TOWARDS DEVELOPING EFFECTIVE DECONTAMINATION PROCEDURES FOR *IN VITRO* CULTURE OF EMBRYONIC AXES EXCISED FROM RECALCITRANT SEEDS

By

Jency Cherian

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PREFACE

The experimental work described in this Master's thesis was carried out in the School of Biological and Conservation Sciences, University of KwaZulu-Natal, Durban, from May 2009 to September 2010, under the supervision of Professor Patricia Berjak and Professor Norman Pammenter.

This study represents original work by the author and has not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others, it is duly acknowledged in the text.

Jency Cherian
December 2013

DECLARATION 1 – PLAGIARISM

I, ...……Jency Cherian…….……., declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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Signed

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ABSTRACT

Control of seed-associated micro-organisms is vital in reducing losses of plants of economic importance. Recalcitrant seeds being metabolically active and able to be stored only under conditions of high relative humidity makes it more difficult to control contaminants. Nevertheless, means need to be developed to eliminate, or at least curtail, seed-associated fungi and bacteria. The use of biological control is a highly recommended alternative to chemical control for reducing the risk of killing beneficial organisms, as well as in terms of health and environmental hazards. Furthermore, when working with seed-derived tissues, it is extremely important to optimise a method or methods to control contamination without compromising the viability or further development of the explants.

The original aim of the present study was to determine whether the biocontrol agents, EcoT® and Eco77® (commercial products of the spores of *Trichoderma harzianum*) would effectively control/eliminate micro-organisms from the embryonic axes of *Trichilia dregeana*, while promoting growth under *in vitro* conditions. Other means were also tested for their efficacy in controlling contaminants; these were application of Benlate®, Nipastat® (a mixture of parabens), anodic water (the anodic fraction of an electrolysed dilute solution of calcium and magnesium chloride), sodium dichloro-isocyanurate (Medi-Chlor®[NaDCC]) and alginate gel encapsulation of the embryonic axes.

Prior to the experiments, fungal contaminants from the embryonic axes were isolated on potato dextrose medium and identified using light microscopy. EcoT and Eco77 were initially individually tested by co-culture as conidial suspensions with the embryonic axes. A further approach used liquid culture (potato dextrose broth) as well as solid culture medium (based on sugarcane bagasse) in/on which the strains of *Trichoderma harzianum* had been grown. This was aimed at testing for the possible presence of compounds released by *T. harzianum* into the media, which might prove to be effective in curtailing/eliminating the axis-associated microflora. Among the different treatments tested, the best method was utilised to decontaminate the embryonic axes prior to minimal-growth storage (hydrated axes encapsulated in alginate gel 'beads').

Penicillium spp. were predominant among the different fungi isolated, which included *Fusarium* spp., *Rhizopus* spp., *Aspergillus niger and Aspergillus flavus*. Co-culturing with *T. harzianum* for 24 h was successful in terms of the survival of the embryonic axes, although the roots produced were shorter than when axes were cultured alone, but had no effect in eliminating the contaminants. Longer periods of co-culturing with *T. harzianum* affected the germination of the embryonic axes of *T. dregeana* compared with axes germinated in the absence of the biocontrol agent (control). The culture filtrate negatively affected germination of the *T. dregeana* embryonic axes, although it was effective against

the associated contaminants. Nipastat was effective in reducing the contamination, and, depending on the concentration, did not affect germination adversely. Medi-Chlor was highly effective in eliminating all the contaminants from axes *in vitro*. Both these treatments were therefore used to decontaminate axes before minimal-growth storage. All the NaDCC-treated, encapsulated axes examined after 14 d hydrated storage [in Magenta boxes] and after 14-42 d in polythene bags survived; however the axes stored in aluminium foil-lined bags and Eppendorf tubes soon lost viability.

The recommendation is therefore made that the decontamination treatment based on use of NaDCC (or other preparations of sodium dichloro-isocyanurate) be tested on embryonic axes of a range of recalcitrant-seeded species, and, if successful, the procedure be introduced into cryopreservation protocols. The use of NaDCC has emerged as a promising method of eliminating contaminating microflora which otherwise compromise *in vitro* procedures, from seed-derived explants. Furthermore, containment of decontaminated encapsulated axes in sealed polythene bags offers an apparently ideal means of temporary storage and dissemination. The results should find considerable applicability when excised embryonic axes representing the germplasm of recalcitrant seeds, are cryo-conserved.

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Units of Measurement

%	Percent
°C	Degree Celcius
cfu	colony forming unit
conc.	Concentration
d	day(s)
g	gram(s)
g g^{-1}	g H_2O per g
g g^{-1}dmb	g H_2O per g of dry matter, dry mass basis
g kg^{-1}	gram per kilogram
h	hour(s)
L	litre(s)
min	minute(s)
mL	milliliter(s)
mm	millimeter(s)
µL	microliter
µm	micron meter
pH	power of Hydrogen
M	Molarity
µM	micromolar
mV	millivolts
mg L	milligrams per litre
PIRG	Percent Inhibition of Radial Growth

ppm	parts pmillion
RH	Relative humidity
v/v	volume per volume
w/v	weight per volume

Abbreviation and Symbols

ABC	ATP-binding cassette
ANOVA	analyses of variance
ATSDR	Agency for Toxic substances and Disease Registry
BASF	Baden Aniline and Soda Factory
BCA	Biocontrol Agent
c.	circa
$Ca(OCl)_2$	Calcium hypochlorite
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
$CaCl_2.2H_2O$	calcium chloride dihydrate
CMC	Carboxymethylcellulose
$CuSO_4.\ 5H_2O$	Copper sulphate pentahydrate
DMSO	dimethyl sulphoxide
dmb	dry mass basis
DW	dry weight
Eco77	*Trichoderma harzianum* strain B77
EcoT	*Trichoderma harzianum* strain Kd
EFSA	Europian Food Safety Authority
EO	Electrolysed oxidising
EPA	Environmental Protection Agency
$FeCl_2$	Ferrous chloride
fmb	fresh mass basis
FW	fresh weight
GA_3	Gibberellic acid
HCl	Hydrogen chloride
$HgCl_2$	Mercuric chloride
HOCl	Hypochlorite
IAA	Indole-3-acetic acid
IAAld	Indole-3-acetaldehyde
IEt	Indole-3-ethanol
ISR	induced systemic resistance

JA	Jasmonic acid
KCl	Potassium chloride
KH_2PO_4,	Ptasium di-hydrogen phosphate
KNO_3	Ptassium nitrate
LSF	liquid state fermentation
MBC	methyl 2-benzimidazole carbamate
$MgCl_2.6H_2O$	Magnesium chloride hexahydrate
$MgSO_4$, $7H_2O$	Magnesium sulphate heptahydrate
$MnSO_4$	Manganese sulphate
MS	Murashige Skoog
n	Number
Na^+	Sodium ion
Na_2SO_4	Sodium sulphate
NADCC	sodium dichloro-isocyanurate
NaOCl	sodium hypochlorite
NaOH	Sodium Hypochloride
NCP	New Chemicals Program
ORP	oxidation-reduction potential
PCNB	Pentachloronitrobenzene
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
pers.comm	personal communication
PI%	percentage of growth inhibition
PIRG	Percentage Inhibition of Radial Growth
PR	pathogenesis-related proteins
®	Registered
R	Radial growth
RPD No	Report Plant Disease
SA	salicyclic acid
SABS	South African Bureau of Standards
SANS	South African National Standards
SAR	systemic acquired resistance
SASRI	South African Sugar Research Institute
SCB	Sugar cane bagasse

SDB	sorghum dextrose broth
spp	species
SSF	solid state fermentation
T	*Trichoderma*
T22 T-22,DRHCI,T77	*Trichoderma* strain
TDB	tapioca dextrose broth
UV	Ultraviolet
WP	wettable powder
WSP	water soluble film
$ZnSO_4$, $7H_2O$	Zinc sulphate heptahydrate
α-L-	Alpha Lyase
β	beta
β-D-	beta deoxy

CHAPTER 1

INTRODUCTION

1.1 Significance of seed infection and its control

Harmful organisms such as fungi, bacteria, viruses, nematodes, etc. cause plant diseases, which can result in losses of crops of economic importance worldwide (Benitez *et al*., 2004), and fungal infection in particular, causes significant problems during fruit and seed storage (Chet *et al.,* 1997). Health and vigour of seedlings and their further growth are dependent on the quality of seeds (Vozzo, 2002) making it important to control seed-associated contaminants in order to sustain the quality and abundance of food, feed, and fibre produced by growers around the world (Chet *et al.,* 1997).

1.2 Origin of seed infection

Fungal propagules can gain access to the ovary, fruits and seeds at any time from flowering via the stigma and style (Marsh and Payne, 1984) to the post-shedding phase (Dhingra *et al*., 2003). Also, infection can originate from the parent plant itself by systemic transmission (Mycock and Berjak, 1992; Kabeere *et al*., 1997), through wounds, and/or when seeds of high moisture content are stored at particular temperatures (Singh and Mathur, 1993). It has been reported that more than 50 seed-borne contaminants were isolated from seeds of different forest trees (Anderson, 1986). Maintenance of seed production areas, seed stands and seed orchards are implemented in agriculture and horticulture to control seed infection (Bhutta and Ahmad, 1991).

The intensity or severity of infection as well as the site of infection of the seeds are important factors that determine the transmission of the infection to the seedling, which is also influenced by environmental factors during germination (Rennie and Cockerell, 2006). If seed germination is rapid under ideal environmental conditions, there is less chance of contaminants affecting seedlings, even when fungal spores are plentiful (Hewett, 1983). However, if the germination is slow, particularly under poor environmental conditions, then a significant loss of yield can take place (Hewett, 1983; Richards 1990). On the other hand, if the contaminant is already present in the embryonic axes, even environmental factors favouring rapid germination will have little effect

in curtailing disease transmission (Rennie and Cockerell, 2006). As examples, embryonic axes of barley seeds infected with *Ustilago segetum* were found to give rise to a population of seedlings with symptoms of loose smut (Rennie and Cockerell, 2006), and systemic transmission of *Aspergillus flavus* var. *columnaris* from infected maize seeds to those of the next generation has been recorded (Mycock and Berjak, 1992). These examples illustrate why it is important to prevent infections at an earlier stage by treatments which do not adversely affect the embryos/embryonic axes of seeds.

1.3 Seed categorization, seed storage and problems with stored seeds

Conservation of germplasm in the form of seeds, tubers, roots, bulbs, corms, rhizomes, buds, cuttings, etc. is a major challenge (Razdan, 2003) and, for recalcitrant-seeded species, is even more difficult (Berjak, 2006). One of the major problems in germplasm storage is posed by micro-organisms associated with seeds and other stock material.

Depending on their storage behavior, Roberts (1973) categorised seeds that dry naturally, and can be further dehydrated to ~5% water content (wet mass basis [wmb] = 0.05 g g^{-1} dry mass basis [dmb]), and can be stored at low temperatures as '*orthodox*'. Seeds which do not naturally dry, and may be sensitive to low temperatures were termed '*recalcitrant'* by Roberts (1973), while Ellis *et al.* (1990) introduced the term, *'intermediate'* for seeds which can tolerate considerable dehydration, but not as much as can orthodox types, and may be chilling-sensitive in the dry state (Hong and Ellis, 1996). Recalcitrant seeds usually have high water contents at maturity and are always sensitive to desiccation. The post-shedding water contents of recalcitrant seeds of a variety of species have been found to be between 0.4 and 4.0 g H_2O g^{-1} (~30 to 80% wmb) (Berjak and Pammenter, 2002). Desiccation-sensitive seeds have further been classified as highly, moderately and minimally recalcitrant (Berjak and Pammenter, 2004). Highly recalcitrant seeds cannot be stored because they are extremely sensitive to desiccation, withstanding only minimal water loss (Farrant *et al.*, 1987), and also because they germinate at the original water content immediately, or very soon, after shedding, e.g. *Avicennia marina* (Farrant *et al.*, 1992). Minimally recalcitrant seeds were considered to be those which are tolerant to more water loss before viability is adversely affected, show a delay in the start of germination, and can be stored for longer periods, e.g. *Podocarpus henkelii* (Farrant *et al*., 1988) and *Quercus* spp. (Finch-Savage and Blake, 1994). Finally, moderately recalcitrant seeds were considered to be those tolerating more water loss than highly recalcitrant types, but not as much as those categorised as

minimally recalcitrant. Seeds so-categorised, e.g. *Trichilia dregeana*, germinate sooner after shedding than minimally recalcitrant types (Kioko, 2003).

There is now, however, agreement that seed behaviour constitutes a continuum, from the most desiccation-tolerant of orthodox types, through the spectrum of those categorised as intermediate, to those classed as highly recalcitrant (Berjak and Pammenter, 1997; 2004; Pammenter and Berjak, 1999; Sun, 1999; Kermode and Finch-Savage, 2002). Categorisation on a species basis is, however, made even more difficult, as provenance (the latitude of the region of seed production) has been reported as having a marked effect on the degree of recalcitrance (Daws *et al.*, 2004; 2006).

Because they are desiccation-sensitive and often also sensitive to chilling, recalcitrant seeds require being stored at the water content at which they were shed, and at moderate (non-chilling) temperatures; both these factors are favourable for fungal proliferation on, and in, the seeds (Schmidt, 2000; Sutherland *et al.*, 2002; Berjak and Pammenter, 2008). Notwithstanding the problems posed by fungi, germination will be initiated without provision of any additional water, and recalcitrant seeds can be stored only in the short- to medium-term (Farrant *et al*., 1988; Berjak *et al*., 1989; Drew *et al.,* 2000; Eggers *et al.,* 2007). The dry state of orthodox seeds, on the other hand, facilitates long-term storage at low relative humidity (RH) and temperatures, thereby lowering, or imposing a stasis on, the activity of associated fungi (Roberts, 1973; Farrant *et al*., 1989; Bewley and Black, 1994; Berjak and Pammenter, 2001). In this regard, is the spectrum of xerotolerant fungi (the so-called storage fungi), which is associated with orthodox seeds, especially those produced in the tropics and sub-tropics (Christensen and Kaufmann, 1974; McLean and Berjak, 1987). Fungal species so-categorised are metabolically active on, and within, the tissues of orthodox seeds at water contents >0.1 g g^{-1} (Mycock and Berjak, 1990). Orthodox seed water content comes into equilibrium with ambient RH, thus those harvested in humid areas and not dried down to <0.1 g g^{-1} will be increasingly at risk of fungal proliferation in storage as the water content increases (Harrington, 1963).

It is extremely rare that recalcitrant seeds of tropical or subtropical provenance are free of fungi (noting that these are the non-xerotolerant, so-called field fungal species) and the mycoflora proliferates in, and on, seeds under the high relative humidity and relatively warm storage conditions that must be used (Sutherland *et al.*, 2002; Berjak, 2006). This is the case for recalcitrant seeds across species, and all are at major risk of early embryo degeneration in storage

(Reed and Chang, 1997). Recalcitrant seeds generally harbour a range of fungal and bacterial propagules even when they have been newly harvested, although there may be no visible proliferation of the micro-organisms in fresh seeds or during early stages of hydrated storage. It is only during longer-term storage that fungal proliferation becomes apparent (Mycock and Berjak, 1990; Calistru *et al*., 2000) adversely affecting the vigour and viability of stored recalcitrant seeds. Although defence mechanisms have been shown to be present in recalcitrant seeds (Calistru, 2004), they are unable to counteract fungal proliferation during storage. While fungal proliferation may initially be superficial, the embryonic axis, which may constitute only a small proportion of the mass and volume of the seed, is ultimately adversely affected (Calistru *et al.*, 2000). Thus fungal inoculum located on the seed surfaces must be eliminated, and precautions to prevent fungi becoming established within the seeds can be minimised by collecting the seeds from the parent plant prior to shedding. As soon as seeds are received, they must be surface decontaminated and stored in pre-sterilised containers (Sutherland *et al*., 2002).

1.4 Methods of storing recalcitrant germplasm

Different methods of storing recalcitrant germplasm are applicable in the context of the required duration of the storage period. For long-term storage of the genetic resources of recalcitrant-seeded species, there is presently no alternative to cryopreservation, generally in liquid nitrogen (Chin and Roberts, 1980; Dumet *et al.*, 1997; Berjak and Pammenter, 1997; 2001; Kioko, 2003; Engelmann, 2011). For short- to medium-term storage, wet/hydrated storage of the seeds or slow growth of seedlings are common practices. Wet storage, more commonly called hydrated storage, is done by storing the surface-decontaminated, fungicide-coated seeds in a saturated atmosphere in closed containers at the lowest temperature that they will tolerate (Berjak *et al.,* 1989; Drew *et al*., 2000). Alternatively the seeds may be stored in heavy polythene bags or even buried in an inert moist medium such as sawdust (Kioko *et al*., 1993). Good quality *T. dregeana* seeds have been successfully stored under hydrated conditions after aril removal and decontamination with little fungal proliferation or viability loss for 91 d (13 weeks); however, fungal contamination was observed on low quality seeds which deteriorated within 28 d under the same storage conditions (Berjak *et al.*, 2004). Attempts have been made to store recalcitrant seeds after partial drying, which have been termed, sub-imbibed storage (King and Roberts, 1980; Pritchard, 1995), and slow growth has been used to provide planting stock for the next season (Tsan *et al.*, 1997; Krishnapillay *et al.*, 1998). Sub-imbibed storage was ineffective in increasing seed life span and quality or even to prevent fungal proliferation: in fact, there was increased fungal proliferation

on/in seeds of *T. dregeana* (Drew *et al*., 2000; Eggers *et al*., 2007) and more rapid viability loss of seeds of *T. dregeana*, *T. emetica*, *Syzygium cordatum* and the gymnosperm, *Podocarpus henkelii*, when stored after an only insignificant proportion of water was removed (Eggers *et al*., 2007).

1.5 Fungal contamination of recalcitrant seeds

Different organisms, including bacteria, fungi, viruses and nematodes, can cause seed infections, but fungal contamination is the most prevalent of these among the recalcitrant seeds of species of tropical, sub-tropical and temperate Southern African origin (Mycock and Berjak, 1990; 1992; Calistru *et al*., 2000; Berjak *et al*., 2004). Although not in the context of recalcitrant-seeded species, Gullino *et al.* (2000) state that fungal contamination can lead to 20% loss of yield in crops worldwide leading to an appreciation of the fact that is vital to control the contamination.

Among the different species of field fungi, *Fusarium* spp. constituted the major contaminant isolated from surfaces or within the tissues of a range of recalcitrant seeds such as *Avicennia marina*, *Castanospermum australe*, *Litchi chinensis*, *Podocarpus henkelii*, *Landolpia kirkii*, *Scadoxus membranaceus* and *Camellia sinensis*, but for *Landolphiakirkii*, species of *Alternaria*, *Cladosporium*, *Aspergillus* and *Penicillium,* in addition to *Fusarium*, were isolated (Mycock and Berjak, 1990). The damage caused to wet-stored *Avicennia marina* seed by *Fusarium* spp. was detailed by Calistru *et al*. (2000), who demonstrated that seed viability could be considerably extended when fungistatic or fungicidal chemicals were periodically applied.

1.6 Control of seed-associated micro-organisms

Various methods are used to suppress, moderate or control plant contaminants, including chemical and biological control. While fungicides, bactericides and nematocides act directly, virus control is via methods that target the vectors, which include insects (Ogawa and English, 1991).

1.6.1 Chemical control and its hazards

Chemical control has been widely used in agriculture because it is highly effective, relatively cheap and easy to use (Xia *et al*., 2006). However, misuse of the chemicals has not only encouraged the development of contaminants resistant to chemicals (Tjamos *et al*., 1992) but also

permanently left toxic substances in plant products and the environment (Petit *et al.*, 2008; EFSA Scientific Report, 2009).

Fungicides are chemical compounds that kill or inhibit fungal contaminants (Gullino *et al*., 2000) especially seed-associated fungal contaminants (Crosier and Patrick, 1946; Siddiqui and Zaman, 2004). Fungicides can be toxic not only to the targeted fungus, but also to mammals (Belpoggi *et al.*, 2002) including humans (Mendes *et al*., 2005). They can also affect plants by altering their metabolic and physiological activities (Garcia *et al*., 2003) including pigment content, photosynthesis, growth and reproduction (Saladin *et al*., 2003). Farmers often rely on chemical fertilizers and pesticides (Pal and Gardener, 2006), which have added significantly to improvements in crop productivity and quality over the past 100 years. However, the environmental pollution caused by abuse of agrochemicals, as well as the rumours spread by some opponents of the application of chemicals, has changed peoples' outlook, particularly towards the use of pesticides in agriculture (Pal and Gardener, 2006). Chemical control must be used judiciously; therefore it is important to follow certain guidelines before using fungicides, such as identifying type of contaminant, site of infection, suitable time for application, maturity of the host, and finally, environmental conditions (Ogawa and Manji, 1984).

An ideal fungicide for seed treatment should be harmless to the seeds/plants and the environment, inexpensive, stable for long periods especially during seed storage, easy to use, should adhere to the seeds well on coating and should be a licensed or registered product (Copeland and McDonald, 2001). Although there are different groups of chemicals for the control of animal pests as well as micro-organisms, fungicides are the most relevant in terms of seed storage (Ogawa and English, 1991).

1.6.1.1 Fungicides

In agriculture, fungicides are used on seeds for three reasons (McMullen and Lamey, 2000): 1. Against soil-borne fungal contaminants that cause seed rots, damping-off, seedling blights and root rot; 2. To control fungal contaminants on the surfaces of seeds or plants that cause e.g., covered smuts of barley and oats and bunt of wheat, black point of cereal grains and seed-borne safflower rust; and 3. To kill the fungal contaminants located within the inner seed tissues causing, e.g., loose smut of cereals (McMullen and Lamey, 2000).

1.6.1.2 Categories of fungicides

There are two categories of fungicide, *viz.* contact and systemic fungicides. Contact fungicides, also known as protectant, non-systemic, or surface fungicides, are applied before host tissues are fungally invaded and act by killing or inhibiting the fungi (Yuste and Gostinear, 1999). The application of protective-contact fungicides alone following surface decontamination is ineffective when, as often occurs with recalcitrant seeds, the inoculum is located within the seed tissues (Berjak and Pammenter, 2004). They are, however, effective when the fungal inoculum is confined to the outer surfaces of seeds. In contrast, systemic fungicides must be taken up by the plants (which includes metabolically-active recalcitrant seeds) and translocated to the site of infection eventually killing fungi which have already penetrated the tissues. The recently developed systemic fungicides can act both as protectants and eradicants (Garcia *et al.,* 2003), but one of the disadvantages of systemic fungicides is their site-specific action on pathogenic fungi, which can lead to the production of resistant strains, if they are not managed appropriately (Dias, 2012). Resistance to systemic fungicides is said to occur due to their specificity in acting against only one of the functions of the contaminants (e.g. benzimidazoles interfere with nuclear division and organophosphate fungicides inhibit chitin synthesis of the contaminants), rather than on a variety of functions. This apparently often leads to the development of new strains of contaminants that are resistant to one or another of the systemic fungicides (Agrios, 2005).

Use of systemic fungicides to extend storage longevity of recalcitrant seeds by minimising fungal proliferation is being tested in our laboratory. However, we are aware that their use may lead to various defects in the resultant plants, e.g. damage to foliar tissue (RPD No.1002, 2005), possible compromise of GA_3 synthesis in wheat seedlings (Gao *et al*., 2000), interference with photosynthesis, as shown for *Vitis vinifera* (Saladin *et al*., 2003), and seed and seedling mortality, as reported for onion (Fullerton *et al*., 1995). Some examples of contact and systemic fungicides are listed in the table below:

Table 1.1 Different types of fungicides with examples (Agrios, 2005; Sambamurty, 2006; Singh and Singh, 2010)

Fungicide type	Group type	Examples
Contact Fungicide	Inorganic compounds	Bordeaux mixture (Biota Agro Solutions, India); Copper carbonate (CTM Supplies, England); bicarbonates of sodium (Brunner Mont, United Kingdom), potassium (Armands Product Company, South Africa); lithium carbonate (Private Account, South Africa); mono and dipotassium phosphates (Akash Purochem Private Limited, India); elemental sulphur (Keg River Chemical Corporation, Canada), Lime sulphur (African Pegmatite, South Africa); mercuric and mercurous chloride (J M Loveridge, United Kingdom) sodium hypochlorite (NCP Chlorchem, South Africa) and calcium hypochlorite (Aquachlor, South Africa).
	Organic compounds	Heterocyclic compounds e.g.: Captan (Universal Crop Protection, South Africa), iprodione (Bayer Crop Science, Canada) and vinclozolin (BASF, United States). Dialkyldithiocarbamates e.g.: Thiram (Bayer Crop Science, United States) , Ferbam (Amvac Chemical Corporation, California), Ziram (Swarup Chemicals, India) Ethylenebisdithiocarbamates e.g.: Zineb (Universal Crop Protection, South Africa), Maneb (Environmental Protection Agency, United States), metiram (Environmental Protection Agency, United States), Mancozeb (DuPont Agricultural products, United States) Aromatic compounds e.g.: PCNB (Uniroyal Chemical, United States), chlorothalonil (Makhteshim Agan, North America) and polychlorinated biphenyl (Chemconserve, Netherlands).
Systemic Fungicide	Local systemic	Imazalil (Makhteshim Agan, North America), Triforine (American Cyanamid Company, United States)
	Partially or fully systemic	Benzimidazole e.g.: Benlate (Villa Crop Protection, South Africa), Carbendazim (BASF, Germany), thiabendazole (Environmental Protection Agency, United States) Other examples of systemic fungicide groups Triazoles (AlliChem, United States), Strobilurins (Du Pont Agricultural products, United States), Oxanthiins (Crompton Chemical, Switzerland), Acyclalanines (Environmental Protection Agency, United States).

Contact fungicides such as sodium hypochlorite (NaOCl) are used to treat post-harvest seeds or are applied as soil treatments for various crops, in view of the ability to kill, rather just minimise, the surface microflora (Ogawa and English, 1991). For most *in vitro* assays embryonic axes are recorded as being surface disinfected with 1% sodium hypochlorite solution containing 2 to 3 drops of a wetting agent Tween 20® before culturing (Dumet *et al*., 1997). The embryonic axes of *T. dregeana* in the present work were also disinfected in this way. Some other examples of other contact fungicides used on seeds are 3–30% hydrogen peroxide (Sutherland *et al*., 1987), calcium hypochlorite and alcohol (Pelczar *et al.*, 1986), and, under laboratory conditions, 0.1% mercuric chloride is also used (Schmidt, 2000). It is also important to ascertain the ideal time of exposure to, and concentration of, the sterilants for seeds of individual species to avoid toxic effects (Schmidt, 2000). One of the advantages of using contact fungicides over systemic fungicides is that they are inexpensive and resistant strains of contaminants are less likely to develop (Dias, 2012). There are, nevertheless, advantages and disadvantages to the use of individual contact fungicides or groups of such preparations. Among the contact fungicides, organic fungicides are more effective and less toxic (Belpoggi *et al.,* 2002; Hunsche *et al.,* 2007). However, studies conducted in 27 countries have reported that dithiocarbamates, which are organic fungicides, were found to leave more residual contamination than any other type of fungicide (EFSA Scientific Report, 2009).

The fungicides that have attracted most attention for the post-harvest control of contaminants are Benlate, thiobendazole, Dichloron and imazalil; however, resistance of contaminants to thiobendazole (Tecto90®, Tecto 500 SC®) and imazalil (Fungiflor®, Magnate®, Deccozil®, Fungazil®, Imazagard®) has been reported (Holmes and Eckert, 1999; Conway *et al*., 1999). Among the systemic fungicides, Benlate is the most used and effective chemical against a wide range of fungi causing plant diseases, and in crop protection (Garcia *et al.,* 2003). It also acts as an effective contact fungicide for stored recalcitrant seeds such as those of *T. dregeana* (dusting on to the seed surfaces [Kioko, 2003]) as well as by coating seeds using a suspension; e.g. 0.3% suspension of Benlate was highly effective in increasing the storage period of *Hevea brasiliensis* (Chin *et al*., 1988). In the present study various sterilants, *viz.* electrolyzed oxidizing water, Nipastat, Medi-chlor and Benlate were used to decontaminate the axes of *T. dregeana.* These treatments were also applied directly to the axenic cultures of fungal contaminants isolated from the axes.

1.6.1.3 Disinfection based on electrochemistry

Electrochemical disinfection refers to the elimination of the micro-organisms using the highly oxidising anodic fraction of an electrolysed, dilute salt solution (Kraft, 2008). Electrolysed oxidising water is reported to be a powerful decontaminant used in the agricultural and food industries (Huang *et al.*, 2008).

The antimicrobial effect of anodic water is due to its oxidation potential as well as the production of hydroxyl radicals (OH·) from hypochlorous acid (HOCl). The free chlorine also acts on the microbes (Koseki and Itoh, 2001). Electrolysed oxidising water has been shown to kill or inactivate some bacteria, e.g. *Escherichia coli* O157:H7 strain, *Salmonella enteritidis* and *Listeria monocytogenes* on lettuce (Park *et al*., 2001), and to inhibit the growth of some fungal species such as *Aspergillus* spp. (Buck *et al*., 2002; Suzuki *et al*., 2002), *Penicillium expansum* (Okull and Laborde, 2004), and species of *Fusarium, Botrytis, Alternaria*, and *Cladosporium* (Buck *et al.,* 2002).

Studies using electrolysed oxidising water on tomatoes inoculated with *Escherichia coli O157:H7, Salmonella,* and *Listeria monocytogenes* showed a significant reduction of colony counts (Bari *et al.*, 2003), and reportedly its application reduced *Salmonella* associated with alfalfa seeds and sprouts (Kim *et al.,* 2003) and *Escherichia coli* O157:H7 (Sharma and Demirci, 2003). However, in the latter case increasing the exposure time adversely affected the germination of the seeds (Sharma and Demirci, 2003). It therefore appears that, although anodic water is considered to be less toxic than any other chemicals, long exposure could affect the vigour and viability of the seeds.

1.6.1.4 Preservatives used as fungicides

Preservatives are antimicrobial agents used for inhibiting the growth of various pathogenic micro-organisms that cause plant diseases as well as food spoilage (Todar, 2001). Although they are not used in controlling post-harvest diseases, they are effective in minimising decay of the produce. Some of the examples include sodium benzoate, propionic acid, sorbic acid, acetic acid, sulphur dioxide (SO_2), nitrites, nitrates, and a few antibiotics such as nisin (Chichester and Tanner, 1972). Nipa esters are one group of antimicrobial preservatives widely used in cosmetics, toiletries and

pharmaceutical products. Nipastat®, a mixture of parabens (see below), is the most effective preservative of all the nipa-ester-based preparations[1].

a) Nipastat®

Material in this section is drawn from information in the footnoted references[1-3]. Nipastat, a mixture of methyl p-hydroxybenzoate, ethyl p-hydroxybenzoate, butyl p-hydroxybenzoate, isobutyl p-hydroxybenzoate and propyl p-hydroxybenzoate, is most commonly used to protect against bacteria, moulds, yeasts, etc. in cosmetics and toiletries[2]. It works well for rinse-off as well as for leave-on formulations. The maximum concentration of Nipastat used for the personal care products is 0.7%, although the recommended level is 0.05-0.3%[3]. The solubility of Nipastat varies depending on the solvent: ~65% in methanol; ~60% in ethanol and acetone; 35% in propylene glycol; ~40% in hexylene glycol; but only ~1% in pure glycerine; ~0.40% in glycerine/water (1:1); <0.10% in liquid paraffin and ~0.14% in water[2]. It is noted that the solubility of Nipastat in water is lower compared with other solvents, but this does not affect its microbicidal activity. However, the relatively insoluble nature of Nipastat in water makes it difficult to add it directly to any product. The solubility of Nipastat in water does increase, however, when it is added to water at a maximum temperature of 60-100°C[2], although the maximum handling temperature is ~80°C[1].

Nipastat is effective against a wide range of contaminants, *viz.* the bacteria *Staphylococcus aureus*, *Escherichia coli*, *Aerobacter aerogenes*, *Proteus vulgaris*, *Serratia marcescens*, *Pseudomonas aeruginosa*, *Salmonella entiridis*, *Lactobacillus buchneri*, *Nocardia asteroids*, *Bacillus cereus*, *Alkaligenes foecalis*, *Streptococcus haemolyticus* and *Micrococcus flavus*[2]. Nipastat also has proven anti-yeast and anti-fungal properties against *Candida albicans*, *Saccharomyces cerevisae*, *Aspergillus brasiliensis*, *Penicillium expansum*, *Mucor racemosus*, *Pediococcus cerevisae*, *Pityrosporium ovale*, and others[2]. In the current study Nipastat was used in attempts to control micro-organisms contaminating *in vitro* cultures of embryonic axes of the recalcitrant-seeded species, *Trichilia dregeana.*

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[1] Anonymous. 2002. Nipa esters the original parabens, preservatives for cosmetics, toiletries and pharmaceuticals, Clariant. http://www.innovadex.com/Personalcare/Detail/1022/42535/Nipastat

[2]Nipastat. 2010. Clariant, Industrial and consumer specialities. preservative for the cosmetic industry, pp. 1-4. http://www.essentialingredients.com/spec/Nipastat.pdf

[3]Nipastat by Clariant, Personal care, innovadex: http://www.innovadex.com

1.6.1.5 Sodium dichloro-isocyanurate (Medi-Chlor®/ NaDCC)

Sodium dichloro-isocyanurate disinfectant tablets are considered to be superior over traditional hypochlorites due to their safety and simple application. They can be used as a wide spectrum disinfectant and are suitable for high, medium and low level disinfection[4]. Sodium dichloro-isocyanurate used in the present study are effervescent tablets which release disinfectant solutions when mixed with water. The tablet utilises the ingredient sodium dichloro-isocyanurate (NaDCC), which is an organic chlorine donor that releases the active chlorine compound hypochlorous acid when mixed with water at known reliable in-use strengths. The chemistry and mode of action of NaDCC is significantly more effective than the hypochlorites particularly when there is organic contamination[4]. According to the SANS report, NaDCC is proven to be bactericidal when tested against *Pseudomonas aeruginosa*, *Eschericia coli* and *Staphylococcus aureus* giving 99.9% mortality. In the present study, NaDCC of 0.5g strength was used at different concentrations to decontaminate the axes of *Trichilia dregeana.*

1.6.1.6 Benlate

Benlate (methyl 1-butyl carbomoyl 2-benzimidazolecarbamate), which is a systemic benzimidazole, was developed in 1959 by Hein L. Klopping[5] and sold as Benlate. It was produced on a large scale for the market in 1969 by DuPont[6]. Benlate is practically insoluble in oil and in water (2 mg L^{-1}[pH 9.0]; 3.6 mg L^{-1}[pH 5.0]) but is soluble in a few solvents at certain concentrations, e.g. 9.4 g 100 mL^{-1} chloroform; 5.5 g 100 mL^{-1} dimethylforamide; 1.8 g 100 mL^{-1} acetone; 1.0 g 100 mL^{-1} xylene; 0.4 g 100 mL^{-1} ethanol; and 0.04 g 100 mL^{-1} heptane[7]. Benlate is highly effective against fungal contaminants such as members of the Ascomycetes, Basidiomycetes and Deuteromycetes that cause plant diseases (Bankole and Adebanjo, 1996), but is inactive against members of the Phycomycetes (Edgington *et al*., 1971; Bollen, 1972). However, an undesirable effect was reported in that Benlate could inhibit arbuscular mycorrhizae (Thingstrup and Rosendahl, 1994; Pedersen and Sylvia, 1997; Schweiger and Jakobsen, 1998) by inhibiting the internal as well as external hyphae, even at a very low concentration (1 µg g^{-1} soil) (Kjoller and Rosendahl, 2000).

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[4]http://www.medihealthdistributers.co.za

[5]http://www.2.dupont.com/Phoenix_Heritage/en_US/1970_detail.html

[6]http://www.epa.gov/oppsrrd1/REDs/factsheets/Benlate_fs.html

[7]http://www.inchem.org/documents/jmpr/jmpmono/v073pr04.html

Benlate is an effective fungicide because of its ability to penetrate the waxy cuticle of the host plant surface and act on the contaminants located under this layer (Ben, 1975). It works by binding to the fungal microtubules (Davidse, 1986) thus interfering with mitosis and other cell activities (El-Katatny *et al.*, 2004). A degradation product of Benlate, methyl 2-benzimidazole carbamate (MBC), is also toxic to fungal contaminants (Clemons and Sisler, 1969; Peterson and Edgington, 1969).

Benlate, is commercially available under various names[8]. It is sold as wettable powder (WP) and water soluble packaging (WSP) film. Both products contain 50% active ingredient[2]. Benlate is used in foliar and seed treatments and pre-harvest spraying and post-harvest dipping or dusting procedures for a variety of crops. It is absorbed by the plant from the point of application and deposited in the veins and at leaf margins[9].

1.6.2 Biological control of plant diseases

One of the most promising approaches to avoid the hazards of fungicides is an ecological approach, exemplified by use of antagonistic microbes (Cook, 1985), and known as biocontrol. The descriptor. "biological control", and its abbreviated synonym "biocontrol", coined first by (Smith, 1919 loc. cit. Schoenly *et al*., 1996), were used in the entomology and plant pathology fields, the former utilising live killer insects, entomopathogenic nematodes, or microbial contaminants to control different pathogenic insects. In plant pathology, biocontrol involves the use of micro-organisms that attack plant contaminants, including host-specific contaminants, to control plant diseases (Pal and Gardener, 2006). The organisms that prevent or control the plant diseases are called biological control agents/biocontrol agents (BCAs), and are most often referred to as antagonists. Various bacteria, fungi, nematodes and protozoa have been used as biocontrol agents: these include arbuscular mycorrhizae, *Pseudomonas fluorescens*, *Trichoderma* spp., *Azetobacter* spp., *Pasteuria penetrans*, *Bacillus* spp., species of yeasts, Rhizobacteria, non-pathogenic strains of *Fusarium* (Duijff *et al.,* 1998; Benhamou *et al*., 2002), and some species of *Penicillium* and *Phoma* (Koike *et al.,* 2001).

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[8] The Agrochemicals Handbook: Third Edition.1991. Royal Society of Chemistry, Unwin Brothers Ltd., Surrey, England.

[9]Forest Service. 1986. Pesticide Background Statements, Volume II: Fungicides and Fumigants. U. S. Department of Agriculture, Agriculture Handbook No. 661.

Biological control agents are either used alone or in combination with low levels of chemicals to achieve best results (Monte, 2001). In an investigation by Calistru *et al*. (1995) two strains of *Trichoderma* spp. were found to have a marked effect against the orthodox-seed-associated xerotolerant fungal species *in vitro*. It was suggested, therefore, that the use of such non-pathogenic fungal antagonists might also find applicability in recalcitrant seed storage. For example, in an investigation by Finch-Savage *et al*. (2003), the use of a broad spectrum *T. virens* as a biological control agent, resulted in protection against fungal infection and in significant reduction of fungal proliferation during storage of *Quercus robur* seeds.

1.6.2.1 Trichoderma *as a biocontrol agent*

Trichoderma species known as teleomorphs (sexual stage) and anamorphs (asexual stage), belong to the family Hypocrea, class Sordariomycetes (Harman *et al*., 2012) and are free living saprophytic fungi that colonise mainly the roots of plants (Harman *et al*., 2004a). Most of the strains of *Trichoderma* that have biocontrol properties exhibit only the asexual stage and hence are anamorphs.

1.6.2.2 Morphology and distribution

Trichoderma cultures mature within 5 d on potato dextrose agar (PDA) and colonies, having a woolly appearance (Fig.1.1), vary in pigmentation according to the species, from dull yellow to dark green or even reddish, while some produce no colour (St-German and Summerbell, 1996; De Hoog *et al*., 2000). Different species of *Trichoderma* show differing colony characteristics, growth rate in culture, pigmentation, arrangement of conidiophores and shape of conidia. The shape of conidia range from globose to ellipsoidal, obvoidal or even short cylindrical with different sizes of conidia up to 3 µm in diameter. The conidia are smooth- to rough-walled and develop at the tip of flask-shaped phialides (Fig.1.2), but these clusters are often damaged while being prepared for microscopical observations (Harman and Kubicek, 1998; Sutton *et al*., 1998).

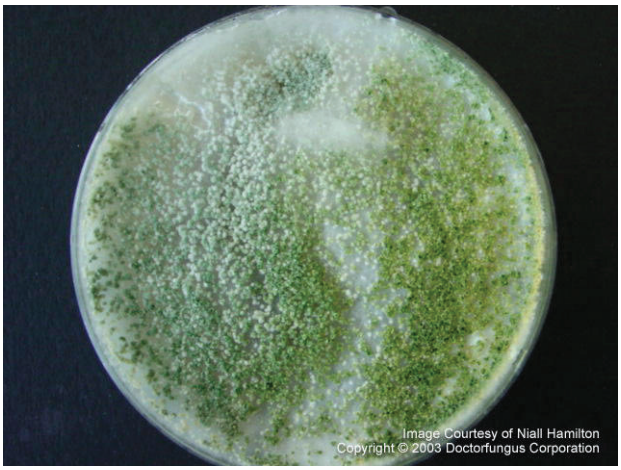

Figure 1.1: *Trichoderma* sp. grown on a potato dextrose agar

(Source:http://www.doctorfungus.org)

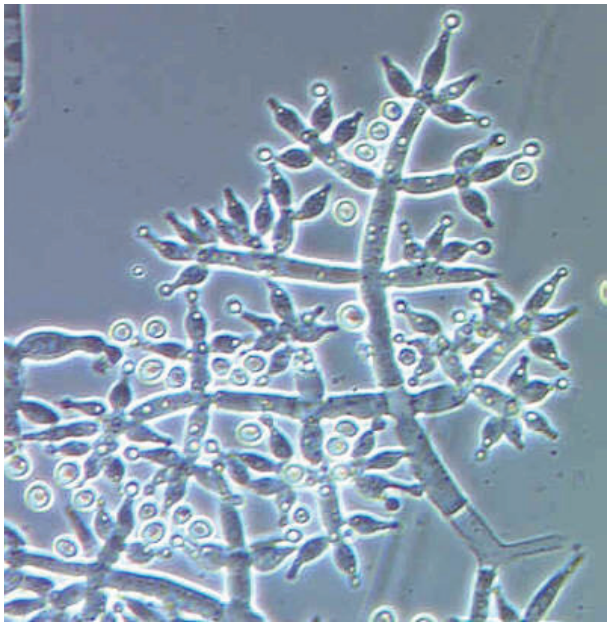

Figure1.2: Microscopical appearance of part of the mycelium of *Trichoderma* sp., with conidia at the tip of phialides from each hyphal branch.

(Source: http://www.ars.usda.gov)

Trichoderma is distributed in almost all types of soils with a pH range of 2.5 to 9.5, but ideally in a slight to moderate acidic environment (Hagedorn, 2004). However, only a few species of *Trichoderma* are able adapt to soil (Friedl and Druzhinina, 2012). *Trichoderma harzianum* and *T. hamatum* are mostly found in soil where they survive by competing with the contaminants for space and nutrients, thereby protecting plants (Bora *et al*., 2000).

1.6.2.3 Biocontrol characteristics of Trichoderma

Trichoderma spp. have a history of more than 70 years as successful biocontrol agents (Harman *et al*., 2008). Among the biocontrol agents, *Trichoderma* as a plant symbiont is considered to be better than other such agents, or chemical control, because of its multiple roles of antagonising plant contaminants, promoting plant growth and facilitating soil remediation (Harman *et al*., 1993; Esposito and da Silva 1998 and Harman *et al*., 2004a). Additionally, those *Trichoderma* spp. present in all soils occur at high densities (Chet *et al*., 1997), are easily able to be cultured on low-cost substrates, grow in most adverse conditions, are efficient in utilisation of nutrients, have the capacity to modify the rhizosphere, act strongly against various phytopathogenic fungi and have resistance against other contaminants (Papavizas, 1985). Therefore, they are highly effective against a wide variety of phytopathogens (Whipps and Lumsden, 2001).

Trichoderma spp. benefit a wide range of host plants, including fruit and vegetable species and ornamentals, by co-existing for up to five years once established in the host; they also work well when applied frequently in high doses (Winter, 2000). *Trichoderma* spp. are non-toxic organisms and, when located in the sub-epidermal cell layers of roots, secrete a variety of compounds that induce localised or systemic resistance responses; this explains their lack of pathogenicity to plants (Harman *et al.,* 2004a). Recent discoveries show that, although *Trichoderma* is an endophytic plant symbiont in the roots, it elicits changes in gene expression in the shoots that include improving abiotic stress resistance, assimilation of nitrogen fertilizer, plant resistance and efficiency of photosynthesis (Hermosa *et al*., 2012). Thus, the changes contributed by *Trichoderma* will promote plant growth and productivity. A study in support of such properties showed that maize plants grown from seeds treated with RootShield® (available commercial strain [T-22] of *T. harzianum* [BioWorks, New York]) gave better yields than plants that were not so-treated (Harman *et al.,* 2004b). The non-toxic nature of *Trichoderma* has been tested and proven by studies of its potential effects on the germination of winter wheat grain (Michalikova and Kohacik, 1992). However, there was a report on *Trichoderma harzianum* being pathogenic to

button mushrooms, *Agaricus bisporus*, which; however, itself is a fungus (Sinden and Hauser, 1953). Spore suspensions as well as culture filtrates of some strains of *T. harzianum* (T969, T447, T614, T678, G525 and *Trichoderma* sp. T [unknown]) have also affected the speed of germination of maize (Hajieghrari, 2010), and culture filtrates of *T. viridae* on cucumber, tomato and pepper seedling germination (Menzies, 1993) and *T. koningii* on onion, chicory and lettuce seeds (Celar and Valic, 2005).

Trichoderma strains are resistant to most of the toxic compounds in the soil, such as herbicides, fungicides, toxins released by the plants and the enzymes of contaminants, as a result of their ATP-binding cassette (ABC) transporter systems (Harman *et al.,* 2004a). These transporters act by reducing the accumulation of various toxins in *Trichoderma* cells (Lanzuise *et al*., 2002), and are also vital for the mycoparasitic interactions with plant fungal contaminants (Ruocco *et al*., 2008). This is evident in ABC-transporter-lacking mutants of *T. atroviride* P1, which was inhibited by plant fungal contaminants such as *Botrytis cinerea*, *Rhizoctonia solani* and *Pythium ultimum* and they also were less effective as fungal parasites (Ruocco *et al*., 2008). The ability to reduce acumulations of toxins makes *Trichoderma* a better antagonist against various contaminants, including *Rhizoctonia solani, Pythium ultimum*, *Sclerotium rolfsii*, *Botrytis cinerea* and species of *Fusarium, Phytophthora, Sclerotinia* and *Verticillium* when they are used alternating with Benlate, Captan, methyl bromide or any other chemicals (Vyas and Vyas, 1995). However, *Trichoderma* spp. can also be used alone to control diseases caused by contaminants such as *Armillaria*, *Botrytis*, *Chondrostereum*, *Colletotrichum*, *Dematophora*, *Diaporthe*, *Endothia*, *Fulvia*, *Fusarium*, *Fusicladium*, *Macrophomina*, *Monilia*, *Nectria*, *Phoma*, *Phytophthora*, *Plasmopara*, *Pseudoperospora*, *Pythium*, *Rhizoctonia*, *Sclerotinia*, *Sclerotium*, *Venturia*, *Verticillium* and wood-rot fungi (Lumsden *et al*., 1993; Monte, 2001). *Trichoderma* also contributes to the bioremediation of the soil by degrading herbicides and pesticides, including insecticides such as organophosphates, organochlorines and carbonates (Ranasingh *et al.,* 2006). *Trichoderma* promotes plant growth by solubilising phosphates and micronutrients and can also increase the number of deep roots, especially of grasses, which thereby help them to resist drought (Ranasingh *et al.,* 2006). It has been reported that after application of *Trichoderma* strain (T22) to corn there was a 40% reduction in the requirement for nitrogen, compared with corn which was not so-treated (Harman, 1997).

1.6.2.4 Mechanisms of biological control

Several mechanisms of action have been described as promoting biological control by *Trichoderma* spp., which include mycoparasitism or direct effects, antibiosis, induced systemic resistance, solubilisation and sequestration of inorganic plant nutrients, and competition.

a) Mycoparasitism

Mycoparasitism by biocontrol agents occurs by direct attack on contaminants and involves physical contact. This is achieved initially by recognition of, and growth towards, the contaminant (Chet *et al.,* 1981). The biocontrol agent then attaches to the contaminant by coiling around it and forming appressoria-like structures, after which cell-wall-degrading enzymes such as chitinases, glucanases, proteases and cellulases are released, as are peptaibol antibiotics (Schirmbock *et al*., 1994; Howell, 2003). As a result of their action, digestion of the cell wall compounds (such as chitin, cellulose and β-1,3 glucan) of the target contaminant occurs, followed by its destruction (Harman *et al*., 2004a). *Trichoderma* spp. have been shown to be very efficient producers of extracellular enzymes, with some of these species being implicated in the biological control of plant diseases (Monte, 2001; Harman, 2006). Examples include mycoparasitism by *T. harzianum* acting against *Fusarium oxyporum, F. roseum, F. solani, Phytophthora colocaciae* and *Sclerotium rolfsii* (Ranasingh *et al.,* 2006).

Studies based on the toxicity of fungal cell-wall degrading enzymes show that they have no adverse effect on plants or animals, including humans. Consequently, registration of *Trichoderma*-based products as biocontrol agents has been approved by the EPA (Environmental Protection Agency) tests done by United States (Monte, 2001). Cell-wall degrading enzymes purified from the culture filtrates of *Trichoderma* are highly recommended for use as commercial products in agriculture due to their stable nature, resistance to drying, freezing, temperatures up to 60°C and broad pH tolerance (Monte, 2001).

Mycoparasitism was studied *in vitro* using various *Trichoderma* species on different plant contaminants. The *in vitro* antagonism by *T. harzianum* and *T. viridae* showed that the former was more aggressive than the latter, although both completely overgrew the fungal contaminant, *Colleotrichum gloeosporioides* (Deshmukh and Raut, 1992). A similar study showed that *T. harzianum* exhibited strong mycoparasitism, completely overgrowing the contaminant, *Sclerotium rolfsii*, while *T. viridae* showed strong antibiosis (Yogendra and Singh, 2002).

b) Antibiosis

Antibiosis describes the release of toxic substances such as antibiotics as well as volatiles and non-volatiles, which kill or suppress contaminants (Tronsmo, 1996). Volatile substances include ethylene, hydrogen cyanide, alcohols, monoterpenes, aldehydes and ketones; and non-volatile metabolites released by *Trichoderma* strains include harzianic acid, alamethicins, tricholin, peptaibols, 6-penthyl-a-pyrone, massoilactone, viridin, gliovirin, glisoprenins and heptelidic acid (Vey *et al.,* 2001). Effects of the volatile as well as the non-volatile compounds have been recorded as reduction in radial growth and colony forming units, and reduced ability for spore formation of target fungi such as *Penicillium expansum, Botrytis cineria* and *Rhizopus stolonifer* (Vinãs, 2004). A recent study suggested that peptaibols, that can cause apoptotic death of the contaminants, are among the most potent anti-fungal agents released by *Trichoderma* (Shi *et al*., 2012).

Studies on *in vitro* dual cultures with *Trichoderma harzianum* show this biocontrol agent was able to produce antibiotic substances that inhibited the growth of *Fusarium* as well as overgrew two-thirds over the contaminant (Michrina *et al*., 1995). An experiment to test the effect of non-volatile substances of *T. harzianum* and *T. viridae* on *Fusarium oxysporum* and *Macrophomina phaeolina* showed that the colony growth of both the contaminants was highly reduced by *T. harzianum* compared with *T. viridae* (Kaur *et al*., 2003)

c) Competition

Trichoderma spp. compete with other organisms for space as well as nutrients in soil (Elad, 1996) and starve the contaminants of the vital substances expelled by the seeds which are essential for the germination of the pathogenic plant fungi (Howell, 2002). They compete mainly for oxygen, infection sites at the root surface of the plants and for nutrients such as carbon, nitrogen and iron. (Paulitz *et al*., 1990; Tronsmo, 1996).

Some of the examples of biocontrol by competition using *Trichoderma harzianum* T-35^{TM} (Makhteshim-Agan Chemicals, Israel), include starving *Botrytis cineria* (Latorre, 2001) and *Fusarium oxysporum* (Sivan and Chet, 1989). In addition to starvation, strains of *Trichoderma* degrade some important enzymes such as pectinases, and others which are necessary for contaminants to invade plants: an example of this is the degradation of the enzymes of *Botrytis cinerea* that facilitate penetration of leaf surfaces (Zimand *et al*., 1996).

d) Solubilisation and sequestration of inorganic plant nutrients

Trichoderma spp. reduce the pH of soil by releasing certain organic acids such as gluconic, citric and or fumaric acid (Vinale *et al*., 2008a) which make phosphates soluble and thus available for plants. These biocontrol agents also make micronutrients and minerals, including iron, magnesium and manganese available from the soil, by secreting diffusible metabolites and siderophores (Jalal *et al*., 1987). Plants are thus able to absorb nutrients more easily (Chet, 2001). This mechanism of solubilisation also reduces the incidence of plant root contaminants (Harman *et al*., 2004a).

e) Induction of plant resistance

Localised and systemic induction of resistance in plants can be caused as a response to attack by contaminants, or to injuries caused by insects or environmental factors, application of chemical inducers or by non-pathogenic Rhizobacteria (Kuc, 2001; Oostendorp *et al.,* 2001; Pal and Gardener, 2006). It has been reported that adding *Trichoderma* strains to the rhizosphere, protected plants against various contaminants including viruses, bacteria and fungi that cause aerial infections, by the induction of systemic acquired resistance and induced systemic resistance in the plants (Harman *et al*., 2004a). There are three different compounds which are released by plants in response to different stimuli, *viz.* salicyclic acid (SA), jasmonic acid (JA) and ethylene. Salicyclic acid is released when attack by a contaminant stimulates the production of pathogenesis-related (PR) proteins, which, in turn, are responsible for systemic acquired resistance (SAR) in plants (Harman *et al.,* 2004a). Pathogenesis-related proteins, first described by van Loon and van Kammen (1970), are a group of enzymes that are responsible for attacking the contaminant as well as strengthening the cell wall boundaries of the host. Jasmonic acid or ethylene is released by the presence of non-pathogenic rhizobacteria, and are responsible for induced systemic resistance (ISR) of plants (Kloepper *et al.,* 1993; Pieterse and van Loon, 1999). Other than rhizobacteria, biocontrol agents such as *Trichoderma*, and non-pathogenic *Fusarium, Rhizoctonia, Penicillium* and *Phoma* also induce resistance in plants (Harman *et al.,* 2004a).

Other mechanisms of biocontrol include changes in the root system anatomy and microbial changes in the rhizosphere. *Trichoderma* strains colonise the outer layers of the root cells (Yedidia *et al*., 1999; Yedidia *et al*., 2000) and enhance the systemic induced resistance of plants (Yedidia *et al*., 2003). Colonisation by *Trichoderma* of the root increases the deep-root intercepts

(used to estimate root-length), which is very well supported by the example of T-22 treated maize plants with twice the number of deep-root intercepts than the control (Harman, 2000). There were also reports of enhanced root biomass production, increased root hair development (Bjorkman *et al*., 1998; Harman *et al.,* 2004b) and increased number of lateral roots (Contreras-Cornejo *et al*., 2009). It is common that an effective biocontrol agent acts through the combination of different mechanisms (Whipps, 2001).

1.6.2.5 Trichoderma harzianum *relative to other* Trichoderma *spp. as a biocontrol agent*

Some of the 89 species of *Trichoderma* are used as biocontrol agents. These include *T. viridae, T. polysporum, T. koningi, T. harzianum, T. longibrachiatum, T hamatum* and *T reesei.* However, the most common BCAs of the *Trichoderma* genus are strains of *T. virens*, *T. viride, T. hamatum* and, above all, *T. harzianum*, which is a species aggregate that includes different strains used as BCAs against phytopathogenic and viral vector fungi (Grondona *et al*., 1997; Harman, 2006). *Trichoderma harzianum* is widely used as biocontrol control agent and there has been extensive research done for the selection, production, formulation and application of efficient strains for control of different plant pathogenic micro-organisms (Tronsmo, 1996).

1.6.2.6 Commercial products of Trichoderma harzianum

A wide variety of products of *T. harzianum* are commercially available for use under field conditions and in greenhouse trials. Some of the products contain only one type of *Trichoderma* species while others are mixtures of two or three species. *Trichoderma harzianum* strains available commercially are used to prevent development of various soil-borne pathogenic fungi. (Shalini *et al*., 2006). *Trichoderma* spp. are commercially available in the form of conidia rather than in the mycelial form, because the former remain more viable and stable for field application (Amsellem *et al.,* 1999), surviving for between 110 and 130 days in soil (Papavizas and Lewis, 1981).

Since 1990, a wide variety of *Trichoderma*-based formulations have been used in different forms such as liquid (water or oil) or dry formulations (granules, pellets and wettable powder): made in large quantities these can be stored for long periods without losing their beneficial properties (Siddiquee *et al.,* 2009). Some of these formulations are: TrichoGreen™, used against *Ganoderma* for basal stem rot of oil palm (Abdullah *et al*., 2003); Trichodex™ which counteracts botrytis responsible for the grey mould of grapevines (O'Neill *et al*., 1996); Supresivit® which is

applied against *Rhizoctonia solani* on peas and *Pythium ultimum* on cucumber (Koach, 1999). Some other products used by the greenhouse growers include Trichoflow (Agrimm, New Zealand), Trichodry (Agrimm, New Zealand), DRHCI (Gro-Chem, New Zealand), TRI-D25 (Agrimm, California) (Nederhoff, 2001). In South Africa, the commonly used *Trichoderma* products are EcoT and Eco77 (Plant Health Products, South Africa). Preparations of *Trichoderma* species can be used alone, in combination with other biological agents, or with low levels of certain chemicals.

1.6.2.7 Strains of Trichoderma harzianum

In the present study two strains of *T. harzianum,* EcoT and Eco77, were used to investigate their effects on controlling contamination as well as promoting germination of the embryonic axes excised from *Trichilia dregeana* seeds. They are commercially sold as wettable powder formulations, the former being strain Kd and the latter the strain B77: one gram of either contains $2x10^9$ conidia.

a) Eco77®

Eco77® colonises pruning wounds and is noted to protect roses, tunnel tomatoes and cucumber plants from wound infections caused by *Botrytis*[10] as well as the pruning wounds of grapevines by *Eutypa lata*[11]. Eco77 can be mixed with water (1 g of Eco77 in 2 L water) and applied directly as a full cover spray or directly on the pruned surfaces. One of the advantages of Eco77 is it provides resistance lasting for more than a year against wound infections compared with chemicals, the effects of which may last for only 10 d[10]. The South African Agricultural Research Council selected the strain Eco77 to control *Botrytis* which was a threat to grapevine plants in greenhouses, as growth of the fungus is favoured in the moist and humid conditions. Eco77 acts by preventing the entry of the contaminants via any wound site, whether on leaves, stem, flowers or fruits[10].

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[10] *Eco77® for* Botrytis *control and pruning wound infections*, http://*www.plant-health.co.za/articles/eco-77_leaflet.pdf*

[11] *Use of Eco77® in pruning wound protection on grapes,* 2003. http://www.plant-health.co.za/articles/Eco-77_Eutypa_flyer.pdf

When Eco77 was applied to the pruning wounds of grapevine and the wounds infected with *Eutypa* 24 h later, the results after one year showed that, although the fungicides such as Benlate and flusilazole were better against the infection compared with Eco77, they were effective for only 10 d, whereas Eco77 remained as an effective barrier against the contaminant even after a whole year. However, when the incubation time was increased to 48 h the results were improved compared to that 24 h during which *Trichoderma* had only just germinated and covered the wound at a minimum rate[11]. Different trials comparing chemicals versus biocontrol showed that products based on *T. harzianum* (T77 [Agricura, Zimbabwe], Vinevax™ (formerly Trichoseal) [Agrimm, New Zealand], Eco77 and Trichoseal-spray [Agrimm, NewZealand]) showed inconsistent results in controlling *Eutypa* infection of grapevines (Halleen *et al.*, 2011). It is thought that one of the reasons why biocontrol products appeared to be less effective was because of the time needed for *Trichoderma* to colonise and exert its biocontrol effects, compared with chemicals which are quick to act (Halleen *et al.*, 2011).

b) EcoT®

Trichoderma in EcoT® colonises the root surfaces of plants and is used to control soil-borne contaminants such as *Rhizoctonia, Pythium, Fusarium* and *Phytophthora* in any crop[12]. EcoT (1 g in 4 L water) can be applied to seeds by using a medium which facilitates the spores sticking to the surfaces of the seeds. Carboxymethylcellulose (1% CMC) or 2 g L^{-1} odourless, flavourless gelatin is used as a sticking agent or as dry powder on moist or damp seeds, or as a drench for seedling trays, seedbeds, potted plants and turf [13]. Trichoderma developing from EcoT applications acts as a symbiont with plants, the Trichoderma depending on the root exudates of the plant for growth while protecting the plant from diseases by competing with contaminants[14].

EcoT application also works well by protecting the plants subjected to various deleterious environmental conditions such as water-logging, drought, pH, hail and nutritional stress[13]. Aside from affording protection to the plant against root infections, as mentioned above for *Trichoderma* spp. generally, another beneficial effect of EcoT is that it can increase the number

[12]*EcoT®, Plant Health Products,* http://*www.tammac.co.za/Pastures/EcoT.pdf*

[13]*Madumbi sustainable agriculture, EcoT®-For larger healthier more effective root systems. (2011). http://www.madumbi.co.za/Product_Eco-T.html*

[14] Anonymous (2008) *Larger, healthier maize roots with EcoT®,* Working with nature. Plant health products Pty,(Ltd). www.plant-health.co.za/articles/Eco-T_maize.pdf

of roots as well as root hairs, providing better root competence for nutrient absorption[15]. The *Trichoderma* from EcoT also provides a trigger for the plant to synthesise the growth regulators, gibberellins and cytokinins, thereby affording better yields[15]. In addition, EcoT also improves seed germination, seedling development and shoot growth, and overall, promotes healthier plants[13]. EcoT was specifically selected by agriculturalists at the University of KwaZulu-Natal after different trials on various biocontrol agents for the control of plant diseases.

1.6.2.8 Cultivation of Trichoderma harzianum *for the production of metabolites*

Trichoderma species in general can utilise a broad variety of inexpensive carbon and nitrogen sources for their growth by releasing lytic enzymes that break down the plant polymers such as cellulose into simple sugars (Kubicek and Penttilä, 1998; Jayalaksmki *et al.,* 2009). Using various enzymes to produce sugars, they are also capable of the metabolism of several other polymers from vegetal and fungal sources such as hemicelluloses, xylan, mannan, arabinan, β-glucan and chitin, among others (Delgado-Jarana *et al*., 2003). Prominent lytic enzymes produced by *Trichoderma* include chitinase and β-1,3 glucanase (Mitchell and Alexander, 1963; Henis and Chet, 1975). These enzymes are responsible for the degradation of the fungal cell wall, which is made up of chitin and β-glucan. Although all the species of *Trichoderma* produce these lytic enzymes, *T. harzianum* is considered to be the best producer (Ojha and Chatterjee, 2011).

Trichoderma spp. can be grown on different nutrient media, for the production of the lytic enzymes that should kill fungal contaminants. In the present study, sugarcane bagasse (SCB) was used for solid state fermentation (SSF) and potato dextrose broth (PDB) was used for liquid state fermentation (LSF). The basis for their use is described below.

a) Sugar cane bagasse for solid state fermentation

Solid state fermentation is the production of compounds by micro-organisms grown on moist (but lacking free water) insoluble substrates (Chahal, 1985; El-Katatny *et al.,* 2000). Solid state fermentation, unlike liquid state fermentation, does not require complex conditions, so it is preferred over LSF (Hesseltine, 1976), but it does have disadvantages (Chahal, 1982) for large scale production of the biomass of *Trichoderma* compared with the latter. However, LSF requires a sophisticated set-up to carry out the mass production of *Trichoderma* (Kanjanamaneeshathian *et*

[15]Mode of action of EcoT® (*Trichoderma harzianum*) Plant health products pty (Ltd). Available online at http://*www.plant-health.co.za/eco-t_mode-of-action.html*

al., 2003). A further advantage of SSF is that lytic enzymes or secondary metabolites are produced at a higher rate in a short period compared with LSF, and the former does not require aseptic conditions (Hesseltine, 1977; Barrios-Gonzalez *et al*., 1988).

The benefit of choosing SCB is because it is cheap, easily available from the sugarcane industry (Dekker, 1989; Pandey *et al.,* 2000) and rich in carbohydrates (cellulose 50%, hemicelluloses 27.9%, lignin 9.8% and cell content 11.3%) (Kewalramani *et al.,* 1988), thus facilitating production of a maximum biomass of spores of *Trichoderma*. An excellent production of lytic enzymes is promoted when SCB is used (Acuna-Arguelles *et al*., 1994; Barrios-Gonzalez and Mejia, 1996; Chaudhari *et al.*, 2011), it has been favoured as the substrate for the production of lytic enzymes in SSF (Couri *et al.,* 2000).

Studies conducted on using SCB as a substrate medium for *T. harzianum L1* showed that, the biocontrol agent could release lytic enzymes such as chitinase, β-1,3 glucanase, protease and xylanase into the medium, without the addition of any inducer (Jayalakshmi *et al.,* 2009)

b) Potato dextrose broth for liquid state fermentation

Liquid state fermentation is the process in which a substrate of small particles is dissolved in a large amount of water (Chahal, 1985). Potato dextrose broth is a liquid medium used for the growth and cultivation of *Trichoderma*. Potato dextrose broth is rich in potato infusion which promotes fungal growth and the carbohydrate, dextrose, is fermented during the reaction, releasing carbon and acting as an energy source. The pH of potato dextrose broth is generally low, to prevent bacterial contamination[16].

A study was conducted with three different liquid media, PDB, tapioca dextrose broth (TDB) and sorghum dextrose broth (SDB) to compare their efficiency for *Trichoderma* growth[16]. It was shown that, although both PDB and TDB supported the growth, sporulation and metabolite production of the *Trichoderma* species equally well, PDB facilitated the maximum conidial count for *T. harzianum* alone, and when combined with *T. virens* (Mathew *et al*., 2007). Culture filtrates of PDB from different *Trichoderma* species have been used to study the inhibitory effects on contaminants that cause plant diseases, and each one differed in their degree of effectiveness

[16]*Potato Dextrose Broth*, *http://*www.condalab.com/pdf/1261.pdf

against contaminants. For example, the culture filtrates of PDB from three different *Trichoderma* species, *T. harzianum, T. virens* and *T. pseudokoningii*, showed that *T. virens* exhibited the highest inhibition followed by *T. harzianum*, with *T. pseudokoningii* giving the lowest inhibition of *Alternaria porri* that causes onion blotch disease (Imtiaj and Lee, 2008). In the present study culture filtrates from PDB and SCB were used to determine their effects on *T. dregeana* embryonic axes, and on the contaminants isolated from *T. dregeana*.

1.7 Encapsulated (minimal-growth) storage of axes of *Trichilia dregeana*

1.7.1 Alginate gel encapsulation

Alginic acid also called algin or alginate, first described by Stanford (Stanford, 1881; Draget, 2000), is a polysaccharide made up of a linear co-polymer chain consisting of (1→4) β-D-mannuronic (M) acid and α-L-glucuronic (G) acid (Huag, 1964). Alginate is extracted from various marine brown algae (family Phaeophyceae), *viz. Laminaria hyperborea*, *Macrocystis pyrifera*, *Laminaria digitata*, *Ascophyllum nodosum*, *Laminaria japonica*, *Eclonia maxima*, *Lessonia nigrescens*, *Durvillea antarctica* and *Sargassum* spp. (Kloareg and Quatrano, 1988) and also from bacteria such as *Azotobacter vinelandii* and *Pseudomonas* spp. (Gorin and Spencer, 1966; Linker and Jones, 1966; Sutherland, 1977; Valla *et al*., 1996). Alginate used in the present study was extracted from *Macrocystis pyrifera*, commonly called giant kelp, which occurs mainly in coastal waters of the eastern Pacific Ocean, from Baja California extending north to South East Alaska, and also in the southern oceans fringing South America, South Africa and Australia.

A variety of natural polymers, *viz.* sodium, potassium, calcium, ammonium salts of alginate, and derivatives extracted from different brown algal species, as well as synthetic polymers, are extensively used as convenient inoculant carriers in medical, industrial, environmental and agricultural applications (Sandford, 1985). Sodium alginate is the most commonly used gel-matrix because of its moderate viscosity, easy gelling properties, non-toxicity, low cost and biocompatibility characteristics (Saiprasad, 2001). According to that author, in agriculture, alginate beads are used to produce artificial seeds (also referred as synthetic seeds, synseeds, clonal seeds, somatic seeds and somseeds). Alginate beads describe artificially encapsulated plant material, which can be developed into a plant under *in vitro* or *ex vitro* conditions (Murashige, 1978). Production of synthetic seeds was initially limited to somatic embryos, but research has now progressed, facilitating encapsulation of non-embryogenic, *in-vitro*-derived plant material such as various types of unipolar vegetative propagules, micro-tubers, micro-bulbs, corms, rhizomes, micro-cuttings: shoots and nodal segments with apical or axillary buds, differentiating

aggregates such as organogenic callus and primordial, polar vegetative propagules, protocorms and protocorm-like bodies (Redenbaugh, *et al.*, 1987; Redenbaugh, 1992; Standardi and Piccioni, 1998). Among the plant materials studied, shoot tips are the best specimens for artificial seed production as a consequence of the mitotic potential of the meristems (Ballester *et al*., 1997).

Alginate coating of specimens affords multiple advantages including ease of handling, potential long-term storage, transport, delivery, low-cost propagation and subsequent production under *in vitro* and/or *ex vitro* conditions (Ghosh and Sen, 1994; Saiprasad, 2001). Two types of artificial seeds can be produced, desiccated or hydrated: the former are produced using specimens encapsulated in polyoxyethylene glycol followed by desiccation. and the latter involves encapsulation in hydrogel, using sodium alginate. The desiccated encapsulation approach is appropriate for orthodox seeds or other desiccation tolerant propagules, while recalcitrant/desiccation sensitive propagules require to be encapsulated using hydrated synthetic seed technology (Ara *et al*., 2000). Hydrated artificial seeds afford the possibility of axenic preservation of viable germplasm of recalcitrant-seeded species (Saiprasad, 2001). Supplementing the gel matrix with nutrients, generally MS salts (Murashige and Skoog, 1962) and a carbon source (Germana *et al*., 1998) such as sucrose (Ma *et al*., 2011), can extend the viability and avoidance of dehydration of plant tissue (Katouzi *et al*., 2011). In addition to the nutrients, antibiotics (Bekheet, 2006), fungicides, pesticides (Bapat and Rao, 1990) and biocontrol agents (Russo *et al.,* 2001; Haverson and Kimbrough, 2002) can be used as supplements to avoid bacterial or fungal contamination of the artificial seeds.

Research has been carried out on various tissues of different plants including somatic embryos of carrot (Gray and Purohit, 1991; Patel *et al*., 2000) micro-bulbs, rhizomes, protocorms of orchids (Standardi and Piccioni, 1998; Datta *et al*., 1999; Saiprasad and Polisetty, 2003), nodal cuttings of cassava (Danso and Ford, 2003) and shoot buds of apple (Uozumi and Kobayashi, 1995; Micheli *et al*., 2002). Encapsulation of the recalcitrant seeds of *Avicennia marina* in a crude alginate gel after pericarp removal not only extended their storage lifespan without fungal contamination but also had no negative impact on the metabolic activity (respiration, protein synthesis) of the seeds compared with non-encapsulated seeds (Motete *et al*., 1997).

In the present study, synthetic seeds containing the embryonic axes of *Trichilia dregeana* were produced. Due to the recalcitrant nature of *T. dregeana* embryos, a calcium alginate hydrogel was used for encapsulation. To form such artificial seeds, the plant material is coated with the sodium alginate and then dropped into a solution of calcium chloride ($CaCl_2.2H_2O$). This forms firm,

clear and round / isodiametric beads as a result of the ion exchange between Na^+ in sodium alginate and Ca^{2+} ions in calcium chloride (Haug, 1964). To maintain viability during the storage period, sucrose was provided as a carbon source in the alginate. The rationale for this study was that, if successful, this approach will be beneficial for short-term maintenance and transport of explants after retrieval from cryo-storage.

1.8 *Trichilia dregeana*

Trichilia, a genus in the Meliaceae family of woody trees and shrubs, is found in Asia, America and Africa. However, *T. dregeana* and *T. emetica* is the only tree species found in South Africa. *Trichilia dregeana* Sond. commonly called the Natal or Forest mahogany, grows well in places of heavy rainfall. The trees are found mostly in southern Africa (Schmidt *et al.*, 2002), in Pondoland and KwaZulu-Natal, Mpumalanga and Limpopo Provinces in South Africa, and in Swaziland and Zimbabwe, although also encountered in the North of tropical Africa (Pooley, 1993). *Trichilia dregeana* can be easily confused with the *T. emetica*, which is very similar in appearance, but there are minor differences.

Trichilia dregeana trees can reach 35 m, with the trunk of about 1.8 m diameter; the leaves are described as dark green, and imparipinnate with 3-5 pairs of leaflets (Fig.1.3) (Pooley, 1993), and the flowers are creamish-white. In Greek *tricho* refers to the three segments, which are observed when the capsule splits. *Trichilia dregeana* was named after Johan Franz Drege, the first person to have collected the seeds (Palmer and Pitman, 1972). Fruit formation occurs between January and May with many capsules opening during May and June, and others later. *Trichilia dregeana* fruits are roughly spherical, with six seeds per capsule (Pooley, 1993). The seed coat of *Trichilia dregeana* is black, and is almost completely covered by an attractive red to scarlet aril (Palgrave and Palgrave, 2002) as can be seen in Fig.1.4.

Figure 1.3: *T. dregeana* tree
(Photo courtesy of Prof. P. Berjak)

Figure 1.4: Naturally dehisced *T. dregeana* capsules revealing the seeds
(Photo courtesy of Prof. P. Berjak)

Every plant part of *T. dregeana* is used for some purpose: the wood for making furniture, carvings, repairing ships; the seeds as food, and for making soaps, body ointment, oil, etc. (Pooley, 1993; Maroyi, 2007). Preparations of the seeds, leaves, bark and the wood are also used for a variety of medicinal purposes (Palgrave, 1977; Grace *et al*., 2003; Eldeen *et al*., 2005; Krief *et al*., 2005; Eldeen *et al*., 2007; Maroyi, 2007).

Trichilia dregeana seeds were first categorised as recalcitrant because of their requirement for high relative humidity (RH) to survive after they are shed, and their high sensitivity to desiccation (Han *et al*., 1997; Kioko *et al*., 1998; Drew *et al*., 2000). Among the recalcitrant-seeded species worked on in our laboratory, good quality *T. dregeana* seeds have been able to be stored for a few

months at 16°C, without any signs of germination (in storage) because they are shed before complete development of the embryonic axis (Goveia *et al*., 2004).

Fungal proliferation on the developing and mature fruits and seeds and shed seeds of *T. dregeana* has been reported, but, with a few exceptions, most of the contaminants did not affect the seeds in the short-term (Sutherland *et al.,* 2002). Different species of fungus have been isolated from fruits and seeds of *Trichilia dregeana* produced by trees growing in KwaZulu-Natal: These were *Alternaria alternata, Collectotrichum gloeosporoides, Penicillium aurantiogriseum, Pestalotiopsis maculans,* and *Rhizopus nigricans,* from the surface of fruits, while *Colletotrichum* spp*., Fusarium semitectum, Fusarium solani, Fusarium subglutinans,* and *Penicillium* spp. and one species each of *Phoma* and *Phomopsis* were isolated from the fruit tissues (Sutherland *et al.,* 2002).

The seed aril of *T. dregeana* harbours many fungal contaminants, but at the same time, this waxy layer prevents water loss from the seeds (Sutherland *et al.,* 2002) which is very important for their post-shedding/-harvest survival (Drew *et al.,* 2000). Seeds of *T. dregeana* are conventionally stored under hydrated conditions in our laboratory. The embryonic axes of low quality seeds of *T. dregeana* were excised and had to be cultured on a medium containing a systemic fungicide and antibiotic to obtain a significant percentage of uncontaminated survivors (Berjak *et al.*, 2004). In this regard, although fungal contamination within the embryonic axes of *T. dregeana* is not usually observed, its prevalence is seasonal and may also be related to the location where the seeds were collected; however, if there is internal infection, seed storage becomes difficult to impossible (Sutherland *et al*., 2002). Hence it is very important to eliminate infections at an early stage to extend the storage life span of the seeds of *T. dregeana* or of any species where the objective is short- to medium-term hydrated storage. It is equally important that seeds to be used as the source of embryonic axes for cryopreservation, are rendered free of contaminants as an *a priori* requirement.

1.9 Aim and objectives of the current study

The original aim of this project was to determine the possibility of the use of *T. harzianum* as a biocontrol agent in relation to *in vitro* cultivation of embryonic axes of *T. dregreana*, particularly as they constitute the ideal explants for cryopreservation of the germplasm of this (and other) recalcitrant-seeded species. However, due to impracticalities of implementing this means of biocontrol, as the study progressed other means of decontaminating seeds and explants were

investigated. These included application of Nipastat, anodic water, NaDCC, Benlate, and alginate gel encapsulation.

The main objectives of this research were as follows:

1. To ascertain the effects of *T. harzianum* on the embryonic axes of *T. dregeana* by co-culturing them *in vitro*, as well as the effects of *T. harzianum* on selected fungal contaminants which could be associated with the seeds.
2. To determine the effects of culture filtrates of *T. harzianum* on both embryonic axes of *T. dregeana* and the selected contaminants.
3. To determine the effects of different fungicides on the embryonic axes of *T. dregeana* as well as on the isolated fungal contaminants
4. To determine the efficacy of alginate encapsulation of optimally decontaminated explants in extending the storage lifespan of the embryonic axes of *Trichilia dregeana.*

1.8.1 Objectives of the current research

The original objective of the current research was to provide insight into an important, but poorly researched, area, *viz.* the use of biocontrol agents *in vitro*. The study was extended to include the application of other unconventional means for eliminating fungal contaminants prior to culture of embryonic axes, as *in vitro* recovery is a vital procedure upon retrieval of cryopreserved specimens.

CHAPTER 2

MATERIALS AND METHODS

Materials

2.1 Seed collection and handling

Mature fruits (capsules) (Fig. 2.1A) of *Trichilia dregeana* Sond. were hand-harvested from trees in Durban, KwaZulu-Natal, South Africa over the May to July period in 2009 and 2010. The capsules were sealed into buckets,which were taken to the laboratory immediately. The seeds (Fig. 2.1B) were extracted from the capsules and the waxy aril and seed coat (which are closely adherent) removed using a scalpel blade. As necessary, embryonic axes with 2 mm segments of each cotyledon attached (Goveia *et al.*, 2004) were immediately excised and plated *in vitro* (Fig. 2.1C) for the different treatments. The remainder of the seeds were decontaminated with 1% (v/v) sodium hypochlorite (NaOCl) for 20 min and rinsed three times with distilled water to remove all the traces of the chemical, and then air-dried on a paper towel overnight. Thereafter seeds were placed in single layers on plastic mesh inside 5 L plastic buckets, both mesh and containers having been previously sterilised with 1% NaOCl and rinsed with ethanol. In order to maintain the high relative humidity (RH) conditions necessary for hydrated storage, the seeds on the mesh were suspended 200 mm above 1% (v/v) NaOCl-soaked paper towel placed in the base of the bucket, and the lids lined with dry paper towel to prevent any condensate formed from dripping back onto the seeds. These buckets were sealed after dusting the seeds with Benlate 500 WP (active ingredient, benzimidazole [500 g kg^{-1}]; Villa Crop Protection, S. Africa) to curtail fungal proliferation, and stored at 16°C for no longer than 91 d (13 weeks) (Berjak *et al*., 2004).

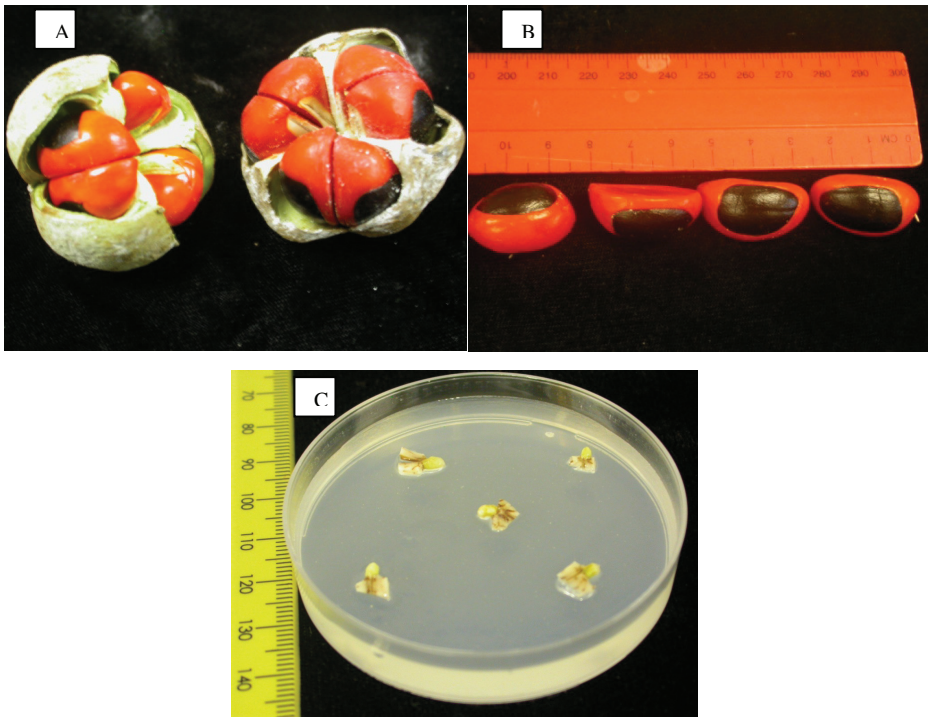

Figure 2.1: *T. dregeana* capsules split open revealing the seeds (A); seeds extracted from the capsule (B); Embryonic axes excised with 2 mm blocks of each cotyledon and plated on half strength Murashige and Skoog (MS) medium (C).

Methods

2.2 Isolation and identification of fungal strains

Fungal contamination assessments were done prior to any further studies. A sample of 20 seeds per treatment was taken immediately after receipt in the laboratory to test for surface contaminants on the axes and/or internal infections. In order to assess surface contaminants, after removal of aril and testa embryonic axes with 2 mm segments of each cotyledon were excised using a sterile scalpel blade, and 10 axes per Petri dish (90 mm) were plated in contact with the medium. Excision and plating of explants (which were not surface-sterilised) on potato dextrose agar (PDA) in Petri dishes was carried out in laminar air-flow. For detection of internal contaminants, the embryonic axes were immersed in 1% (v/v) NaOCl for 10 min and then rinsed in sterile water. Each embryonic axis was then halved longitudinally and both halves of five axes were plated on 90 mm PDA under aseptic conditions. The plates were sealed with Parafilm (American National Can Co., New Orleans) and incubated at 24±1°C in the dark for 10 d to facilitate establishment of fungal colonies. It was realised retrospectively that fungal inoculum

from the seed surface could have contaminated axes during the excision process (Berjak, pers.comm.[17]).

To obtain and maintain pure cultures of the fungi isolated from the embryonic axes, a small portion of the medium with an established fungal colony, or a part of the infected embryonic axis, was excised using a sterile scalpel blade. The fungal colonies were suspended in sterile distilled water and a loopful of the suspension was streaked onto a PDA plate. The plate was then incubated in dark for 5 d at 24±1°C. If the fungal culture obtained was not pure, it was re-streaked until an axenic culture was obtained (Jha, 1995). The fungi were then identified using macroscopic features and microscopically. Identification to genus level was assisted by Makhathini (pers.comm.[18])

For macroscopical identification, the colonies of fungi were distinguished by different growth pattern and colours (spores or colony pigmentation). For microscopical identification, one or two drops of lactophenol-cotton-blue solution were placed on a clean glass slide with the aid of a dropper. A little of the mycelium was removed using an inoculation loop and placed in the drop of the stain. The material was then gently teased out with sterile needles and covered with a glass cover slip with little gentle pressure to eliminate any air-bubbles (James and Natalie, 2001). The preparation was observed using a compound microscope (Carl Zeiss, Germany), first under 100x magnification, to observe the thinner parts of the preparation (generally around the edges of the mounted material) and then under 400x magnification. The fungi isolated were identified macroscopically and microscopically with reference to Raper and Fennel (1965); Ellis (1971); Domsch *et al*. (1980); and Nelson *et al*. (1983), with the five most prevalent, *viz. Rhizopus* sp.; *Penicillum* sp.; *Fusarium* sp.; *A. flavus* and *A. niger*, being selected as the test isolates.

2.3 Standardisation of surface decontamination using sodium hypochlorite, mercuric chloride and calcium hypochlorite

In order to ascertain, and then standardise, the best surface disinfection procedure for the embryonic axes of *T. dregeana*, three decontaminants were tested, *viz.* 1% NaOCl (v/v) (10 min), 0.1% mercuric chloride (w/v) (5 min) and 1% calcium hypochlorite (w/v) (10 min) to each of

[17] Prof. Patricia Berjak, School of Life Sciences, University of KwaZulu-Natal, Durban

[18] Ms Aneliswa Phumzile Makhathini, Faculty of Applied Sciences, Biotechnology Department, Durban University of Technology, Durban

which 2 to 3 drops of Tween 20 were added to reduce the surface tension. Each treatment was followed by rinsing the axes three times with sterile distilled water to remove all traces of the sterilising agent and blotting them on sterile filter paper to remove surface moisture. The efficacy of decontamination protocol was checked by transferring samples of the treated axes on to the germination medium (Section 2.4). Cultures were incubated under a 16 h/8h light/dark photoperiod at 24±1°C. The axes were checked after 1 to 2 weeks for possible contamination. Twenty axes were used for each decontaminant trial and each was replicated thrice.

2.4 Preparation of the growth medium

Germination of the embryonic axes was carried out on half strength (2.21 g L^{-1}) MS (Murashige and Skoog, 1962) medium containing 30 g L^{-1} sucrose and 8 g L^{-1} agar, at pH 5.6-5.8. After autoclaving at 121°C for 15-20 mins, medium was poured into 90 mm Petri dishes under sterile conditions.

2.5 Effect of other decontamination protocols for the embryonic axes of *T. dregeana*

2.5.1 Disinfection using electrolysed oxidising (EO) water

Oxidising water was generated by electrolysis of a solution containing 0.5 µM $CaCl_2.2H_2O$ and 0.5 mM $MgCl_2.6H_2O$ (CaMg), the original CaMg solution having been developed by Mycock (1999). Oxidising water was generated in one of two beakers (linked by a salt bridge [see below]) containing 200 mL sterile CaMg into which the anode had been introduced, the cathode being immersed in the solution in the second beaker.The connecting salt bridge was constituted by saturated potassium chloride (KCl) and agar (3 g KCl and 0.3 g agar in 10 mL sterile distilled water). Electrolysis was effected using a power pack (Bio-Rad™, United States of America) delivering 60 V for 1 h. Electrolysed oxidising (EO) water at a pH *c*. 2.4 was obtained from the solution in which the anode had been immersed (while reduced water, pH *c.* 11.2, was generated in the cathodic chamber). The anodic water fraction was used for disinfecting 20 embryonic axes of *T. dregeana*, which were immersed in the solution in which they were mechanically agitated for 10, 20, 30, 40, 60, 90 and 120 min. Axes were then blotted using sterile filter paper, plated aseptically on half strength MS medium in 90 mm sterile Petri dishes and incubated under 16 h photoperiod conditions at 24±1°C. The axes were monitored daily for contamination, with the final percentages of germinating and contaminated axes being recorded after 3 weeks.

2.5.2 Disinfection using Toxicity method

The potential toxicity (anti-fungal activity) of the various chemical preparations and biological filtrates tested for their potential to curb proliferation of test isolates was assessed *in vitro* after their inclusion in a PDA-based medium: Preparations tested included Nipastat® (a mixture of methyl-, butyl-, ethyl-, propyl- and isobutyl-paraben [Clariant Chemicals Ltd, India]) or Benlate 500 WP (Villa Crop Protection, S. Africa; active ingredient, benzimidazole [500 g kg^{-1}]), as well as culture filtrates of the *Trichoderma harzianum* strains from EcoT® and Eco77® (Powder formulations of *Trichoderma* spp. [$2x10^{9}$$cfug^{-1}$] were obtained from Plant Health Products, KwaZulu- Natal, South Africa, and stored in sealed polyethylene bags at 4°C).

2.5.2.1 Preparation and application of Nipastat

Nipastat was applied by immersing 20 embryonic axes per concentration, *viz.* 0.1%, 0.14% and 0.2%, with 10 axes per 100 mL Erlenmeyer flask each containing 50 mL of aqueous solution for 10 and 20 min, and the treatments were replicated thrice. For the controls, 20 embryonic axes were immersed in sterile distilled water under similar conditions. At ambient temperature, Nipastat is very sparingly soluble in water to a maximum of 0.14%, achieved with constant stirring for 20 min[1,2]. However, a 0.2% solution was able to be made by adding Nipastat to water pre-heated to 80°C in a water bath[1,2]. Axes were plated out on half strength MS medium and assessed for contamination every day and the final results for germination and contamination were recorded after 3 weeks in culture.

As an alternative means of provision of Nipastat, it was added at concentrations of 0.01, 0.02, 0.03 and 0.04% to half strength MS medium by dissolving the appropriate concentration in 10 mL of sterile water, with the solutions subsequently sterilised using 0.22 µm Millipore (Millipore Ireland Ltd, Ireland) membrane filters. These were then added to the autoclaved molten half strength MS medium at 40±3°C to get final concentrations of 0.01, 0.02, 0.03 and 0.04% before it was poured and solidified in 90 mm Petri dishes. For the controls, 10 mL of sterile distilled water were added to the autoclaved medium. Twenty embryonic axes per concentration were used for the experiment and control, with 10 axes in each Petri dish plated under sterile conditions. The percentage germination and the number of contaminated axes were recorded after 3 weeks. In a similar way, an agar disc (5 mm diameter) with fungal mycelium (from 4-d-old cultures) was placed in the centre of each Nipastat-enriched and control PDA medium plate. Plates were incubated at 24±1°C for 7 d, after which the surface area from two radial measurements of the

colonies of the test isolate of controls and treatments was measured. The percentage of growth inhibition (PI) was recorded as follows (Etebarian, 2006):

$$PI\% - (A-B)/Ax100$$

where A = colony area of the test isolate in the presence of culture filtrate, and B = colony area of the test isolate on the corresponding control plate. The treatment and controls per test isolate were replicated three times and the experiment was repeated thrice.

2.5.2.2 Preparation and application of sodium dichloro-isocyanurate (Medi-Chlor®)

Sodium dichloro-isocyanurate (NaDCC) as Medi-Chlor is effervescent, and was dissolved in distilled water to obtain concentrations of 0.1, 0.2 and 0.3%. To evaluate the effect of NaDCC on contamination, 20 embryonic axes for each concentration were immersed in the solutions of NaDCC, or in sterile distilled water (control) for 10 or 20 min, blotted dry and plated aseptically on half strength MS medium under sterile conditions. The effects of exposure to NaDCC on axes plated on half strength MS medium were assessed after 3 weeks in culture. The experiments were repeated thrice.

2.5.2.3 Preparation and application of Benlate

To make up the concentration of 100 ppm (0.01%) of benomyl in the medium, Benlate, a chemical with poor water solubility, was first dissolved in 10 mg mL^{-1} dimethyl sulphoxide (DMSO; Sigma-Aldrich, Germany) to make a stock solution (Straight and Murray, 1997), sterilised by filtration, and then added to 100 mL of PDA medium which was near boiling to avoid Benlate precipitation. The medium was then allowed to cool slowly to room temperature (Shonn *et al.*, 2000; Calhelha *et al*., 2006). Twenty embryonic axes were placed on this Benlate-incorporating half strength MS medium to check the effects on contaminating fungi and bacteria. Similarly, 5 mm diameter mycelial agar discs of 7-d-old cultures of test isolates were cut out of Petri dishes using a sterile needle, and each was placed in the centre of a plate containing the 100 ppm (0.01%) Benlate-MS medium. The inhibition percentages were recorded (as described in section 2.5.2.1). Different concentrations, *viz.* 0.05, 0.1, 0.5 and 1%, were also used, although Benlate from the stock solutions of these concentrations precipitated once added to the medium. Higher concentrations were tested to standardise the best concentration required to counteract fungal proliferation. Three replicates were used per isolate per concentration of the Benlate.

2.6 Biocontrol of contaminants (isolated from embryonic axes of *T. dregeana*) by *Trichoderma harzianum*

Two preparations of the biocontrol agent, *Trichoderma harzianum,* EcoT and Eco77, were used to study their effects on checking or inhibiting the growth of test isolates.

2.6.1 Culture characteristics and growth rate of two isolates of T. harzianum *(EcoT and Eco77)*

Culture differences between the two isolates of *T. harzianum* afforded by EcoT and Eco77, respectively, were studied by placing the mycelial agar discs in the centre of three plates and incubating them on PDA medium in the dark for 14 d at 24±1°C. Differences in pigmentation on the top and underside of the PDA medium by the coloration of the colonies of *T. harzianum* strains themselves were recorded.

The growth rates of the isolates of EcoT and Eco77 were studied by placing a plug of each at the centre of a PDA plate, after which the growth of each isolate per day, was measured and the number of days which the colony took to cover the entire plate was recorded. Both experiments for culture differences and growth rates for EcoT and Eco77 were repeated thrice.

2.6.2 The effect of directly diffusible metabolites produced by T. harzianum *cultures on test isolates*

The two variants of the commercially-available *T. harzianum* preparations, EcoT and Eco77, were screened individually for their mycoparasitic ability using a dual culture method (Dennis and Webster, 1971a). The percentage inhibition of radial growth of the test isolates by EcoT and Eco77, and the number of days taken for the *T. harzianum* to overgrow the test fungal colony totally (colony degradation period [Siddiquee *et al*., 2009]), were calculated.

Dual cultures (Fig. 2.2) were carried out on 20 mL of sterile PDA medium solidified in 90 mm Petri dishes. A 5-mm diameter disc from 5-d-old cultures of each of the test isolates, *Penicillium* sp.*, A. flavus, A. niger* and *Fusarium* sp. was placed 10 mm from the edge of the plate, and on the opposite side, a disc (5 mm diameter) taken from the edge of a 5-d-old pure culture of *T. harzianum* was placed in such a way that it was 55 mm from the test isolate disc. However, in case *of Rhizopus* sp.*,* the *Trichoderma*-bearing disc was first placed 10 mm from one edge of the plate and only after 48 h, was a disc of *Rhizopus* sp. placed 10 mm from the opposite edge of the

plate. This was done to afford the time for metabolites of *T. harzianum* to diffuse into the medium, because the growth rate of the *Rhizopus* sp. was much faster than that of the biocontrol agent. For the controls, the test isolates were introduced 10 mm from the edge of the plate, but without the *T. harzianum* discs. The cultures were incubated at 24±1°C in the dark. The *T. harzianum*/test-isolate dual cultures and controls were replicated three times and the experiment was repeated thrice. After 7 d the radial growth of the test isolates (*Penicillium* sp., *A. flavus, A. niger, Fusarium* sp. and *Rhizopus* sp.) in the presence of the fungal antagonist and on the control plates was measured. The data from the control and test were used to calculate the percentage inhibition of radial growth using the following formula (Hajieghrari *et al.*, 2008):

$$\% \text{ PIRG} = [(R1-R2)/R1] \times 100$$

Where, PIRG = Percentage Inhibition of Radial Growth; R1 = Radial growth of the test isolate in the absence of the antagonist (control); R2 = Radial growth of test isolate in the presence of the antagonist.

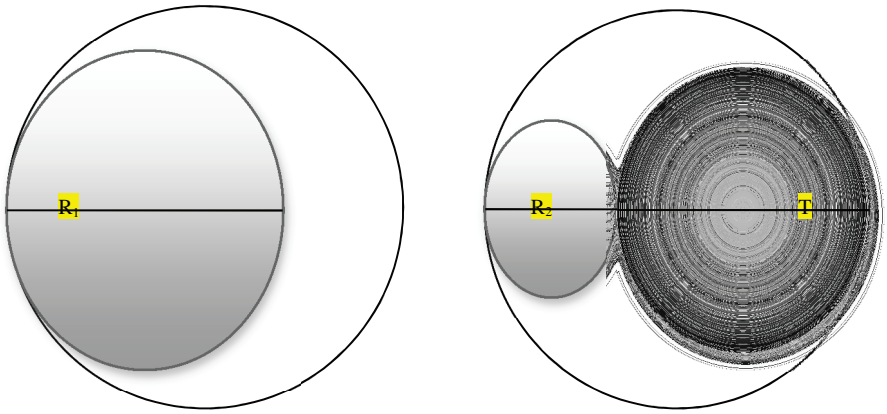

Figure 2.2: Diagrammatic representation of dual culture test showing control (R_1 – test isolate alone introduced at one side) and treatment (R_2 test isolate with *Trichoderma* (T) opposite). The diagram is an example of class 4 type of antagonism explained below.

The plates were observed daily for 7 d, and degree of antagonism between the biocontrol agent and test isolates scored on a scale of 1-5, modified from the scale suggested by Bell *et al.* (1982). Class 1 = antagonist completely overgrew the test isolate by covering the entire plate (100% overgrowth).

Class 2 = antagonist overgrew at least three-quarters of the surface of the test isolate (75% overgrowth).

Class 3 = antagonist overgrew half of the surface of the test isolate (50% overgrowth).

Class 4 = test isolate and the antagonist locked at the point of contact, both appearing to withstand encroachment.

Class 5 = The test isolate completely overgrew the antagonist.

2.6.3 The effect of directly diffusible metabolites produced by T. harzianum *cultures on embryonic axes of* T. dregeana

Decontaminated embryonic axes of *T. dregeana* were used to study the possibility that the diffusible metabolites of *Trichoderma* might affect germination as well as root length, as done for *Arabidopsis* seedlings by Contreras-Cornejo *et al*., (2009) with some modifications. The tests were done with 10 freshly excised embryonic axes as well as with embryonic axes cultured for a week on half strength MS medium. In both cases, five embryonic axes per Petri dish per treatment (EcoT/Eco77) were placed on one side of the plate. A *Trichoderma* (EcoT/Eco77) mycelial plug was placed on the medium opposite the axes. Control axes were cultured without plugs of *Trichoderma*. Results were recorded for germination and root length after 1, 2 and 3 weeks. The experiments were replicated thrice and were repeated thrice.

2.6.4 The effect of volatile metabolites of T. harzianum *against test isolates*

The effect of volatile metabolites evolved by *T. harzianum* on the test isolates was tested using the method described by Dennis and Webster (1971a). Agar discs (5 mm diameter) excised from the edge of 4-d-old pure cultures of each of the *T. harzianum* strains were placed at the centre of PDA plates. Next, a disc of the same size was taken from each test isolate (*Penicillium* sp.*, A. flavus, A. niger, Fusarium* sp.) except for that of *Rhizopus* sp. and placed in a similar way on another agar plate. The lids were removed and each of the test isolate plates was inverted over a plate on which the antagonist had been cultured, and sealed with Parafilm, noting that the head-space between the plates prevented any physical contact between the test isolate and antagonist (Fig. 2.3). In the case of *Rhizopus* sp., (for the reason given in 2.6.2) the antagonist alone was plated first and, after 48 h, the plate on which the *Rhizopus* sp. plug had been introduced was inverted over the antagonist plate and sealed as described above. As controls, the test isolate plates were inverted over uninoculated plates (Dennis and Webster, 1971a). The assessment per isolate per treatment (EcoT/Eco77) and controls was done in triplicate, and the experiment repeated thrice. All plates were incubated for 96 h at 24±1°C with 12 h/12 h light/dark

photoperiod. After 96 h, colony diameters of the test isolates in all the treatments and the control were measured, and PIRG was calculated using the formula given above (section 2.6.2).

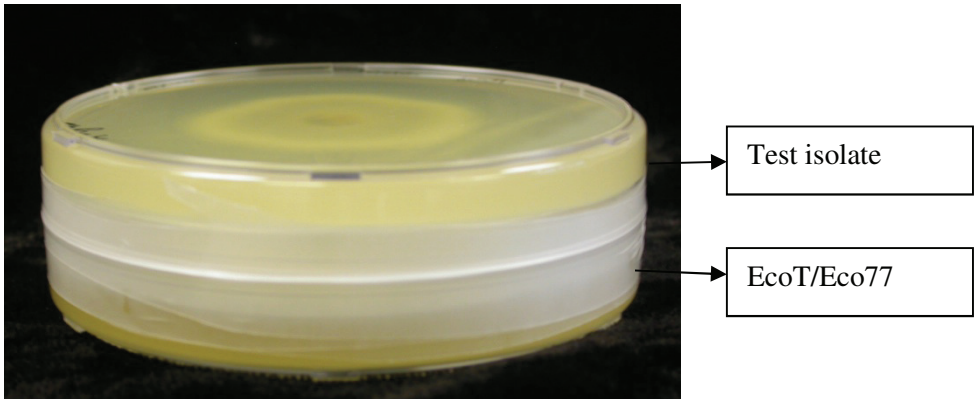

Figure 2.3: Set-up to test for the effects of volatile metabolites produced by *Trichoderma* on test isolates.

2.6.5 The effect of non-volatile metabolites of T. harzianum *against test isolates*

Cultures of *T. harzianum* (EcoT/Eco77) were grown on potato dextrose broth (PDB) and sugarcane bagasse (SCB) medium in order to harvest the non-volatile metabolites produced by the *Trichoderma* strains.

2.6.5.1 Extraction of non-volatile substances using sugarcane bagasse (SCB) and their antifungal effects

Extraction of non-volatile metabolites using SCB was carried out by the method described below (Jayalaksmi *et al*., 2009) with some modifications. Sugarcane bagasse (a fibrous waste product from the sugar refining industry) was collected from the South African Sugar Research Institute (SASRI). It was first washed with distilled water, after which it was dried at 80°C for 24 h and crushed using a blender (Waring®, USA).

The SCB was then passed through a 125 µm sieve (Star Screens Test Sieve, South Africa) and 10 g of the powder transferred to each of six 250 mL Erlenmeyer flasks, three for each of EcoT and Eco77, moistened with 10 mL of mineral salts solution in distilled water (KH_2PO_4, 2.5 g L^{-1}; KNO_3, 5.0 g L^{-1}; $MgSO_4$, $7H_2O$, 1.0 g L^{-1}; Na_2SO_4, 1.0 g L^{-1}; $FeCl_2$, 0.02 g L^{-1}; $ZnSO_4$, $7H_2O$, 0.0015 g L^{-1}; $CuSO_4$. $5H_2O$, 0.003 g L^{-1} and $MnSO_4$, 0.001 g L^{-1}), sterilised twice by autoclaving (as done by Jayalakshmi *et al*. [2009]), cooled and inoculated with 1 mL of conidial suspension of

either EcoT or Eco77 ($4x10^7$ conidia mL^{-1}). The flasks were incubated at 28±2°C for 7, 14, 21 and 28 d under dark conditions to allow *Trichoderma* growth (Fig. 2.4). According to Jayalakshmi *et al.* (2009), enzymes such as β-glucanase, chitinase, protease and xylanase would be extracted from the SCB-based medium after the incubation period. Any enzymes so-produced by *T. harzianum* were extracted twice from the medium with 50 mL of 50 mM sodium acetate buffer (pH 6.5), this buffer being recommended due to its extraction efficiency for enzymes (D'Souza *et al*., 1999; Heck *et al*., 2002; Rezaei *et al*., 2011) and the contents squeezed twice through wet muslin cloth and later filtered twice through Whatman No. 1 filter paper. The pooled extracts were centrifuged (15 min at 15,000 g) at 4°C. The clear supernatant was collected and used as the source of enzymes extracted from *T. harzianum*, and the pellet discarded. The supernatant was sterilised using 0.22 µm Millipore membrane filters, and used for the treatments described below. The enzyme extracts were used to treat the embryonic axes and the test isolates. The same procedure was carried out for the control (SCB without *T. harzianum* [EcoT/Eco77]).

The sterilised filtrate was incorporated into autoclaved PDA in the ratio of 1:1 before the medium was solidified. Under sterile conditions, 20 mL of the filtrate-PDA mixture was then poured into each of 60 (90 mm) Petri dishes, three for each of five test isolates per incubation period. Tests were performed using this *Trichoderma* culture filtrate for the treatment and *Trichoderma*-free culture filtrate for the controls, with assessments being made in terms of the growth inhibition of test isolates. This was done by placing a mycelial plug of each test isolate on the centre of a plate of PDA medium and the tests were replicated thrice for each test isolate for each incubation time and the experiments were repeated thrice, to ascertain what incubation period was best to achieve the greatest inhibition of the test isolates.

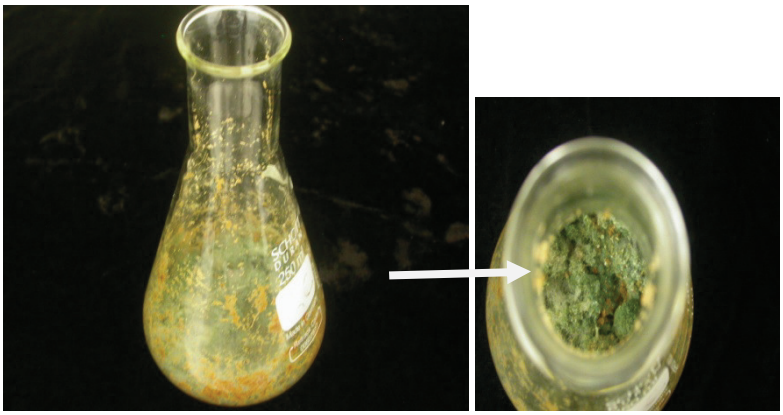

Figure 2.4: Sugarcane bagasse medium with *Trichoderma* mycelium in 250 mL volumetric flask

2.6.5.2 Extraction and assessment of anti-fungal effects of non-volatile compounds from potato dextrose broth (PDB)

Extraction of non-volatile antibiotics using PDB was carried out according to El-Katatny *et al*., (2000) and Imtiaj and Lee (2008). Erlenmeyer flasks (100 mL capacity) each containing 20 mL of PDB were sterilised by autoclaving for 20 min, cooled to room temperature and inoculated with six discs (each 5 mm in diameter) per flask (Fig. 2.5A) of 5-d-old mycelium of EcoT or Eco77 previously grown on PDA at 24±1°C. After inoculation, the Erlenmeyer flasks were incubated on a rotary shaker at 150 rpm at 24±1°C for time intervals varying from 7 to 28 d. After incubation, the cultures were filtered through three layers of Whatman No. 1 filter paper to remove the mycelium. The filtrate was then passed through 0.22 µm Millipore filters to obtain spore-free culture filtrates (Fig. 2.5B). The control (culture) filtrate was not inoculated.

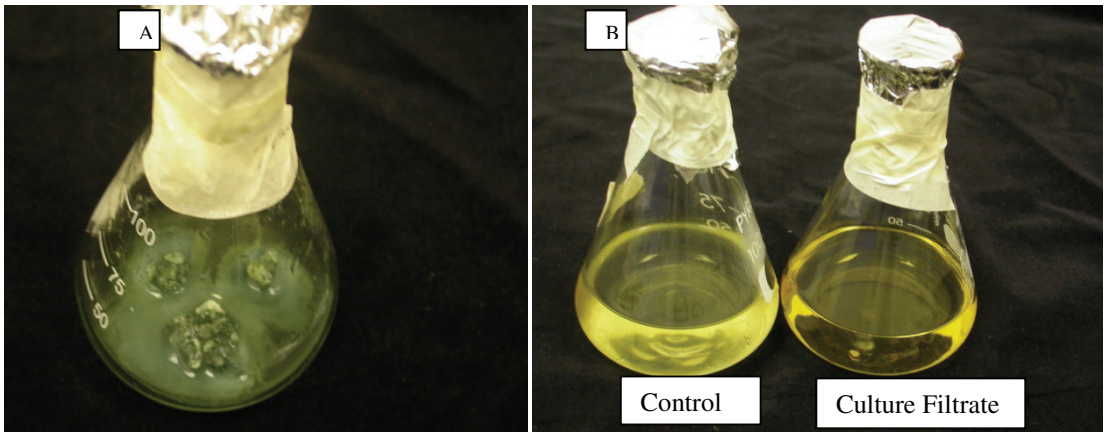

Figure 2.5: Potato dextrose broth (PDB) with *Trichoderma* mycelial plugs in 250 mL conical flasks before extraction (A), and (B) after extraction showing the control and culture filtrate.

Filtrate (10 mL) in which *T. harzianum* from EcoT or Eco77 had been cultured was introduced into 90 mm sterile Petri dishes, immediately followed by 10 mL of molten PDA at 40±3°C, and well-mixed to obtain a final concentration of 50% (v/v). After the agar solidified, 5-mm-wide mycelial discs of the test isolates (*Penicillium* sp., *A. flavus, A. niger, Fusarium* sp. and *Rhizopus* sp.) were placed at the centre of three Petri dishes per isolate and incubated at 24±1°C for 7 d in the dark. Potato dextrose agar with non-inoculated filtrates served as the control. The surface area from two radial measurements of the colonies of the test isolate of controls and treatments was measured and calculated on the seventh day. The percentage inhibitions (PI%) were recorded (as

described in section 2.5.2.1). Three Petri dishes per isolate were assessed and the experiment was repeated thrice.

2.6.6 The effect of non-volatile metabolites of T. harzianum *on the embryonic axes of* T. dregeana

Effects of culture filtrates on the embryonic axes of *T. dregeana* were studied in terms of axis germination and the percentage contaminated, on media incorporating the culture filtrates, as well as after axis immersion in the culture filtrates prepared by both the PDB and SCB methods.

To study the effects of the culture filtrates on proliferation of contaminants from the embryonic axes of *T. dregeana*, two different experiments were performed. For the first, the culture filtrate was incorporated in the half strength MS medium; 20 embryonic axes for the control were not decontaminated with 1% NaOCl, but rinsed thrice with distilled water, blotted dry and plated on half strength MS medium in which control filtrate (i.e. which had not been inoculated with *T. harzianum*) had been incorporated. To test the effects of non-volatile metabolites of *T. harzianum*, 20 embryonic axes for the treatment were similarly not decontaminated and plated on the medium enriched with the *T. harzianum* filtrate. The experiment per treatment (EcoT/Eco77) was repeated thrice. For the second experiment, without prior decontamination as above, 20 embryonic axes for each time interval (1, 2, 3 and 4 h) per treatment (EcoT/Eco77) were immersed into control filtrate or in the culture filtrates of *T. harzianum* in 100 mL Ehlenmeyer flasks. The Ehlenmeyer flasks containing 20 mL of culture filtrate with 10 axes were rotated on an orbital shaker to allow aeration, for 1, 2, 3 and 4 h. Thereafter, the embryonic axes were blotted dry and 10 embryonic axes per 90 mm Petri dish were plated on half strength MS medium (as described above) under sterile conditions. Observations of contamination and germination were made daily, the final percentages being recorded after 3 weeks and all treatments were repeated thrice.

In order to evaluate the effect of the culture filtrate on germination alone, both control and the test embryonic axes were decontaminated with 1% NaOCl for 10 min, rinsed three times with sterile distilled water and blotted dry. Twenty embryonic axes were plated under sterile conditions on half strength MS medium incorporating either the control or *T. harzianum* filtrates in 90 mm Petri dishes, which were then maintained under 16 h/8 h light/dark photoperiod conditions at 24±1°C. Twenty decontaminated embryonic axes for each time interval were also immersed in control or in *T. harzianum* culture filtrates for 1, 2, 3 and 4 h, blotted dry and plated on half strength MS

medium. Observations of germination were made daily, and final percentages recorded after 3 weeks; each treatment was repeated thrice.

2.6.7 Inoculation of embryonic axes of T. dregeana *with conidia of* Trichoderma

The strains of *Trichoderma harzianum*, used in bio-control studies are generally applied as conidial preparations (Coley-Smith *et al.*, 1991 and Papavizas, 1992). Conidial suspensions of *T. harzianum* (EcoT/Eco77) were prepared by mixing 1 g ($2x10^9 cfug^{-1}$) of the powder formulation in 10 mL of sterile distilled water in a test tube (the suspension containing ~$2x10^8$ conidia [cfu] mL^{-1}). This suspension was then serially diluted to obtain different dilutions, *viz*. $1x10^8$, $2x10^7$, $1x10^7$, $2x10^6$ and $1x10^6$ conidia mL^{-1}.

In a preliminary experiment aimed at ascertaining the most appropriate dilution of the conidial suspension for exposure of the explants, embryonic axes of *T. dregeana* were first excised using a sterile scalpel blade. Twenty embryonic axes for each dilution ($2x10^8$, $1x10^8$, $2x10^7$, $1x10^7$, $2x10^6$ and $1x10^6$ conidia mL^{-1}) were surface decontaminated with 1% NaOCl and were immersed in the *Trichoderma* conidial suspension for 10 min. For each conidial preparation, 20 axes were immersed as a sample in 20 mL. Axes were then rinsed three times with sterile distilled water. For the equivalent controls, 20 axes were immersed into sterile distilled water; 20 axes were used for each experiment and control and each was repeated thrice. Among the dilutions tested $1x10^6$ conidia mL^{-1} was standardised for the following steps.

2.6.7.1 Inoculation

The embryonic axes were inoculated with *Trichoderma* using different methods: first, by placing the axes (which had been decontaminated with 1% NaOCl) in contact with medium flooded with *Trichoderma* suspension for different time intervals (24, 48 and 72 h). This was done by introducing 1 mL of $1x10^6$ conidia mL^{-1} (see below) of EcoT or Eco77 using a 1 mL pipette into 65 mm Petri dishes containing 15 mL solidified water agar, five embryonic axes were plated on each Petri dish with 20 axes per treatment (EcoT/Eco77). Water agar was made by adding 8 g L^{-1}agar to distilled water, after adjusting (pH 5.6–5.8) using (0.1 M) NaOH or (0.1 M) HCl, autoclaved and poured into 65 mm Petri dishes under sterile conditions. A concentration of $1x10^6$ conidia mL^{-1} was ultimately chosen for the suspension after trials to check the concentration to avoid overgrowth of the axes by *Trichoderma*: these trials involved immersing 20 embryonic axes for 10 min in 10 mL conidial dilutions of $2x10^8$, $1x10^8$, $2x10^7$, $1x10^7$, $2x10^6$, $1x10^6$ and

$2x10^5$ conidia mL^{-1} followed by plating on half strength MS medium. Observations were made daily and the results for overgrowth by *Trichoderma* was recorded after 3 days.

As a second approach, 20 embryonic axes (which had been previously decontaminated with 1% NaOCl) per treatment (EcoT/Eco77) with five plated on each 65 mm Petri dish were set to germinate for 24, 48 and 72 h on a water agar medium which was previously inoculated with the biocontrol agent 5 d previously by placing a mycelial plug of 5-d-old *Trichoderma* in the centre of the plate.

The third method involved immersing 10 embryonic axes (which had been previously decontaminated with 1% NaOCl) in 10 mL conidial suspension at a concentration of $1x10^6$ conidia $mL^{-1}$for different times ranging from 10, 20 and 30 min, to ensure that adequate time was allowed for conidia to adhere to all axes; 30 min immersion was ultimately chosen. Axes were then plated on half strength MS medium.

2.6.7.2 Co-cultivation step

Following the *Trichoderma* (EcoT/Eco77) inoculation phase, 20 axes each from all three trials were cultured on half strength MS medium for 24, 48 and 72 h. The axes from each treatment and each time interval were either rinsed with sterile distilled water or surface decontaminated with 1% NaOCl, plated on half strength MS medium and successful inoculation confirmed by the growth of *Trichoderma* around the axes. The trial in which axes were immersed for 30 min with *Trichoderma* suspension and co-cultivated for 24 h were used for further tests.

2.6.8 Light microscopy

A sample of five embryonic axes from the co-cultivation step of the standardised (third) trial was used for microscopical studies. Segments of the radicle and cotyledon were finely cut with a sterile scalpel blade and fixed by dipping in a mixture of ethanol:dichloromethane (3:1, v/v) containing 0·15% trichloroacetic acid for 24 h at 24±1°C (Anguelova-Merhar *et al.*, 2003). The fixed tissues were washed twice with 50% aqueous ethanol for 15 min each, then twice with 0.05 M NaOH for 15 min each, and thrice with water, followed by immersion in 0·1 M Tris–HCl buffer (pH 8.5) for 30 min. The specimens were then stained for 5 min with 0·1% Uvitex 2B (Ciba Geigy, Basel, Switzerland) in 0·1 M Tris–HCl buffer (pH 8.5) following the method of Rohringer *et al*. (1977), but replacing Calcofluor stain with Uvitex 2B (Anguelova-Merhar *et al*.,

2003). The specimens were washed four times with distilled water (10 min each) and then immersed in 25% glycerol for 30 min. Specimens were stored in 50% glycerol (containing a trace of lactophenol as a preservative) until they were examined with a Nikon E- 400 microscope equipped with epifluorescence optics. The filter combination, UV 2-A (excitation filter 330-380 nm and barrier filter 420 nm) was used to visualise the fungal structures by their light blue fluorescence, while the B2-A combination (excitation filter 450-490 nm and barrier filter 490 nm) was used for autofluorescence assessment. Images were captured at 400x magnification. Five embryonic axes from each time interval in the co-cultivation step from the third trial were used and the experiment was repeated three times.

2.6.9 Inhibition of external proliferation of Trichoderma

Following the co-cultivation step, all 20 embryonic axes were sub-cultured on a Benlate-containing medium (100 ppm Benlate incorporated into half strength MS medium as described in section 2.5.2.3). This was done to inhibit the further proliferation of *Trichoderma* (Khan and Shahzad, 2007), which could have otherwise have led to its overgrowing the axes.

2.6.10 Growth and biomass studies

2.6.10.1 Assessment of length and dry weight of roots and seedlings

Root lengths were recorded for seedlings that germinated from the embryonic axes treated with *Trichoderma*, as well as for the controls. Seedlings and roots were sampled for biomass assessments 6 weeks (42 d) after the axes were cultured. As required, five seedlings and roots which were separated from the piece of cotyledon and shoot were taken for water content determination, weighed individually on aluminium-foil weighing boats using a Mettler AE 240 5-place balance and the fresh weight (FW) recorded. Thereafter, specimens were dried to constant mass in an oven at 80°C for 48 h and the dry weight (DW) of each was determined. Water content was expressed on dry mass basis as grams water per gram dry mass (g g^{-1}). The experiment for roots and seedlings were repeated thrice.

2.6.11 Inoculation of the Trichoderma*-treated embryonic axes of* T. dregeana *with* Penicillium *sp.*

Two different tests were conducted, the first by infecting 20 embryonic axes (which were previously decontaminated with 1% NaOCl) with *Penicillium* sp., after completely inhibiting the growth of *Trichoderma* with which they had been co-cultivated for 24 h, with Benlate as in 2.6.9.

For the second test, 20 embryonic axes which had been co-cultivated with *Trichoderma* for 24 h were infected with *Penicillum* sp. but without completely inhibiting the growth of *Trichoderma.* The infected axes were then plated on half strength MS medium for further 24 h (allowing time potentially for *Trichoderma* to completely inhibiting the growth of the *Penicillium* sp.) and then transferred to the Benlate-enriched medium. Both experiments were repeated thrice.

2.6.11.1 Preparation of spore suspension of Penicillium *sp. and inoculation*

The *Penicillium* sp. was incubated in the dark on potato dextrose agar (PDA) medium at 25°C in 90 mm Petri dishes. After 3 weeks of growth the spore suspension was prepared by pouring 10 mL sterile water with few drops of Tween 20 on to the culture and scraping the spores from the plate using a sterile inoculation loop. The number of spores in the suspension obtained was determined using a haemocytometer and adjusted with sterile distilled water to 1×10^6 spores mL^{-1} (assisted by Singh [pers.comm.[19]]). Twenty embryonic axes were inoculated by hypodermic injection (Salama, 1979) of 10 µL spore suspension containing 1×10^6 spores mL^{-1} into the epicotyl.

2.6.12 Effect of Trichoderma *inoculation in the context of contamination of the embryonic axes of* T. dregeana

In order to ascertain the effect of *Trichoderma* inoculation on inherent contamination, 20 non-decontaminated embryonic axes were rinsed with distilled water (three times) before *Trichoderma* inoculation by immersion in $1x10^6$ conidia mL^{-1} for 30 min, blotted dry and plated on half strength MS medium. After 24 h incubation, the axes were plated on Benlate-enriched PDA medium. Twenty non-decontaminated axes constituting the control sample were rinsed with distilled water and immersed further with distilled water for 30 min. Although Benlate is said to be effective against *Penicillium* spp. (Harding, 1968; Spalding *et al*., 1969; Asare-Bediako *et al*., 2007), *Rhizopus* spp. are not completely inhibited (Gilman, 1957; Fry, 1982; Eckert and Ogawa, 1988). (Note that while the present study (described in 2.5.2.3 for the test isolates) showed that 0.01% (100 ppm) Benlate-enriched PDA medium did not inhibit the species of *Penicillium* or *Rhizopus*, but *Fusarium* sp., *A. niger* and *A. flavus* were completely inhibited). The experiment was replicated three times.

[19] Dr. Nisha Singh, School of Life Sciences, University of KwaZulu-Natal, Durban Durban, South Africa.

2.7 Storage of the embryonic axes of *T. dregeana* using alginate gel encapsulation

Ten embryonic axes of *T. dregeana* per treatment were decontaminated using the standardised decontaminants individually (NaDCC [0.2 and 0.3% for 20 min] and Nipastat [0.2% for 10 min]), while control axes were exposed to sterile distilled water. Axes were then blotted dry using sterile filter paper and encapsulated with alginate gel prior to storage.

2.7.1 Alginate gel encapsulation, storage and assessment

Alginate gel encapsulation was carried out in two steps. Initially, ten decontaminated embryonic axes were immersed in a medium of 2% (w/v) sodium alginate (Sigma-Aldrich®, Germany) either containing 0.5% sucrose (w/v) or without sucrose, for 5 min. The embryonic axes were individually collected on the tip of a 5mm-diameter pipette and immersed in a 0.1M $CaCl_2$ solution for 1 h to allow polymerisation of the alginate gel. The beads were then blot dried with sterile filter paper and stored moist in monolayers on a grid suspended over 1% NaOCl-moistened paper towel in a closed container at 16±2°C. Ten axes were assessed for viability and contamination after three 14 d intervals to 42 d. The experiments were replicated thrice and repeated thrice.

2.7.2 The effect of the use of different storage containment on alginate-encapsulated axes of T. dregeana

In order to compare the survival of encapsulated axes stored in a saturated atmosphere (as described in section 2.7.1) to those stored without humidification, an experiment was conducted using different types of air-tight containment. Four types of storage containment were used in this study, *viz.* Magenta boxes® (hydrated storage) as described above, Eppendorf tubes® (0.5 mL) leaving minimal headspace, plastic bags (Ziploc®, Johnson & Son, South Africa (Pty) Ltd) and foil-lined bags (165x280 mm; Tear Nick metalized pouches, Laboratory and Analytical Supplies, Durban, South Africa). Both the plastic and the foil-lined bags were cut to size and tightly sealed using a heat sealer, leaving minimal air space. All the containers used for storing the encapsulated axes in the present study were irradiated under UV light for 30 min prior to the experiment. Ten embryonic axes were excised and treated with either with NaDCC or distilled water (control), and encapsulated (as described in section 2.7.1) in calcium alginate containing 0.5% sucrose (Fig. 2.6) and 10 non-encapsulated axes (Fig. 2.7) were blotted with sterile filter paper. All samples (n=10) were stored at 16±2°C as three separate lots in each type of containment (n=10; total 30 per containment-type). Ten axes were assessed for viability and contamination after three 14 d

intervals up to 42 d. Five embryonic axes from both encapsulated and non-encapsulated axes after the NaDCC treatment were sampled at the end of storage period (42 d), to ascertain the water contents after storage in the different containers. The experiments were repeated thrice.

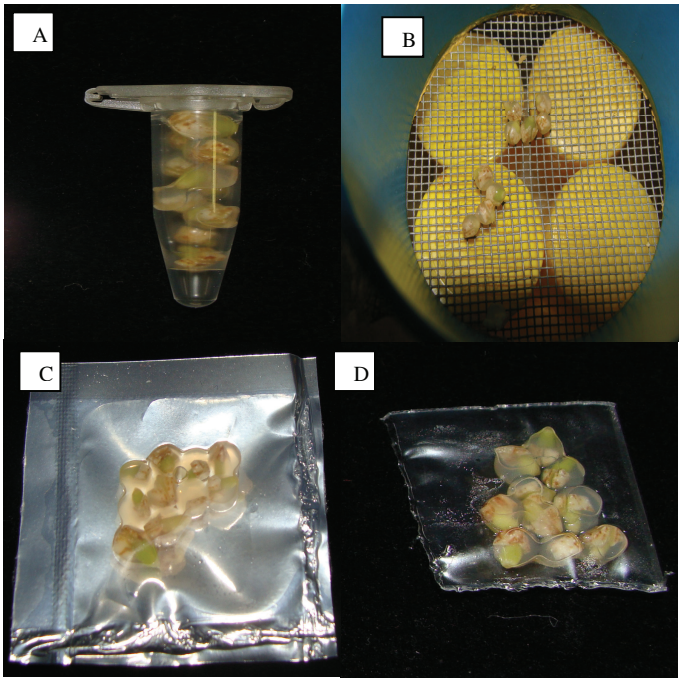

Figure 2.6: Alginate encapsulated embryonic axes of *T. dregeana* within a 0.5 mL Eppendorf tube (A), a Magenta box (B), a foil-lined bag (C) and a polythene bag (D), are illustrated.

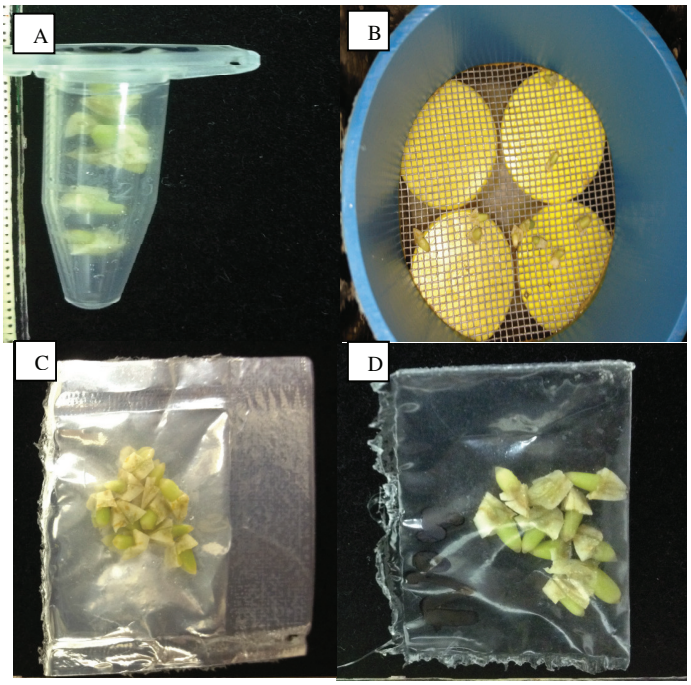

Figure 2.7: Non-encapsulated embryonic axes of *T. dregeana* stored in a 0.5 mL Eppendorf tube (A), a Magenta box (B), a foil-lined bag (C), and a polythene bag (D) are illustrated.

2.8 Statistical analysis

The data obtained were analysed using SPSS 19.0 for Windows 7. The data in percentages that were not normally distributed were subjected to arcsine transformation before analysis, but the original data (untransformed) are presented in the results. One-way and two-way analyses of variance (ANOVA) were used to analyse the data and the means were compared using Duncan's multiple range test ($p \leq 0.05$). T-test and Chi square tests were used where necessary to test the differences between two samples.

CHAPTER 3

RESULTS

3.1 Isolation of microflora from *T. dregeana*

Freshly harvested embryonic axes of *T. dregeana* harboured both fungi and bacteria on their surfaces (Fig. 3.1); no contaminants were isolated from the internal tissues (Table 3.1). Ultimately, five fungal species from the surface of the embryonic axes, *viz. A. niger*, *A. flavus*, *Fusarium* sp., *Penicillium* sp. and *Rhizopus* sp. were cultured axenically, identified and the effects of different decontaminants tested to achieve best results.

When fungi were isolated from the surfaces of embryonic axes excised from newly-harvested seeds of *T. dregeana,* only 1.7–2.8% showed contamination by *Aspergillus niger, A. flavus* and *Fusarium* spp. (Table 3.1). *Rhizopus* spp. were isolated from 24.4% of axes, while 47.8% were contaminated with *Penicillium* spp. Fungal proliferation from contaminated axes was visible from the third day, except in the case of *Rhizopus* spp., where growth was observed from the first day. Bacterial contamination was also evident on the surface of the embryonic axes but neither bacteria nor fungi were isolated from the internal tissues from the excised embryonic axes from newly-harvested seeds. However, embryonic axes excised from the seeds stored for more than three months were internally infected by *Penicillium* spp., which had emerged as the dominant surface contaminant of axes from fresh seeds.

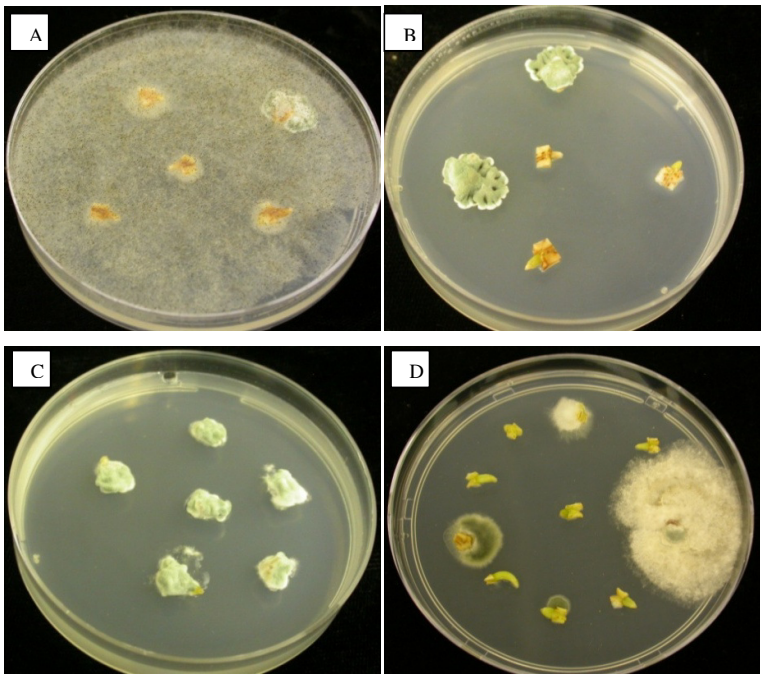

Figure 3.1: Embryonic axes of *T. dregeana* after 2 weeks in half strength MS medium (90 mm Petridish) culture showing contamination of *Rhizopus* spp. and *Penicillium* spp. (A), *Penicillium* spp. alone (B&C), *Penicillium* spp. and *Fusarium* spp. (D).

Table 3.1: Incidence of fungi on the surfaces and internal tissues of embryonic axes from freshly-harvested seeds of *T. dregeana* before storage and after 3 weeks in culture, (n = 60). Mean values followed by the different letters within columns are significantly different (Dependent variables were subjected to Arcsine transformation, one-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$).

Microflora isolated		% of axes showing surface contamination by fungi	% of axes showing internal infection
Fungi	*A. niger*	2.8^{ad}	0.0^{a}
	A. flavus	1.7^{a}	0.0^{a}
	Fusarium spp.	1.7^{a}	0.0^{a}
	Pencillium spp.	47.8^{b}	0.0^{a}
	Rhizopus spp.	24.4^{c}	0.0^{a}
	Unknown isolates	8.3^{d}	0.0^{a}

Axenic cultures (test isolates) were prepared and first identified macroscopically by colour and sometimes texture: *Penicillium* sp. was greenish or blue-green (Fig. 3.2A); colonies of *Rhizopus* sp. were very fast growing, covering the entire plate, with an initially white, cottony/fuzzy mycelium (Fig. 3.2B). As the colonies of *Fusarium* sp. matured, the colour changed from white to pink (Fig. 3.2C, the test isolate chosen), while colonies of other *Fusarium* species developed purple or yellow coloration. Originally white colonies of *Aspergillus* spp. became greenish-blue to yellow (*A. flavus*) (Fig. 3.2D) or black (*A. niger*) (Fig. 3.2E) as they matured.

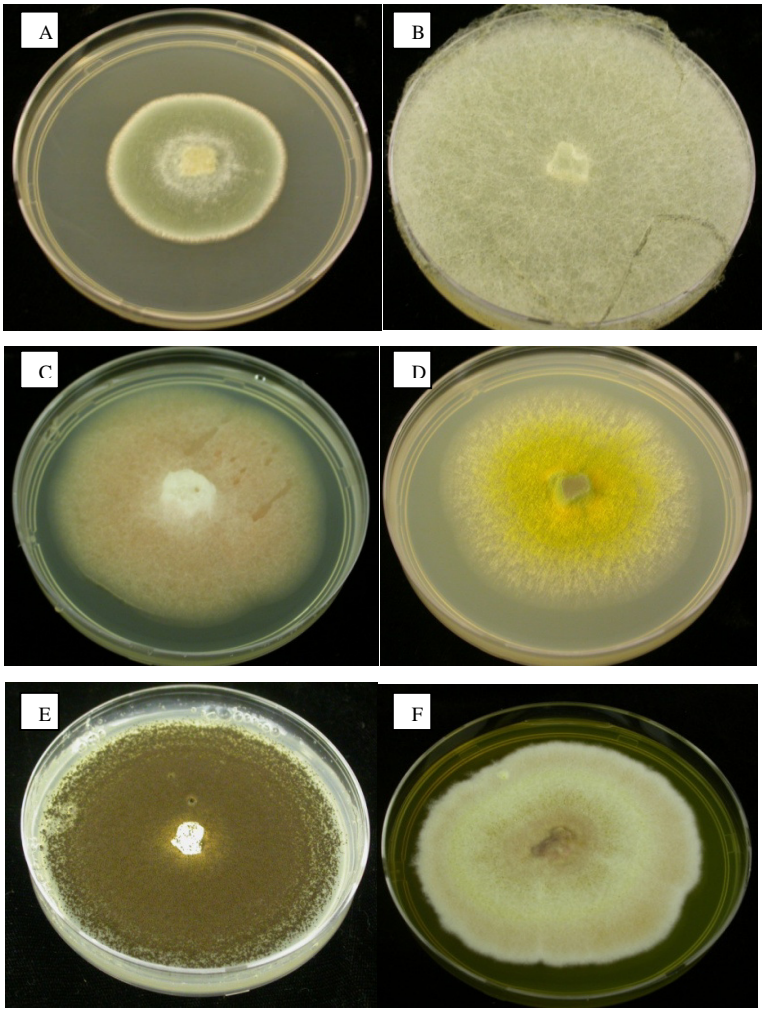

Figure 3.2: Pure cultures of fungi isolated from the embryonic axes of *T. dregeana* grown on PDA medium in 90mm Petri dishes for 2 weeks: *Rhizopus* sp. [A]; *Penicillum* sp. [B]; *Fusarium* sp. [C]; *A. flavus* [D]; *A. niger* [E]; Unknown isolate (F).

All the test isolates were also identified microscopically. Some of the features used for identifications are shown in Figure 3.3 and described in the caption.

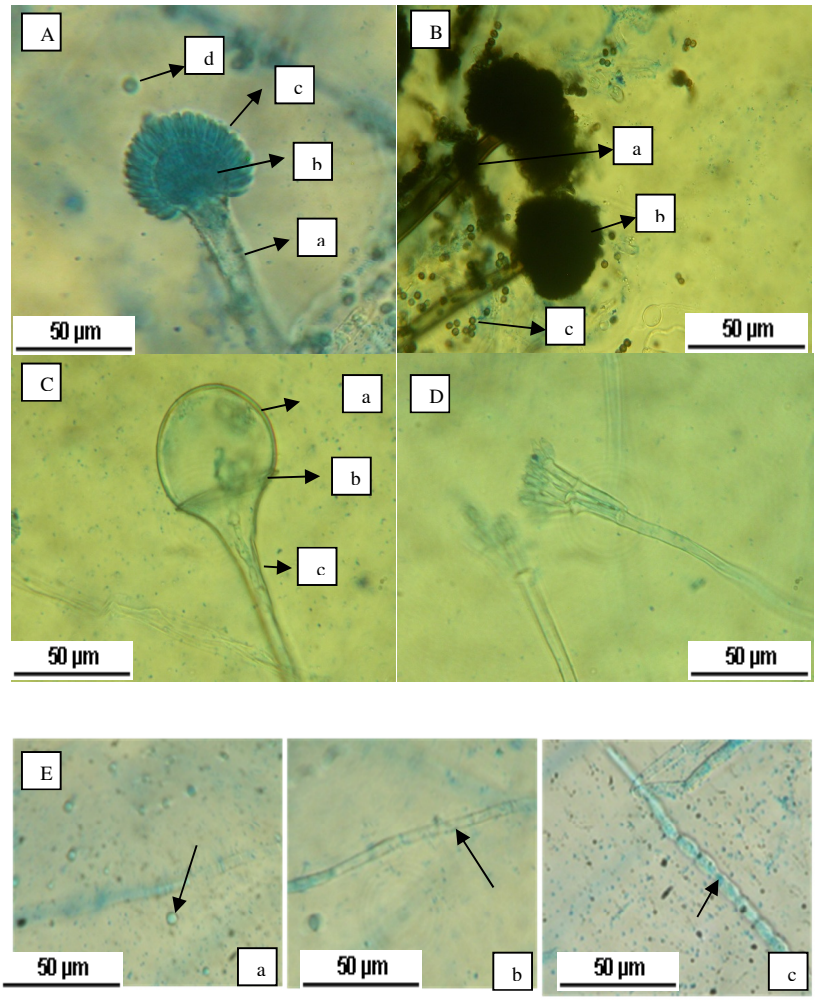

Figure 3.3: *Aspergillus flavus* (Fig. 3.3A) showing a conidiophore (a) with a conidial head with globose to subglobose, uncoloured vesicle (b) having radiating phialides (c) with conidia dispersed–off the phialides are globose to ellipsoidal in apperarance (d); *Aspergillus niger* (Fig. 3.3B), showing a conidiophore (a) bearing a conidial head with large, globose and dark brown to black vesicle (b) having radiating phialides (not apparent due to the dark color) with conidia (c) dispersed-off the phialides are brown to black, rough and globose in appearance; *Rhizopus* sp. (Fig. 3.3C), showing a spherical structure called sporangium (a) where the sporangiospores are produced (sporangiospores at the tip of sporangia are not apparent), sporangia is supported by hemispherical columella (b), atop a long stalk, sporangiophore (c) ; *Penicillium* sp. (Fig. 3.3D), showing conidiophores with brush-like clusters of phialides bearing conidia at the tip; *Fusarium* sp. (Fig. 3.3E) showing ovoid microconidia (a), thread-like hyphae (b) and thick walled chlamydospores in chains (c), macroconidia not visible.

3.2 Effect of surface decontaminants on *T. dregeana*

A comparative study of different decontaminants, *viz*. 0.1% (w/v) mercuric chloride ($HgCl_2$), 1% (v/v) sodium hypochlorite (NaOCl) and 1% (w/v) calcium hypochlorite ($Ca(OCl)_2$ was carried out (Table 3.2) on the basis of reducing contamination and their effect on the viability of the embryonic axes. The percentage of contaminated axes was significantly reduced by all the decontaminants compared with the control (Table 3.2). Although treatment with calcium hypochlorite showed no residual fungal contamination, bacterial contamination of 11.7% of axes was revealed (Table 3.3).

Table 3.2: Effect of different decontaminants on the viability and residual contamination of the embryonic axes of *T. dregeana* after 3 weeks in culture. Mean values followed by the different letters within columns are significantly different, (n = 60). (Dependent variables were subjected to Arcsine transformation, one-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$).

Treatment with:	Germination (%)	Axes contamination (%)
Distilled H_2O (control)	31.7^{a}	68.3^{a}
$HgCl_2$ (0.1%)	90.0^{b}	0.0^{b}
NaOCl (1%)	100.0^{c}	0.0^{b}
$Ca(OCl)_2$ (1%)	93.3^{b}	11.7^{c}

Table 3.3: Different contaminants isolated from embryonic axes of *T. dregeana* after decontamination and 3 weeks in culture, (n = 60). Mean values followed by the different lower case letters within columns and different uppercase letters in each row are significantly different. (Dependent variables were subjected to Arcsine transformation, two-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$).

Treatment with:	Percentage of axes contaminated by:			
	Penicillium spp.	*Rhizopus* spp.	Unknown isolate	Bacteria
Distilled H_2O (control)	28.3^{aA}	21.7^{aA}	3.3^{aB}	15.0^{aC}
$HgCl_2$ (0.1%)	0.0^{bA}	0.0^{bA}	0.0^{aA}	0.0^{bA}
NaOCl (1%)	0.0^{bA}	0.0^{bA}	0.0^{aA}	0.0^{bA}
$Ca(OCl)_2$ (1%)	0.0^{bA}	0.0^{bA}	0.0^{aA}	11.7^{aB}

In order to check the effect of the three decontaminants, *viz*. sodium hypochlorite, calcium hypochlorite and mercuric chloride on the vigour of the embryonic axes (Table 3.4), radicle growth *in vitro* (measured as lengths attained) were compared. The vigour of the axes treated with mercuric chloride was compromised, as can be seen from the results after 3 weeks, where radicle length was only 9 mm, whereas radicle lengths of embryonic axes treated with sodium and calcium hypochlorite were 21.4 mm and 19 mm, respectively. Although mercuric chloride was a good surface decontaminant, it was also observed that the embryonic axes showed a slight decline in total germination in addition to the marked decline in the vigour compared with the axes treated with 1% NaOCl. Thus 1% v/v NaOCl was chosen as the best treatment for the surface decontamination of the embryonic axes.

Table 3.4: Effect of different decontaminants on the vigour of the embryonic axes of *T. dregeana* assessed by radicle growth after 7, 14 and 21 d in culture from an initial length of 2 mm, (n = 15). Mean values followed by the different letters within columns are significantly different (one-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$).

	Length of radicles developing from axes after 7, 14 & 21 d in culture		
Treatment with:	7 d	14 d	21 d
NaOCl (1%)	7.6[a]	13.7[a]	21.4[a]
$Ca(OCl)_2$ (1%)	7.0[a]	11.9[a]	19.0[a]
$HgCl_2$ (0.1%)	3.8[b]	5.4[b]	9.0[b]

3.3 Effect of different decontaminants on the micro-organisms associated with the embryonic axes of *T. dregeana*

3.3.1 Electrochemical or electrolytic disinfection by anodic water

Exposure of *T. dregeana* axes to anodic water did not emerge as an effective decontamination treatment as both fungal and bacterial contamination persisted (Table 3.5). In fact, a negative correlation was observed between the immersion time of the embryonic axes and the proliferation of contaminants for after 72 to 96 h of incubation (results not shown). That is, fungal proliferation was evident only after 72 (*Rhizopus* spp.) to 96 h (*Penicillium* spp.) when the embryonic axes were immersed in anodic water for 30, 40, 60, 90 and 120 min, whereas the embryonic axes immersed in anodic water for 10 or 20 min and in distilled water for 10 min showed fungal proliferation within 24 (*Rhizopus* spp.) to 48 h (*Penicillium* spp.). Even after 120 h immersion in anodic water a total of 40% of axes were revealed as contaminated by fungi and 10% by bacteria. Depending on the exposure time to anodic water, 40–20% of axes were contaminated by *Penicillium* spp., 26.7-20.0% by *Rhizopus* spp. and 13.3–6.7% by bacteria. There were also some unidentified fungi observed to be

contaminating 10–3.3% embryonic axes treated with either anodic water or distilled water (control). Except in the case of the *Penicillium* spp., anodic water treatment neither reduced nor eliminated contamination compared with control through the immersion intervals and was therefore considered not an effective decontaminant for the embryonic axes of *T. dregeana*.

Table 3.5: Effect of anodic water on contamination and viability of embryonic axes of *T. dregeana* after 3 weeks in culture, (n = 30). Mean values followed by the different letters within columns are significantly different (Dependent variables were subjected to Arcsine transformation, one-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$).

Treatment with:	Time (min)	Germination (%)	Percentage of axes contaminated by:			
			Rhizopus spp.	*Penicillium* spp.	Unknown isolates	Bacteria
NaOCl (1%)	10	100.0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
Distilled H_2O (control)	10	23.3^{b}	23.3^{b}	40.0^{b}	3.3^{ab}	10.0^{ab}
Anodic water	10	23.3^{b}	26.7^{b}	40.0^{b}	3.3^{ab}	6.7^{ab}
	20	26.7^{b}	26.7^{b}	36.7^{b}	10.0^{b}	13.3^{b}
	30	30.0^{b}	20.0^{b}	33.3^{bc}	6.7^{ab}	10.0^{ab}
	40	43.3^{c}	20.0^{b}	26.7^{cd}	0.0^{a}	10.0^{ab}
	60	46.7^{c}	20.0^{b}	23.3^{d}	0.0^{a}	10.0^{ab}
	90	50.0^{c}	20.0^{b}	20.0^{d}	0.0^{a}	10.0^{ab}
	120	50.0^{c}	20.0^{b}	20.0^{d}	0.0^{a}	10.0^{ab}

3.3.2 Chemical disinfection

3.3.2.1 Effect of exposure to Nipastat on the embryonic axes and contaminants

After 0.1% Nipastat exposure for 10–20 min, 35–30% of axes remained contaminated, but this treatment was relatively effective compared with the control, where contamination was associated with a total of 68.4% of axes. The contaminants isolated were *Rhizopus* spp., *Penicillium* spp. and bacteria. Effective decontamination resulted when embryonic axes were immersed in Nipastat at a concentration of 0.14% for 10 and 20 min with 91.7–95% germination: only 8.3–5% of axes retained residual contaminants (Table 3.6). Exposure to 0.2% Nipastat for 10 min eliminated all fungal contaminants, and bacterial contamination was associated with only 1.7% of the axes, while these

residual bacterial contaminants were no longer in evidence after 20 min exposure. However, the longer treatment with 0.2% Nipastat was also toxic - or at least, inhibitory – to the embryonic axes, as only 40% had germinated after 3 weeks in culture (Table 3.6).In those treatments with Nipastat where contaminants were not eliminated, their proliferation was delayed for up to 8 d in culture, compared with the control where fungal proliferation, especially of *Rhizopus* spp., occurred from the first day (data not shown).

Table 3.6: Effect of exposure to different concentrations of Nipastat on the embryonic axes and associated contaminants of *T. dregeana* after 3 weeks in culture, (n = 60). Mean values followed by the different letters within columns are significantly different (Dependent variables were subjected to Arcsine transformation, one-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$).

Treatment with:	Conc: (%)	Time (min)	Germination (%)	Percentage of axes contaminated by:			
				Rhizopus spp.	*Penicillium* spp.	*A. niger*	Bacteria
NaOCl	1	10	100.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}
Distilled H_2O (control)	-	10	31.7^{b}	21.7^{b}	31.7^{b}	1.7^{a}	13.3^{b}
Nipastat	0.1	10	65.0^{c}	13.3^{c}	15.0^{c}	0.0^{a}	6.7^{c}
		20	70.0^{c}	10.0^{c}	13.3^{c}	0.0^{a}	6.7^{c}
	0.14	10	91.7^{d}	1.7^{a}	3.3^{a}	0.0^{a}	3.3^{ac}
		20	95.0^{de}	1.7^{a}	0.0^{a}	0.0^{a}	3.3^{ac}
	0.2	10	100.0^{ae}	0.0^{a}	0.0^{a}	0.0^{a}	1.7^{a}
		20	40.0^{f}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}

3.3.2.2 Effect of Nipastat-enriched medium on the embryonic axes and associated contaminants

Embryonic axes were plated on half strength MS medium incorporating different concentrations of Nipastat, *viz.* 0.01, 0.02, 0.03 and 0.04%. Although significantly reduced, contaminants were not eliminated by Nipastat to the concentration of 0.03% (Table 3.7), and no axes showed residual contamination when cultured in the presence of 0.04% Nipastat. However, exposure to both 0.03% and 0.04% Nipastat in the culture medium was toxic – or at least inhibitory – with no embryonic axes germinating within three weeks (Table 3.7). Studies on the effect of Nipastat on different contaminants (Table 3.8) of embryonic axes showed that there was a significant reduction of *Penicillium* spp. compared with the control from when the concentration was 0.02%, whereas the incidence of axis contamination by *Rhizopus* spp. and bacteria was significantly reduced only after exposure to 0.03% and 0.4% Nipastat.

Table 3.7: Effect of the different concentrations of the Nipastat incorporated half strength MS medium on germination and residual contamination of the embryonic axes of *T. dregeana* after 3 weeks in culture, (n = 60). Mean values followed by the different letters within columns are significantly different (Dependent variables were subjected to Arcsine transformation, one-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$).

Treatment with:	Concentration (%)	Germination (%)	Axis contamination (%)
Distilled H_2O (control)	0	36.7^{a}	63.3^{a}
Nipastat	0.01	38.3^{a}	61.7^{a}
	0.02	48.3^{b}	51.7^{b}
	0.03	0.0^{c}	30.0^{c}
	0.04	0.0^{c}	0.0^{d}

Table 3.8: Percentage of embryonic axes of *T. dregeana* on Nipastat-enriched medium showing inhibition of individual contaminants 3 weeks after plating, (n = 60). Mean values followed by the different letters within columns are significantly different (Dependent variables were subjected to Arcsine transformation, one-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$).

Treatment with:	Percentage of axes contaminated by:		
	Rhizopus spp.	*Penicillium* spp.	Bacteria
Distilled H_2O (control)	26.7[a]	30.0[a]	6.7[a]
Nipastat 0.01%	31.7[a]	21.7[b]	8.3[a]
Nipastat 0.02%	26.7[a]	20.0[b]	5.0[a]
Nipastat 0.03%	15.0[b]	15.0[b]	0.0[b]
Nipastat 0.04%	0.0[c]	0.0[c]	0.0[b]

3.3.2.3 Effect of Nipastat on test isolates

When the five test isolates from the embryonic axes of *T. dregeana*, *A. niger, A. flavus*, *Fusarium* sp., *Rhizopus* sp. and *Penicillium* sp., were incubated on the plates containing PDA enriched with concentrations of Nipastat from 0.01–0.05%, the fungal proliferation was either reduced or completely inhibited (Table 3.9). On plates incorporating 0.01% Nipastat, growth of *A. niger* and *A. flavus* was inhibited by 44.3 and 46.7%, respectively, while proliferation of *Penicillium* sp. was inhibited by only 12.7%, and there was no inhibition of *Rhizopus* sp. In contrast, there was hardly any growth of the cultures of *Fusarium* sp. Proliferation of the test isolates was increasingly inhibited with increasing concentrations of Nipastat in the medium. Growth of the colonies of four of the fungal species was completely (or almost completely [*A. niger*, 99.8%]) inhibited in the presence of 0.04% Nipastat, the exception being *Penicillium* sp. where there was an 80.4% inhibition. Proliferation of *A. niger* was, however, completely inhibited in the presence of 0.05% Nipastat, and on this medium growth of *Penicillium* sp. was almost completely inhibited (94.7%).

Table 3.9: Effect of different concentrations of Nipastat on test fungal isolates, assessed after 7 d incubation, (n = 9). Mean values followed by the different letters within rows are significantly different (Dependent variables were subjected to Arcsine transformation, one-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$).

	Nipastat concentration (%) in medium				
	0.01	0.02	0.03	0.04	0.05
Test isolate	Inhibition of fungal growth (%)				
A. niger	44.3[a]	88.6[b]	97.8[c]	99.8[c]	100.0[c]
A. flavus	46.7[a]	86.2[b]	97.0[c]	100.0[c]	100.0[c]
Penicillium sp.	12.7[a]	46.9[b]	73.5[c]	80.4[d]	94.7[e]
Rhizopus sp.	0.0[a]	0.0[a]	89.6[b]	100.0[c]	100.0[c]
Fusarium sp.	95.8[a]	100.0[b]	100.0[b]	100.0[b]	100.0[b]

3.3.2.4 The effects of sodium dichloro-isocyanurate (Medi-Chlor/NaDCC) on the embryonic axes and associated contaminants of T. dregeana

Embryonic axes were immersed in solutions containing 0.1% (1000 ppm) to 0.3% (3000 ppm) of NaDCC for 10 or 20 min (Table 3.10). The results indicated that NaDCC was effective against the fungal contaminants after exposure to 0.2% (2000 ppm) for 10 min. However, bacterial contamination persisted, becoming apparent after 6 d, but there was 100% germination of the embryonic axes including those which were contaminated with bacteria. When NaDCC was used at 0.2% for 20 min, there was neither bacterial nor fungal contamination. Furthermore, NaDCC had no adverse effects on germination when used as a 0.3% solution.

Table 3.10: Concentration and exposure treatment time-related effects of sodium dichloro-isocyanurate (NaDCC as Medi-Chlor) on the embryonic axes and associated contaminants of *T. dregeana* assessed after 3 weeks in culture, (n = 30). Mean values followed by the different letters within columns are significantly different (Dependent variables were subjected to Arcsine transformation, one-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$).

Treatment with:	Conc. (%)	Time (min)	Germination (%)	Percentage of axes contaminated by:			
				Rhizopus spp.	*Penicillium* spp.	*Fusarium* spp.	Bacteria
Distilled H_2O (control)	0.0	10	20.0^{a}	23.3^{a}	46.7^{a}	0.0^{a}	10.0^{a}
NaOCl*	1.0	10	100.0^{b}	0.0^{b}	0.0^{b}	0.0^{a}	0.0^{a}
NaDCC	0.1	10	73.3^{c}	0.0^{b}	10.0^{b}	6.7^{a}	10.0^{a}
		20	93.3^{b}	0.0^{b}	6.7^{b}	0.0^{a}	30.0^{b}
	0.2	10	100.0^{b}	0.0^{b}	0.0^{b}	0.0^{a}	30.0^{b}
		20	100.0^{b}	0.0^{b}	0.0^{b}	0.0^{a}	0.0^{a}
	0.3	10	100.0^{b}	0.0^{b}	0.0^{b}	0.0^{a}	0.0^{a}
		20	100.0^{b}	0.0^{b}	0.0^{b}	0.0^{a}	0.0^{a}

*Previously found to be effective (Table 3.2), and used here as a comparison

3.3.2.5 Effect of Benlate on test isolates

Growth of the test isolates, *A. niger*, *A. flavus*, and *Fusarium* sp., was completely inhibited by all concentrations of Benlate incorporated in the medium (Fig. 3.4). In contrast, however, proliferation of *Penicillium* sp. and *Rhizopus* sp. was not affected by Benlate concentrations <0.05%. The most effective inhibition of *Rhizopus* sp. and *Penicillium* sp. was when 1% Benlate was incorporated in the medium, but even at this concentration, proliferation, especially of *Rhizopus* sp., was not effectively achieved.

The effect of Benlate on the embryonic axes showed that at 0.01% it was not toxic, but, as indicated above, was not an effective decontaminant. Increasing the concentration of Benlate in the medium was toxic to the embryonic axes of *T. dregeana* (results not shown).

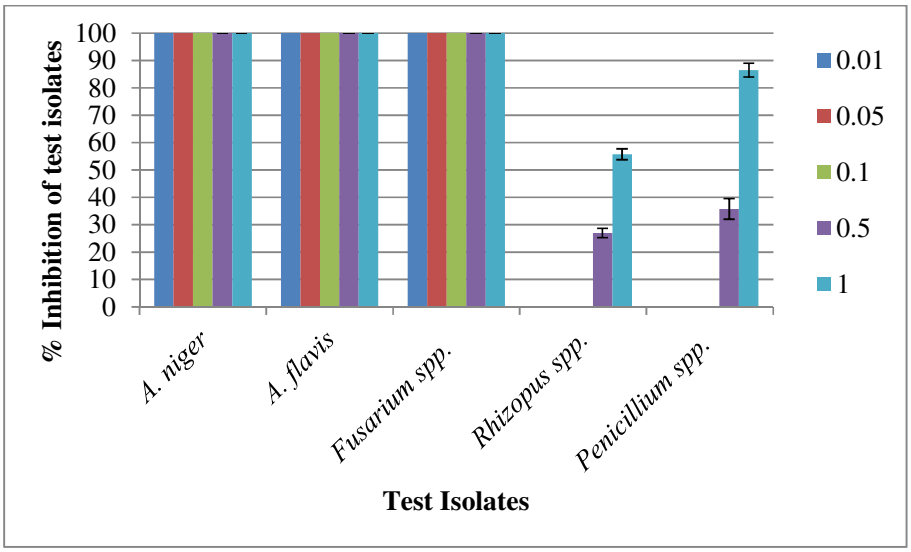

Figure 3.4: Effect after 3 weeks in culture of different concentrations of Benlate incorporated in the medium, on test isolates (n = 9). Bars indicate standard deviation of the mean

3.4 Biocontrol agents

3.4.1 Cultural characteristics and growth rate of two isolates of T. harzianum *(EcoT and Eco77)*

The two different strains of *T. harzianum* showed different cultural characteristics based on their pigmentation. The mycelium of both strains initially appeared to be colourless, but became yellowish green for EcoT and whitish green for Eco77 as the colonies grew and matured (Fig. 3.5). The coloration of the reverse sides of the plates was whitish yellow for EcoT and whitish green for Eco77.

The strains of *T. harzianum* in Eco77 grew slightly faster than that of EcoT (Table 3.11). Studies on their daily growth showed that on Day 1, EcoT had grown by 4.4 mm only, in contrast to the 6.9 mm shown by Eco77. It also took only 3 d for Eco77 to cover the entire plate, in contrast to 4 d taken by EcoT.

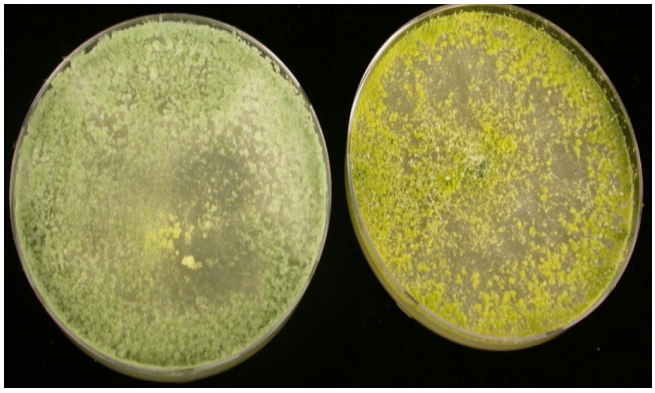

Figure 3.5: Strains of *T. harzianum* characteristic of Eco77 (left) and EcoT (Right) grown for 14 d on potato dextrose agar (PDA) medium in 90 mm Petri dishes

Table 3.11: Comparison of growth rate of EcoT and Eco77 on potato dextrose agar (when mycelial disc of EcoT or Eco77 placed in the centre of the plate) after 1, 2 and 3 d incubation, (n = 9). Mean values followed by the different letters within columns are significantly different (Chi-squared test and t-test, $p \leq 0.05$).

T. harzianum isolates	Total growth after 1, 2 & 3 d (mm per day)			Days to cover the entire plate
	Day 1	Day 2	Day 3	
EcoT	4.4[a]	15.3[a]	28.8[a]	4
Eco77	6.9[b]	22.3[b]	35.0[b]	3

3.4.2 Plate assays for evaluation of biocontrol properties of T. harzianum *(EcoT and Eco77) on contaminants (test isolates).*

3.4.2.1 Effect of directly diffusible metabolites produced by EcoT and Eco77 on the percentage inhibition of radial growth (PIRG) and colony overgrowth of test isolates

In studying *T. harzianum* isolates and the test isolates in dual culture (Fig. 3.6) both EcoT and Eco77 had inhibitory effects on the growth of the latter (Table 3.12). Comparing the two *Trichoderma* isolates, EcoT showed significantly greater inhibition against *A. niger* and slightly higher degree of inhibition against *Penicillium* sp. than Eco77. However, Eco77 caused a significantly greater degree of inhibition of *A. flavus* and *Rhizopus* sp. than EcoT. Both EcoT and Eco77 inhibited the *Fusarium* sp. to the same degree.

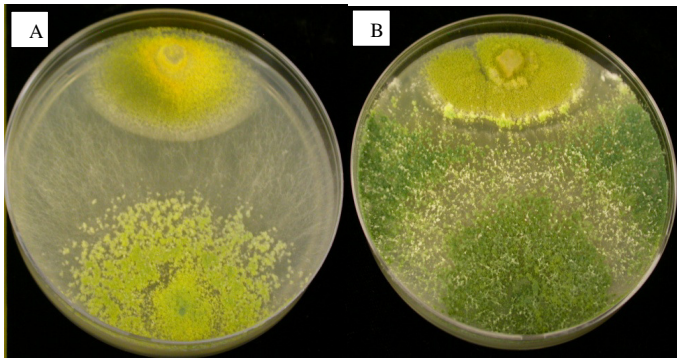

Figure 3.6: An example of dual culture tests between *A. flavus* and Eco77 (A) and EcoT (B) after 7 d in 90 mm Petri dishes. In both cases, the colony at the top of the plate is *A. flavus*.

Table 3.12: Percent inhibition of mycelial growth (PIMG) of test isolates in dual culture after 7 d of incubation by EcoT and Eco77, (n = 9). Mean values followed by the different letters within columns are significantly different. Data were expressed as percentage of control colonies without antagonist and values are average of nine replicates (Chi-squared test and t-test, $p < 0.05$).

Antagonist:- *T. harzianum* from:	Percent inhibition of mycelial growth (PIMG)				
	A. flavus	*A. niger*	*Fusarium* sp.	*Rhizopus* sp.	*Penicillium* sp.
Eco77	63.4[a]	61.3[a]	60.0[a]	55.0[a]	59.6[a]
EcoT	56.8 [b]	66.6[b]	60.0[a]	50.8[b]	65.4[a]

Evaluation of *T. harzianum* isolates against fungal contaminants by dual culture using the scale of Bell *et al.* (1982) showed that in most cases, the antagonist (EcoT/Eco77) overgrew the contaminant and covered the entire medium surface (Table 3.13). In the cases of dual cultures of EcoT and *A. flavus* and Eco77 and *A. niger*, after 7 d complete overgrowth by *Trichoderma* had not yet occurred. In general though, this study suggests that metabolites released by these strains of *T. harzianum* were toxic and fungistatic to all the test isolates.

Table 3.13: Antagonistic effect of *T. harzianum* on growth of *test isolates* after 7 d evaluated using the scale of Bell *et al.* (1982), (n = 9).

Antagonist:- *T. harzianum* from:	Test Isolates	Bell's Scale (after 7 d)	Days at which the antagonist grew over the test isolates and covered the plate
EcoT	*A. niger*	1	7.0
	A. flavus	2	10.0
	Fusarium sp.	1	6.0
	Rhizopus sp.	1	7.0
	Penicillium sp.	1	7.0
Eco77	*A. niger*	3	13.0
	A. flavus	1	6.0
	Fusarium sp.	1	6.0
	Rhizopus sp.	1	6.0
	Penicillium sp.	1	6.0

3.4.2.2 The effect of directly diffusible metabolites produced by EcoT and Eco77 on embryonic axes of T. dregeana

Dual culture performed with the excised embryonic axes from freshly-harvested seeds (Fig. 3.7) and those pre-germinated for 1 week (Fig. 3.8) revealed that neither EcoT nor Eco77 had an effect in terms of increasing the root length of the axes. In fact, after 2 weeks in dual culture both strains of *T. harzianum* appeared to have markedly depressed radicle extension (Table 3.14). The overall impression was that once the mycelium of *Trichoderma* was in contact with the radicles, particularly of newly-excised axes, a stasis was imposed on their growth (*cf.* radicle lengths after 1 and 2 weeks [Table 3.14]). It was also noticed for the pre-germinated axes that there was a decline in the growth of the radicles when co-cultured with either EcoT or Eco77, relative to those of the controls (Table 3.15). It cannot presently be said whether further growth of the radicles would have been inhibited had the co-cultures been evaluated after more than 2 weeks.

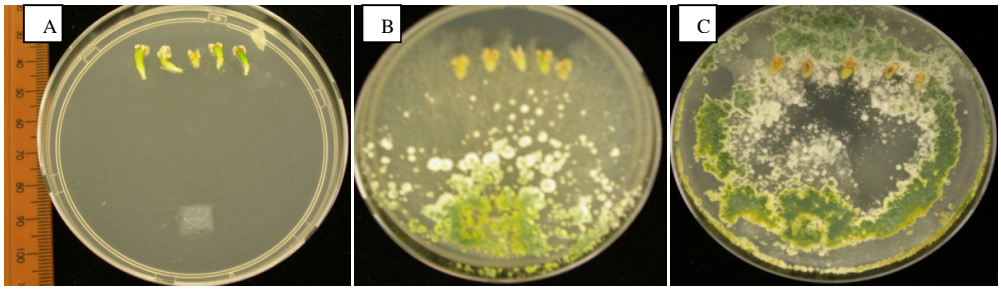

Figure 3.7: Effect on root elongation of EcoT and Eco77 on freshly excised embryonic axes of *Trichilia dregeana* after 1 week in dual culture: (A) Control (no *Trichoderma* plug); (B) Eco77; and (C) EcoT

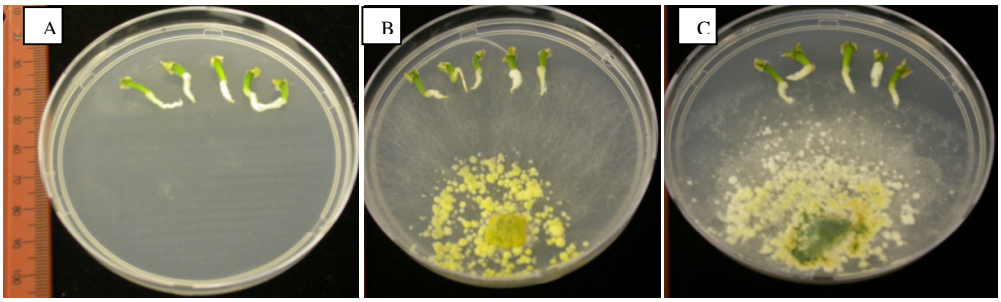

Figure 3.8: Effect on root elongation of EcoT and Eco77 on 1-week-old embryonic axes of *Trichilia* dregeana after 1 week in dual culture: (A) Control (no *Trichoderma* plug); (B) Eco77; and (C) EcoT.

Table 3.14: Effect on root elongation by EcoT and Eco77 of embryonic axes excised from fresh seeds of *Trichilia dregeana* after 0-14 d in dual culture, (n = 30). Mean values followed by the different letters within columns are significantly different. (one-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$).

Treatment with:	Radicle length of axes after 0, 7& 14 d in culture		
	0 d	7 d	14 d
Distilled H_2O (control)	2.0^{a}	7.2^{a}	13.6^{a}
EcoT	2.0^{a}	4.3^{b}	4.3^{b}
Eco77	2.0^{a}	5^{b}	5^{b}

Table 3.15: Effect on root elongation by EcoT and Eco77 of 1-week-old (i.e. pre-germinated) embryonic axes of *Trichilia dregeana* after 0-14 d in dual culture, (n = 30). Mean values followed by the different letters within columns are not significantly different. (one-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$).

Treatment with:	Radicle length of axes after 0, 7 & 14 d in culture		
	0 d	7 d	14 d
Distilled H_2O (control)	6.1[a]	13.9[a]	18.0[a]
EcoT	6.3[a]	10.0[b]	12.0[b]
Eco77	6.7[a]	9.3[b]	12.5[b]

3.4.2.3 The effect of volatile metabolites of T. harzianum *against test isolates*

The results for volatile metabolites produced by EcoT and Eco77 (Fig. 3.9) indicated that there was a relatively low inhibitory effect on proliferation of the test isolates (Fig. 3.10). The inhibition varied from 5.7 to 23.9% for EcoT and 4.0 to 30% for Eco77. Between volatile and direct diffusible metabolites (REF. Table 3.12) both EcoT and Eco77 showed higher inhibition percentages for the latter.

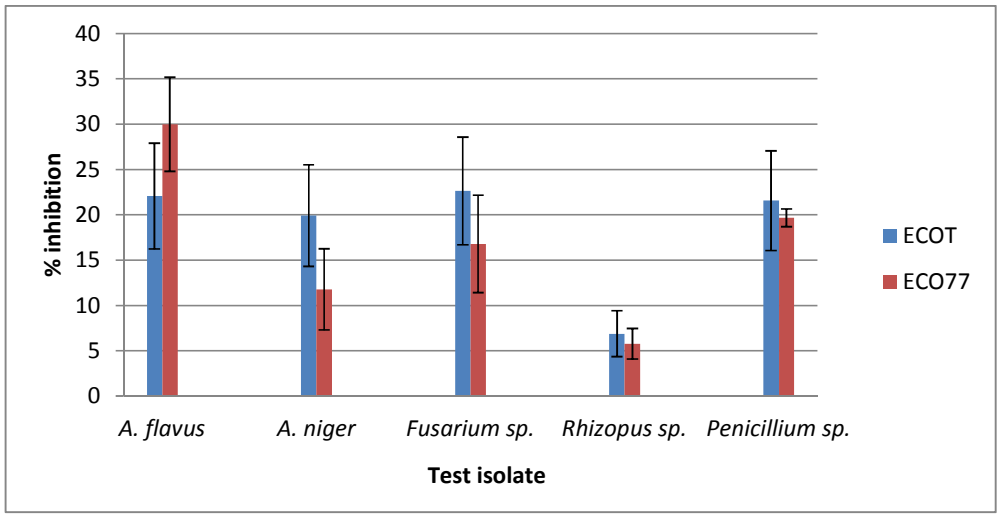

Figure 3.9: Inhibitory effects of volatile metabolites produced by EcoT and Eco77 on test isolates after 4 d in culture. (n = 9). Bars indicate one standard deviation about the mean.

3.4.2.4 Effect of non-volatile compounds using solid and liquid state culture filtrates of T. harzianum *on different test isolates*

a) The effect of non-volatile metabolites of T. harzianum *from Solid state fermentation (SSF) on test isolates*

Culture filtrates of EcoT and Eco77 were extracted after different incubation times (Tables 3.16 and 3.17) and assessed for their effects on the test isolates. The proliferation of all was inhibited by more than 80% when exposed to the culture filtrate of *T. harzianum* from EcoT obtained after 14 d of incubation on the solid Sugar Cane Bagasse medium (SCB) (Fig. 3.10). The percentage inhibition obtained declined when filtrates obtained after longer incubation periods (21 and 28 days) were applied (Table 3.16), and a similar trend was observed when SCB culture filtrates of the strain of *Trichoderma* from Eco77 was used (Table 3.17). Therefore the culture filtrates obtained after culturing *Trichoderma* for 14 d were used for further investigations.

Table 3.16: Inhibition of the test isolates by the SCB culture filtrate after 7-28 d culture of *Trichoderma* from EcoT, (n = 9). Mean values followed by the different letters within rows are significantly different (Dependent variables were subjected to Arcsine transformation, one-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$).

Test Isolate	Inhibition (%) of test isolates upon exposure to culture filtrates of *Trichoderma**from EcoT after 7, 14, 21 & 28 d growth on SCB			
	7	14	21	28
A. niger	64.0[a]	88.2[b]	60.1[a]	48.4[c]
A. flavus	50.0[a]	82.4[b]	58.1[c]	41.9[d]
Penicillium sp.	63.2[a]	74.7[b]	64.9[a]	33.8[c]
Rhizopus sp.	96.2[ab]	98.6[a]	92.7[b]	84.7[c]
Fusarium sp.	81.4[a]	90.8[b]	89.8[b]	84.5[a]

*There was no growth inhibition of control cultures of any of the test isolates – i.e. when exposed to *Trichoderma*-free culture filtrate

Table 3.17: Inhibition of the test isolates by the SCB culture filtrate after 7-28 d culture of *Trichoderma* from Eco77, (n = 9). Mean values followed by the different letters within rows are significantly different (Dependent variables were subjected to Arcsine transformation, one-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$).

Test Isolate	Inhibition (%) of test isolates upon exposure to culture filtrates of *Trichoderma* from EcoT after 7, 14, 21 & 28 d growth on SCB			
	7	14	21	28
A. niger	73.6^{ab}	78.9^{a}	70.2^{b}	54.2^{c}
A. flavus	81.8^{a}	90.6^{b}	64.9^{c}	56.8^{d}
Penicillium sp.	68.1^{a}	75.6^{b}	62.8^{c}	45.4^{d}
Rhizopus sp.	90.9^{a}	97.9^{b}	86.5^{a}	73.1^{c}
Fusarium sp.	80.2^{a}	89.1^{b}	87.7^{bc}	82.7^{ac}

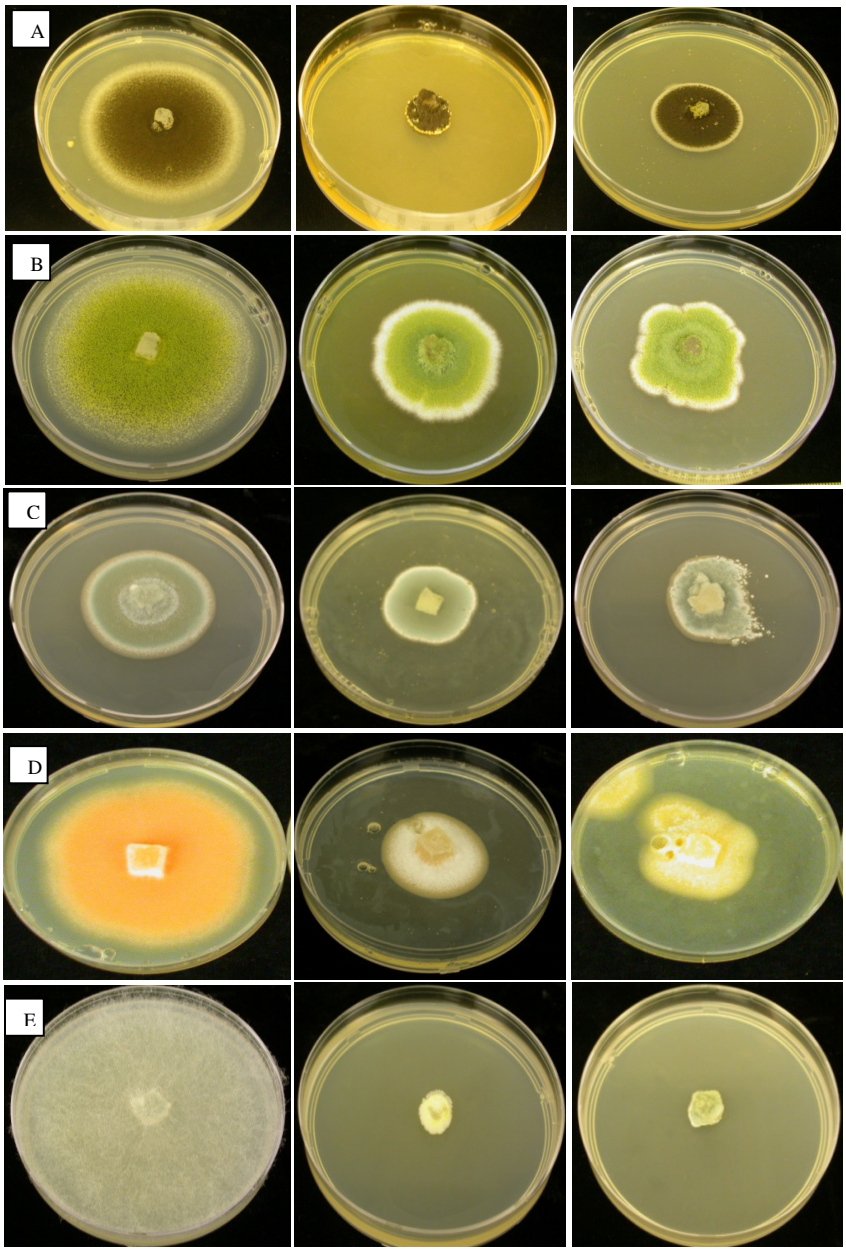

Figure 3.10: Effect of culture filtrates obtained after the 14 d incubation on SCB of *Trichoderma harzianum* from EcoT (middle row), Eco77 (right-hand row) compared with *Trichoderma*-free culture filtrates (control, left-hand row) incorporated on a PDA medium in 90 mm Petri dishes on different test isolates. (A) *A. niger*, (B) *A. flavus*, (C) *Penicillium* sp., (D) *Fusarium* sp. and (E) *Rhizopus* sp.

b) Effect of culture filtrates (SCB) on contamination and germination of T. dregeana *embryonic axes*

Although the percentage of contamination was less in the embryonic axes immersed in the culture filtrates compared with the controls, there was still a high percentage of contaminated axes irrespective of immersion time and there was no significant difference between the EcoT- and Eco77-treated axes in terms of germination and contamination percentage (Table 3.18). Overall, axis contamination by *Rhizopus* spp. was reduced after 60 min immersion and that of both *Rhizopus* spp. and *Penicillium* spp. was reduced with increasing immersion times from 120-180 min in the culture filtrates of SCB-grown strains of *Trichoderma* from both EcoT and Eco77, compared with axes exposed to distilled water (Table 3.19). It was noted that when the levels of fungal contamination reduced, bacteria proliferated. Although the culture filtrate reduced contamination of embryonic axes compared with the control, it had a negative impact on the vigour of the embryonic axes as the immersion time increased. Additionally, vigour and viability (especially after immersion for 180 min/3 h) of embryonic axes that were not contaminated was also affected (Fig. 3.11) and those that germinated showed stunted growth.

Table 3.18: Percentages of germination and residual contamination of axes of *T. dregeana* immersed for different time intervals in distilled water, NaOCl (1%), or the culture filtrates of EcoT and Eco77 from SCB, assessed after 3 weeks in culture, (n = 60). Mean values followed by the different lower case letters within columns and different upper case letters in each row of individual parameters (Germination/Axis contamination) between different time intervals (60, 120 and 180 d) are significantly different (Dependent variables were subjected to Arcsine transformation,two-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$).

Treatment with:	Germination (%) of axes immersed for 60, 120 or 180 min				Axis contaminaton (%)			
	10*	60	120	180	10*	60	120	180
Distilled H_2O (control)	-	13.3^{aA}	13.3^{aA}	18.3^{aA}		86.7^{aA}	86.7^{aA}	81.7^{aB}
NaOCl (1%)	100.0				0.0			
EcoT	-	36.7^{bA}	41.7^{bA}	40.0^{bA}		63.3^{bA}	58.3^{bA}	50.0^{bB}
Eco77	-	38.3^{bA}	36.7^{bA}	40.0^{bA}		61.7^{bA}	63.3^{bA}	55.0^{bB}

*Immersion time of 10 min not used, except for NaOCl

Table 3.19: Percentage of axes of *T. dregeana* from which different microflora were isolated between different time intervals (10, 60, 120 and 180 min) immersion in distilled water, NaOCl (1%), or the culture filtrates of EcoT and Eco77 from SCB, (n = 60). Mean values followed by the different lower case letters within columns of each test isolate between different treatments and different uppercase letters in each row are significantly different (two-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$).

Treatment with:	Contaminant	Percentage of axes immersed for 60, 120 or 180 min			
		10*	60	120	180
NaOCl (1%)	*Rhizopus* spp.	0			
Distilled H_2O (control)			31.7^{aA}	38.3^{aB}	31.7^{aA}
EcoT			11.7^{bA}	6.7^{bB}	3.3^{bC}
Eco77			15.0^{bA}	11.7^{bAB}	8.3^{cB}
NaOCl (1%)	*Penicillium* spp.	0			
Distilled water			40.0^{aA}	40.0^{aA}	35.0^{aA}
EcoT			33.3^{bA}	23.3^{bB}	21.7^{bB}
Eco77			33.3^{bA}	23.3^{bB}	20.0^{bB}
NaOCl (1%)	Bacteria	0			
Distilled water			15.0^{aA}	8.3^{aB}	15.0^{aA}
EcoT			18.3^{bA}	28.3^{bB}	30.0^{bB}
Eco77			13.3^{abA}	28.3^{bB}	26.7^{bA}

*Immersion time of 10 min not used, except for NaOCl

Figure 3.11: Effect of vigour assessed in terms of root growth/length after 6 weeks (42 d) in relation to different immersion intervals of uncontaminated *T. dregeana* axes from 1 to 3 h of EcoT-/Eco77-free SCB culture filtrate (A: 1 h; B: 2 h and C: 3 h); EcoT culture filtrate (D: 1 h; F: 2 h and H: 3 h) and culture filtrate of Eco77 (E: 1 h; G: 2 h; and I: 3 h).

The embryonic axes plated on the culture-filtrate-enriched medium (Table 3.20) showed a significant reduction of contamination by *Penicillium* spp. and *Rhizopus* spp.; however, bacterial contamination was high. Overall, germination percentages of axes plated on medium enriched with culture filtrate of EcoT was somewhat higher compared with medium enriched with Eco77 culture filtrate, but germination percentages on both these media was significantly higher than that of the control. The effect of culture filtrate on the germination alone of the embryonic axes was studied by decontaminating them prior to plating. As was found when axes were immersed, vigour was severely affected by the culture filtrate in the medium (Table 3.21; Fig. 3.12).

Table 3.20: Effect of (solid state) culture-filtrate-enriched medium on the germination of embryonic axes of *T. dregeana* assessed after 3 weeks in culture, (n = 60). Mean values followed by the different letters within columns are significantly different (one-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$).

Treatment with:	Germination (%)	Percentage of axes contaminated by:		
		Rhizopus spp.	*Penicillium* spp.	Bacteria
DistilledH_2O (control)	26.7[a]	35.0[a]	30.0[a]	8.3[a]
EcoT	58.3[b]	6.7[b]	18.3[b]	16.7[b]
Eco77	53.3[c]	10.0[b]	16.7[b]	20.0[b]

Table 3.21: Effect of the culture-filtrate-enriched medium on growth potential/vigour of the embryonic axes of *T. dregeana* after 7-21 d in culture, (n = 30). Mean values followed by the different letters within columns are significantly different (one-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$).

Treatment with:	Germination (%)	Root length of axes after 7, 14 or 21 d in culture		
		7d	14 d	21 d
Distilled H_2O (control)	100.0	8.0[a]	13.0[a]	17.2[a]
EcoT	100.0	3.0[b]	5.6[b]	7.2[b]
Eco77	100.0	2.8[b]	5.8[b]	6.4[b]

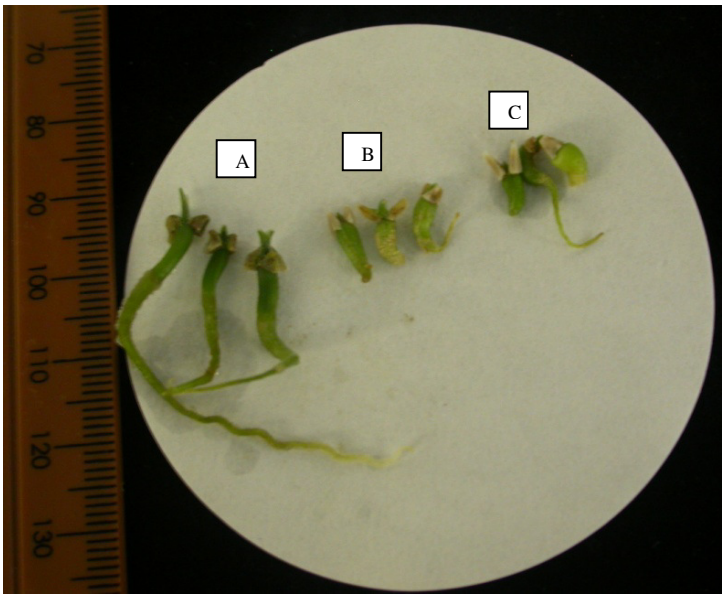

Figure 3.12: Effect on vigour assessed by root length of the embryonic axes after 6 weeks (42 d) being plated on media incorporating A) culture-filtrate alone; B) EcoT-SCB culture filtrate; and C) Eco77-SCB culture filtrate.

c) Liquid state fermentation on potato dextrose broth

Liquid state fermentation was carried out for periods from 7 to 28 d. In all cases, inhibition of the test isolates was highly effective when exposed to culture filtrates incubated for 14 d on PDB, increasing slightly after 21 d and then remaining essentially constant (Tables 3.22 and 3.23; Fig. 3.13). Therefore, culture filtrates extracted after the two strains of *Trichoderma* had been maintained in PDB for 21 d were used for the treatment of embryonic axes.

Table 3.22: Inhibition of the test isolates by the potato dextrose broth culture filtrate after 7-28 d culture of *Trichoderma* from EcoT, (n = 9). Mean values followed by the different letters within columns are significantly different (one-way ANOVA, Duncan's Multiple Range Test, p ≤ 0.05).

Test Isolate	Inhibition (%) of test isolates upon exposure to culture filtrates of *Trichoderma**from EcoT after 7, 14, 21 & 28 d growth on PDB			
	7	14	21	28
A. niger	68.2[a]	95.1[b]	98.7[c]	94.0[b]
A. flavus	94.7[a]	95.9[a]	96.0[a]	95.0[a]
Penicillium sp.	81.9[a]	97.0[b]	98.94[b]	98.0[b]
Rhizopus sp.	100.0[a]	100.0[a]	100.0[a]	100.0[a]
Fusarium sp.	91.7[a]	95.5[b]	99.0[c]	98.2[c]

*There was no growth inhibition of control cultures of any of the test isolates – i.e. when exposed to *Trichoderma*-free culture filtrate

Table 3.23: Inhibition of the test isolates by the potato dextrose broth culture filtrate after 7-28 d culture of *Trichoderma* from Eco77, (n = 9). Mean values followed by the different letters within columns are significantly different (one-way ANOVA, Duncan's Multiple Range Test, p ≤ 0.05).

Test Isolate	Inhibition (%) of test isolates upon exposure to culture filtrates of *Trichoderma**from Eco77 after 7, 14, 21 & 28 d growth on PDB			
	7	14	21	28
A. niger	58.9[a]	92.4[b]	97.1[c]	96.1[bc]
A. flavus	97.4[a]	98.9[a]	99.9[a]	98.6[a]
Penicillium sp.	80.0[a]	91.1[b]	94.9[b]	94.7[b]
Rhizopus sp.	100.0[a]	100.0[a]	100.0[a]	100.0[a]
Fusarium sp.	89.1[a]	94.9[b]	97.3[b]	97.9[b]

*There was no growth inhibition of control cultures of any of the test isolates – i.e. when exposed to *Trichoderma*-free culture filtrate

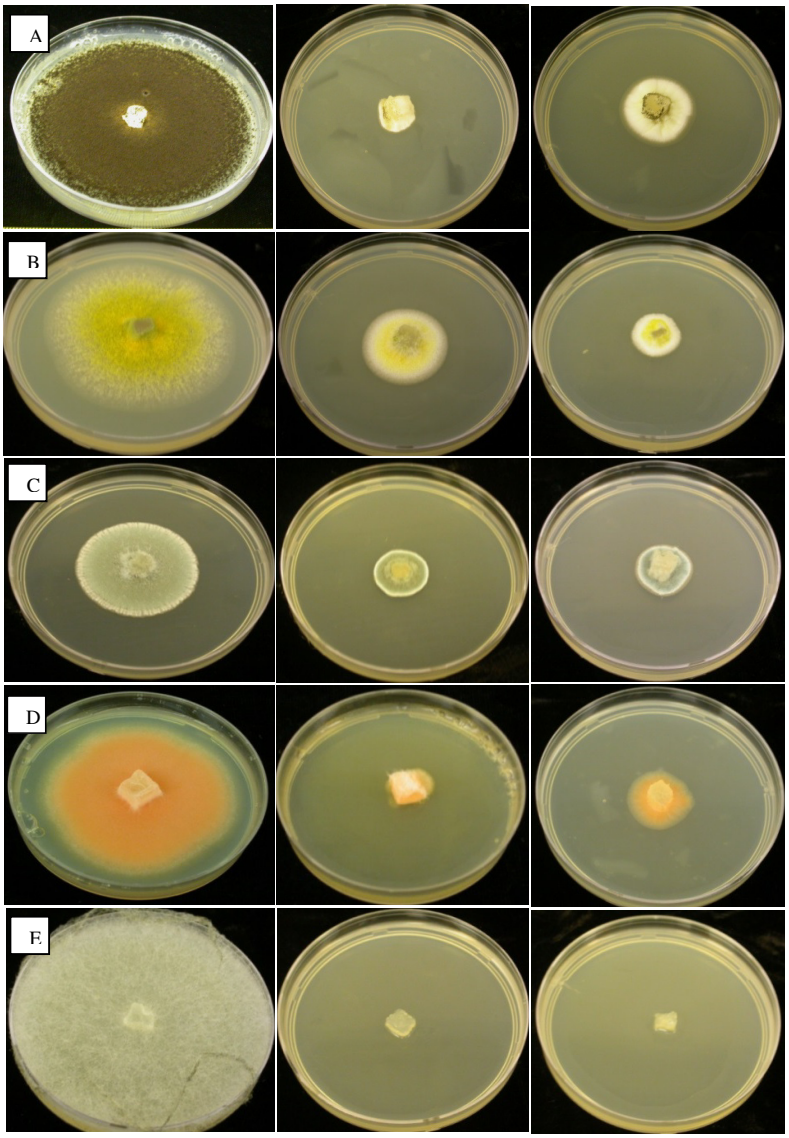

Figure 3.13: Effect of culture filtrates extracted from PDB after 14 d incubation of *T. harzianum* strains from EcoT (middle), Eco77 (right) and control PDB (left) incorporated with the PDA medium in 90 mm Petri dishes on different test isolates. (A) *A. niger*, (B) *A. flavus*, (C) *Penicillium* sp., (D) *Fusarium* sp. and (E) *Rhizopus* sp.

d) Effect of culture filtrate (PDB) on contamination and germination of T. dregeana *embryonic axes*

Germination percentages of embryonic axes immersed in PDB extracts of EcoT and Eco77 (Table 3.24) from 60 to 180 min increased significantly compared with control especially by increasing the immersion time in the PDB culture filtrate (from 55 to 68.3% and 55 to 65%), which probably was due to reduction of contamination. There was no apparent contamination of axes by *Rhizopus* spp. after immersion in extracts of either EcoT or Eco77 for 180 min (Table 3.25). However, some of the

embryonic axes were still contaminated by *Penicillium* spp. and bacteria, although there was a marked decline of contamination compared with axes simply immersed in distilled water (control). Immersing embryonic axes for a longer time decreased the contamination, but also affected the vigour of the embryonic axes as indicated by stunting or abnormal growth (Fig. 3.14).

Table 3.24 Percentage germination and residual contamination of PDB culture-filtrate-treated embryonic axes of *T. dregeana* after 3 weeks in culture, (n = 60). Mean values followed by the different lower case letters within columns and different uppercase letters in each row of individual parameters (Germination/Axes contamination) between different time intervals (60, 120 and 180 min) are significantly different. (Dependent variables were subjected to Arcsine transformation, two-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$).

Treatment with:	Germination (%) of axes immersed for 60, 120 or 180 min				Axes contamination (%)			
	10*	60	120	180	10*	60	120	180
NaOCl (1%)	100.0				0.0			
Distilled H_2O (control)		16.7^{aA}	20.0^{aA}	18.3^{aA}		83.3^{aA}	80.0^{aA}	81.7^{aA}
EcoT		55.0^{bA}	60.0^{bAB}	68.3^{bB}		45.0^{bA}	40.0^{bA}	31.7^{bB}
Eco77		40.0^{bA}	53.3^{cB}	65.0^{bC}		60.0^{cA}	46.7^{bB}	35.0^{bC}

*Immersion time of 10 min not used, except for NaOCl

Table 3.25: Percentage of axes of *T. dregeana* from which different microflora were isolated after immersion for different time intervals (10, 60, 120 and 180) in distilled water, NaOCl (1%), or the culture filtrates of EcoT and Eco77 from PDB after 3 weeks in culture, (n = 60). Mean values followed by the different lower case letters within columns of each isolate between different treatments and different uppercase letters in each rows are significantly different (Dependent variables were subjected to Arcsine transformation, two-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$).

Treatment with:	Contaminant	Percentage of axes immersed for 60, 120 or 180 min			
		10*	60	120	180
NaOCl (1%)	*Rhizopus* spp.	0			
Distilled H_2O (control)			33.3^{aA}	30.0^{aA}	31.7^{aA}
EcoT			6.7^{bA}	3.3^{bA}	0.0^{bA}
Eco77			11.7^{bA}	3.3^{bB}	0.0^{bB}
NaOCl (1%)	*Penicillium* spp.	0			
Distilled H_2O (control)			36.7^{aA}	35.0^{aA}	41.7^{aA}
EcoT			20.0^{bA}	16.7^{bA}	15.0^{bA}
Eco77			26.7^{bA}	15.0^{bB}	11.7^{bB}
NaOCl (1%)	Bacteria	0			
Distilled H_2O (control)			13.3^{aA}	15.0^{aA}	8.3^{aA}
EcoT			18.3^{abA}	20.0^{abA}	16.7^{bA}
Eco77			21.7^{bA}	28.3^{bB}	23.3^{bAB}

*Immersion time of 10 min not used, except for NaOCl

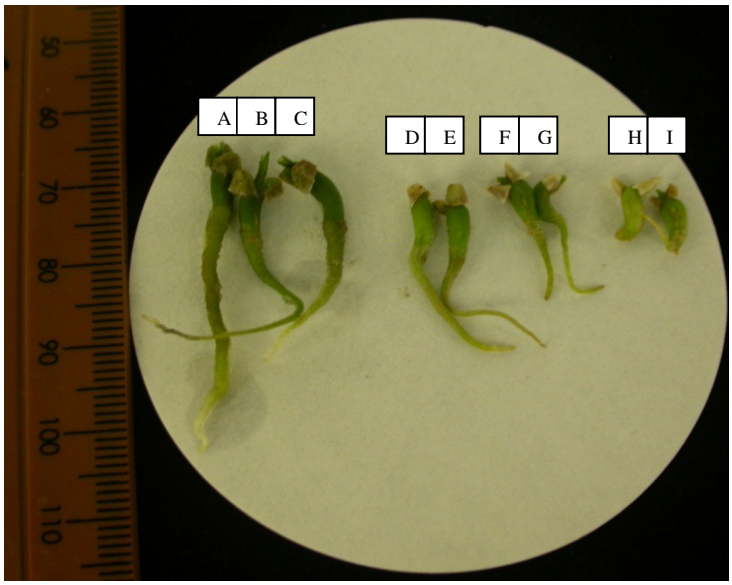

Figure 3.14: Effect of vigour (assessed in terms of root growth/length after 6 weeks (42 d) in relation to different immersion intervals of *T. dregeana* axes from 1 to 3 h of EcoT-/Eco77-free PDB culture filtrate (A: 1 h; B: 2 h and C: 3 h); EcoT culture filtrate (D: 1 h; F: 2 h and H: 3 h) and culture filtrate of Eco77 (E: 1 h; G: 2 h; and I: 3 h).

Culture filtrate incorporated in half strength MS medium was effective for the reduction of axis contamination (Table 3.26) compared with the control axes on medium not incorporating the filtrate. However, the embryonic axes plated on the PDB culture-filtrate-enriched medium appeared to have reduced root hair formation, and the vigour was compromised compared with the control (Table 3.27; Fig. 3.15). It was noted that proliferation of the contaminants started on the surfaces of the embryonic axes which were not in contact with the medium.

Table 3.26: Effect of PDB culture-filtrate-enriched medium on the embryonic axes of *T. dregeana* after 3 weeks in culture, (n = 60). Mean values followed by the different letters within columns are significantly different (Dependent variables were subjected to Arcsine transformation,one-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$).

Treatment with:	Germination (%)	Percentage of axes contaminated by:		
		Rhizopus spp. (%)	*Penicillium* spp. (%)	Bacteria (%)
Distilled H_2O (control)	19.6[a]	33.1[a]	41.7[a]	5.7[a]
EcoT	71.7[b]	0.0[b]	20.0[b]	8.3[a]
Eco77	61.6[c]	0.0[b]	31.7[c]	6.7[a]

Table 3.27 Effect on vigour indicated by root length of the embryonic axes of *T. dregeana* on PDB culture-filtrate-enriched medium after 3 weeks in culture, (n = 30). Mean values followed by the different letters within columns are significantly different (one-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$).

Treatment with:	Germination (%)	Vigour of axes assessed by root length (mm) after 7, 14 or 21 d in culture		
		7d	14 d	21 d
Distilled H_2O (control)	100.0	7.6^a	12.6^a	16.6^a
EcoT	100.0	3.6^b	6.6^b	7.8^b
Eco77	100.0	3.2^b	6.6^b	7.0^b

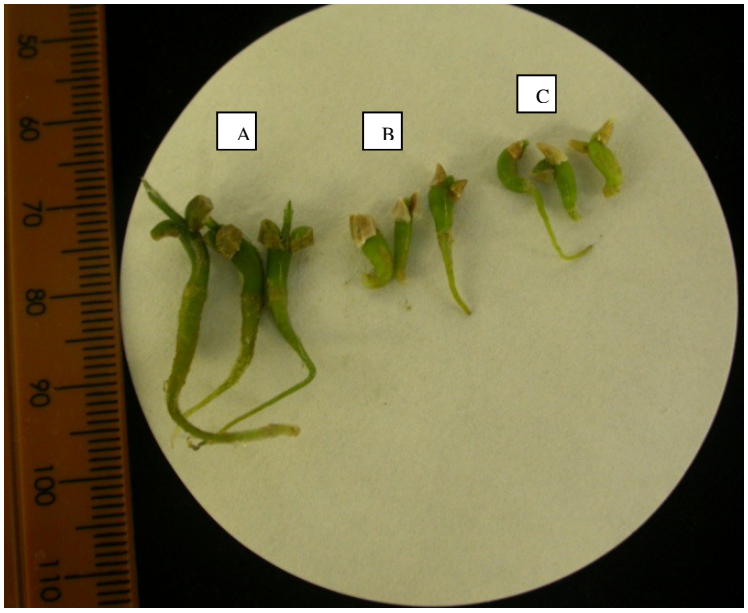

Figure 3.15: Effect on vigour assessed by root length of the embryonic axes after 6 weeks (42 d) being plated on A) medium free of EcoT/Eco77 PDB culture filtrate (control), B & C) media incorporating EcoT culture filtrate and C) Eco77 culture filtrate, respectively.

3.4.3 Trichoderma harzianum *as a biocontrol agent in tissue culture methods*

Among the different spore dilutions tested (Table 3.28), by the third day overgrowth of the embryonic axes by *Trichoderma* was observed when using dilutions of $2x10^8$ to $2x10^6$ mL^{-1} which was associated with 0% germination of the embryonic axes. In order to obtain 100% germination of embryonic axes, a dilution $1x10^6$ mL^{-1} of spores of *Trichoderma* was chosen after the trial (Table 3.28), noting though,

that not all axes showed associated proliferation of the biocontrol agent after 3 d (Fig. 3.16A). Although there was also little overgrowth of the axes by *Trichoderma* within 3 d, eventually this did occur as the biocontrol agent proliferated over the entire plate within 7 d (Fig. 3.16B). This could not be avoided despite modifications of the medium (use of ¼ strength MS medium and sugar-free MS medium). The only way to avoid overgrowth may have been to use a nutrient-free water agar medium, but this was considered as not realistic, as embryonic axes (which have no cotyledonary reserves) require a nutrient-rich medium for germination and subsequent growth.

Table 3.28: Effect of 10-min immersion of embryonic axes of *Trichilia dregeana* in different dilutions of *T. harzianum* spores from both EcoT and Eco77 after 3 d, (n = 60). Mean values followed by the different letters within columns are significantly different (Dependent variables were subjected to Arcsine transformation, one-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$).

Antagonist: - *Trichoderma* from:	Spores (mL^{-1})	Axes showing *Trichoderma* growth (%)	Axes overgrown by *Trichoderma* (%)
EcoT and Eco77	$2x10^8$	100.0^a	100.0^a
EcoT and Eco77	$1x10^8$	100.0^a	100.0^a
EcoT and Eco77	$2x10^7$	100.0^a	100.0^a
EcoT	$1x10^7$	100.0^a	76.7^b
Eco77		100.0^a	71.7^b
EcoT	$2x10^6$	100.0^a	43.3^c
Eco77		100.0^a	38.3^c
EcoT	$1x10^6$	60.0^b	0.0^d
Eco77		46.7^c	0.0^d

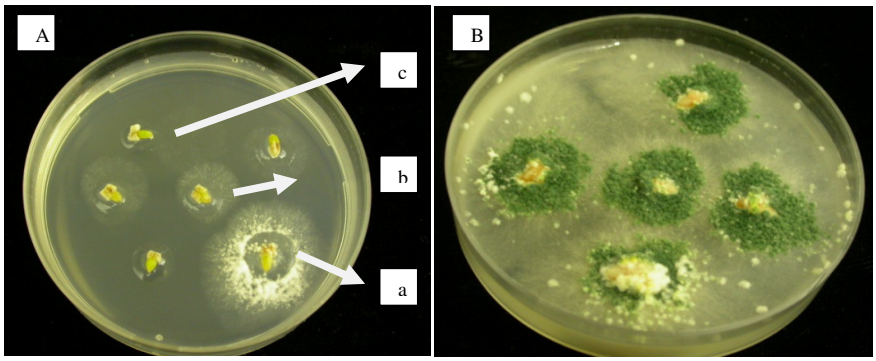

Figure 3.16: Embryonic axes co-cultivated on half strength MS medium in 65 mm Petri dishes with *T. harzianum* after exposure to 1 x 10^6 mL^{-1} spore suspension after 3 d (A): the biocontrol agent overgrew from axes (a), proliferated from some axes (b), but there were also axes from which no obvious proliferation occurred (c); (B). In all instances, however, after 7 d, *Trichoderma* proliferated over the entire plate.

a) Infection and co-cultivation

Whether or not *Trichoderma* had penetrated axes was checked by its proliferation originating from axes which had been immersed in 1% NaOCl before plating on half strength medium, and by fluorescence microscopy (see Fig. 3.21, later). In the first experiment the axes were plated on water agar, on the surface of which l mL of the 1x10^6 mL^{-1} *Trichoderma* spore suspension had been layered (Fig. 3.17). After 72 h, there was evidence of inoculation of all axes by *Trichoderma*, with the exception of those exposed to spores of the strain of *Trichoderma* derived from EcoT, where the figure was 91.7% (Table 3.29).

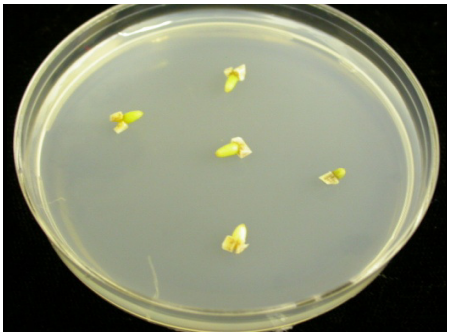

Figure 3.17: Embryonic axes on water agar in a 65 mm Petri dish with *Trichoderma* suspension.

Table 3.29: Percentage of embryonic axes (initially decontaminated with 1% NaOCl) showing *Trichoderma* penetration after 24, 48 and 72 h co-culture on water agar with 1 x 10^6 mL^{-1} spore suspension of EcoT and Eco77, respectively, (n = 60). Mean values followed by the different letters within columns at each co-cultivation time are significantly different between EcoT, Eco77 and control. (Dependent variables were subjected to Arcsine transformation, one-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$).

Antagonist: - *T. harzianum* from:	Time of co-cultivation (h)	Surface decontaminant	Axes inoculated with *Trichoderma* (%)
EcoT	24	Distilled water	16.7[a]
Eco77			20.0[a]
No antagonist (control)			0.0[b]
EcoT		1% NaOCl	0.0[b]
Eco77			0.0[b]
No antagonist (control)			0.0[b]
EcoT	48	Distilled water	41.7[a]
Eco77			43.3[a]
No antagonist (control)			0.0[c]
EcoT		1% NaOCl	20.0[b]
Eco77			26.7[b]
No antagonist (control)			0.0[c]
EcoT	72	Distilled water	100.0[a]
Eco77			100.0[a]
No antagonist (control)			0.0[c]
EcoT		1% NaOCl	91.7[b]
Eco77			100.0[a]
No antagonist (control)			0.0[c]

The second mode of exposure involved plating axes on water agar inoculated 7 d previously with a plug of *Trichoderma* (Fig. 3.18), which effectively ensured that 100% of the axes had been penetrated by *Trichoderma* within 24 h (Table 3.30).

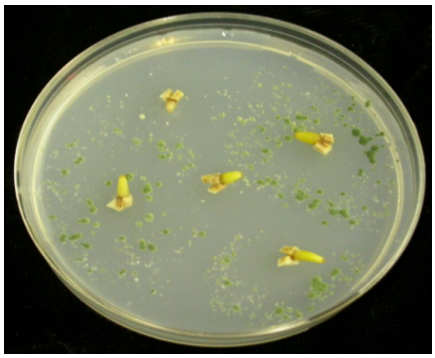

Figure 3.18: Embryonic axes after 72 h plated on water agar in a 65 mm Petri dish previously inoculated with *Trichoderma*.

Table 3.30: Percentage of embryonic axes (initially decontaminated with 1% NaOCl) penetrated by *Trichoderma* after 24, 48 and 72 h co-culture on water agar medium inoculated 7 d previously with EcoT or Eco77, (n = 60). Mean values followed by the different letters within columns at each co-cultivation time between EcoT, Eco77 and control are significantly different (Dependent variables were subjected to Arcsine transformation, one-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$).

Antagonist: - *T. harzianum* from:	Time of co-cultivation (h)	Surface decontaminant	Axes inoculated with *Trichoderma* (%)
EcoT	24	Distilled water	100.0[a]
Eco77			100.0[a]
No antagonist (control)			0.0[b]
EcoT		1% NaOCl	100.0[a]
Eco77			100.0[a]
No antagonist (control)			0.0[b]
EcoT	48	Distilled water	100.0[a]
Eco77			100.0[a]
No antagonist (control)			0.0[b]
EcoT		1% NoOCl	100.0[a]
Eco77			100.0[a]
No antagonist (control)			0.0[b]
EcoT	72	Distilled water	100.0[a]
Eco77			100.0[a]
No antagonist (control)			0.0[b]
EcoT		1% NaOCl	100.0[a]
Eco77			100.0[a]
No antagonist (control)			0.0[b]

In the third instance, axes had been immersed for 10, 20 or 30 min in the 1 x 10^6 mL^{-1} suspension of *Trichoderma* spores (Table 3.31) before plating on half strength MS medium (as described above). Following 30 min of immersion in the spore suspension, after 24 h in culture all axes were shown to have been successfully inoculated (Table 3.32). Therefore embryonic axes co-cultivated for 72 h (the first trial), 24 h (the second and third trials) were selected for further studies.

Table 3.31: Relationship between immersion time in 1 x 10^6 mL^{-1} *Trichoderma* suspensions and persistence of this biocontrol agent in the embryonic axes of *Trichilia dregeana* after 3 d in culture, (n = 30). Mean values followed by the different letters within columns and within each time interval are significantly different (Dependent variables were subjected to Arcsine transformation, one-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$).

Antagonist: - *T. harzianum* from:	Immersion time (min)	Axes showing *Trichoderma* growth (%)
EcoT	10	35.0[a]
	20	81.7[b]
	30	100.0[c]
Eco77	10	40.0[a]
	20	76.7[b]
	30	100.0[c]

Table 3.32: Percentage of embryonic axes (initially decontaminated with 1% NaOCl and immersed for 30 min with EcoT/Eco77) penetrated by *Trichoderma* after 24, 48 and 72 h co-culture on half strength MS medium, (n = 60). Mean values followed by the different letters within columns at each co-cultivation time between EcoT and Eco77 are significantly different (Dependent variables were subjected to Arcsine transformation, one-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$).

Antagonist: - *T. harzianum* from:	Time of co-cultivation (h)	Surface decontaminant	Axes inoculated with *Trichoderma* (%)
EcoT	24	Distilled water	100.0[a]
Eco77			100.0[a]
No antagonist (control)			0.0[b]
EcoT		1% NaOCl	100.0[a]
Eco77			100.0[a]
No antagonist (control)			0.0[b]
EcoT	48	Distilled water	100.0[a]
Eco77			100.0[a]
No antagonist (control)			0.0[b]
EcoT		1% NaOCl	100.0[a]
Eco77			100.0[a]
No antagonist (control)			0.0[b]
EcoT	72	Distilled water	100.0[a]
Eco77			100.0[a]
No antagonist (control)			0.0[b]
EcoT		1% NaOCl	100.0[a]
Eco77			100.0[a]
No antagonist (control)			0.0[b]

Fluorescence microscopy confirmed the presence of hyphal structures of *Trichoderma* penetrating the internal tissues of radicles which had been inoculated at 24 to 48 h, with the absence of *Trichoderma* in the control (Fig. 3.19 A-D).

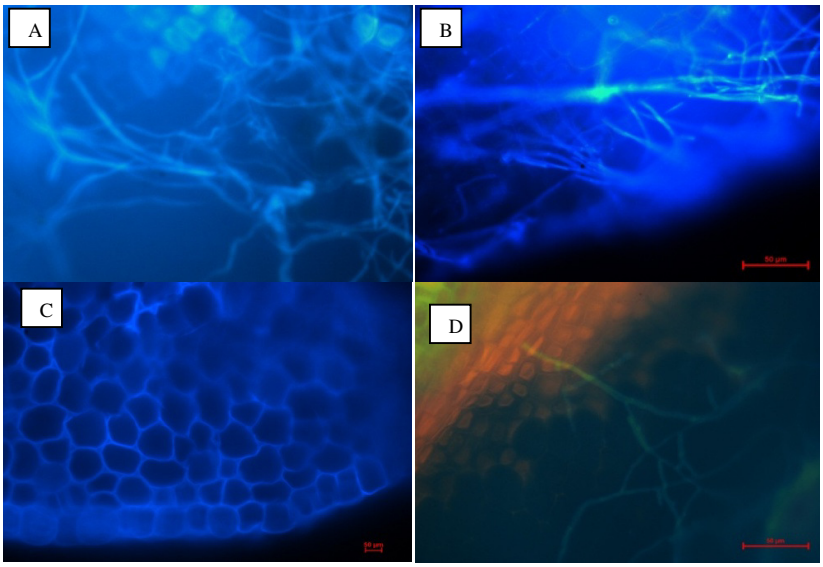

Figure 3.19: Fluorescence microscopy of hand-cut sections of radicle of control (A); and *Trichoderma*-treated axes showing hyphal penetration within 24 h (B), 48 h (C) and 72 h (D), which were previously inoculated by 30 min immersion in 1 x 10^6 mL^{-1} of *Trichoderma* suspensions and co-cultured for 24 h on half strength MS medium.

b) Inhibition of external proliferation of Trichoderma

Once *Trichoderma* penetration had been established, embryonic axes inoculated by the three different procedures were plated on half strength medium incorporating different agents with the potential to inhibit further proliferation of either strain of *Trichoderma*, i.e. that derived from EcoT and from Eco77 (Table 3.33). Embryonic axes were taken (a) after 72 h of co-cultivation on medium layered with the spore suspension; (b) 24 h after plating on water agar previously inoculated with a plug of *Trichoderma*; and (c) after 24 h *in vitro* following immersion in the 1 x 10^6 mL^{-1} suspension of *Trichoderma* spores. In all cases, 100 ppm (0.01%) of benomyl inhibited proliferation of *Trichoderma* around all the axes without any discernible adverse effects on any of the axes.

Among the different trials carried out to inhibit *Trichoderma* proliferation (Table 3.33), in most cases where the agent used was effective, it was also detrimental to the embryonic axes. The surface decontaminant, 1% v/v NaOCl, reduced the growth of *Trichoderma* for only 2 d, after which it over-grew the plate. Although mercuric chloride was effective in curbing *Trichoderma* proliferation, it also had adverse effects on the embryonic axes, confirming what had been previously ascertained (data not shown). Culture filtrates of both the *Trichoderma* isolates (EcoT and Eco77) from liquid and solid state fermentation were tested to check if further proliferation could be 'auto-inhibited'; however, neither filtrate brought about any inhibition at all (Table 3.33). Different concentrations of Nipastat and Benlate were incorporated into the half strength MS medium and tested for their effectiveness

against *Trichoderma*. Nipastat at a concentration of 0.02% and Benlate 0.01% were effective and killed the *Trichoderma* that has penetrated into the embryonic axes. Of the two, Benlate was selected as the best means of inhibiting *Trichoderma* proliferation, as, at 0.02%, Nipastat was toxic to, or delayed germination of, the embryonic axes (Table 3.33).

Table 3.33: Percentage of axes of *T. dregeana* showing inhibition by selected agents (incorporated in the medium) of *Trichoderma* growth after 1 week in culture which were surface decontaminated with 1% NaOCl, (n = 20). Mean values followed by the different letters within columns are significantly different (Dependent variables were subjected to Arcsine transformation, one-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$).

Antagonist: - *T. harzianum* from:	Agent applied to inhibit *Trichoderma* proliferation	Conc. (%)	Germination (%)	Axes from which *Trichoderma* proliferation inhibited (%)
EcoT	Nipastat	0.01	100.0[a]	0.0[a]
		0.02	0.0[b]	100.0[b]
		0.03	0.0[b]	100.0[b]
Eco77		0.01	100[a]	0.0[a]
		0.02	0.0[b]	95.0[b]
		0.03	0.0[b]	100.0[b]
EcoT Eco77	Benlate	0.01	100.0[a]	100.0[b]
EcoT Eco77	Culture filtrate (PDB)	100	100.0[a]	0.0[a]
EcoT Eco77	Culture filtrate (PDB)		100.0[a]	0.0[a]
EcoT Eco77	Culture filtrate (SCB)		100.0[a]	0.0[a]
EcoT Eco77	Culture filtrate (SCB)		100.0[a]	0.0[a]
EcoT Eco77	Culture filtrate (SCB)		100.0[a]	0.0[a]

The assessments described above were done on surface-decontaminated embryonic axes in order to be sure that the fungal penetration observed was only by *Trichoderma*. Additionally, these trials also afforded the opportunity of assessing the effects of co-cultivation with *Trichoderma* on the embryonic axes, other than whether they would germinate or not. In all instances, except for those axes immersed in the spore suspension and then co-cultivated with *Trichoderma* for 24 h, development was seriously compromised (Fig. 3.20). Thus only embryonic axes exposed to the spore suspension and subsequently cultured for 24 h, were selected for further tests.

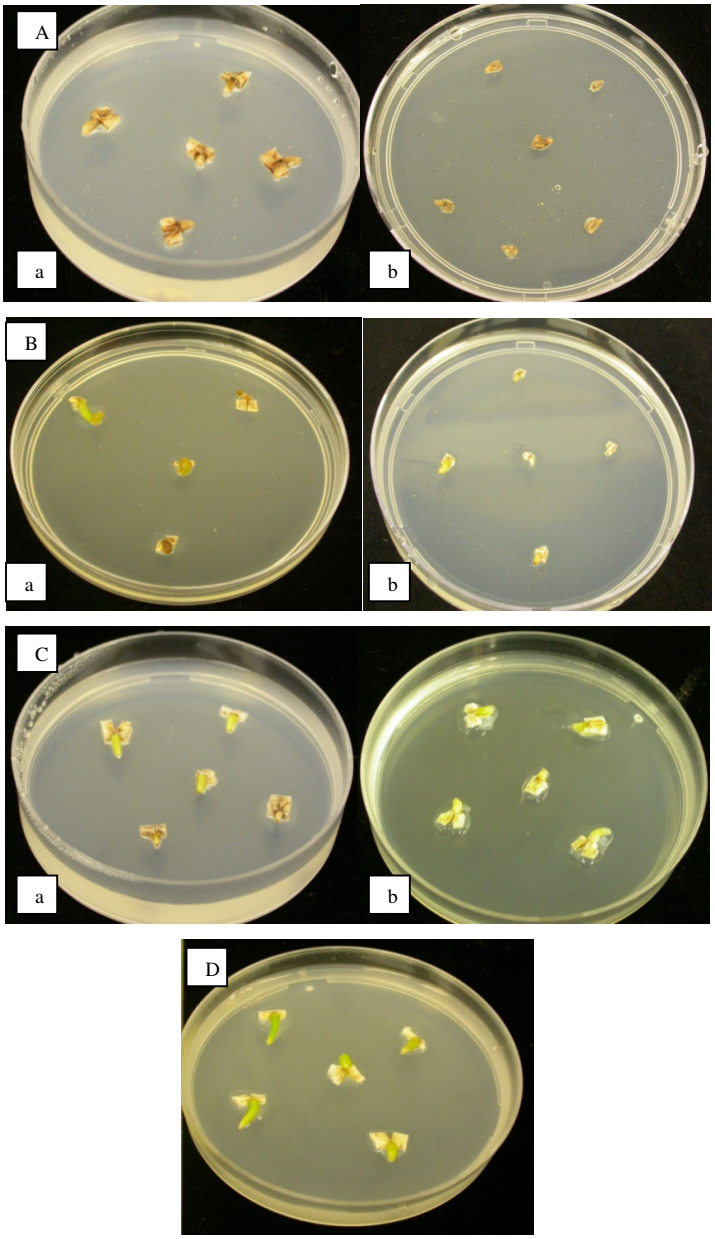

Figure 3.20A. Embryonic axes on water agar in 65 mm Petri dishes pre-inoculated with EcoT (a) and Eco77 (b) plated on half strength MS medium for 24 h, shown after 7 d on Benlate-enriched MS medium; B. embryonic axes plated on half strength MS medium in which spore suspension of EcoT (a) and Eco77 (b) had been layered, plated on half strength MS medium, followed by Benlate-enriched half strength MS medium after 24 h; (C). axes immersed with spore suspension of EcoT (a) and Eco77 (b) for 30 min and plated on half strength MS medium for 24 h followed by Benlate-enriched MS medium after 7 d; (D). axes which had not been exposed to *Trichoderma*, after 7 d on Benlate-enriched half strength MS medium.

3.4.4 Effect of Trichoderma *on the biomass of the embryonic axes of* T. dregeana *at 24 h co-cultivation period*

Studies based on the root length of seedlings (Fig. 3.21) and biomass of the roots and seedlings showed that there was a significant difference in the root length of seedlings and, root or seedling dry mass of those developing after the embryonic axes had been treated with the *Trichoderma* and the control, where axes had not been exposed to *Trichoderma* (Table 3.34). This could be because *Trichoderma* penetration had adversely affected the growth potential (vigour) of the embryonic axes.

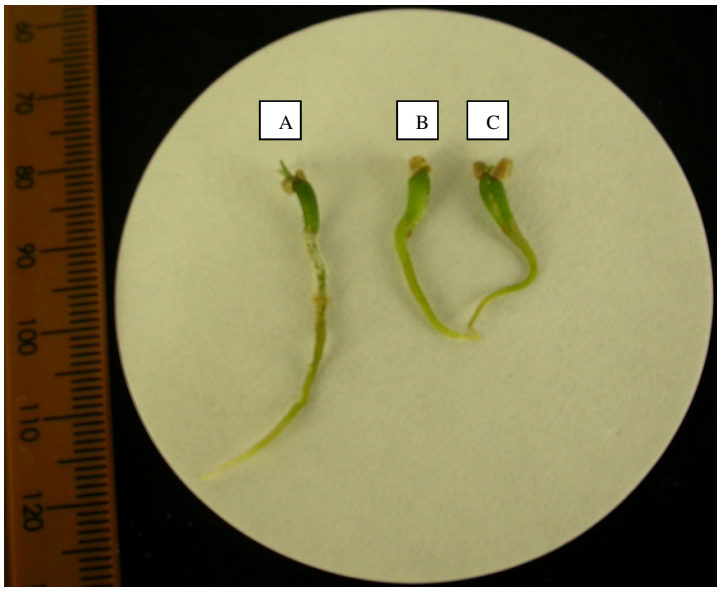

Figure 3.21: Effect on root growth of seedlings after 6 weeks in culture when axes were not exposed to *Trichoderma* (A; control) or exposed to spore suspensions from the third trial for 24 h co-cultivation with EcoT (B) or Eco77 (C).

Table 3.34: Effect of *T. dregeana* axis exposure to *Trichoderma* suspensions from the third trial for 24 h co-cultivation on root growth and dry mass of seedlings after 6 weeks in culture, (n = 15). Mean values followed by the different letters within columns are significantly different (one-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$).

Treatment with:	Root length (mm)	Root Fresh mass (mg)	Root dry mass (mg)	Seedling fresh mass (mg)	Seedling dry mass (mg)
Distilled H_2O (control)	26^a	0.0496^a	0.0055^a	0.0569^a	0.0085^a
EcoT	13.6^b	0.0380^b	0.0037^b	0.0493^b	0.0072^b
Eco77	13^b	0.0357^b	0.0037^b	0.0463^b	0.0063^b

3.4.5 Effect of Inoculating Trichoderma-*treated embryonic axes of* T. dregeana *with* Penicillium *sp.*

A trial was then conducted to check for the effect of contamination by infecting the embryonic axes with the axenic isolate of *Penicillium* sp. The axes, which had been previously co-cultivated (after dipping in the spore suspension) for 24 h with *Trichoderma*, were infected either before (first test), or after (second test), inhibiting *Trichoderma* by Benlate exposure. It was found that all the embryonic axes became contaminated by *Penicillium* spp. once the *Trichoderma* was killed. The first test was done on embryonic axes which had been co-cultivated with *Trichoderma* for 24 h, infected with the *Penicillium* sp., and then cultured on half strength MS medium for a further 24 h before being transferred to Benlate-enriched medium. None of the embryonic axes showed contamination by *Penicillium* sp. However, none of the embryonic axes from this first test survived, which was probably due to the longer co-cultivation period with *Trichoderma*. The second test was done by infecting the axes which had been co-cultivated with *Trichoderma* for 24 h and then transferred to the Benlate-enriched medium for another 24 h before infecting, with the *Penicillium* sp. on a benlate-free half strength MS medium for 5 d. This test was also not successful, as all the axes became infected by the *Penicillium* sp. (data not shown).

3.4.6 Effect of Trichoderma *inoculation in the context of contamination of the embryonic axes of* T. dregeana

In order to ascertain whether there was an effect on contamination after axis inoculation by immersing in spore suspensions of EcoT and Eco77, embryonic axes were not surface-decontaminated prior to the co-cultivation step. The results showed that somewhat lower levels of contaminated axes than in the control (treated with distilled water) persisted after inoculation with *Trichoderma* (Table 3.35). In particular, however, contamination by *Rhizopus* spp. was not significantly diminished, although *Penicillium* spp. were isolated from significantly fewer axes. However, concomitantly, bacteria proliferated from more of the axes (Table 3.36) despite co-cultivation with *Trichoderma*, particularly when the strain was obtained from Eco77.

Table 3.35: Effect of *Trichoderma* suspensions from the third trial for 24 h co-cultivation on inherent infections of embryonic axes of *T. dregeana* after 3 weeks in culture, (n = 60). Mean values followed by the different letters within columns for each co-cultivation period for different treatments are significantly different (one-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$).

Treatment with:	Co-cultivation period (h)	Germination (%)	Axes contaminated (%)	Mortality (%)
Distilled H_2O (control)	24	48.3^a	51.7^a	51.7^a
EcoT		56.7^b	43.3^b	43.3^b
Eco77		53.3^b	46.7^b	46.7^b

Table 3.36: Percentage of axes showing contamination after exposure to spore suspensions from the third trial after 24 h co-cultivation with EcoT and Eco77 after 3 weeks in culture, (n = 60). Mean values followed by the different letters within columns at each interval are significantly different (one-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$).

Treatment with:	Co-cultivation period (h)	Percentage of axes contaminated by:			
		Rhizopus spp.	*Penicillium* spp.	Unknown fungi	Bacteria
Distilled H_2O (control)	24	6.7^a	26.7^a	6.7^a	18.3^a
EcoT		3.3^a	16.7^b	0.0^b	23.3^a
Eco77		5.0^a	18.3^b	0.0^b	23.3^a

3.5 Viabilty of embryonic axes of *T. dregeana* in storage after alginate gel encapsulation

3.5.1 Alginate gel encapsulation, storage and assessment under hydrated conditions

Nipastat (the mixture of methyl-, butyl-, ethyl-, propyl- and isobutyl-paraben) and NaDCC (sodium dichloro-isocyanurate), both of which were highly effective in inhibiting proliferation of the test isolates, were used to decontaminate embryonic axes of *T. dregeana* before encapsulation and storage at 16°C (Fig. 3.22).

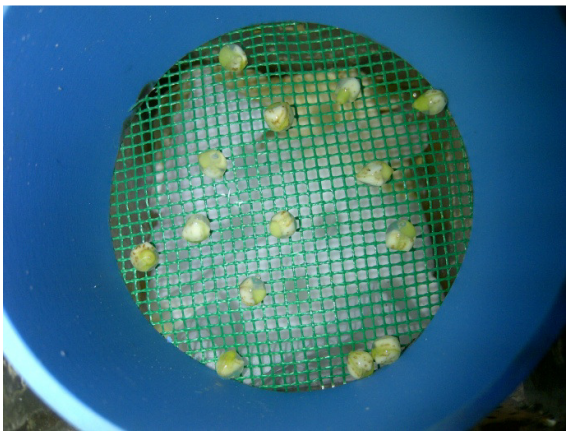

Figure 3.22: Embryonic axes of *T. dregeana* encapsulated with alginate gel and stored hydrated on a grid in a magenta box.

Prior to alginate coating, embryonic axes were immersed in Nipastat at 0.2% for 10 min or NaDCC at 0.2% (2000 ppm) for 20 min and then stored on grids in magenta boxes. However, after 14 d storage, fungal contamination was seen to have persisted, the major contaminant being *Penicillium* spp. for axes treated with both Nipastat (Tables 3.37 and 3.38) and NaDCC (Tables 3.39 and 3.40) prior to alginate encapsulation. Additionally, the embryonic axes had not retained viability (Fig. 3.23), whether treated with Nipastat, NaDCC, or distilled water (control). It was considered that axis viability loss could have been due to the absence of a carbon source. When Nipastat was used as the decontaminant (Tables 3.37 and 3.38), persistence of the contaminants was greatly reduced in the encapsulated axes compared with those exposed to distilled water prior to encapsulation. Unfortunately, the concentration of Nipastat could not be increased above 0.2% because of apparent toxicity/inhibitory effects on the embryonic axes. However, survival of the embryonic axes (Fig. 3.24A) was increased by incorporating 0.5% sucrose in the alginate, as was the case when NaDCC was used for decontamination (Table 3.39). In the case of NaDCC, persistence of the contaminants was resolved by increasing the concentration to 0.03% (3000 ppm) for the same time (20 min) and viability retention (as indicated by germinability after 14 d storage) was enhanced, all axes remaining germinable (Fig. 3.24B) when 0.5% sucrose was incorporated in the alginate gel (Tables 3.39 and 3.40). Although the viability of axes so-encapsulated was lost after 28 d in storage, this clearly was not associated with residual fungal contamination (Table 3.39).

Table 3.37: Viability and contamination after storage of axes of *T. dregeana* encapsulated in alginate gel (incorporating sucrose or not) after treatment for 10 min with 0.2% Nipastat, (n = 30). Mean values followed by the different lower case letters within columns and different uppercase letters in each row of individual parameters (Germination/Contamination) between different time intervals (14, 28 & 42 d) are significantly different. (Dependent variables were subjected to Arcsine transformation, two-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$).

Treatment with:	Sucrose (%)	% Germination of axes assessed after 14, 28 and 42 d in culture			% Axes showing contamination		
		14	28	42	14	28	42
Distilled H_2O (control)	0.0	0.0^{aA}	0.0^{aA}	0.0^{aA}	40.0^{aA}	60.0^{aB}	60.0^{aB}
	0.5	60.0^{bA}	0.0^{aB}	0.0^{aB}	46.7^{aA}	66.7^{aB}	66.7^{aB}
Nipastat	0.0	0.0^{aA}	0.0^{aA}	0.0^{aA}	13.3^{bA}	26.7^{bB}	33.3^{bC}
	0.5	86.7^{cA}	0.0^{aB}	0.0^{aB}	13.3^{bA}	20.0^{bB}	26.7^{bC}

Table 3.38: Contaminants isolated after storage for 14, 28 & 42 d of embryonic axes of *T. dregeana* encapsulated in alginate (incorporating sucrose or not) after treatment with 0.2% Nipastat for 10 min, (n = 30). Mean values followed by the different lower case letters within columns for each isolate between different treatments and different uppercase letters in each row are significantly different. (Dependent variables were subjected to Arcsine transformation, two-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$).

Treatment with:	Sucrose (%)	Contaminant	% Axes showing contamination		
			14 d	28 d	42 d
Distilled H_2O (control)	0	*Pencillium* spp.	40.0^{aA}	56.7^{aB}	46.7^{aC}
	0.5		40.0^{aA}	53.3^{aB}	66.7^{bB}
Nipastat	0		26.7^{bA}	26.7^{bA}	33.3^{cA}
	0.5		13.3^{cA}	20.0^{bB}	26.7^{cC}
Distilled H_2O (control)	0	*Fusarium* spp.	0.0^{aA}	13.3^{aB}	13.3^{aA}
	0.5		0.0^{aA}	6.7^{aB}	0.0^{bB}
Nipastat	0		0.0^{aA}	0.0^{bA}	0.0^{bA}
	0.5		0.0^{aA}	0.0^{bA}	0.0^{bA}
Distilled H_2O (control)	0	Bacteria	0.0^{aA}	0.0^{aA}	0.0^{bB}
	0.5		6.7^{bA}	6.7^{bA}	0.0^{aA}
Nipastat	0		0.0^{aA}	6.7^{bB}	0.0^{aA}
	0.5		0.0^{aA}	0.0^{aA}	0.0^{aA}

Table 3.39: Viability and contamination after 14, 28 & 42 d of axes of *T. dregeana* encapsulated in alginate (incorporating sucrose or not) after treatment with 0.2% or 0.3% NaDCC for 20 min. Control axes were exposed to distilled water before encapsulation, (n = 30). Mean values followed by the different lower case letters within columns and different uppercase letters in each row of individual parameters (Germination/Contamination) between different time intervals (14, 28 & 42 d) are significantly different. (Dependent variables were subjected to Arcsine transformation, two-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$).

Treatment with:	Conc:	Sucrose (%)	% Germination of axes			% Axes showing contamination		
			14 d	28 d	42 d	14 d	28 d	42 d
Distilled H_2O (control)		0.0	0.0^{aA}	0.0^{aA}	0.0^{aA}	33.3^{aA}	60.0^{aB}	60.0^{aB}
		0.5	53.3^{bA}	0.0^{aB}	0.0^{aB}	46.7^{bA}	60.0^{aB}	66.7^{aC}
NADCC	0.2	0.0	0.0^{aA}	0.0^{aA}	0.0^{aA}	20.0^{cA}	33.4^{bB}	46.6^{bC}
		0.5	73.3^{cA}	0.0^{aB}	0.0^{aB}	26.7^{cA}	47.0^{cB}	47.0^{bB}
	0.3	0.0	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.0^{dA}	0.0^{dA}	10.0^{cB}
		0.5	100.0^{dA}	0.0^{aB}	0.0^{aB}	0.0^{dA}	0.0^{dA}	13.3^{cB}

Table 3.40: Contaminants isolated after storage for 14, 28 & 42 d of embryonic axes of *T. dregeana* encapsulated in alginate (incorporating sucrose or not) after 20 min treatment with 0.2 or 0.3% NaDCC, (n = 30). Mean values followed by the different lower case letters within columns for each isolate between different treatments and different uppercase letters in each row are significantly different. (Dependent variables were subjected to Arcsine transformation, two-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$)

Treatment with:	Sucrose (%)	Contaminant	% Axes showing contamination		
			14 d	28 d	42 d
Distilled H_2O (control)	0.0	*Pencillium* spp.	40.0^{aA}	53.3^{aB}	66.7^{aC}
	0.5		33.3^{aA}	53.3^{aB}	60.0^{aC}
NADCC (0.2%)	0.0		26.7^{bA}	26.7^{bA}	33.3^{bB}
	0.5		20.0^{bA}	33.3^{bB}	40.0^{bC}
NADCC (0.3%)	0.0		0.0^{cA}	0.0^{cA}	10.0^{cB}
	0.5		0.0^{cA}	0.0^{cA}	13.3^{cB}
Distilled H_2O (control)	0.0	*Fusarium* spp.	0.0^{aA}	6.7^{aB}	0.0^{aA}
	0.5		0.0^{aA}	0.0^{bA}	6.7^{bB}
NADCC (0.2%)	0.0		0.0^{aA}	0.0^{bA}	0.0^{aA}
	0.5		0.0^{aA}	0.0^{bA}	0.0^{aA}
NADCC (0.3%)	0.0		0.0^{aA}	0.0^{bA}	0.0^{aA}
	0.5		0.0^{aA}	0.0^{bA}	0.0^{aA}
Distilled H_2O (control)	0.0	*A. niger*	0.0^{aA}	0.0^{aA}	13.3^{aB}
	0.5		0.0^{aA}	6.7^{bB}	0.0^{bA}
NADCC (0.2%)	0.0		0.0^{aA}	0.0^{aA}	13.3^{aB}
	0.5		6.7^{bA}	13.3^{cB}	6.7^{cA}
NADCC (0.3%)	0.0		0.0^{aA}	0.0^{aA}	0.0^{bA}
	0.5		0.0^{aA}	0.0^{aA}	0.0^{bA}
Distilled H_2O (control)	0.0	Bacteria	6.7^{aA}	6.7^{aA}	0.0^{aB}
	0.5		0.0^{bA}	0.0^{bA}	0.0^{aA}
NADCC (0.2%)	0.0		0.0^{bA}	6.7^{aB}	0.0^{aA}
	0.5		0.0^{bA}	0.0^{bA}	0.0^{aA}
NADCC (0.3%)	0.0		0.0^{bA}	0.0^{bA}	0.0^{aA}
	0.5		0.0^{bA}	0.0^{bA}	0.0^{aA}

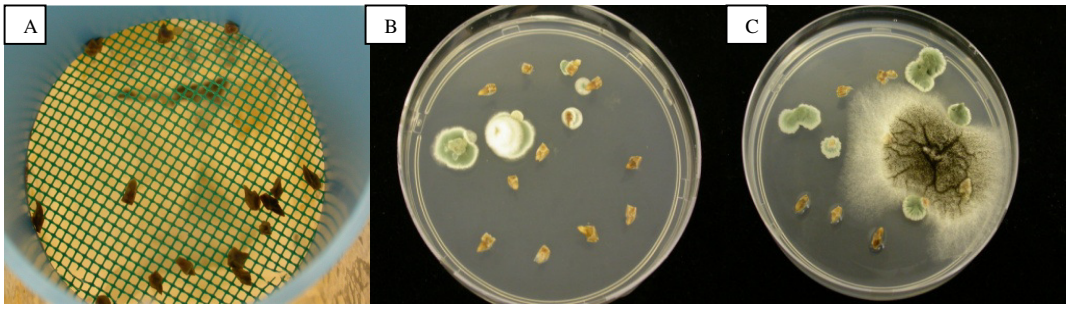

Figure 3.23: (A) Necrotic appearance after 14 d of embryonic axes coated with alginate gel, but not provided with sucrose; (B & C) embryonic axes, none of which germinated, showing contamination when plated on half strength MS medium in 90 mm Petri dishes. Axes had been decontaminated with Nipastat (B) and distilled water control (C) prior to encapsulation.

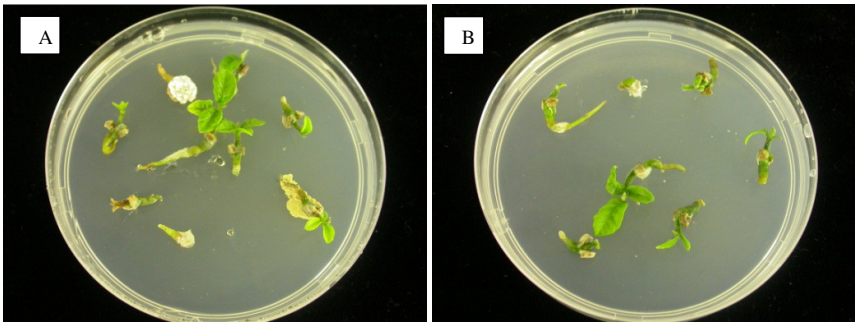

Figure 3.24: Seedlings developed after 3 months on half strength MS medium in 90 mm Petri dishes from embryonic axes treated with Nipastat (A) or NaDCC (B) which was stored encapsulated in alginate gel incorporating 0.5% sucrose for 14 d.

3.5.2 The effect of the use of different storage containment on alginate-encapsulated axes of T. dregeana

The viability and water content of the axes varied over the storage period with the different types of containment. Encapsulated axes which had initially been decontaminated with NaDCC (Fig. 3.25; Table 3.41) and stored in polythene bags maintained 100% germinability for all three time intervals (14, 28 and 42 d). It was also observed that the alginate capsules remained intact throughout the storage period. Seedlings established from encapsulated axes stored for 42 d in polythene bags were as vigorous as those developing from encapsulated axes that were not stored (Fig. 3.25). Viability of axes stored in Magenta boxes, remained high for 14 d, but the alginate capsule appeared to have thinned, when sampled at 28 and 42 d, no axes had remained encapsulated and none were viable (Fig. 3.26). In contrast, the alginate coating of the axes contained in from Eppendorf tubes and foil-lined aluminium bags remained intact; however, the viability (germinability) of the axes was compromised at all sampling intervals (Table 3.41). Loss of viability was not associated with micro-organisms, as contamination after NaDCC treatment was effectively zero. In contrast, those axes not NaDCC-

decontaminated prior to alginate encapsulation showed bacterial and fungal contamination, with bacteria being the dominant contaminant Table 3.41).

Figure 3.25: Seedlings developed from encapsulated axes after 6 weeks on half strength MS medium. Encapsulation was in alginate gel incorporating 0.5% sucrose, the beads being stored for 42 d in a Magenta box (A), a foil-lined bag (B), an Eppendorf tube (C), a polythene bag (D) and a seedling developed from an unstored bead (control) (E).

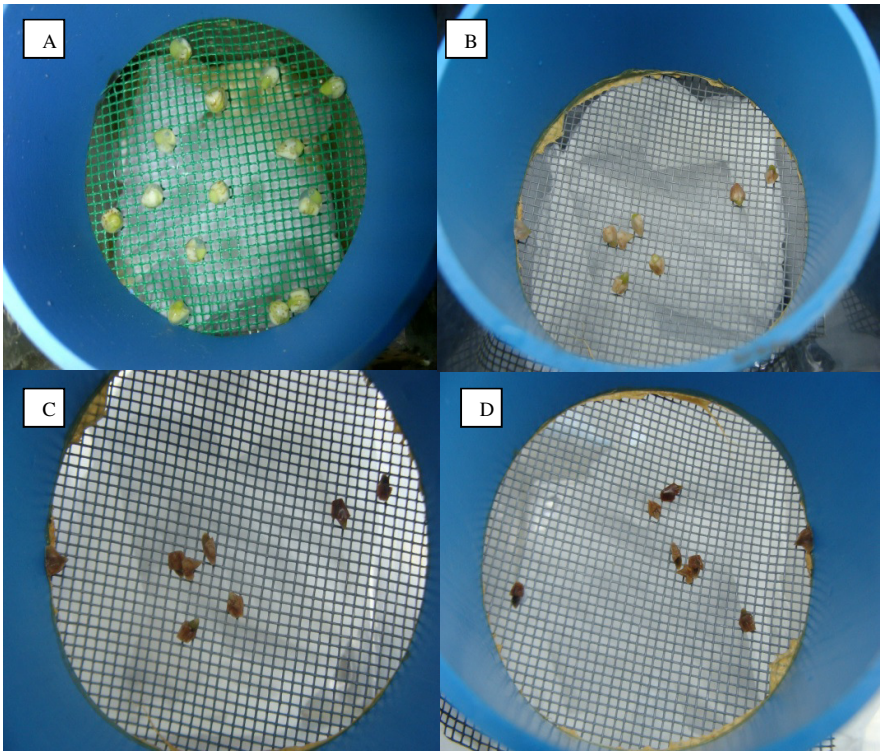

Figure 3.26: Alginate encapsulated embryonic axes of *T. dregeana* in Magenta boxes before storage (A), after 14 d (B), 28 d (C) and 42 d (D).

Table 3.41: Viability and contamination of *T. dregeana* axes (initially decontaminated or not, with NaDCC) encapsulated in alginate and stored in the different types of containment for 14-42 d. Control axes were not stored, (n = 30). Mean values followed by the different lower case letters within columns (Germination/Axis contamination) and different upper case letters in each row different contaminants, are significantly different (Dependent variables were subjected to Arcsine transformation, two-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$).

Treatment	Containment	Storage period (days)	Germination (%)	Percentage of axes contaminated by:			
				Bacteria	*Rhizopus* spp.	*Penicillium* spp.	*Fusarium* spp.
Encapsulated;with NaDCC pre-treatment	No storage	0	100^{a}	0^{aA}	0^{aA}	0^{aA}	0^{aA}
	Magenta box	14	93.3^{a}	0^{aA}	0^{aA}	0^{aA}	0^{aA}
		28	0^{e}	0^{aA}	0^{aA}	0^{aA}	0^{aA}
		42	0^{e}	0^{aA}	0^{aA}	0^{aA}	0^{aA}
	Foil-lined bag	14	46.7^{b}	6.7^{abA}	0^{aA}	0^{aA}	0^{aA}
		28	6.7^{d}	0^{aA}	0^{aA}	0^{aA}	0^{aA}
		42	0^{e}	0^{aA}	0^{aA}	0^{aA}	0^{aA}
	Polythene bag	14	100^{a}	0^{aA}	0^{aA}	0^{aA}	0^{aA}
		28	100^{a}	0^{aA}	0^{aA}	0^{aA}	0^{aA}
		42	100^{a}	0^{aA}	0^{aA}	0^{aA}	0^{aA}
	Eppendorf tube	14	6.7^{de}	0^{aA}	0^{aA}	0^{aA}	0^{aA}
		28	0^{e}	0^{aA}	0^{aA}	0^{aA}	0^{aA}
		42	0^{e}	0^{aA}	0^{aA}	0^{aA}	0^{aA}
Encapsulated;without NaDCC pre-treatment	No storage	0	43.3^{b}	36.7^{bA}	13.3^{aB}	6.7^{aB}	0^{aB}
	Magenta box	14	16.7^{de}	80^{efgA}	0^{aB}	3.3^{aB}	0^{aB}
		28	0^{e}	50^{cA}	0^{aB}	0^{aB}	0^{aB}
		42	0^{e}	26.7^{bA}	0^{aB}	3.3^{aB}	16.7^{bAB}
	Foil-lined bag	14	10^{de}	73.3^{defA}	10^{aB}	0^{aB}	0^{aB}
		28	10^{de}	93.3^{fgA}	0^{aB}	0^{aB}	0^{aB}
		42	0^{e}	93.3^{fgA}	0^{aB}	0^{aB}	0^{aB}
	Polythene bag	14	23.3^{cd}	60^{cdeA}	10^{aB}	6.7^{aB}	0^{aB}
		28	10^{de}	90^{fgA}	0^{aB}	0^{aB}	0^{aB}
		42	33.3^{bc}	53.3^{cdA}	6.7^{aB}	6.7^{aB}	0^{aB}
	Eppendorf tube	14	0^{e}	100^{gA}	0^{aB}	0^{aB}	0^{aB}
		28	0^{e}	76.7^{efA}	0^{aB}	0^{aB}	0^{aB}
		42	0^{e}	90^{fgA}	0^{aB}	0^{aB}	0^{aB}

Non-encapsulated axes that were previously decontaminated with NaDCC (Fig. 3.27; Table 3.42) and stored in polythene bags maintained germination percentages of 96.7-93.3 over the 14–42 d of storage, The germinability of decontaminated axes stored in foil-lined bags had dropped to 70% after 14 d, declining to zero by day 42. When contained in Magenta boxes, after 14 d only 46.7% of axes remained viable, with none germinating after 28 or 42 d. Axes stored in Eppendorf tubes also showed a significant loss of viability within 14 d, when only 36.7% germinated, and none remained viable after 28 d Table 3.42). It was observed that, although >90% of axes stored in polythene bags germinated, radicles were somewhat shorter than those developed by non-stored axes (Fig. 3.27). Viability of unencapsulated axes not decontaminated with NaDCC was compromised from the outset, irrespective of the containment, or if not stored. Both bacteria and fungi proliferated on unencapsulated, non-decontaminated axes, whereas there was no contamination of NaDCC-treated, unencapsulated axes (Table 3.42).

Figure 3.27: Seedlings developed after 6 weeks on half strength MS medium, from non-encapsulated axes stored for 42 d in a Magenta box (A), a foil-lined bag (B), an Eppendorf tube (C), a polythene bag (D) and an unstored (control) axis (E).

Table 3.42: Viability and contamination of *T. Dregeana* axes (initially decontaminated or not, with NaDCC) that were non-encapsulated and stored in the different types of containment for 14-42 d. Control axes were not stored, (n = 30). Mean values followed by the different lower case letters within columns (Germination/Axis contamination) and different upper case letters in each row for different contaminants are significantly different (Dependent variables were subjected to Arcsine transformation, two-way ANOVA, Duncan's Multiple Range Test, $p \leq 0.05$).

Treatment	Containment	Storage period (days)	Germination (%)	Percentage of axes contaminated by:			
				Bacteria	*Rhizopus* spp.	*Pencillium* spp.	*Fusarium* spp.
Non-encapsulated; NaDCC pre-treatment	No storage	0	100^{a}	0^{aA}	0^{aA}	0^{aA}	0^{aA}
	Magenta	14	46.7^{c}	0^{aA}	0^{aA}	0^{aA}	0^{aA}
		28	0^{i}	0^{aA}	0^{aA}	0^{aA}	0^{aA}
		42	0^{i}	0^{aA}	0^{aA}	0^{aA}	0^{aA}
	Foil	14	70^{b}	0^{aA}	0^{aA}	0^{aA}	0^{aA}
		28	36.7^{d}	0^{aA}	0^{aA}	0^{aA}	0^{aA}
		42	0^{i}	0^{aA}	0^{aA}	0^{aA}	0^{aA}
	Plastic	14	96.7^{a}	0^{aA}	0^{aA}	0^{aA}	0^{aA}
		28	93.3^{a}	3.3^{abA}	0^{aA}	0^{aA}	0^{aA}
		42	93.3^{a}	6.7^{abA}	0^{aA}	0^{aA}	0^{aA}
	Eppendorf	14	36.7^{d}	0^{aA}	0^{aA}	0^{aA}	0^{aA}
		28	0^{i}	0^{aA}	0^{aA}	0^{aA}	0^{aA}
		42	0^{i}	0^{aA}	0^{aA}	0^{aA}	0^{aA}
Non- encapsulated; without NADCC pre-treatment	No storage	0	13.3^{gh}	20^{bcA}	26.7^{bA}	36.7^{cdA}	3.3^{aB}
	Magenta	14	20^{fgh}	23.3^{bcA}	6.7^{abB}	46.7^{dA}	3.3^{aB}
		28	0^{i}	13.3^{abcA}	0^{aB}	36.7^{cdC}	0^{aB}
		42	0^{i}	13.3^{abcA}	3.3^{abA}	36.7^{cdB}	6.7^{aA}
	Foil	14	10^{hi}	30^{cA}	10^{abB}	46.7^{dA}	3.3^{aB}
		28	13.3^{gh}	26.7^{bcA}	13.3^{abAC}	43.3^{dB}	3.3^{aC}
		42	0^{i}	23.3^{bcA}	0^{aB}	43.3^{dC}	10^{aB}
	Plastic	14	26.7^{def}	16.7^{abcA}	6.7^{abA}	50^{dB}	0^{aA}
		28	23.3^{efg}	26.7^{bcA}	6.7^{abB}	43.3^{dC}	0^{aB}
		42	16.7^{d}	26.7^{bcA}	6.7^{abB}	50^{dC}	0^{aB}
	Eppendorf	14	0^{i}	70^{dA}	13.3^{abB}	10^{abB}	6.7^{aB}
		28	0^{i}	70^{dA}	10^{abB}	20^{bcBC}	0^{aB}
		42	0^{i}	83.3^{dA}	0^{aB}	16.7^{abC}	0^{aB}

The water content of encapsulated axes (decontaminated with NaDCC) (Fig. 3.28) had increased the most was after 42 d when the axes were stored in aluminium foil-lined bags, followed by those contained in polythene bags. Water content of encapsulated exes contained in Eppendorf tubes for 42 d had also increased relative to that of the control axes (0 day storage), while axes stored in Magenta

boxes had lost water relative to the unstored, control axes. The water content of axes that were not encapsulated was essentially similar after 42 d (and somewhat lower than the unstored control axes), irrespective of the containment.

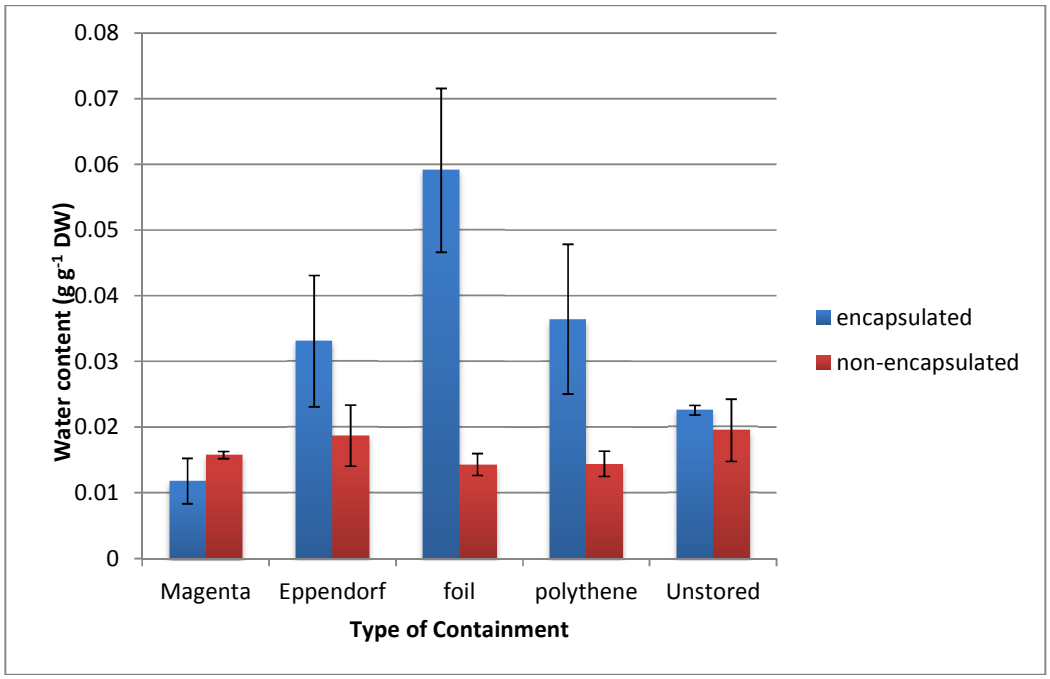

Figure 3.28: Water contents of non-encapsulated and encapsulated axes of *T. dregeana* in alginate gel after 42 d of storage in the different types of containment (n = 5). Bars indicate one standard deviation about the mean.

CHAPTER 4

DISCUSSION

4.1 The need for optimising an ideal decontamination protocol for the embryonic axes of *Trichilia dregeana*

Microbial contamination constitutes a significant problem in plant tissue culture. Seed surfaces carry a wide variety of microbial contaminants especially fungal species. In order to avoid microbial contamination, seed tissues should be surface decontaminated before placing on the culture medium. It is necessary to render the seeds contamination-free so as to protect the inner tissues from fungal or bacterial penetration which will eventually make it difficult or even impossible to eliminate the infection (Sutherland *et al*., 2002). These problems are particularly pertinent when the aim is cryopreservation of the embryonic axes of recalcitrant-seeded species (Berjak *et al*., 1999a,b).

Trichilia dregeana seeds, being recalcitrant, are shed at high water content, making them more prone to invasion by fungi once on the ground and within storage containers in which saturated relative humidity must be maintained (Berjak and Pammenter, 2004). If the seeds are collected from the ground and immediately surface decontaminated, it should avoid the risk of superficially located fungi from invading the inner tissues (Sutherland *et al*., 2002). It is also of utmost importance to choose a surface decontaminant which is not toxic to the tissues of the seeds and ultimately, the explants. Thus the concentration of the surface decontaminant and the time period taken for the treatment should be chosen to eliminate contaminants, but to avoid – or at least, to minimise – injury to the plant tissues. The present study was carried out on freshly harvested embryonic axes of *T. dregeana* and the effectiveness of surface decontamination was based on the percentage showing residual contamination, and on retention of axis vigour and viability.

4.2 Effect of different surface decontaminants on excised axes of *T. dregeana*

The surface decontaminants, 1% w/v calcium hypochlorite ($Ca(OCl)_2$), 1% v/v sodium hypochlorite (NaOCl) and 0.1% w/v mercuric chloride ($HgCl_2$) were used for sterilising the embryonic axes. It was shown that exposure to 1% (v/v) sodium hypochlorite for 10 min or 0.1% (w/v) mercuric chloride for 5 min were effective as decontaminating treatments. However, mercuric chloride had adverse effects on the axes in terms of their compromised vigour. Mercury is widely acknowledged to be highly toxic (ATSDR, 2001), which could be the basis of the deleterious effects on the *T. dregeana* axes. Similar results were obtained when mercuric chloride was used on barley seeds (Jonathan *et al*., 2002) and also when applied to the axes of *Andrographis paniculata* (Talei *et al*., 2011). The toxic effects of mercuric chloride depend on two factors, *viz*. exposure time and concentration used: in the present study although the concentration of mercuric chloride was low (0.1%) and the exposure time was only 5 min, the treatment nevertheless compromised the vigour of the embryonic axes of *T. dregeana*. Although there was no residual fungal contamination after exposure to calcium hypochlorite, bacterial contamination was still prevalent. Hypochlorites generally, are effective against micro-organisms, even at low concentrations. In the current study, sodium hypochlorite was finally chosen for surface decontamination of the embryonic axes as it eliminated both the fungi and bacteria, and did not have a negative impact on axis vigour or viability. Use of sodium hypochlorite has been highly recommended compared with other sterilants because of its widespread availability, low cost, high efficiency, and the ease with which it can be diluted to any required concentration (Talei *et al.*, 2011).

4.3 Optimising different methods of decontamination

Different methods of decontamination using anodic water, chemical disinfection using Nipastat and Benlate, and exposure to non-volatile compounds elaborated by the biocontrol agents were all tested for their effectiveness against contamination and possible impact on axis germinability.

4.3.1 Electrolysed oxidising (EO) water

Electrolysed oxidising (EO) water is being used as a disinfectant in Japanese hospitals and dental clinics, and for food (Izumi, 1999; Venkitanarayanan *et al*., 1999a, 1999b) as well as in the agriculture industry (Grech and Rijkenberg, 1992; Yamaki, 1998; Bonde *et al*., 1999). The sanitising effect of EO water depends on two factors, *viz*. oxidation-reduction potential (ORP)

and pH. In some cases the high ORP has been suggested to be responsible for reduction of contamination; an example is the reduction of teliospore germination on *Tamerindus indica* seeds (Bonde *et al*., 1999). In other cases both high ORP and low pH were considered together to be responsible for reducing contamination. As an example, the EO water at pH 3 and ORP +1165 mV was found to be more effective against the brown rot disease of peach than the EO water at pH 6.8 and oxidation reduction potential +940 mV (Al-Haq *et al*., 2001).

In the present study, however, despite the properties of EO water as a sterilant, it did not effectively eliminate the contamination but only delayed proliferation of the contaminants present on the surfaces of the embryonic axes of *T. dregeana* for 3 to 4 d compared with control axes. Even increasing the time of exposure to EO water from 10 to 120 min, did not result in any significant effect against fungal contaminants. Therefore, in the present study the treatment with EO water was shown to be only fungistatic but not fungicidal. This could be because the HOCl in EO water, although being a good oxidant, is considered to act first against inorganic and organic material, the debris, including that at plant wound sites, rather than on the micro-organisms: in studies on fruit, these factors were suggested to decrease its effectiveness against microflora partially or fully embedded in the (fruit) tissues (Eckert and Sommer, 1967; Dychdala, 1991). The present results are in agreement with studies on the fungistatic effect of EO water on brown rot of peach (*Prunus persica*), in that although it was an effective surface sanitiser, it delayed the disease development for only 7 d (Al-haq *et al*., 2001). It therefore appears that, although EO water may be an effective decontaminant in the short-term, it cannot be recommended as an appropriate sterilant for axes which are to be germinated *in vitro*.

4.3.2 Nipastat

The recommended usage of Nipastat as a preservative is as at concentrations of 0.05 to 0.3%. Studies have shown that some of the common moulds and yeasts are killed by Nipastat at a concentration of 0.13% as are bacteria using 0.1 and 0.13% concentrations, when exposed for different time periods[2]. In the current study, *Penicillium* spp. and *Rhizopus* spp. were significantly inhibited only at Nipastat concentrations of 0.03% or higher in the fungal toxicity tests. Although Nipastat proved to be a good fungicide at higher concentrations, it was found to be toxic to the *T. dregeana* embryonic axes after effective exposure times. Germination of the embryonic axes placed on a 0.03% Nipastat-enriched half strength MS medium was adversely affected, while it was not an effective decontaminant at concentrations 0.01 to 0.02%. It was also noted that

Penicillium spp. and *Rhizopus* spp. persisted even when the medium incorporated 0.03% Nipastat. On other hand, when embryonic axes were exposed to higher concentrations for longer times, as in the case of culture on Nipastat-enriched medium, the chemical not only killed the fungi and bacteria associated with the embryonic axes, but also impacted negatively on axis germination. However, the latter may have been the effect of delaying germination within the 3-week culture period presently used, as Motete *et al.* (1997) showed that when *Avicennia marina* seeds were encapsulated in a Nipastat-containing alginate gel, fungal proliferation was inhibited and germination in hydrated storage was significantly delayed. Nipastat has also proven to be effective as rinse-off formulations[3], as was the case presently when used as solutions of different concentrations in which the embryonic axes were immersed for varying time periods. It was presently shown that immersing embryonic axes in 0.2% aqueous Nipastat solutions for 10 min was effective in eradicating the contaminants and at the same time was not toxic to the embryonic axes.

4.3.3 Medi-Chlor® (sodium dichloro-isocyanurate [NaDCC])

Medi-Chlor has been used for water purification and has proven to be effective especially in the rural parts of India (Bachhuber *et al*., 2008). The active ingredient in Medi-Chlor, sodium dichloro-isocyanurate (NaDCC) is considered to be more effective compared with the hypochlorites[4]. Presently NaDCC eliminated all the fungal contaminants from the embryonic axes of *T. dregeana* even when used for 10 min at the low concentration of 0.2%, compared with NaOCl used at 1%. The results showed that although exposure to 0.2% NaDCC for 10 min did eradicate the fungi, bacterial contamination was observed after 6 d, suggesting perhaps that NaDCC suppressed, rather than eliminated, the bacterial contaminants for 6 d, but lost its effectiveness later. However, this seemingly had no effect on the embryonic axes, all of which germinated. Furthermore, there was 100% seedling establishment by all the bacterially-contaminated as well as the uncontaminated axes. When NaDCC was used at 0.3% for 20 min, there was neither bacterial nor fungal contamination, with 100% of the axes germinating. Thus Medi-Chlor appears to be an effective bactericide for explants prior to *in vitro* culture, as shown in the present study when used at an appropriate concentration for a suitable time. These observations are in agreement with information contained in the test report by the South African Bureau of Standards (SABS) indicating the effectiveness of NaDCC against the bacteria, *Pseudomonas aeruginosa, Staphylococcus aureus* and *Eschericia coli*[4]. The present results indicating the efficacy of NaDCC in eliminating both fungi and bacteria, and its non-toxic nature,

provide a good argument for its use in place of either of the hypochorites or of mercuric chloride for plant tissue culture. This could be of particular significance when excised embryonic axes are to be cryopreserved, making testing of the use of NaDCC in such endeavours a logical next step.

4.3.4 Benlate

Benlate is considered to be an effective fungicide, due to its selective binding to fungal microtubules, which are essential structures involved in intracellular transport, cell division and cell wall synthesis, therefore interfering with fungal cell functionality[20 1] (Benomyl, 1994). However, in the present study Benlate was not universally effective against the fungi isolated from embryonic axes of *T. dregeana* when used at various concentrations from 0.01 to 1%. *Aspergillus niger, A. flavus* and *Fusarium* spp. were completely inhibited by Benlate, even at lower concentrations, but the major contaminants, *Penicillium* spp. and *Rhizopus* spp. were resistant. At the highest concentration, Benlate (1%in PDA medium) inhibited the *Penicillium* spp. significantly but not the *Rhizopus* spp. These results are in agreement with those of Eckert and Ogawa (1988) in terms of the lack of efficacy of Benlate against some of the major post-harvest contaminants, *Rhizopus* spp., *Mucor* spp., *Phytophthora* spp., *Alternaria* spp., *Geotrichum* spp., and soft rot bacteria. The lack of ability of Benlate to inhibit the growth of *Rhizopus stolonifer* completely was reported by Asare-Bediako *et al*. (2007) as being due to the lack of sensitivity to the fungicide. This is in agreement with the earlier work of Fry (1982) who reported that benomyl, as a systemic fungicide, while being especially effective against Ascomycetes was not effective against *R. stolonifer*, a phycomycete (Gilman, 1957). Although Benlate is highly effective against *Penicillium* spp. (Harding, 1968; Spalding *et al*., 1969), some strains of the species – notably *P. digitatum* and *P. italicum* in citrus fruits, and *P. expansum* in pome and stone fruits – have developed resistance to Benlate (Barkai-Golan, 2001). This is ascribed to selection pressures on contaminant populations associated with the repeated use of Benlate for long periods (Georgopoulos, 1977).

In the present study Benlate was also used to kill the *T. harzianum* associated with embryonic axes originally inoculated with the biocontrol agents; there was 100% inhibition for both strains of *T. harzianum*, i.e. from EcoT and Eco77, when 0.01% (100 ppm) of Benlate was used. A study conducted on the effect of Benlate against *Trichoderma* by Khan and Shahzad (2007) similarly

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[20] http://www.inchem.org/documents/pds/pds/pest87

indicated that Benlate completely inhibited the growth of *T. harzianum* when used at concentrations of 0.01%, 0.1% (1000 ppm) and 1.0% (10000 ppm).

4.3.5 Biocontrol agents and their effects

Controlling plant diseases using chemicals is a challenge in view of their toxic effects, high cost and development of resistant strains of contaminants. Thus the need for an eco-friendly approach for plant protection is crucial, and the use of biological control agents is the best alternative for replacing chemical control. In the current research one of the most efficient of biological control agents, *T. harzianum*, was studied with strains originating from the two preparations, EcoT and Eco77. The antagonistic quality of this fungus derives from its ability to release metabolites (Schirmbock *et al*., 1994), and cell wall degrading compounds which are effective against fungal contaminants (Chet *et al*., 1967; Elad *et al*., 1982; Kucuk and Kivanc, 2003).

4.3.5.1 Effect of direct diffusible metabolites of EcoT and Eco77

Based on dual culture tests the two *Trichoderma harzianum* isolates, *viz*. EcoT and Eco77, were found to inhibit the growth of five test isolates strongly, which is suggested to be through mycoparasitism, competition and antibiosis based on PIRG (Percent Inhibition of Radial Growth) values. The main factor that appeared to contribute to the inhibition of the test isolates was the competition imposed by the *Trichoderma* strains as the result of their rapid colonisation of the medium, compared with that of the isolates: this has been held to occur even before the release of mycotoxins by *Trichoderma* takes place (Dennis and Webster, 1971b; Simon and Sivasithamparam, 1988; Schirmbock *et al*., 1994). All test isolates and those of *Trichoderma* were inoculated at the same time, except in the case of *Rhizopus* sp., which grew faster than *Trichoderma*, as also observed by Tello *et al*. (2009). Therefore plates were inoculated with *Trichoderma* first and after 2 d *Rhizopus* sp. was introduced, to study factors other than growth rate that could contribute to its inhibitory effects. Although microscopical observations were not made, it is possible that inhibition of *Rhizopus* sp. could have been due to mycoparasitism, by *Trichoderma* hyphae coiling round those of *Rhizopus* sp., and enzymatically penetrating the cell wall of the latter (Chaturvedi and Dwivedi, 1983).

The major cell wall degrading enzymes reported for *T. harzianum* are chitinase and β-1,3 glucanase (Sivan *et al*., 1984). The *Trichoderma* strain from EcoT was a better antagonist against *A. niger* and *Penicillium* sp. than the strain from Eco77 in terms of its higher inhibition ability

and the shorter time taken to overgrow (cover the plate) the test isolates. However, the strain of *Trichoderma* from Eco77 was more effective against *A. flavus* and *Rhizopus* sp., and both the strains of *T. harzianum* were equally good antagonists against *Fusarium* sp. This varying degree of inhibition is held to be related to the different antagonistic potential of different strains and species of *Trichoderma* (Roiger and Jeffers, 1991).

Studies based on the dual culture methods with *Arabidopsis* seedlings and *Trichoderma harzianum* or *Trichoderma virens* showed increase in growth and development of lateral roots of the seedlings compared with those not exposed to the *Trichoderma* spp. The changes in the growth and development of the seedlings were found to be due to the influence of auxin signalling, as well as the ability of the *Trichoderma* species to produce indole compounds, *viz.* indole-3-acetic acid (IAA), indole-3-acetaldehyde (IAAld), and indole-3-ethanol (IEt), which were associated with mediating plant growth promotion (Contreras-Cornejo *et al*., 2009). In contrast, in the present study of dual culture of the axes of *T. dregeana* with EcoT or Eco77, there were significant decreases in root length compared with growth parameters of the control axes. It is possible that the diffusible metabolites which inhibited the growth of the fungal agents also may have affected the growth and development of the axes. It is also possible that the duration of exposure of the axes to *Trichoderma* may have impacted negatively on their growth. Transferring axes from dual cultures (between 1 to 3 d, shortly before contact is established between the *Trichoderma* and the axes) onto a fresh half strength MS medium without *Trichoderma,* may negate the negative effects presently reported. However, further studies to elucidate the negative effects of diffusible metabolites of *Trichoderma* on root length need to be undertaken.

4.3.5.2 Effect of volatile metabolites

Most of the *Trichoderma* spp. are believed to produce volatile secondary metabolites that have a negative impact on the growth of plant contaminants, e.g. *Fusarium oxysporum, Rhizoctonia solani, Curvularia lunata, Bipolaris sorokiniana, Collectotrichum* spp. and others (Xiao-Yan *et al*., 2006; Zivkovic *et al*., 2010). Studies have revealed that volatile metabolites, *viz.* ethylene, hydrogen cyanide, aldehydes and ketones evolved by *Trichoderma* spp. act against contaminants (Vey *et al*., 2001). Additionally, some *Trichoderma* spp. were found to produce dermadin (Pyke and Dictz, 1960), and others, trichodermin (Godtfredsen and Vagedal, 1965), as the main volatile antibiotics which inhibited many pathogenic organisms. However, the current study showed that volatile metabolites of EcoT and Eco77 either were not produced, or, if present, were not inhibitory to any of the test isolates. Similar results were obtained for *Pythium afertile*, which was

not affected by the volatile metabolites of *T. harzianum*, *T. konengii* or *T. viridae* (Gildiyall and Pandey, 2008). However, no qualitative tests were performed which would have revealed whether or not the strains of *Trichoderma* produced volatile metabolites under the culture conditions used in the present study. Thus it cannot be conclusively stated that the lack of inhibition of the test isolates was due to the ineffectiveness of volatile metabolites.

4.3.5.3 Effect of non-volatile metabolites

Culture filtrates were used in the present study to demonstrate the possible presence and role of non-volatile metabolites in the antagonistic behaviour of *T. harzianum*. The results showed the best incubation time of *Trichoderma* giving the highest inhibition of the test isolates was 14 d, beyond which there was a decline in inhibition. Previous studies on analyses of culture filtrate from *Trichoderma harzianum* L1 have shown loss of production of the lytic enzymes, ß-1, 3-glucanase, chitinase, xylanase and protease, when incubation time at 40°C exceeded 120 h (Jayalksmi, *et al*., 2009). In the present study, 14 d incubation at 28±2°C of *Trichoderma* to obtain effective culture filtrates was found to be best. In this context, it is suggested that the loss of lytic enzyme activity under culture conditions at 40°C reported by Jayalksmi *et al.* (2009) might have been the consequence of thermal denaturation, which did not occur at 28±2°C in the present investigation. It is suggested that the decline in inhibition observed after 14 d in the present study (and perhaps that reported by Jayalksmi *et al.* [2009]) might have been the result of a negative feed-back – i.e. beyond a threshhold, there might be a reduced production of non-volatile metabolites. The effect of the culture filtrates of *Trichoderma* on growth inhibition of other fungi could have been due to the presence of antibiotics. Antibiotics (trichodermin, trichodermol, harzianum A, dermadin and harzianolide) in the culture filtrates of *Trichoderma* spp. have been well documented (Dennis and Webster, 1971b; Claydon *et al*., 1987; Simon and Sivasithamparam, 1988; Schirmbock *et al*., 1994). *Trichoderma* spp. also produce cell wall-degrading enzymes such as chitinase and ß-1,3 glucanase, ß-1,6 glucanases and proteases when they are grown on media containing polysaccharides, cell walls of fungal contaminants or even sterilised or heat-killed mycelium as a carbon source (Lorito, 1998; Kucuk *et al*., 2007; Kucuk and Kivanc, 2008; Singh *et al*., 2008). Among the enzymes, chitinases and ß-glucanases are mainly involved in the inhibition of fungal contaminants by destroying the skeletal polysaccharides of the fungal cell wall, *viz*. chitin and ß-1,3 glucan, thereby destroying cell wall integrity (Elad, 2000).

Studies have shown that *T. harzianum* releases extracellular glucanases and chitinase when grown in liquid medium (Kucuk and Kivanc, 2004). Most of the biocontrol agents secrete ß-glucan-degrading enzymes with extracellular ß-1,3 glucanases being secreted by most strains into the medium (Cook and Vesth, 1991). These enzymes could also have contributed to the inhibitory effects of the culture filtrates obtained from both SCB and PDB in the present study. Studies on the inhibitory effect of culture filtrates of *Trichoderma* on different plant contaminants have been reported: the culture filtrate of *T. harzianum* was effective against *Pythium aphanidermatum* (but in comparison, the filtrate from *T. hamatum* showed low inhibition for the same contaminant [Sivan *et al*., 1984]); the culture filtrates of *T. viridae* and *T. polysporum* on *Ceratocystis paradoxa* (Eziashi *et al*., 2006); culture filtrates of *T. viridae* and *T. harzianum* on *F. moniliforme* (Calistru *et al*., 1997) and culture filtrate of *T. viridae* on *Sclerotinia sclerotiorum* (Lee and Wu, 1984). A study on the inhibitory property of the culture filtrates of *T. harzianum* on *Fusarium oxysporum f*. sp. *ciceris* causing chick pea wilt was also supportive (Dubey and Suresh, 2006).

In the present study, culture filtrates of both strains of *Trichoderma* had a negative impact on germination and on seedling root development. In contrast, metabolites from culture filtrates of *Trichoderma* were reported to have increased the germination rate of maize seeds (Gupta and Sharma, 1995; Celar and Valic, 2005). However, there are also reports of *Trichoderma* having a negative influence on seed germination when applied as culture filtrates as well as when applied as spore suspensions. For example, the culture filtrates of *Trichoderma harzianum* T969, *T. harzianum* T447, *Trichoderma hamatum* T614, *Trichoderma roseum* T678, *Gliocladium virens* G525 and an unknown *Trichoderma* species isolate, (*Trichoderma* sp. T), were associated with a significant reduction of the rate of germination of maize (*Zea mays* L. cultivar B73) when compared with the uninoculated control, but had no influence at the totality of germination (Hajieghrari, 2010). Other studies in which the inhibitory action of culture filtrates of *Trichoderma* spp. have been demonstrated include: the effects of the culture filtrate of *T. viride* on cucumber, tomato and pepper seed germination (Menzies, 1993); the culture filtrate of *T. viride* on onion seed germination; and that of *T. koningii* on onion, chicory and lettuce seeds (Celar and Valic, 2005).

The negative influence of culture filtrates of *Trichoderma* on germination may be due to the production of some compounds such as species-/strain-specific auxin-like, and/or auxin inducer, compounds that have inhibitory effects at higher concentrations than the optimal (Vinale *et al*., 2008a; 2008b). The optimal concentration of such compounds which might impact positively on

seed germination may also be different for different plant species, as may be the types of compounds released by individual *Trichoderma* spp. This obviously is an area requiring further study.

4.3.4.4 Effect of Trichoderma *isolates as conidial suspensions on* Trichilia dregeana *axes*

Although different strains and species of *Trichoderma* have been used as biocontrol agents for many crops against many plant contaminants, no other work has been done on their effect *in vitro* on axes excised from recalcitrant seeds. In view of the significant problems posed by fungi in embryo culture, the present investigation aimed at testing the effect of EcoT and Eco77 on germination, as well as on the contamination status of embryonic axes of *T. dregeana in vitro*.

a) Effect of Trichoderma *on the viability of the embryonic axes of* Trichilia dregeana *plated on water agar*

Inoculum of *Trichoderma*, introduced as a spore suspension in water, was co-cultivated with the embryonic axes on water agar for 24 to 72 h. After 24 h, no *Trichoderma* penetration of axes had occurred, and, even when the co-cultivation time was increased, only a few were revealed as having been penetrated by the biocontrol agent. This might have been the result of the high spore concentration – which is in agreement with the inhibition of germination of *Trichoderma hamatum* spores on water agar, especially when the spore count was more than 10^8 spores mL^{-1} (Nol and Henis, 1987). Although in the present study $1x10^6$ conidia mL^{-1} were introduced, a contributory factor might have been that germination of *Trichoderma* spores, even in lower concentrations than $1x10^8$ mL^{-1}, is retarded on water agar due to lack of nutrients. The minimum period taken for the conidia of the *Trichoderma* spp. to germinate is >10-14 h at 26°C on a nutrient rich medium (Lifshitz and Baker, 1986). However, there was a negative effect on the vigour of embryonic axes plated for longer periods with *Trichoderma* suspension. It is possible that competition between the axes and spores for space on the agar surface might have affected the vigour of the co-cultivated embryonic axes. It is also possible – that some material exuded from the ungerminated spores could have had adverse effects on the co-cultured axes. However, both these explanations are necessarily conjectural at this stage.

Axes were plated on water agar which had been previously inoculated with *Trichoderma* for 7 d, for 24 to 72 h. All axes showed *Trichoderma* penetration after 24 h, but axis vigour and viability were seriously compromised. The negative influence on the growth and development of the axes

might have been as a result of the organic acids secreted by the *Trichoderma* strains, *viz.* gluconic, citric or fumaric acid which acidify the surrounding environment (Gómez-Alarcón and de la Torre, 1994). The present results are in agreement with the studies on potted maize (*Zea mays* L. Cultivar B73) in which no seedlings emerged from the soil which was previously inoculated with various isolates of *Trichoderma* (*T. harzianum* [T969], *T. harzianum* [T447], *T. hamatum* [T614], *T. roseum* [T678], *Gliocladium virens* [G525] and an unknown *Trichoderma* species isolate [*Trichoderma* sp. T]) (Hajieghrari, 2010). As seedlings from the seeds potted in non-inoculated soil emerged after 30 d, that author suggested that the bio-control agents could actually have become parasitic (see below) after seed germination in the inoculated soil, resulting in pre-emergence damping-off (Hajieghrari, 2010).

b) Embryonic axes immersed in Trichoderma *spore suspension and then cultured on half strength MS medium*

Embryonic axes were immersed in *Trichoderma* conidial suspensions for 30 min and then co-cultivated (i.e. axes together with adherent spores) on half stength MS medium for times from 24 to 72 h. Axes germinated readily on the half strength MS medium after immersion in the spore suspension for 24 h, but after immersion for 48 h and 72 h the germinability of the axes was compromised. However, although after the 24 h immersion period all axes appeared to have been inoculated with *Trichoderma*, it was also noted that there was reduction, but not complete elimination of the inherent contamination compared with the control axes (Table 3.35). This result is in agreement with the trials conducted on the pruning wounds of vine against *Eutypa lata* by Eco77, where it was recorded that after 24 h, colonisation of the vine by the just-germinated *Trichoderma* spores was minimal with consequent protection against the contaminant being inadequate[10].

Some studies on the effects of various species of *Trichoderma* have indicated that they have the potential to increase seed germination totality (germination percentage) and productivity of the resultant plants, including maize (Harman, 2006) pigeon pea (Manju and Mall, 2008); cotton seedlings (Hanson, 2000); rice (Mishra and Sinha, 2000); Okra (Mukhtar, 2008); Cabbage and Lettuce (Rabeendran *et al*., 2000); chickpea (Dubey *et al*., 2007). Several other studies also reported that *T. harzianum, T. viride* and *T. pseudokoningii* not only promoted seed germination, but also improved the vigour of the pea (Zheng and Shetty, 2000), and watermelon (Bharath *et al*., 2006), seedlings with diminished occurrence of seed-borne fungal contaminants. In all these cases a symbiotic relationship between the *Trichoderma* species and the treated seeds/seedlings is

assumed. However, there are also examples of *Trichoderma* spp. becoming parasitic rather than a symbiotic (Hajieghrari, 2010) when the conidial suspensions (10^6-10^7 spores per mL-1) of *Trichoderma harzianum* T969, *T. harzianum* T447, *Trichoderma hamatum* T614, *Trichoderma roseum* T678, *Gliocladium virens* G525 and the unknown *Trichoderma* species isolate (*Trichoderma* sp. T) were used to treat the seeds. In that study, it was found that seed germination as well as the rootlet and shoot development of maize caryopses (*Zea mays* L. cultivar B73) was adversely affected, apparently by necrosis of the inner seed tissues by *Trichoderma* (Hajieghrari, 2010). That author reported that culture filtrates of the various species of *Trichoderma* also had adverse effects (see above).

The inhibitory effect of *Trichoderma* has been further documented in various other studies, e.g. the effects of *Trichoderma* spp. on maize seed and seedlings (Sutton, 1972; Mc-Fadden and Sutton, 1975) and also on cucumber, pepper and tomato seedlings (Menzies, 1993). Most of the *Trichoderma* spp. penetrate only on the root epidermis without causing damage and the further penetration of the hyphae is suggested to be blocked by the deposition of callose by the neighbouring cells (Yedidia *et al*., 1999). But in the present study even at 24 h of co-cultivation with the *Trichoderma*, retarded growth and reduced biomass of the *T. dregeana* axes was recorded. This could be due to the penetration of the hyphae of *Trichoderma* into the tissues below the root epidermis of the germinating axes, on the assumpton that blocking by callose was inadequate. Alternatively, inhibition of axis germination could have been due to increasingly anoxic conditions developing during axis immersion, despite the process having been carried out on a shaker. It must be stressed that axes excised from recalcitrant seeds are metabolically-active (Berjak and Pammenter, 2004; 2008), hence they would be sensitive to anoxia, in contrast to orthodox seeds, which would be metabolically inert at least early in an immersion period.

In the current study it was shown that if the *Trichoderma* was not killed (by application of Benlate) once successful inoculation was achieved, the fungus would overgrow and kill the embryonic axes.

4.3.4.5 Effect of Trichoderma *on root growth and biomass*

Trichoderma spp. are known for their beneficial effects of increasing the plant growth and productivity in soils (Chang *et al.*, 1986; Yedidia *et al*., 2001; Adams *et al*., 2007). Studies showed that when EcoT was applied to soybean seeds in combination with silicon, the root area, root biomass, root length as well as the shoot biomass was increased compared with the control (Bosse *et al*., 2011). Other studies have shown that *Trichoderma* spp. increased the seedling growth, primary root-length and root branching in maize and beans by inducing lateral root growth and increased germination rate for maize; but, there was no significant influence on the emergence of bean seedlings (Okoth *et al*., 2011). There are also other examples where *Trichoderma* was associated with increased root and shoot biomass of willow (Adams *et al*., 2007), cucumber (Chaur-Tsuen and Chien-Yih, 2002), bean (Hoyos-Carvajal *et al*., 2009), and lettuce (Ousley *et al*., 1994).

In the present study, however, root growth was reduced even after the 24 h co-cultivation period with *Trichoderma*, although there was 100% germination of the *T. dregeana* embryonic axes. Studies of inoculation with a *Trichoderma harzianum* strain (T22) on *in vitro*-cultured shoots of GiSeLa6® (*Prunus cerasus x Prunus canescens*) and GF677 (*Prunus amygdalus x Prunus persica*), important commercial rootstocks utilised for stone fruits, were reported by Sofo *et al.* (2010). Those authors showed that early inoculation with the T-22 strain had a negative influence in damaging the shoot tips by acting as a competitor for the nutrients in the medium, and then behaved as a saprophyte rather than a symbiont (Sofo *et al*., 2010). However, when shoots were inoculated with the T22 strain of *T. harzianum* 7 d after they had been cultured on a root inducing medium, root and shoot development were improved, as indicated by enhanced root lengths and basal diameters of the stem (Sofo *et al*., 2010). It is possible, therefore, that the early inoculation of *T. dregeana* axes with EcoT or Eco77 could account for the adverse effects on germination and growth.

However, it was decided that use of *Trichoderma* spp. as biocontrol agents for axes (whether ultimately cryopreserved or not) which were to be developed further *in vitro* was not a practical proposition, as the resultant problems by far out-weighed any potential benefits.

4.4 Minimal growth storage of the embryonic axes using alginate gel encapsulation

The decontaminants, NaDCC and Nipastat, which proved to be effective pre-treatments for axes to be germinated in vitro, were used for minimal growth storage. It showed that the contamination of axes coated with alginate gel after treatment with Nipastat were further reduced compared with axes treated with Nipastat alone. Increasing the concentration of Nipastat more than 0.2% could have been effective in terms of contamination-free embryonic axes; however, as mentioned earlier, Nipastat was toxic to the embryonic axes when used at higher concentrations than 0.2%, or even by increasing the immersion time from 10 to 20 min. Exposure to NaDCC at a concentration of 0.2% for 10 min and longer prior to alginate gel encapsulation was shown to be effective against fungal contamination, but a small proportion of axes had retained bacterial contaminants. However, this problem was solved by increasing the NaDCC concentration to 0.3%, to which the axes were exposed for 20 min prior to encapsulation.

When included in a PDA medium, the fungicidal/fungistatic effectiveness of an alginate gel (in that case a crude cell wall extract of *Ecklonia maxima* but preserved with Nipastat [concentration unknown]) was demonstrated by Myeza (2005). However, the results obtained in the current study indicated that the gel (presently made up as a preparation of 2% sodium alginate [Sigma-Aldrich, Germany] in distilled water) was ineffective if used without the decontaminating pre-treatment of the *T. dregeana* axes with NaDCC or Nipastat. In the present study the gel-encapsulated embryonic axes of *T. dregeana* which had not been decontaminated with either Nipastat or NaDCC showed low level of contamination after two weeks, with fungal contamination of an increasing proportion of axes becoming evident after four and six weeks of storage. This was essentially similar to the results of Motete *et al*. (1997), who showed that encapsulation with the same Nipastat-preserved gel used by Myeza (2005) was associated with reduced fungal contamination of hydrated *Avicennia marina* seeds over a prolonged storage period.

Alginate gel encapsulation after treatment of the axes with Nipastat or NaDCC was effective in controlling contamination. However, only those embryonic axes encapsulated with sucrose incorporated in the bead germinated, none doing so without the inclusion of this sugar. Considering that the excised recalcitrant embryonic axes – which are metabolically active – are virtually devoid of a carbon source, inclusion of sucrose in the protective alginate gel coating appeared to be imperative for viability retention. However, 100% survival was obtained only for

axes decontaminated with 0.5% NaDCC and then encapsulated with sucrose-containing alginate gel, and only when assessed after 14 d storage: none appeared to have survived for 28 or 42 d. Already after 14 d, the alginate capsules had changed markedly, appearing 'thinned out'. It is suggested that relatively soon within the storage period the alginate coating could have become increasingly ineffective to the point where (by 28 and 42 d of storage) it would no longer have constituted an effective barrier (see also below). This, in turn, could have led to water loss from the desiccation-sensitive axis tissues to the point that viability was lost. If this proves to be the case, in subsequent trials re-encapsulation of the axes might afford a solution which effectively could extend the storage life of the axes. Metabolism-linked dehydration damage (Pammenter *et al*., 1998; Walters *et al*., 2001) has been shown to be associated with progressive failure of the endogenous anti-oxidant system of axes in slowly-dried T. dregeana seeds (Varghese et al., 2011). Although axis water contents were determined after 42 d storage in the various types of containment (see below), in the preliminary experiment axis water contents during the storage period were not monitored, therefore the progression of hydration/dehydration which might occur needs to be assessed when the studies are taken further. An additional modification of the encapsulating gel (initially, and if re-applied) by using cathodic water which has powerful reducing properties (Berjak *et al*., 2011) in place of distilled water, might prove effective.

It is also possible that depletion of the sucrose by the axes occurred within, or shortly after, 14 d in storage. Increasing the sucrose concentration in the gel above 0.5% might increase the storage life span of the embryonic axes, provided the alginate coating remains effective. However, this could have one of two consequences: either osmotic dehydration of the axes, or initiation of germination during storage. The latter argument is supported by the results from work in which alginate encapsulated axenic shoot tips and nodal segments of *Eclipta alba* were exposed to 3% sucrose during storage, when random shoot formation resulted and the explants were not able to be stored beyond four weeks (Ray and Bhattacharya, 2010). Similar results were observed for the encapsulated shoot tips of cassava (Danso and Ford-Lloyd, 2003).

One of the major limitations of encapsulation is that the explants could grow out of the gel matrix. In the present study, the temperature at which the alginate-coated embryonic axes were stored was 16°C. Most of the storage reported for of alginate gel encapsulated synseeds was at 4°C. However, shoot tips of several tropical tree species have been encapsulated and successfully stored at 20 to 25°C (Maruyama *et al*., 1997). In the case of the *T. dregeana* axes, storage at 16°C was considered to be best: lower temperatures could not be used in view of the demonstrated

chilling-sensitivity of the seeds (Kioko, 2003), and higher temperatures might well have favoured germination in storage. Although presently, the axes did not grow out of the encapsulating gel, at 16°C, their ongoing metabolism could well have depleted the carbon source provided as sucrose in the gel. The disappearance of the alginate gel around the axes may have been due to deliquescence; that is, the alginate encapsulation absorbed water from the surrounding environment as a consequence of being stored in a saturated atmosphere (Magenta box). Thus the axes would effectively no longer have been encapsulated.Other factors that can have an effect on the stability of the alginate beads include the chelating ion (Ca^{2+} ions being the most commonly used), alginate concentration, metal ion chelators such phosphate, citrate and lactate, alginate composition (α-L- guluronic acid content >70%), molecular weight of the alginate polymers (modified from Endress, 1994). A variety of other parameters could, therefore, have an influence and need to be tested for the development of the most effective gel encapsulation of embryonic axes.

With respect to axis cryostorage, encapsulation could precede cryogen exposure (although this might have an adverse effect in retarding cooling rate to cryogenic temperatures), or could be utilised after the retrieval and decontamination with NaDCC of cryopreserved explants. The latter is presently suggested as a promising means of curtailing/eliminating fungal and bacterial inoculum, so resulting in explant recovery and further development *in vitro*.

The fate of axes stored under hydrated and non-hydrated conditions varied significantly. In the case of axes stored in Magenta box, as described above the alginate coating thinned out ultimately seeming to disappear as the storage period progressed, which, in turn, led to water loss from, and death of, the axes. In the case of axes stored in Eppendorf tubes, viability could have been adversely affected by anaerobic conditions. According to Smith (1995) when recalcitrant seeds are stored under anaerobic conditions or an anoxic atmosphere in air impermeable containers, respiration will be curtailed, which could have been the case for the axes stored in the Eppendorf tubes. Oxygen has been shown to be vital for the viability retention of the recalcitrant seeds of *Araucaria hunsteinii* (Tompsett, 1983).

The viability of the axes contained in polythene bags remained unaffected, with 100% germinating throughout the storage period, as opposed to those stored in foil-lined bags where only 46.7% germinated after 14 d, and none thereafter. This may be have been because the polythene bags were relatively permeable thus not restricting ongoing respiration of the axes,

whereas the foil-lined bags could have been completely impermeable. In addition, aluminium has been shown to be lethal to plant tissues, which might also have contributed to the death of the axes. However, the inner side of the aluminium foil bags used in the present study was lined with a thin layer of polythene, which explains that the axes were not in direct contact with the aluminium. In the present study containment of axes in aluminium foil-lined bags adversely affected the viability by the end of their 42 d storage period for both encapsulated and non-encapsulated axes. In contrast, whether encapsulated or not, axes stored in polythene bags maintained high viability and established normal seedlings.

For orthodox seeds, fluctuations in water content have been shown to occur depending on the storage conditions, type of containment used, and the kind of seeds; these are all factors which influence the migration of moisture from the air to the seed and *vice-versa* (Gómez-Campo, 2006; Walters, 2007). In the case of recalcitrant seeds – or axes, as presently used – water loss is generally lethal, especially if dehydration is slow (Berjak and Pammenter, 2008). In contrast, gain of water by the axes should hasten germinative metabolism. Axes of *T. dregeana* stored in Magenta boxes lost water, which is suggested to have been caused by the deterioration of the alginate capsule and the volume (and therefore airspace) of the containers. Similarly, unencapsulated axes in Magenta boxes became more dehydrated relative to the control. It therefore appears that slow dehydration from the outset of the storage period was the major lethal factor. In contrast, the water content of encapsulated axes increased when they were contained in Eppendorf tubes, polythene bags and especially in foil-lined bags, while water content of similarly stored unencapsulated axes remained relatively constant. The major source of extraneous water in all these cases, would have been the alginate capsule itself. It was observed (results were not recorded) that the axes stored in polythene bags germinated more rapidly than the control (unstored axes). This was probably due to the elevated water content and accessibility of oxygen (on the assumption of the permeability of the bags), which could have facilitated more rapid development of axes towards germination. It appears therefore, that the water content of encapsulated axes may have equilibrated with that of the alginate, although the significantly higher gain in water by axes contained in the foil-lined bags remains difficult to explain.

4.5 Concluding summary

In the present study various contaminants were isolated from the embryonic axes of *T. dregeana*, the results showing that the predominant contaminants of the control, decontaminated and alginate-gel encapsulated embryonic axes were *Penicillium* spp., although other fungi such as *A.*

niger, *A. flavus*, and species of *Fusarium* and *Rhizopus* were also observed. It was also observed that contamination by *Penicillium* spp. was manifested by embryonic axes of seeds stored at 16°C for more than three months. The tests done to check the effect of EcoT and Eco77 in dual culture on different axenic isolates showed that both strains of *Trichoderma harzianum* gave similar results. Filtrates from the two strains of SCB- and PDB-cultured *Trichoderma* were much more effective in inhibiting the growth of test isolates compared with the EcoT and Eco77 mycelia.

The current research showed that co-culturing *Trichoderma* strains from both EcoT and Eco77 with the embryonic axes of *T. dregeana* was not effective. Although co-culturing for 24 h did not affect the survival of the embryonic axes, this had no effect on increasing their growth or eliminating the contaminants. Longer periods of co-culturing with *T. harzianum* affected germination and seedling establishment of the embryonic axes of *T. dregeana* which showed loss of viability and reduced root growth of germinated axes, respectively, compared with those germinated in the absence of the biocontrol agent (control). In effect, *T. harzianum* strains from both EcoT and Eco77 had a negative influence on the growth of the embryonic axes. Establishing the appropriate inoculation method for explants and its timing is important in obtaining a symbiotic relationship between the biocontrol agent and the explant. It was also observed that *Trichoderma* was more effective, when it was alive than it was when killed after becoming established in the axes. Benlate was effective in killing the *Trichoderma* without damaging the embryonic axes. Therefore, it is also necessary to devise a method where the *Trichoderma* and embryonic axes germinate together effectively. In the present study the culture filtrates of both EcoT and Eco77 from solid and liquid state fermentation, had a negative influence on axis vigour and viability, rather than improving their development.

Among the fungicides, Nipastat and NaDCC had a significant effect in curtailing contamination, without affecting the germination of the embryonic axes when used at specific concentrations. These fungicides were also used for minimal growth storage of axes encapsulated within calcium alginate gel ‘beads’. It was shown that 100% of the embryonic axes pre-treated with NaDCC and encapsulated with 0.5% sucrose incorporated in the alginate gel, survived for 14 d without any (or only minimal) contamination being manifested. However, survival of encapsulated axes stored for longer than 14 d was not obtained. It is suggested that the alginate coating may have become ineffective as the axes expanded, possibly accompanied by a lethal degree of slow dehydration. Improving the parameters for alginate encapsulation, including introducing a second coating, are suggested as possible means for prolonging viability retention, However, as the objective of

alginate encapsulation was to facilitate axis germination and seedling establishment in vitro without contamination, extending the life span of stored, encapsulated axes is a secondary consideration – except for providing a means by which the germplasm can be safely consigned to distant laboratories or planting sites. In this regard, implementing encapsulation following axis retrieval from, and recovery after, cryostorage is seen as a priority.

The use of NaDCC – or other preparations containing sodium dichloro-isocyanurate – has emerged as a promising method of eliminating contaminating microflora which otherwise compromise *in vitro* procedures, from seed-derived explants. The results should find considerable applicability when excised embryonic axes representing the germplasm of recalcitrant seeds, are cryo-conserved. In conclusion then, the present study on storage of encapsulated axes has shown that, if effectively decontaminated, alginate beads afford a promising means of temporary storage and dissemination of explants such as recalcitrant embryonic axes, upon retrieval from cryostorage. The study has further indicated that the means of containment is critical, to maximise vigour and viability retention by the encapsulated explants. Other factors, including controlling the water content of the calcium alginate capsule itself, may need to be optimised to prevent excessive further hydration of the explant.

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