tabacum* were observed using cryo-scanning electron microscopy techniques (see section 2.10) after growth of the plant tissues on media containing various doses of filtrates produced by the antagonist *T.viride* strain 24039 (Plates 5.3.1 and 5.3.2). Untreated callus tissues (grown on extract-free media) exhibited evenly distributed and regular cell configurations, clear areas of inter-cellular space, and cell walls of width 3-5um (Plate 5.3.1a). In contrast, callus cells grown on media containing 5% v/v *T.viride* filtrate appeared more compact and irregularly distributed. Cell shrinkage was also evident in these samples and cell walls were thinner, between 1 and 2um (Plate 5.3.1b). At higher filtrate doses (10 and 20% v/v) reduced cell densities and distorted cellular forms were evident (Plates 5.3.2a&b). The sheet-like structures apparent within and around the cells may be precipitates of cytoplasmic constituents which have been forced out of solution by dehydration effects (compression of the cytoplasm due to cell shrinkage) (B.Grout, pers.comm.).

**Plate 5.3.1**

Scanning electron micrographs of cryogenic freeze-fractured 28 d old callus cultured cells of *N.tabacum* (transverse section X 280).

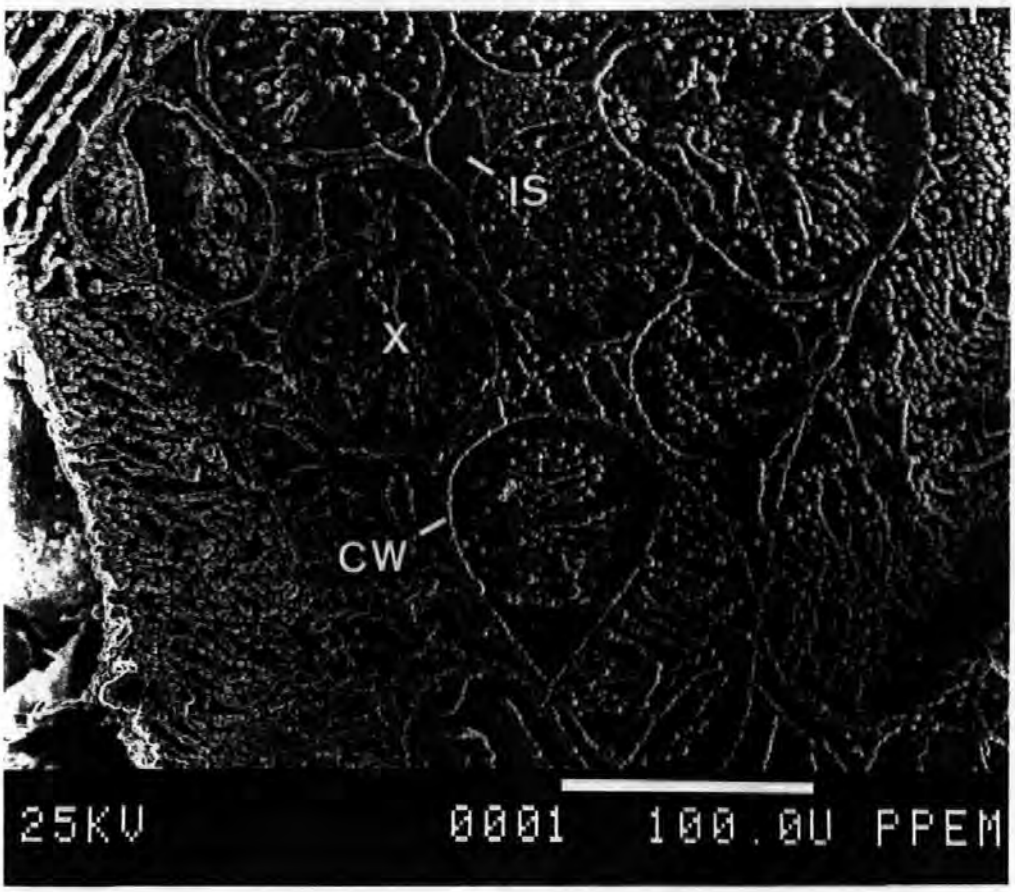
A. Untreated tissue

B. Tissue grown on media incorporated with a 5% v/v dose of filtrates produced by *T.viride* strain 24039

CW, cell wall; IS, inter-cellular space; CS, cell shrinkage

X, precipitated cytoplasmic constituents;

A




B

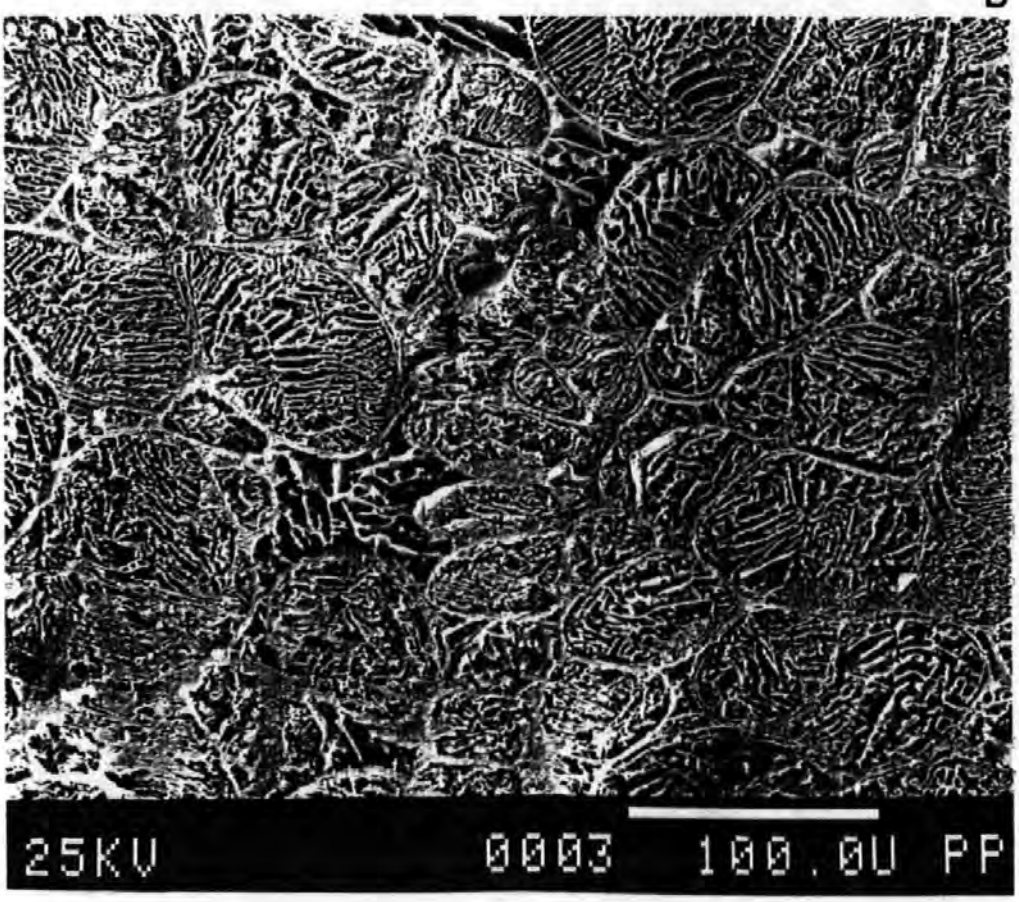


**Plate 5.3.2**

Scanning electron micrographs of cryogenic freeze-fractured 28 d old callus cultured cells of *N.tabacum* (transverse section X 280).

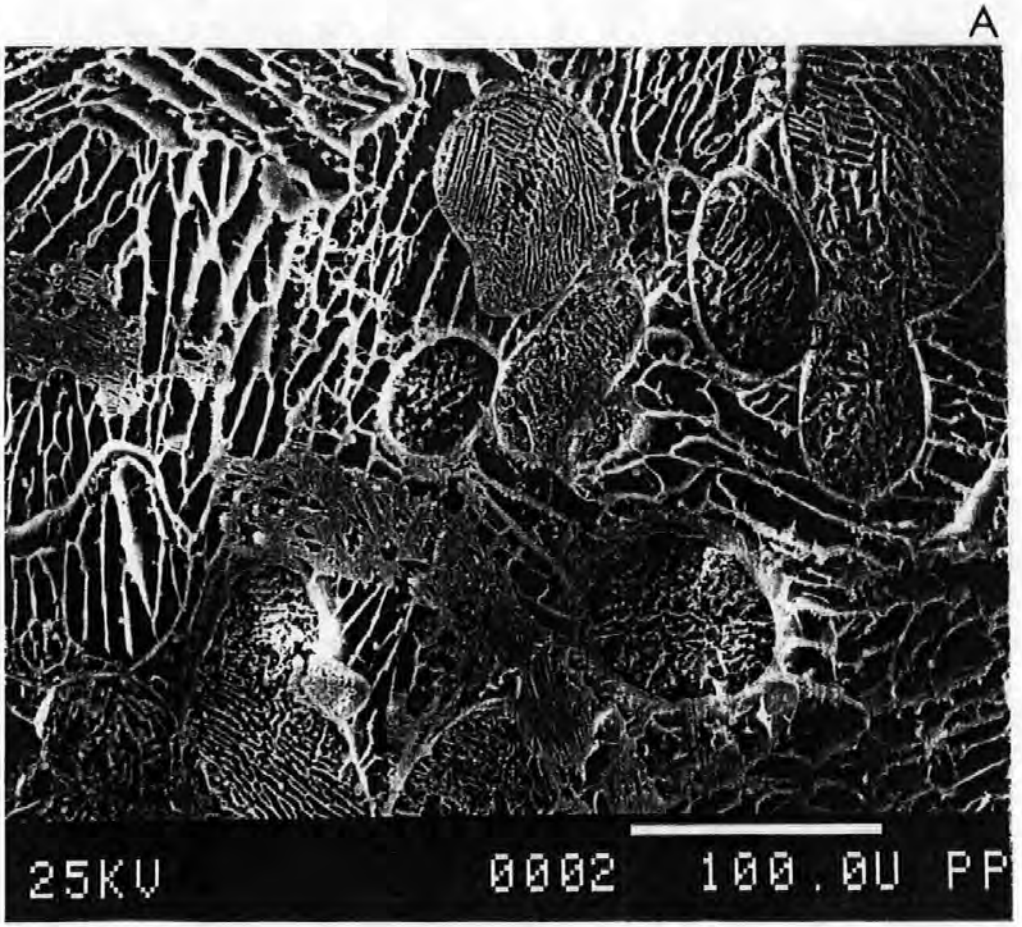
A. Tissue grown on media incorporated with a 10% v/v dose of filtrates produced by *T.viride* strain 24039

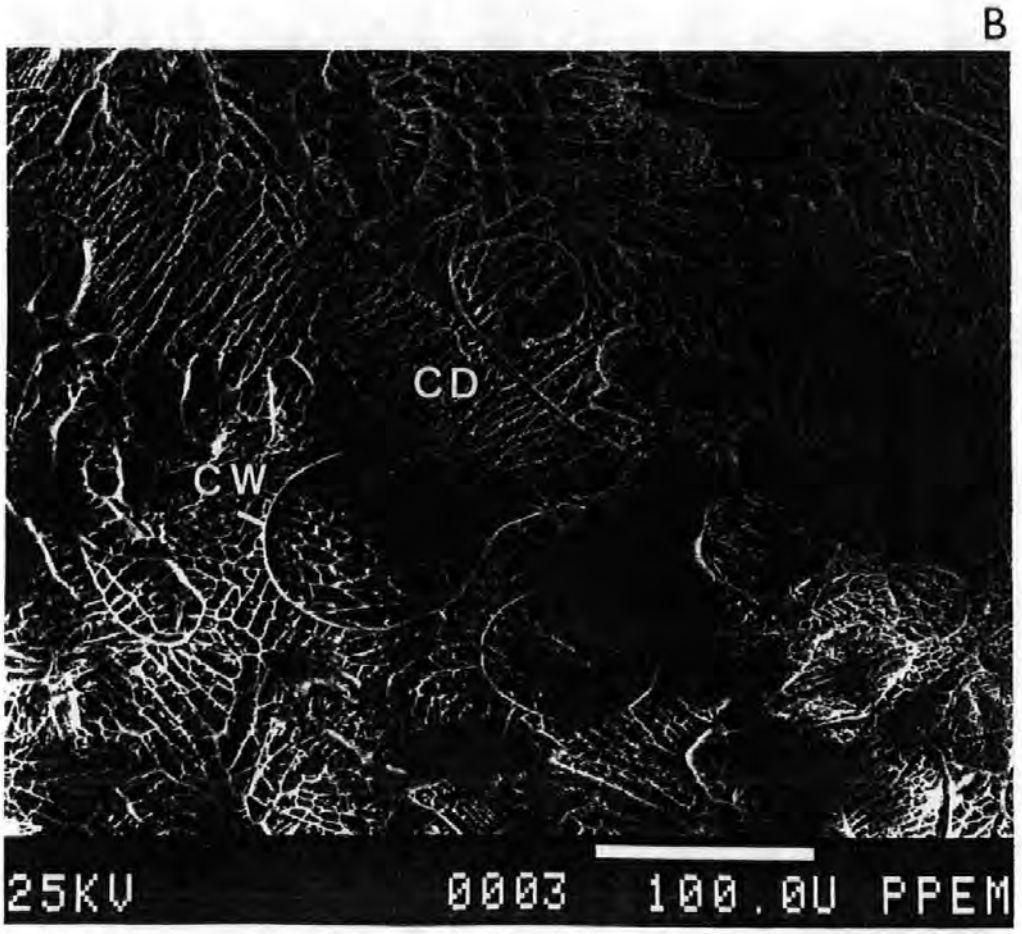
B. Tissue grown on media incorporated with a 20% v/v dose of filtrates produced by *T.viride* strain 24039

CW, cell wall; SI, sublimation of ice crystals;

CD, cellular distortion; LD, low cell density;

X, precipitated cytoplasmic constituents;


A
25KV
0002
100.0U PP


B
CD
CW
25KV
0003
100.0U PPEM

**Discussion**

If anti-microbial culture filtrates produced by antagonistic micro-organisms are to be successfully employed as 'plant-friendly' alternatives to conventional antibiotic compounds for contamination control in plant tissue culture systems, then it is obvious that their phytotoxicity must be less than that of the antibiotics currently being employed for similar purposes. Investigations conducted in this report attempted to establish the maximum permissive doses of each of the culture filtrates studied which could be incorporated in to the growth media of a range of plant tissue cultures without exhibiting deleterious effects on those systems. The recovery capacity of those plant species exhibiting reduced growth when cultured in media containing a level of filtrate greater than the maximum permissive dose was assessed when the plant cultures were subsequently transferred to extract-free media and grown over several passages. An examination of filtrate phytotoxicity at the cellular level was also conducted.

The experiments conducted in this investigation established that the test plant culture species displayed different responses to the various microbial culture filtrates studied. Some of the filtrate dosage-levels investigated proved totally unsuitable by killing the plant cultures, inducing deformed growth at the cellular level, or generally inhibiting development and proliferation. Other filtrates had no observable effects on the plant cultures studied when incorporated in to growth media at specific levels. In general, those filtrates produced by the *P.fluorescens* and *G.roseum* isolates exhibited the greatest phytotoxicity, whereas those produced by the *T.viride* and *B.subtilis* isolates were tolerated at higher levels by the various plant species.

Phytotoxic effects induced by conventional antibiotics have been reported by various authors. Thurston *et al.* (1979) screened 25 fungicidal, bactericidal and dual-action compounds in various combinations for *in vitro* phytotoxicity using orchid seedlings. Some compounds proved totally

unsuitable for incorporation in to plant tissue culture growth media for contamination control, killing the seedlings, inducing deformed growth, or generally inhibiting development. Some had no observable effect, and several enhanced growth. No such growth enhancement was observed when the plant tissue cultures employed in this study were treated with cell-free culture filtrates of antagonistic micro-organisms. Bastiens *et al.* (1983) noted reduced proliferation rates and fresh weights of *Fiscus benjamina* and *Cordyline terminalis* when grown on media containing a range of antibiotic compounds at concentrations greater than their maximum permissive dose. In this thesis, as in those investigations contained within this report, the test plant cultures displayed different responses to the compounds tested. Dodds & Roberts (1981) and Eichholtz *et al.* (1982) noted that inhibition of callus formation and adventitious shoot formation in cultured tissues of a variety of plant species (including tobacco callus, salpiglosis leaf discs, romaine lettuce and Jerusalem artichoke tubers) was observed when these tissues were exposed to excess doses of the bactericidal antibiotic, gentamicin sulphate. Again, similar deleterious effects caused by exposure to antagonistic filtrates were sometimes observed in the present investigations noted within this study. Pollock *et al.* (1983) assessed the comparative toxicities of an array of conventional antibiotics on protoplasts derived from *Nicotiana plumbaginifolia* and determined the least toxic antibiotics which could be employed as broad-spectrum antimicrobial agents without significant toxicity to the plant cells. However, as illustrated by the experiments described above, phytotoxicity may vary greatly between different cultured species and therefore such isolated studies are of limited usefulness when attempting to derive a universally-employed antimicrobial agent.

In contrast to the numerous phytotoxicity studies conducted on conventional antibiotics, there are relatively few reports of such studies relating to culture filtrates produced by antagonistic micro-organisms. This is perhaps

not so suprising, since there are few examples of the successful and commercially feasible application of culture filtrates for plant disease/contamination control. An extensive study was however conducted by Broadbent *et al.* (1971) on the antagonism of fungal root pathogens by organisms isolated from Australian soils. Phytotoxicity assessments of filtrates from *Pseudomonas* isolates revealed a reduction in germination rates and root growth of Antirrhinum seedlings when the filtrates were employed to control fungal pathogens. In experiments conducted by Hijwegen (1989) on the effect of culture filtrates of an array of fungicolous fungi on sporulation of cucumber powdery midew, plants treated with filtrates of the antagonists *Aphanocladium album*, *Calcarisporium arbuscula*, *Paecilomyces farinosus* and *Verticillium lecanii* all exhibited phyto-toxic symptoms. These experiments illustrate the importance of establishing levels of anti-microbial agents which can be tolerated by plant tissues prior to their application. Equally important is an evaluation of the recovery capacity of treated tissues when subsequently sub-cultured on to extract-free media. Both of these assessments have been conducted for the culture filtrates being employed on a range of plant tissue culture systems in this report. It is therefore now possible to apply this knowledge of maximum permissive doses of the various filtrates, and the recovery capacities of the test plant tissues, when employing these anti-microbial extracts for contamination control in plant tissue culture systems.

Critical growth point?

**Figure 5.1.1**

Evaluation of the phytotoxic effects of antimicrobial culture filtrates produced by antagonistic organisms on *Nicotiana* shoot cultures after 6 weeks growth.

A. Effects on number of shoots (N)
B. Effects on length of shoots (L)
C. Effects on fresh weight of cultures (FW)

All data represents the mean of 3 replicates. Values in the same data series followed by the same letter do not differ at $p < 0.05$ (Duncan test).

1 - *T.viride*
2 - *B.subtilis*
3 - *P.fluorescens*
4 - *G.roseum*

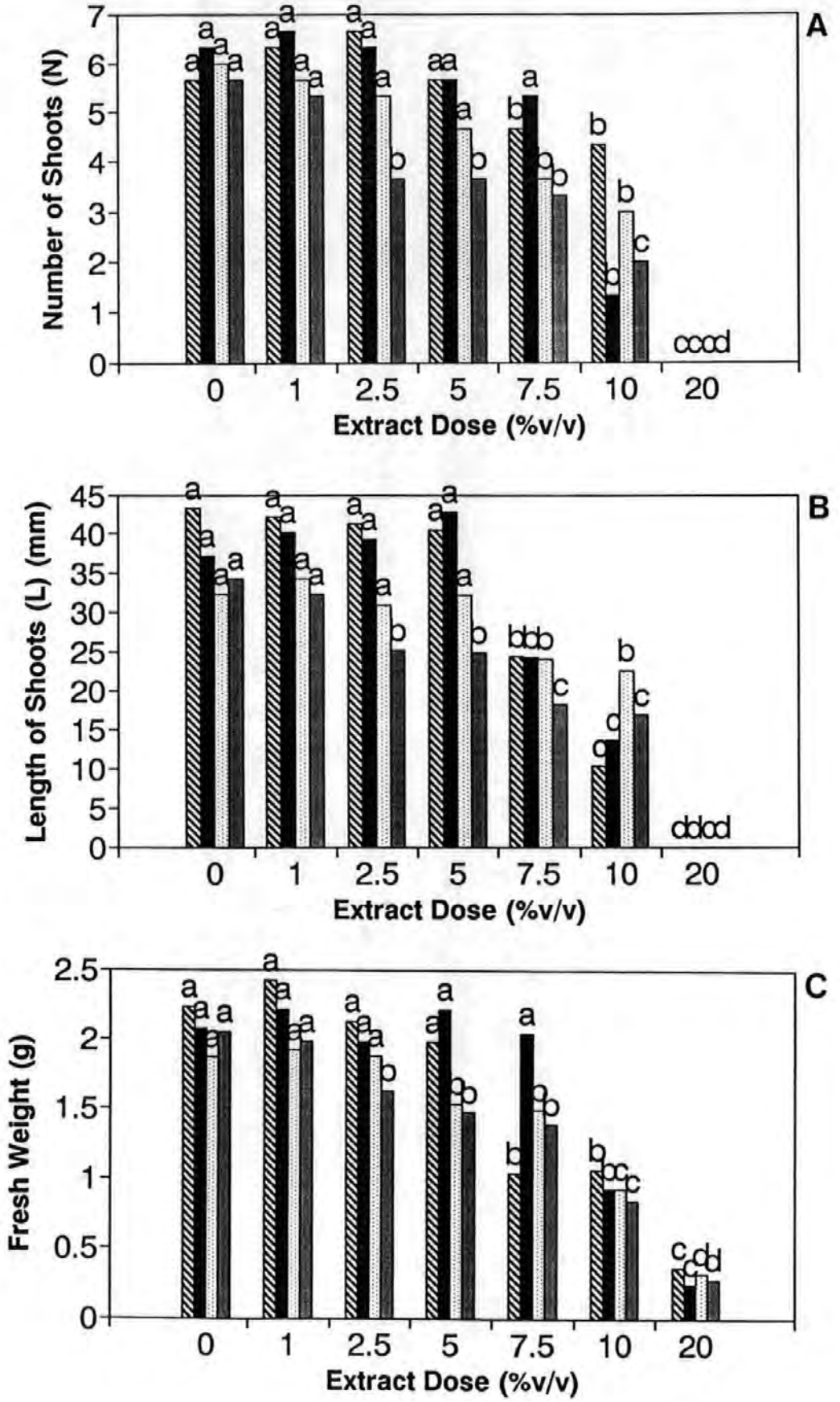

A
Number of Shoots (N)
0
1
2
3
4
5
6
7
0
1
2.5
5
7.5
10
20
Extract Dose (%v/v)
B
Length of Shoots (L) (mm)
0
5
10
15
20
25
30
35
40
45
0
1
2.5
5
7.5
10
20
Extract Dose (%v/v)
C
Fresh Weight (g)
0
0.5
1
1.5
2
2.5
0
1
2.5
5
7.5
10
20
Extract Dose (%v/v)
1
2
3
4

**Figure 5.1.2**

Evaluation of the phytotoxic effects of antimicrobial culture filtrates produced by antagonistic organisms on *Nicotiana* root cultures after 6 weeks growth.

A. Effects on number of roots (N)
B. Effects on length of roots (L)
C. Effects on fresh weight of cultures (FW)

All data represents the mean of 3 replicates. Values in the same data series followed by the same letter do not differ at $p < 0.05$ (Duncan test).

1 - *T.viride*
2 - *B.subtilis*
3 - *P.fluorescens*
4 - *G.roseum*

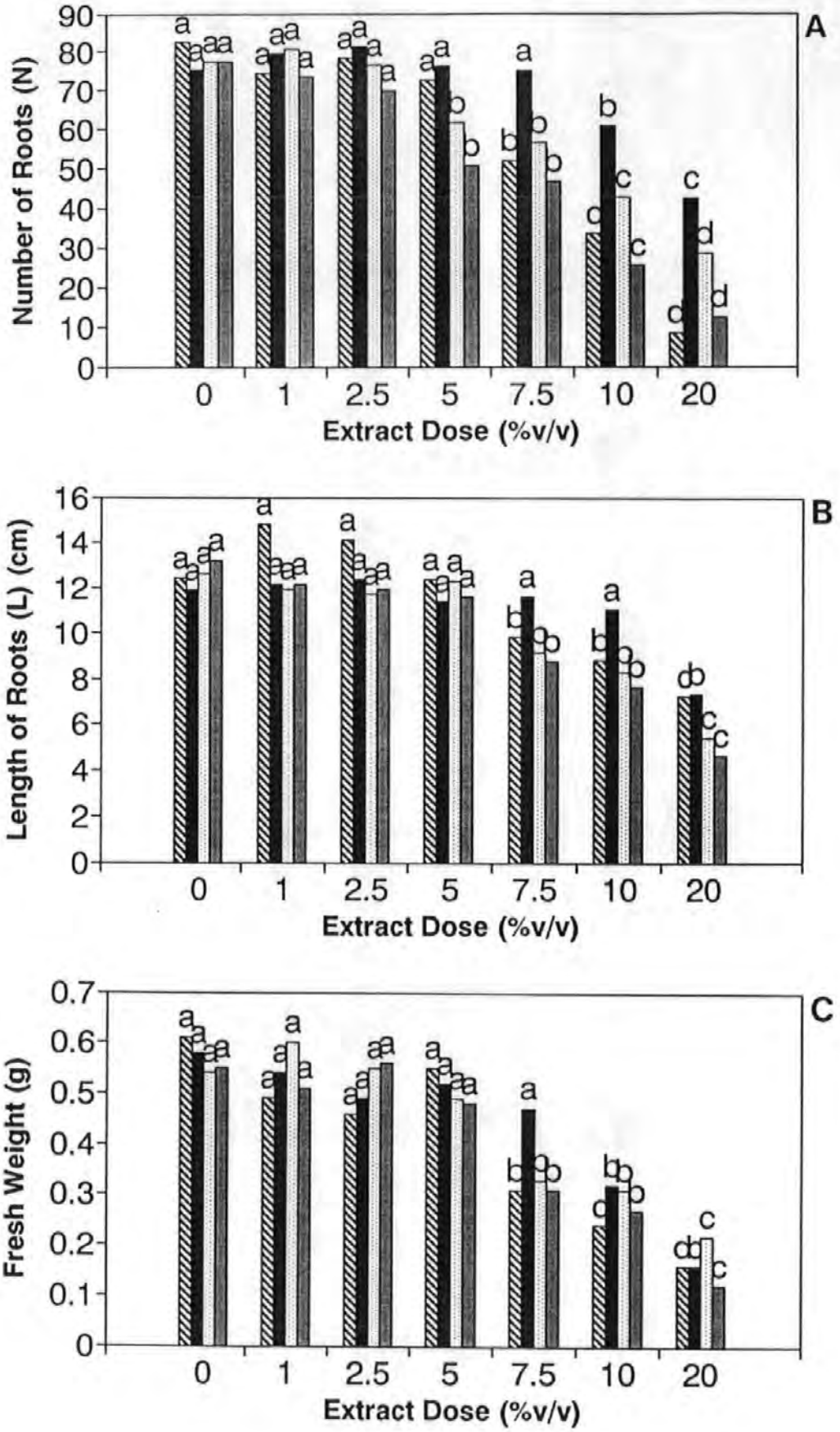

A
Number of Roots (N)
90
80
70
60
50
40
30
20
10
0
0
1
2.5
5
7.5
10
20
Extract Dose (%v/v)
B
Length of Roots (L) (cm)
16
14
12
10
8
6
4
2
0
0
1
2.5
5
7.5
10
20
Extract Dose (%v/v)
C
Fresh Weight (g)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1
2.5
5
7.5
10
20
Extract Dose (%v/v)
1
2
3
4

**Figure 5.1.3**

Evaluation of the phytotoxic effects of antimicrobial culture filtrates produced by antagonistic organisms on *Hamamelis* shoot cultures after 6 weeks growth.

A. Effects on number of shoots (N)
B. Effects on length of shoots (L)
C. Effects on fresh weight of cultures (FW)

All data represents the mean of 3 replicates. Values in the same data series followed by the same letter do not differ at $p<0.05$ (Duncan test).

1 - *T.viride*
2 - *B.subtilis*
3 - *P.fluorescens*
4 - *G.roseum*

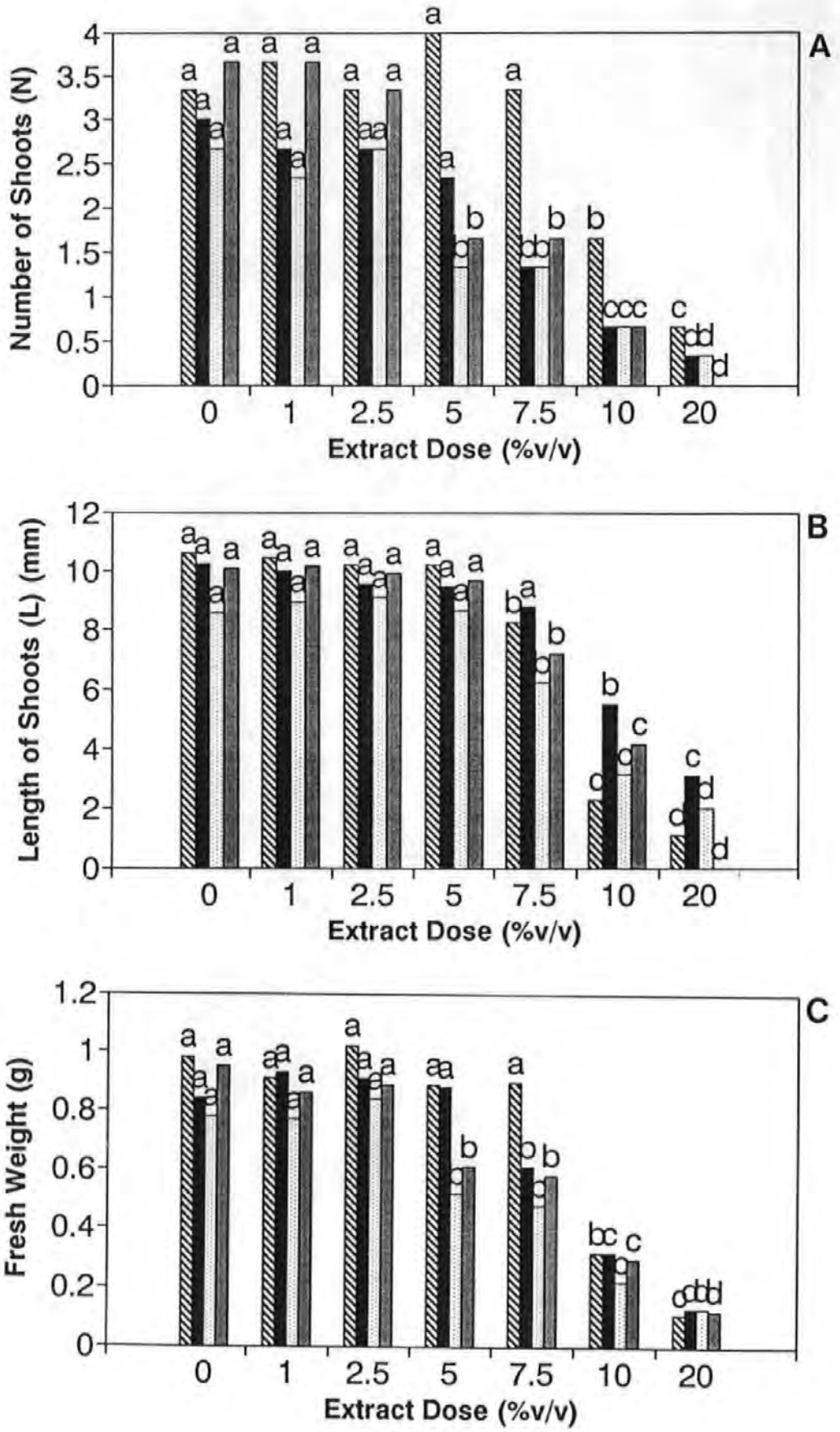

A
Number of Shoots (N)
0
0.5
1
1.5
2
2.5
3
3.5
4
0
1
2.5
5
7.5
10
20
Extract Dose (%v/v)
B
Length of Shoots (L) (mm)
0
2
4
6
8
10
12
0
1
2.5
5
7.5
10
20
Extract Dose (%v/v)
C
Fresh Weight (g)
0
0.2
0.4
0.6
0.8
1
1.2
0
1
2.5
5
7.5
10
20
Extract Dose (%v/v)
1
2
3
4

**Figure 5.1.4**

Evaluation of the phytotoxic effects of antimicrobial culture filtrates produced by antagonistic organisms on *Betula* shoot cultures after 6 weeks growth.

A. Effects on number of shoots (N)
B. Effects on length of shoots (L)
C. Effects on fresh weight of cultures (FW)

All data represents the mean of 3 replicates. Values in the same data series followed by the same letter do not differ at $p < 0.05$ (Duncan test).

1 - *T.viride*
2 - *B.subtilis*
3 - *P.fluorescens*
4 - *G.roseum*

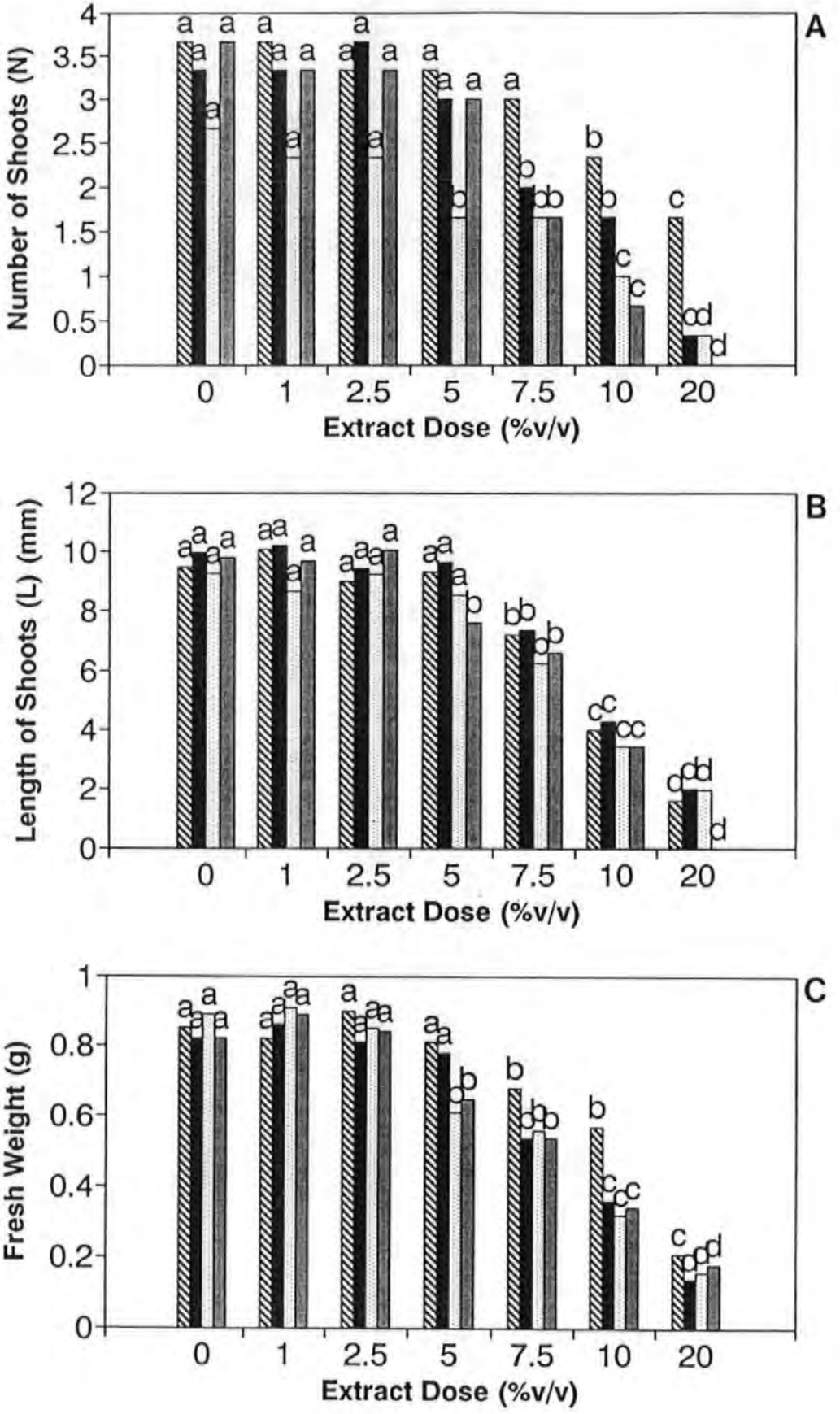

A
Number of Shoots (N)
0
0.5
1
1.5
2
2.5
3
3.5
4
0
1
2.5
5
7.5
10
20
Extract Dose (%v/v)
B
Length of Shoots (L) (mm)
0
2
4
6
8
10
12
0
1
2.5
5
7.5
10
20
Extract Dose (%v/v)
C
Fresh Weight (g)
0
0.2
0.4
0.6
0.8
1
0
1
2.5
5
7.5
10
20
Extract Dose (%v/v)
1
2
3
4

**Figure 5.1.5**

Evaluation of the phytotoxic effects of antimicrobial culture filtrates produced by antagonistic organisms on *Dalbergia* shoot cultures after 6 weeks growth.

A. Effects on number of shoots (N)
B. Effects on length of shoots (L)
C. Effects on fresh weight of cultures (FW)

All data represents the mean of 3 replicates. Values in the same data series followed by the same letter do not differ at $p < 0.05$ (Duncan test).

1 - *T.viride*
2 - *B.subtilis*
3 - *P.fluorescens*
4 - *G.roseum*

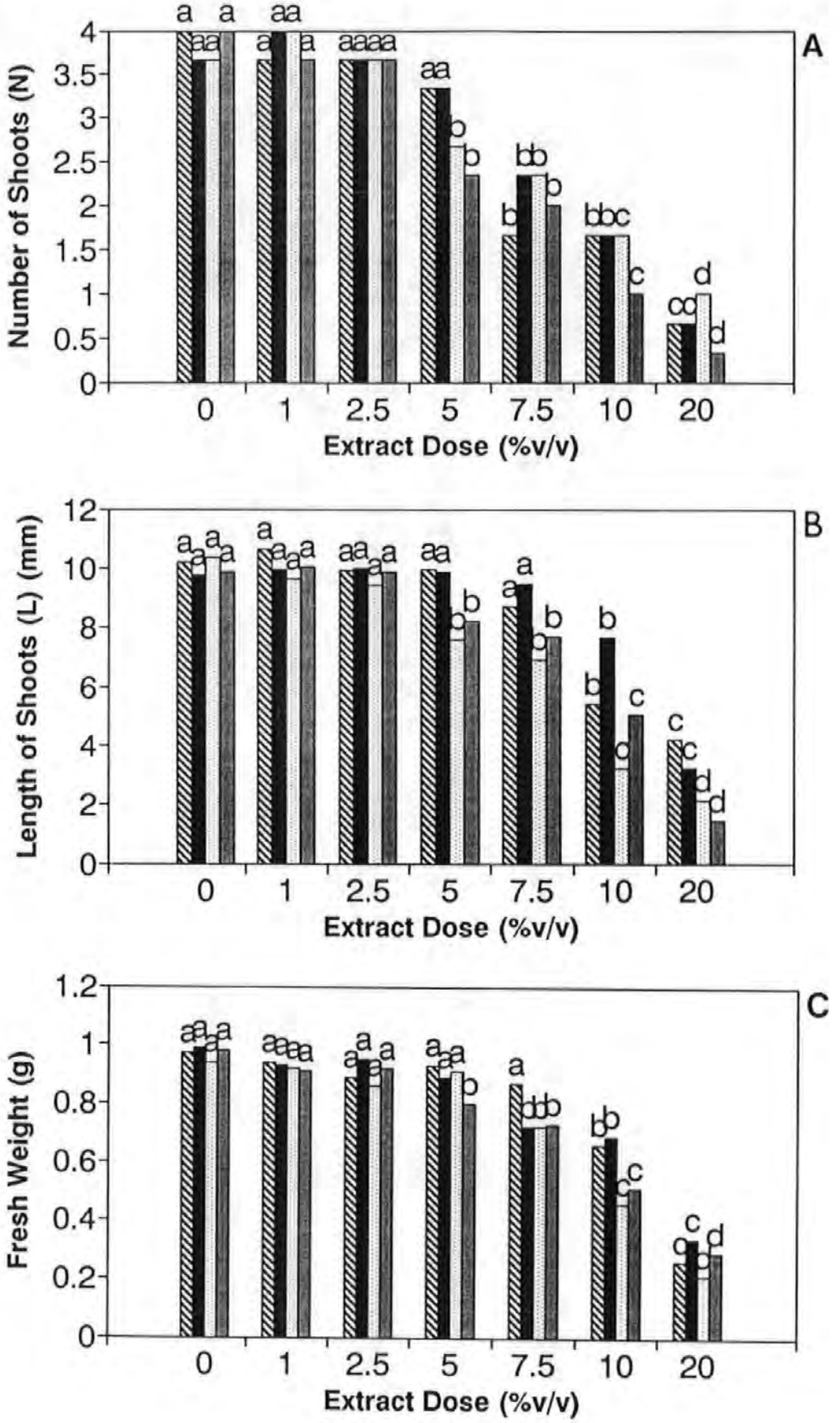

A
Number of Shoots (N)
0
0.5
1
1.5
2
2.5
3
3.5
4
0
1
2.5
5
7.5
10
20
Extract Dose (%v/v)
B
Length of Shoots (L) (mm)
0
2
4
6
8
10
12
0
1
2.5
5
7.5
10
20
Extract Dose (%v/v)
C
Fresh Weight (g)
0
0.2
0.4
0.6
0.8
1
1.2
0
1
2.5
5
7.5
10
20
Extract Dose (%v/v)
1
2
3
4

**Figure 5.2.1**

Recovery capacity of shoot cultures of *Nicotiana* after exposure to excessive doses of antimicrobial culture filtrates produced by antagonist organisms.

Plant cultures were grown in extract-free media for up to four passages after previously being grown in media containing extract doses greater than the maximum permissive levels. All data represents the mean of 3 replicates.

A. Effects on number of shoots (N)

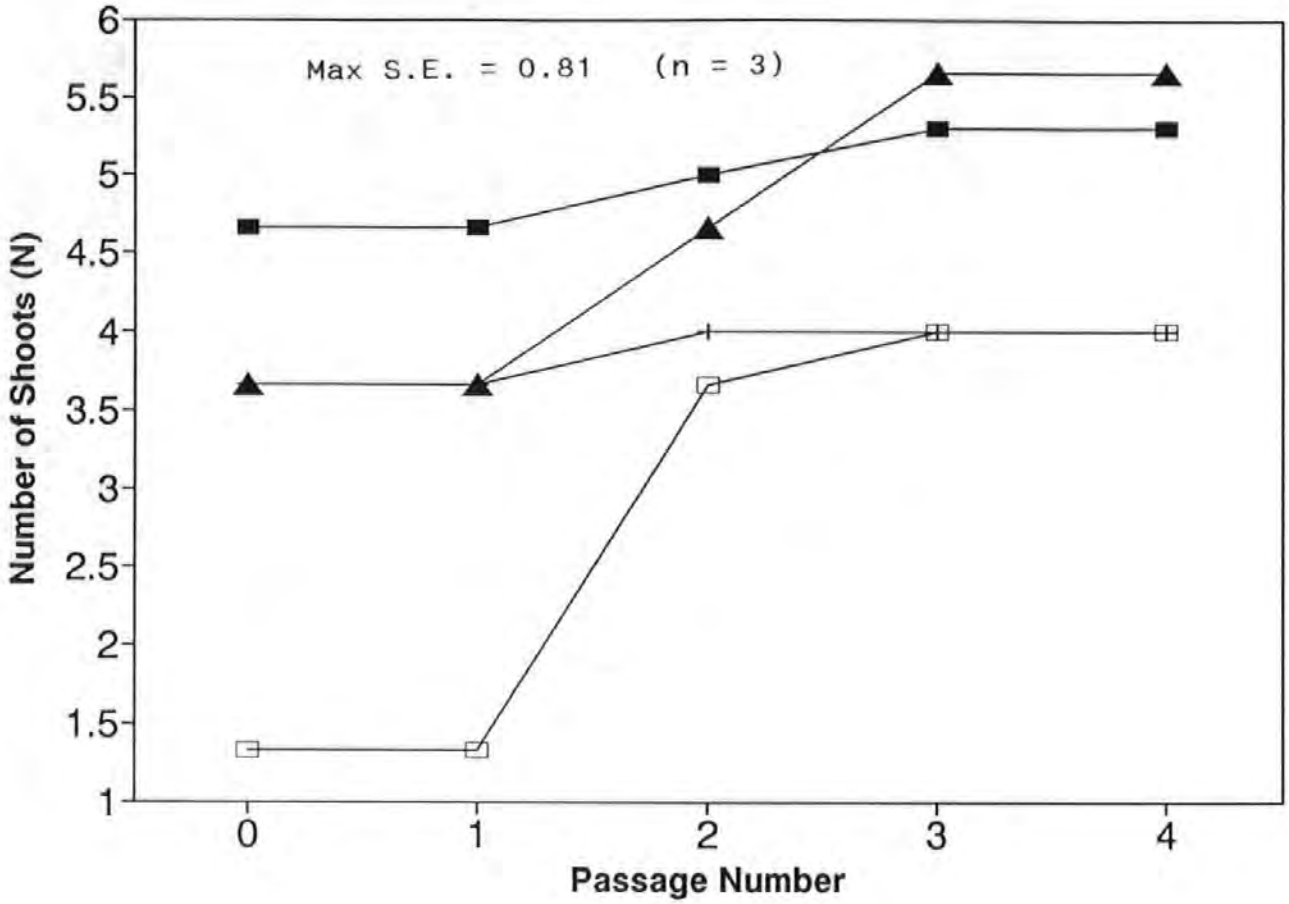
B. Effects on length of shoots (L)

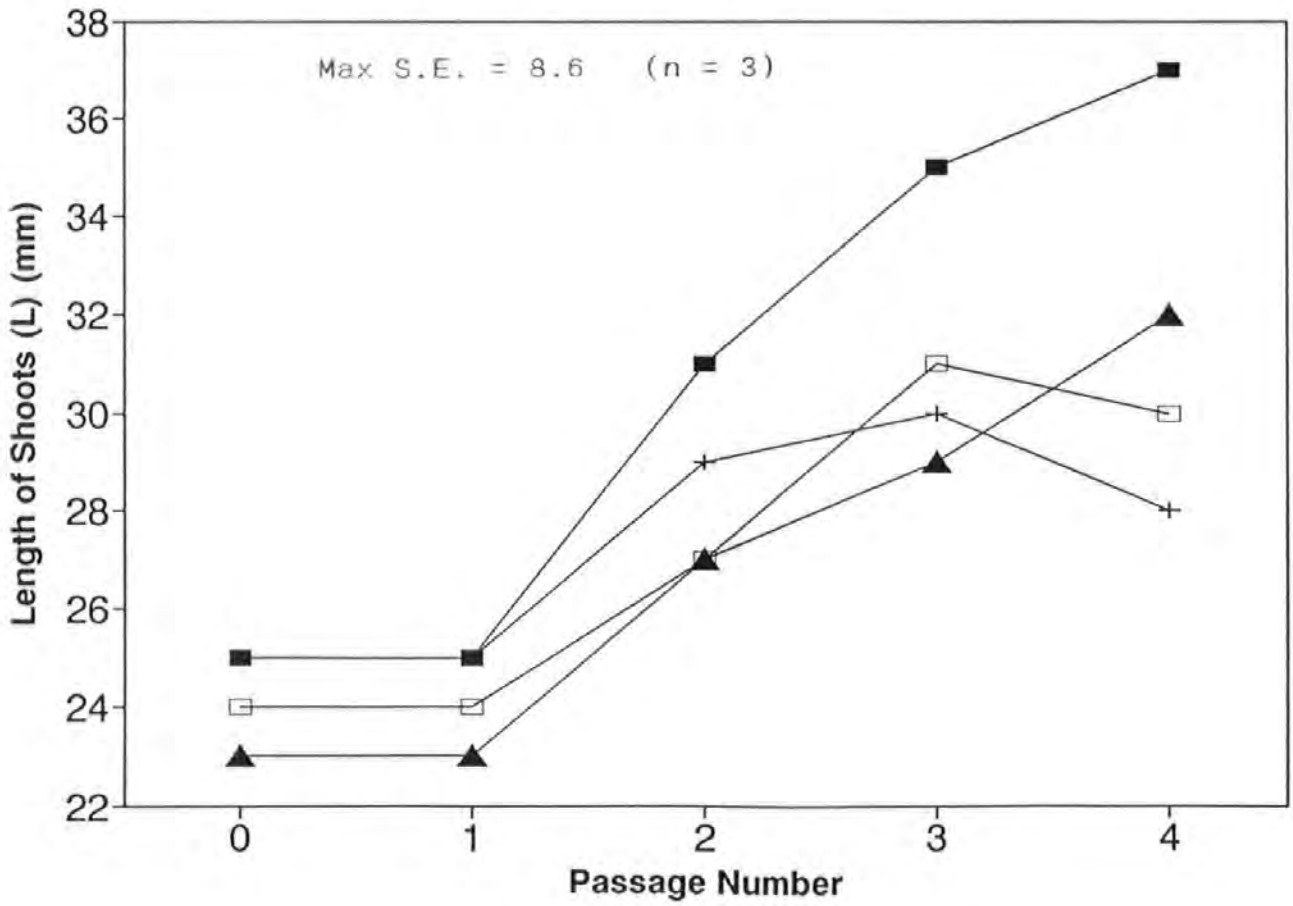
1 - *T.viride*

2 - *B.subtilis*

3 - *P.fluorescens*

4 - *G.roseum*


Max S.E. = 0.81 (n = 3)
Number of Shoots (N)
6
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0
1
2
3
4
Passage Number


Max S.E. = 8.6 (n = 3)
Length of Shoots (L) (mm)
38
36
34
32
30
28
26
24
22
0
1
2
3
4
Passage Number

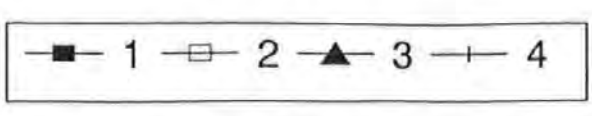

1
2
3
4

**Figure 5.2.2**

Recovery capacity of root cultures of *Nicotiana* after exposure to excessive doses of antimicrobial culture filtrates produced by antagonist organisms.

Plant cultures were grown in extract-free media for up to four passages after previously being grown in media containing extract doses greater than the maximum permissive levels. All data represents the mean of 3 replicates.

A. Effects on number of roots (N)

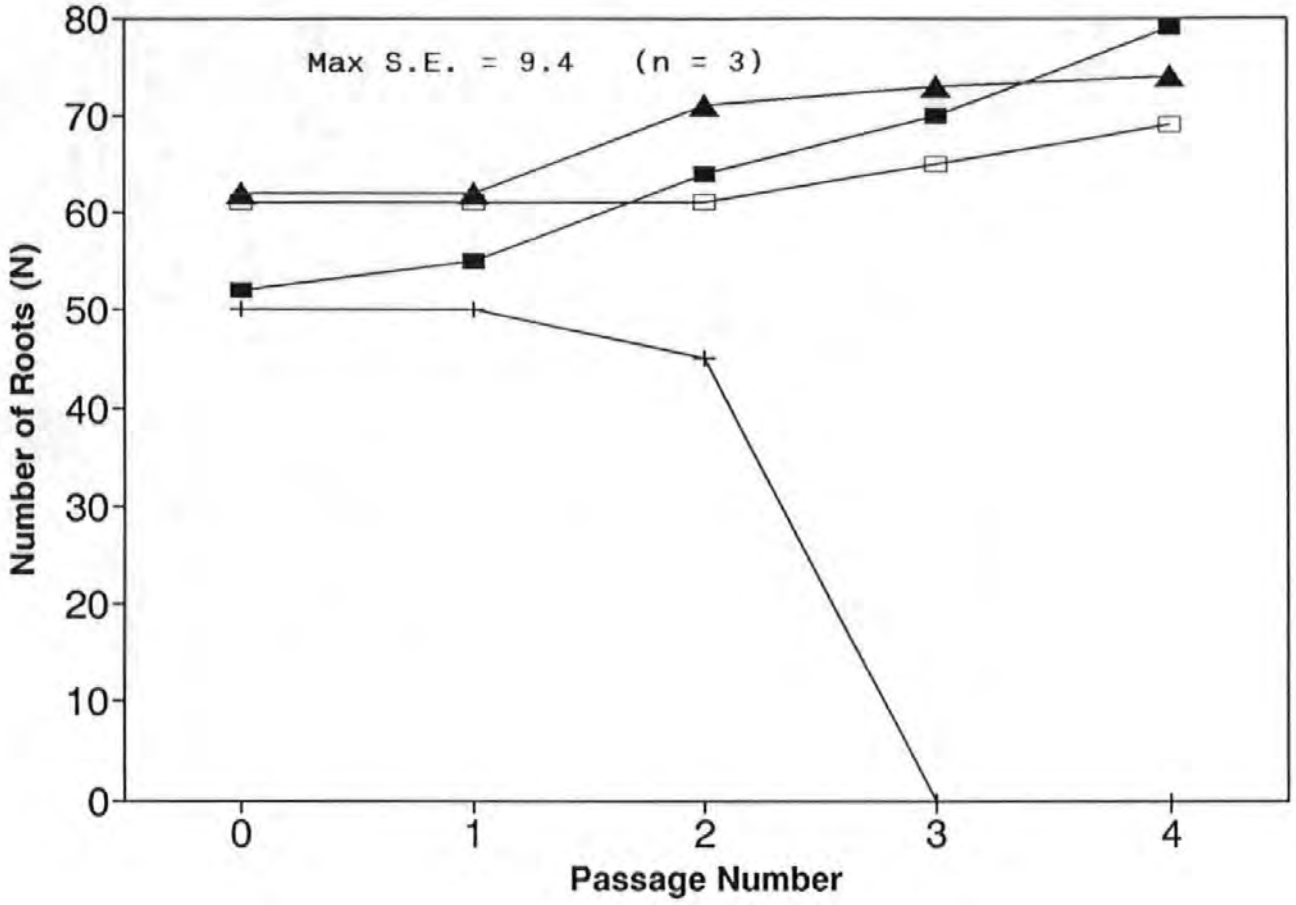
B. Effects on length of roots (L)

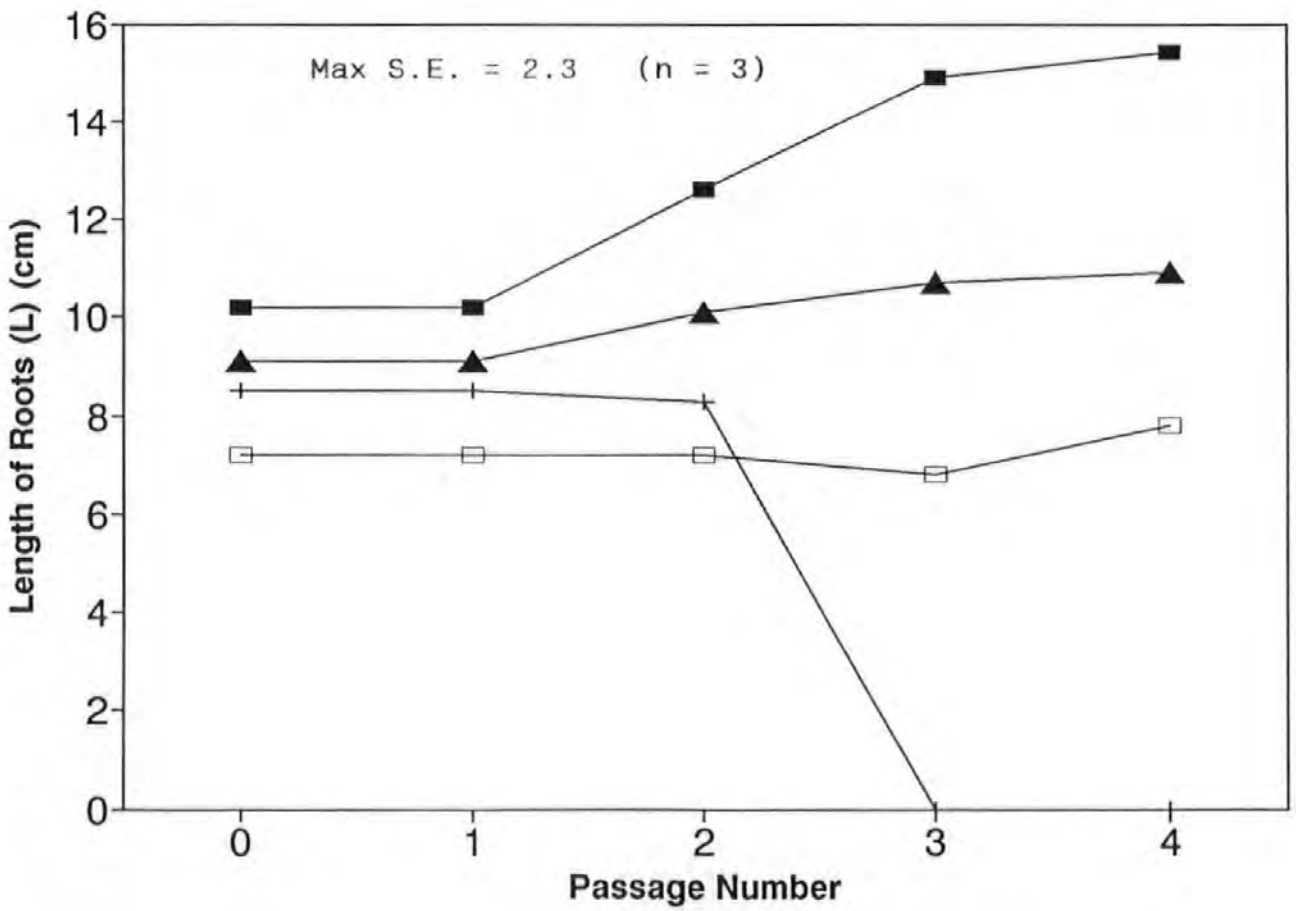
1 - *T.viride*

2 - *B.subtilis*

3 - *P.fluorescens*

4 - *G.roseum*


Max S.E. = 9.4 (n = 3)
Number of Roots (N)
80
70
60
50
40
30
20
10
0
0
1
2
3
4
Passage Number


Max S.E. = 2.3 (n = 3)
Length of Roots (L) (cm)
16
14
12
10
8
6
4
2
0
0
1
2
3
4
Passage Number

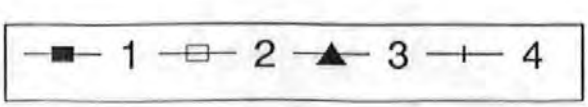

1
2
3
4

**Figure 5.2.3**

Recovery capacity of shoot cultures of *Hamamelis* after exposure to excessive doses of antimicrobial culture filtrates produced by antagonist organisms.

Plant cultures were grown in extract-free media for up to four passages after previously being grown in media containing extract doses greater than the maximum permissive levels. All data represents the mean of 3 replicates.

A. Effects on number of shoots (N)

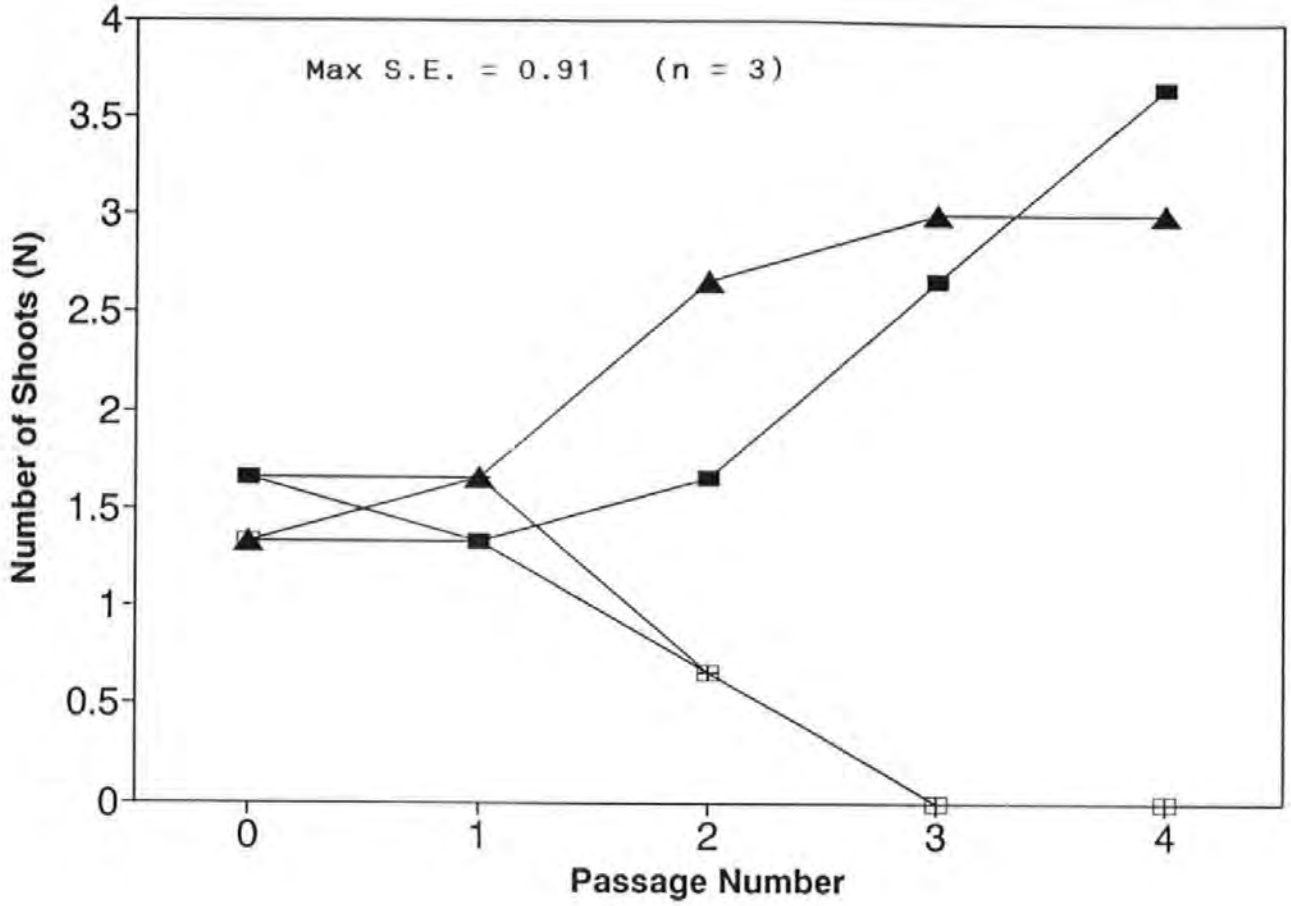
B. Effects on length of shoots (L)

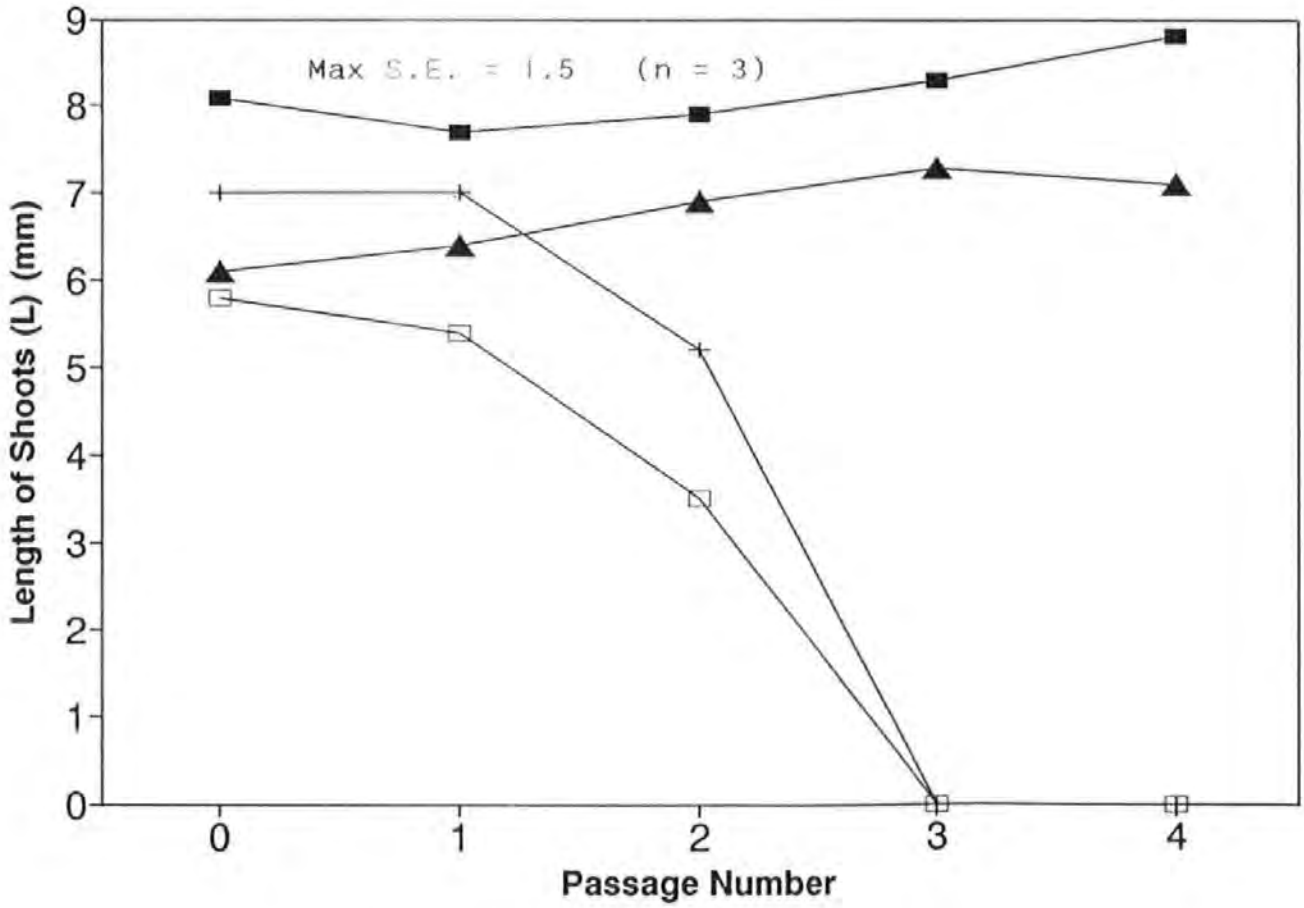
1 - *T.viride*

2 - *B.subtilis*

3 - *P.fluorescens*

4 - *G.roseum*


Max S.E. = 0.91 (n = 3)
Number of Shoots (N)
Passage Number
0
0.5
1
1.5
2
2.5
3
3.5
4
0
1
2
3
4


Max S.E. = 1.5 (n = 3)
Length of Shoots (L) (mm)
Passage Number
0
1
2
3
4
5
6
7
8
9
0
1
2
3
4

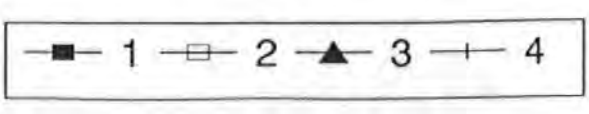

1
2
3
4

**Figure 5.2.4**

Recovery capacity of shoot cultures of *Betula* after exposure to excessive doses of antimicrobial culture filtrates produced by antagonist organisms.

Plant cultures were grown in extract-free media for up to four passages after previously being grown in media containing extract doses greater than the maximum permissive levels. All data represents the mean of 3 replicates.

A. Effects on number of shoots (N)

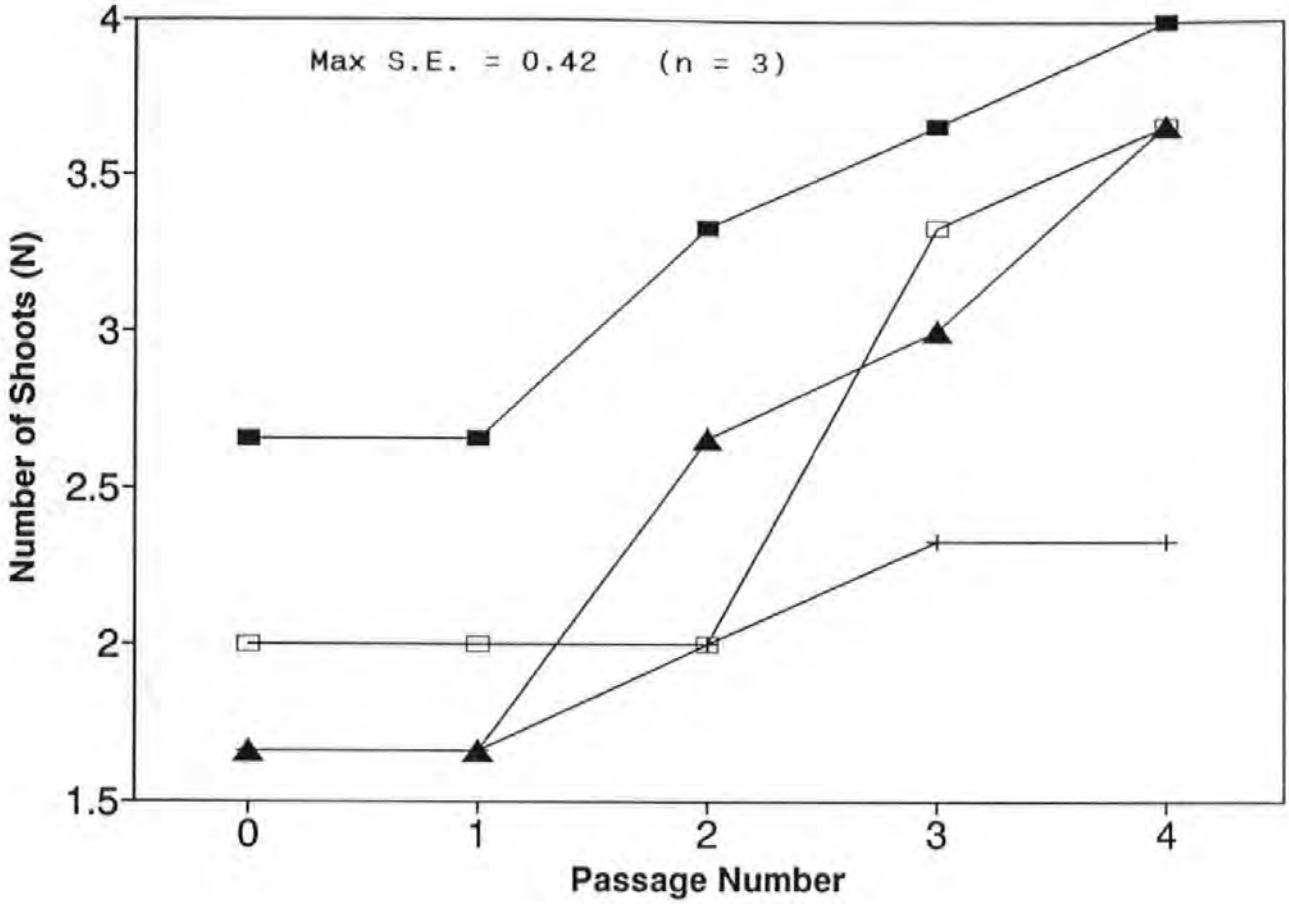
B. Effects on length of shoots (L)

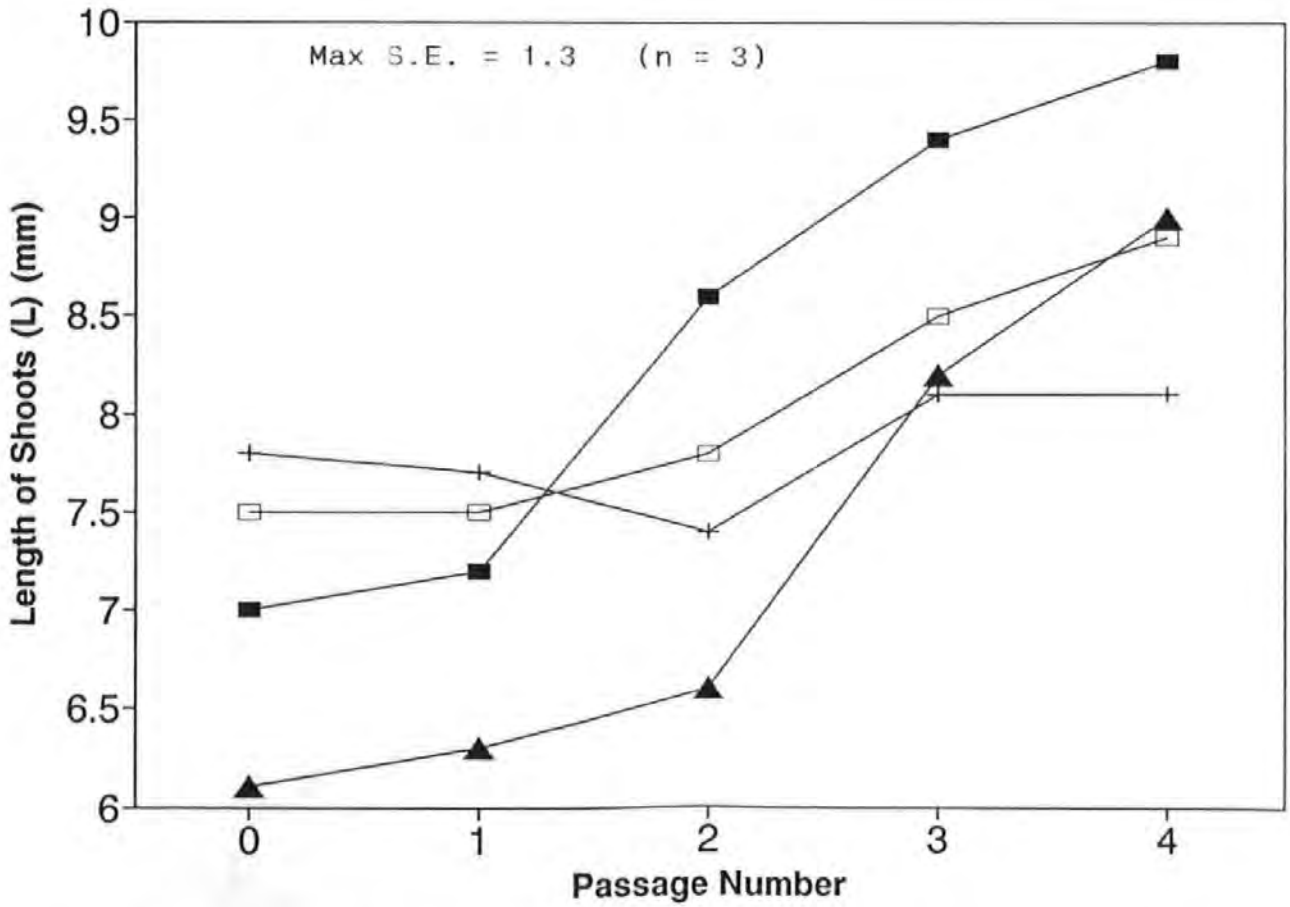
1 - *T.viride*

2 - *B.subtilis*

3 - *P.fluorescens*

4 - *G.roseum*


Max S.E. = 0.42 (n = 3)
Number of Shoots (N)
4
3.5
3
2.5
2
1.5
0
1
2
3
4
Passage Number


Max S.E. = 1.3 (n = 3)
Length of Shoots (L) (mm)
10
9.5
9
8.5
8
7.5
7
6.5
6
0
1
2
3
4
Passage Number

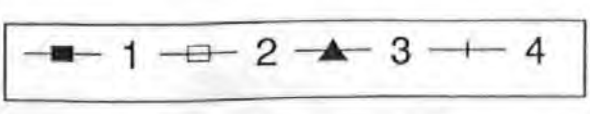

1
2
3
4

**Figure 5.2.5**

Recovery capacity of shoot cultures of *Dalbergia* after exposure to excessive doses of antimicrobial culture filtrates produced by antagonist organisms.

Plant cultures were grown in extract-free media for up to four passages after previously being grown in media containing extract doses greater than the maximum permissive levels. All data represents the mean of 3 replicates.

A. Effects on number of shoots (N)

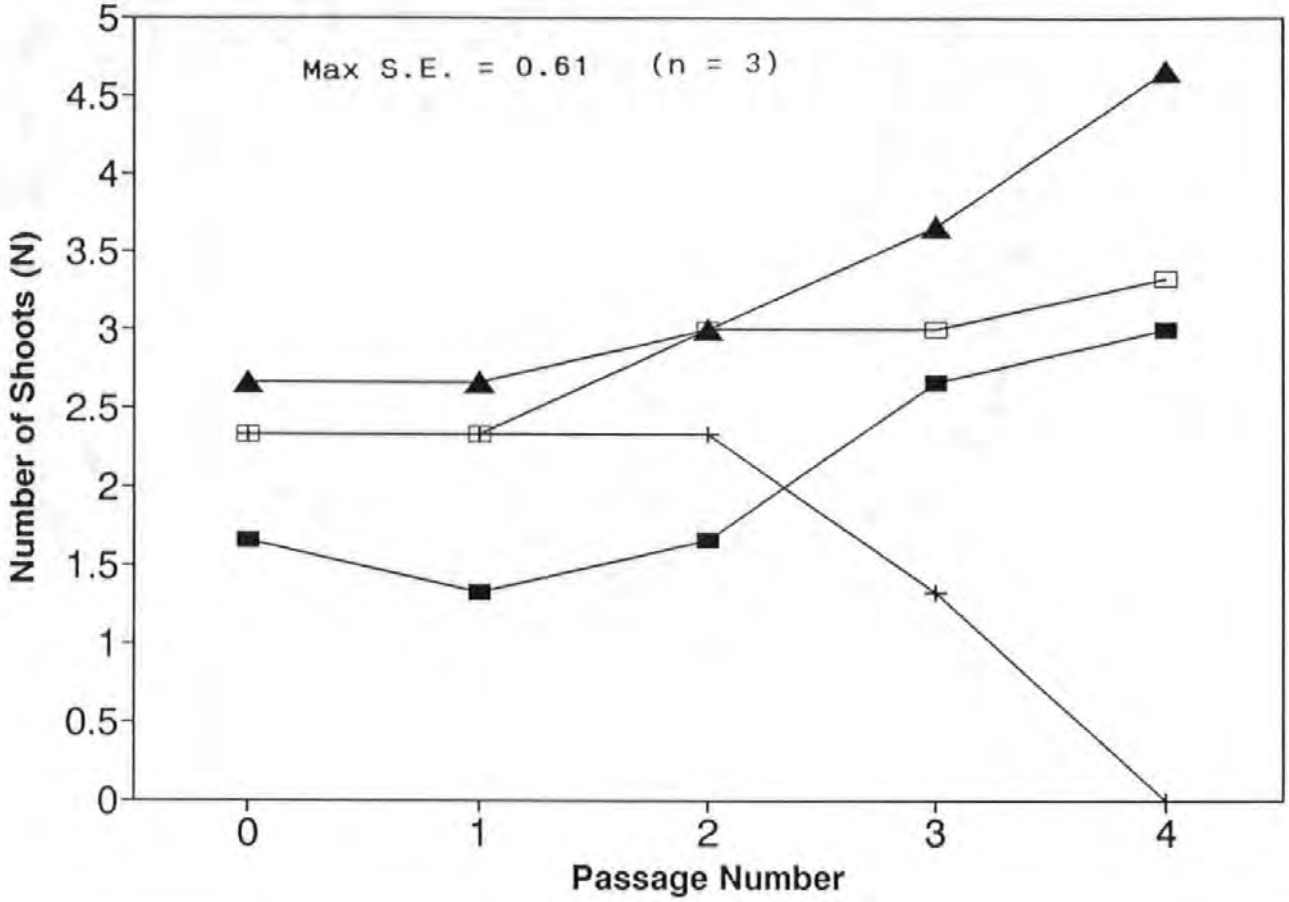
B. Effects on length of shoots (L)

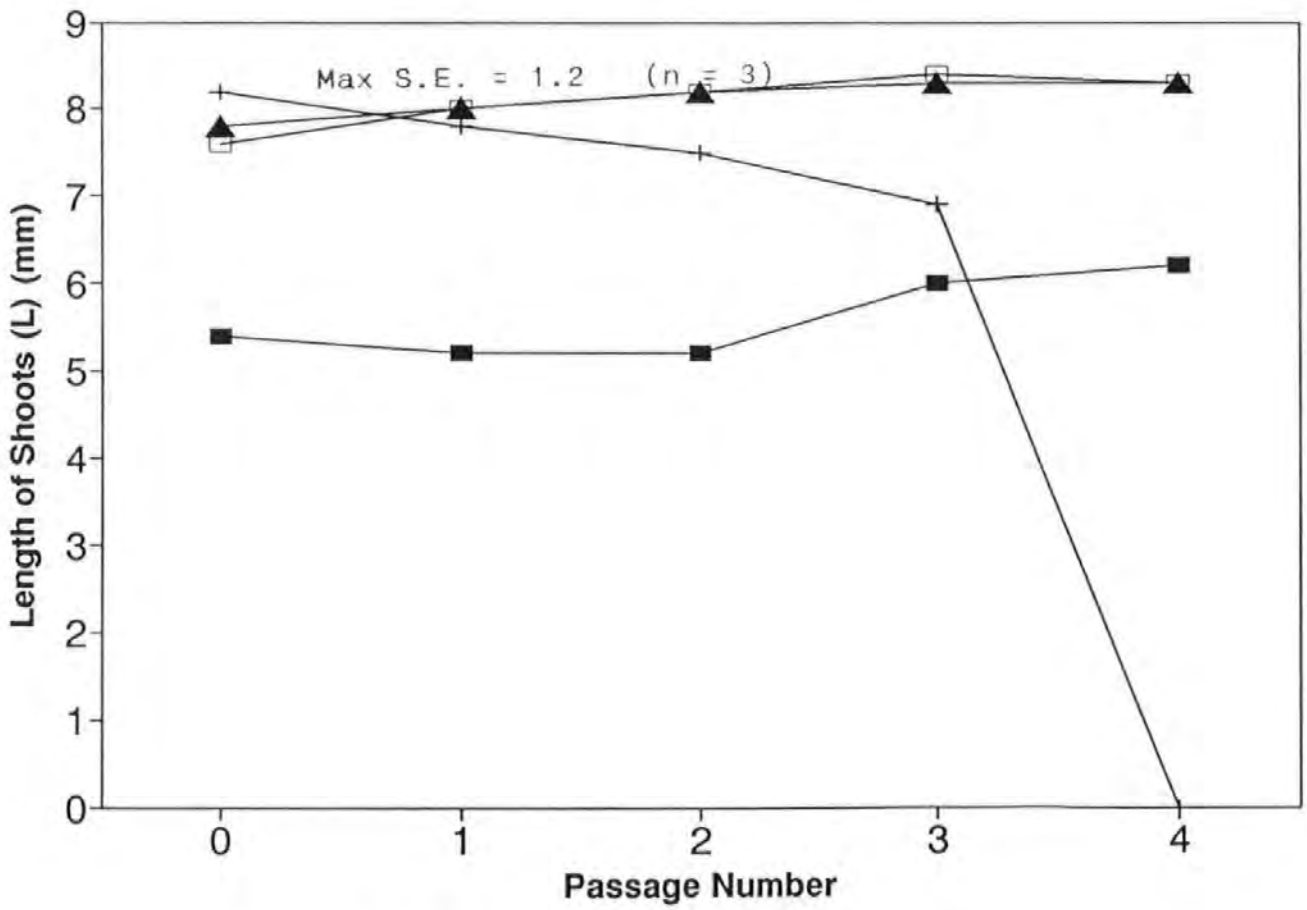
1 - *T.viride*

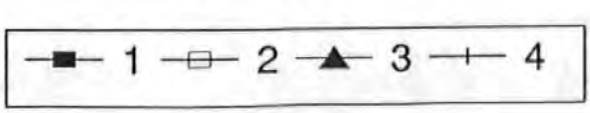
2 - *B.subtilis*

3 - *P.fluorescens*

4 - *G.roseum*


Max S.E. = 0.61 (n = 3)
Number of Shoots (N)
Passage Number
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
0
1
2
3
4


Max S.E. = 1.2 (n = 3)
Length of Shoots (L) (mm)
Passage Number
0
1
2
3
4
5
6
7
8
9
0
1
2
3
4


1
2
3
4

CHAPTER 6

# EXTRACT ACTIVITY IN CONTAMINATED PLANT TISSUE CULTURE SYSTEMS

Plant tissue culture media is conducive to the growth of microbial contaminations, which often multiply and overgrow the explant. Although aseptic conditions are usually implied, many plant cultures are not, or do not, stay aseptic *in vitro* (Boxus & Terzi, 1987). The two main sources of contamination are poor aseptic technique and latent endogenous bacterial infections which are particularly a problem in woody plant cultures (Young *et al.*, 1984) and may only become apparent after a number of passages in culture.

As previously discussed (see chapter 5) the application of traditional antibiotic compounds for contamination control is not generally recommended. It has been observed within this study (see chapter 3) that several groups of antagonistic micro-organisms exhibited growth inhibition of test contaminations when bioassayed on *in vitro* test plates. Obviously it would be naive to extrapolate from inhibition of microbial lawns to activity against contaminated plant tissue culture systems. Therefore the purpose of this study was to evaluate the *in vitro* activity of antimicrobial extracts for the control of accidental contaminations and latent infections in woody and herbaceous plant tissue culture systems.

## 6.1 Activity against accidental contaminations

Maximum permissive doses of selected antimicrobial extracts (*T.viride* strain 24039, *B.subtilis* strain SR2, *P.fluorescens* strain 95.6, and *G.roseum*) were incorporated into plant tissue culture media (section 2.1.2) which was subsequently deliberately contaminated by exposure to a non-sterile microbiological laboratory environment. Three replicate experiments were conducted. The effects of the extracts on the proliferation of contaminations in the test media after a 2 week incubation period is shown in Table 6.6.1. Contaminant density was estimated by employing an arbitrary assessment scale, where 0 indicates a complete absence of contaminations and 5 indicates mass overgrowth. All untreated (control) test media exhibited massive overgrowth by contaminations after the incubation period (Table 6.1.1). When incorporated in to media at their maximum allowed dosage, only those extracts produced by the *T.viride* and *B.subtilis* isolates were capable of reducing contaminant density in plant tissue culture media (a reduction from an arbitrary contaminant density rating of 5 to one of 4). When incorporated in to media at doses greater than this maximum allowed dose, media containing extracts produced by the *P.fluorescens* isolate also exhibited reduced contaminant densities (rating = 4). At these higher doses, the extracts from the *T.viride* isolate brought about a further reduction in the density of fungal contaminations on test media (rating = 3). Media containing extracts produced by the *G.roseum* isolate did not inhibit reduced contaminant densities relative to control media, irrespective of the dosage applied.

Effects on the extent of microbial growth in deliberately contaminated tobacco and birch explant shoot cultures of incorporating the maximum allowed doses of the various anti-microbial extracts into growth media are illustrated in Figures 6.1.1 and 6.1.2. The various anti-microbial extracts which were incorporated in to growth media produced a similar response in terms of the extent of accidental contamination (an arbitrary estimate where 0 indicates

Table 6.1.1

Effects of anti-microbial agents on proliferation of contaminations in tissue culture media. Various doses of anti-microbial extracts were incorporated in to plant tissue culture media which was subsequently exposed to a non-aseptic microbiological laboratory environment. The frequency of contaminations present in the media was scored after a 2 week incubation period.

| Extract | Treatment [Y] | Dose %v/v | Contaminant Density [Z] | |
|---|---|---|---|---|
| | | | Bacterial | Fungal |
| *T.viride* | A | 5.0 | 4 (4) | 4 (4) |
| | B | 7.5 | 4 (4-5) | 3 (3-4) |
| *B.subtilis* | A | 7.5 | 4 (4) | 5 (5) |
| | B | 10.0 | 4 (4) | 4 (4-5) |
| *P.fluorescens* | A | 2.5 | 5 (5) | 5 (5) |
| | B | 5.0 | 5 (5) | 4 (4-5) |
| *G.roseum* | A | 2.5 | 5 (5) | 5 (5) |
| | B | 5.0 | 5 (5) | 5 (5) |

[Y] Treatment 'A' corresponds to an extract dose equivalent to the maximum permissive dose.
Treatment 'B' corresponds to an extract dose greater than the maximum permissive dose.

[Z] Arbitary estimates of contaminant density after 48 h growth in plant tissue culture media (where 0 indicates a complete absence of contaminations and 5 indicates massive overgrowth). Figures represent mean values [n=3] followed (in parentheses) by the variation between 3 replicate experiments.

(Untreated control media exhibited severe contamination after the test period).

a complete absence of contamination and 10 indicates mass overgrowth) in both of the test plant species (Figs. 6.1.1 & 6.1.2). Tobacco and birch shoot cultures grown on untreated (control) media, and media incorporated with extracts from the *G.roseum* isolate, all exhibited mass overgrowth by contaminations after the 2 week incubation period. Contaminations present were predominantly *Penicillium*, *Aspergillus*, and *Botrytis* fungal species, and *Bacillus*, *Pseudomonas*, and *Erwinia* bacterial species. Shoot cultures grown on media incorporated with extracts produced by the *T.viride* and *B.subtilis* isolates exhibited a reduction in the extent of contamination by both the *Penicillium* and the *Aspergillus* species. This reduction was more pronounced in the birch shoot cultures ( a 20% reduction in the extent of contamination by both *Penicillium* and *Aspergillus* species, as opposed to a 10% reduction of these contaminations in the tobacco shoot cultures). Extracts produced by the *P.fluorescens* isolates proved effective at reducing the extent of contamination by *Penicillium* species only in the birch shoot cultures (a 10% reduction).

## 6.2 Activity against latent infections

The effect of pre-infection (prophylactic) treatment of woody plant culture explants with antimicrobial extracts for the preventative control of latent endogenous infections is illustrated in Figure 6.2.1. Shoot cultures of witch-hazel and rosewood species grown on untreated (control) media and on media incorporated with extracts from the *G.roseum* isolate all exhibited 100% contamination by *Bacillus*, *Pseudomonas*, and *Erwinia* species after the 8 week incubation period. Extracts produced by the bacterial isolates *B.subtilis* and *P.fluorescens* were more effective for the control of latent infections when employed in rosewood shoot culture media (a 20% reduction in *Bacillus* contaminations, and a 10% reduction in *Pseudomonas* and *Erwinia* shoot contaminations, respectively) than when these extracts were applied to witch-hazel systems (a 10% reduction in *Bacillus* contaminations, and no reduction

in shoot contamination, respectively). Extracts produced by the *T.viride* isolate had a similar prophylactic effect in both of the plant species tested (a 20% reduction in shoot contamination by *Bacillus* species in both types of plant culture).

When the antimicrobial extracts were applied as post-infection treatments in woody plant cultures, those extracts produced by the *P.fluorescens* and *G.roseum* isolates proved to be totally ineffective for the eradication of latent infections (Figure 6.2.2). In witch-hazel cultures a 10% reduction in the number of contaminated shoots was noted after a second growth passage on growth media incorporated with *T.viride* and *B.subtilis* extracts, due to the partial control of *Bacillus* and *Pseudomonas* contaminant species.. In the rosewood cultures only those extracts from the *B.subtilis* isolate were able to bring about a reduction in the number of infected shoots (10% reduction in *Bacillus* contaminants) when grown in subsequent culture.

### 6.3 Efficacy of combination treatments

The effects of extract combination treatments on the extent of accidental contaminations and latent infections in shoot cultures of tobacco and witch-hazel are shown in Figures 6.3.1 and 6.3.2, respectively. Combinations of antimicrobial extracts 'B' and 'E' were equally effective in reducing the extent of accidental contaminations (a 20% reduction) in tobacco shoot cultures (Fig. 6.3.1). Combination 'D' (comprising the two bacterial extracts) did not reduce the extent of contamination relative to untreated (control) cultures, whereas treatment of shoot cultures with extract combination 'C' resulted in a 10% reduction in contaminant density (Fig. 6.3.1). With reference to the control of latent infections in witch-hazel shoot cultures, extract combinations 'B' and 'E' were again equally effective in reducing the number of contaminated shoots (a 20% reduction) when they were grown in subsequent culture (Fig. 6.3.2). Extract combinations 'C' and

'D' were equally effective contamination control agents (both treatments resulting in a 10% reduction in the number of contaminated shoots).

The relative abundance of various species groups of accidental contaminations in tobacco shoot cultures, and latent infections in witch-hazel shoot cultures, are illustrated in Figures 6.3.3 and 6.3.4. Combination treatments comprising extracts from the *T.viride* and *B.subtilis* isolates (i.e. combinations 'V' and 'Z') produced a reduction in overall contamination density of 20% relative to untreated controls in the tobacco cultures (Fig. 6.3.3). Where extracts of the *T.viride* isolate were absent from growth media, those cultures displayed mass overgrowth by contaminations (i.e. 100% contamination). *Penicillium* species were by far the most abundant of the accidental contaminations present in all tests, irrespective of the combination of anti-microbial extracts employed in the growth media. All combination treatments did, however, reduce the density of the *Penicillium* species relative to untreated (control) cultures. A consequence of this controlling action was the appearance of bacteria less susceptible to extract treatment, including *Bacillus* and *Pseudomonas* species, as observed in treatments 'W', 'Y', and 'Z' (Fig. 6.3.3). When extracts produced by the *P.fluorescens* isolate were employed as control agents, bacterial contamination by *Erwinia* species was not evident. Extracts of the *B.subtilis* isolate did not appear to exert any specific controlling effect against any of the accidental contaminations, but rather worked in combination with extracts produced by the *T.viride* isolate as a general anti-microbial agent. With reference to the abundance of latent bacterial infections in untreated (control) witch-hazel cultures, all specimens were contaminated, with *Bacillus* species being predominant (Fig. 6.3.4). Growth of the shoot cultures on media incorporated with extract combinations 'V' and 'Z' lead to a reduction in the number of contaminated shoots of up to 20%. A reduction in the extent of contamination by the *Bacillus* species was

accompanied by the development of less susceptible infections, including *Erwinia*, *Pseudomonas*, and unidentified Gram negative bacterial species.

**Discussion**

If cell-free culture filtrates produced by antagonistic micro-organisms are to be effectively employed for contamination control in plant tissue cultures, then the antimicrobials must be compatible with such systems so as to assure their activity. Investigations conducted in this study attempted to establish the inhibitory activity, and the spectrum of activity, of such anti-microbials against opportunist contaminations and latent infections when the filtrates were incorporated in to plant tissue culture growth media.

Only those extracts produced by the *T.viride* and *B.subtilis* isolates were capable of producing a reduction in the density of opportunist contaminations of growth media when they were employed at their maximum permissive dosage. Extracts from the *P.fluorescens* isolate were only effective when employed at phytotoxic levels (see chapter 5) and were therefore unsuitable for application as control agents in the systems under study. The activity of those extracts produced by the *G.roseum* isolate proved to be totally ineffective and therefore were excluded from further studies on contamination control in subsequent chapters.

When applied for the control of accidental contaminations of the test plant cultures themselves, the activity of extracts from the *T.viride* and *B.subtilis* isolates was of a similar magnitude. Extracts from the *P.fluorescens* isolate, however, displayed a different degree of activity when incorporated in to the different plant culture systems (i.e. activity was plant species-specific). It is possible that this specificity was a result of interactions between this antimicrobial and the constituents of the various media (see review by Falkiner, 1990).

Because of their intended preventative role, it is more appropriate to consider the use of antimicrobials as a form of prophylaxis rather than treatment. In plant tissue culture, however, it is difficult to apply the criteria for prophylaxis (i.e. pathogen-specificity, sustained pathogen-sensitivity, and minimum exposure during maximum risk period) because a variety of micro-organisms are often present and because the antimicrobial is incorporated in to the media for the duration of the incubation period (Greenwood, 1989). Despite these draw-backs, antimicrobial extracts of *T.viride*, *B.subtilis*, and *P.fluorescens* were applied, and did offer a modest level of prophylactic control in the two woody plant species tested in these studies. Post-treatment sterilant activity in the same woody systems was minimal, and only exhibited by extracts from the *T.viride* and *B.subtilis* isolates.

When extracts produced by the *T.viride* and *B.subtilis* isolates were applied together for the control of accidental contaminations and latent infections, the magnitude of their combined activity was comparable to that of their individual activities (i.e. there was no evidence of synergistic activity between these two anti-microbials). Assays which illustrate synergistic activity have been described by Kavanagh (1972) and may be employed to verify the absence of synergistic activity between extracts employed in this study. When *B.subtilis* and *P.fluorescens* extracts were applied in combination, however, the resulting effect was zero antimicrobial activity. This suggests that these extracts were incompatible in the test systems studied, since *B.subtilis* extracts were inhibitory when applied exclusively for contamination control. Combinations of bacterial extracts were modestly effective against latent infections, whereas they were totally ineffective against some of the accidental contaminations (i.e. the bacterial extracts exhibited target specificity).

Studies on the abundance of various contaminations revealed that certain extracts exhibiting growth inhibition of specific contaminations in bioassay tests (see chapter 3) displayed a similar specificity in plant tissue

culture systems. Extracts of *T.viride* prevented the mass overgrowth of plant cultures by the ubiquitous *Penicillium* species. However, the partial control of this susceptible species allowed the growth of more tolerant types which were less vulnerable to control by the anti-microbial extracts. The frequency of *Bacillus* species was restricted by the presence of *T.viride* and *B.subtilis* extracts in the growth media. Extracts of the *P.fluorescens* isolate were effective for the eradication of *Erwinia* species, but only when employed at phytotoxic levels.

To summarize, the culture extracts of 2 of the 4 microbial isolates screened for anti-microbial activity in 'clean' and infected herbaceous and woody plant tissue culture systems lowered the frequency of contaminations when employed at their maximum permissive dosage. The use of combinations of extracts was only advantageous when specific types of contaminations were present. The application of extracts as post-infection sterilants, exclusively or in combination, was generally ineffective over the test period.

Therefore it is feasible that anti-microbial extracts might be employed for partial contamination prevention and/or control in plant tissue culture systems. However, for such a programme to be viable it is clear that the activity of extracts must be at least comparable to that of conventional antibiotics which are currently employed. Such comparative studies are considered in chapter 7. It is also feasible that extract activity may be limited by non-active components present within the extracts themselves. Therefore further purification of the antagonistic extracts may increase the usable maximum permissive dosage range.

Figure 6.1.1

**Extract activity against accidental contaminations in tobacco shoot culture explants.**

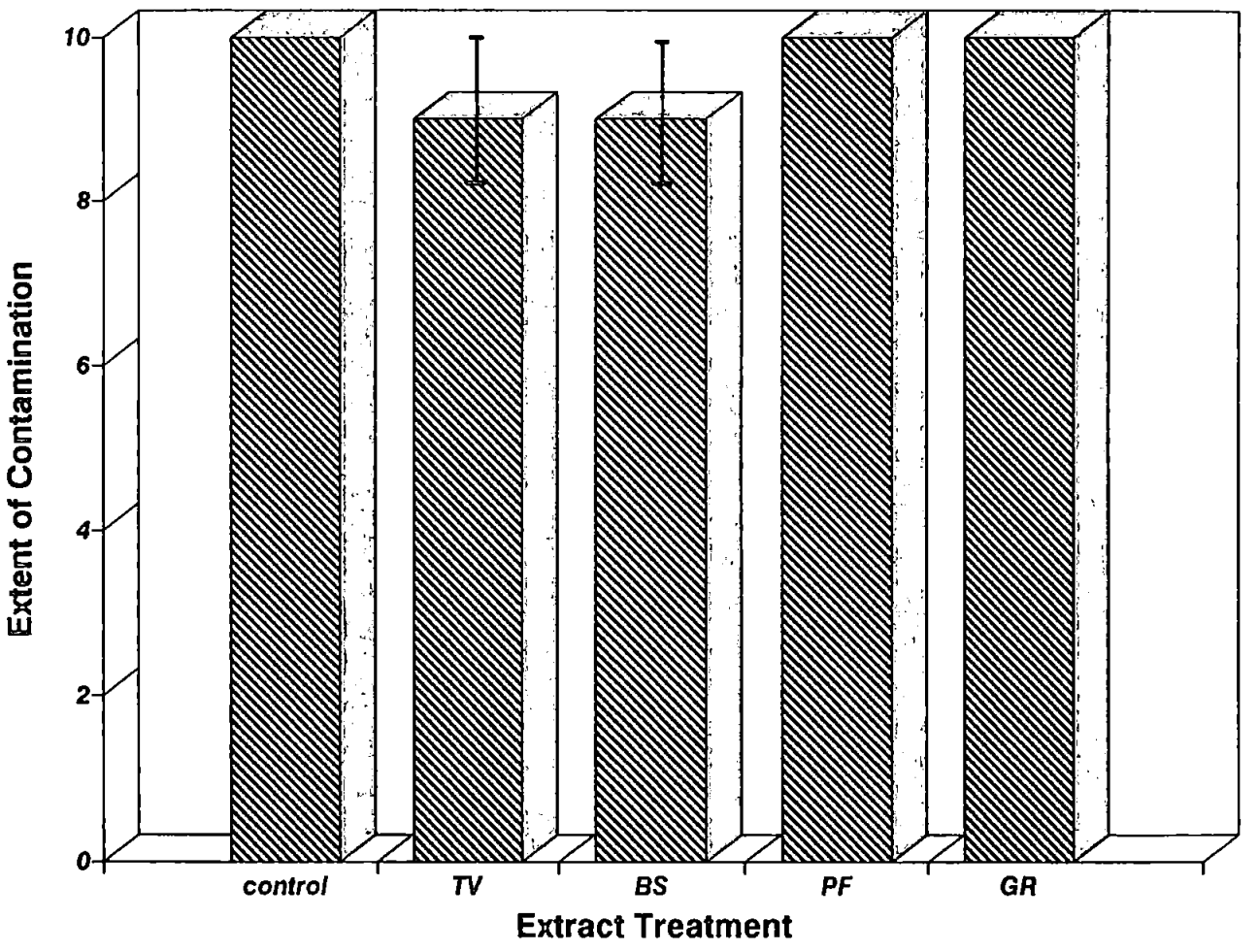
Previously determined maximum permissive doses of extracts were incorporated in to plant tissue culture growth media. The plant cultures were deliberately contaminated by exposure to a non-sterile microbiological laboratory environment. The extent of contamination (an arbitary estimate where 0 indicates complete absence of contamination and 10 indicates mass overgrowth) in the tissue culture explants was observed after 2 weeks incubation at 25$^{O}$C.

TV, *T.viride* (5 %v/v)

BS, *B.subtilis* (7.5 %v/v)

PF, *P.fluorescens* (5 %v/v)

GR, *G.roseum* (1 %v/v)




Vertical line = range of estimates (n = 3)

Figure 6.1.2

**Extract activity against accidental contaminations in birch shoot culture explants.**

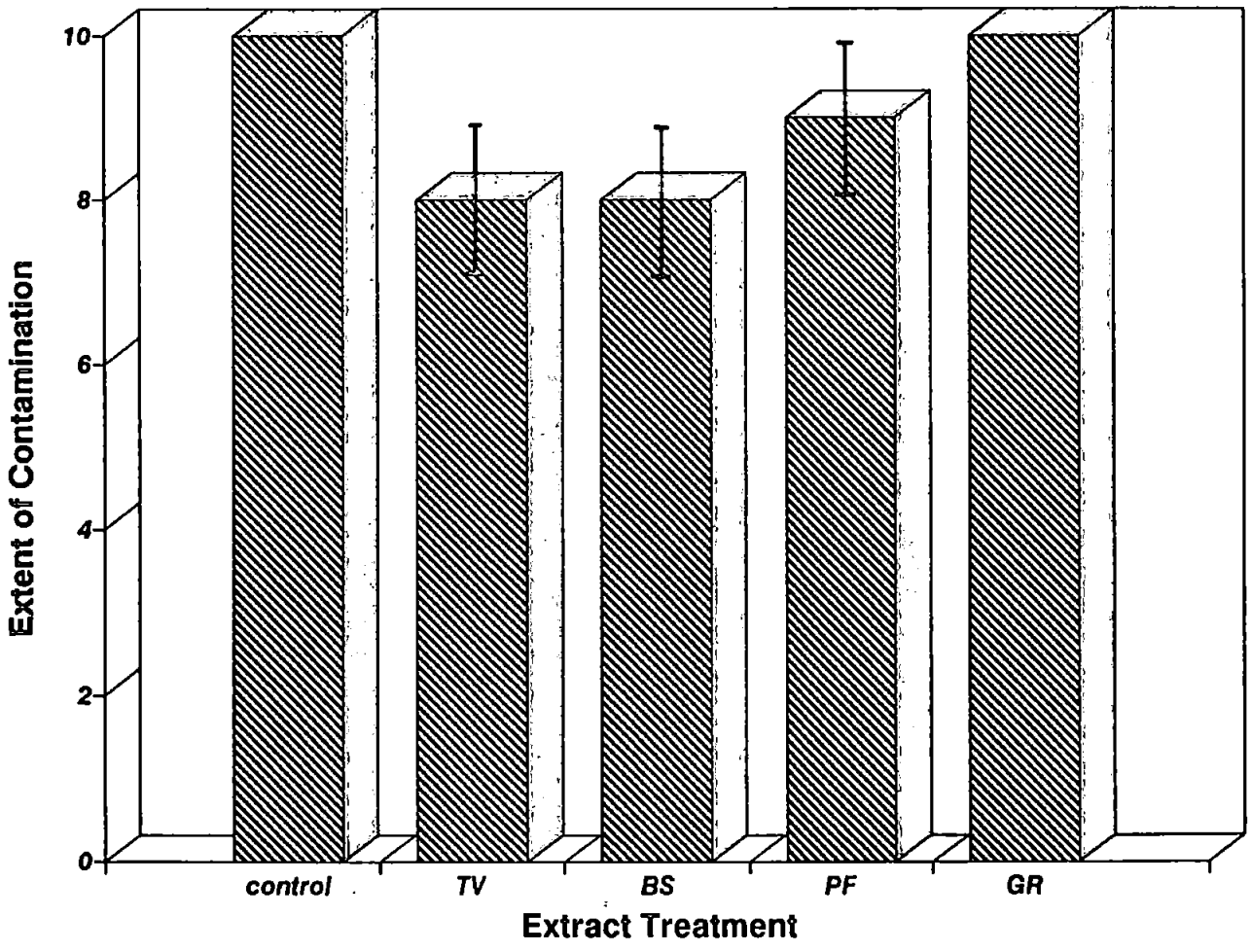
Previously determined maximum permissive doses of extracts were incorporated in to plant tissue culture growth media. The plant cultures were deliberately contaminated by exposure to a non-sterile microbiological laboratory environment. The extent of contamination (an arbitary estimate where 0 indicates complete absence of contamination and 10 indicates mass overgrowth) in the tissue culture explants was observed after 2 weeks incubation at 25$^{O}$C.

TV, *T.viride* (5 %v/v)

BS, *B.subtilis* (7.5 %v/v)

PF, *P.fluorescens* (5 %v/v)

GR, *G.roseum* (1 %v/v)




Vertical line = range of estimates (n = 3)

Figure 6.2.1

**Pre-infection (prophylactic) treatment of woody plant culture explants with anti-microbial extracts for preventative control of latent endogenous contaminations.**

Previously determined maximum permissive doses of extracts were incorporated in to plant tissue culture growth media of the following explant species:

A Witch-hazel

B Rosewood

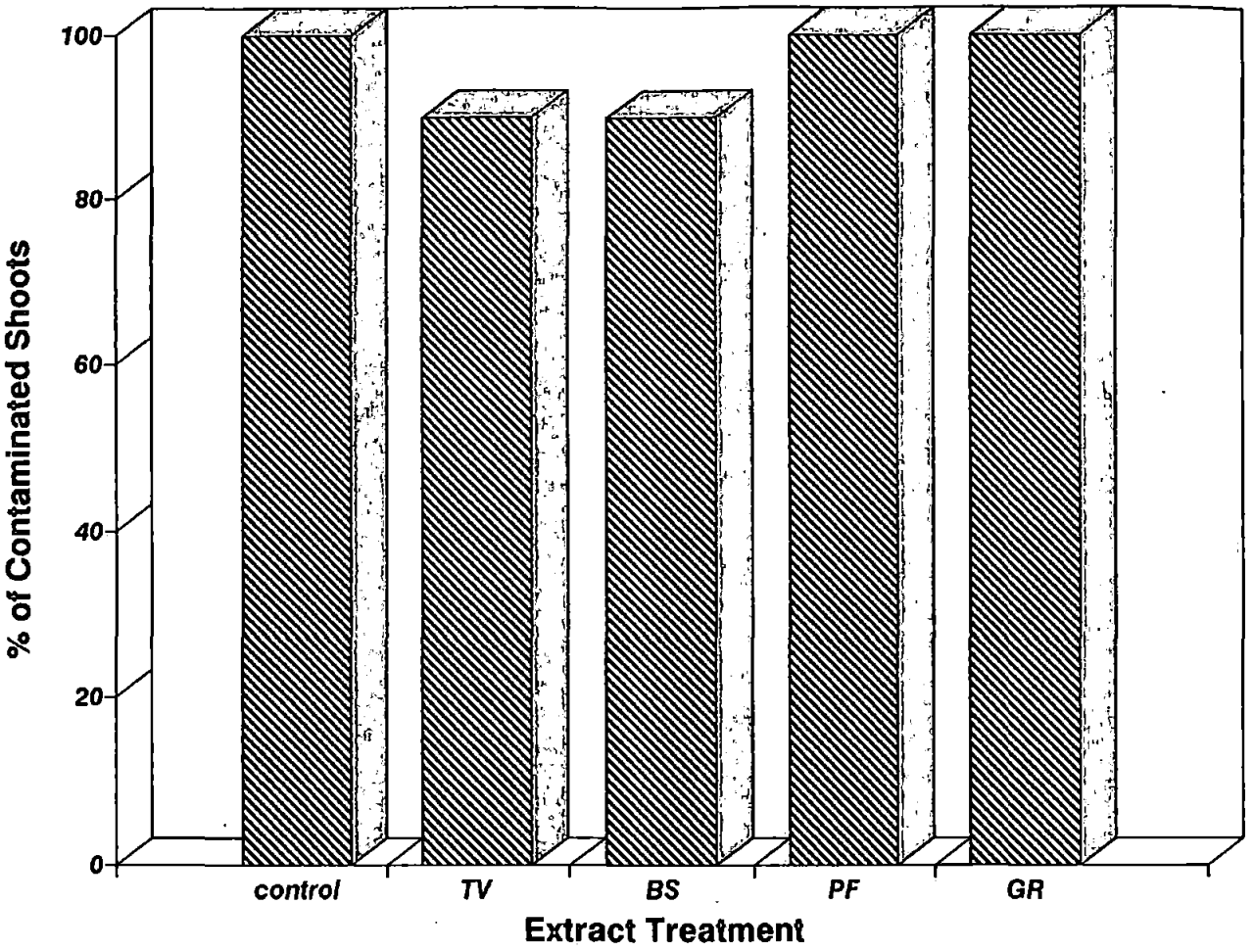
The number of shoot cultures (expressed as a percentage) contaminated by latent bacterial infections was noted after 8 weeks incubation at 25$^{o}$C.

TV, *T.viride* (5 %v/v)

BS, *B.subtilis* (7.5 %v/v)

PF, *P.fluorescens* (5 %v/v)

GR, *G.roseum* (1 %v/v)




Vertical line = range of estimates (n = 3)

B

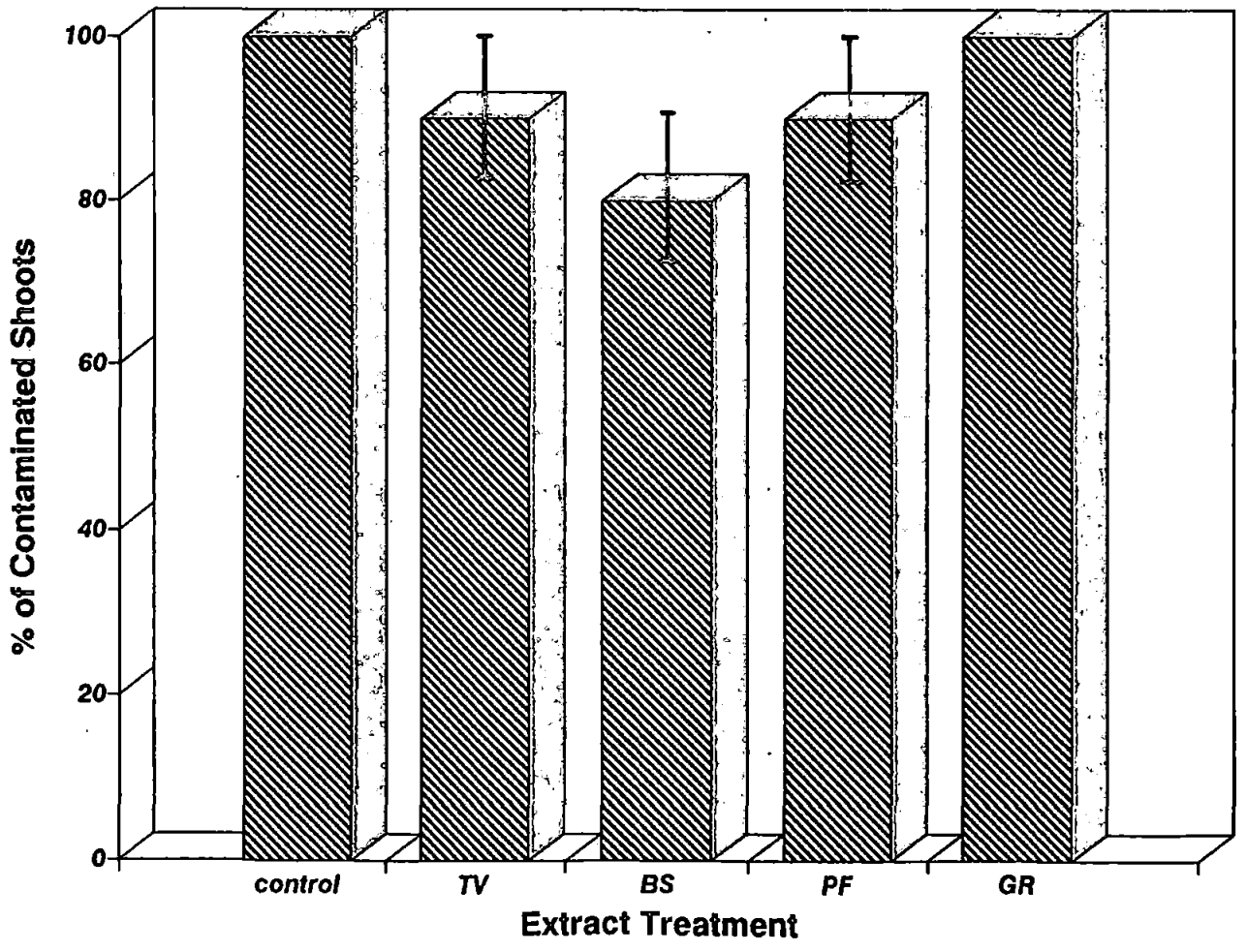


Figure 6.2.2

**Containment and eradication potential of anti-microbial extracts when applied as post-infection treatments in woody plant culture species.**

Explants exhibiting latent infection were sub-cultured on to extract-incorporated growth media. The percentage of contaminated shoot cultures observed after this subsequent passage in culture was noted.

A Witch-hazel

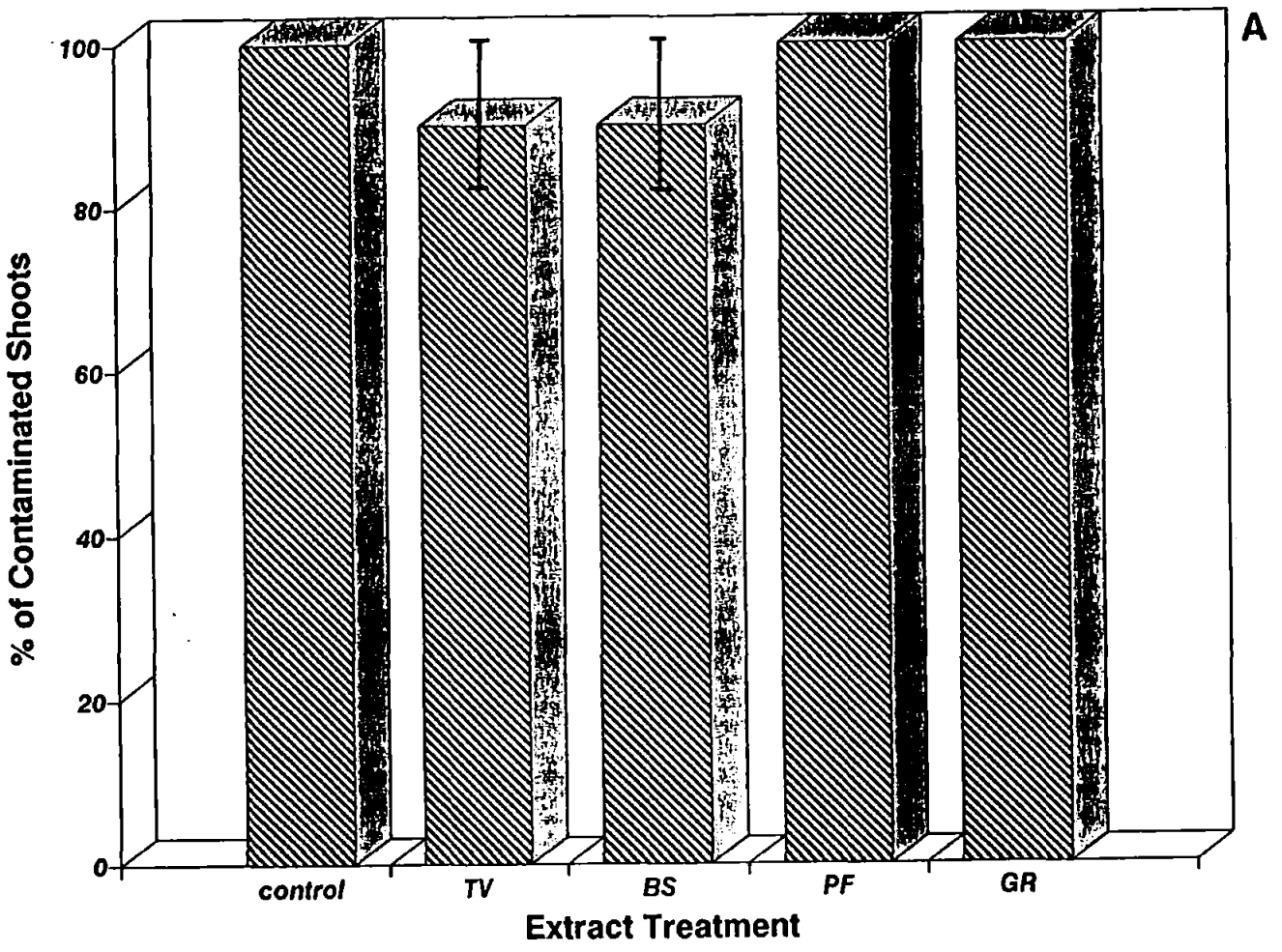
B Rosewood

TV, *T.viride* (5 %v/v)

BS, *B.subtilis* (7.5 %v/v)

PF, *P.fluorescens* (5 %v/v)

GR, *G.roseum* (1 %v/v)




Vertical line = range of estimates (n = 3)

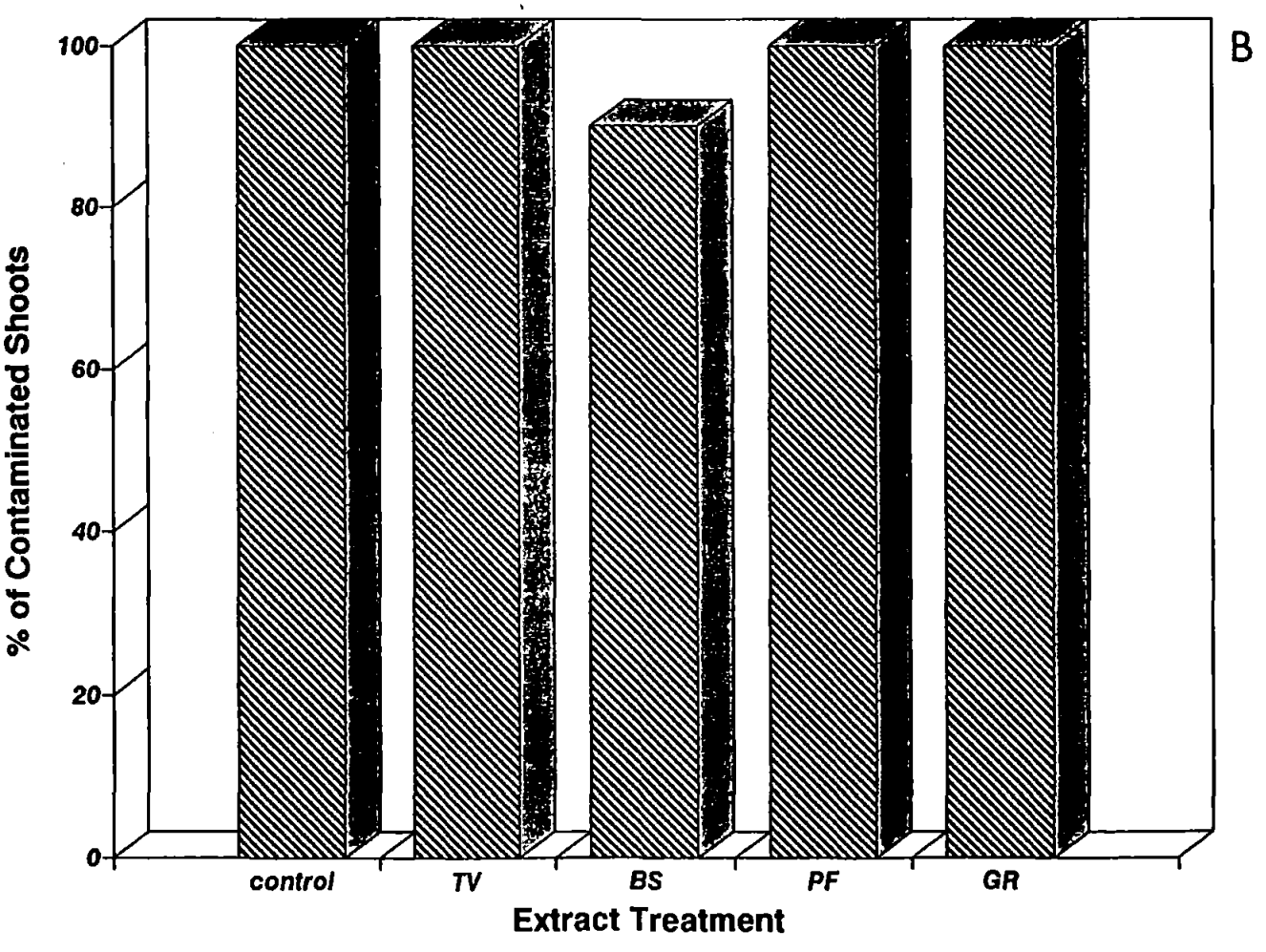


**Figure 6.3.1**

**Effect of extract combination-treatments on the extent of accidental contaminations in tobacco shoot cultures.**

Combinations of extracts were incorporated into plant tissue growth media. The explants were deliberately contaminated by exposure to a non-sterile microbiological laboratory environment. The extent of contamination (an arbitrary estimate where 0 indicates complete absence of contamination and 10 indicates mass overgrowth) in the tissue culture explants was observed after 2 weeks incubation at 25$^{O}$C.

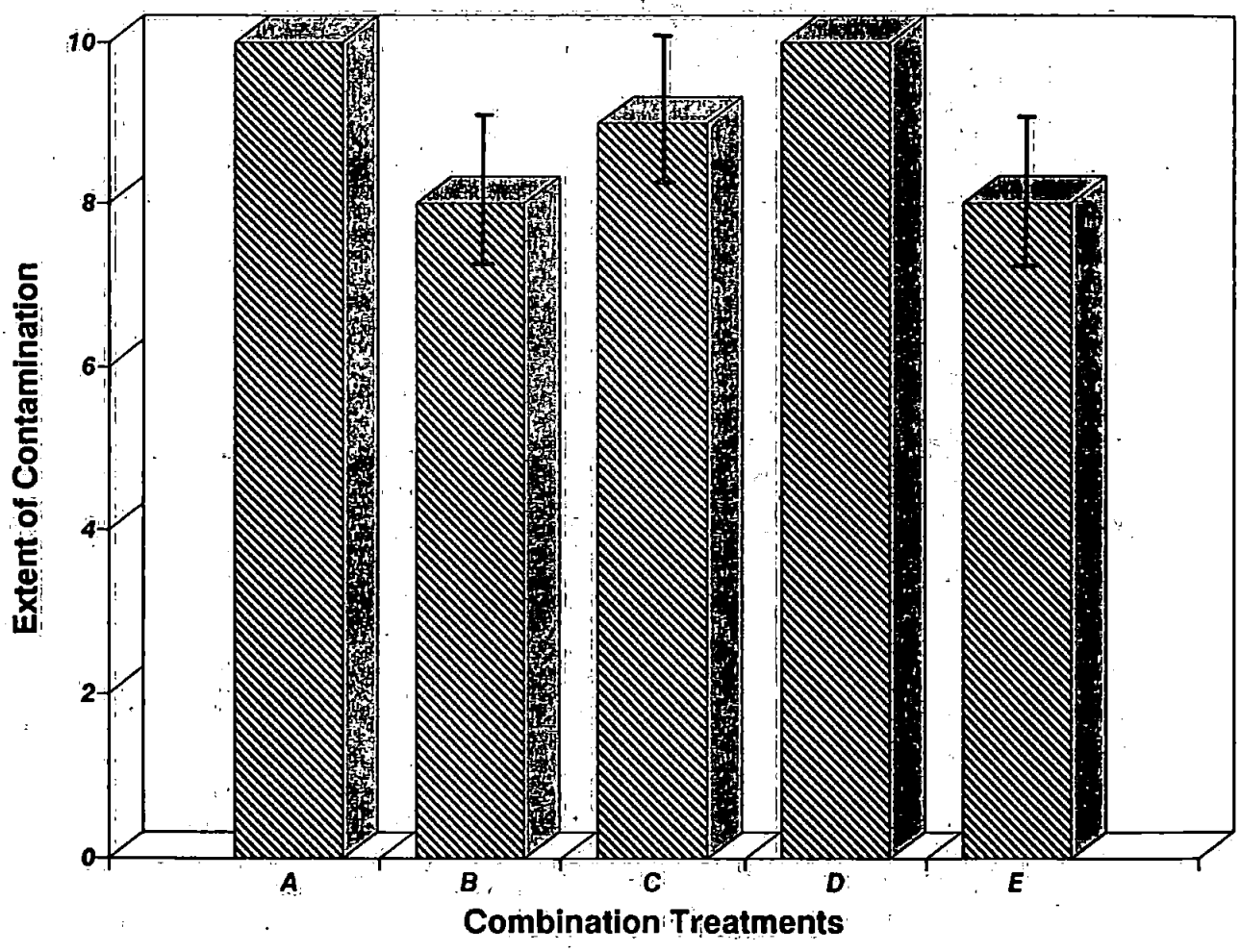
A Untreated controls

B *T.viride* (5 %v/v) + *B.subtilis* (7.5 %v/v)

C *T.viride* (5 %v/v) + *P.fluorescens* (5 %v/v)

D *B.subtilis* (7.5 %v/v) + *P.fluorescens* (5 %v/v)

E *T.viride* (5 %v/v) + *B.subtilis* (7.5 %v/v) + *P.fluorescens* (5 %v/v)




Vertical line = range of estimates (n = 3)

Figure 6.3.2

**Effect of extract combination-treatments on the extent of latent infections in witch-hazel shoot cultures.**

Explants exhibiting latent infections were sub-cultured on to extract-incorporated growth media. The percentage of contaminated shoot cultures observed after this subsequent passage in culture was recorded.

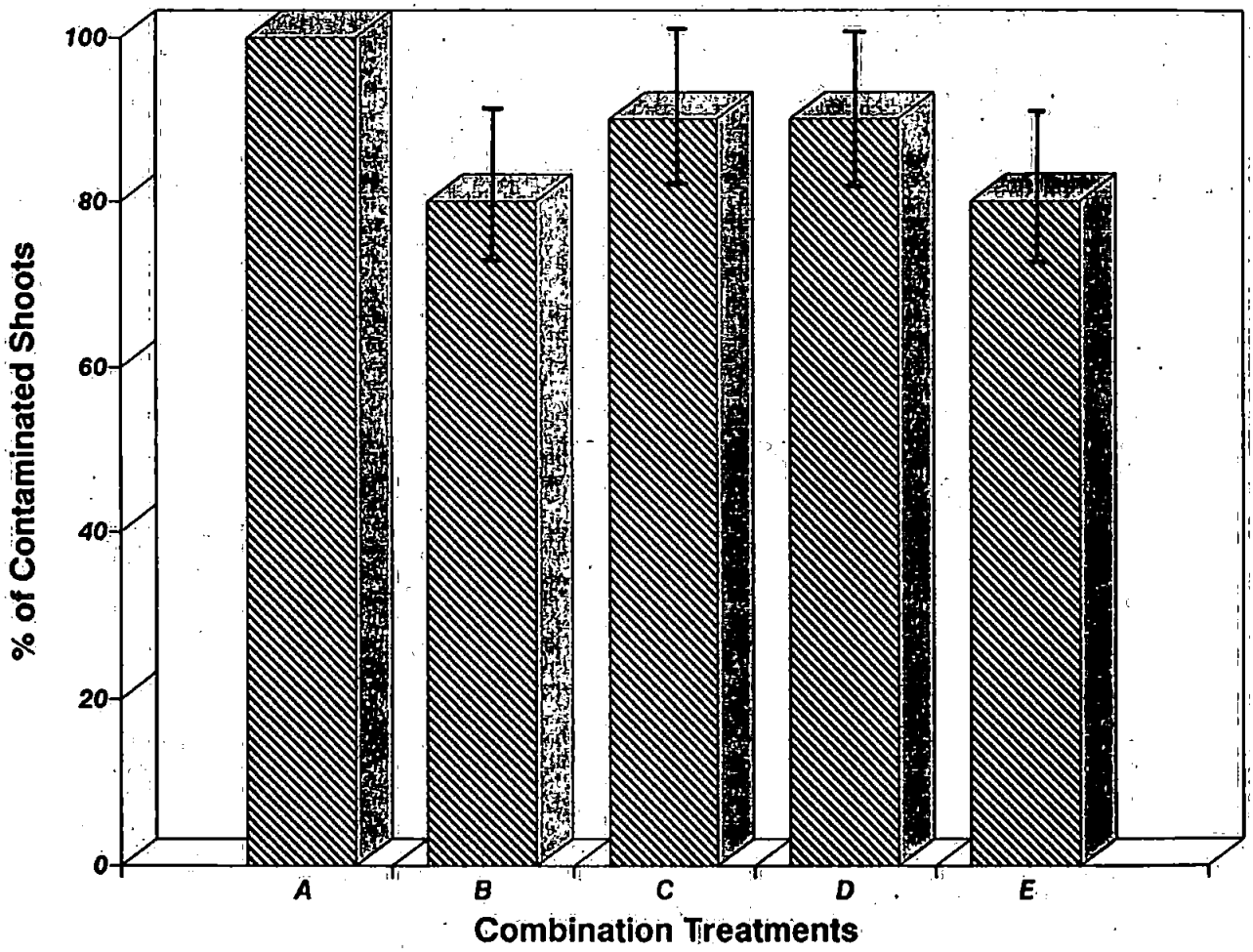
A Untreated controls

B *T.viride* (5 %v/v) + *B.subtilis* (7.5 %v/v)

C *T.viride* (5 %v/v) + *P.fluorescens* (5 %v/v)

D *B.subtilis* (7.5 %v/v) + *P.fluorescens* (5 %v/v)

E *T.viride* (5 %v/v) + *B.subtilis* (7.5 %v/v) + *P.fluorescens* (5 %v/v)



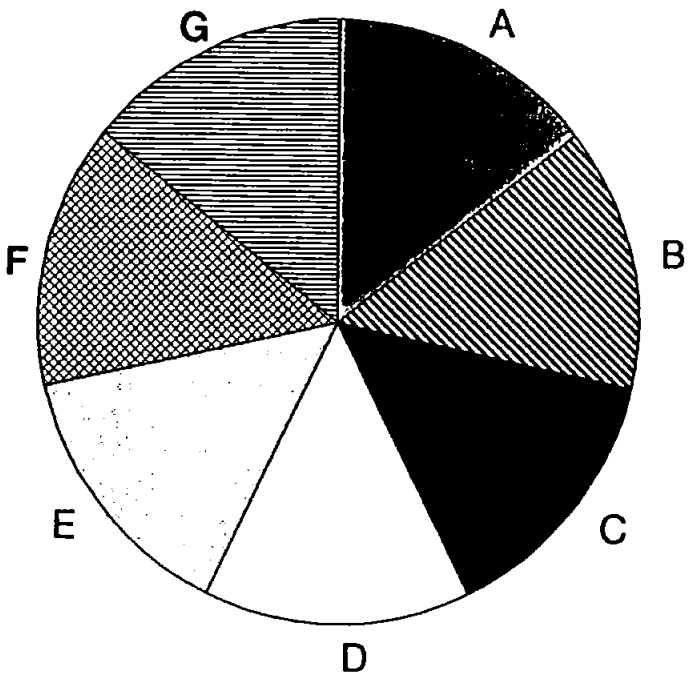

Vertical line = range of estimates (n = 3)

**Figure 6.3.3**

**Relative abundance of accidental contaminations in tobacco shoot cultures grown on media incorporated with a combination of anti-microbial extracts (after 8 weeks growth at 25$^{o}$C).**

Values represent the average percentage contaminant density observed visually in 10 replicate shoot culture systems.

Key to charts:




A *Penicillium*

B *Aspergillus*

C *Botrytis*

D Uncontaminated

E *Erwinia*

F *Bacillus*

G *Pseudomonas*

**With reference to the pie diagrams overleaf:-**

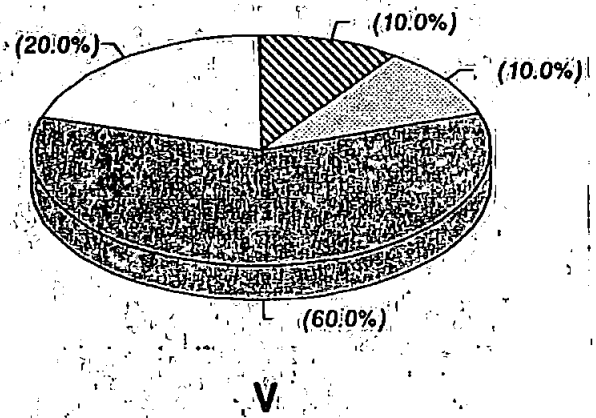
(V) *T.viride* (5 %v/v) + *B.subtilis* (7.5 %v/v)

(W) *T.viride* (5 %v/v) + *P.fluorescens* (5 %v/v)

(X) Untreated controls,

(Y) *B.subtilis* (7.5 %v/v) + *P.fluorescens* (5 %v/v)

(Z) *T.virlde* (5 %v/v) + *B.subtilis* (7.5 %v/v) + *P.fluorescens* (5 %v/v)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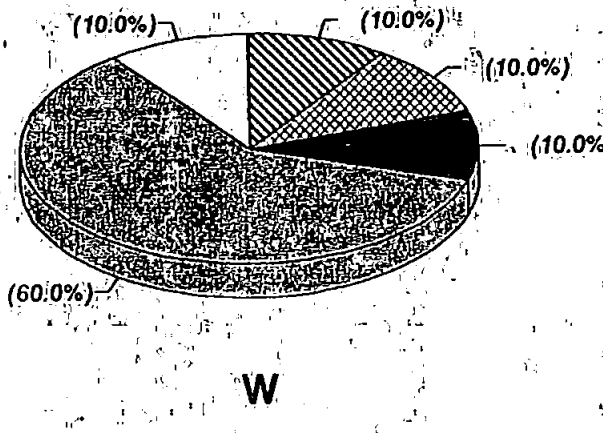
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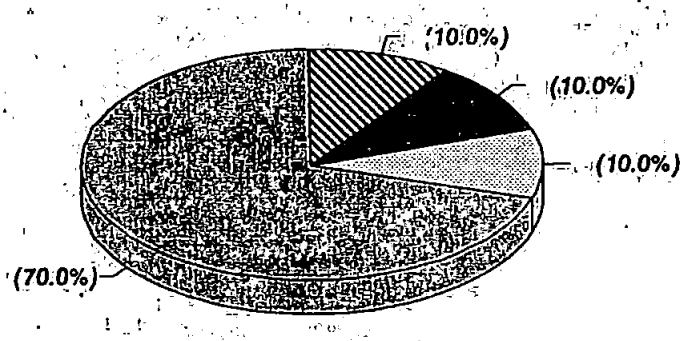
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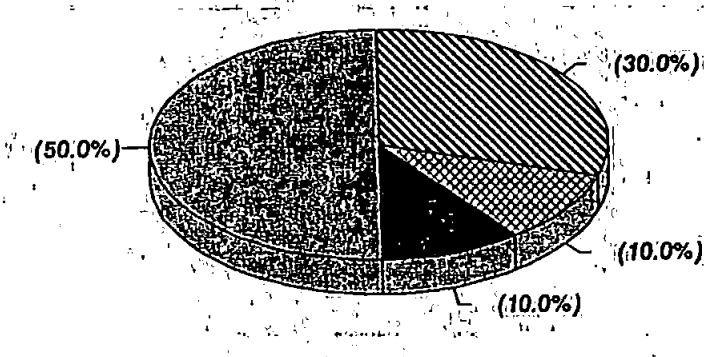
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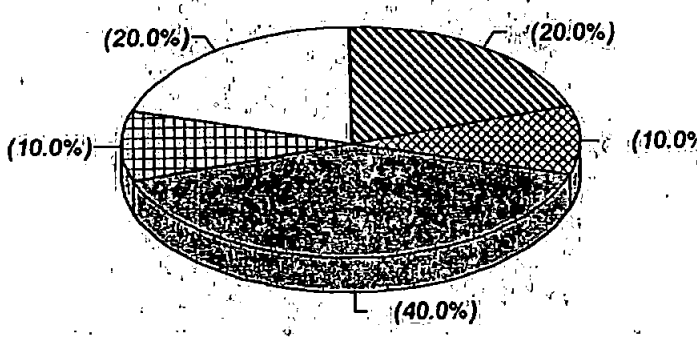
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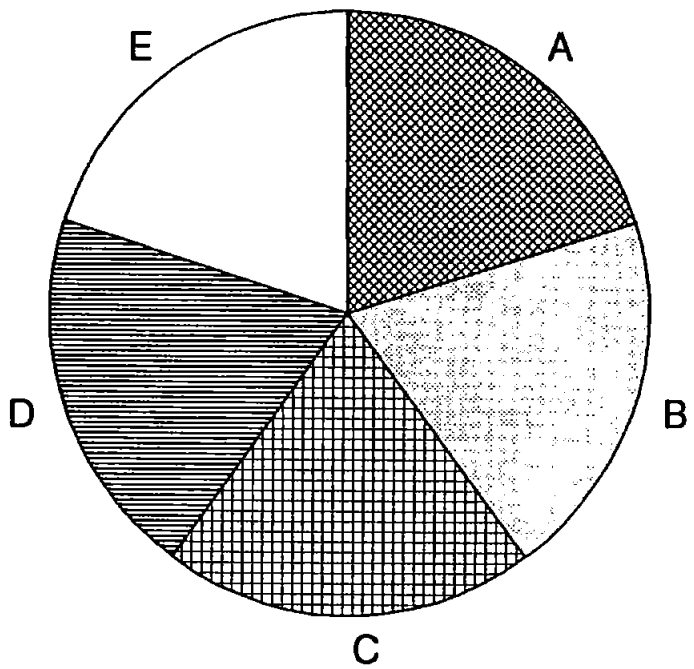
**Figure 6.3.4**

**Relative abundance of latent infections in witch-hazel shoot cultures grown on media incorporated with a combination of anti-microbial extracts (after 8 weeks growth at 25$^{o}$C).**

Values represent the average percentage contaminant density observed in 10 replicate shoot culture systems.

Key to charts:




A *Bacillus*

B *Erwinia*

C *Pseudomonas*

D Unidentified Gram negative bacteria

E Uncontaminated

**With reference to the pie diagrams overleaf:-**

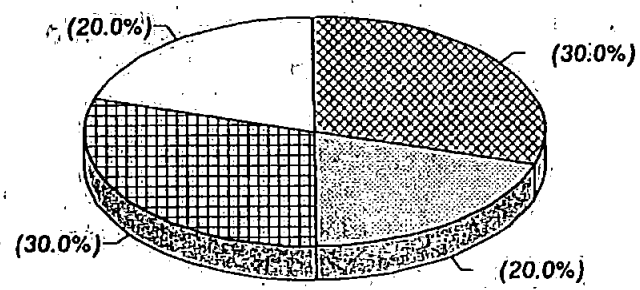
(V) *T.viride* (5 %v/v) + *B.subtilis* (7.5 %v/v)

(W) *T.viride* (5 %v/v) + *P.fluorescens* (5 %v/v)

(X) Untreated controls,

(Y) *B.subtilis* (7.5 %v/v) + *P.fluorescens* (5 %v/v)

(Z) *T.viride* (5 %v/v) + *B.subtilis* (7.5 %v/v) + *P.fluorescens* (5 %v/v)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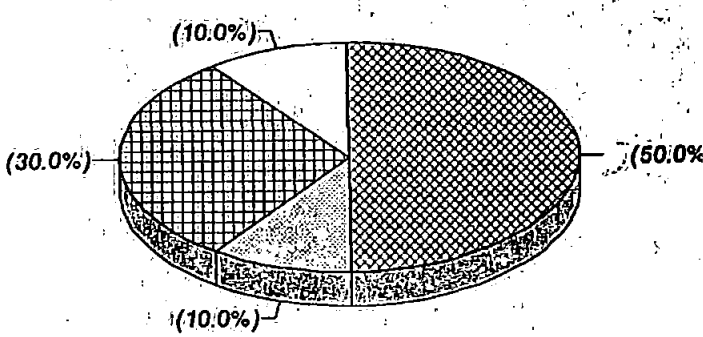
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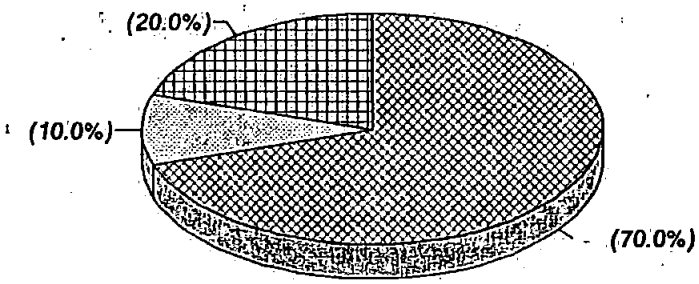
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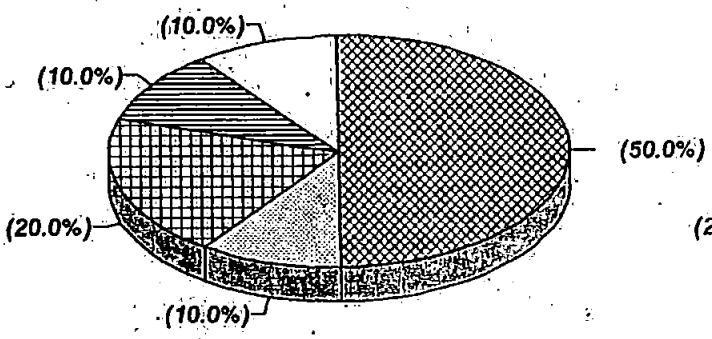
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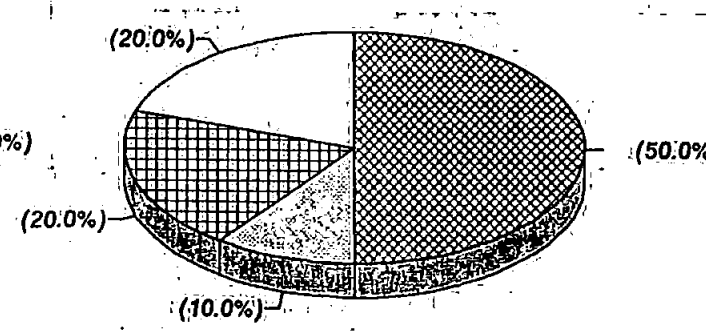
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CHAPTER 7

# EFFICACY OF ANTIMICROBIAL AGENTS FOR INFECTION CONTROL AND DECONTAMINATION IN PLANT TISSUE CULTURE SYSTEMS

As previously mentioned (see chapter 5) the use of antibiotics in plant tissue culture is not generally recommended, largely because of the possibility that inclusion of such antimicrobial agents in plant culture medium may adversely affect cell growth. Surface-sterilization of the plant parts to be cultured, in combination with careful aseptic technique, is satisfactory for the majority of applications, where a proportion of infected cultures can be tolerated. However, in certain circumstances, endemic bacterial contamination may dictate the use of antibacterial agents as a last resort. Although for many cultures bacterial or fungal contamination is rare, for particular plant species, especially woody types (see Young *et al*, 1984; Wilkins *et al*, 1985), cultures may become systemically contaminated with bacteria. Susceptible explants cannot be identified prior to culture by casual or microscopic examination, but on excision and transfer of explants to culture medium, massive bacterial contamination invariably occurs within 24-36 h.

Surface-sterilization procedures themselves may be a problem. Cultures which become contaminated are generally discarded because re-sterilization of *in vitro* cultures is usually severely damaging (Thurston *et al*, 1979). Such procedures are even more problematic when employed for the decontamination of delicate organ explants such as shoot-tips. Elaborate procedures must often be employed for this purpose (Arditti, 1977), and for many plant species surface sterilization may be a limiting factor (Stewart & Button, 1975). The need for surface-sterilization could be reduced or eliminated if explants were cultured on media containing anti-contaminants (as

often is the case with animal tissue and cell cultures). Eliminating the requirement for asepsis in the establishment of *in vitro* plant cultures would have immense commercial applications, such as facilitating mechanization and robotization techniques for *in vitro* culture, procedures which are particularly prone to accidental contamination.

The aim of the present study was to screen a range of antimicrobial agents, including antagonistic extracts, antibiotics, and chemotherapeutic agents (both exclusively and in combination), for the control and eradication of accidental contaminations and latent infections in established cultures and short-term explants of herbaceous and woody plant culture systems. The efficacy of the various agents for the decontamination of explant material and for the treatment of donor plant material prior to establishment of cultures was also evaluated.

### 7.1 Efficacy of antimicrobials in contaminated systems

Maximum permissive doses of selected anti-microbial extracts (*T.viride* strain 24039 and *B.subtilis* strain SR1) and recommended concentrations of conventional antibiotics (section 1.4.1) were incorporated (exclusively and in combination) into tobacco and witch-hazel shoot culture growth media (section 2.1.2). The tobacco cultures were subsequently deliberately contaminated by exposure to a non-sterile microbiological laboratory environment. The effects of the antimicrobial treatments on the proliferation of accidental contaminations in the tobacco cultures, and on the control of latent infections in the witch-hazel cultures, following incubation periods of 4 and 8 weeks at 25$^{o}$C, respectively, are shown in Tables 7.1.1. and 7.1.2. In both series of tests contamination density was estimated by employing an arbitrary assessment scale, where 0 indicates a complete absence of contaminations and/or latent infections and 5 indicates mass overgrowth. The phytotoxicity of

the antimicrobials was determined visually by checking for necrosis, browning, chlorosis, and morphological changes. All tests were repeated once.

With regards to the deliberately contaminated tobacco cultures, bacterial isolates observed included species of *Bacillus*, *Pseudomonas* and *Erwinia*. Fungal contaminations which were present were predominantly *Penicillium*, *Aspergillus* and *Botrytis* species. All untreated (control) cultures exhibited mass overgrowth by contaminations after the incubation period. No single antimicrobial tested was effective against all of the contaminations employed. Extracts produced by the *T.viride* and *B.subtilis* antagonists both produced only a minimal reduction in the extent of each of the fungal contaminations and were even less effective against the bacterial isolates. Rifampicin was bactericidal against *Bacillus* species at both of the concentrations employed. Amphotericin B was fungicidal against the *Penicillium* and *Aspergillus* species and fungistatic against the *Botrytis* species. Both of the conventional antibiotics tested were less effective when employed at the lower concentrations. When applied exclusively at their higher concentrations, all of the antimicrobials exhibited various degrees of phytotoxicity.

The efficacy of a combination of the extracts produced by the microbial antagonists (combination A) was similar to when each of these extracts were applied exclusively (i.e no synergistic activity). A combination of all of the antimicrobials employed at their higher doses and concentrations (combination B) was the most effective means of controlling the various groups of contaminations in these studies, reducing the severity of infection by all contaminations. However, this combination was highly phytotoxic to the tobacco shoot cultures.

With regards to the control of latent bacterial infections in the witch-hazel shoot-tip cultures (Table 7.1.2), bacterial isolates observed after the 8 week incubation period included *Bacillus*, *Pseudomonas*, and *Erwinia* species.

No fungal contaminations were evident in any of the witch-hazel shoot-tip cultures. All untreated cultures exhibited mass overgrowth by the various latent infections. No single antimicrobial was effective against all of the types of bacterial isolates present. Extracts from the 2 microbial antagonists produced a minimal reduction in the extent of latent infections (rating = 9), irrespective of the extract dosages applied. Rifampicin was bactericidal against *Bacillus* species and bacteriostatic against *Pseudomonas* and *Erwinia* species. The magnitude of activity was more pronounced when this antibiotic was employed at the higher concentration (50 $mgL^{-1}$), but at this concentration severe phytotoxic effects were observed in the witch-hazel cultures. Amphotericin B was totally ineffective for the control of the latent infections at both of the concentrations employed. All of the antimicrobials exerted varying degrees of phytotoxicity when employed at the higher dosages and concentrations.

A combination of the microbial extracts produced a reduction in bacterial density in the witch-hazel systems greater than the reduction observed when these 2 extracts were applied exclusively. This suggests enhanced activity and/or synergism between the extracts in this culture system. A combination of all of the antimicrobials (combination B) produced a reduction in bacterial density of a similar magnitude to that of rifampicin when the latter was applied exclusively at its higher concentration. Combination B was severely phytotoxic to the witch-hazel shoot-tip cultures.

### 7.2 Control of bacterial growth in short-term tobacco explant cultures

Tobacco stem explant cultures were placed onto growth media (section 2.1.2) incorporated with various antimicrobial agents. The cultures were then deliberately contaminated with 2 types of bacterial suspensions (as described in section 2.8). The effect of the antimicrobial agents on the proliferation of the contaminating bacterial inocula relative to untreated (control) cultures was assessed after a 48 h incubation period. An arbitrary estimate of bacterial

density was made, where 0 indicated a complete absence of contaminating bacteria and 5 indicated mass overgrowth. The fresh weight of non-contaminated explants exposed to the various antimicrobial agents was determined after 4 weeks incubation. The ability of the explant cultures to synthesize chlorophyll when illuminated was assessed visually. All experiments were repeated twice.

All untreated explants exhibited severe bacterial contamination after the 48 h incubation period (Table 7.2.1). Explants grown on media containing the higher doses of the *T.viride* and *B.subtilis* extracts exhibited a very modest reduction in the extent of contamination by the *Bacillus* infections only (rating = 4 in both cases). At these higher doses, however, extracts from the *B.subtilis* isolate were inhibitory to chlorophyll formation in the explant cultures and both extracts produced a reduction in the fresh weight of non-contaminated explants after a 4 week incubation period. A combination of the 2 antagonistic extracts employed at their lower dosages (combination A) proved more successful for the reduction of bacterial density in the explant cultures than when they were employed exclusively. This extract combination did not inhibit chlorophyll formation and did not reduce the fresh weight of non-contaminated explant cultures to any great extent relative to untreated control cultures.

The antibiotic rifampicin displayed a wide range of activity and was bacteriostatic to both types of contaminations at $25mgL^{-1}$ and bacteriocidal at $50mgL^{-1}$. At the higher concentration, however, the antibiotic inhibited chlorophyll formation and reduced the fresh weight of non-contaminated explants by 20% relative to untreated controls. A combination of the 3 antimicrobial agents employed at their higher concentrations and dosages (combination B), was bacteriocidal to both types of contaminations. This combination inhibited chlorophyll formation and reduced the fresh weight of non-contaminated explant cultures by 20% relative to controls.

### 7.3 Tobacco stem explant sterilization

Tobacco stem explants were obtained from donor plant material and exposed to a range of potential surface sterilant treatments prior to incubation on appropriate growth media (section 2.1.2). The efficacy of the various agents as sterilants and their effects on the growth of the explant cultures is described (Table 7.3.1). An arbitrary estimate of the extent of contamination was made, where 0 indicated zero contamination and 10 indicated mass overgrowth by various bacterial and fungal contaminations. No specific distributions or frequencies of contaminations were observed.

Of all of the antimicrobial agents and chemotherapeutic agents assessed, only ethanol (70%) and sodium hypochlorite (5%) proved to be totally effective as sterilising agents. Large percentage growth increases were observed in explants exposed to both of these sterilants (211% and 187%, respectively). These two agents are recognized as standard sterilant treatments in plant tissue culture protocols and as such may be considered as standard control treatments against which the efficacy of the other sterilants may be assessed.

The antibiotic rifampicin and the chemotherapeutic agents silver nitrate, hydrogen peroxide and mercuric chloride all produced contamination ratings of between 2 and 3 in the explant cultures when applied at the stated concentrations. However, the percentage growth increases noted for these test explants ranged from only 13% to 78% when treated with these sterilant agents.

All tested dosages of the two antimicrobial agents and the antibiotic Amphotericin B were generally ineffective as sterilising agents. Only the higher doses of the *T.viride* extract and the antibiotic produced any reduction in the extent of contamination (rating =4). All of these agents severely reduced the growth of the explant cultures (less than 13% growth in all cases).

## 7.4 Treatment of donor plants

Witch-hazel stock plant material was treated with antibiotics, antimicrobial extracts and chemotherapeutic agents (as described in section 2.9). Subsequent levels of latent infections in *in vitro* shoot-tip cultures were assessed following explant sterilization and incubation for 4 weeks at 25$^{O}$C (Table 7.4.1).

Only high concentrations of sodium hypochlorite (20%) and rifampicin (25mgL$^{-}$1) and combination treatments containing the antibiotic were able to reduce the frequency of contaminated witch-hazel shoot-tip cultures when they were employed as donor plant pretreatments (90% contamination in all cases). However, in all of these cases severe phytotoxic effects were observed in the explant cultures. Treatment of donor plant material with extracts of the *T.viride* and *B.subtilis* isolates and with the antibiotic Amphotericin B did not reduce the frequency of contaminated cultures relative to untreated controls (i.e. 100% contamination in all cases).

## Discussion

The aim of this study was to identify substances which can be incorporated into plant tissue culture growth media to reduce the dependence on sterilization procedures for eliminating contaminations. Various publications have reported attempts to combat contamination problems in plant tissue cultures using an array of conventional antibiotics (see Venis, 1967; Sood & Pillay, 1972; Watts & King, 1973; Phillips *et al*, 1981). However, this is the first attempt to assess bacterial and fungal culture filtrates with recognized antimicrobial activity (see Herman, 1987; Kope & Fortin, 1989) for their ability to control contaminations in plant culture systems, both exclusively and in combination with antibiotic preparations.

In the present study no single antimicrobial agent was effective against all of the contaminations observed in infected tobacco and witch-hazel cultures. The antibiotic rifampicin ($50mgL^{-1}$) was bacteriocidal but at this concentration it exhibited phytotoxic effects. Studies by Phillips *et al* (1981) have shown rifampicin to be highly effective at suppressing the growth of bacterial contaminants when applied at similar concentrations (by inhibition of RNA synthesis) without such deleterious effects on the growth of herbaceous plant cultures, highlighting the variability of antimicrobial agent phytotoxicity in different plant culture systems. Amphotericin B ($10mgL^{-1}$) was found to be fungistatic, a result which is consistent with those reported by Thurston *et al* (1979) on studies relating to the application of anti-contaminants in orchid culture media. The extent of contamination control exhibited by the antimicrobial extracts, applied exclusively or in combination at non-phytotoxic levels, was not substantial enough to warrant further studies relating to their commercial application as decontaminants in infected systems. There was no evidence of growth stimulation by any of the antimicrobials employed in this study when incorporated into growth media.

With regards to bacterial growth inhibition in short-term tobacco explant cultures, again only rifampicin displayed effective control. The extent of contamination control was proportional to the concentration employed, but at the higher concentration phytotoxcity was observed in the explants. Extracts produced by the microbial antagonists were largely ineffective for bacterial decontamination, though their activity was marginally enhanced when lower, non-phytotoxic doses of extracts were applied in combination. Here the relative ineffectiveness of the antimicrobial extracts is not so unexpected, as the deliberate addition of a large population of actively growing bacteria presents a more severe challenge than is likely to be normally encountered.

When compared to a range of antibiotic treatments and chemotherapeutic agents, the antimicrobial extracts did not compare favourably for the surface sterilization of tobacco explant material. Of the potential sterilants employed only 70% ethanol and 5% sodium hypochlorite were favourable with respect to both efficiency of explant decontamination and absence of growth retardation. The antimicrobial extracts displayed levels of explant growth inhibition much greater than those levels observed when the extracts were incorporated into the growth media of established explants, suggesting greater explant susceptibility to phytotoxic damage during the preliminary stages of *in vitro* culture.

The two antimicrobial extracts were totally ineffective when applied as donor plant treatments for the reduction of latent bacterial infection rates in subsequent witch-hazel shoot-tip cultures. This may have been a result of low extract activity or possibly poor penetration of the donor plant tissues, as the established antibacterial agents (rifampicin and sodium hypochlorite) only produced minor reductions in infection frequencies themselves, suggesting deficiencies in the application technique itself.

This series of experiments has unquestionably illustrated the chasm which must be traversed when attempting to extrapolate from preliminary

observations on *in vitro* growth inhibition by antimicrobial agents in bioassay tests to activity in contaminated culture systems. Therefore it appears that non-phytotoxic doses of the antimicrobial agents employed in this study could not efficiently substitute for good sterile technique but their use should still be considered, together with antibiotics, for the disinfection of hard-to-sterilize tissues or in attempting to rid irreplaceable cultures from contamination.

Table 7.1.1

Comparative activity against accidental contaminations and phytotoxicity of antimicrobial agents to tobacco plant shoots *in vitro*. Shoots were grown on media incorporated with antimicrobials for 4 weeks.

| Antimicrobial Treatment | Concentration | Extent of Contamination [Y] | Phytotoxicity [Z] |
|---|---|---|---|
| **Control** | - | 10 (10) | - |
| *T.viride* | 5.0 %v/v | 9 (9-10) | - |
| | 7.5 %v/v | 8 (8-10) | + |
| *B.subtilis* | 7.5 %v/v | 9 (9-10) | - |
| | 10 %v/v | 9 (9-10) | + + |
| **Rifampicin** | 25 $mgL^{-1}$ | 7 (7-8) | - |
| | 50 $mgL^{-1}$ | 6 (5-6) | + + |
| **Amphotericin B** | 5 $mgL^{-1}$ | 5 (5-6) | - |
| | 10 $mgL^{-1}$ | 4 (4-6) | + |
| **Combination A** | [W] | 8 (8-10) | - |
| **Combination B** | [X] | 4 (3-5) | + + |

[W] *T.viride* 5.0 %v/v + *B.subtilis* 7.5 %v/v

[X] *T.viride* 7.5 %v/v + *B.subtilis* 10 %v/v + Rifampicin 50 $mgL^{-1}$ + 10 $mgL^{-1}$ Amphotericin B

[Y] Arbitrary estimate of contaminant density, where 0 indicates zero contamination and 10 indicates mass overgrowth by contaminations. Figures represent mean values, followed (in parentheses) by variation between experiments.

[Z] Strong phytotoxicity is indicated by + +, mild toxicity by +, and no toxicity by - .

Table 7.1.2

Comparative activity against latent infections, and phytotoxicity of antimicrobial agents to witch-hazel shoot tip cultures *in vitro*. Shoots were grown on media incorporated with antimicrobials for 8 weeks.

| Antimicrobial Treatment | Concentration | Bacterial Density [Y] | Phytotoxicity [Z] |
|---|---|---|---|
| **Control** | - | 10 (10) | - |
| *T.viride* | 5.0 %v/v | 9 (9-10) | - |
| | 7.5 %v/v | 9 (9-10) | + |
| *B.subtilis* | 7.5 %v/v | 9 (9-10) | - |
| | 10 %v/v | 9 (9-10) | + |
| **Rifampicin** | 25 $mgL^{-1}$ | 5 (5-6) | - |
| | 50 $mgL^{-1}$ | 3 (2-5) | + + |
| **Amphotericin B** | 5 $mgL^{-1}$ | 10 (10) | - |
| | 10 $mgL^{-1}$ | 10(10) | + |
| **Combination A** | [W] | 8 (8-10) | - |
| **Combination B** | [X] | 3 (2-5) | + + |

[W] *T.viride* 5.0 %v/v + *B.subtilis* 7.5 %v/v

[X] *T.viride* 7.5 %v/v + *B.subtilis* 10 %v/v + Rifampicin 50 $mgL^{-1}$ + 10 $mgL^{-1}$ Amphotericin B

[Y] Arbitrary estimate of bacterial density, where 0 indicates zero infection and 10 indicates mass overgrowth by contaminations. Figures represent mean values, followed (in parentheses) by variation between experiments.

[Z] Strong phytotoxicity is indicated by + +, mild toxicity by +, and no toxicity by - .

Table 7.2.1

Effects of antimicrobial agents on growth of short-term tobacco stem explants and proliferation of contaminating bacterial inocula.

| Treatment | Concentration | Fresh Weight (g) [Y] | Chlorophyll formation | Bacterial Density [Z] *Bacillus* | *Pseudomonas* |
|---|---|---|---|---|---|
| Control | - | 3.12 (0.17) | + | 5 (5) | 5 (5) |
| *T.viride* | 5.0 %v/v | 3.02 (0.12) | + | 5 (5) | 5 (5) |
| | 7.5 %v/v | 2.76 (0.15) | + | 4 (4-5) | 5 (5) |
| *B.subtilis* | 7.5 %v/v | 3.07 (0.17) | + | 5 (5) | 5 (5) |
| | 10.0 %v/v | 2.65 (0.26) | - | 4 (4-5) | 5 (5) |
| Rifampicin | 25 $mgL^{-1}$ | 3.22 (0.08) | + | 3 (3-4) | 2 (2-4) |
| | 50 $mgL^{-1}$ | 2.54 (0.15) | - | 0 (0) | 0 (0) |
| Combination A | [W] | 3.09 (0.15) | + | 4 (4-5) | 5 (5) |
| Combination B | [X] | 2.51 (0.13) | - | 0 (0) | 0 (0) |

[W] *T.viride* 5.0 %v/v + *B.subtilis* 7.5 %v/v

[X] *T.viride* 7.5 %v/v + *B.subtilis* 10 %v/v + Rifampicin 50 $mgL^{-1}$

[Y] Mean and standard deviation of explant fresh weight after 4 weeks incubation of non-contaminated explants.

[Z] Arbitrary estimate of bacterial density after 48 h incubation in contaminated explants, where 0 indicates a complete absence of contaminating bacteria and 5 indicates mass overgrowth. Figures represent mean values [n=3] followed (in parentheses) by the variation between 3 replicate experiments.

Table 7.3.1

Antimicrobial agents and chemotherapeutic agents for tobacco stem explant sterilization.

| Treatment | Concentration | Exposure Time (mins) | % Growth Increase [Y] | Extent of Contamination [Z] |
|---|---|---|---|---|
| *T.viride* | 5.0 %v/v | 15 | 4 (2-7) | 5 (5) |
| | 7.5 %v/v | 5 | 7 (5-11) | 4 (4-5) |
| *B.subtilis* | 7.5 %v/v | 15 | 3 (0-6) | 5 (5) |
| | 10.0 %v/v | 5 | 2 (0-4) | 5 (5) |
| Rifampicin | 25mgL-1 | 10 | 78 (54-92) | 2 (2-4) |
| Amphotericin B | 5 mgL-1 | 10 | 13 (3-17) | 4 (4-5) |
| Ethanol | 70% | 15 | 211 (171-234) | 0 (0) |
| Sodium hypochlorite | 5% | 15 | 187 (152-196) | 0 (0) |
| Silver nitrate | 1% | 10 | 24 (17-39) | 2 (2-3) |
| Hydrogen peroxide | 5% | 10 | 17 (9-27) | 3 (3-4) |
| Mercuric chloride | 0.5% | 10 | 13 (5-21) | 2 (2-3) |

[Y] Percentage growth increase in fresh weight of explant tissue after 14 d incubation at 25°C. Figures represent mean values [n=3] followed (in parentheses) by the variation between 3 replicate experiments.

[Z] Arbitrary estimate of the extent of contamination, where 0 indicates zero contamination and 5 indicates mass overgrowth by contaminations. Figures represent mean values followed (in parentheses) by variation between experiments.

**Table 7.4.1**

Effect of treatment of witch-hazel donor plants with antimicrobial agents on subsequent levels of contamination in shoot-tip *in vitro* cultures (after 4 weeks incubation at 25°C).

| Antimicrobial Treatment | Concentration | % Contaminated Shoot-tip cultures [W] |
|---|---|---|
| *T.viride* | 5 %v/v | 100 (100) |
| | 20 %v/v | 100 (100) |
| *B.subtilis* | 7.5 %v/v | 100 (100) |
| | 20 %v/v | 100 (100) |
| Rifampicin | 25 $mgL^{-1}$ | 100 (100) |
| | 250 $mgL^{-1}$ | 90 (90-100) [Z] |
| Amphotericin B | 5 $mgL^{-1}$ | 100 (100) |
| | 50 $mgL^{-1}$ | 100 (100) |
| Sodium hypochlorite | 5 %v/v | 100 (100) |
| | 20 %v/v | 90 (90-100) [Z] |
| Combination A | [X] | 90 (90-100) [Z] |
| Combination B | [Y] | 90 (90-100) [Z] |

[W] Figures represent mean values [n=10], followed (in parentheses) by variation between 2 experiments. *Bacillus*, *Pseudomonas*, and *Erwinia* species were present in all cultures, irrespective of treatment.

[X] T.viride 20 %v/v + Rifampicin 250 mgL-1

[Y] B.subtilis 20% v/v + Rifampicin 250 mgL-1

[Z] Shoot-tip cultures exhibiting severe phytotoxic effects.

(Untreated [control] cultures all exhibited 100% contamination following the incubation period).

CHAPTER 8

## CONCLUDING REMARKS

Contamination of *in vitro* plant cultures by microorganisms can be a major problem in micropropagation programmes and can result in extensive culture losses. Considerable effort has been directed towards the disinfestation of explant material and the elimination of systemic contaminants using highly toxic sterilising agents and antibiotics (Thurston *et al*, 1979; Phillips *et al*, 1981; Bastiens *et al*, 1983, Pollock *et al*, 1983; Falkiner, 1990). These attempts at producing axenic cultures (i.e. cultures free of microbial contamination) have often proved unsuccessful and even catastrophic. This is, perhaps, not surprising when it is considered that commercial antibiotics were developed via many thousands of screenings, for the control of human and animal pathogens rather than plant pathogens.

The experiments carried out during the cause of this study have covered a number of subject areas and have entailed the use of a wide range of techniques. They have evaluated the application of antimicrobial culture filtrates produced by antagonistic microorganisms for the control of accidental contaminations and latent infections in plant tissue culture systems as a 'plant-friendly' alternative to potentially harmful conventional antibiotics.

The culture techniques and the methods employed in these studies showed antimicrobial activity to be evident in 13 of the antagonistic microorganisms originally screened for *in vitro* antagonism in bioassay trials. These extracts are able to restrict the growth of a range of test contaminants and latent infections commonly isolated from diseased plant tissue cultures, and alter the morphology of some of the phytopathogens. Those extracts produced by the *T.viride* and *B.subtilis* isolates are generally the most effective, both groups exhibiting low *in vitro* specificity and sustained growth

inhibition of phytopathogens. Those extracts produced by the *A.quisqualis*, *G.roseum* and *E.aerogenes* isolates are also inhibitory to the growth of contaminants, though to a lesser extent than the *T.viride* and *B.subtilis* isolates.

Optimization studies on selected extracts showed that microbial filtrates with the greatest inhibitory activity are produced when antagonists are cultured within an initial media pH range of 5-7, and over a temperature range of 20$^{o}$C - 30$^{o}$C, and 25$^{o}$C - 35$^{o}$C, for the fungal and bacterial isolates respectively. Fermenter studies have demonstrated that the bulk-volume production of inhibitory filtrates by the antagonist *T.viride* is achievable by optimization of pH, temperature, and aeration conditions within the fermentation system. Therefore the production of large quantities of inhibitory filtrates can be facilitated should they be employed on a commercial scale as contamination control agents.

Stability studies have shown the filtrates to be stable up to 70$^{o}$C which therefore favours some form of heat sterilization and also eliminates the requirement for expensive sterile-membrane filter systems for ensuring the asepsis of filtrates. Temporal stability of the filtrates may present a problem should they be employed in a commercial situation as their activity decreases dramatically only a few weeks after production.

Phytotoxicity studies conducted in this investigation have established that plant culture species display different responses to the various microbial culture filtrates studied. Such experiments illustrate the need for broad-range phytotoxicity screens to establish the maximum allowed dosage in a wide variety of plant tissue culture systems if a universally-employed antimicrobial agent is to be derived.

When employed at non-phytotoxic levels, those filtrates produced by the *T.viride* and *B.subtilis* isolates are capable of producing a reduction in the density of opportunist contaminations of herbaceous plant tissue culture systems. Filtrates of the microbial antagonists are only effective contamination control agents when employed at phytotoxic levels and are therefore unsuitable for application as control agents in the plant systems reviewed in this study. Experiments also showed that the *T.viride* and *B.subtilis* isolates can offer a modest degree of prophylactic protection in woody plant tissue cultures, but are ineffective as post-infection sterilants in the same systems.

The ultimate assessment of novel antimicrobial substances as contamination control agents is by comparing their efficacy with that of established agents such as conventional antibiotics. When non-phytotoxic doses of a typical plant tissue culture antibiotic, rifampicin, were directly compared with microbial culture filtrates for their ability to (1) control contamination levels in herbaceous and woody plant cultures, (2) restrict bacterial growth in short-term explant cultures, and (3) disinfect donor plant material, the antibiotic was far more effective in all cases. Therefore, despite the ability of the filtrate preparations to inhibit the growth of test contaminations in bioassay trials, it appears that non-phytotoxic doses of these filtrates cannot efficiently substitute for conventional antibiotics that are normally employed as contamination control agents.

It is probable that further work on the subject of contamination control by microbial culture filtrates in plant tissue culture systems will prove to be most productive in the area of filtrate purification. The presence of antimicrobial activity as been conclusively demonstrated in a number of the antagonistic filtrates studied. However, it is feasible that extract activity may be limited by the presence of non-active components within the extracts themselves. Therefore further purification of the antagonistic filtrates may improve the efficacy of the microbial filtrates for contamination control.

## 6.1 Activity against accidental contaminations

Maximum permissive doses of selected antimicrobial extracts (*T.viride* strain 24039, *B.subtilis* strain SR2, *P.fluorescens* strain 95.6, and *G.roseum*) were incorporated into plant tissue culture media (section 2.1.2) which was subsequently deliberately contaminated by exposure to a non-sterile microbiological laboratory environment. Three replicate experiments were conducted. The effects of the extracts on the proliferation of contaminations in the test media after a 2 week incubation period is shown in Table 6.6.1. Contaminant density was estimated by employing an arbitrary assessment scale, where 0 indicates a complete absence of contaminations and 5 indicates mass overgrowth. All untreated (control) test media exhibited massive overgrowth by contaminations after the incubation period (Table 6.1.1). When incorporated in to media at their maximum allowed dosage, only those extracts produced by the *T.viride* and *B.subtilis* isolates were capable of reducing contaminant density in plant tissue culture media (a reduction from an arbitrary contaminant density rating of 5 to one of 4). When incorporated in to media at doses greater than this maximum allowed dose, media containing extracts produced by the *P.fluorescens* isolate also exhibited reduced contaminant densities (rating = 4). At these higher doses, the extracts from the *T.viride* isolate brought about a further reduction in the density of fungal contaminations on test media (rating = 3). Media containing extracts produced by the *G.roseum* isolate did not inhibit reduced contaminant densities relative to control media, irrespective of the dosage applied.

Effects on the extent of microbial growth in deliberately contaminated tobacco and birch explant shoot cultures of incorporating the maximum allowed doses of the various anti-microbial extracts into growth media are illustrated in Figures 6.1.1 and 6.1.2. The various anti-microbial extracts which were incorporated in to growth media produced a similar response in terms of the extent of accidental contamination (an arbitrary estimate where 0 indicates

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## APPENDIX I

### Range of microorganisms surveyed

### and considered for screening programmes

A Mycoparasitic Antagonists

**i) Internal types**

*Rozella cladochytri, Olpidiopsis incrassata, Chytridium parasiticum, Woronia spp., Pleopidium spp., Pleotrachelus spp., Rhizidiomyces spp.*

**ii) Contact types**

*Gonatorrhodiella highlei, Gonatobotrys simplex, Gonatobotryum fuscum, Calcarisporium parasiticum, Stephanoma phaesospora.*

**iii) Haustorial types**

*Piptocephalis virginiana, P.benjaminii, P.xenophila, Syncephalis nodosa, Dimargaris verticillata, Dispira cornuta, D.simplex, D.parvispora*

B Bacterial Antagonists

*Agrobacterium radiobacter, A.tumefaciens,, Corynebacterium michiganens, C.insidiosum, Erwinia caratovora, E.chrysanthemi, E.herbicola, E.salicis, E.uredovora, Pseudomonas fluorescens, P.aptata, P.lachrymans, P.glycinea, P.syringae, P.phaseolicola, Xanthomonas albilineans, X.betilcola, X.juglandis, X.phaseoli.*

## APPENDIX II

Statistical treatment of data

In all cases where results were replicated, means, standard deviations and standard errors were calculated. Appropriate statistical analyses were applied to the data to determine the level of significance of the results. These analyses took the form of paired data t-tests and one- or two-way analyses of variance. Typical examples of the procedures employed are given in the following pages, together with significance figures for presented data.

1. Paired data t-tests

NULL HYPOTHESIS: There is no significant difference between the mean extent of contamination in untreated tabacco shoot culture media and in similar media treated with extracts of *T. viride*.

DATA

Mean estimate of extent of contamination

| Control | TV | d (TV - CON) |
|---|---|---|
| 10 | 7 | 3 |
| 10 | 9 | 1 |
| 10 | 11 | 1 |
| $\bar{X}_C = 10$ | $\bar{X}_{TV} = 9$ | |

$$\bar{d}_{(TV - CON)} = \bar{X}_{TV} - \bar{X}_{CON} = -1$$

$$S^2 = d - \bar{d}^2 / N - 1 = 2$$

$$T = \frac{\bar{d}}{S^2 / N}$$

Therefore for CON / TV : $t = 2.08$

By tables, $t_{0.1}$, 2 df = 1.88

Therefore the Null Hypothesis is rejected ($p < 0.1$). Therefore there are significant differences between the mean extent of the contamination in control media and in the extract-treated media.

2. <u>One-way analysis of variance</u>

NULL HYPOTHESIS: Culture medium pH causes no significant change in inhibition zone diameter.

<u>DATA</u>

| | Culture medium pH | | | | | | |
|---|---|---|---|---|---|---|---|
| | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| Replicate | | | | | | | |
| 1 | 0 | 3 | 10 | 18 | 22 | 17 | 12 |
| 2 | 0 | 4 | 10 | 16 | 25 | 14 | 13 |
| 3 | 0 | 3 | 11 | 18 | 23 | 16 | 11 |
| | — | — | — | — | — | — | — |
| t = | 0 | 10 | 31 | 54 | 70 | 47 | 36 |

$T = t_1 + t_2 + t_3 \ldots\ldots\ldots t_7 = 248$

$x_2 = 4072 \qquad N = 21$

Correction term, $C = T^2 / N = (248)^2 / 21 = 2929$

Total sum of squares : $TSS = x_2 - C = 1143$

Total degrees of freedom : $Tdf + N - 1 = 20$

pH sum of squares : pHSS = $t^2 / n_t$ - C

= $0^2 / 3$ + $10^2 / 3$ + $31^2 / 3$ + ..... - 2929

= 4127.2 - 2929 = 1198

pH degrees of freedom : pHdf = 7 - 1 = 6

Within groups sum of squares : WGSS = TSS - pHSS

= 1143 - 1198 = -56

Within groups degrees of freedom : WGdf = Tdf - pHdf

= 20 - 6 = 14

pH variance estimate = WGSS / WGdf = 55 / 20 = 2.75

F (pH) ratio:

= pH variance estimate / Within groups variance estimate

= 200 / 2.75 = 72.7

By tables, $F_{.001}$, 6df = 5.9

Therefore the null hypothesis is rejected - culture medium pH does cause significant changes in inhibition zone diameter ($p < 0.001$).

3. Two-way analysis of variance

NULL HYPOTHESIS: There is no interaction between incubation temperature and culture medium pH on inhibition zone diameter.

DATA

| | pH | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| TEMP°C | 2 | 3 | 4 | 5 | 6 | 7 | 8 | Total |
| 15 | 0,0,0 | 0,0,0 | 3,1,2 | 2,2,2 | 3,4,2 | 2,2,2 | 3,5,1 | 36 |
| 20 | 0,0,0 | 1,2,0 | 3,4,5 | 4,4,4 | 7,6,5 | 7,7,4 | 4,3,2 | 72 |
| 25 | 0,0,0 | 3,1,2 | 4,4,4 | 7,4,4 | 9,6,6 | 8,7,6 | 8,4,3 | 90 |
| 30 | 0,0,0 | 2,0,1 | 6,2,1 | 3,4,2 | 6,5,1 | 5,3,1 | 4,2,0 | 48 |
| 35 | 0,0,0 | 0,0,0 | 3,0,0 | 2,1,0 | 4,0,2 | 1,2,0 | 0,0,0 | 15 |
| Total | 0 | 12 | 42 | 45 | 66 | 57 | 39 | |

$N = 105$ $T = 261$ $x^2 = 1215$

Correction term, $C = T^2 / N = 261^2 / 105 = 648.8$

Total sum of squares : $TS = x_2 - C = 566.2$

Total degrees of freedom : $Tdf = N - 1 = 79$

Total variance of estimate : T var. est. $= TSS / Tdf = 7.2$

TEMP sum of squares : $TEMPSS = t^2 / n_t - C$

$= [36^2 / 3 + 72^2 / 3 + 90^2 / 3 + 48^2 / 3 + 15^2 / 3] - 648.8$

$= 5703 - 648.8 = 5054.2$

TEMP degrees of freedom : TEMPdf = 5 - 1 = 4

TEMP variance estimate : TEMP var. est. = TEMPSS / TEMPdf

= 1263.6

pH sum of squares : pHSS + $t^2 / n_t$ - C

= $[0^2 / 3 + 12^2 / 42^2 / 3 + .....]$. - 648.8

= 4353 - 648.8 = 3704.2

pH degrees of freedom : pHdf = 7 - 1 = 6

pH variance estimate : pH var. est. = 617.3

pH x TEMP interaction effect:

Totals

Totals

| | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|
| 15 | 0 | 0 | 6 | 6 | 9 | 6 | 9 |
| 20 | 0 | 3 | 12 | 12 | 18 | 18 | 9 |
| 25 | 0 | 6 | 12 | 15 | 21 | 21 | 15 |
| 30 | 0 | 3 | 9 | 9 | 12 | 9 | 6 |
| 35 | 0 | 0 | 3 | 3 | 6 | 3 | 0 |

pH x TEMP = $t^2 / n_t$ - (pHSS + TEMPSS + C)

= 1101 - 9407.2 = -8306.2

pH x TEMPdf = 6 x 4 = 24

pH x TEMP var. est. = 346.1

Within cells sum of squares : WCSS = TSS - all other SS

= 566.2 - [5054.2 + 3704.2 + -8306.2]

= 114

Within cells df : WCdf = Tdf - all other df

= 79 - [6 + 4 + 24]

= 45

Within cells variance estimate = 114 / 45 = 2.53

F values :

For temperature : F = 1263.6 / 2,53 = 499.4 4, 45 df

For pH : F = 617.3 / 2.53 = 243.9 6, 45 df

For TEMP / pH interaction : F = 346.1 / 2.53

= 136.8 24, 45 df

By tables, for 4, 45 df F .001 = 5.7

for 6, 45 df F .001 = 4.7

for 24, 45 df F .001 = 3.0

Therefore, the null hypothesis is rejected - there are significant changes induced by both pH ($p < 0.001$) and temperature ($p < 0.001$). There is a significant interaction effect ($p < 0.001$).

4. Significance figures for presented data

Key

TV - *T. viride*; BS - *B. subtilis*; PF - *P. fluorescens*; A0 - *A. quisqualis*; GR - *G. roseum*; EA - *E. aerogenes*; TV1 - *T. viride* strain 24039; TV2 - *T. viride* strain 153416; TV3 - *T. viride* strain 170657; TV4 - *T. viride* strain 45553ii; SR1 - *B. subtilis* strain SR1; SR2 - *B. subtilis* strain SR2; 95.6 - *P. fluorescens* strain 95.6; 106.6 - *P. fluorescens* strain - 106.6; Temp - Temperature; pH - pH; Z - Inhibition zone; B - % Biomass; CON - Control;

| Figure | Test Applied | Significance |
|---|---|---|
| 4.1.1 a | 2-way $ANOVAR_{TEMP}$ | $p < 0.01$ |
| | 2-way $ANOVAR_{pH}$ | $p < 0.001$ |
| | 2-way $ANOVAR^{INT}$ | $p < 0.01$ |
| 4.1.1 b | 2-way $ANOVAR_{TEMP}$ | $p < 0.01$ |
| | 2-way $ANOVAR_{pH}$ | $p < 0.001$ |
| | 2-way $ANOVAR_{INT}$ | $p < 0.01$ |
| 4.1.2 a | 2-way $ANOVAR_{TEMP}$ | $p < 0.1$ |
| | 2-way $ANOVAR_{pH}$ | $p < 0.001$ |
| | 2-way $ANOVAR_{INT}$ | $p < 0.01$ |
| 4.1.2 b | 2-way $ANOVAR_{TEMP}$ | $p < 0.001$ |
| | 2-way $ANOVAR_{pH}$ | $p < 0.001$ |
| | 2-way $ANOVAR_{INT}$ | $p < 0.001$ |
| 4.1.3 a | 2-way $ANOVAR_{TEMP}$ | $p < 0.001$ |
| | 2-way $ANOVAR_{pH}$ | $p < 0.001$ |
| | 2-way $ANOVAR_{INT}$ | $p < 0.01$ |

| | | |
|---|---|---|
| 4.1.3 b | 2-way $ANOVAR_{TEMP}$ | $p < 0.001$ |
| | 2-way $ANOVAR_{pH}$ | $p < 0.01$ |
| | 2-way $ANOVAR_{INT}$ | $p < 0.01$ |
| 4.1.4 a | 1-way $ANOVAR_{SR1}$ | $p < 0.001$ |
| | 1-way $ANOVAR_{SR2}$ | $p < 0.001$ |
| | 1-way $ANOVAR_{95.6}$ | $p < 0.001$ |
| | 1-way $ANOVAR_{106.6}$ | $p < 0.001$ |
| | 1-way $ANOVAR_{EA}$ | $p < 0.01$ |
| 4.1.4 b | 1-way $ANOVAR_{TV1}$ | $p < 0.001$ |
| | 1-way $ANOVAR_{TV2}$ | $p < 0.001$ |
| | 1-way $ANOVAR_{TV3}$ | $p < 0.001$ |
| | 1-way $ANOVAR_{TV4}$ | $p < 0.001$ |
| | 1-way $ANOVAR_{AQ}$ | $p < 0.1$ |
| | 1-way $ANOVAR_{GR}$ | $p < 0.01$ |
| 4.2.3 | 1-way $ANOVAR_{C}$ | $p < 0.001$ |
| | 1-way $ANOVAR_{B}$ | $p < 0.001$ |
| 4.3.1 | 1-way $ANOVAR_{TV}$ | $p < 0.01$ |
| | 1-way $ANOVAR_{BS}$ | $p < 0.01$ |
| | 1-way $ANOVAR_{PF}$ | $p < 0.001$ |
| | 1-way $ANOVAR_{AQ}$ | $p < 0.01$ |
| | 1-way $ANOVAR_{GR}$ | $p < 0.05$ |
| | 1-way $ANOVAR_{EA}$ | $p < 0.05$ |

| | | |
|---|---|---|
| 4.3.2 a | 1-way $ANOVAR_{TV}$ | $p < 0.001$ |
| | 1-way $ANOVAR_{BS}$ | $p < 0.001$ |
| | 1-way $ANOVAR_{PF}$ | $p < 0.001$ |
| | 1-way $ANOVAR_{AQ}$ | $p < 0.001$ |
| | 1-way $ANOVAR_{GR}$ | $p < 0.001$ |
| | 1-way $ANOVAR_{EA}$ | $p < 0.001$ |
| 4.3.2 b | 1-way $ANOVAR_{TV}$ | $p < 0.001$ |
| | 1-way $ANOVAR_{BS}$ | $p < 0.001$ |
| | 1-way $ANOVAR_{PF}$ | $p < 0.001$ |
| | 1-way $ANOVAR_{AQ}$ | $p < 0.001$ |
| | 1-way $ANOVAR_{GR}$ | $p < 0.001$ |
| | 1-way $ANOVAR_{EA}$ | $p < 0.001$ |
| 4.3.3 a | 1-way $ANOVAR_{TV}$ | $p < 0.001$ |
| | 1-way $ANOVAR_{BS}$ | $p < 0.001$ |
| | 1-way $ANOVAR_{PF}$ | $p < 0.001$ |
| | 1-way $ANOVAR_{AQ}$ | $p < 0.001$ |
| | 1-way $ANOVAR_{GR}$ | $p < 0.001$ |
| | 1-way $ANOVAR_{EA}$ | $p < 0.001$ |
| 4.3.3 b | 1-way $ANOVAR_{TV}$ | $p < 0.001$ |
| | 1-way $ANOVAR_{BS}$ | $p < 0.001$ |
| | 1-way $ANOVAR_{PF}$ | $p < 0.001$ |
| | 1-way $ANOVAR_{AQ}$ | $p < 0.001$ |
| | 1-way $ANOVAR_{GR}$ | $p < 0.001$ |
| | 1-way $ANOVAR_{EA}$ | $p < 0.001$ |

| | | |
|---|---|---|
| 5.2.1 a | 1-way $ANOVAR_{TV}$ | $p > 0.01$ |
| | 1-way $ANOVAR_{BS}$ | $p < 0.001$ |
| | 1-way $ANOVAR_{PF}$ | $p > 0.05$ |
| | 1-way $ANOVAR_{GR}$ | $p < 0.01$ |
| 5.2.1 b | 1-way $ANOVAR_{TV}$ | $p > 0.01$ |
| | 1-way $ANOVAR_{BS}$ | $p < 0.01$ |
| | 1-way $ANOVAR_{PF}$ | $p > 0.05$ |
| | 1-way $ANOVAR_{GR}$ | $p < 0.01$ |
| 5.2.2 a | 1-way $ANOVAR_{TV}$ | $p > 0.01$ |
| | 1-way $ANOVAR_{BS}$ | $p < 0.01$ |
| | 1-way $ANOVAR_{PF}$ | $p > 0.05$ |
| | 1-way $ANOVAR_{GR}$ | $p < 0.001$ |
| 5.2.2 b | 1-way $ANOVAR_{TV}$ | $p > 0.01$ |
| | 1-way $ANOVAR_{BS}$ | $p < 0.01$ |
| | 1-way $ANOVAR_{PF}$ | $p > 0.05$ |
| | 1-way $ANOVAR_{GR}$ | $p < 0.001$ |
| 5.2.3 a | 1-way $ANOVAR_{TV}$ | $p > 0.01$ |
| | 1-way $ANOVAR_{BS}$ | $p < 0.001$ |
| | 1-way $ANOVAR_{PF}$ | $p > 0.01$ |
| | 1-way $ANOVAR_{GR}$ | $p < 0.001$ |
| 5.2.3 b | 1-way $ANOVAR_{TV}$ | $p > 0.01$ |
| | 1-way $ANOVAR_{BS}$ | $p < 0.001$ |
| | 1-way $ANOVAR_{Pf}$ | $p > 0.01$ |
| | 1-way $ANOVAR_{GR}$ | $p < 0.001$ |

| | | |
|---|---|---|
| 5.2.4 a | 1-way ANOVAR$_{TV}$ | $p > 0.05$ |
| | 1-way ANOVAR$_{BS}$ | $p < 0.01$ |
| | 1-way ANOVAR$_{PF}$ | $p > 0.05$ |
| | 1-way ANOVAR$_{GR}$ | $p < 0.001$ |
| 5.2.4 b | 1-way ANOVAR$_{TV}$ | $p > 0.01$ |
| | 1-way ANOVAR$_{BS}$ | $p < 0.01$ |
| | 1-way ANOVAR$_{PF}$ | $p > 0.05$ |
| | 1-way ANOVAR$_{GR}$ | $p < 0.001$ |
| 5.2.5 a | 1-way ANOVAR$_{TV}$ | $p < 0.001$ |
| | 1-way ANOVAR$_{BS}$ | $p < 0.05$ |
| | 1-way ANOVAR$_{PF}$ | $p > 0.05$ |
| | 1-way ANOVAR$_{GR}$ | $p < 0.001$ |
| 5.2.5 b | 1-way ANOVAR$_{TV}$ | $p < 0.01$ |
| | 1-way ANOVAR$_{BS}$ | $p < 0.01$ |
| | 1-way ANOVAR$_{PF}$ | $p > 0.01$ |
| | 1-way ANOVAR$_{GR}$ | $p < 0.001$ |
| 6.1.1 | Paired data t-test $_{CON/TV}$ | $p < 0.1$ |
| | Paired data t-test $_{CON/BS}$ | $p < 0.01$ |
| | Paired data t-test $_{CON/PF}$ | $p < 0.05$ |
| | Paired data t-test $_{CON/GR}$ | $p > 0.05$ |
| 6.1.2 | Paired data t-test $_{CON/TV}$ | $p < 0.05$ |
| | Paired data t-test $_{CON/BS}$ | $p < 0.05$ |
| | Paired data t-test $_{CON/PF}$ | $p > 0.05$ |
| | Paired data t-test $_{CON/GR}$ | $p > 0.05$ |

| | | |
|---|---|---|
| 6.2.1 a | Paired data t-test $_{CON/TV}$ | $p < 0.01$ |
| | Paired data t-test $_{CON/BS}$ | $p < 0.01$ |
| | Paired data t-test $_{CON/PF}$ | $p > 0.05$ |
| | Paired data t-test $_{CON/GR}$ | $p > 0.05$ |
| 6.2.1 b | Paired data t-test $_{CON/TV}$ | $p < 0.05$ |
| | Paired data t-test $_{CON/BS}$ | $p < 0.01$ |
| | Paired data t-test $_{CON/PF}$ | $p < 0.05$ |
| | Paired data t-test $_{CON/GR}$ | $p > 0.05$ |
| 6.2.2 a | Paired data t-test $_{CON/TV}$ | $p < 0.05$ |
| | Paired data t-test $_{CON/BS}$ | $p < 0.05$ |
| | Paired data t-test $_{CON/PF}$ | $p > 0.05$ |
| | Paired data t-test $_{CON/GR}$ | $p > 0.05$ |
| 6.2.2 b | Paired data t-test $_{CON/TV}$ | $p > 0.05$ |
| | Paired data t-test $_{CON/BS}$ | $p < 0.01$ |
| | Paired data t-test $_{CON/PF}$ | $p > 0.05$ |
| | Paired data t-test $_{CON/GR}$ | $p > 0.05$ |
| 6.3.1 | Paired data t-test $_{A/B}$ * | $p < 0.01$ |
| | Paired data t-test $_{A/C}$ * | $p < 0.05$ |
| | Paired data t-test $_{A/D}$ * | $p > 0.05$ |
| | Paired data t-test $_{A/E}$ * | $p < 0.01$ |
| 6.3.2 | Paired data t-test $_{A/B}$ * | $p < 0.01$ |
| | Paired data t-test $_{A/C}$ * | $p < 0.05$ |
| | Paired data t-test $_{A/D}$ * | $p < 0.05$ |
| | Paired data t-test $_{A/E}$ * | $p < 0.01$ |

* Refer to the relevant figure for key.