

**The effects of *Trichoderma* (Eco-T[®]) on biotic and
abiotic interactions in hydroponic systems.**

by

Brendon John Neumann

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School of Applied Environmental Sciences
Faculty of Science and Agriculture
University of Natal
Pietermaritzburg
Republic of South Africa*

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Abstract

The following body of research provides a detailed overview of the interactive effects of biocontrol agents and environmental factors and how these influence both the host plant and pathogen populations within hydroponic systems.

Pythium and other zoosporic fungi are pathogens well suited to the aquatic environment of hydroponics. Motile zoospores facilitate rapid dispersal through fertigation water, resulting in *Pythium* becoming a yield reducing factor in most hydroponic systems and on most crops. With increasing trends away from pesticide use, biocontrol is becoming an ever more popular option. Unfortunately, much of our knowledge of biocontrol agents and their formulation can not be directly transferred to the widely differing environments of hydroponic systems. Paulitz (1997) was of the opinion that if biocontrol was to be successful anywhere, it would be in hydroponics. This is primarily due to the increased ability, in hydroponics, to control the growing environment and to differentiate between the requirements of the pathogen versus those of the host plant and biocontrol agent. Key environmental factors were identified as soil moisture, root zone temperature, form of nitrogen and pH.

A review of the literature collated background information on the effects of biocontrol agents and environmental manipulation on plant growth and disease severity in hydroponic systems.

A commercial formulation of *Trichoderma* (Eco-T^{®1}) was used as the biocontrol agent in all trials. Dose responses in *Pythium* control and plant growth stimulation in lettuce were first determined using a horizontal trough system (closed system). In such systems optimum application rates were found to be lower than in field application (1.25×10^5 spores/ml). This is probably because *Trichoderma* conidia are not lost from the system, but re-circulate until being transported into the root zone of a host plant. No significant growth stimulation was observed, although at high doses (5×10^5 and 2.5×10^5 spores/ml)

¹Plant Health Products (Pty) Ltd. Box 207, Nottingham Rd., 3280, South Africa

a significant reduction in yield was recorded. Possible reasons for this growth inhibition are suggested and a new theory is proposed and investigated later in the thesis. In an open system of cucumber production (drip irrigated bag culture) no statistically significant results were initially obtained, however, general trends still showed the occurrence of positive biocontrol activity. The initial lack of significant results was mostly due to a poor knowledge of the horticulture of the crop and a lack of understanding of the epidemiology behind *Trichoderma* biocontrol activity. These pitfalls are highlighted and, in a repeat trial, were overcome. As a result it could be concluded that application rates in such systems are similar to those used in field applications.

Management of soil moisture within artificial growing media can aid in the control of *Pythium* induced reductions in yield. A vertical hydroponic system was used to determine the interactive effects of soil moisture and *Trichoderma*. This system was used because it allowed for separate irrigation regimes at all 36 stations, controlled by a programmable logic controller (PLC). With lettuce plants receiving optimum irrigation levels, no significant reduction in yield was observed when inoculated with *Pythium*. However, after *Pythium* inoculation, stresses related to over- or under-watering caused significant yield losses. In both cases, *Trichoderma* overcame these negative effects and achieved significant levels of disease control, especially under higher soil moisture levels. Growth stimulation responses were also seen to increase with increasing soil moisture. Similar results were obtained from strawberry trials. These results show that *Pythium* control is best achieved through the integration of *Trichoderma* at optimum soil moisture. However, where soil moisture is above or below optimum, *Trichoderma* serves to minimize the negative effects of *Pythium*, providing a buffering capacity against the effects of poor soil moisture management.

Pythium, root zone temperature and form of nitrogen interact significantly. In greenhouse trials using horizontal mini troughs with facilities for heating or cooling recirculating water, nitrate fertilizer treatments resulted in statistically significant results. Lettuce growth was highest at 12°C, although no significant differences in yield were observed between 12-24°C. *Pythium* was effective in causing disease over the same temperature range. *Pythium* inoculation did not result in yield reduction at 6 and 30°C.

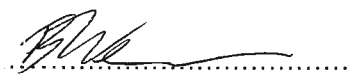
Trichoderma showed a slight competitive advantage under cooler temperatures (i.e., 12°C), although significant biocontrol occurred over the 12-24°C range. Ammonium fertilizer trials did not generate statistically significant data. This is possibly due to complex interactions between root temperature, ammonium uptake, and competitive exclusion of nitrification bacteria by *Trichoderma*. These interactions are difficult to replicate over time and are probably influenced by air temperature and available light which are difficult to keep constant over time in the system used. However, the data did lead to the first clues regarding the effects of *Trichoderma* on nitrogen cycling as plants grown with a high level of ammonium at high temperatures were seen to suffer more from ammonium toxicity when high levels of *Trichoderma* were added.

In further trials, conducted in the recirculating horizontal mini trough system, it was determined that *Trichoderma* applications resulted in an increase in the percentage ammonium nitrogen in both the re-circulating solution and the growing medium. This was a dose-related response, with the percentage ammonium nitrogen increasing with increasing levels of *Trichoderma* application. At the same time an increase in ammonium in the root tissue was observed, corresponding with a decrease in leaf nitrate levels and an increase in levels of Cu, Na, Fe and P in leaf tissue. In independent pot trials, populations of nitrifying bacteria in the rhizosphere were also seen to decrease with increasing *Trichoderma* application rates. This led to the conclusion that the increase in ammonium concentration was as a result of decreased nitrification activity due to the competitive exclusion of nitrifying bacteria by *Trichoderma*. The possibility that *Trichoderma* functions as a mycorrhizal fungus and so increases the availability of ammonium for plant uptake is not discarded and it is thought that both mechanisms probably contribute.

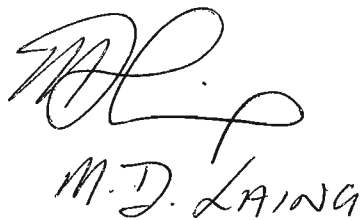
Water pH provides the most powerful tool for enhancing biocontrol of *Pythium* by *Trichoderma*. *Trichoderma* shows a preference for more acidic pHs while *Pythium* prefers pHs between 6.0 and 7.0. *In vitro* tests showed that *Trichoderma* achieved greater control of *Pythium* at pH 5.0, while achieving no control at pH 8.0. In greenhouse trials with the recirculating horizontal mini trough system, yield losses resulting from *Pythium* inoculation were greatest at pH 6.0 and 7.0, with no significant reduction in yield at pH 4.0. Biocontrol activity showed an inverse response with greatest biocontrol at pH 5.0.

Declaration

I, Brendon John Neumann, declare that the research reported in this thesis, except where otherwise indicated, is my own original research. This thesis has not been submitted for any degree or examination at any other university.



Brendon John Neumann



M.D. LAING

Foreword

All research presented in this thesis was conducted at the University of Natal, Pietermaritzburg, RSA. The work presented is the culmination of four years of research with what began as a Masters degree and evolved into a Doctorate. The first year of the project was spent largely on repairing, adapting, and upgrading the hydroponic facilities at the University. Some completely new systems were also installed.

As part of the Biocontrol for Africa programme, the main emphasis of the research was in determining the efficacy of *Trichoderma* (biocontrol agent) in a hydroponic environment. *Pythium* was used as the pathogen in all trials as it is considered the most widespread pathogen in hydroponics although also being one which many farmers are not aware of. In most instances it causes minor, yet consistent, infections resulting in uniform reduction in yield with few visible symptoms. The control of such a sub-lethal pathogen would give noticeable increases in yield in most instances, increasing the productivity of hydroponic systems.

Unfortunately much of our knowledge of biocontrol agents and formulations cannot be directly transferred to hydroponics due to the widely differing environments presented by many systems. In order to accurately formulate *Trichoderma* for use in hydroponics, an understanding of the effects of the various environmental factors was deemed necessary. The approach in this research was thus to determine the following factors:

- i) The application rates and efficacy of *Trichoderma* (Eco-T®), in terms of *Pythium* control and plant growth, in both open and closed hydroponic systems.
- ii) The effects of soil moisture, root zone temperature, form of nitrogen, and pH on plant growth and disease severity with a particular focus on how these factors interact with biocontrol agents and thus influence biocontrol and growth stimulation activity.

As this was the first research of this type to be conducted in South Africa, and a fairly new topic world wide, the initial approach was to screen a large number of interactions

at a basic level. It was hoped that this would help identify key areas of research for future studies which could then be tackled in a more detailed study. Furthermore, as the aim of the Biocontrol for Africa programme was to develop marketable biocontrol products, much of the research done at this stage was aimed at the end user (i.e., the growers). For this reason in many trials only mean total yields were recorded (as wet weights), as it is the increase or decrease in yield (as seen by the grower) which will determine the success of the biocontrol product. Dry weights were used only where wet weight results were clearly being skewed by increased water uptake (visible in lettuce by differential leaf colouring).

After three years of mostly field trial work several interesting interactions were identified. Of greatest significance was the frequent observation of growth inhibition under high levels of *Trichoderma* application. This apparent phytotoxic effect was seen to be enhanced under conditions of high root temperatures and ammonium concentrations. The final year of study thus focussed on a possible mechanism behind these observations and it is in this that the most novel aspects of this work can be found.

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Contents

Abstract	i
Declaration	iv
Foreword	v
Acknowledgements	vii
Contents	viii
Chapter 1	
Literature review	
The effects of biological control agents on biotic and abiotic interactions in hydroponic systems	1
1.1 Introduction	1
1.2 Novel features of hydroponic systems	2
1.3 <i>Pythium</i> spp. as major pathogens in hyroponics	6
1.4 Biocontrol in hydroponic systems	7
1.5 Biocontrol and growth stimulation by <i>Trichoderma</i>	7
1.6 Effects of biocontrol agents and soil moisture	11
1.7 Effects of biocontrol agents and root zone temperature ..	16
1.8 Effects of biocontrol agents and pH	19
1.9 Effects of biocontrol agents and nutrition	21
1.10 Concluding Remarks	28
1.11 References	29

Chapter 2

Construction of a Hydroponic Research Facility	40
2.1 Introduction	40
2.2 Greenhouse construction	40
2.3 Verti-gro® system	40
2.4 Horizontal mini troughs	41
2.5 Temperature trials	42
2.6 Bag culture trials	42

Chapter 3

Dose effects of <i>Trichoderma</i> on <i>Pythium</i> disease severity and plant growth in Open and Closed Hydroponic Systems	45
--	----

Abstract	45
----------------	----

3.1 Introduction	45
------------------------	----

① 3.2 Trial 1 - The effect of dosage rates of Eco-T®, in a recirculating (closed) hydroponics system, on <i>Pythium</i> control and plant growth stimulation	47
--	----

3.2.1 Introduction	47
--------------------------	----

3.2.2 Materials and methods	48
-----------------------------------	----

3.2.3 Results	51
---------------------	----

3.2.4 Discussion	56
------------------------	----

② 3.3 Trial 2 - The effect of dosage rates of Eco-T®, in cucumber bag culture (open system), on <i>Pythium</i> control and plant growth stimulation	57
---	----

3.3.1 Introduction	57
--------------------------	----

3.3.2 Materials and Methods	57
-----------------------------------	----

3.3.3 Results	60
3.3.4 Discussion	62
3.4 Trial 3 - The effect of <i>Trichoderma</i> (Eco-T®) in cucumber bag culture - some pitfalls overcome	64
3.4.1 Introduction	64
3.4.2 Materials and methods	64
3.4.3 Results	65
3.4.4 Discussion	67
3.5 Conclusions	68
3.6 References	69

Chapter 4

<i>Trichoderma</i> and Soil Moisture	72
--	----

Abstract	72
----------------	----

4.1 Introduction	72
4.2 Trial 1: Determining the optimum range of soil moisture for hydroponically grown butter lettuce	74
4.2.1 Introduction	74
4.2.2 Materials and Methods	74
4.2.3 Results	75
4.2.4 Conclusions	77
4.3 Trial 2: Effects of <i>Trichoderma</i> inoculation under varying soil moisture conditions	78
4.3.1 Introduction	78
4.3.2 Materials and Methods	78
4.3.3 Results:	81
4.3.4 Discussion	85
4.4 Conclusions	88

4.5 References	89
 Chapter 5	
<i>Trichoderma</i> and Form of Nitrogen	90
 Abstract	90
 5.1 Introduction	90
5.2 The role of <i>Trichoderma</i> in N nutrition: Inconclusive temperature trials reveal one of <i>Trichoderma</i> 's secrets	91
5.2.1 Introduction	91
5.2.2 Materials and Methods	93
5.2.3 Results	94
5.2.4 Discussion	100
5.3 <i>Trichoderma</i> and nitrogen nutrition - effects on growth promotion/inhibition	102
5.3.1 Introduction	102
5.3.2 Materials and Methods	102
5.3.3 Results	104
5.3.4 Discussion	109
5.4 <i>Trichoderma</i> and N cycling	111
5.4.1 Introduction	111
5.4.2 Materials and Methods	111
5.4.3 Results	112
5.4.4 Discussion	115
5.5 Conclusions	117
5.6 References	118

Chapter 6

<i>Trichoderma</i> and pH effects	120
Abstract	120
6.1 Introduction	120
6.2 <i>In vitro</i> screening of pH effects on <i>Trichoderma</i> conidial germination, and root colonizing ability	122
6.2.1 Introduction	122
6.2.2 Materials and Methods	122
6.2.3 Results	123
6.2.4 Discussion	125
6.3 Effects of pH and sugar availability on spore germination and sporulation of <i>Trichoderma</i>	126
6.3.1 Introduction	126
6.3.2 Materials and Methods	127
6.3.3 Results	127
6.3.4 Discussion	129
6.4 Interactions between <i>Trichoderma</i> and pH in the biological control of <i>Pythium</i>	130
6.4.1 Introduction	130
6.4.2 Materials and Methods	130
6.4.3 Results	131
6.4.4 Discussion	132
6.5 <i>Pythium</i> control by <i>Trichoderma</i> under varying pHs. ...	133
6.5.1 Materials and Methods	133

6.5.2 Results	134
6.5.3 Discussion	137
6.6 Conclusions	138
6.7 References	138

Chapter 7

Thesis Overview	140
7.1 Introduction	140
7.2 Efficacy of Eco-T® in hydroponic systems	141
7.2.1 Current understanding	141
7.2.2 Future research	142
7.3 Soil moisture effects	143
7.3.1 Current understanding	143
7.3.2 Future research	143
7.4 Water temperature and form of nitrogen effects	143
7.4.1 Current understanding	143
7.4.2 Future research	144
7.5 pH effects	145
7.5.1 Current understanding	145
7.5.2 Future research	145
7.6 Overall conclusion	146
7.7 References	146

Chapter 1

Literature review

The effects of biological control agents on biotic and abiotic interactions in hydroponic systems

1.1 Introduction

*'Great fleas have little fleas upon their backs to bite 'em,
And little fleas have lesser fleas, and so on ad infinitum'*

(Augustus de Morgan, 1871, cited by Jarvis, 2001)

This could well be the first written record of the concept of biological control, although more recent definitions are more precise. Baker and Cook (1974) defined biocontrol as, 'the reduction of inoculum density or disease-producing activity of a pathogen or parasite in its active or dormant state, by one or more organisms, accomplished naturally, or through manipulation of the environment, host, or antagonist, or by mass introduction of one or more antagonists'.

Hydroponic culture also has some distant references and can be traced back as far as the hanging gardens of Babylon. A little closer to date, however, Dr. W.F. Gericke, defined the term in the 1930s in stating that 'hydroponics is the science of growing plants with the use of a substrate to which is added a nutrient solution containing all the essential elements needed by the plant for its normal growth and development' (Le Pivert, 1996).

Through the incorporation of the concept of manipulating the environment, host or antagonist, in biocontrol, this definition in itself alludes to the fact that such biocontrol may be far easier to achieve in hydroponic systems. This is especially true when considering the manipulation of the environment in terms of abiotic factors such as pH, root zone temperature, N nutrition, and soil moisture. These manipulations should

however, not be seen as a one way path. Not only is it important to understand how the environment can be manipulated to enhance biocontrol activity, but also to understand how biocontrol agents themselves interact with their environment thus effecting their own efficacy as well as the growth of host plants.

Hydroponic systems provide for a range of novel plant disease problems. Stanghellini and Rasmussen (1994) pointed out that, with the exception of *Fusarium oxysporum* f. sp. *radicis-lycopersici* Jarvis and Schoemaker, most of the destructive root diseases in hydroponics have been attributed either directly or indirectly to the genera *Pythium*, *Phytophthora*, *Plasmopara* and *Oidium*. All these fungi produce motile zoospores, are favoured by aquatic environments, and spread within hydroponic systems by re-circulation of zoospore infested nutrient solution. These pathogens can have profound effects on plant growth and yield in such systems, if left unchecked.

The following review thus focusses on the four way interactions between the hydroponics environment, the addition of a biocontrol agent and the combined effects on disease severity and plant growth. Novel features of hydroponic systems are highlighted (including novel pathogen problems) and both direct effects of biocontrol agents and the interactive effects with controllable elements of the hydroponic environment are discussed in detail.

1.2 Novel features of hydroponic systems

Hydroponic systems provide growers with a number of advantages over conventional farming methods. Resh (1995) listed the following advantages of using soilless culture versus soil culture (see Table 1.2.A)

Table 1.2.A Advantages of using soilless over soil culture (from Resh, 1995)

Cultural Practice	Soil	Soilless
Sterilization of growing media	Steam or chemical fumigants - labour intensive and time consuming	Steam, chemical fumigants or simply bleach or HCl - less time consuming
Plant nutrition	Highly variable with localised deficiencies. Often unavailable to plants due to poor soil structure/pH. Difficult to sample, test and adjust	Controlled, relatively stable and homogeneous to all plants. Easily tested, sampled and adjusted
Plant spacing	Limited by soil nutrition and available light	Limited only by available light therefore closer spacing possible
Weed control	Weeds always present	No weeds
Diseases and soil inhabitants	Many soilborne diseases, nematodes and insects which can attack crop	No diseases, insects and nematodes if suitable sterilization is achieved
Water	Often poor soil-water relations. Soil structure and water holding capacity leading to water stress. Water use is often inefficient due to percolation past the root zone and evaporation from the soil surface	No water stress. More efficient use of water through system mechanization and the use of moisture sensors and feedback controllers. If managed properly very little water loss due to percolation and evaporation
Fruit quality	Often soft or puffy due to K or Ca deficiencies. Poor shelf life	Firm fruit with long shelf life
Fertilizers	Inefficient use due to broadcast applications resulting in non-uniform distribution and leaching of over 50% past the root zone	Efficient use of small quantities which are uniformly distributed to all plants with no leaching beyond the root zone
Plant maturity	Seasonal length	With adequate light conditions, plant can mature faster than in soil conditions
Yields	Greenhouse tomatoes in soil achieve 6.8-9.1 kg/year/plant.	11.4-15.9 kg/year/plant.

The above advantages all assume that the soilless culture is being run at optimum potential and it must be realised that not all these advantages are as noticeable in all systems.

Besides the advantages listed above, the growth in popularity of hydroponics in many countries can be attributed to environmental concerns. In Belgium, the change-over from soil to soilless culture was induced largely due to the problems associated with methyl bromide residues seen in traditional farming practices. Steam treatment of hydroponic substrates was seen as an efficient and rational substitute (Benoit and Ceustermans, 1995).

Considering the environmental advantage of hydroponics, closed systems have been seen as even more favourable as they limit the incidence of ground and surface water pollution from fertilizers, chemical sterilants and pesticides. Van Os (1995) pointed out that to achieve a more efficient use of water, nutrients and pesticides, and to decrease emissions to the environment, the traditional open systems are slowly changing to closed systems. However, economic aspects are delaying the further introduction of closed systems. Economics are all important and one of the major drawbacks in many hydroponic systems (especially closed systems) is that of capital investment. Lataster *et al.* (1993) proved that factors such as number of plants per m², increase in production and utilization of space all determine whether a change to a closed system of hydroponics is viable.

Besides the many novel advantages to the use of hydroponic systems there are also a number of novel problems which arise from their use. Wood and Laing (1992) pointed out that hydroponics provides ideal conditions for certain fungi, in particular, water borne pathogens such as *Pythium* and *Phytophthora* spp. The range of genera and species of pathogens and host plants present ensure that any pathogenic opportunity is rapidly exploited.

Paulitz (1997) provided a number of reasons why disease control is a particular problem in hydroponics:

1. In soilless, greenhouse systems plants are often genetically identical and can thus be uniformly susceptible. Furthermore these systems often employ high planting densities which facilitate the movement of pathogens from infected to healthy plants.
2. The physical environment, especially temperature and humidity, can be favourable for the pathogens.
3. In closed systems with recirculating nutrient solution, pathogen propagules can be easily spread.
4. Soilless substrates lack the microbial diversity and biological "buffering" found in natural soils.

van Assche and Vangheel (1994) stated that- "in transferring a plant from the soil with its natural buffering against physico-chemical and biological pathogen influences to some 'biological vacuum' has even increased the chance of epidemics".

Stanghellini and Rasmussen (1994) pointed out that these factors have resulted in rapid development of a plant disease being a characteristic of root or below ground infectious agents in hydroponic environments. Prior to hydroponics these characteristics were regarded as unique to foliar or above ground infectious agents.

Stanghellini and Rasmussen (1994) gave the following example to demonstrate the reproductive capabilities of zoosporic fungi and the effects they can have in a recirculating hydroponic system: About 40 sporangia of *Plasmopara lactucae-radicis* Stangh and Gilb. are produced on 1cm of an infected lettuce root. Each sporangium produces about 100 zoospores resulting in the release of approximately 4000 zoospores per centimetre of root. A single mature lettuce plant has about 2000 cm of roots, so assuming uniform infection of the plant, about 8 million zoospores could be released from a single plant.

1.3 *Pythium* spp. as major pathogens in hydroponics

Moulin *et al.* (1994) assayed 39 isolates of *Pythium* spp. for their ability to cause damping off in cucumber seedlings. These authors established that *P. aphanidermatum* (Edson) Fitzp., *P. irregulare* Buisman, *P. sylvaticum* Campb. and Hendrix and *P. ultimum* Trow were highly pathogenic on cucumber plants grown in a sand-peat medium. However, *P. aphanidermatum* was the only species that was pathogenic under soilless culture conditions. Utkhede *et al.* (2000) monitored populations of *Pythium* spp. at two commercial greenhouses over a four month period. Consistent populations (1×10^3 to 13×10^3 CFU/100g) of *P. aphanidermatum* were found in nutrient storage tanks at both locations.

Stanghellini *et al.* (1998) were the first to report root rot of hydroponically grown lettuce caused by *P. myriotylum* Drechsler. This species was consistently isolated from a commercial hydroponic facility and Koch's postulates were performed to confirm its pathogenicity. The severity of this pathogen was clearly demonstrated by the fact that over a two week period approximately 50% of the 30000 plants in the commercial system were destroyed (Stanghellini *et al.*, 1998).

Rey *et al.* (1997) reported on another group of *Pythium* spp. which do not cause the devastation of those species mentioned above and yet are possibly more important in terms of total yield losses. *Pythium* F was found to represent 82% of all the *Pythium* spp. isolated in soilless culture systems. This minor pathogen was shown to cause host cell damage responsible for yield losses in tomato plants even though the roots looked macroscopically healthy (Rey *et al.*, 1997). The lack of visible symptoms means that this pathogen often goes unnoticed while continually limiting the productivity of commercial hydroponic systems.

1.4 Biocontrol in hydroponic systems

Paulitz (1997) pointed out that biological control should be ideally suited to soilless culture in closed structures and that if biocontrol is to be successful anywhere, it will be under these conditions. This is primarily due to the more consistent and monitorable conditions found in the hydroponics environment. Another advantage of the hydroponics environment is that most systems employ the use of substrates which are, to a large extent, sterile at the beginning. Thus, it is easy to establish high populations of the biocontrol agent before an increase in the population of competitors occurs. The application of the agents is seen as being easier as well, because most agents can simply be added to the nutrient solution. The higher economic value of most hydroponic crops also justifies the larger expense which may be involved with biocontrol (Paulitz, 1997). Biocontrol could also give a marketing advantage to hydroponic growers, as consumers may favour crops grown without pesticides.

Gullino and Garibaldi (1994) were of a similar opinion regarding the prospects for biocontrol in hydroponics. They also noted the possibilities for regulation of environmental factors such as temperature in favour of biocontrol agents as seen in the above definition. Despite the advantages to biocontrol in hydroponics, Paulitz (1997) pointed out that comparatively little research has been done in this field and what has been done focusses mostly on the use of rhizobacteria such as species of *Pseudomonas* and *Bacillus*. Many of the mechanisms involved in biocontrol responses are still not completely understood and one must ask whether the responses studied can be directly translated into the widely differing environments of hydroponic systems.

1.5 Biocontrol and Growth stimulation by *Trichoderma*

Weindling (1932) was the first person to report on the ability of *Trichoderma lignorum* Harz to parasitize *Rhizoctonia solani* Kühn isolated from damped-off citrus seedlings. As a result of these observations Weindling (1932) suggested the possibility of controlling certain soil pathogens by the abundant inoculation of soil with *Trichoderma*. Today this possibility has been turned into reality and many reports exist in which

biocontrol of a number of soil pathogens, in numerous crops, has been achieved with *Trichoderma*. These include the control of *Sclerotium rolfsii* Sacc. and *Rhizoctonia solani* in beans (Elad *et al.*, 1980), *Pythium aphanidermatum* and *Fusarium graminearum* Schwabe induced stalk rot of maize (Jie *et al.*, 1999) and damping-off of tomato caused by *Pythium aphanidermatum* (Gnanavel and Jayaraj, 2003).

GuoJing *et al.* (2001) found that the application of *Trichoderma* at 10^7 CFU/g to lettuce seedlings in nutrient film (NFT) hydroponic systems alleviated infection by root pathogens, promoting lettuce growth as a result. These authors concluded that *Trichoderma* treatments have potential as economic methods of root disease control in commercial NFT systems.

Caron *et al.* (2002) evaluated the ability of a strain of *T. harzianum* Rifai to control five plant pathogens on greenhouse cucumber and tomato. This strain significantly reduced the incidence of *Pythium ultimum* and *Rhizoctonia solani* on both cucumber and tomato as well as *Fusarium oxysporum* f.sp. *radicis-lycopersici* on tomato. The strain was further noted to stimulate plant growth of cucumber plants in the absence of any pathogens (Caron *et al.*, 2002).

A number of authors have reviewed the mechanisms employed by *Trichoderma* species in the biological control of plant diseases (Papavizas, 1985; Hjeljord and Tronsmo, 1998; Howell, 2003). The following mechanisms are generally recognised:-

- a) mycoparasitism,
- b) enzyme production,
- c) antibiotic (toxin) production
- d) rhizosphere competence and competitive displacement,
- e) systemic acquired resistance,
- f) metabolism of germination stimulants.

Mycoparasitism refers simply to the physical parasitism of other fungi by *Trichoderma* as first described by Weindling (1932). This parasitism involves the coiling of *Trichoderma* hyphae around the hyphae of the other fungus followed by penetration of the fungus and finally the dissolution of the hosts cytoplasm (Howell, 2003). The

production of enzymes such as chitinase, and glucanase, although proposed as a separate mechanism, can aid in mycoparasitism by helping to disrupt cell wall integrity.

Antibiotic production or antibiosis refers to the ability of *Trichoderma* spp. to produce various antibiotic substances such as gliotoxin which are inhibitory to plant pathogens. Wilhite *et al.* (1994) demonstrated that the loss of gliotoxin production in *T. virens* (Mill, Giddens and Foster) Arx mutants reduced the efficacy of these mutants as biocontrol agents thus highlighting the importance of antibiosis in some biocontrol interactions.

Rhizosphere competence refers to the ability of a biocontrol agent to grow in the rhizosphere environment. Isolates of *Trichoderma* that are more rhizosphere competent will have a greater ability to grow and compete for both space and nutrients within the rhizosphere, competitively excluding other organisms, including pathogens.

Yedidia *et al.* (1999) looked at the ability of *Trichoderma harzianum* T-203 to induce plant defence responses in hydroponically grown cucumber seedlings. Throughout the experiment it was found that *Trichoderma*-treated plants were more developed than non-treated plants. Electron microscopy of *Trichoderma*-treated roots showed penetration of *Trichoderma* into the roots, and strengthening of the epidermal and cortical cell walls, as well as the deposition of newly formed barriers. Inoculation with *Trichoderma* also resulted in increased peroxidase and chitinase activity within 48 and 72 hrs, respectively. As a result of these findings it was concluded that *Trichoderma harzianum* may induce systemic resistance mechanisms in cucumber plants.

Howell (2002) found that *T. virens* was able to metabolize germination stimulants released by cotton seeds which normally induced pathogen propagules to germinate. In so doing mutants that were deficient for mycoparasitism, antibiotic production, and induction of terpenoid synthesis in cotton roots were still able to provide control of damping-off.

Plant growth promotion effects are also commonly reported as a result of *Trichoderma* application. Growth promotion has been attributed to mechanisms including the control of sub-lethal pathogens, a reduction in oxidative damage to roots (Björkman *et al.*,

1998), plant hormone and vitamin production (Kampert and Strzelezyk, 1975a&b; Reddy and Reddy, 1987) and the solubilization of sparingly soluble materials (Altomare *et al.*, 1999).

Despite these promising responses there have also been occasions in which biocontrol agents were found to reduce plant yields. Ousley *et al.* (1993) concluded that growth promotion by *Trichoderma* could be a balance between growth promotion and growth inhibition. MacKenzie *et al.* (2000) came to similar conclusions with regards to the effects of *Trichoderma* on growth response of unrooted chrysanthemum cuttings. MacKenzie *et al.* (2000) suggested that growth inhibition is related to application rates and that phytotoxic effects are possible with higher rates. A possible explanation for this phytotoxicity is provided by Cutler *et al.* (1986) and Cutler and Jacyno (1991) who studied metabolites with phytotoxic activities produced by strains of *Trichoderma*. These included 6-pentyl- α -pyrone (Cutler *et al.*, 1986) as well as (-)harzianopyridone, Koninginin A and Koninginin B (Almassi *et al.*, 1991) (cited by MacKenzie *et al.*, 2000).

Trichoderma-based biocontrol agents for the control of root pathogens have been commercially developed in several countries around the world. Possibly one of the best known and researched products currently available is Rootshield® which is registered in the USA and manufactured by BioWorks, Inc.² This product comes in both drench and granule formulations containing 1×10^7 CFU/g of *Trichoderma harzianum* strain T-22. Eco-T® is a similar product developed and registered in South Africa by Plant Health Products (Pty) Ltd. Eco-T® is formulated as a wettable powder containing 2×10^9 conidia/g of a locally (South African) isolated strain of *T. harzianum*. The product is registered for the control of root diseases and growth enhancement of a wide variety of vegetables, crops, ornamentals and Eucalyptus species and can be applied as either a drench or seed treatment.

²BioWorks, Inc. 345 Woodcliff Drive, First Floor, Fairport, NY, 14450

1.6 Effects of biocontrol agents and soil moisture

For the purpose of this review soil moisture refers simply to the percentage water found within a growing medium. This is because in hydroponics, soil as such is not used. Soil moisture is affected by both the medium used and the irrigation schedules. One cannot view the optimization of irrigation practices without considering the medium through which the water is to be applied. Coupled with the supply of water, is the supply of air. Plants need both these resources in certain quantities and it is the growing medium which supports the roots which determines whether the supply of these resources is at an optimum. Therefore, in order to optimise the supply of these resources, one needs to understand the physical properties of growing media.

Growing media consist of large numbers of different sized particles. Between these particles are spaces, called pores, which at any one time contain either water or air. It is through these pores that air and water move into and through a medium. Water is attracted to the surfaces of the particles in a growing medium. This results in water being held on those surfaces and in the pores between them. The smaller the pores, the more tightly they hold water. It is due to these forces of attraction that all water does not drain from a medium. Below a plant container there is just one large pore (the atmosphere) and the forces which it applies are far less than the attractive forces of the particles holding water in the pore spaces. Thus, when one applies water to a medium in a container it will drain to a certain point and then no further. This is said to be the container capacity. The amount of water held by the medium at this point is the water holding capacity. Container capacity is slightly higher than field capacity shown in natural soils. This is because natural soils are far deeper and the underlying layers exert more pull on the water in the pores than the atmosphere does in potted media. This means that media in containers have a greater capacity for water and thus a lower capacity for holding air. For this reason media for use in containers must have a higher proportion of larger pores if the plants are to get enough oxygen (Handreck and Black, 1994).

When a medium is at container capacity there is plenty of water in the pores and the demands of the plant roots are easily met. The water in the large pores is the first to be removed. As removal continues, the remaining water is held in smaller and smaller pores and higher suction pressures need to be applied by the plant roots in order to remove this water. This extra suction pressure comes from a further decrease in the water content of the plant cells which leads to a slight wilting in the plant. When this wilting gets to an irreversible stage, the remaining water in the soil is of no use to the plant as it is held too tightly in the pores and thus the plant will die. This is termed the 'permanent wilting point'. The water held in the medium between container capacity and the permanent wilting point is termed available water. This is the maximum amount of water which that medium can supply to plants growing in it (Handreck and Black, 1994).

Plant roots not only need water but oxygen too. If the oxygen supply to a plant's roots is completely cut off, root growth stops within minutes. Nutrient uptake is almost stopped and the ability of water to enter the roots is decreased three fold (Handreck and Black, 1994). Oxygen enters growing media mainly through diffusion. As the concentration of oxygen in the air in a medium is decreased through use by plant roots and soil microbes, more diffuses in from the atmosphere. The larger the pores within the medium, the quicker the oxygen will be able to move. Oxygen diffuses through air at about 10 000 times the rate that it moves through water. Thus, a waterlogged soil will have a seriously reduced ability to supply oxygen to plant roots. The more larger pores within a medium, the less water it will hold at container capacity. This in turn means that there will be more space for air to occupy and thus more oxygen. The amount of air in a medium at container capacity is termed the 'air-filled porosity' of the medium. Handreck and Black (1994) showed how all parameters of growth increase with increasing air-filled porosity in Table 1.4.A, reproduced from Flocker *et al.* (1959).

Table 1.4.A The effect of air filled porosity in a potted medium on tomato growth (From Flocker *et al.*, 1959)

Air filled porosity (volume %)	Days to emergence	Germination (%)	No. of flower buds at 5 weeks	Height (cm)
38	8.5	98	3.5	29
33	8.8	98	4.5	29
30	9.2	85	3	27
26	10.6	85	2.5	25
15	11.3	55	0	20
7	12.5	53	0	13
3	12.8	50	0	8

Handreck and Black (1994) provided the following guidelines for the air-filled porosity requirements of plants in container growing media (Table 1.4.B).

Table 1.4.B Summary of the air-filled porosity requirements for plants in container growing media (From Handreck and Black,1994)

Plants and situation	Air-filled porosity (volume %)
Wetland plants	less than 5
Large containers, low growth rates	5-10
Plants destined for planting into a harsh environment	10-15
General nursery mixes: lower end suitable for short term growth; upper end preferred for longer-term growth and when high growth rates are essential.	10-25
Propagation mixes, heavy rainfall areas	25-30
Some propagation mixes; frequent watering needed	30-40
Epiphytes	40-50

Verdonck *et al.* (1983) claimed that for optimal growth conditions it is necessary that in a substrate there is at the same time 20% volume of air and 20-30% volume of easily available water. These figures are supported by the findings of a number of authors. Fakhri *et al.* (1995) found that *Gerbera* plants grown in peat-perlite mixed at 1:1 ratio, performed better than plants grown in ordinary perlite or pumice media. When looking at this in terms of volume percentage air and easily available water, of the three media tested, they found that the peat-perlite mix came the closest to the ratios proposed by Verdonck *et al.* (1983). Further support can be seen in the experiments of Reis *et al.* (1995). These authors concluded that a pine bark-peat mix at a ratio of 1:3 showed a tendency to improve geranium growth. This they attributed to its excellent equilibrium of volume percent air and easily available water.

Handreck (1983) investigated the relationship between particle size and the above properties. He concluded that for very open mixes, no more than about 10% of the material should be in the particle size range of 0.1 - 0.5mm. In slightly closer mixes with air fill porosities of 15 to 25%, the volume ratio of particles larger than 0.5mm to those in the range 0.1 - 0.5mm can be in the range 2:1 to 3:1.

It is thus clear that the percentage soil moisture has a direct impact on the health and productivity of a plant and for a given crop, it is possible to determine what the optimal levels are so as to be able to increase the health of the crop as a whole.

Bedasie and Stewart (1987) looked at watering regimes in nutrient film technique (NFT) production of lettuce. In such systems one is able to remove the effects of the medium as none is being used. Instead, issues such as flow rate and depth of nutrient solution in the channels become important. Bedasie and Stewart (1987) looked at intermittent flow of nutrient solutions as a further factor for consideration in NFT. These authors found that where continuous flow was maintained prior to lettuce hearting, followed by intermittent flow thereafter, no significant difference in final fresh weight was recorded. The use of intermittent flow after hearting was found to significantly reduce electrical consumption, thus improving the profitability of the crop.

An increase in disease severity at higher soil moistures is typically seen with soil borne pathogens. Sippell and Hall (1982) found that root rot of beans, caused by *Pythium* and *Fusarium* spp., was greatest in wetter soils. This result appeared contradictory at first as fusarium root rot of beans is typically referred to as dry root rot. The authors explained this in terms of the effect of the fungus on plant yields appearing more dramatic in drier years due to the greater water stress already on the plant. The fact remains that the growth of the pathogen and the incidence of disease increase at higher moisture levels. This was also supported by Pieczarka and Abawi (1978) who studied the effects of soil moisture and temperature on root rot of snap beans caused by *Pythium ultimum*, and by Cappaert and Powelson (1990) who studied *Verticillium* on potato.

In looking at the positive effects of growth promoting and biocontrol fungi one finds a similar response. Wakelin *et al.* (1999) found that *Trichoderma koningii* Oudem in sterile soil showed greatest saprophytic growth at 70% soil water holding capacity (WHC) with little saprophytic growth occurring below 20% WHC. *Trichoderma* spp. can adapt to poor moisture conditions to some extent through cytoplasmic translocation. Under poor moisture conditions cell wall synthesis continues while cytoplasm synthesis stops and cytoplasm is relocated to growing tips as the hyphae extend (Paustian and Schnürer , 1987a,b cited by Hjeljord and Tronsmo, 1998). No information was available as to whether the increase in growth of beneficial microbes with increasing soil moisture was proportional to that of the pathogens.

Azcón *et al.* (1992) reported increases in proline in *Glomus fasciculatum* (an arbuscular mycorrhizal fungus) inoculated plants. Free proline levels have been used as a criterion for the selection of drought tolerant plants. Enhanced CO₂ assimilation and increased proline levels are considered to protect various enzyme systems of nitrogen metabolism against various negative effects including drought stress (Paleg *et al.*, 1984 cited by Azcón *et al.*, 1992). Inoculation with organisms that in some way increase free proline levels within host plants could thus help protect plants from drought and other stresses.

1.7 Effects of biocontrol agents and root zone temperature

The effect of nutrient solution temperature in a hydroponic system is seen as important as, in most systems, it is this which will govern the root zone temperature of the plant. Root zone temperatures in turn have a wide spectrum of effects on plant growth.

Kafkafi (2001) measured a 250% increase in water flow rate through tomato stems when increasing the root temperature from 12°C to 20°C, at constant light radiation, air humidity and shoot temperature. The decline in sap flow from 20°C to 12°C is explained by: 1) increase in water viscosity and 2) decline in water permeability due to change in the root membranes viscosity (Kuiper, 1964) or closure of water channels in the root (Johansson *et al.*, 1998, Carvajal *et al.*, 1999)

Daskalaki and Burrage (1998) also showed that the uptake of nutrients by cucumber plants increased sharply when root temperatures were raised from 12°C to 20°C. The restricted uptake of ions at low temperatures was seen to limit plant growth. Water uptake, on the other hand, was seen to increase at higher temperatures with a maximum at 28°C. At higher temperatures ions of phosphate were found to accumulate in the root. It was suggested that the steady increase of levels of P in the roots with increasing temperature is indicative of the fact that sufficient P uptake for the plant had occurred at the lower range of the temperature scale.

In some instances, complex manipulations of water temperature are proposed. Benoit and Ceustermans (1986) give the following practical advice for the growing of tomatoes in NFT: The NFT temperature is set at 23°C until the roots of the tomato plants meet. The temperature of the recirculating solution is then lowered to between 18 and 16°C depending on the tomato variety used. Just before the opening of the 7th cluster the temperature is again raised to 23°C to stimulate new root formation and to assist the crop over its maximum energy consuming stage. After the first truss is picked the temperatures can then be reduced to 16 -18°C again. This range of 16 -18°C is critical, as Giacomelli and Janes (1986) are of the opinion that tomato roots exposed to root temperatures below 15°C for extended periods suffer negatively in terms of yield. This

serves to show how complex the influences of temperature can be with a number of factors such as nutrition and stage of growth interacting.

Macduff *et al.* (1986) looked at the more direct responses of the plant roots to temperature in oilseed rape and barley grown in flowing solution culture. Plants were grown at a range of root temperatures ranging from 3-25°C, with a common shoot temperature. Root length of barley was seen to increase with temperature over the entire range tested. Changes were also noted in the length and number of root hairs. In rape, the density of root hairs increased by a factor of four between 3 and 25°C while in barley, the highest density was at 9°C. These experiments serve to show the effects of changes in temperature on different crop plants. It is important to note that the optimal temperature is different for each crop and that, as a grower, one needs to know these optima.

Kennedy *et al.* (1993) found that the growth of roots of healthy and *Phytophthora cryptogea* Pethylor. and Laff infected tomato plants was higher at root temperatures of 25°C than at 15°C. Other authors have noted similar responses in terms of increases in fruit yields, shoot growth and transpiration. It is, however, unclear as to whether the increased root temperature results in a direct effect on yield through increased nutrient uptake and photosynthetic efficiency together with a suppression of the pathogen or an indirect response as a result of new root initiation and growth (Kennedy *et al.*, 1993).

Stanghellini and Rasmussen (1994) were of the opinion that nutrient solution temperatures are the most important environmental factor governing the onset and prevalence of root diseases caused by specific zoosporic pathogens. They gave several examples as proof of this statement. *Pythium aphanidermatum*, which attacks cucumbers, tomatoes and spinach, is most destructive when the nutrient solution is above 25°C while it is not of economic importance below 20°C. In contrast to this, *Phytophthora cryptogea*, a pathogen of tomatoes, is most destructive at 15°C and causes little to no damage at 25°C and above. In both of the above examples, it was found that lack of disease at a certain temperature was associated with an inhibition of, or reduction in, the production of zoospores (i.e., no secondary inoculum).

Schuerger and Mitchell (1992) found similar responses when looking at the effect of nutrient solution temperature on the attachment of *Fusarium solani* f. sp. *phaseoli* Snyder and Hansen macroconidia to mung bean roots in hydroponics. In these experiments it was found that the role of temperature and pH in the binding of macroconidia to roots was not limited to a preventative one. The binding reaction was found to be reversible where plants inoculated at 25°C and pH 5 were moved to a solution at 35°C or pH 7. Schuerger and Mitchell (1992) highlighted a further difference between pathogenesis in conventional soil crops and hydroponics in pointing out that attachment of fungi to root surfaces may not be a prerequisite for pathogenesis in soil crops. This is because the proximity of infection propagules to root surfaces is maintained by the soil matrix. In recirculating hydroponics systems (especially NFT), however, the attachment of non-motile propagules (as found in *Fusarium*) is essential in causing disease.

Gold and Stanghellini (1985) looked at the effect of nutrient solution temperature on two different *Pythium* species. They concluded that the differences in pathogenicity at specific temperatures give a temporary competitive advantage to the favoured species with respect to rapidity of host colonisation and subsequent fungus reproduction. This could prove useful if one were to look at it in terms of biological control. If a biocontrol agent were to have a different optimum temperature, this could be used to aid in the establishment and efficacy of the agent. Wakelin *et al.* (1999) found that the saprophytic growth of *Trichoderma koningii* in sterile soil increased with temperature from 5°C, with the optimum being 25°C and no growth occurring at 30°C. No literature was found in which temperature was used in an attempt to optimise *Trichoderma* or other biocontrol agents (BCA's) control efficacy.

1.8 Effects of biocontrol agents and pH

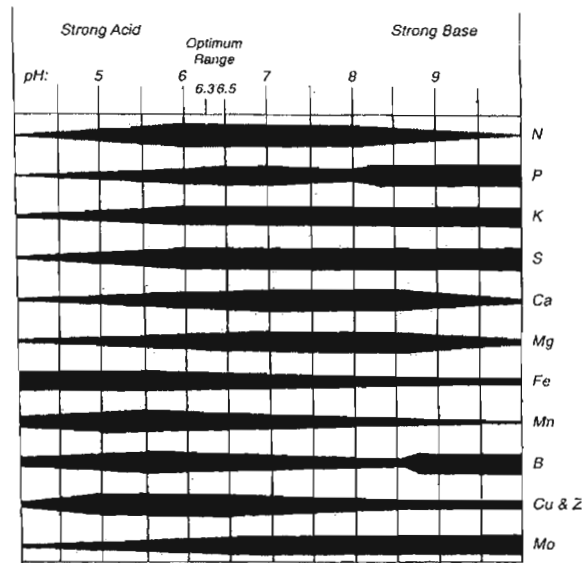


Fig. 1.6.A The effect of pH on the availability of plant nutrients (Resh, 1995)

Resh (1995) provided a basic explanation into the effects of pH on the availability of various elements in plant nutrition. In his book it is pointed out that pH is a logarithmic function and that a 1 fold increase in pH is therefore a 10 fold increase in hydrogen ion concentration. Any change in pH can thus have a large effect on the availability of ions to the plant. Fig 1.6.A (from Resh, 1995) shows the effect of pH on the availability of essential elements. From this it is understandable why most plants have an optimum pH range between 5.5 and 7.0.

However, the effects of pH are not limited to plant growth. pH has a major effect on microbial activity as well. Jones and Woltz (1972) found that the incidence of *Verticillium* wilt on tomato was increased while that of *Fusarium* wilt decreased by raising the soil pH from 6.0 to 7.0 or 7.5. They proposed that the suppression of *Fusarium* wilt was due to the induced deficiency of micronutrients associated with liming and not a direct pH response. Schuerger and Mitchell (1992) found when looking at macroconidium attachment of *Fusarium solani* f. sp. *phaseoli* to mung bean roots in hydroponics, disease was greatest when roots were inoculated at a pH of 4.0. Plants inoculated at pH 7.0 were found to be no different to the uninoculated controls. In a flowing nutrient solution the attachment of non-motile propagules (macroconidia) is essential if the

organism is to remain in contact with the root for sufficient periods so as to allow germination of the conidia and subsequent infection. As a result of this work, Jones and Waltz (1972) suggested that the manipulation of pH in nutrient solutions may prove an inexpensive disease management tool for hydroponic systems.

Harman and Taylor (1988) looked at the effects of pH in matrix priming with various biocontrol agents. They noted that *Trichoderma* species grew well at low pHs (around 3.0) and that this pH should be inhibitory to most other micro-organisms. As tomato seeds are naturally very acidic this was seen as the major reason for *Trichoderma* having a competitive advantage on tomato but not on cucumber, which has a more alkaline seed. In further experiments, HCl was added to seed treatments of cucumber to make the seed pH 3.7 or 3.1. It was found that these treatments markedly improved the ability of *Trichoderma* to control seed rot in cucumber seed. These treatments were, however, found to be phytotoxic after a couple of days of storage but it is suggested that other acidic *Trichoderma* seed treatments might give improved seed protection (Harman and Taylor, 1988).

The varying effects of pH were further highlighted when it was shown that *Enterobacter cloacae* was ineffective when matrix priming was done with Agro-lig (pH = 4.1) while its activity was markedly better when priming was done with a bituminous coal with a pH of 6.6. One of the major differences between the two priming solutions was the pH and the results make sense when considering that *E. cloacae* is favoured by conditions approaching neutrality as opposed to the acidic conditions favoured by *Trichoderma* (Harman and Taylor, 1988)

Jeong *et al.* (1997) investigated the effects of pH on the antagonistic activity and rhizosphere competence of biocontrol agents, including *Gliocladium virens* and *Trichoderma harzianum*. It was found that the rhizosphere competence of both these fungi improved in soils at pH 5.0 and 6.0 compared with soils at pH 7.0. Similarly, mycelial growth of *Pythium ultimum* and *Rhizoctonia solani* was strongly inhibited by the above antagonists at these more acidic soil levels. Abdelzaher *et al.* (1997) showed that for *P. aphanidermatum* and *P. oligandrum* as well as *Pythium* "group F", the optimum pH for mycelial growth and zoospore production was 7.0.

Looking at the practicalities of controlling pH, many growers find it laborious and time consuming and very few are prepared to monitor and alter pH on a consistent basis. Righetti *et al.* (1991) provided a practical alternative to hydroponic growers in terms of pH control in the form of macroreticulate buffers. These buffers consist of amphoteric resins made with buffering and titrant groups, simultaneously affixed to the matrix. The resulting beads possess a very precise isoelectric point (pI) and are able to maintain the solutions pH close to their pI values for extended growing periods (Righetti *et al.*, 1991). These buffers can be produced with any desirable pH within the range 2.5 - 11. The advantage of such buffers is that they work over an extended period of time. This results in the absence of pH spiking which is commonly seen in hydroponic systems where growers check and adjust pH at weekly intervals only. Use of such buffers helps to provide a more stable and conducive environment in which the plant can grow.

1.9 Effects of biocontrol agents and nutrition

In hydroponics the nutrient solution which is added to the crop forms the only source of nutrition for the plants. This is different to fertilization of soil grown crops in which certain elements are already available in the soil. Thus, a hydroponics solution needs to be complete, containing every element needed for plant growth in its most soluble form. Such a solution can either be mixed by the grower from its basic components or it can be purchased as a complete product. When mixing nutrients, it is critical that one is aware of which elements are compatible in concentration. Certain components precipitate when they come together and these elements need to be mixed separately into a working solution and then brought together in their dilute form. It is for this reason that many of the commercial solutions are sold in twin packs.

Another factor for consideration is the change in nutrient requirements as the plants go through their developmental sequence. Plants require different nutrients at different concentrations depending on whether they are undergoing vegetative growth or are investing energy in flower and fruit formation. Most commercial solutions thus come in 'grow and bloom' formulations. Nutrient optimization is not only about finding the correct levels of different elements to be given to the plant at its different stages of

development, but also about maintaining these levels. Measuring the amount of nutrients in the irrigation water is normally done by measuring the Electrical Conductivity (EC) of the solution. Distilled or de-ionised water will conduct virtually no electricity and will thus have an EC value of 0. As salts are dissolved in the water so the conductivity of the water increases, i.e., the conductivity of a solution is a measure of its strength as indicated by the actual amount of salts dissolved in it. Once a certain solution has been chosen for a specific crop it is necessary to mix standard solutions of known concentrations so that the relationship between EC and exact concentration can be calculated. This provides the grower with an optimum EC reading at which the nutrient solution can be maintained.

A final factor to be considered is the relationship between the medium used and the delivery of nutrients. As with water and air, the nutrients are dependent on the growing medium being used for their delivery to the plants. Certain media are known to absorb some elements leaving them unavailable to the plants. Ansermino *et al.* (1995) found that composted pine bark (CPB) released less ammonia and phosphorus than peat as they were strongly bound by bark. They concluded that a starter charge of ammonium and phosphate, and liquid phosphate and nitrate application would be beneficial. This is supported by the findings of D'Angelo *et al.* (1995) who concluded that in the growing of cyclamen, more N and P was required when using a pine medium as apposed to peat.

The above information provides some basic pointers to plant nutrition in hydroponics. The details of crop nutrition have been extensively studied and well documented for a wide variety of crops. For the purpose of this review it is not practical to look at the effects of all nutrients on plant growth and health. It is noted that there are optimal requirements for the different nutritional elements and these optima differ from plant to plant. However, the form of nitrogen (i.e., ammonium vs. nitrate) will be discussed in detail. The effects which the form of nitrogen has on both plant growth and microbial survival and performance often involves complex interactions.

Haynes (1986) pointed out that the tolerance of plants to an ample supply of ammonium is low, while the tolerance for nitrate is high. Nitrate can be accumulated and transported

through the plant with few toxic effects, while ammonium toxicity occurs readily. Haynes (1986) outlined the major reasons for this toxicity as presented below.

a) *Increase in rhizosphere pH* - A preferential uptake of NH_4^+ results in the excess uptake of cations and H^+ production during NH_4^+ assimilation by root tissue. This leads to an enhanced net extrusion of H^+ and a decrease in rhizosphere pH (Neumann and Romheld, 2002). This form of acidification has been shown to be toxic to many plant species (Maynard *et al.*, 1966; Maynard and Barker, 1969).

b) *Induced cation deficiencies* - The increased uptake of NH_4^+ results in the reduced uptake of cations such as K^+ and Ca^+ due to ionic competition either with NH_4^+ ions *per se* or with H^+ ions excreted during active NH_4^+ uptake (Haynes, 1986). As a result K and Ca deficiencies are commonly cited in association with NH_4^+ toxicity.

c) *Induced water stress* - NH_4^+ nutrition has been shown to cause a decrease in water uptake, xylem exudation, and leaf water potential (Quebedeaux and Ozbun, 1973; Pill and Lambeth, 1977, 1980; Pill *et al.*, 1978).

d) *Increased carbohydrate metabolism* - Detoxification of ammonium in the roots results in a demand for carbon skeletons within the plant. These are supplied mainly by intermediates in glycolysis and the tricarboxylic acid cycle (Givan, 1979 cited by Haynes, 1986). This in turn may result in an increase in respiration in order to rapidly turn over the carbon skeletons needed for NH_4^+ assimilation (Givan, 1979). This increased respiration rate and high demand for stored carbohydrates in ammonium fed plants is particularly damaging when plants begin to translocate excess ammonium to the shoots. Ammonium accumulation in the shoots results in the inhibition of photosynthesis and thus carbohydrate production (Goyal *et al.*, 1982).

Lee *et al.* (1991) looked at the effect of the nitrate to ammonium ratios on the growth, mineral content and yield of tomatoes in hydroponics. It was found that the highest ratio of nitrate to ammonium (93:7) resulted in better growth and productivity of plants in all parameters measured. The higher levels of nitrate were also seen to reduce the incidence of blossom-end rot and resulted in a far slower decrease in solution pH over

time when compared with the 50:50 ratio. Similar responses were described by Simonne *et al.* (1992) for hydroponic watermelon, Elia *et al.* (1996) for egg plant and by Gimenez *et al.* (1996) for melon and watermelon seedlings in soilless culture. Santamaria *et al.* (1999) found similar responses in swiss chard but found fennel and celery to be quite unresponsive to nitrogen (N) form. Chance *et al.* (1999) also found similar responses in zucchini squash with plant growth being better when nitrate was the sole form of N used. It was proposed that increased fruit yield could be obtained by using a predominantly nitrate fertilizer through the vegetative growth stage and then shifting the nitrate:ammonium ratio to one of more ammonium (3:1) during the reproductive phase.

It is important to note that despite the negative aspects of ammonium in culture solutions, some ammonium can be beneficial to plant growth. Haynes (1986) pointed out that at sufficiently low levels of ammonium, the depression of nitrate uptake is less than the rapid uptake of ammonium and as a result the total intake of N, plant protein content, and growth rate increase.

Elia *et al.* (1996) found that the daily Ca and Mg uptake increased linearly when nitrate was the prevailing form of nitrogen added (Mg increased from 1 to 5 mg/plant while Ca increased from 3 to 28 mg/plant when $\text{NO}_3\text{-N}$ was increased from 0 to 100% of total N). Potassium on the other hand was seen to increase quadratically with increasing $\text{NO}_3\text{-N}$.

Jeong and Lee (1996), working with *Ageratum* and *Salvia* plants in hydroponic culture found that the uptake of potassium, dihydrogen phosphate, sulfate, manganese and zinc was significantly enhanced in solutions containing 9mM NH_4^+ compared with solutions containing either 15mM NO_3^- or combined 9mM NH_4^+ + 9mM NO_3^- .

Lasa *et al.* (2000) found that sunflower plants grown in hydroponic culture with 5mM ammonium as nitrogen source resulted in a reduction in dry matter accumulation and CO_2 assimilation when compared with plants grown with 5mM nitrate as N source. Ammonium fed plants also showed a greater content of free amino acids, soluble proteins, Rubisco and anions, and a lower content of cations, especially Mg^{2+} .

Kafkafi (2001) pointed out that the responses to the form of nitrogen can be temperature sensitive. Nitrogen as ammonium ($\text{NH}_4\text{-N}$) can be beneficial when root zone

temperatures are low, but detrimental when they are high. This is because ammonium is completely metabolised in the root while nitrates are only partly reduced in the roots, with the larger part of their metabolism taking place in the leaves. With increasing root temperatures, respiration rates increase, consuming sugars. At high temperatures (32°C) no sugar is available for the metabolism of ammonium in the root and the resulting ammonia, which is produced in the cytoplasm, kills the root.

These complex interactions are highlighted by the works of Borowski and Michalek (1995) who looked at the interaction between the form of nitrogen and solution temperature in hydroponically grown lettuce. It was determined that these two factors are interdependent, with ammonium having a beneficial effect on lettuce plants grown at low temperatures (7 and 14°C). At higher temperatures yields were higher in plants receiving nitrate nitrogen in all cases.

A further example is provided by Zornoza *et al.* (1995) who looked at the interaction between form of nitrogen and light intensity in two cultivars of tomato. Here an even more complex interaction was noted as there was a differential response between cultivars as well. Cultivar Marglobe, under conditions of high light intensity, fared better when grown with nitrate alone rather than a combination of nitrate and ammonium. The second cultivar, Carmelo, showed no sign of light dependence in terms of its ammonium tolerance. This cultivar was unaffected by both light intensity and form of nitrogen.

The complexities of interactions between forms of nitrogen and plant growth is furthered by the effects which the form of nitrogen has on microbial populations and activity. Jayaraj and Ramabadran (1998) looked at the effect of certain nitrogenous sources on the *in vitro* growth, sporulation and production of antifungal substances by *T. harzianum*. Of the seven nitrogenous salts tested, ammonium nitrate, ammonium sulphate and sodium nitrate recorded the maximum increase of the above factors, respectively. Jayaraj and Ramabadran (1998) also studied the effect of nitrogenous fertilizers on the survival and competitive saprophytic ability of *Trichoderma* in soil. Here it was found that ammonium sulphate enhanced the growth and survival of *T. harzianum* to the maximum extent, followed by urea and ammonium chloride. This finding was supported by

Wakelin *et al.* (1999) who showed that N added as ammonium increased the saprophytic growth of *T. koningii* in a soil sandwich bioassay. Nitrogen added as nitrate was conversely seen to decrease saprophytic growth of the fungus.

Other nutritional elements have also been seen to affect both microbial pathogens and antagonists in their interactions with host plants. Forster *et al.* (1998) looked at the effects of phosphite on tomato and pepper plants and on the susceptibility of pepper to *Phytophthora* root and crown rot in hydroponic culture. In these experiments the authors used phosphate or technical and commercial formulations of phosphite as phosphorus nutrition. They found that the plants treated with phosphite showed significantly less growth than those treated with phosphate and that the plants receiving phosphite showed typical symptoms of phosphorus (P) deficiency. When looking at disease levels it was found that the incidence of crown rot was significantly lower in phosphite treated plants when compared with plants receiving phosphate or no phosphorus at all. Although *Phytophthora* was isolated from the plants treated with phosphite (supplied as phosphoric acid) disease symptoms were not observed in most plants over the four week trial period. The observations of Forster *et al.* (1998) are supported by other authors such as Ouimette and Coffey (1989) and Smillie *et al.* (1989) who also found phosphonates to be useful as antifungal compounds against Oomycetous fungi.

Dhanvantari and Papadopoulos (1995) looked at the effect of potassium-nitrogen (K/N) ratios in the suppression of bacterial stem rot (*Erwinia carotovora* subsp. *carotovora* (Jones) Berg.) in hydroponically grown tomatoes. Low, medium and high K/N ratios were supplied as 300:300, 400:200 and 480:120 ppm respectively. Constant levels of P, Magnesium (Mg), Calcium (Ca) and micronutrients were maintained as well as pH and EC. The spread of stem rot was significantly lower in the case of the 4:1 K/N ratio. It is suggested that such a nutritional regime may help suppress bacterial stem rot in hydroponic tomatoes.

Cherif and Belanger (1992) looked at the effects of potassium silicate amendments to suppress *Pythium ultimum* on long english cucumber in recirculating nutrient solutions. In these experiments, it was found that supplementing the nutrient solutions with 100

or 200 ppm of silicate significantly reduced mortality, root decay and yield losses attributed to infection by *P. ultimum*. Treating inoculated plants with potassium silicate increased root dry weight and number of fruit. Silicon alone did not improve yields in non-infected plants and the results were most significant when conditions were such as to promote the spread of *P. ultimum*. These facts led the authors to conclude that the results were due to disease suppression rather than a fertilization effect.

In the above cases the alteration of nutrient solutions was found to have direct effects on plant diseases. Many biocontrol and growth promoting fungi and bacteria can be enhanced by the alteration of nutrient solutions as well. Duffy and Defago (1997) reported on the ability of zinc (Zn) to improve the biocontrol of *Fusarium* root and crown rot of tomatoes by *Pseudomonas fluorescens* Migula. It was found that this biocontrol agent provided only moderate control of *Fusarium oxysporum*. Similarly, a once-off application of Zn at 33ug/ml to hydroponic nutrient solution in rockwool culture had no effect on disease. However, when this application was made in combination with the biocontrol agent, a 25% decrease in disease was noted. *In vitro* studies showed that Zn in concentrations as low as 10ug/ml abolished the production of fusaric acid (a *Fusarium* pathogenicity factor). It was suggested that Zn improved the biocontrol activity by reducing fusaric acid production by the pathogen, enabling increased antibiotic production by the biocontrol agent.

Added to the effects which nutrition has on biocontrol fungi and the pathogens they control is the reverse phenomenon in which fungi alter the nutritional status of the soil or growing medium. Altomare *et al.* (1999) looked at the ability of *T. harzianum* Rifai 1295-22 (T-22) to solubilize, *in vitro*, some insoluble or sparingly soluble minerals. Three possible mechanisms were proposed. These were: acidification of the medium, production of chelating metabolites, and redox activity. Acidification of the medium was ruled out as a possible mechanism, as in the solubilization of MnO sub(2), metallic zinc, and rock phosphate, the pH of the culture never fell below 5. It was concluded that the solubilization of these phosphates and micronutrients by *Trichoderma* involves both chelation and reduction. It is also proposed that this activity might explain, at least partially, the ability of *Trichoderma* to increase plant growth.

Mycorrhizal fungi are those fungi that share a symbiotic association with the roots of a plant (Agrios, 1997). Haynes (1986) pointed out that mycorrhizal fungi are known to prefer $\text{NH}_4\text{-N}$ to $\text{NO}_3\text{-N}$ and that it appears possible that mycorrhizal fungi may act as biocontrol agents of nitrification under vegetated conditions. This suggestion goes some way towards demonstrating the complexity of interactions which may result from the addition of a biocontrol agent into a closed system, especially if these organisms show mycorrhizal-like tendencies. Any effects on nutrient cycling would result in associated indirect effects on plant growth as well as other factors such as root zone pH.

1.10 Concluding Remarks

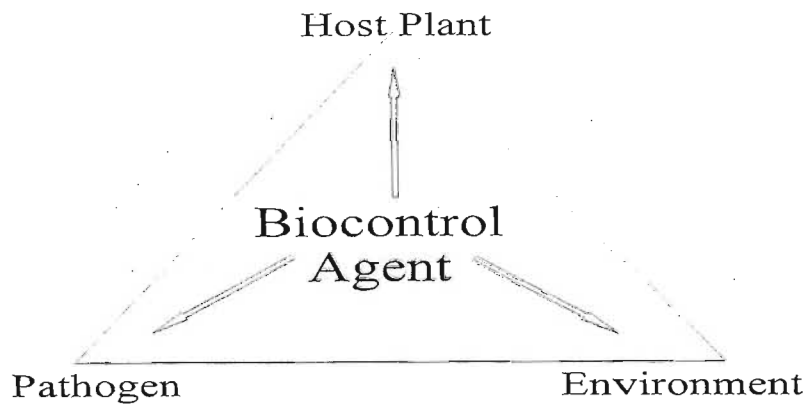


Fig. 1.8.A. Diagrammatic representation of interactions studied

Figure 1.8.A. shows a diagrammatic representation of the interactions covered in this review. From this work it is clear to see that the effects of biocontrol agents within any environment can be extremely varied and complex. Not only can the biocontrol agent affect the host, pathogen and environment but each of these can indirectly affect each other and can influence the functioning of the biocontrol agent in reverse. These interactions and effects can be even more noticeable in a hydroponic environment in which many of the interactions are largely unbuffered. It is thus important to understand when adding a biocontrol agent to such a system, how far reaching the consequences may be. The research which follows is thus aimed at identifying some of the effects which *Trichoderma* (biocontrol agent) may have when added to a hydroponic environment as well as some possible mechanisms behind these effects.

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Chapter 2

Construction of Hydroponic Research Systems

2.1 Introduction

Over six months were spent constructing the systems necessary for hydroponic research. This included re-building a greenhouse to house several hydroponic systems. The large number of systems used were necessary in order to facilitate studies involving a number of environmental parameters.

2.2 Greenhouse construction

A standard, 30m long, 8m wide and 4m high, hooped tunnel was covered with polycarbonate sheeting due to the risk of hail damage which had interfered with earlier trials. A wet wall (7m x 1.5m) was fitted on the south wall and three fans on the north. An automated curtain of white shade cloth was fitted on the outside of the tunnel and three Tempadair 45 watt fan heaters along the inside of the eastern wall. These all aided in providing a research facility capable of optimal temperature control. Maximum daily temperatures inside the tunnel never rose above 32°C, with a mean daily maximum of approximately 28°C and little seasonal variation. Night time minimums were more varied, depending on seasons, although not dropping below 5°C during any trials, with a mean night time minimum of 16°C in summer months and 10°C in winter months.

2.3 Verti-gro® system

Thirty six independent Verti-gro® stacks were constructed (Fig. 2.3.A). Each of these consisted of a 20ℓ black plastic trough (reservoir) linked to the water mains via a ball and float valve. Each reservoir was fitted with a NatHura Re-Action IP 1000 submersible pump and a Natural 50 watt aquarium heater. The reservoirs were covered with white

mulch plastic to prevent solar heating. A plant pot containing coarse river sand was placed in each reservoir to function as a filter. Above this, four Verti-gro® pots (volume = 4ℓ) were stacked. The Verti-gro® pots were held in place by a central, poly-vinyl-chloride (PVC) pipe, supported from above by horizontal wire braces. The number of pots per stack, growing medium and nutrient solution could be altered with each experiment. A maximum of 6 pots could be placed in each stack.

A flexible pipe was fitted to each pump to carry water from the reservoir to the top of the stack, where it was connected to a specially designed irrigation ring. This ring was made from a piece of spaghetti tube (a piece of tubing with an internal diameter of 2mm) bent into a circle with 10 evenly spaced holes drilled in it. These rings ensured even distribution of nutrient solution to all parts of the top pot at a rate of 2ℓ/min.

The irrigation of each stack was individually controlled by a Keyence programmable logic controller (PLC) linked to a Kessler Ellis Products (KEP) keypad interface. This allowed for 36 verti-gro® stacks each capable of being run with a different irrigation regime, essential for use in soil moisture trials.

2.4 Horizontal mini troughs

Troughs (2m x 180mm) were constructed from 1mm black plastic, folded and held in place in a wooden frame. The wooden frames were mounted on tables so that plants were at waist height for easier working arrangement (Fig. 2.4.A). Nine troughs were arranged per table, with four tables giving a total of 36 troughs. The troughs ran in alternating directions as the reservoirs would not fit next to each other if they all ran in the same direction. Reservoirs (8ℓ buckets) were linked to the water mains by ball and float valves. Each reservoir contained a 50 watt Natural aquarium heater and a Project Powerhead 100 submersible pump. Pumps ran continually, and were fitted to supply lines which carried water to the top end of the troughs from where it flowed through the troughs and back to the reservoirs. Seedlings of test plants (e.g. lettuce) were placed directly into the troughs in Speedling®-24 trays (24 cell seedling trays with each cell having a volume of 37mℓ) containing pine bark medium.

2.5 Temperature trials

Temperature trials were conducted in troughs similar to those described in Section 2.4. Two tables were used, providing 18 troughs. Troughs were shortened (1m long) to reduce the distance over which the water could change temperature. Polystyrene (1cm thick) was placed on top and below each trough as insulation, with a hole cut in the top piece into which a Speedling®-24 tray (24 cells of 37ml each) could be snugly fitted (Fig. 2.5.A).

Reservoirs containing heaters for warm nutrient solution treatments (24°C or 30°C) were placed at the ends of the respective troughs. The reservoirs for cold nutrient solution treatments were arranged in 3 fridge/freezers (KIC, Vari Freeze) set at different temperatures (6°C, 12°C and 18°C). The lids were removed from the freezers and replaced with wooden lids, lined with polystyrene. A hole was cut into each lid into which a 100mm diameter plastic pipe was inserted to allow for entry of delivery and return pipes as well as electric cables for the submersible pumps. All external pipes ran through insulating foam sheaths. Each fridge/freezer contained three reservoirs allowing for three treatments at each temperature.

2.6 Bag culture trials

Cucumber trials were conducted in a separate glasshouse fitted with a wet wall and fans. Plants were arranged in six single rows of 10 plants each with an in-row spacing of 0.5m and a between-row spacing of 1m. Individual seedlings were transplanted into Fifteen-litre bags filled with pine wood shavings. For each bag there was a 2l/h dripper manifold connected by spaghetti tube to an arrow dripper which was inserted into the pine bark at the base of the plant. Irrigation was controlled by a Hardie™ Rain Dial 900 irrigation controller with fertilizer (Ocean Agriculture 3:1:3 (38) complete³ at 1g/l) being applied via a Dosatron® system.

³Ocean Agriculture (Pty) Ltd., P.O. Box 742, Muldersdrift, 1747, South Africa



Fig. 2.3.A. Single Verti-gro[®] stack



Fig. 2.4.A Horizontal mini trough system



Fig. 2.4.B Horizontal mini trough system with plants

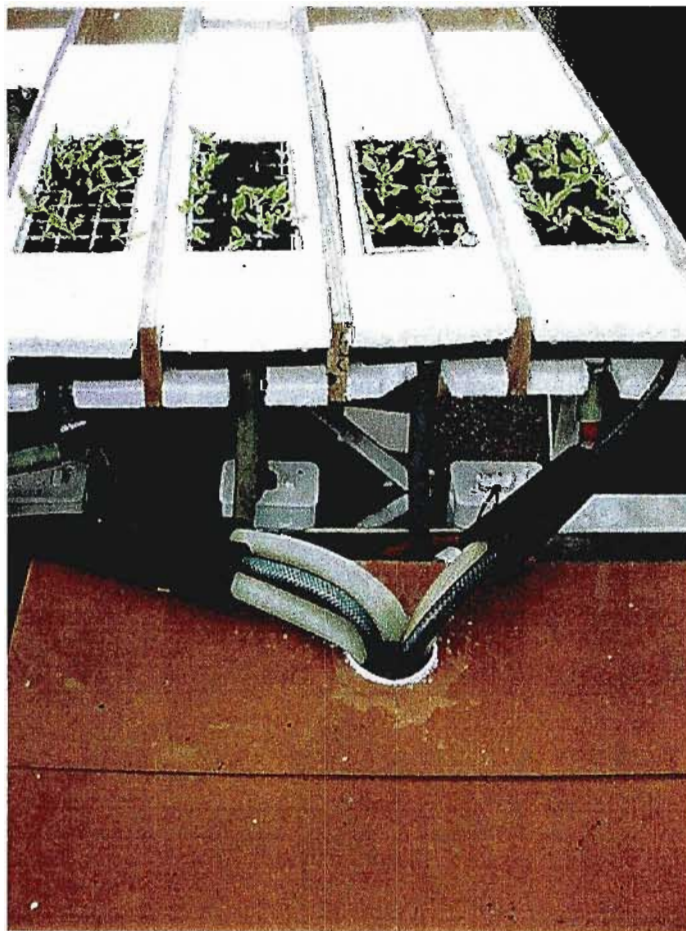


Fig. 2.5.A Temperature trial system

Chapter 3

Dose effects of *Trichoderma* on *Pythium* disease severity and plant growth in Open and Closed Hydroponic Systems

Abstract

Trials were conducted to determine the effect of application rates, of a commercial *Trichoderma* formulation (Eco-T[®]), on biocontrol and growth stimulation activity in both open and closed hydroponic systems. In closed systems *Trichoderma* was effective when added directly to the recirculating nutrient solution. Optimum application rates were lower in these systems (0.25g/l nutrient solution) as conidia are re-circulated and can be transported into the root zone, to germinate over time. Significant levels of biological control were recorded at all application rates (1-0.0625g/l). No significant growth stimulation was recorded. Cucumber trials in open hydroponics were at first unsuccessful in generating statistically significant results, mostly due to a poor knowledge of the horticulture of the crop. However, some general trends were observed from the graphs and it appeared that application rates in such systems would be similar to field applications. These conclusions were supported in repeat trials in which statistically significant results were obtained. Errors in methodology from the first trial are explained.

3.1 Introduction

Paulitz (1997) commented that biological control would appear to be ideally suited for soilless systems in closed structures, and claimed that if biocontrol is to be successful anywhere, it will be in such systems. This is largely due to the ability to control, and maintain, relatively constant environmental conditions. The low level of naturally occurring microbes in many artificial media is also seen as providing an ideal window for biocontrol agents establishment in the initial absence of competition.

Trichoderma is well known for its biological control potential, which has been documented by numerous authors (Chet and Baker, 1981; Migheli *et al.* 1995; Lewis *et al.*, 1996; Lo *et al.*, 1996). However, mechanisms of control are still not entirely known. Ousley *et al.* (1993) pointed out that biocontrol activity may involve competition for infection sites or substrates on the plant surface, physical restriction, antibiosis and/or the production of lytic enzymes. Howell (2003) provided a more comprehensive list of possible mechanisms in his review of the mechanisms employed by *Trichoderma* species in the biological control of plant diseases. These include:-

- a) Mycoparasitism and antibiotic (toxin) production,
- b) competition and rhizosphere competence,
- c) enzyme production,
- d) systemic acquired resistance,
- e) metabolism of germination stimulants.

Plant growth stimulation is less well documented and understood. In many instances, it has been shown to occur primarily due to the control of sub-lethal pathogens. Ousley *et al.* (1993) maintained, however, that direct stimulation of plant growth by *Trichoderma* could not be ruled out. Other mechanisms commonly proposed for plant growth promotion include increased uptake of minerals, plant hormone and vitamin production and the solubilization of sparingly soluble materials. Plant growth inhibition by *Trichoderma* has also been reported (Ousley *et al.*, 1993). The mechanisms governing these negative responses are at present largely unknown. One possible explanation for this reduced plant growth is provided by Cutler *et al.* (1986) and Cutler and Jacyno (1991) who studied metabolites with phytotoxic activities produced by strains of *Trichoderma*. These included 6-pentyl- α -pyrone (Cutler *et al.*, 1986) as well as (-)harzianopyridone, Koninginin A and Koninginin B (Almassi *et al.*, 1991) (cited by MacKenzie *et al.*, 2000).

Eco-T[®] is a *Trichoderma* formulation, manufactured in South Africa by Plant Health Products (Pty) Ltd., and tested under hydroponic conditions as part of its proposed registration trials. The need for separate trials in different systems was highlighted by Hoitink and Boehm (1999) who investigated biocontrol as a substrate-dependent phenomenon. For example, fresh hardwood bark was seen to stimulate populations of

Trichoderma. These elevated levels of *Trichoderma* were, however, not capable of controlling *Rhizoctonia* damping-off. On composted bark, although *Trichoderma* populations remained low, *Rhizoctonia* was controlled and readily eradicated (Hoitink and Boehm, 1999). Thus one cannot assume that a *Trichoderma* formulation will work in a hydroponics environment on the basis of seedling or even field trial data.

Even if functional in the new environment, application rates are likely to differ in different systems, especially with regard to open and closed systems. In closed systems, *Trichoderma* conidia circulate in the nutrient solution and can inoculate roots over an extended period of time. However, open systems are more like field applications, in which the *Trichoderma* conidia have a single chance at germinating and becoming established in the root-zone before being leached beyond the roots.

3.2 Trial 1 - The effect of dosage rates of Eco-T[®], in a recirculating (closed) hydroponics system, on *Pythium* control and plant growth stimulation.

3.2.1 Introduction

Pythium spp. form one of the major genera of zoosporic fungi causing root rot diseases in hydroponics, with the other major genera being *Phytophthora* and *Olpidium* (Paulitz, 1997). These fungi are most suited to an aquatic environment due to their ability to produce asexual, motile zoospores. These spores enable active dispersal and infection in an aquatic environment, and demonstrate chemotaxis (direct movement towards root exudates) (Paulitz, 1997).

van Assche and Vangheel (1994) pointed out that in the absence of potential antagonists (as in most hydroponic media) such well-suited pathogenic organisms, once in the system, hardly meet any obstruction and rapidly multiply and spread. This results in some pathogens, considered as minor pathogens in traditional cultivation, developing

into major pathogens in hydroponics. *Pythium* is a prime example of this. In traditional cultivation it primarily causes soft rots, damping off and root rot in seedlings. However, in hydroponics it causes crop losses in most crops and at most stages of growth.

In many cases *Pythium* spp. can result in decreased yields without forming visible symptoms of disease. Rey *et al.* (2001) found that extensive root infections were not correlated with severe damage. This was due to a large percentage of *Pythium* isolates being of the minor pathogen, *Pythium* Group F. This fungus did, however, penetrate cortical root areas and induce alterations in cell walls and cytoplasmic contents of invaded host cells. These damages led to noticeable yield losses although (macroscopically) the roots looked healthy (Rey *et al.*, 1997)

A recirculating system was used in which *Pythium* would be expected to become established at high levels due to its ability to spread and reinfect through the recirculating nutrient solution. Eco-T[®] was added directly to the nutrient solution. It was hoped that it would then be transported with the water, into the root zone. This method of application was seen as the least labour intensive, and therefore most likely to gain support by growers, if functional. It was hypothesised that application rates would be lower in a recirculating system for reasons already discussed.

3.2.2 Materials and methods

Speedling[®]-24's were planted with lettuce seed (Mayford - All Year Round) in pine bark medium. The seeds were left to germinate for two days in the potting shed. Thereafter, one tray was placed into each of the 36 horizontal mini troughs (Fig. 3.2.2.A). Three replicates of each of the 11 treatments (Table 3.2.2.A) were carried out and these were arranged in a randomised block design.

Table 3.2.2.A Treatments used in Trial 1

Treatment	<i>Pythium</i> added	<i>Trichoderma</i> (g/l)	<i>Trichoderma</i> (conidia/l)
1	no	0	0
2	yes	0	0
3	yes	1	2×10^9
4	yes	0.5	1×10^9
5	yes	0.25	5×10^8
6	yes	0.125	2.5×10^8
7	no	1	2×10^9
8	no	0.5	1×10^9
9	no	0.25	5×10^8
10	no	0.125	2.5×10^8
11	no	0.0625	1.25×10^8

The Eco-T[®] formulation used consisted of 2×10^9 conidia/g. The reservoirs for each trough contained 7ℓ of nutrient solution and *Trichoderma* was added directly to the reservoirs along with fertilizer. Plants were inoculated with *Pythium* by means of 25mm² agar blocks taken from a one week old culture grown on potato dextrose agar (PDA) The agar blocks containing *Pythium* growth were placed on the surface of the growing medium next to each emerging seedling 24 hours after Eco-T[®] treatments.

The *Pythium* isolate used in this and all subsequent trials was isolated from a commercial hydroponic system where both lettuce and strawberry plants were being grown. Samples of growing medium (50mℓ) were placed in 250mℓ beakers to which 150mℓ of distilled water was added. Citrus leaf discs were floated on the surface as a selective bait. Leaf discs were removed after two days, dried on paper towel, and placed onto water agar. Fungal growth was identified microscopically after five days as being a *Pythium* species. This identification was later confirmed by the Plant Protection

Research Institute⁴ (PPRI) as being *Pythium myriotylum* Drechsler (PPRI accession number 04169). The isolate was stored both on agar plugs in sterile distilled water and in double autoclaved sand to prevent attenuation.

Water temperature was maintained at $\pm 26^{\circ}\text{C}$. Ocean Agriculture 3:1:3 (38) Complete fertilizer was used (1g/l), with the nutrient solution maintained at an electrical conductivity (EC) of 1.8 ms (millisiemens). pH was amended once a week using nitric acid or potassium hydroxide to maintain the pH at 6.0. Plants were harvested once a visual difference in plant size was observed between treatments (24 days).

Once the trial was completed, all 36 troughs were emptied and the troughs and components washed down with, or rinsed in, a solution of Sporekill[®] (a quaternary ammonium compound (QAC) sterilant)⁵. The troughs were then reassembled and the experiment repeated. However, a few important changes were made. When planting, two seeds were placed in each cell of the seedling tray. The seeds were then allowed to germinate in the potting shed for two days, as before. They were then allowed to grow for 10 days in the seedling greenhouse. On transferring the trays to the hydroponic troughs, the trays were thinned out, or made up, to 24 plants each. This provided a more equal and comparable base between replicates at the start of the trial. In the repeat trial the formulation of Eco-T[®], supplied by Plant Health Products (Pty) Ltd, had been diluted to 5×10^8 conidia/g on the basis of earlier dose trials. An extra dose ($0.0625\text{g/l} = 3.125 \times 10^7$ conidia/l) was used in the biocontrol treatments, giving 12 treatments in total.

In both trials, total shoot wet weight was recorded for each replicate and statistical analysis was done using the SAS system for Windows 98, Version 6.1. Analysis of variance (ANOVA) and the Student-Newman-Keuls test were conducted. The mean wet weight for each treatment was calculated and graphed.

⁴ARC-Plant Protection Research Institute, Private Bag X134, Pretoria, 0001, South Africa

⁵Hygrotech Seeds, P.O. Box 21880, Mayors Walk, 3208, South Africa.

3.2.3. Results

In Trial 1a. (Fig. 3.2.3.A and Table 3.2.3.A), significant yield losses were achieved by inoculation with *Pythium* (inoculated control). Significant levels of biocontrol were recorded at all doses when compared with this inoculated control. However, at all doses except 0.25g/l, yield was still below that of the uninoculated control. At 0.25 g/l yield was significantly higher than in the uninoculated control, although the difference was only approximately three grams in mean total wet weight.

In Trial 1b. (Fig. 3.2.3.B and Table 3.2.3.B) the same trends were recorded with the only difference being that at 0.5g/l *Trichoderma* resulted in yields not significantly different to the uninoculated control.

In growth stimulation Trial 1a. (Fig. 3.2.3.C and Table 3.2.3.C) no significant changes in yield were recorded under the different treatments.

In Trial 1b the same trends can be seen when comparing Figs. 3.2.3.C and D. In this repeat trial, however, statistically significant differences were obtained. At *Trichoderma* doses of 1 and 0.5 g/l yield was seen to be significantly lower than for the uninoculated control (Table 3.2.3.D). This was a significant yield reduction response. No significant growth stimulation was recorded at any of the doses.

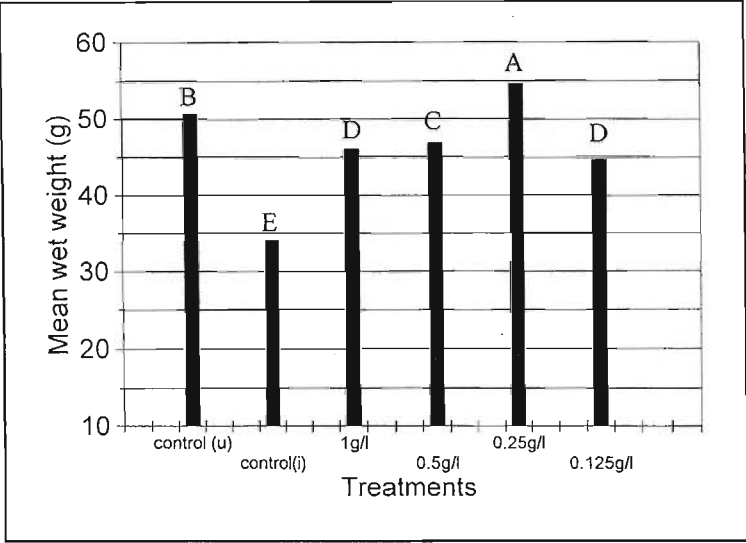


Fig. 3.2.3.A - Biocontrol dose response Trial 1a - *Trichoderma* vs. *Pythium* on lettuce. Treatments with the same letter are not significantly different at $p = 0.05$

Key to Figs. 3.2.3.A and B
Control (u) = Uninoculated control
Control (i) = Inoculated control (inoculated with *Pythium*)
All other treatments were inoculated with *Pythium* and treated with the dose of Eco-T® shown in g/l

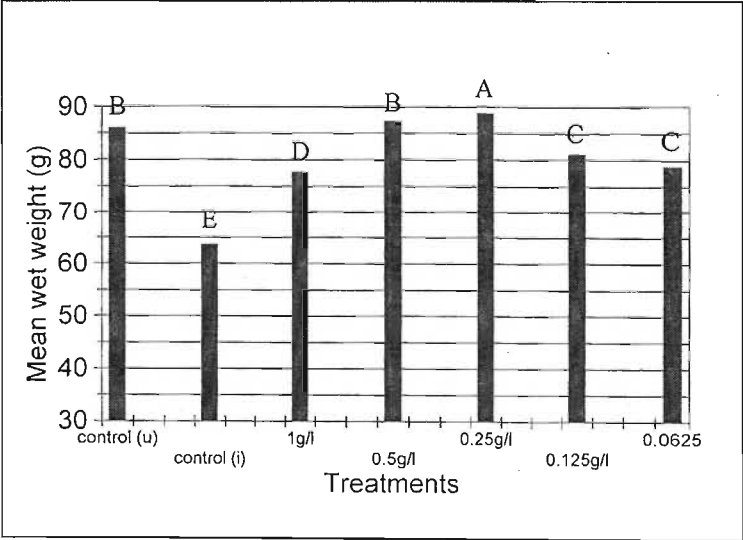


Fig. 3.2.3.B - Biocontrol dose response Trial 1b - *Trichoderma* vs. *Pythium* on lettuce. Treatments with the same letter are not significantly different at $P = 0.05$

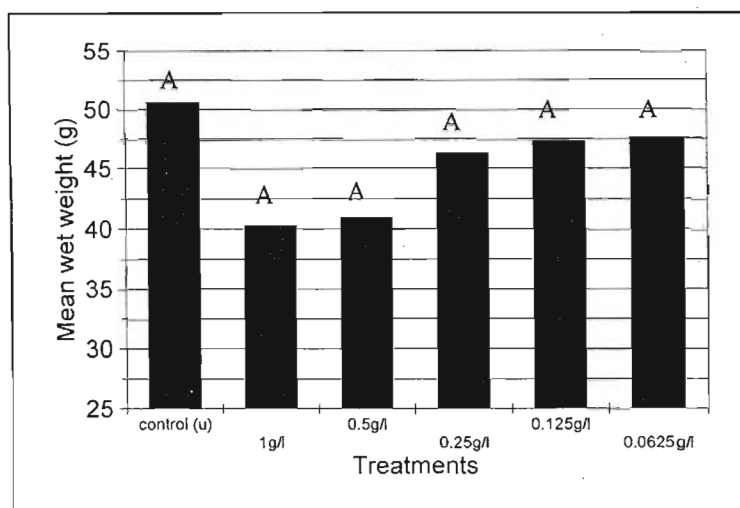


Fig. 3.2.3.C Growth stimulation dose response - Trial 1a. Treatments with the same letter are not significantly different at P = 0.05

Key to Figs. 3.2.3.C and D

Control (u) = Uninoculated control

All other treatments received only the dose of Eco-T[®] shown in g/l

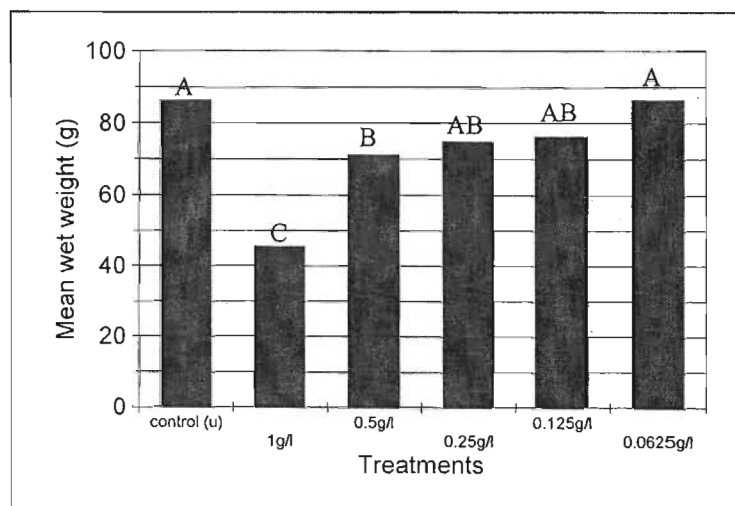


Fig. 3.2.3.D Growth stimulation dose response - Trial 1b. Treatments with the same letter are not significantly different at P = 0.05

Table 3.2.3.A Summary of results for Trial 1a - biocontrol dose response

treatment	<i>Pythium</i>	Eco-T® (g/l)	mean wet weight (g)	rank	Student-Newman-Keuls (SNK) grouping
1	no	0	50.67	2	b
2	yes	0	34.00	6	e
3	yes	1	44.33	5	d
4	yes	0.5	47.67	3	c
5	yes	0.25	53.33	1	a
6	yes	0.125	44.67	4	d
		f = 114.36	P=0.05	CV (%) = 2.38	

* Treatments sharing the same Student-Newman-Keuls (SNK) letter do not differ significantly

Table 3.2.3.B Summary of results for Trial 1b - biocontrol dose response

treatment	<i>Pythium</i>	Eco-T® (g/l)	mean wet weight (g)	rank	SNK grouping
1	no	0	86.05	3	b
2	yes	0	63.58	7	e
3	yes	1	76.32	6	d
4	yes	0.5	87.25	2	b
5	yes	0.25	88.77	1	a
6	yes	0.125	81.2	4	c
7	yes	0.0625	80	5	c
		f = 319.94	P=0.05	CV (%) = 1.04	

* Treatments sharing the same Student-Newman-Keuls (SNK) letter do not differ significantly

Table 3.2.3.C Summary of results for Trial 1a - growth stimulation dose response

treatment	Eco-T® (g/l)	mean wet weight (g)	rank	SNK grouping
1	0	50.67	1	a
2	1	40.33	6	a
3	0.5	41.00	5	a
4	0.25	46.33	4	a
5	0.125	47.33	3	a
6	0.0625	47.67	2	a
		F = 1.86	P=0.05	CV (%) = 11.33

* Treatments sharing the same Student-Newman-Keuls (SNK) letter do not differ significantly

Table 3.2.3.D Summary of results for Trial 1b - growth stimulation dose response

Treatment	Eco-T (g/l)	Mean wet weight (g)	rank	SNK grouping
1	0	86.33	2	a
2	1	45.67	6	c
3	0.5	71.33	5	b
4	0.25	75.00	4	ab
5	0.125	76.33	3	ab
6	0.0625	86.67	1	a
		F = 23.08	P=0.05	CV (%) = 7.36

* Treatments sharing the same Student-Newman-Keuls (SNK) letter do not differ significantly

3.2.4 Discussion

In both trials Eco-T[®] was seen to achieve significant disease control at all doses, when compared with the inoculated controls (Tables 3.2.3.A and B). However, it is evident that at high concentrations, *Trichoderma* does not function at an optimum. In both trials 0.25g/l gave the best results, although in the second trial, in which the formulation was diluted four fold, 0.5g/l functioned noticeably better than in the first. This is advantageous for manufacturers, as it allows dilution of the product while achieving better control, increasing the likelihood for economic competitiveness and success.

No significant growth stimulation was recorded (Figs. 3.2.3.C and D). Instead, *Trichoderma* applied in the absence of the pathogen resulted in a decrease in yield at most doses. These reductions in yield were significant in the repeat trial at 1 and 0.5g/l (Table 3.2.3.D). A possible explanation for this phytotoxicity is provided by Cutler *et al.* (1986) and Cutler and Jacyno (1991) who studied metabolites, with phytotoxic activities, produced by strains of *Trichoderma*. These included 6-pentyl- α -pyrone (Cutler *et al.*, 1986) as well as (-)-harzianopyridone, Koninginin A and Koninginin B (Almassi *et al.*, 1991, cited by MacKenzie *et al.*, 2000).

Another possible explanation could be related to the use of fertilizer containing high levels of ammonium nitrogen (NH₄-N). In a medium like composted pine bark, much of this NH₄-N would normally be converted to nitrate nitrogen (NO₃-N) by nitrification bacteria. *Trichoderma* shows a preference for NH₄-N (Wakelin *et al.*, 1999) and could, in large artificial populations, compete with these bacteria and reduce the nitrification process. This would result in an abnormally high level of NH₄-N in the root zone and could lead to ammonia toxicity. This theory is covered in greater depth in Chapter 5.

A further mechanism could be linked to the secretion of plant growth hormones by *Trichoderma*. This would have obvious benefits if *Trichoderma* were to be considered as a mycorrhizal fungus (i.e., increased growth of the host allows for increased growth of the mycorrhizal organism). In large artificial populations it might be possible that the production of growth hormones by *Trichoderma* would be in such large concentrations that it would result in inhibitory responses.

3.3 Trial 2 - The effect of dosage rates of Eco-T® in cucumber bag culture (open system), on *Pythium* control and plant growth stimulation

3.3.1 Introduction

Tomato, cucumber and pepper production under plastic, in South Africa, is typically in 15ℓ black polyethylene bags containing pine wood shavings. Seedlings are planted in wood shavings and are drip irrigated. Bags stand on raised, plastic covered ridges and excess nutrient solution runs to waste. This is a typical open system of hydroponics, with no recirculation of nutrient solution. Although possibly less favourable to zoosporic fungi than the closed systems, the medium is watered regularly and remains consistently moist, thus enabling these soil pathogens to thrive. Spread of disease is less threatening than with recirculating nutrient solutions, but poor drainage, algal build-up, and the presence of fungus gnats shown to vector *Pythium* spp. (Jarvis *et al.* 1993) result in *Pythium* diseases still being a large problem.

3.3.2 Materials and Methods

A separate glasshouse (10m x 6m) with a wet wall and fans was used. Two-week old cucumber seedlings (cultivar Ashley) were planted into 15ℓ black polyethylene bags containing pine wood shavings obtained from a local sawmill. Cucumber plants were inter-cropped with tomato plants from another trial, due to lack of space. Each bag was connected to the fertigation supply via a 2ℓ/h dripper manifold, with an arrow dripper placed into the wood shavings at the base of each plant. Irrigation was controlled by a Hardie™ Rain Dial 900 irrigation controller. Nutrient stock solution (Ocean Agriculture 3:1:3 (38) Complete) was prepared in a 200ℓ stock tank, with fertilizer application carried out through an in-line Dosatron system. The fertigation water reaching the plants was thus maintained at an EC of 1.8ms and pH at 6.0. Plants were arranged in six rows with in-row spacings of 0.5m and between-row spacings of 1m. Treatments were arranged in a randomised block design with three replicates and 18 treatments (Table 3.3.2.A).

Eco-T® treatments were applied at transplanting at a rate of 250ml per plant of the relevant dilution (Table 3.3.2.A). *Pythium* (PPRI 04169, stored on sterilized sand) was grown up on V8 agar plates. Spores were washed from the plates and diluted to a concentration of 5×10^4 spores/ml. Ten millilitres of this spore solution was applied to the base of relevant plants 24 hours after Eco-T® treatments.

Seedlings were planted into bags on the 7 March 2001. Plants were trained and pruned to single stems every three days. All fruit was removed up to the 6th node, after which they were left to grow. All plants were terminated at the 15th node which, for most plants, was at the height of the overhead wire. As the cultivar used was not a commercial tunnel grown cultivar, male flowers were formed. The number of flowers was recorded as an extra variable. Flowers were harvested every 2nd day for three weeks beginning on 03 April 2001. Cucumbers were harvested once a week for three weeks beginning 24 April 2001. All fruit over 20cm in length were harvested and total weights per plant recorded. At the end of the trial, stems were cut at soil level and total shoot wet weight recorded. Total fruit and shoot weights were averaged for the three replicates and graphed (Figs. 3.3.3.A and B). Analysis of variance (ANOVA) and Student-Newman-Keuls mean tests were conducted using the SAS system for Windows 98, Version 6.1. A summary of statistical results is presented in Table 3.3.3.A.

Table 3.3.2.A Treatments used in cucumber biocontrol and growth stimulation trial.

Treatment	<i>Pythium</i> added	<i>Trichoderma</i> (g/l) Formulation = 5×10^8 spores/g	frequency
Uninoculated control	no	0	-
Inoculated control	yes	0	-
P+T1	yes	1	at planting
P+T2	yes	0.5	at planting
P+T3	yes	0.25	at planting
P+T4	yes	0.125	at planting
P+T1	yes	1	monthly
P+T2	yes	0.5	monthly
P+T3	yes	0.25	monthly
P+T4	yes	0.125	monthly
T1	no	1	at planting
T2	no	0.5	at planting
T3	no	0.25	at planting
T4	no	0.125	at planting
T1	no	1	monthly
T2	no	0.5	monthly
T3	no	0.25	monthly
T4	no	0.125	monthly

3.3.3 Results

All of the results were non-significant, with high CV (%) and low F values (Table 3.3.3.A).

Mean shoot wet weights (Fig. 3.3.3.A) were mostly higher with single applications of *Trichoderma* compared with monthly applications, for both disease control and growth stimulation treatments. No growth stimulation was recorded, with all weights being below that of the uninoculated control.

Mean fruit weight (Fig. 3.3.3.B) shows high levels of disease control at 1g/l Eco-T[®] (single application) and 0.5g/l Eco-T[®] (monthly application). Growth stimulation in this case seems higher in plants receiving monthly applications of Eco-T[®].

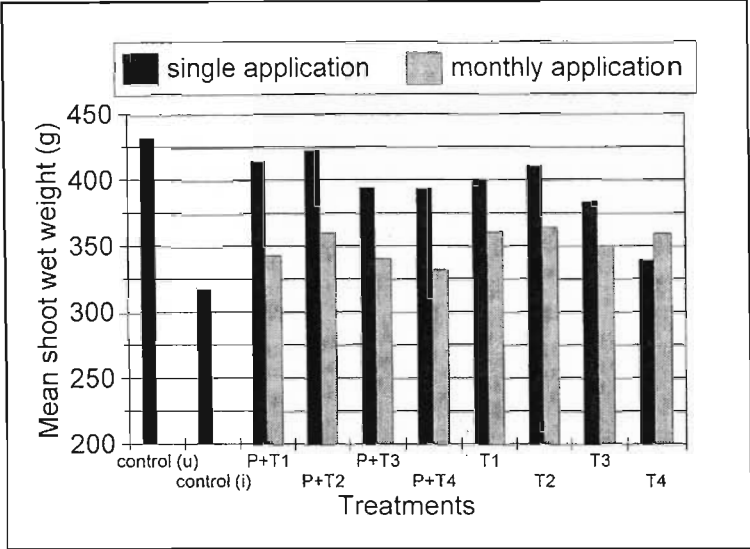


Fig. 3.3.3.A Effect of Eco-T® dose rates and frequency of application on *Pythium* control and growth stimulation as shown by mean total shoot wet weight in cucumber. These results were not statistically significant at P = 0.05

Key to Figs. 3.3.3.A and B

Control (u) = Uninoculated control

Control (i) = Inoculated control, inoculated with *Pythium*

P+T1, P+T2, P+T3 and P+T4 were all inoculated with *Pythium* and dosed with 1, 0.5, 0.25 and 0.125 g/l Eco-T, respectively (i.e., biocontrol).

T1, T2, T3 and T4 were all uninoculated, but treated with 1, 0.5, 0.25 and 0.125 g/l Eco-T®, respectively (i.e., growth stimulation).

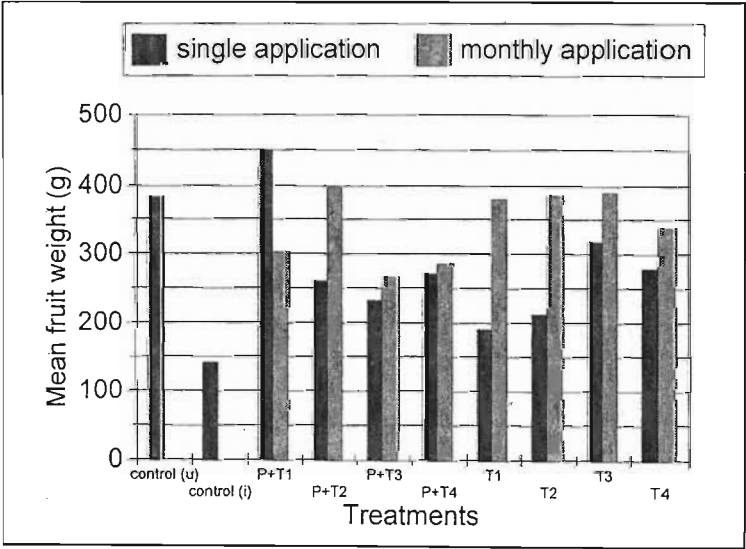


Fig. 3.3.3.B Effect of Eco-T® dose rates and frequency of application on *Pythium* control and growth stimulation as shown by mean total fruit weight in cucumbers. These results were not statistically significant at P = 0.05

Table 3.3.3.A Summary of results for cucumber trial 1.

test	F (treatment)	CV (%)
Growth stimulation (mean total shoot weight (g))	1.62	10.45
Growth stimulation (mean total fruit weight (g))	0.75	49.87
Biocontrol (mean total shoot weight (g))	0.79	52.43
Biocontrol (mean total fruit weight (g))	0.98	19.3

3.3.4 Discussion

Statistical results (Table 5.3.3.A) show that this trial was unsuccessful. In all parameters measured, for both biocontrol activity and growth stimulation, no significant differences were recorded, with high CV (%) values in all cases. There are a number of reasons for this, mostly related to an initial poor understanding of the effects of *Pythium* on cucumber growth. During the course of the trial it was observed that the main effect of *Pythium* was during the two to four week stage. This resulted in a time dependent reduction in yield as growth rates were slowed during this initial stage when *Pythium* was not suitably controlled. It is thus necessary to compare the development of fruit at set nodal positions at set time periods rather than measuring fruit weight of any fruit over 20cm in each week as was done in this trial.

Another source of error was in the choice of cultivar, which was purchased as a commercially used cultivar under the advice of the local nursery. Commercial cultivars used in tunnel growing do not produce male flowers, and are indeterminate in growth. Ashley produced numerous male flowers and comparatively little fruit.

Plant spacing was also seen as a limiting factor. The trial was inter-cropped with a tomato trial, due to lack of space. Although tomatoes are slower growing than cucumbers and should not have interfered with cucumber growth, this did result in the cucumber plants growing closer together than optimally. Combined with this was a significant shading effect generated by the wet wall (which in this tunnel runs along the East wall) and a significant yield gradient occurred. Plants on the West side developed

on average a week faster than those on the East. The close plant spacing also made effective spraying difficult once the plants reached the overhead supports. This led to sporadic infestations of white fly and red spider mite, and a constant powdery mildew problem. All these factors combined to result in non-significant ($P = 0.05$) data.

Despite the statistical data, if one looks at the mean results (Fig. 3.3.3.A and B), many of the general trends are the same as observed in the lettuce trial (Section 3.2). Looking at mean total plant weights (Fig. 3.3.3.A) biocontrol activity can be noted. Optimum dose rates for biocontrol appear between 1 and 0.5g/l (P+T1 and P+T2) with single applications at planting giving better control than monthly applications. The higher optimum dose rates compared with the lettuce are, as expected, due to the differences in the system used. Some of the conidia would have been leached beyond the root zone by subsequent irrigations and would be lost to the system, unlike a recirculating system in which conidia have numerous other chances to establish in the root zone.

The lower plant weights with monthly applications of Eco-T[®] could be due to certain phytotoxic mechanism as previously mentioned in Section 3.2.4. *Pythium* inoculation was only done at transplanting. A single application of Eco-T at the same time would have largely reduced the infection caused by this inoculation. Subsequent applications of Eco-T[®] would have resulted in high levels of *Trichoderma* in a relatively sterile medium, with little buffering capacity. Resulting low levels of nitrification bacteria would have further resulted in higher levels of ammonium in the root zone, leading to ammonia toxicity in the roots. A similar response can be seen in the growth stimulation trials where, in all cases, growth inhibition occurred.

Monthly applications of Eco-T[®] produced better yields of fruit in most cases. It is thought that if ammonium toxicity was responsible for decreased plant weight, then the increased consumption of sugars necessary for ammonium metabolism would have resulted in decreased sugar availability for fruit production. Fruit yield data was, however, random and more accurate data is presented in Section 3.4.

3.4 Trial 3 - The effects of *Trichoderma* (Eco-T®) in cucumber bag culture - some pitfalls overcome

3.4.1 Introduction

It is important in any trial in which plant-microbe interactions are being studied that the effects and epidemiology of the interactions are understood. Section 3.3 revealed some problems which may be encountered when there is an insufficient understanding of the trial system under investigation. Cucumber yields were not found to differ significantly when fruit weights were collected from any fruit over 20cm each week for a period of three weeks. This was probably because stunted fruit that were not harvested in Week One were left to grow for a further week. They were then measured in Week Two when they had increased in size considerably. It was thus concluded that the effect of *Pythium* on fruit yield was a time dependent variable. In this repeat trial, fruit from set nodal positions were thus picked at set time intervals regardless of minimum sizes. This ensured more significant responses in analysed data.

3.4.2 Materials and Methods

Cucumber seedlings (cultivar Cadiz) were grown in Speedling®-24 trays. At two weeks seedlings were transplanted into 7ℓ bags containing wood shavings as medium. Twelve treatments were used (Table 3.4.2.A) with each treatment being replicated four times. Forty-eight plants were thus used. These were placed in four rows of 12 plants each with between row spacing of 1m and within row spacing of 75cm. The trial was laid out using a randomized blocks design. Eco-T® treatments (250ml per bag of relevant dilution) were applied at transplanting. The pathogens, *Pythium myriotylum* (PPRI accession number 04169) and *Rhizoctonia solani* (PPRI accession number 03212), were grown on potato dextrose agar and after seven days growth were inoculated onto relevant plants as a 1cm² agar block, buried 1cm below the medium surface 5cm from the stem base, two days after Eco-T® treatments. Both treatments and pathogen inoculation were repeated at four weeks from transplanting. Pruning of tendrils and

lateral branches was done weekly. The first 2 nodes were pruned of fruit as well. Fruit wet weights were recorded as follows:

Week 8 = Nodes 3 and 4

Week 9 = Nodes 5 and 6

Week 10 = Nodes 7 and 8

The mean fruit wet weight was calculated for each plant. These results were graphed and analyzed statistically using the SAS system for Windows 1998, Version 6.1.

Table 3.4.2.A – Summary of treatments used in repeat cucumber trial

Treatment	<i>Pythium</i>	<i>Rhizoctonia</i>	Eco-T® (g/l)	Eco-T® conidia/l
1	No	No	0	0
2	Yes	No	0	0
3	No	Yes	0	0
4	Yes	No	2	1×10^9
5	Yes	No	1	5×10^8
6	Yes	No	0.5	2.5×10^8
7	Yes	No	0.25	1.25×10^8
8	Yes	No	0.125	6.25×10^7
9	No	Yes	2	1×10^9
10	No	Yes	1	5×10^8
11	No	Yes	0.5	2.5×10^8
12	No	Yes	0.25	1.25×10^8

3.4.3. Results

Both *Pythium* and *Rhizoctonia* inoculated controls showed significant reductions in yield compared with the uninoculated control. The *Rhizoctonia* inoculated control gave much lower yields as in two out of four replicates the seedlings were killed within a week of inoculation and therefore recorded zero yields. *Pythium* inoculation did not result in the death of seedlings but some fruit abortion was noted. *Trichoderma* treatments of 2g/l and 1g/l both achieved significant control of both diseases with yields under such treatments being similar to those of the uninoculated control. Although at 0.5g/l significant disease control was recorded, the levels of control under this dose were significantly lower than under the two higher doses.

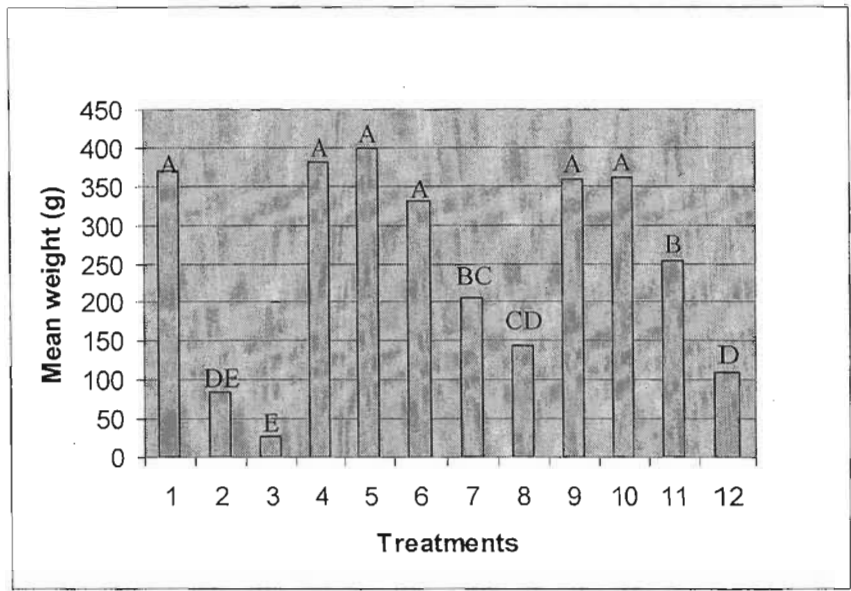


Fig. 3.4.3.A Effect of *Trichoderma* application rates on the biological control of *Pythium* and *Rhizoctonia* in an open hydroponics system of cucumber production, determined on the basis of fruit weight. Treatments with the same letter are not significantly different at $P = 0.05$

Key to Fig. 3.4.3.A

- 1 = uninoculated control
- 2 = inoculated with *Pythium*
- 3 = inoculated with *Rhizoctonia*
- 4-8 = inoculated with *Pythium* and treated with 2, 1, 0.5, 0.25, and 0.125g/l Eco-T
- 9-12 = inoculated with *Rhizoctonia* and treated with 2, 1, 0.5 and 0.25 g/l Eco-T

Table 3.4.3.A. Summary of results for cucumber trial 2.

Treatment	mean fruit weight	rank	SNK grouping
1 = Uninoculated control	369.5	3	a
2 = inoculated (<i>Pythium</i>)	85.25	11	de
3 = inoculated (<i>Rhizoctonia</i>)	26.75	12	e
4 = <i>Pythium</i> + Eco-T [®] 2g/l	382.25	2	a
5 = <i>Pythium</i> + Eco-T [®] 1g/l	399.25	1	a
6 = <i>Pythium</i> + Eco-T [®] 0.5g/l	331.25	6	a
7 = <i>Pythium</i> + Eco-T [®] 0.25g/l	205.75	8	bc
8 = <i>Pythium</i> + Eco-T [®] 0.125g/l	145	9	cd
9 = <i>Rhizoctonia</i> + Eco-T [®] 2g/l	359	5	a
10 = <i>Rhizoctonia</i> + Eco-T [®] 1g/l	361.5	4	a
11 = <i>Rhizoctonia</i> + Eco-T [®] 0.5g/l	253.5	7	b
12 = <i>Rhizoctonia</i> + Eco-T [®] 0.25g/l	110.25	10	d
		F=34.42	P=0.05

* Treatments sharing the same Student-Newman-Keuls (SNK) letter do not differ significantly

3.4.4. Discussion

In this trial, *Pythium* and *Rhizoctonia* inoculation resulted in significant reductions in yield. *Rhizoctonia* controls showed very low yields as some of the seedlings were killed and thus recorded zero growth. *Pythium* was not seen to cause seedling death but stunted seedlings and delayed fruit development.

High application rates of Eco-T[®] (2 and 1 g/l = 1×10^9 and 5×10^8 conidia/l) gave the best levels of control for both diseases with no statistically significant differences in mean fruit wet weights when compared with the uninoculated controls (P=0.05). These application rates are much higher than the optimum rates observed in closed systems. These results were to be expected because in open systems much of the *Trichoderma* inoculum may be leached from the system during subsequent irrigation cycles before the conidia can germinate. A good understanding of the epidemiology of the system

under investigation is necessary before decisions can be made regarding variables to be measured. In the case of this trial the time dependent nature of pathogen induced yield reductions was critical in obtaining significant data.

Koch (1999) recorded similar levels of control of *Rhizoctonia solani* in greenhouse grown pea seedlings treated with the commercial product Soilgard⁶ (containing *Trichoderma virens* at 1×10^6 conidia/g). In these trials Soilgard was mixed directly with the potting mix at a rate of 1g/l. Differences in application rates and efficacy of *Trichoderma*-based products against different pathogens and on different crops are common. Such differences were highlighted in the comparative trials conducted by Koch (1999) in which four commercial *Trichoderma* products were screened against *Pythium ultimum* on cucumbers and *R. solani* on peas. The concentrations of the commercial products (CFU/g) varied by three orders of magnitude contributing to the considerable variations in efficacy. Other contributing factors included the biological properties of the *Trichoderma* strain used as well as the nature of the formulations. These differences can be further compounded by both biotic (e.g. plant species, pathogen virulence) and abiotic (e.g. water potential, substrate temperature) factors leading Koch (1999) to conclude that biocontrol products must be rigorously tested in different crops, pathogens and environments before accurate recommendations can be made to growers.

3.5 Conclusions

Eco-T[®] has potential for use as a biocontrol agent in hydroponics. It is important, however, that formulations and application instructions be correctly adjusted to suit the various available systems. In closed systems, Eco-T[®] is functional when added directly to the recirculating nutrient solution, but at lower doses than in normal drench applications. This is probably because recirculating conidia can be taken into the root zone over an extended period of time without being lost from the system. Growth inhibition, rather than stimulation, was recorded in many cases where Eco-T[®] was applied in the absence of *Pythium* infection. Exact mechanisms involved are unknown,

⁶Thermo Trilogy, 7379 Route 32, Columbia, MD 21044, USA.

and mostly speculative, although a new suggestion is that it could be related to form of nitrogen and nitrogen cycling, as discussed in Chapter 5.

In open systems, application rates are higher than for closed systems and resemble field based application rates. This is probably due to the potential for *Trichoderma* conidia to be leached beyond the reach of the root zone while in closed systems they can be re-circulated and have more opportunities to become established in the root zone.

A good understanding of the crop and the host-pathogen interactions is required in order to establish the correct method of data collection for such trials. Incorrect data collection could lead to insignificant results. This can be costly in terms of research time and money.

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Chapter 4

Trichoderma and Soil Moisture

Abstract

Management of soil moisture levels within artificial growing media can aid in the control of *Pythium* induced reductions in yield. Optimum soil moisture levels were calculated for lettuce production in the Verti-gro® system. The effects of over- and under-watering on plants, *Pythium* disease severity, and biocontrol activity of Eco-T® were determined. In lettuce trials no significant differences were noted between uninoculated controls and all other treatments at optimum soil moisture. Stresses related with over- and under-watering resulted in *Pythium* inoculation causing significant yield losses. In both cases Eco-T® overcame these negative responses, achieving significant biological control. In terms of both biocontrol and growth stimulation, *Trichoderma* functions better in wetter soils/media. Similar trends were observed in strawberry trials although greater differences were recorded under optimal soil moisture conditions in terms of disease severity and growth stimulation. Lowest yields were recorded in over- and under-watered plants inoculated with *Pythium*. Biological control and growth stimulation activity was low in under-watered plants and increased with increasing soil moisture. Highest yields were achieved with optimal irrigation combined with Eco-T® as a growth stimulant. These results show that *Pythium* can be best controlled through the integration of Eco-T® at optimal soil moisture. Where soil moistures are above or below optimum, Eco-T® serves to minimize the negative effects caused by the presence of *Pythium* in the growing system. In this way *Trichoderma* provides a buffering capacity against the direct and indirect negative effects of poor soil moisture management.

4.1 Introduction

Plants require both air and water to survive. If water levels drop too low, the plants wilt and will eventually die. As the pores between soil particles can be occupied by either water or air, an increase in the amount of water in a medium will result in a decrease in the amount of air. If the oxygen supply to the roots is completely cut off, root growth

stops within minutes, nutrient uptake is considerably reduced, and the ability of water to enter the roots is decreased by about three times. An optimum level of soil moisture must therefore exist between the two extremes where optimum amounts of water and oxygen are supplied to the roots. Verdonck *et al.* (1983) claimed that for optimal growth conditions, it is necessary that there is at the same time 20% volume of air and 20-30% volume of easily available water in a growing medium.

The Verti-gro® system was used to conduct the following trials. This was because a local farmer using this system for the commercial production of strawberries was unable to manage soil moisture levels properly, due to incorrect growing medium choice, resulting in increased levels of soft rot infections. The Verti-gro® system consists of a series of stackable polystyrene pots (see Fig.2.3.A). Pots are irrigated from the top and water flows down through to the lower pot and is collected at the bottom for recirculation. The medium used is thus largely responsible in creating a column in which the soil moisture can be consistently managed throughout. Much of the information obtained from these trials can be applied to other hydroponic systems; e.g., bag culture of cucumbers or tomatoes in wood shavings.

In the Verti-gro® system, combinations of coir and coarse potting mix (CPM) were used to get a range of media exhibiting a full spectrum of physical properties. These were considered the most practical and affordable media for use on a large scale in South Africa. In bag culture, various sizes of wood shavings or combinations of wood shavings and sawdust could be used in order to obtain the same result. In a hydroponics system, such as the Verti-gro® one, the optimum levels of water and oxygen can be obtained through manipulation of both the physical properties of the growing medium and the irrigation regime used which includes duration and frequency of irrigation.

The aim of these trials was to establish the optimum level of soil moisture and then to test the effects of *Trichoderma* on plant growth and *Pythium* disease severity under optimum as well as over- and under-watered conditions.

4.2 Trial 1: Determining the optimum range of soil moisture for hydroponically grown butter lettuce

4.2.1 Introduction

Before one can determine the effects of *Trichoderma* under varying soil moistures, it is necessary to establish the optimum soil moisture range for the crop concerned.

4.2.2 Materials and Methods

Butter-lettuce (Mayford, cultivar All Year Round) seeds were planted in Speedling® -24's and allowed to germinate for 48 hrs in the potting shed. The trays were then moved to the seedling greenhouse to grow for two weeks. Verti-gro® stacks were used with only one pot in each stack (pot volume = 4ℓ). Six different media were used. These were selected for their range in air filled porosity and water holding capacities which were determined by the methods described in Handreck and Black (1994) and are listed in Table 4.2.2.A.

Table 4.2.2.A Physical characteristics of media used.

coarse potting mix (composted pine bark) (volume%)	coir (volume%)	water holding capacity (%)	air filled porosity (%)
100	0	33.2	46.5
80	20	37.5	37.7
70	30	46.6	27.3
40	60	55.3	16.9
30	70	56.3	14.5
0	100	68.7	10.9

Six pots were filled with each of these media. Four, two-week old lettuce seedlings were placed in each pot. Six different irrigation regimes were applied (Table 4.2.2.B) and repeated for each of the six media used.

Table 4.2.2.B Irrigation regimes used

irrigation regime	no. of minutes	times per day
a	60	12
b	5	24
c	5	12
d	5	6
e	5	3
6	5	2

Irrigation regimes were based on a 12 hour day commencing at 6:00 and ending at 18:00. The flow rate for all irrigation was approximately 2ℓ/min. The nutrient solution was made up to an electrical conductivity (EC) of 1.8ms using Ocean Agriculture's 3:1:3 (38) Complete at approximately 1g/ℓ. The EC and pH were measured and adjusted weekly. pH was adjusted using either nitric acid or potassium hydroxide. The nutrient solution temperature was set at 26°C.

After four weeks the plants were harvested and the total wet weight per pot was recorded. For each medium used, the resulting wet weights were plotted against irrigation (Figs. 4.2.3A-F). For each medium the irrigation regime resulting in maximum growth was identified. Soil samples were taken from that pot before and after irrigation in order to determine maximum and minimum soil moistures and air filled porosities supporting the best growth in each medium. These results were tabulated in order to determine the optimum range best suited to lettuce production (Table 4.2.3 A).

4.2.3 Results

Results are presented in graphic (Figs. 4.2.3. A - F), and tabular (Table 4.2.3.A) form. With six media and six irrigation regimes, providing 36 treatments, there was no room for replications. This has resulted in some aberrant results, visible in Figs. 4.2.3. A-F. Possible explanations include temperature gradients resulting from wet wall and afternoon sun positions. However, if all graphs are viewed and compared with each other, it is possible to extrapolate where the optimum levels should be and which results are obviously incorrect.

Key to Figs. 4.2.3.A-F

CPM = coarse potting mix

SGM = seedling growers mix

Irrigation regimes are as follows:-

a = continuous

b = 5 min. every half hour

c = 5 min. every hour

d = 5 min. every two hours

e = 5 min. every four hours

f = 5 min. every six hours

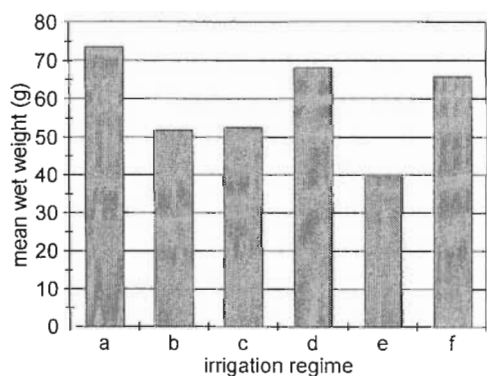


Fig. 4.2.3.A - Effect of irrigation regime on lettuce growth in 100% CPM

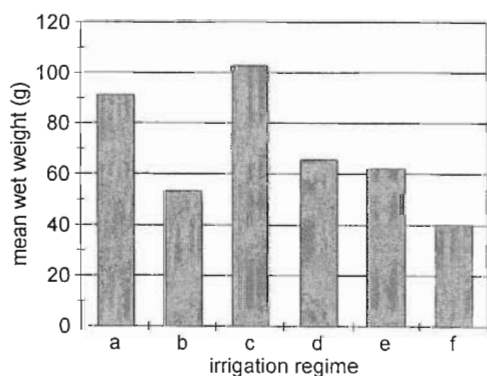
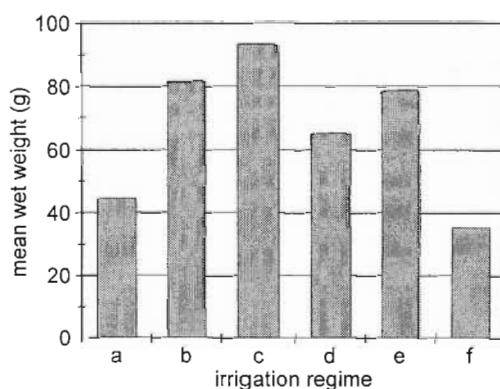


Fig. 4.2.3.C - Effect of irrigation regime on lettuce growth in 70% CPM, 30% coir

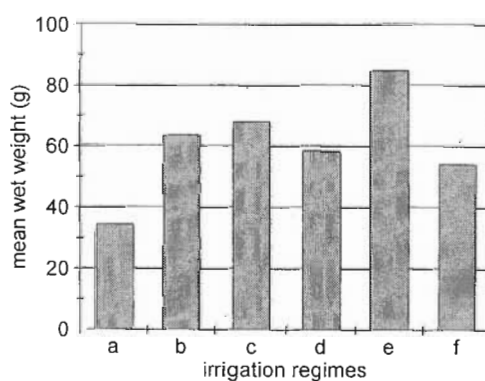


Fig. 4.2.3.D - Effect of irrigation regime on lettuce growth in 100% seedling growers mix (SGM)

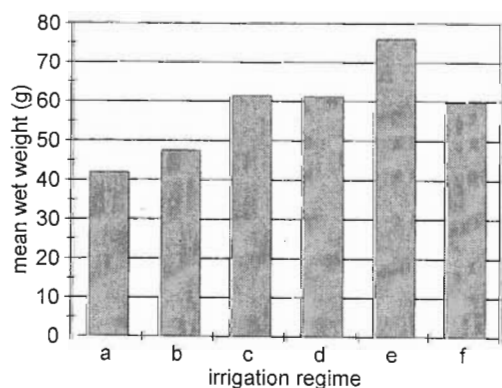


Fig. 4.2.3.E - Effect of irrigation regime on lettuce growth in 40% CPM, 60% coir

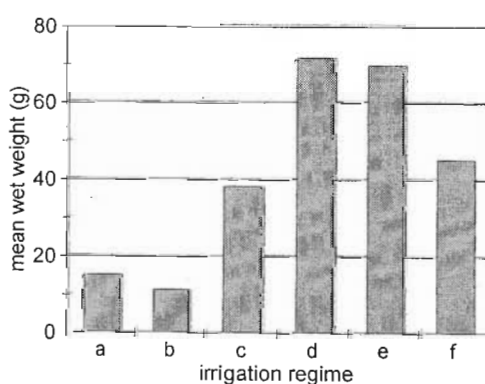


Fig. 4.2.3.F - Effect of irrigation regime on lettuce growth in 100% coir

Table 4.2.3. A Soil moisture and air filled porosity ranges under optimum irrigation regimes in each medium

medium (% CPM by volume)	irrigation regime	soil moisture maximum (%)	soil moisture minimum (%)	air filled porosity maximum (%)	air filled porosity minimum (%)
100	a	46.85	46.85	32	32
80	c	47.3	45.1	30.2	27.7
70	c	51.7	44	29.9	22.2
40	e	50.4	45.1	27.1	21.8
30	e	51.6	45.3	28.9	22.4
0	e	65.8	46.1	26.8	13.8

4.2.4 Conclusions

For the cultivar of butter lettuce used, the optimum soil moisture content was between 44 - 52%, with an optimum air filled porosity range of between 22 - 30%. When irrigation regimes resulted in soil moisture levels outside these limits (above or below) plant growth decreased. This is probably directly related to the physical stresses of insufficient water or oxygen. These limits appear a bit higher than those proposed by Verdonck *et al.* (1983) who claimed that for optimal growth conditions it is necessary that in a substrate there is at the same time 20% volume of air and 20-30% volume of easily available water. However, the soil moisture values of 44 - 52% refer to total water content and not only easily available water.

In order to test the interactive effects of soil moisture and *Trichoderma* on plant health, the optimum range identified in this first trial was used as a base level for future irrigation regimes. Other regimes were then chosen to give greater or lesser soil moisture, to ascertain the effects of these stresses.

4.3 Trial 2: Effects of *Trichoderma* inoculation under varying soil moisture conditions

4.3.1 Introduction

When looking at biological control, the interactive effects of soil moisture and the biocontrol agent on plant yields and pathogen severity is of utmost importance.

For most soil fungi, disease severity increases with increasing soil moisture. This observation is supported by the work of Pieczarka and Abawi (1978) who studied the effects of soil moisture and temperature on root rot of snap beans caused by *Pythium ultimum*; and also by Sippell and Hall (1982) who evaluated the effects of soil moisture on root rot of beans, caused by *Pythium* and *Fusarium* spp.

Wakelin *et al.* (1999) found that *Trichoderma koningii* in sterile soil showed greatest saprophytic growth at 70% soil water holding capacity (WHC), with little saprophytic growth occurring below 20% WHC. However, no literature could be found in which the interactive effects of soil moisture on these two biotic responses (infection and biocontrol) was reported.

4.3.2 Materials and Methods

Verti-gro® stacks consisting of four pots (4ℓ) each were used. All pots were filled with 70% CPM 30% coir mixed on a volume to volume basis (% v/v). A single stack was set up first and irrigated till the medium was saturated. Irrigation was then stopped and soil samples were taken every hour until the soil had dried out to around 44% WHC (identified in Trial 1 as the bottom of the optimum range). The stack was then irrigated for 5 min. and the process repeated. The time taken for the medium to dry out to 44% WHC the second time was used as the timing between irrigations. Irrigation duration was set at five minutes per irrigation. Using this process, an optimum irrigation regime of five minutes (at a flow rate of 2ℓ/min) every 90 minutes between 6am and 6pm was obtained.

Four 2-week old lettuce seedlings were placed in each pot. The plants were allowed to acclimatize for one week with standard irrigation in all stacks (six irrigations of five minutes each). The EC in each stack was checked weekly and adjusted to 1.8ms. The pH was also checked weekly and maintained between 5.0 and 7.0 through the addition of either nitric acid or potassium hydroxide. The water temperature ranged between 24°C and 27°C and was largely dependent on air temperature, despite the use of aquarium heaters. The treatments applied are shown in Table 4.3.2.A.

Table 4.3.2.A Treatments used in trial 2

treatment	irrigation	<i>Pythium</i>	<i>Trichoderma</i>
1	optimum	no	no
2	optimum	yes	no
3	optimum	yes	yes
4	optimum	no	yes
5	double	no	no
6	double	yes	no
7	double	yes	yes
8	double	no	yes
9	half	no	no
10	half	yes	no
11	half	yes	yes
12	half	no	yes

Each treatment was replicated three times in randomised blocks. Eco-T[®] was added at 1g/l and 50ml per plant, applied at planting. *Pythium* was grown up on V8 agar and applied as a 1cm² block to the base of each plant 24 hrs. after Eco-T[®]. After five weeks, plants were harvested and the total wet weights for each stack were graphed and compared (Figs. 4.3.3 A and C). Statistical analysis was done using the SAS system for Windows 98, Version 6.1.

The trial was repeated using strawberry plants. All pots were cleaned and sterilized in Plasdip (6% CuOCl in PVA paint). Pumps, reservoirs, heaters, etc. were rinsed in a solution of Sporekill (a QAC sterilant). Stacks were reassembled using a 40% CPM and 60% coir (% $\frac{v}{v}$) mix. Strawberry cuttings, (cultivar Chandler) rooted one month prior to the trial were planted out into pots with four plants in each pot. Although the stacks consisted of four pots each, only Pots two and four of each stack were used. All stacks received standard fertigation of six cycles of five minutes each for the first two weeks. The EC in the reservoirs was adjusted to 1.8ms every week and water temperature was maintained at 24-27°C. The pH was recorded weekly and adjusted to pH 6.0. After the 2-week acclimatization period, the three irrigation regimes were set. The three different irrigation regimes used were - three times a day (half), six times a day (optimum) and 12 times a day (double). This was based on a 12 hour day starting at 6am. Treatments were the same as those used in the lettuce trial. Harvesting and weighing of ripe fruit started four weeks later and continued twice a week for eight weeks. Fruit weights were totalled and compared. Statistical analysis was done using the SAS System for Windows 98, Version 6.1. Both analysis of variance (ANOVA) and Student-Newman-Keuls tests were conducted.

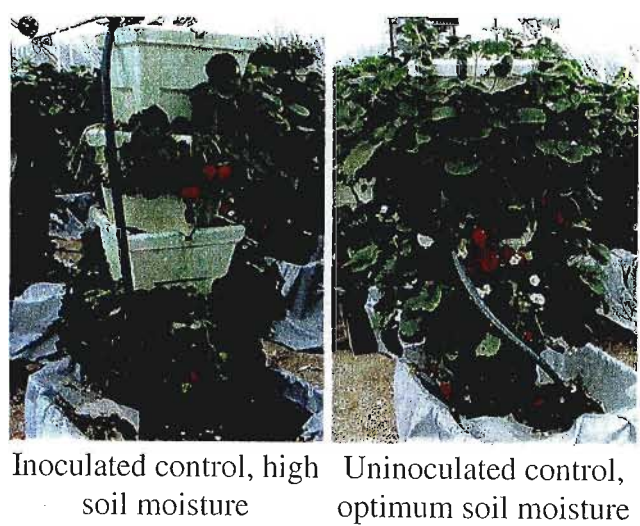


Fig. 4.3.3.E *Pythium* disease severity as affected by soil moisture

4.3.3 Results:

Results are presented graphically in Figs. 4.3.3 A-F and a summary of data and statistics is provided in Tables 4.3.3A and B.

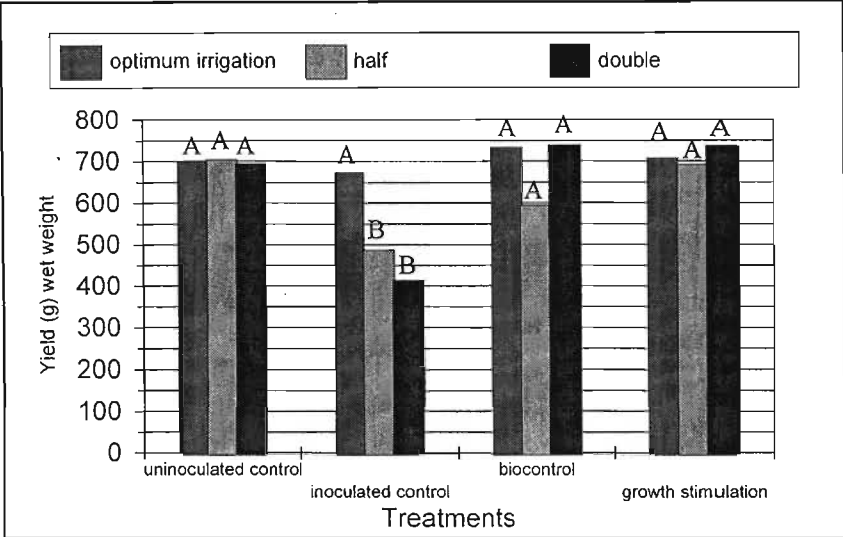


Fig. 4.3.3.A Effect of soil moisture on biological interactions in lettuce. Treatments with the same letter are not significantly different at $P = 0.05$

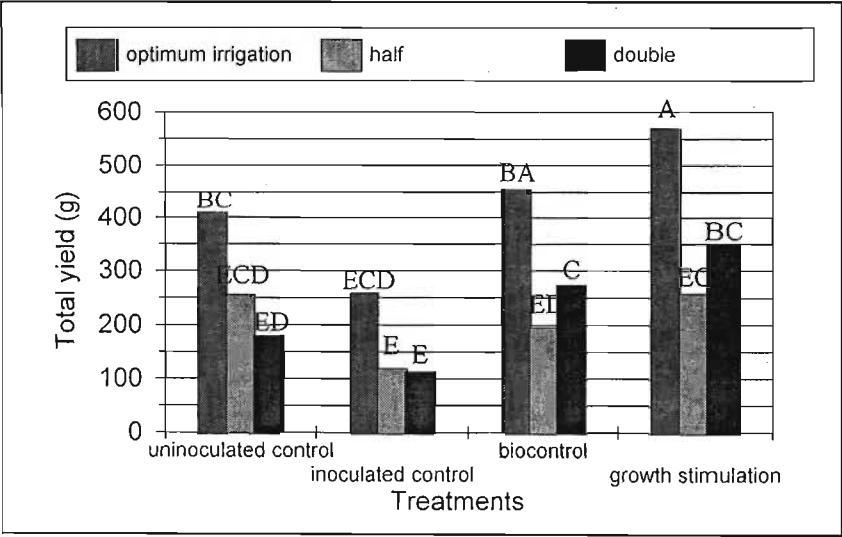


Fig. 4.3.3.B Effect of soil moisture on biological interactions in strawberries. Treatments with the same letter are not significantly different at $P = 0.05$

Key to graphs 4.3.3.C and E

Yield of uninoculated control = x

Yield of inoculated control = y

yield of biocontrol treatments = z

yield of growth stimulation treatments = q

% yield loss = $100 - (y/x \times 100)$

% biocontrol = $(z/x \times 100) - (y/x \times 100)$

% growth stimulation = $(q/x \times 100) - 100$

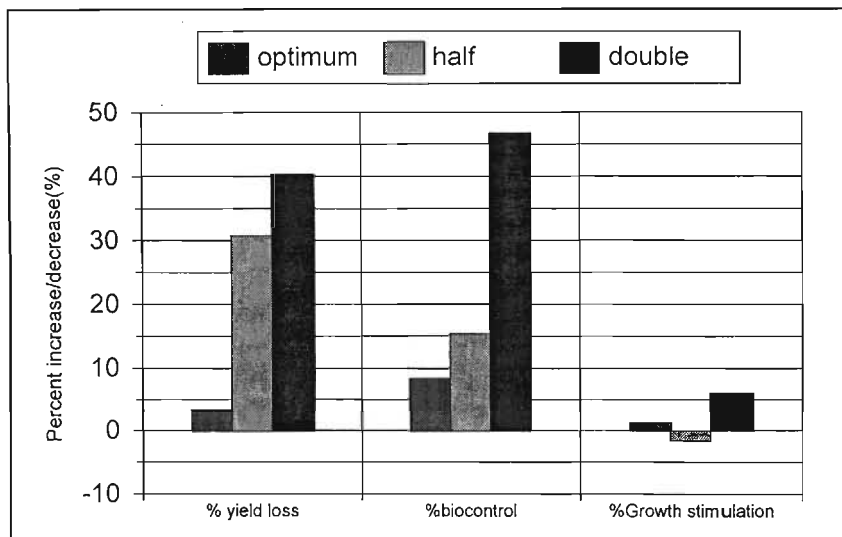


Fig. 4.3.3.C Percent increase or decrease in yield resulting from various treatments and soil moisture levels in lettuce

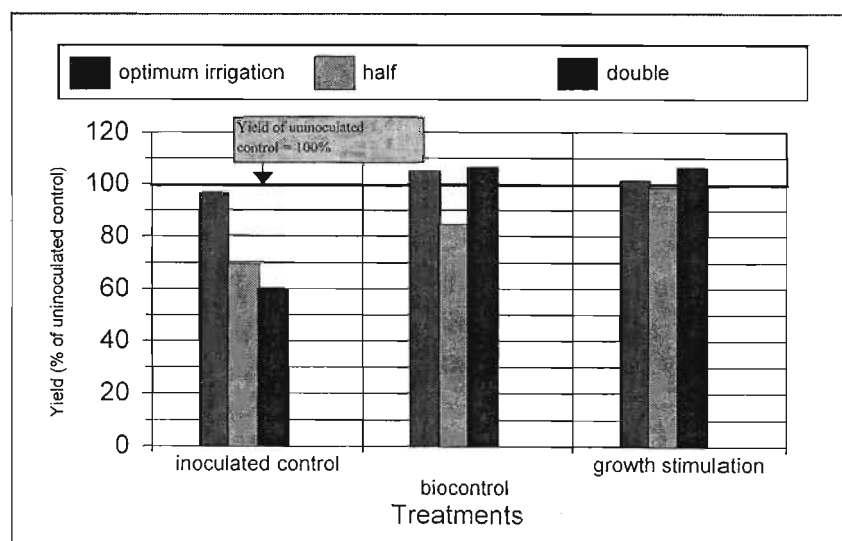


Fig. 4.3.3.D Yield obtained under various treatments of lettuce, expressed as percent yield of uninoculated controls

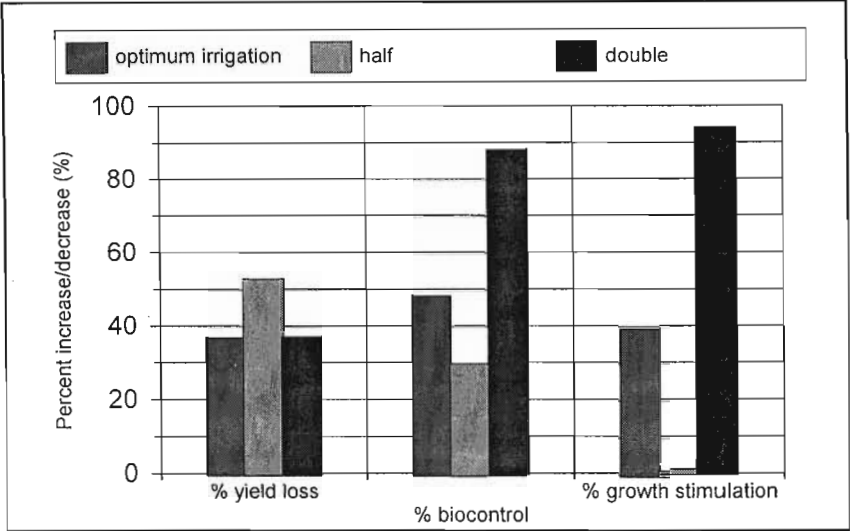


Fig. 4.3.3.E Percent increase or decrease in yield under various treatments and soil moisture levels of strawberries

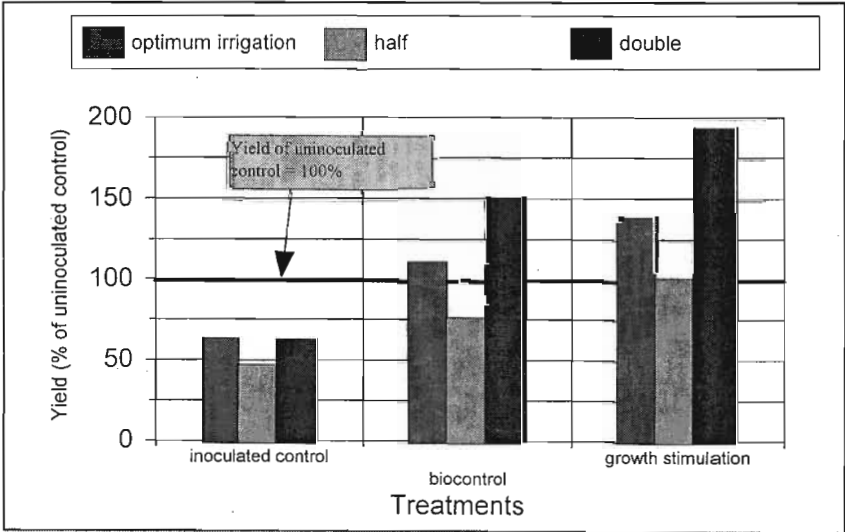


Fig. 4.3.3.F Yield obtained under various treatments of strawberries, expressed as percent yield of uninoculated controls

Table 4.3.3.A Summary of results from lettuce trial

Treatment	Mean total weights per column (g)	rank
1. Optimum irrigation - Uninoculated control	698.33 a	6
2. Optimum irrigation - Inoculated control	674 a	9
3. Optimum irrigation- <i>Pythium</i> + <i>Trichoderma</i>	732 a	3
4. Optimum irrigation - <i>Trichoderma</i> only	707 a	4
5. Double irrigation - Uninoculated control	694.67 a	7
6. Double irrigation - Inoculated control	413.67 b	12
7. Double irrigation - <i>Pythium</i> + <i>Trichoderma</i>	738.33 a	1
8. Double irrigation - <i>Trichoderma</i> only	737 a	2
9. Half irrigation - Uninoculated control	703.33 a	5
10. Half irrigation - Inoculated control	487.33 b	11
11. Half irrigation - <i>Pythium</i> + <i>Trichoderma</i>	594 a	10
12. Half irrigation - <i>Trichoderma</i> only	692.33 a	8
	F=9.13 P=0.05 CV (%)=8.45	

* Treatments with the same letter do not differ significantly for P=0.05.

Table 4.3.3.B Summary of results from strawberry trial

Treatment	Mean total weights per column (g)	rank
1. Optimum irrigation - Uninoculated control	408.47 bc	3
2. Optimum irrigation - Inoculated control	257.72 ecd	7
3. Optimum irrigation- <i>Pythium</i> + <i>Trichoderma</i>	455.47 ba	2
4. Optimum irrigation - <i>Trichoderma</i> only	569.12 a	1
5. Double irrigation - Uninoculated control	180.7 ed	10
6. Double irrigation - Inoculated control	113.61 e	12
7. Double irrigation - <i>Pythium</i> + <i>Trichoderma</i>	273.4 ecd	5
8. Double irrigation - <i>Trichoderma</i> only	351.12 bcd	4
9. Half irrigation - Uninoculated control	254.56 ecd	8
10. Half irrigation - Inoculated control	119.85 e	11
11. Half irrigation - <i>Pythium</i> + <i>Trichoderma</i>	195.44 ed	9
12. Half irrigation - <i>Trichoderma</i> only	257.83 ecd	6
	F =12.1 P=0.05 CV (%)=33.18	

* Treatments with the same letter do not differ significantly for P=0.05.

4.3.4 Discussion

Hendrix and Campbell (1973) were of the opinion that *Pythium* spp. are more demanding of suitable soil conditions (mostly temperature) than most root pathogens. Many species exhibit very narrow ranges of optimal conditions, although as a genus, they are able to colonise almost any soil type. However, higher soil moistures are generally favoured by most species. Pieczarka and Abawi (1978) cited a number of authors in stating that high soil moisture has been reported as necessary for survival, spore germination and saprophytic growth of *Pythium* spp. and that wet soils provide an ecological advantage for *Pythium* spp. which are, otherwise, poor competitors. The general results in these trials are seen to follow these same trends, with the worst yields being recorded in plants inoculated with *Pythium* at high soil moistures. The poor competitive nature of *Pythium* is also highlighted in terms of the ability of *Trichoderma* to achieve significant disease control at almost all moisture levels.

The findings of Wakelin *et al.* (1999) are also supported regarding the fact that *Trichoderma* appears to favour the wetter soil conditions as well. In both trials the greatest levels of biocontrol activity and growth stimulation were achieved at higher soil moistures and the lowest levels in the drier soils (Figs. 4.3.3.C-F).

Lettuce trial - No significant differences were observed in plant growth in the absence of disease under varying soil moisture conditions (Table 4.3.3.A). This is thought to be because the WHC of the medium used was 46.6%. This made it difficult to over-water to any level much beyond the range favoured by butter-lettuce as shown in Trial 1.

The difference in soil moisture levels did, however, result in a significant difference as soon as the pathogen was introduced to the system. Where the plants were under no water stress, they showed only a slight (non-significant) reduction in yield compared with the over- and under-watered plants which show marked (significant) reductions (approximately 40 and 31% respectively). Although *Pythium* is typically associated with moist soil conditions, in these experiments it also caused significant reductions in yield

in under-watered plants. This is probably because these plants were under stress from the lack of water, and thus were more susceptible to infection by the pathogen.

In all cases, *Trichoderma* successfully overcame the negative effects of water stress, with treated plants showing no significant differences in yield compared with the uninoculated controls (Fig. 4.3.3.A).

No significant growth stimulation was recorded. This is probably because all plants received high levels of recirculating nutrients. The mineralisation of nutrients by the fungus would therefore not result in any significant advantage to the host plants as they all received optimal levels of nutrients. The fact that little evidence of growth stimulation was noted in hydroponic systems throughout the course of this research does provide indirect evidence that a major mode of action behind growth stimulation is the mineralisation of otherwise limiting nutrients, a characteristic which would benefit plants in a soil environment.

Strawberry trial - In the strawberry trials, the 60% coir 40% CPM was used in an attempt to allow for a greater degree of over-watering and thus a wider range of soil moisture values. It is apparent that this achieved the desired results, as noted in the differences between the uninoculated controls at the three different soil moistures (Fig 4.3.3.B). The significantly low yields in the over and under-watered plants might indicate the presence of fungi entering the system from other sources. Although not inoculated, it was not possible to keep these stacks completely free of pathogens. Such pathogens would have caused greater disease in the water stressed conditions of the host plants.

The same general trends were noted in the strawberry trial as in the lettuce trial. Statistically, however, the strawberry results showed some problems. There is a much more significant difference between replicates and a high CV% value. This is probably because the strawberry plants require a longer growing season before results can be obtained. This means that there is more time for light and temperature gradients to affect the growth of the plants. Other external factors such as insect pests (red spider mite and aphids) affected the growth and yield of some plants more than others.

Although preventative spraying was done, it did not prevent two outbreaks of these pests during the three month duration of the trial.

A greater degree of disease was noted under optimum levels of soil moisture. This was probably because the crop was harvested over a period of eight weeks. This would have allowed more time for the pathogen to have an effect on plant yield. Considering the growth rate of the two plants, it was less likely that the strawberry plants were able to outgrow the effects of the *Pythium* infections, as was probably the case in the lettuce trial.

In the optimum and over-watered stacks, biocontrol activity was high and plant yields were higher than in the uninoculated controls (Fig.4.3.3.B). It appears that there was not only a disease control response but also a growth stimulation response in the presence of the pathogen. This was not the case in the under-watered stacks where only a 60% increase in yield was achieved between inoculated controls and biocontrol stacks. This serves to demonstrate that *Trichoderma* is more active at the higher levels of soil moisture. This is further highlighted when looking at the growth stimulation results. In the absence of the pathogen, high levels of growth stimulation resulted from *Trichoderma* treatments in both optimum and over-watered stacks. However, in the under-watered stacks, no significant growth stimulation was observed, even in the absence of the pathogen.

This confirmed the findings of Wakelin *et al.* (1998) who showed that the saprophytic growth of *Trichoderma* in soils increased with increasing soil moisture. Their results show an initial increase between 10 to 20% soil WHC, of almost five fold increase in *Trichoderma* growth (500 - 2500mm² of filter membrane colonised by *Trichoderma*). After this initial rapid increase, a slower, yet consistent increase in growth occurred with increasing soil water holding capacity up to 70%. The same pattern of results is evident in these trials in terms of growth stimulation activity (Figs. 4.3.3.C and E). These graphs show the percentage mean increase in growth between the uninoculated controls and the *Trichoderma* treated stacks in both lettuce and strawberries, respectively. As the soil moisture levels increased so the percentage increase in yield through growth stimulation by *Trichoderma* also increased. This response was a lot more significant in the

strawberry trials, although the trends were the same for both. The greater significance in the strawberry trials was probably due to the initially low yields of the uninoculated strawberry controls at the higher soil moisture level, as discussed.

4.4 Conclusions

Plants have an optimum soil moisture at which they grow best. In some crops, such as lettuce, where these optimum levels are maintained, the plant health in general improves. The plant is less susceptible to yield losses caused by *Pythium* infections. This is probably due to the ability of the plant to outgrow the influences of the pathogen and the fact that the pathogen is negatively affected by the lower soil moisture in terms of its competitive ability.

Pythium spp. generally cause more disease at higher soil moistures, although disease levels are still significant in drier soils probably due to the stressed nature of the host plants. Fortunately, in this instance the *Trichoderma* also shows a preference for moist soil conditions. This means that, although soil moisture cannot be used to give *Trichoderma* a competitive advantage over *Pythium* in hydroponic systems, *Trichoderma* can reduce the negative effects of *Pythium* where optimum soil moisture management is difficult to achieve. This information is useful in terms of field applications as well. Where soils are naturally waterlogged (due to high clay content or rainfall) it is possible that *Trichoderma* could reduce these influences and achieve highly significant control of root rot diseases.

4.5 References

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Chapter 5

Trichoderma and Form of Nitrogen

Abstract

Inconclusive trials investigating the interactive effects of root zone temperature and *Trichoderma* application revealed that under conditions of high $\text{NH}_4\text{-N}$ and high temperatures, the addition of *Trichoderma* may increase yield losses. The symptoms shown by these trial plants resembled NH_4 toxicity. These observations prompted research into the interactions between *Trichoderma* and nitrogen cycling. It was concluded that when *Trichoderma* is added at artificially high population densities, it interferes with the normal process of nitrification. This occurs either through the competitive exclusion of nitrifying bacteria in the root zone or through enhanced ammonium uptake facilitated by *Trichoderma* in a mycorrhizal type of association. As a result, the risk of ammonium toxicity and associated growth inhibition is increased. Other side effects of high $\text{NH}_4\text{-N}$ were also recorded in terms of medium acidification and a reduction in K^+ and Ca^+ in leaf tissue. *Trichoderma* applications to soils in the absence of host plants still resulted in changes in the ammonium:nitrate ratios. The levels of $\text{NH}_4\text{-N}$ in the medium were shown to be inversely related to population size of nitrifying bacteria. This indicates that the primary mechanism involves the exclusion of nitrifying bacteria, although the facilitated uptake of ammonium by plants can not be ruled out.

5.1 Introduction

The ratios of $\text{NH}_4\text{-N}$ to $\text{NO}_3\text{-N}$ nitrogen can be an important influence on plant yields. Certain levels of $\text{NH}_4\text{-N}$ can be beneficial to plant growth and the ecosystem as a whole because $\text{NH}_4\text{-N}$ can be directly channelled into protein synthesis, while the assimilation of $\text{NO}_3\text{-N}$ requires a considerable amount of reducing equivalents and energy to bring about the reduction of $\text{NO}_3\text{-N}$ to $\text{NH}_4\text{-N}$ (Haynes, 1986). However, the advantages of $\text{NH}_4\text{-N}$ are seldom observed, primarily because $\text{NH}_4\text{-N}$ is toxic to plants at considerably

lower concentrations than is $\text{NO}_3\text{-N}$ (Haynes, 1986). In most soil environments $\text{NH}_4\text{-N}$ is thus oxidized to $\text{NO}_3\text{-N}$ by the process of nitrification. Considering both the advantages and disadvantages of $\text{NH}_4\text{-N}$ in plant nutrition, Alexander (1965) concluded that 'Nitrification is a mixed blessing and possibly a frequent evil' (cited by Haynes, 1986). Haynes (1986) suggested that mycorrhizae may act as agents of biological control of nitrification under vegetated conditions. If *Trichoderma* is viewed in this light then it goes some way to explaining the switch from growth promotion to growth inhibition with increasing application rates of *Trichoderma*.

5.2 The role of *Trichoderma* in N nutrition: Inconclusive temperature trials reveal one of *Trichoderma*'s secrets

5.2.1 Introduction

The following trials were initially conducted as part of an investigation into the interactive effects of *Trichoderma* and root-zone temperature on plant growth and *Pythium* disease severity. Although much of the data on this topic was inconclusive, this trial is included as it was from this work that the first associations between *Trichoderma* and nitrogen nutrition were revealed.

Economakis and Chartzoulakis (1997) studied the effect of root-zone temperature on growth and water uptake by lettuce plants in solution culture. Butterhead lettuce (cv. Tardisix), grown at solution temperatures of 10, 15 and 20°C, showed significant increases in shoot fresh and dry weights with increasing temperature. Cumulative water uptake and mean number of leaves per plant also increased with increasing solution temperature. Kafkafi (2001) suggested that root temperature controls the root's resistance to water flow. This is a direct response which Kafkafi (2001) explained as being mainly a physical phenomenon, occurring due to:-

- 1) increase in water viscosity and

2) decline in water permeability, due to change in the root membranes viscosity (Kuiper, 1964) or closure of water channels in the root (Johansson *et al.*, 1998; Carvajal *et al.*, 1999), at lower temperatures.

Kafkafi (2001) reported a 250% increase in the rate of water flow through tomato stems when increasing root temperatures from 12°C to 20°C, while maintaining constant light radiation, air humidity and shoot temperature.

Borowski and Michalek (1995) looked specifically at the response of lettuce to form of nitrogen at different solution temperatures. For lettuce cultivar Alka, they concluded that the effect of nitrogen (N) form on dry mass yield depended on plant age and the temperature of the nutrient solution. $\text{NH}_4\text{-N}$ had a beneficial effect on two-week old plants at 7°C and 14°C and on four-week old plants at 7°C. In all other cases, yields were higher in plants receiving $\text{NO}_3\text{-N}$. Kafkafi (2001) pointed out that nitrogen as ammonium ($\text{NH}_4\text{-N}$) can be beneficial when root zone temperatures are low, but detrimental when they are high. This is because ammonium is completely metabolised in the root while nitrates are only partly reduced in the roots, with the larger part of their metabolism taking place in the leaves. With increasing root temperatures, respiration rates increase, consuming sugars. At high temperatures (32°C) no sugar is available for the metabolism of ammonium in the root, resulting in ammonium toxicity.

Abdelzaher *et al.* (1997) found that different *Pythium* spp. had different optimum temperatures for zoospore production. *Pythium aphanidermatum* and *P. oligandrum* both produced zoospores most abundantly at 15°C, while *Pythium* "Group F" preferred 25°C. Optimum temperature for hyphal growth was 30°C for all species tested. Although no literature could be found on *Pythium* species preference for form of N, Abdelzaher *et al.* (1997) did find that the optimum pH for mycelial growth and zoospore production was 7. Considering the root-zone acidification effect of $\text{NH}_4\text{-N}$ (Kafkafi, 2001), *Pythium* would probably function better in the root environment when receiving $\text{NO}_3\text{-N}$. *Trichoderma*, however, shows a preference for $\text{NH}_4\text{-N}$ (Wakelin *et al.*, 1999).

5.2.2 Materials and Methods

Trials were conducted in a 6m x 6m glasshouse equipped with a fan and pad cooling system. A bench was set up with 15 horizontal troughs, each trough measuring 1m long x 180mm wide. Each trough was insulated top and bottom with polystyrene with a slot cut in the top into which a Speedling®-24 tray could be snugly inserted (Fig. 2.5.A). Three variable setting chest freezer/fridges (KIC, Vari Freeze) were placed at the ends of the troughs with three buckets in each. The lids were removed from the freezers and replaced with polystyrene lined wooden lids into which a hole was cut to allow the insertion of delivery and return pipes as well as electric cables to the submersible pump in each bucket. Freezer temperatures were set at 6, 12, and 18°C. Another six buckets were placed independently, one at the end of each of six randomly selected troughs. Three troughs contained heaters set at 24°C and three at 30°C.

Lettuce seeds were planted, in Speedling®-24's, in Perlite. Perlite was used due to its added insulatory value and to reduce surface heating through solar radiation. Seeds were allowed to germinate in the planting room for 48 hrs and then moved to the seedling greenhouse for 10 days. After this time trays were made up to exactly 24 plants each and one tray was placed into each trough. The electrical conductivity (EC) in the buckets was adjusted to 1.8ms using Ocean Agriculture's 3:1:3 (38) Complete fertilizer at 1g/l. Pumps were turned on and plants were given 24 hrs to acclimatize before heaters/fridges were turned on. The *Pythium* isolate (PPRI 04169) was removed from storage under sterile distilled water and grown on PDA for 1 week. Eco-T® was added to the biocontrol treatments at a rate of 0.5g/l (2.5×10^8 conidia/g). A 25mm² agar block of *Pythium* was then added to the first 12 seedlings in the inoculated controls and *Trichoderma* experiments at each temperature set.

The trial was run for a period of 21 days during which time the EC was adjusted to 1.8ms every second day. After 21 days, the plant shoots were harvested and wet weights were recorded (Ohaus® precision plus scale accurate to two decimal places). The experiment was replicated, over time, three times using Ocean Agriculture 3:1:3 (38) Complete (a fertilizer containing 51% nitrogen as ammonium) and three times using Hydrogro® combined with calcium nitrate (giving a predominantly nitrate based fertilizer).

Dry weights were also measured in ammonium treated trials due to a noticeable difference in leaf colour (indicative of differential uptake of nutrients and water). Dry weights were measured after the shoots were dried for 72 hrs in an oven at 60°C. After each replicate was completed, plants were weighed and the old trays discarded. Sporekill® (a QAC sterilant) was added to the buckets at 1ml/l and allowed to circulate through the system for 24 hrs. This water was then drained out and fresh water was used to rinse the systems before the new trial was set up. New 10 day old seedlings were moved into the systems and fresh nutrient solution was made up. The temperatures of the fridges were swapped to randomise the trial. One extra trough was used into which seedlings of the same age were placed. After about 18 days, one of these seedlings was harvested daily and its weight recorded. This was done to allow harvesting at comparative stages of growth in all replicates.

Statistical analysis (analysis of variance and Student-Newman-Keuls test) was done using the SAS System for Windows 98, Version 6.1.

5.2.3 Results

When using nitrate fertilizer, no significant differences in yield were recorded in the uninoculated controls between 12°C and 24°C, although the highest yield was recorded at 12°C. At 6°C and 30°C yields decreased significantly (Fig. 5.2.3.B and Table 5.2.3.A).

At 6°C and 30°C no significant decrease in yield resulted from *Pythium* inoculation. However, *Pythium* inoculation did result in significant decreases in yield at temperatures between 12°C and 24°C (Fig 5.2.3.B and C and Table 5.2.3.A). Similarly, significant biocontrol activity was recorded in the same temperature ranges (Fig. 5.2.3.B and D and Table 5.2.3.A). No significant results were obtained when using high ammonium fertilizer, seen by the high CV value (37.04%) (Table 5.2.3.B).

Results from trials with high ammonium fertilizer levels were not statistically significant (Fig. 5.2.3.E and F and Table 5.2.3.B). However, some interesting trends were observed. These trends are discussed and the possible implications considered.

Key to Figs. 5.2.3.B, E and F

control(u) = uninoculated control

control(i) = inoculated control (inoculated with *Pythium*)

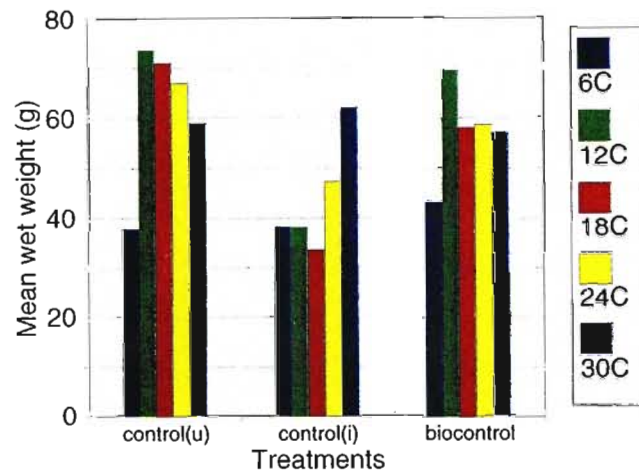


Fig. 5.2.3.B Effect of water temperature on biological interactions in hydroponic lettuce when using fertilizer with a low NH_4 nitrogen content. Treatments with the same letter are not significantly different at $P = 0.05$.

P = *Pythium* added T = *Trichoderma* added

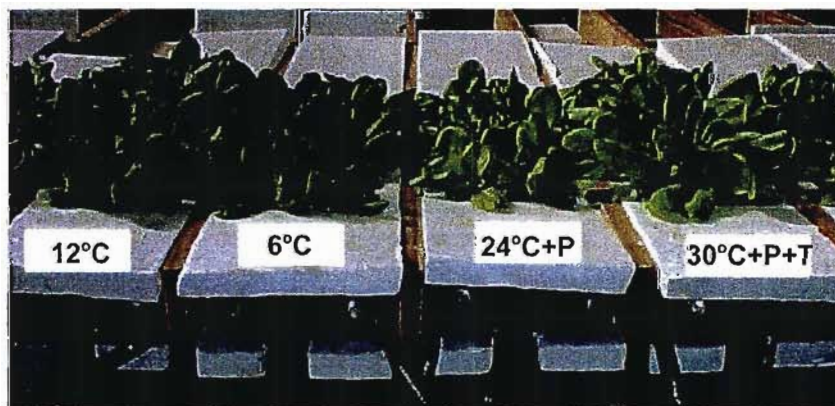


Fig. 5.2.3.A Effect of water temperature and nitrate fertilizer on biological interactions

Key to graphs 5.2.3.C and D
Yield of uninoculated control = x
Yield of inoculated control = y
Yield of biocontrol treatments = z
% yield loss = $100 - (y/x \times 100)$
% biocontrol = $(z/x \times 100) - (y/x \times 100)$

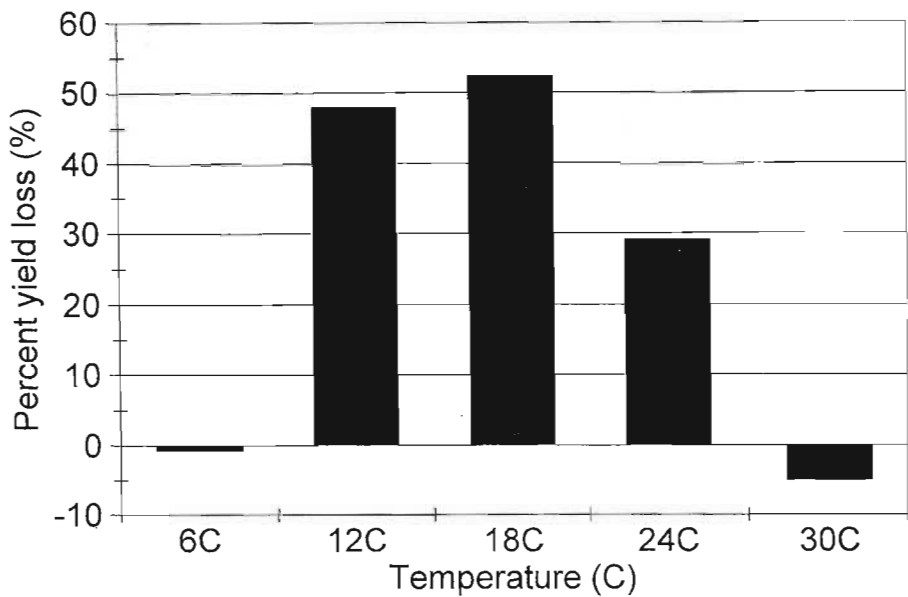


Fig. 5.2.3.C Percent yield losses resulting from *Pythium* infection when fertilizer has a low NH₄ nitrogen content.

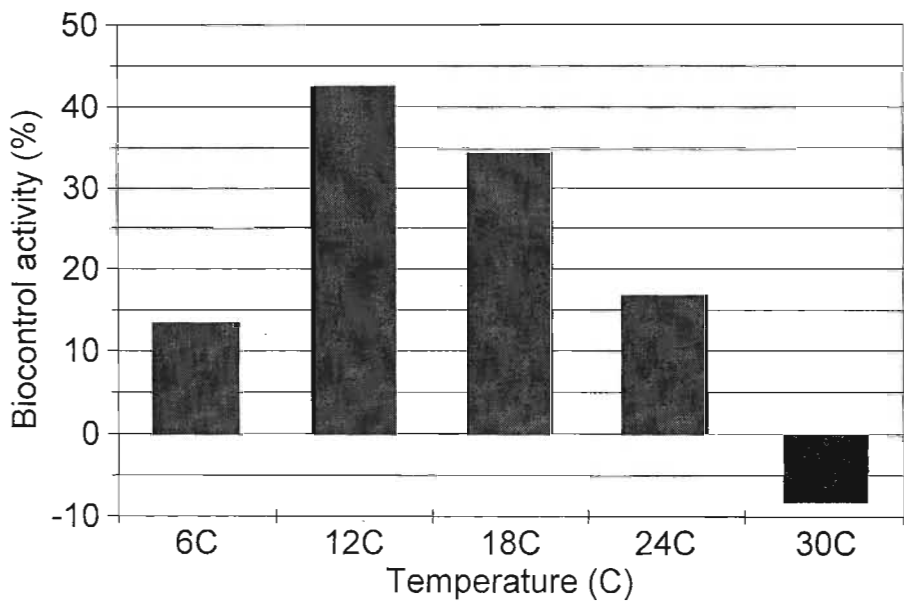


Fig. 5.2.3.D Percent biocontrol activity achieved by *Trichoderma* vs. *Pythium*, when the fertilizer has a low NH₄ nitrogen content

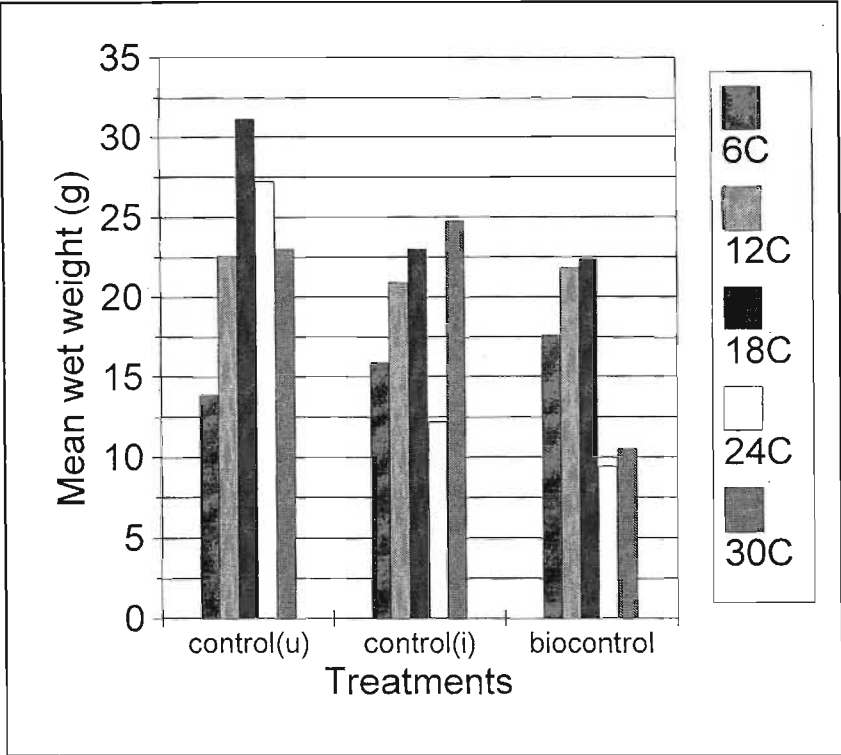


Fig. 5.2.3.E Effect of water temperature on biological interactions in hydroponic lettuce (wet weight) when using fertilizer with a high NH_4 nitrogen content. The above differences were not significant at $P = 0.05$.

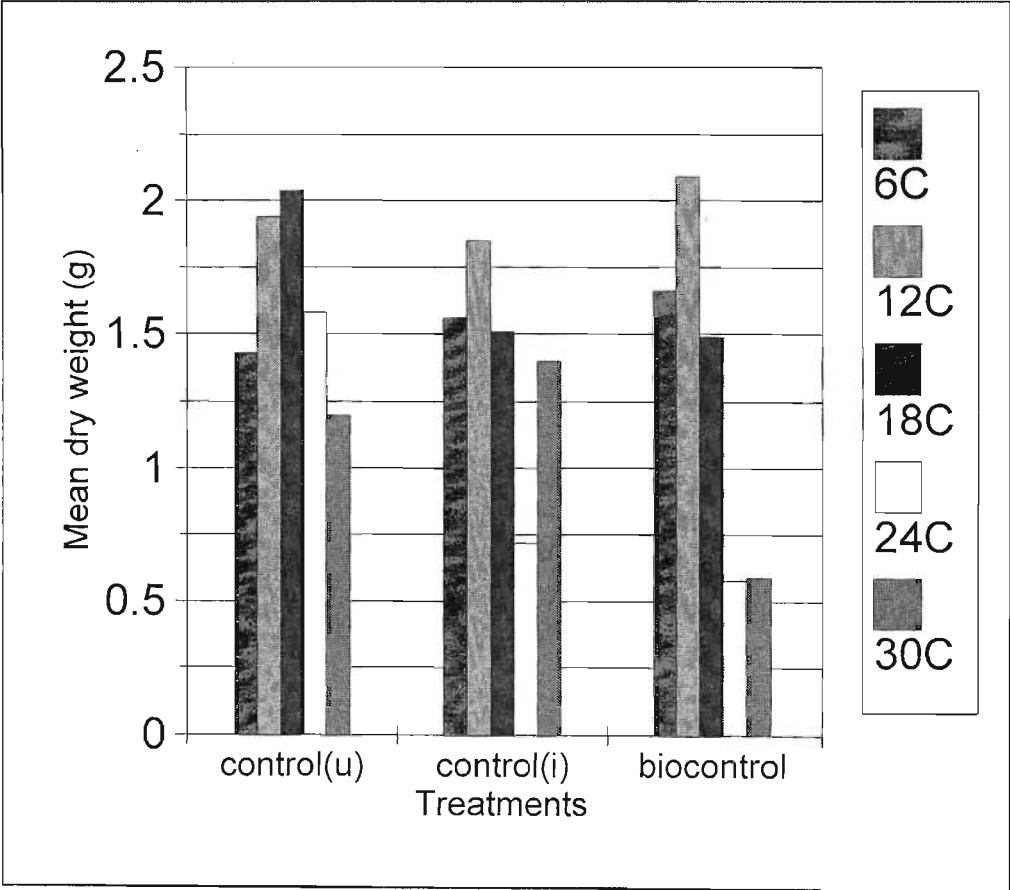


Fig. 5.2.3.F Effect of water temperature on biological interactions in hydroponic lettuce (dry weight) when using fertilizer with a high NH_4 nitrogen content. The above differences were not significant at $P = 0.05$.

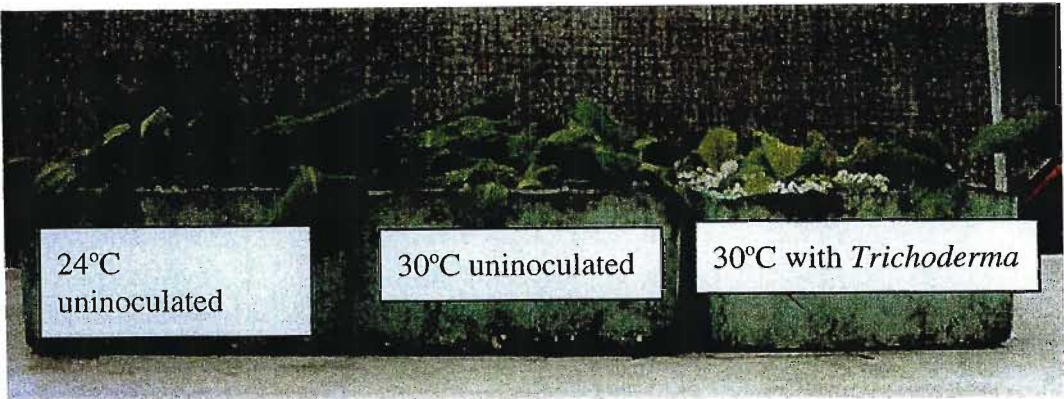


Fig. 5.2.3.G The effects of temperature and *Trichoderma* when using a fertilizer with a high $\text{NH}_4\text{-N}$

Table 5.2.3.A Summary of results for temperature effects, using 49% nitrogen as Nitrate

temperature	<i>Pythium</i> added	<i>Trichoderma</i> added	mean weight (g)	SNK grouping	rank
6	no	no	37.9	de	14
6	yes	no	38.21	de	12
6	yes	yes	43.3	de	11
12	no	no	73.52	A	1
12	yes	no	38.19	de	13
12	yes	yes	69.47	ab	3
18	no	no	71.09	a	2
18	yes	no	33.76	e	15
18	yes	yes	58.25	c	8
24	no	no	66.99	abc	4
24	yes	no	47.4	d	10
24	yes	yes	58.72	c	7
30	no	no	59.01	c	6
30	yes	no	62.05	bc	5
30	yes	yes	57.15	c	9
		F = 33.33	P=0.05	CV (%) = 7.45	

*Treatments with the same letter are not significantly different for P=0.05.

Table 5.2.3.B Summary of results for temperature effects, using 51% nitrogen as ammonium

temperature	<i>Pythium</i> added	<i>Trichoderma</i> added	mean weight (g)	SNK grouping	rank
6	no	no	14	a	10
6	yes	no	16	a	9
6	yes	yes	17.33	a	8
12	no	no	22.67	a	5
12	yes	no	20.67	a	7
12	yes	yes	22	a	6
18	no	no	31.33	a	1
18	yes	no	22.67	a	5
18	yes	yes	22.67	a	5
24	no	no	27.33	a	2
24	yes	no	12	a	11
24	yes	yes	9.33	a	13
30	no	no	23	a	4
30	yes	no	24.67	a	3
30	yes	yes	10.33	a	12
F = 2.28 P=0.05 CV (%) = 37.04					

*Treatments with the same letter are not significantly different for P=0.05

5.2.4 Discussion

When using nitrate fertilizer, no significant differences in yield were recorded in the uninoculated controls between 12°C and 24°C, although the highest yield was recorded at 12°C. At 6°C and 30°C yields decreased significantly.

At 6°C and 30°C no significant decrease in yield resulted from *Pythium* inoculation. However, *Pythium* inoculation did result in significant decreases in yield at temperatures between 12°C and 24°C (Figs. 5.3.3.B and C, and Table 5.3.3.A). Similarly, significant biocontrol activity was recorded in the same temperature ranges (Fig. 5.3.3.B and D, and Table 5.3.3.A). Eco-T® might function at temperatures outside the 12°C - 24°C range, but in the absence of disease, this activity was not detected.

There is thus very little that can be gained practically in terms of the interactive effects of *Trichoderma* and root zone temperature as it is not possible to differentiate between the optimum temperature of *Trichoderma* and *Pythium* in this instance. It is however, important to note that *Trichoderma* overcame the negative effects of *Pythium* at all temperatures at which *Pythium* caused significant yield losses. This has important implications for the marketing of Eco-T® which had previously only been recommended for use in the summer months as it was thought that cold winter temperatures might impact on efficacy.

The most interesting result arose from the temperature trials in which a high level of ammonium fertilizer was applied. Although these trial results were not statistically significant they did serve to raise some interesting questions with regards to the interactions between form of nitrogen, *Trichoderma* application and root zone temperature.

Dry weights (Fig. 5.2.3.F) showed that yield of uninoculated controls were highest at 12°C and 18°C. This is in accordance with Kafkafi (2001) who concluded that the presence of ammonium in the nutrient solution is beneficial at low root zone temperatures, but might be detrimental at high root temperatures. The wet weights (Fig. 5.2.3.E) show higher yields at 24°C and 30°C, compared with those at 12°C. A larger

percentage of this wet weight was water, with plants visibly lacking in sugars (i.e., pale in colour). This increased water uptake was due to reasons explained in the introduction.

Pythium inoculation only resulted in yield decreases at 18°C and 24°C. At 30°C *Pythium* infection resulted in a yield increase. This is possibly because the C leakage induced by *Pythium* infection resulted in an increase in microbial populations and N cycling, converting more ammonium to nitrate, as proposed by Naseby *et al.* (2000), and thus reducing ammonium toxicity induced losses in yield.

The high decrease in yield which occurred when Eco-T[®] was added at 24°C and 30°C can be explained in the same manner. The presence of Eco-T[®] might reduce the damage caused by *Pythium* and therefore the amount of C leakage. It is also possible that *Trichoderma* prevents the establishment of a functioning population of nitrification bacteria by direct competition. Haynes (1986) cited Verstraete (1981) as having indicated that mycorrhizae may act as agents of biological control of nitrification under vegetated conditions (i.e. in soils in which plants are present). *Trichoderma* has been shown to have mycorrhizal-like properties (Kleifeld and Chet, 1992) and it is thus possible that it may act as a biocontrol agent of nitrification. *Trichoderma* has a preference for ammonium nitrogen supply, which increases its competitive ability. It would thus seem likely that, in a soil environment, it would compete directly with the nitrification bacteria, maintaining a high level of ammonium in the soil. Perlite, unlike pine bark used in other trials, does not contain any natural microflora. *Trichoderma* was therefore in a position to become established, largely to the exclusion of these bacteria. This would have resulted in reduced nitrification and even higher levels of ammonium in the root zone, resulting in increased root death from ammonium toxicity.

5.3 *Trichoderma* and nitrogen nutrition - effects on growth promotion/inhibition

5.3.1 Introduction

On the basis of the previous trial it was decided to further investigate the effects which *Trichoderma* has on form of nitrogen in hydroponic systems. It has already been ascertained that high levels of $\text{NH}_4\text{-N}$ can have negative effects on plant growth. The fact that the addition of *Trichoderma* to plants receiving high levels of $\text{NH}_4\text{-N}$, at high soil temperatures, appeared to compound these negative effects, suggested that *Trichoderma* in some way impacts on the level of $\text{NH}_4\text{-N}$ available to the plant.

An understanding of the mechanisms of ammonium toxicity was seen as necessary in attempting to understand, and measure, the effects which *Trichoderma* might be having. These mechanisms were summarised by Haynes (1986) and include: a) acidification of the plant rhizosphere, b) induced cation deficiencies, c) enhanced plant water stress, and d) increased carbohydrate metabolism associated with the detoxification of ammonium in the roots (Section 1.7).

On the basis of these mechanisms it was decided that rhizosphere pH, cation content and photosynthetic activity were all variables which needed to be measured, along with soil and root $\text{NH}_4\text{-N}$ and $\text{NO}_3\text{-N}$ levels, in order to establish the effects of *Trichoderma* on N nutrition.

5.3.2 Materials and Methods

Fifteen horizontal mini troughs were used. Three-week old lettuce seedlings (cultivar all year round) were transplanted into 10 pots containing perlite as a medium. Six plants were placed in each mini trough and the irrigation in the troughs was started and run for 24 hrs. before the addition of fertilizer. Fertilizer (Ocean Agriculture 3:1:3 (38) complete) was added at 1g/l, the resulting pH was 5.8 and was not amended in any way. All plants were left to acclimatize for a further 48hrs before the treatments were added. Five

treatments were used and are summarised in Table 5.3.2.A. All treatments were replicated three times. The treatments were obtained by diluting Eco-T[®] (2x10⁹ conidia/g) in tap water. Final concentrations were varified under the light microscope using a counting chamber.

Table 5.3.2.A Treatments used to determine the effects of *Trichoderma* on nitrogen nutrition

Treatment	<i>Trichoderma</i> Eco-T [®] (conidia/ml)
1	0
2	2.5 x 10 ⁵
3	5 x 10 ⁵
4	1 x 10 ⁶
5	2 x 10 ⁶

Initial NH₄-N and NO₃-N levels were recorded. The plants were left to grow for 14 days before destructive measurements were taken. After 7 days levels of NH₄-N and NO₃-N in the recirculating nutrient solution were measured. A plant was removed from each trough and root NH₄-N and shoot NO₃-N were recorded. The pH of the perlite medium from these pots was also determined and compared. This was done by diluting the perlite at a 1:1 ratio (by volume) with distilled water and measuring the pH with a pH meter. During the 14 days water was added when needed with all additions of water being recorded. On day eight an additional 5g of fertilizer was added to each trough. During the overall trial period a total of 16ℓ of water was added to each trough together with 15g of fertilizer (10g at the start of the trial and a further 5g at day 8).

At the end of 14 days NH₄-N and NO₃-N levels were again measured in both the recirculating nutrient solution and the growing medium. Water and medium pH were also measured. Plants were harvested and a series of destructive measurements were taken as follows.

Rhizosphere acidification was observed by embedding plant roots in PDA (39g/ℓ water) containing Bromocresol Purple (0.03g/ℓ agar mix) and observing for colour changes at

the root-agar interface. Roots were detached from the plants, dusted free of growing medium and immediately embedded in the agar just prior to the agar setting. Shoot-root ratios were determined on a dry weight basis using two plants from each replication. A third plant was used to determine $\text{NH}_4\text{-N}$ levels in the roots and $\text{NO}_3\text{-N}$ in the shoots. This was done by macerating the tissue with a pestle and mortar and diluting the resulting liquid in distilled water. $\text{NH}_4\text{-N}$ and $\text{NO}_3\text{-N}$ levels were then measured using relevant meters. In all cases ammonium and nitrate were measured using meters from Hanna⁷ instruments (Nitrate meter = HI 93728 Nitrate ISM and ammonium meter = HI 93715 Ammonium High Range ISM).

The final two plants from each replication were used for leaf tissue analysis. This was performed by the Plant Laboratory at the KwaZulu-Natal Department of Agriculture and Environmental Affairs.

5.3.3 Results

Table 5.3.3.A Effects of *Trichoderma* application rates on plant growth parameters.

<i>Trichoderma</i> (spores/ml)	Mean shoot wet weight (g)	Mean shoot dry weight (g)	Mean root dry weight (g)	Shoot:Root ratio (dry weight)
0	14.1 (b)	1.11 (b)	0.22 (b)	5.14 (a)
2.5×10^5	17.02 (a)	1.37 (a)	0.34 (a)	4.05 (a)
5×10^5	12.84 (bc)	1.08 (b)	0.23 (b)	4.67 (a)
1×10^6	10.01 (d)	0.88 (c)	0.20 (b)	4.34 (a)
2×10^6	11.65 (cd)	0.95 (bc)	0.22 (b)	4.50 (a)
	F = 20.1 P = 0.05	F = 18.2 P = 0.05	F = 8.15 P = 0.05	F = 3.17 P = 0.05

* Treatments sharing the same letter (brackets) do not differ significantly at the 95% confidence level for the variable in question determined using the Student-Newman-Keuls test..

⁷Hanna Instruments Inc. Woonsocket, Rhode Island, 02895, USA.

Table 5.3.3.B Effects of *Trichoderma* application rates on nitrogen nutrition at seven days from treatment

<i>Trichoderma</i> (spores/ml)	NO ₃ -N in solution (mg/l)	NH ₄ -N in solution (mg/l)	% NH ₄ -N in solution	NO ₃ -N in shoots (mg/l)	NH ₄ -N in roots (mg/l)
0	104.33 (a)	68 (a)	39.4 (c)	1600	106
2.5x10 ⁵	97.3 (a)	67.7 (a)	41.0 (c)	1400	110
5x10 ⁵	76.3 (b)	67.7 (a)	47.0 (b)	1600	137
1x10 ⁶	70 (bc)	68 (a)	49.3 (ba)	1700	145
2x10 ⁶	63.7 (c)	67.3 (a)	51.4 (a)	1700	138
	F = 47.99***	F = 0.03 (NS)	F = 27.19**		

* Treatments sharing the same letter (brackets) do not differ significantly at the 95% confidence level for the variable in question determined using the Student-Newman-Keuls test.

By Day 7, NO₃-N levels in the nutrient solution had dropped from an initial level of 120mg/l to a mean of 63.7mg/l in those troughs receiving Eco-T[®] at four times the recommended dose. At the same time NO₃-N levels in the uninoculated controls had only dropped to a mean of 104.3mg/l. Ammonium levels were fairly constant irrespective of the treatments used although, as a result, the percentage NH₄-N of total nitrogen can be seen to rise with increasing levels of *Trichoderma* (Table 5.3.3.B and Fig. 5.3.3.A). These results were statistically significant at the 95% confidence level.

This increase in NH₄-N concentration in terms of the amount of NH₄-N relative to the total N is inversely related to plant growth in terms of shoot wet and dry weights recorded after 14 days (Fig. 5.3.3.A). The level of NH₄-N in the plant roots was also seen to increase with increasing levels of *Trichoderma*, while leaf NO₃-N levels did not differ significantly (Table 5.3.3.B).

By Day 14, differences in terms of NO₃-N levels in the recirculating solution were no longer significant and as a result no significant differences in terms of the %NH₄-N in the solutions were measured. NH₄-N concentrations in the roots had also stabilized around a mean of 112mg/l. No trends in terms of the effects of *Trichoderma* application

Key to Figs. 5.3.3.A,B and C

Treatment 0 = No *Trichoderma* added

Treatments 0.5 - 4 = 0.5, 1, 2 or 4 times the recommended dose of *Trichoderma* (i.e., 2.5×10^5 , 5×10^5 , 1×10^6 , and 2×10^6 spores/ml respectively)

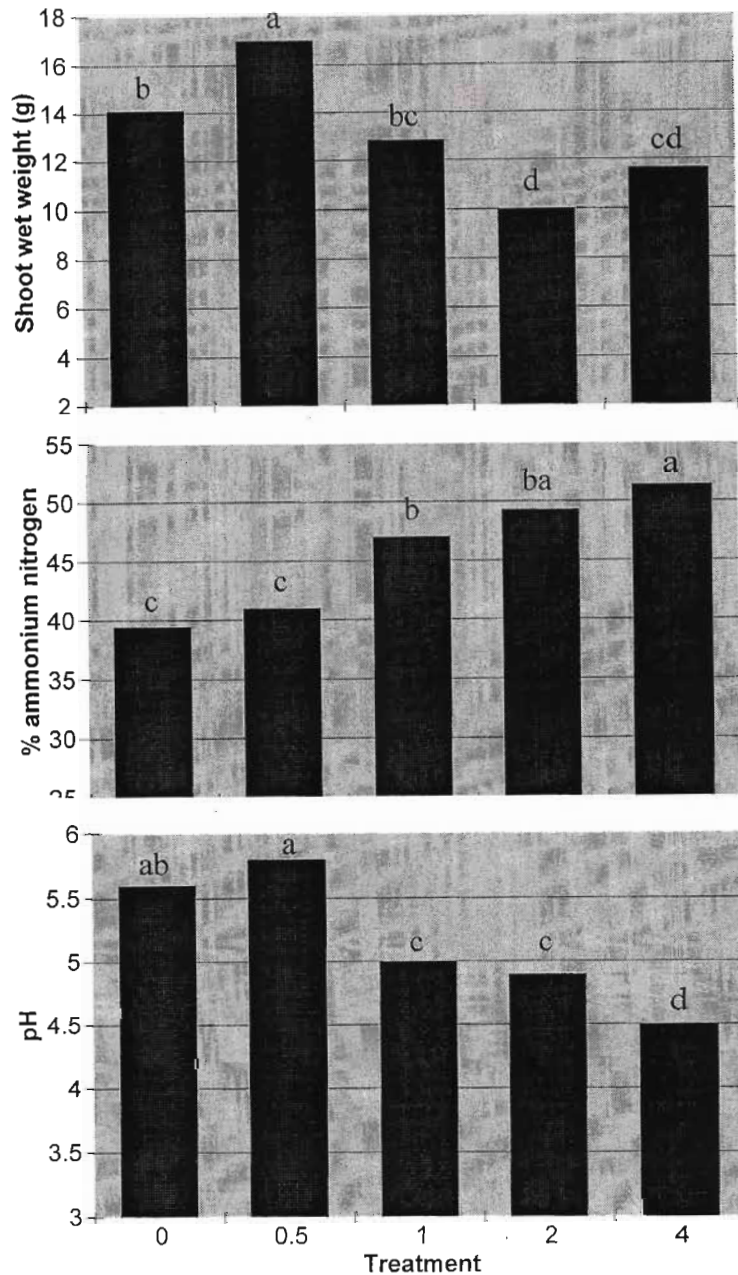


Fig. 5.3.3.A. Effect of *Trichoderma* treatments on medium pH and % $\text{NH}_4\text{-N}$ in recirculating hydroponic solution (seven days after treatment) relative to plant growth (recorded as shoot wet weight 14 days from treatment). For each variable, treatments with the same letter are not significantly different at $P = 0.05$.

on N nutrition could be observed although the effects of the earlier differences were still expressed in terms of the yield differences.

Medium pH on Day 7 decreased from 5.6 in the untreated plants to 4.5 in those treated with 2×10^6 spores/ml (Table 5.3.3.C and Fig.5.3.3.A). By Day 14 the medium pH levels had stabilized to some degree although acidification of the perlite medium was still evident in those pots treated with high levels of *Trichoderma*. Rhizosphere acidification was not demonstrated by the Bromocresol Purple agar.

Table 5.3.3.C Effect of *Trichoderma* application rates on medium pH over time

<i>Trichoderma</i> (spores/ml)	pH of perlite at day 7	pH of perlite at day 14
0	5.6	5.8
2.5×10^5	5.8	5.8
5×10^5	5	5.6
1×10^6	4.9	5.8
2×10^6	4.5	5.2

Table 5.3.3.D Effect of *Trichoderma* application rates on ion concentration in shoots

<i>Trichoderma</i> (spores/ml)	Ca %	K %	Na mg/kg	Fe mg/kg	P %	Cu mg/kg	Mn mg/kg
0	0.3	4.08	1329.4	99	0.67	23.8	42
2.5×10^5	0.28	4.12	1443.2	119	0.7	27.5	49
5×10^5	0.23	4.19	1515.5	124	0.75	32.6	63
1×10^6	0.23	3.75	1858.2	135	0.76	44.4	61
2×10^6	0.21	3.8	1844.5	138	0.74	45.1	57

The addition of *Trichoderma* resulted in a decrease in Ca and K levels while Na, Fe, P Cu and Mn were all seen to increase (Fig. 5.3.3.B and Table 5.3.3.D). The most noticeable responses were in terms of Na, Fe and Cu.

Key to Figs. 5.3.3.A,B and C
 Treatment 0 = No *Trichoderma* added
 Treatments 0.5 - 4 = 0.5, 1, 2 or 4 times the recommended dose of *Trichoderma* (i.e., 2.5×10^5 , 5×10^5 , 1×10^6 , and 2×10^6 spores/ml respectively)

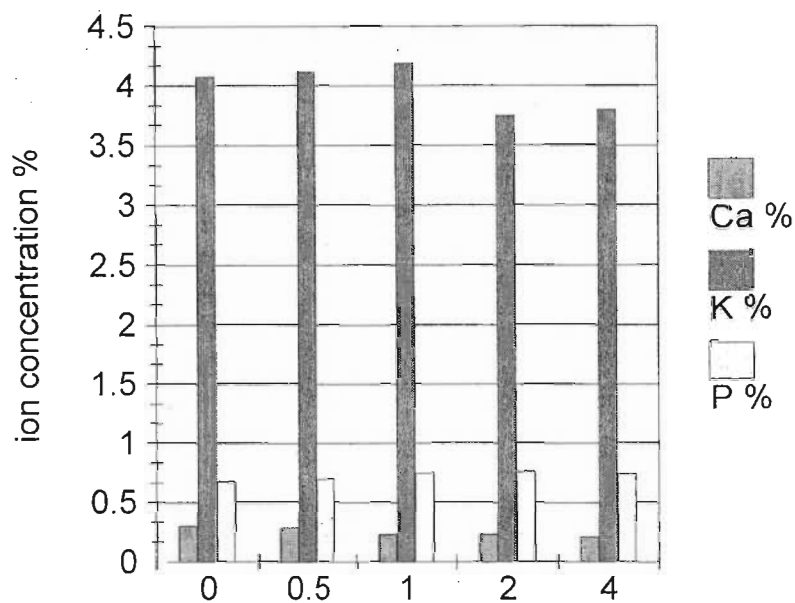


Fig. 5.3.3.B. Effect of *Trichoderma* application rates on Ca, K, and P concentration within lettuce leaf tissue (%)

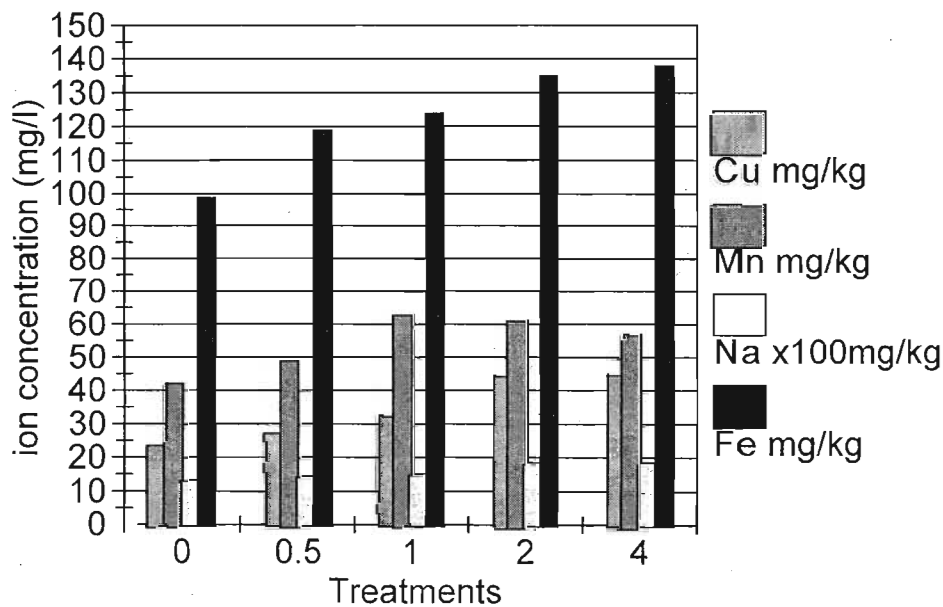


Fig. 5.3.3.C. Effect of *Trichoderma* application rates on Cu, Mn, Na, and Fe concentration in lettuce leaf tissue (mg/kg)

5.3.4 Discussion

Trichoderma clearly has some influence on the levels of $\text{NO}_3\text{-N}$ in the recirculating nutrient solution seven days after *Trichoderma* treatments are applied. Nitrate levels typically decrease with increasing levels of *Trichoderma*. Ammonium levels were not seen to change. This could either be due to differential usage (i.e., in untreated plants more $\text{NH}_4\text{-N}$ is converted to $\text{NO}_3\text{-N}$ by nitrification while in treated plants the $\text{NH}_4\text{-N}$ is used by *Trichoderma* and the plants) or because $\text{NH}_4\text{-N}$ is strongly bound to the medium particles and therefore not available for accurate measurement. Despite these reservations, the % $\text{NH}_4\text{-N}$ relative to the total N in solution is affected by the change in the $\text{NO}_3\text{-N}$ levels. The increase in the % $\text{NH}_4\text{-N}$ also appears to be inversely related to yield as shown in Fig. 5.3.3.A. This response is to be expected in terms of the negative effects of ammonium toxicity as discussed in Section 1.7 and conforms with the results of Borowski and Michalek (1995).

Medium pH is similarly seen to decrease with increasing $\text{NH}_4\text{-N}$ concentrations. This was measured in the perlite medium seven days after treatment. The cause of this acidification is explained in terms of the excess uptake of cations and H^+ production during NH_4^+ assimilation by root tissue which leads to an enhanced net extrusion of H^+ and a decrease in rhizosphere pH (Neumann and Romheld, 2002). Root zone acidification was not clearly demonstrated with the use of Bromocresol Purple agar and this technique will need to be refined or substituted in further experiments.

The decrease in leaf K^+ and Ca^+ provides further proof of ammonium toxicity related stresses. Haynes (1986) explained that the increased uptake of NH_4^+ results in the reduced uptake of cations such as K^+ and Ca^+ due to ionic competition either with NH_4^+ ions *per se* or with H^+ ions excreted during active NH_4^+ uptake. The increase in Cu^+ , Mn^+ , Fe^+ , Na^+ and P^+ can also be explained in terms of increased NH_4^+ concentration. Jeong and Lee (1996) recorded similar increases of Cu^+ , Mn^+ , Fe^+ and P^+ in shoot tissue of ageratum and salvia plants, with increasing $\text{NH}_4\text{-N}$ levels. A possible explanation for this is linked to the acidification effect as all the above elements are more soluble at lower pH levels.

The effect of *Trichoderma* on $\text{NH}_4\text{-N}$ can go some way to explaining a number of other effects of *Trichoderma* treatment. Growth stimulation could in part be a response to optimum $\text{NH}_4\text{-N}$ uptake and use. Haynes (1986) pointed out that the assimilation of $\text{NO}_3\text{-N}$ by plants requires a considerable amount of reducing equivalents and energy to bring about the reduction of $\text{NO}_3\text{-N}$ to $\text{NH}_4\text{-N}$. Ammonium on the other hand can be directly channelled into protein synthesis.

Increased $\text{NH}_4\text{-N}$ concentration could also be proposed as another mechanism by which *Trichoderma* could influence disease levels. Haynes (1986) cited Henis (1976) who reported suppression by $\text{NH}_4\text{-N}$ and resulting low pH for *Phymatotrichum omnivorum* in cotton, *Thielaviopsis basicola* in tobacco, and *Ophiobolus dahliae* and *Verticillium albo-atrum* in tomato, eggplant and potato.

It is interesting to note that *Trichoderma* caused no significant effects on N nutrition and medium pH 14 days after treatment with *Trichoderma*. This suggests that the system is largely self stabilizing and that an equilibrium is re-established within 14 days. The effects of the early imbalance are, however, longer lasting as observed in the plant yield and leaf tissue analysis results at harvest (14 days). The damage done within the first seven days puts the plant behind in terms of development. It is not known whether these plants could make up this early loss if the time to harvest was prolonged.

5.4 *Trichoderma* and N cycling

5.4.1 Introduction

From the previous experiment it is apparent that *Trichoderma* in some way influences the level of $\text{NH}_4\text{-N}$ available to the plant. This could either be through improved ammonium uptake facilitated by *Trichoderma* mycorrhizal type association with the plant, or through its indirect influences over the nitrogen cycle. One suggestion is that *Trichoderma* in high populations competes with nitrifying bacteria. This prevents the conversion of $\text{NH}_4\text{-N}$ to $\text{NO}_3\text{-N}$ and thus artificially high levels of $\text{NH}_4\text{-N}$ accumulate in the rhizosphere. In order to test this hypothesis the following trials were conducted in the absence of plant roots in order to determine the direct effects of *Trichoderma* applications on soil microbial populations and nitrogen cycling.

5.4.2 Materials and Methods

One litre plant pots were filled with growing medium (nine with pinebark and nine with perlite) and placed in two-litre ice-cream tubs. Each pot was watered with a solution containing 500ml water, 5ml Voermolas⁸ (an animal energy supplement based on molasses and used to stimulate microorganism growth) and 0.5g Ocean Agriculture 3:1:3 (38) complete fertilizer. Prior to watering, solutions were amended with either 0, 0.125 or 0.5g of Eco-T[®]. Each treatment was replicated three times with each medium.

Pots were left to stand for 4 days after which growing medium samples were analysed for $\text{NH}_4\text{-N}$ and $\text{NO}_3\text{-N}$ concentrations as well as pH. For determining growing medium $\text{NH}_4\text{-N}$ and $\text{NO}_3\text{-N}$ levels the Jenway Aquanova 6300 spectrophotometer⁹ was used together with the relevant colorimetric test kits. Ten grams of medium was added to 100ml of distilled water. The flasks were sealed with parafilm and placed on a horizontal shaker at 25°C for 30minutes. Samples were then filtered through a 45µm filter before analysing. Results were averaged from the three replicates of each treatment.

⁸Voermol feeds (Pty) Ltd, Box 13, Maidstone, 4380, South Africa.

⁹www.Jenway.com

pH was determined on a 1:1 dilution basis (by volume) using a standard pH metre. It is recognised that there are more accurate means of determining soil pH but for comparative means this method was deemed suitable.

Ten grams of medium was placed in a flask with 100ml of sterile distilled water and 20 glass beads. The flasks were then placed on a horizontal shaker for 30 minutes at 25°C. Dilution series were made and a 0.1ml sample of each dilution was spread onto agar plates containing a medium selective for nitrifying bacteria (Ball, 1997) or tryptone soy agar. Each dilution was replicated three times with each of the agar types. These plates were then incubated for 6 days at 25°C after which CFU values were determined.

5.4.3 Results

In the pinebark medium the initial level of $\text{NO}_3\text{-N}$ (20 minutes after experiment was commenced) was 460µg per gram of medium. After four days the level of $\text{NO}_3\text{-N}$ had increased in the untreated pots to 490µg/g, while decreasing to 310µg/g in the pots receiving four times the recommended dose of *Trichoderma*. $\text{NH}_4\text{-N}$ levels were not seen to change significantly and were measured at approximately 25µg/g, with initial levels of 60µg/g. This means that the percentage of $\text{NH}_4\text{-N}$ had increased from 4.9% to 7.5% after the addition of *Trichoderma*. In the perlite medium the percentage $\text{NH}_4\text{-N}$ increased from 20.8% (without *Trichoderma*) to 36% (four times *Trichoderma* dose). These results are summarised in Tables 5.4.3.A and B.

Key to Fig. 5.4.3.A

Treatment 0 = No *Trichoderma* added

Treatment 1 = Recommended dose of *Trichoderma* (5×10^5 spores/ml)

Treatment 4 = Four times the recommended dose (2×10^6 spores/ml)

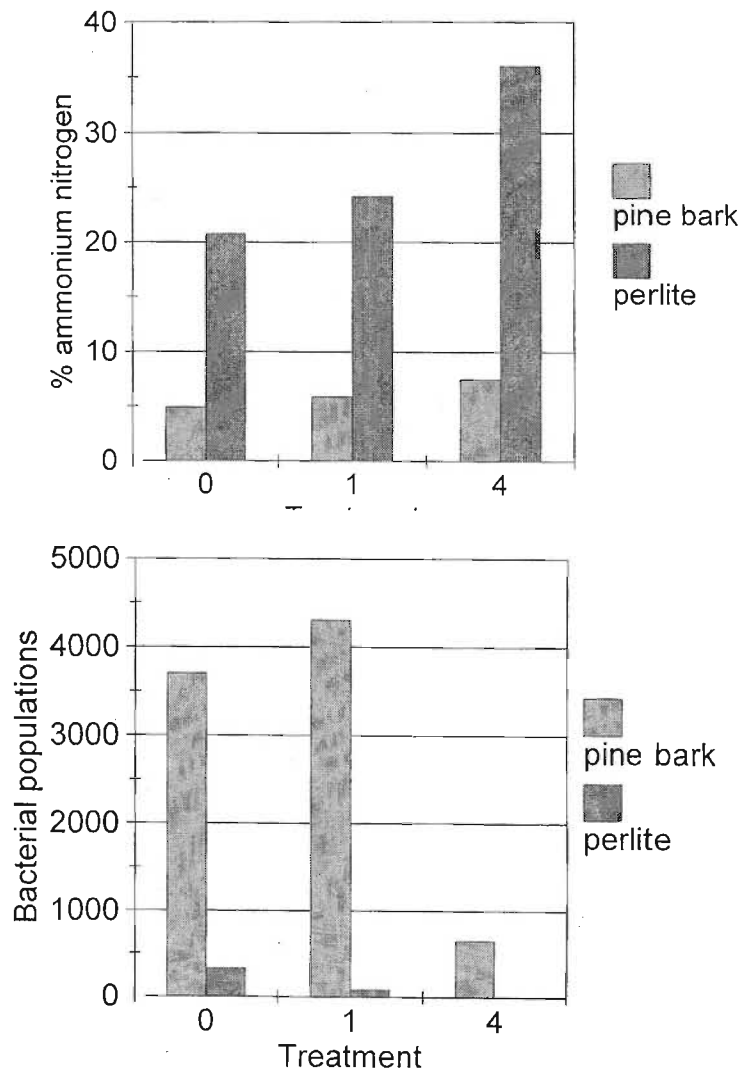


Fig. 5.4.3.A The effect of *Trichoderma* application rates on percentage $\text{NH}_4\text{-N}$ and population size of nitrifying bacteria within different media (For actual population sizes the figures on the Y-axis need to be multiplied by 10^6).

Table 5.4.3.A Effects of *Trichoderma* dose on nitrogen form in pine bark media

<i>Trichoderma</i> application rate (spores/ml)	NH ₄ -N (µg/g soil)	NO ₃ -N (µg/g soil)	%NH ₄ -N
0	25 (a)	490 (a)	4.9% (b)
5x10 ⁵	24 (a)	380 (b)	5.9% (ab)
2x10 ⁶	25 (a)	310 (c)	7.5% (a)
	F = 0.06 CV% = 16.05	F = 67.55 CV% = 4.86	F = 6.26 CV% = 14.81

* Treatments sharing the same letter (brackets) do not differ significantly at the 95% confidence level for the variable in question determined using the Student-Newman-Keuls test.

Table 5.4.3.B Effects of *Trichoderma* dose on nitrogen form in perlite medium

<i>Trichoderma</i> application rate (spores/ml)	NH ₄ -N (µg/g soil)	NO ₃ -N (µg/g soil)	%NH ₄ -N
0	42 (a)	160 (a)	20.8% (c)
5x10 ⁵	51 (a)	160 (a)	24.2% (b)
2x10 ⁶	45 (a)	80 (b)	36% (a)
	F = 3.94 CV% = 8.7	F = 24.68 CV% = 12.08	F = 99.67 CV% = 5.17

* Treatments sharing the same letter (brackets) do not differ significantly at the 95% confidence level for the variable in question determined using the Student-Newman-Keuls test.

The total bacterial populations in perlite media without the addition of *Trichoderma* averaged 6.3x10¹⁰ CFU's on tryptone soy agar at 25°C, while the addition of *Trichoderma* at 2x10⁶ spores/ml resulted in a reduction of total bacteria to 2.1x10⁸ CFU's. The number of CFU's on nitrifying bacteria agar (Ball, 1997) were similarly seen to decrease from 3.3x10⁸ to 4.5x10⁶ with the addition of *Trichoderma*. Similar trends were observed in the pine bark medium although in general bacterial populations were higher. Results of spore counts are shown in Tables 5.4.3.C and D.

No differences were observed with respect to medium pH as influenced by *Trichoderma* application rates.

Table 5.4.3.C The effect of *Trichoderma* on total bacterial population numbers in different media (measured as CFU's on tryptone soy agar at 25°C after six days)

Medium	<i>Trichoderma</i> (spores/ml)	Bacterial CFU's/10g growing medium
Perlite	0	6.3×10^{10}
Perlite	5×10^5	2.3×10^{10}
Perlite	2×10^6	2.1×10^8
Pine bark	0	1.5×10^{11}
Pine bark	5×10^5	3.3×10^{11}
Pine bark	2×10^6	5.7×10^9

Table 5.4.3.D The effect of *Trichoderma* on nitrifying bacteria population numbers in different media (measured as CFU's on nitrifying bacteria agar at 25°C after six days)

Medium	<i>Trichoderma</i> (spores/ml)	Nitrifying bacteria (CFU's/10g growing medium)
Perlite	0	3.3×10^8
Perlite	5×10^5	8.5×10^7
Perlite	2×10^6	4.5×10^6
Pine bark	0	3.7×10^9
Pine bark	5×10^5	4.3×10^9
Pine bark	2×10^6	6.5×10^8

5.4.4 Discussion

In the perlite medium the percentage $\text{NH}_4\text{-N}$ was much higher in all cases than in the pine bark medium. This is largely because the composted pine bark has a high level of initial $\text{NO}_3\text{-N}$ unlike perlite which has no initial nitrogen of its own. Furthermore, perlite is considerably more sterile to begin with, and thus it would be expected that nitrification would take longer to occur as populations of nitrifying bacteria have to get established first.

The method of determining $\text{NH}_4\text{-N}$ levels should be improved as much of the $\text{NH}_4\text{-N}$ probably remains strongly bound to the medium particles and is thus not measured. The method was however adequate for comparative means.

As seen by the bacterial population counts, *Trichoderma* appears to displace some bacteria, probably purely on a basis of competition for food resources. Both total bacterial populations and nitrifying bacteria are seen to decrease with the addition of *Trichoderma*. The fact that the bacterial populations appear to increase with the addition of *Trichoderma* at optimum rates (i.e., 5×10^5 spores/ml) in the pinebark medium is unexplainable. In both cases however, this result was not statistically significant at the 95% confidence level and could well be a result of experimental error.

The percentage $\text{NH}_4\text{-N}$ in the medium was inversely related to the size of the nitrifying bacteria population (Fig. 5.4.3.A). This indicated that the inhibition of nitrification is a means by which the addition of *Trichoderma* at excessively high rates can result in ammonium toxicity within the host plant. However, the improved availability of ammonium through mycorrhizal type of associations cannot be ruled out at this stage.

The fact that no acidification of the medium resulted from variable *Trichoderma* application rates in the absence of a host plant indicates that this phenomenon is dependent on the host plant in some way. The most probable explanation is that medium acidification only occurs at the root interface through the uptake of $\text{NH}_4\text{-N}$ by the plant. When $\text{NH}_4\text{-N}$ is available in abundance plants absorb more cations than anions. As a result plant growth results in a net efflux of H^+ ions into the rhizosphere causing a drop in the pH.

5.5 Conclusions

There is a very dynamic inter-relationship between *Trichoderma* and nitrogen nutrition. *Trichoderma* has the ability to out-compete nitrifying bacteria within relatively unbuffered hydroponic environments. This can result in a reduction in the conversion of potentially toxic ammonium to nitrate with the end result being an increased risk of ammonium toxicity associated with high application rates of *Trichoderma*. It is also possible that *Trichoderma* functions as a mycorrhizal organism (Kleifeld and Chet, 1992) and can thus actively increase the uptake of otherwise sparingly soluble and unavailable ammonium with the same negative risks. Furthermore, under conditions of high $\text{NH}_4\text{-N}$ nutrition root zone acidification occurs which can have further detrimental effects on plant growth and uptake of essential elements.

In applied terms it is thus important that growers are aware of these potential risks and use fertilizers with very low levels of $\text{NH}_4\text{-N}$. The risks are even greater under conditions of elevated root temperatures. High root temperatures occur readily during summer months when recirculating nutrient solutions can be warmed to $\pm 28^\circ\text{C}$. Being aware of these interactions can help explain some instances in which *Trichoderma* fails to operate or is seen to have negative effects on plant growth. In this light a thorough knowledge of these dynamic effects is essential in enabling biocontrol technologists to make informed recommendations regarding products such as Eco-T®.

5.6 References

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Chapter 6

Trichoderma and pH effects

Abstract

In vitro trials showed that conidial germination, sporulation, root colonization and biocontrol activity, of Eco-T[®], were all pH dependent, with optimum activity exhibited under acidic conditions. In liquid culture, conidial germination and subsequent root colonization were not observed at pH levels above 3.0. In artificial media, qualitative trials showed germination to occur over a wider pH range (3.0-8.0). Possible reasons for this are explored. In a recirculating hydroponic system, *Pythium*-induced reductions in yield were greatest at high pHs (6 and 7) with greater biocontrol activity at acidic pHs (4 and 5). The integration of pH management and Eco-T[®] applications can thus be used to obtain enhanced control of *Pythium* in hydroponics.

6.1 Introduction

Resh (1995) pointed out that pH is a logarithmic function and that a 1-fold increase in pH results in a 10-fold increase in hydrogen ion concentration. Any change in pH can thus have a large effect on the availability of ions to the plant, thereby affecting plant yields.

Again, effects are not limited to direct effects on plant growth, as pH has a major effect on microbial activity. Schuerger and Mitchell (1992) found, when looking at macroconidium attachment of *Fusarium solani* f. sp. *phaseoli* to mung bean roots in hydroponics, that disease was greatest when roots were inoculated at a pH of 4. Plants inoculated at pH 7 were found to be no different to the uninoculated controls. They pointed out that in soils the attachment of fungi to root surfaces may not be a prerequisite for pathogenicity as the proximity of infectious propagules is maintained by the stability of the soil matrix. In a flowing nutrient solution, however, the attachment of nonmotile propagules (macroconidia) is essential if the organism is to remain in contact

with the root for sufficient periods so as to allow germination of the conidia and subsequent infection. The manipulation of pH in nutrient solutions may, therefore, prove an inexpensive disease management tool for hydroponic systems.

Sharma and Gupta (1999) found sporangial germination in *Pythium ultimum* to be pH dependent. Germination occurred over a pH range of 5.6 to 7.0, with maximum germination at 6.4. *Trichoderma* on the other hand favours more acidic pH levels with Harman and Taylor (1988) having noted that *Trichoderma* species grew well at pHs around 3.0. Jeong *et al.* (1997) used these facts to improve the rhizosphere competence of *Trichoderma harzianum* at a practical level. Mycelial growth of *Pythium ultimum* was seen to be strongly inhibited by *Trichoderma harzianum* under acidic conditions (pH 4.5 and 6) compared with soils at pH 7.0.

Harman and Taylor (1988) used pH to improve the efficacy of *Trichoderma* in matrix priming. They noted that *Trichoderma* species grew well at low pHs (± 3.0) and that this pH should be inhibitory to most other microorganisms. The acidity of tomato seeds was seen as the major reason for *Trichoderma* having a competitive advantage on tomato but not on cucumber. In further experiments hydrochloric acid (HCl) was added to seed treatments of cucumber so as to make the seed pH 3.7 or 3.1. It was found that these treatments markedly improved the ability of *Trichoderma* to control seed rot in cucumber. These treatments were, however, found to be phytotoxic after a couple of days of storage, but it is suggested that other acidic *Trichoderma* seed treatments might give improved seed protection (Harman and Taylor, 1988).

6.2 *In vitro* screening of pH effects on *Trichoderma* conidial germination, and root colonizing ability.

6.2.1 Introduction

Before *Trichoderma* can have an effect on plant growth (biocontrol or growth stimulation) it needs to germinate and develop within the root zone. The effect of pH on both these processes was investigated.

Marschner and Römheld (1983) showed that form of nitrogen influenced the pH within the root zone. With ammonium the pH declines while with nitrate it increases. This effect impacts on a very narrow band immediately surrounding the root in the root-soil interface. This fact makes it difficult to study the effect of pH on biological interactions in the root zone when using media. Setting the nutrient solution pH is easy, but will not necessarily reflect the pH about the root, where the pathogens and biocontrol agents need to function in order to affect plant growth. For this reason it was initially thought that all pH trials should be conducted in pure liquid culture so that it could be certain that the pH of the recirculating nutrient solution reflected that of the root zone as closely as possible.

However, the work of Schuerger and Mitchell (1992) would suggest that the ability of *Trichoderma* to colonise roots in the absence of a solid medium (i.e., in liquid culture) would be more pH dependent than if a medium was present. This is due to the lack of motile spores. For this reason *in vitro* screening of pH effects on germination and colonization ability was done both with solid and liquid rooting media.

6.2.2 Materials and Methods

Six 500ml beakers were half filled with water containing fertilizer (Ocean Agriculture 3:1:3 (38) Complete) adjusted to an E.C. of 2.0ms. The pH in each beaker was set to a different level (3.0, 4.0, 5.0, 6.0, 7.0, 8.0) using either nitric acid or potassium

hydroxide, and 0.25g Eco-T[®] (5×10^8 conidia) was added to each. A circular polystyrene disk was cut to fit into each beaker, with a hole in the centre through which a three week old lettuce seedling could be inserted. Disks, together with seedlings were placed into the beakers, with disks about 2cm above the water level and seedling roots suspended in the solution. The beakers were covered in black paper, to prevent light reaching the roots, and placed on a flask shaker to produce movement of nutrient solution about the roots, as in liquid culture. Roots were sampled after 24, 48 and 72hrs, rinsed under distilled water and examined under the light microscope for spore attachment and germination.

For tests involving growing media, segments of onion were placed in perlite filled petri dishes. Onion was used due to the transparency of the tissue which allowed for easy microscopic observation. Perlite was moistened with nutrient solution containing Eco-T[®] (1g/l) adjusted to different pHs (3, 4, 5, 6, 7, 8). Two segments of onion were used, with the first being sampled after 48hrs and the second after 72hrs. Sampling was done by removing the thin outer skin of the onion segment and viewing this under the compound microscope for spore attachment and development.

6.2.3 Results

In liquid culture Eco-T[®] was only able to germinate and colonise lettuce roots at pH 3 (Figs. 6.2.3.A and B). Plants left for up to two weeks at higher pH levels showed no signs of *Trichoderma* conidial germination until the pH was dropped to 3.0, after which extensive root colonization was observed after 48hrs.

In media, *Trichoderma* germinated and colonised onion tissue at all pH levels although more rapidly at lower pHs. These observations were purely qualitative.

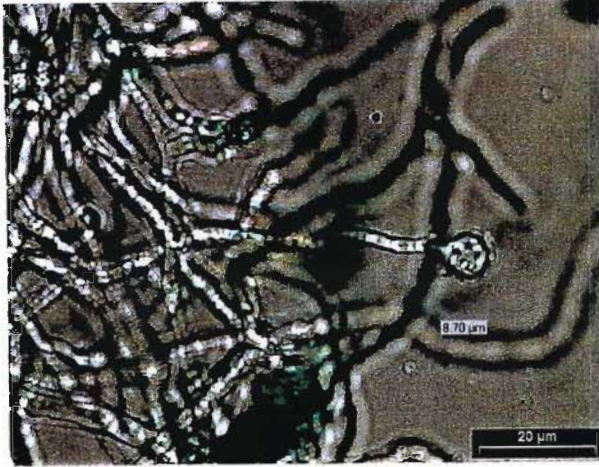


Fig. 6.2.3.A *Trichoderma* conidium germinating at pH3 after 24 hrs

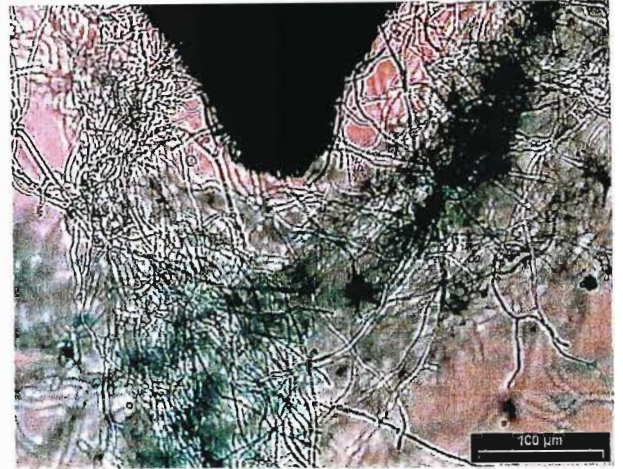


Fig. 6.2.3.B Root tip colonization by *Trichoderma* at pH 3 after 48 hrs



Fig. 6.2.3.C Root colonization by *Trichoderma* at pH 3 after 48 hrs

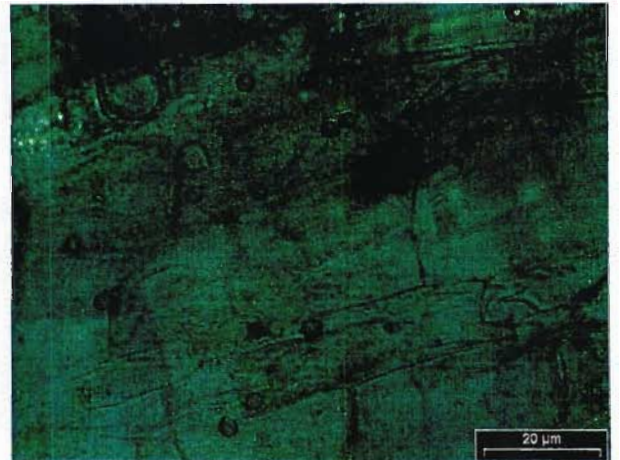


Fig.6.2.3.D *Trichoderma* conidia on root surface at pH 4 after 48hrs

6.2.4 Discussion

From these experiments it can be concluded that *Trichoderma* (Eco-T[®]) would not work well in liquid culture systems due to *Trichoderma*'s inability to germinate and colonise roots in flowing nutrient solutions at pHs above 3.0. This is because it is not practical to lower the pH to 3.0 in commercial systems because this is well below the optimum range of crop plants. Trials designed to study the effect of pH on *Trichoderma* biocontrol or growth stimulation activity should thus not be conducted in such systems.

In the presence of a medium, *Trichoderma* is capable of germinating and colonising roots at much higher pH levels. The mechanisms behind this differential response are unknown at present. Carlile and Watkinson (1994) reported on the mechanisms responsible for the stimulation of spore germination. In general, most fungi require water (either liquid or high relative humidity) and oxygen. A requirement for CO₂ is also common and temperature limits for spore germination are often narrower than for vegetative growth (Carlile and Watkinson, 1994). The presence of nutrients such as sugars and amino acids can indicate locations suitable for fungal growth (eg., root exudates) and could thus provide useful signals for spore germination in saprophytic and root infecting fungi (Carlile and Watkinson, 1994). In many plant pathogens, germination may be stimulated by chemicals emitted at low concentrations by potential hosts. These include substances such as hydrocarbons, alcohols, aldehydes, ketones and terpenes. Carlile and Watkinson (1994) stated that many of the chemicals that break the dormancy of fungal spores are lipophilic, and changes in the permeability of the plasma membrane, or of internal membrane systems, may be widely involved in the breaking of dormancy. In studies on the activation of ascospores of *Neurospora* it was found that effective agents resulted in a change in permeability of lipoprotein membranes, allowing trehalase to act on trehalose, a major nutrient reserve (Carlile and Watkinson, 1994).

It is thus possible that, in a recirculating nutrient solution, chemical stimulants are constantly washed from the root surface and diluted to such an extent that germination of Eco-T[®] conidia is not stimulated. At pH 3.0 either the need for stimulants is negated, or the permeability of the conidial membranes is suitably altered so as to allow

stimulation at much lower concentrations. The observation that *Trichoderma* conidia germinate in nutrient solution in the absence of plant roots may indicate that the change in permeability of membranes at pH 3.0 might, in itself, enable increased metabolic pathways and resulting germination.

When using media, chemical stimulants collect in the pore spaces about the plant roots in higher concentrations. At such concentrations it is possible that these stimulants function independently of pH.

These experiments highlight the difficulties in establishing the effect of pH in field conditions. Media have to be used in order to get a true reflection of pH responses with *Trichoderma*. The difficulty is in measuring the pH immediately around the root zone as it is this pH which will affect the functioning of *Trichoderma* and not that of the nutrient solution used.

6.3 Effects of pH and sugar availability on spore germination and sporulation of *Trichoderma*

6.3.1 Introduction

Assuming that available nutrients for *Trichoderma* growth act as stimulants for germination, it was decided to test the interactive effects between such stimuli and pH levels. If a pH of 3.0 results in spore germination by negating the need for such stimuli then it would be expected that such a pH response would only be observed under conditions of limiting nutrient supply.

6.3.2 Materials and Methods

Solutions were made up containing 1g/l of fertilizer (Ocean Agriculture 3:1:3 (38) complete) mixed with distilled water. The pH of the solutions were amended using either nitric acid or potassium hydroxide to provide a range of pH values (3.0, 4.0, 5.0, 6.0 and 7.0). For each pH, solutions were made containing different levels of available sugars through the addition of either 0g/l, 0.5g/l or 4g/l of Voermolas. *Trichoderma* was added to each of the solutions at 1g/l (2x10⁹ conidia/l), and 10ml of each of the resulting solutions was placed in a petri dish and incubated at 25°C. After 24hrs., samples of each of the solutions were observed under the compound microscope and the percentage germination and mean hyphal length were recorded for each pH and sugar concentration combination. Plates were then incubated for a further 48hrs., after which the extent of sporulation was observed and photographed.

6.3.3 Results

In the absence of suitable nutrients no germination of *Trichoderma* spores occurred within 24hrs. Some germination was however observed after 72hrs., although at very low levels. With a limiting sugar supply (0.5g/l Voermolas) germination was only observed at pH 4.0 and 3.0. Under conditions of ample sugar supply, germination occurred at all pHs. The length of the hyphae after 24hrs was however seen to differ with pH. The maximum hyphal growth was observed at pH 4.0.

Sporulation was seen to be inhibited by pH values of 6.0 and above, while being considerably higher at pHs of 5.0 and below.

Table 6.3.3.A Effect of pH and available sugar on *Trichoderma* spore germination and growth.

Voermolas (g/l)	pH	% germination (24hrs.)	Hyphal length (µm)
0	7	0	0
0	6	0	0
0	5	0	0
0	4	0	0
0	3	0	0
0.5	7	0	0
0.5	6	0	0
0.5	5	0	0
0.5	4	8.5	63
0.5	3	12.2	85
4	7	43.6	100
4	6	45.7	95
4	5	58.1	198
4	4	44.8	258
4	3	42.5	235

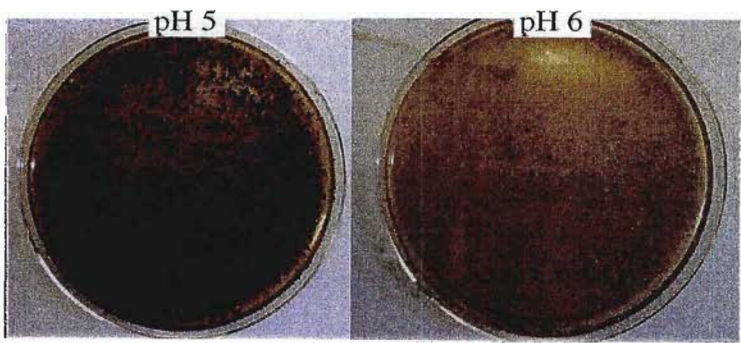


Fig. 6.3.3.A Effect of pH on sporulation of *Trichoderma* (Eco-T®)

6.3.4 Discussion

The effect of pH on conidium germination is most pronounced under conditions of no or very limiting sugar supply. Where sugar supply is not limiting pH does not affect spore germination although it does affect the rate of hyphal growth and the level of sporulation.

These results suggest that conidium germination in *Trichoderma* is dependent on the availability of a suitable source of nutrition for further growth and development. Low pH levels appear to reduce this dependence and allow for some germination at very low nutritional levels.

It is questionable whether this information can be used in the practical implementation of *Trichoderma* in the field. Formulations could be pre-mixed at low pHs and allowed to stand for several hours prior to application. This would result in a higher percentage germination of conidia but should these conidia be faced with adverse conditions at, or shortly after application, the survival of the conidia would be less assured. Pre-mixing also involves a further step for the farmer which detracts from the acceptability of the product by the end-user.

Where a knowledge of pH effects could be applied is in the manufacture of the biocontrol agents. These results clearly demonstrate the importance of maintaining pH levels of 5.0 or below in *Trichoderma* growing media in order to achieve a maximum harvest of conidia.

6.4 Interactions between *Trichoderma* and pH in the biological control of *Pythium*.

6.4.1 Introduction

Despite the difficulties established by the above experiments, it is still possible to determine the effect of pH on the ability of *Trichoderma* to control *Pythium*. This would then give an idea of the pH at which growers should attempt to maintain their plant roots to combat *Pythium*-induced crop losses.

Dual culture tests has been used extensively as a means of *in vitro* screening for biocontrol activity.

6.4.2 Materials and Methods

Bell tests (Bell *et al.*, 1982) are simply dual culture tests (Whipps, 1987) which are rated according to the scale developed by Bell *et al.* (1982). This *in vitro* screening method involves inoculating agar plates simultaneously with a pathogen and antagonist placed at opposite ends of the plate. The growth of both the antagonist and pathogen is then observed and rated over time. The rating scale proposed by Bell *et al.* (1982) provides for the following rating scores:

- 1 = *Trichoderma* completely overgrows the pathogen, covering 100% of the plate
- 2 = *Trichoderma* inhibits pathogen growth, covering 66% of the plate
- 3 = *Trichoderma* and the pathogen each cover half of the plate
- 4 = the pathogen outgrows *Trichoderma* and covers 66% of the plate
- 5 = The pathogen overgrows *Trichoderma* and covers 100% of the plate.

The tests were performed using half strength PDA adjusted to pH 5.0, 6.0, 7.0, or 8.0 by adding either nitric acid or potassium hydroxide. Half strength PDA was chosen as a medium which would provide an intermediate between nutrient rich and nutrient poor growing conditions, the importance of which was highlighted by Whipps (1987). For each pH the Bell tests were replicated three times, with both *Pythium* (PPRI 04169) and

Rhizoctonia (PPRI 03212) (pathogens) being tested against Eco-T® (biocontrol agent). Tests were rated according to the scale, established by Bell *et al.* (1982). Mean ratings were tabulated (Table 6.4.3.A) and pictures of plates taken (Fig. 6.4.3.A)

6.4.3 Results

Table 6.4.3.A Effect of pH on Bell rating of *Trichoderma* against *Pythium* and *Rhizoctonia*

pH	Bell Rating	
	<i>Rhizoctonia</i>	<i>Pythium</i>
5	2.67	2.33
6	3.33	3
7	4	3.67
8	4.67	4.33

Ratings were approximately the same for both pathogens tested, although only *Rhizoctonia* plates are shown (Fig.6.4.3.A) as *Pythium* growth on water agar was not clearly visible when photographed. All ratings were relatively low, but biocontrol activity clearly increased with decreasing pH (Table 6.4.3.A and Fig. 6.4.3.A).

Trichoderma on left, *Rhizoctonia* on right of each plate

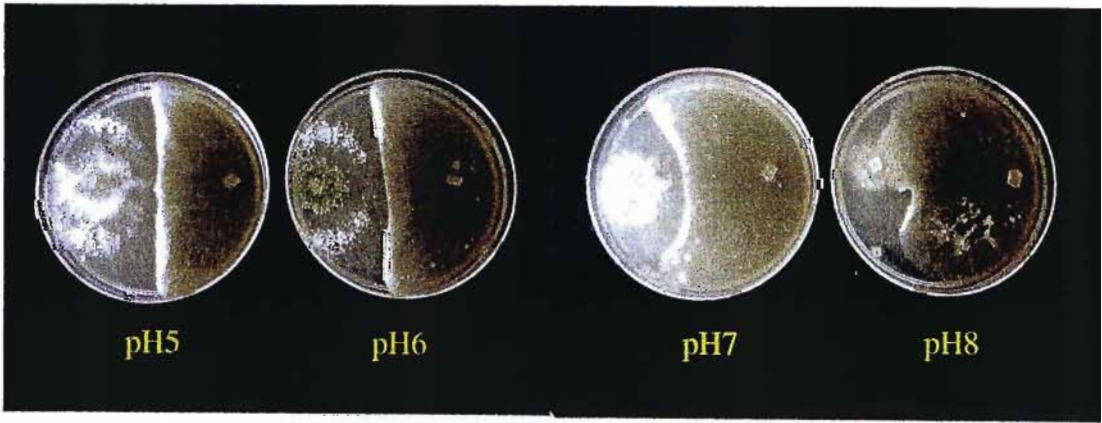


Fig. 6.4.3.A Effect of pH on Eco-T® biocontrol of *Rhizoctonia* as demonstrated by Bell tests

6.4.4 Discussion

Dual culture analysis (Whipps, 1987) is one of the most common methods for screening for levels of biocontrol activity. Whipps (1987) demonstrated the importance of choosing a suitable growing medium for such tests by using three media with varying nutritional status (nutrient poor tap water agar, soil extract agar having no added nutrients and the nutrient rich potato dextrose agar). The results (Whipps, 1987) showed that the medium choice had a significant effect on the growth rate and morphology of the fungi being screened. Medium choice also significantly affected the production of volatile and non-volatile antibiotics by the antagonist and the response of the pathogens to these antibiotics. In the above experiments half strength PDA was used as an intermediate between a nutrient rich and a nutrient poor medium. It was hoped that this would go some way towards representing the nutritional status of the rhizosphere within a hydroponic system. Future screening may however be necessary with a wider range of agar media so as to facilitate a better understanding of pH-nutrition interactions.

All Bell ratings appear low, possibly due to the low nutrient status of the medium used. However, biocontrol activity clearly increases with decreasing pH. At a practical level nutrient solution pH cannot be taken below 5.0 due to the direct negative effects that more acidic pH levels would have on plants.

The work of Jeong *et al.* (1997) and Sharma and Gupta (1999) would suggest that, in the control of *Pythium*, a pH level of approximately 5.5 would be ideal in providing *Trichoderma* with a competitive advantage. What needs to be established is whether the resulting increase in biocontrol activity will outweigh the possible reduction in yield resulting from reduced nutrient availability to the host plant.

6.5 *Pythium* control by *Trichoderma* under varying pHs

6.5.1 Materials and Methods

Lettuce seeds (All Year Round) were planted in Perlite, in speedling®-24's. Trays were left in the potting shed for 24 hours and then moved to the seedling tunnel where they were left for 10 days. Trays were then made up to 24 plants each and moved into the mini trough system. Twelve treatments were used (Table 6.5.1.A) each replicated three times.

Table 6.5.1.A Treatments used in pH trial

Treatment	pH	<i>Pythium</i> used	<i>Trichoderma</i> used
1	4	no	no
2	4	yes	no
3	4	yes	yes
4	5	no	no
5	5	yes	no
6	5	yes	yes
7	6	no	no
8	6	yes	no
9	6	yes	yes
10	7	no	no
11	7	yes	no
12	7	yes	yes

Nutrient solution was made up using Hydrogro® (1g/l) and calcium nitrate (0.6g/l). The electrical conductivity (EC) was adjusted to 1.8ms weekly and pH was adjusted to the relevant levels every second day using nitric acid or potassium hydroxide. *Trichoderma* was added, on transferral of plants to the mini troughs, at 0.5g/l (2.5×10^8 conidia/g). *Pythium* was isolated from infected soil one week prior and bulked up on water agar. Agar blocks (25mm²), with *Pythium* mycelial growth, were placed (fungal growth down) on the perlite, at the base of seedlings to be inoculated, two days after *Trichoderma*

application. Harvesting was done three weeks later and total shoot wet weight was recorded for each replicate. Mean total shoot wet weight was calculated for each treatment and graphed (Fig. 6.5.2.A). Statistical analysis (ANOVA and Student-Newman-Keuls tests) was done using the SAS system for Windows 98, Version 6.1.

6.5.2 Results

In the uninoculated controls maximum yield was achieved at pH 6.0. This was significantly higher than the yields at pH 4.0 and 5.0 with the minimum yield at pH 4.0 (Fig. 6.5.2.A). In the inoculated controls (inoculated with *Pythium*) highest yields were recorded at pH 4.0 and 5.0 (Fig. 6.5.2.A). The greatest loss in yield, resulting from *Pythium* inoculation, was seen at pH 6.0 (Fig. 6.5.2.B). Yield losses caused by *Pythium* inoculation were significant at all pHs, except 4.0 (Table 6.5.2.A)

Biocontrol activity of Eco-T[®] was greatest at pH 5.0 and decreased with increasing pH with a minimum activity at pH 7.0 (Fig. 6.5.2.B). Biocontrol activity was, however, significant at all pH levels.

Table 6.5.2.A Summary of statistical results from pH trial

pH	<i>Trichoderma</i> added	<i>Pythium</i> Added	Mean wet weight (g)	Student-Newman- Keuls (SNK) grouping
4	no	no	125	cd
4	no	yes	115.67	de
4	yes	yes	125.33	cd
5	no	no	138	abc
5	no	yes	117	de
5	yes	yes	140.33	ab
6	no	no	152.33	a
6	no	yes	105.33	e
6	yes	yes	142	ab
7	no	no	150.67	a
7	no	yes	105.67	e
7	yes	yes	133.67	bc
			F = 20.81*** P=0.05 CV (%) = 4.74	

*Treatments with the same letter are not significantly different at P = 0.05

Key to Figs. 6.5.2.A and B

control (u) = uninoculated control

control (i) = inoculated control (inoculated with *Pythium*)

biocontrol = inoculated with *Pythium* and treated with Eco-T®

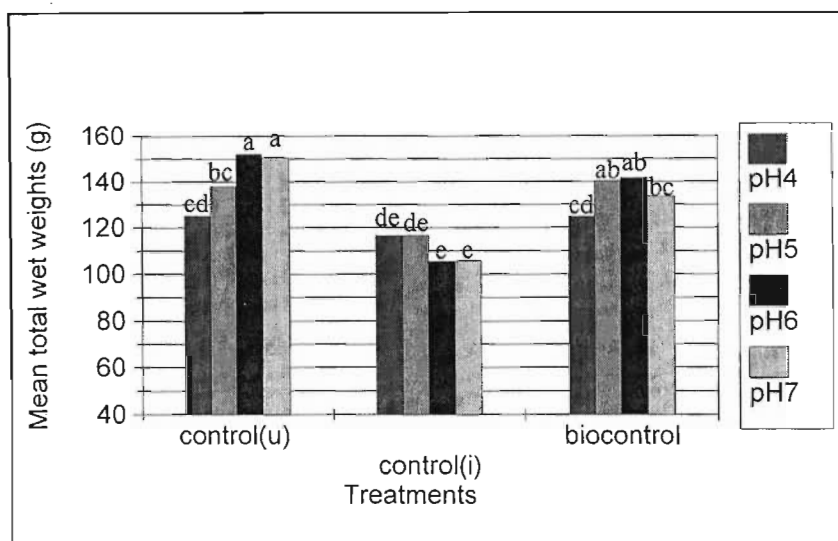


Fig. 2.5.2.A Effect of pH on disease severity and biocontrol activity in hydroponic lettuce. Treatments with the same letter are not significantly different at $P = 0.05$.

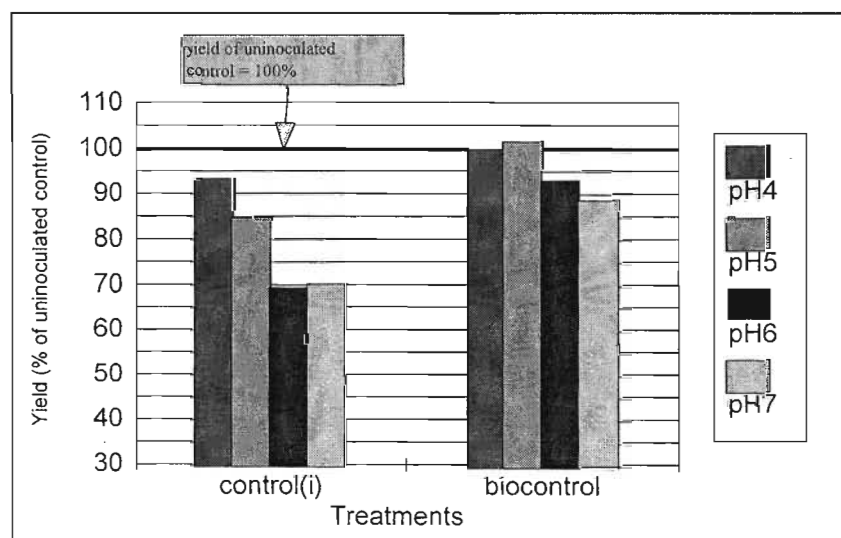


Fig.6.5.2.B Yield obtained under various treatments expressed as percent yield of uninoculated controls

6.5.3. Discussion

Optimum plant growth in uninoculated controls was at pH 6.0-7.0, as was expected given the effect which pH has on the availability of ions to the plant, as summarised in Fig.1.6.A (from Resh, 1995). The optimum range for the availability of most essential elements lies between pH 6.0 and 6.5.

The greater reductions in yield resulting from *Pythium* inoculation at higher pHs is supported by the works of Abdelzaher *et al.* (1997) and Sharma and Gupta (1999). Abdelzaher *et al.* (1997) found that for three species of *Pythium* tested, optimum pH for mycelial growth and zoospore production was 7.0. Sharma and Gupta (1999) showed that sporangial germination of *P. ultimum* occurred at a pH range of 5.6-7.0 with a maximum at 6.4. In the light of these publications it would have been expected that no reductions in yield would be recorded at pH levels of 4.0 and 5.0. This was true at pH 4.0, where the slight reduction in yield observed was not statistically significant. However, at pH 5.0 *Pythium* inoculation resulted in a significant reduction in yield. This may have been due to strain specificity, with the strain of *Pythium* used in these trials being functional over a wider pH range. Another explanation is that raised pH levels about the root zone resulted from using mostly nitrate based fertilizers.

Eco-T[®] treatments functioned best at lower pHs, with the highest level of biological control activity being seen at pH 5.0. At pH 4.0 the positive effects of *Trichoderma* were not as noticeable due to the direct negative effects which the low pH levels have on plant growth. The decreasing efficacy of Eco-T[®] with increasing pH is supported by the findings of Harman and Taylor (1988) and Jeong *et al.* (1997). A narrower range of pH levels needs to be examined in future trials to find the optimum pH for integration with Eco-T[®] use, which appears to lie between 5.0 and 6.0.

Future studies also need to look at the interactions between pH, form of nitrogen, and *Trichoderma* application. It is known that ammonium nitrogen reduces the pH about the root zone while nitrate nitrogen increases it. These facts could be used to formulate fertilizers which result in optimum pH levels and increased biocontrol activity. It must also be established whether *Trichoderma*'s liking for ammonium nitrogen is a direct response or an indirect one linked with the resulting decrease in root zone pH.

6.6 Conclusions

Trichoderma application would not appear practical in liquid culture systems as germination of Eco-T[®] conidia, in a purely aquatic environment, does not occur at pHs above 3.0. Where artificial media are used germination occurs up to pH 7.0 although maximum germination, sporulation and mycelial growth are still favoured by more acidic pH levels.

Pythium is favoured by higher pH levels (around 7.0) and does not cause significant levels of disease at pHs below 5.0. The combination of Eco-T[®] at a pH of about 5.0 results in increased levels of *Pythium* control.

6.7 References

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Chapter 7

Thesis Overview

7.1 Introduction

The world population in 1997 was estimated to be 5.8 billion and an increase in world population of 49% over the next 25 years was predicted by Skeen (1997). This increase in world population and the resulting decrease in availability of arable land, means that more intensive food production methods are essential. Hydroponics can be seen as one such method.

When managed properly, hydroponics can provide a number of financial and environmental benefits. A more efficient use of water and fertilizers helps increase yields while reducing the agricultural impact on the environment. Plant spacing is limited only by light availability, therefore allowing a closer plant spacing and a resulting increase in yield per unit area. Hydroponic systems are sustainable in that soil fertility and structure are not limiting factors. In traditional cultivation systems, crop rotation is essential with many crops. This results in large tracts of land standing unused for relatively long periods. The ability to sterilize growing media in hydroponic systems means that this inefficient use of space is not necessary. Furthermore, hydroponics can be practised independently of the need for arable land.

One of the major disadvantages in hydroponics is the development of pathogens, considered as minor pathogens in traditional cultivation systems, into major pathogens. This is mostly due to the absence of soil microbes, which in traditional systems provide an antagonistic buffering capacity. *Pythium* is one such pathogen. With motile zoospores capable of directional movement through water towards host roots (chemotaxis), this fungus is ideally suited to the aquatic environment of hydroponic systems. Although in many cases not causing dramatic or even noticeable disease in host plants, many *Pythium* spp. are minor pathogens, causing relatively low yet significant and consistent reductions in yield. In an attempt to maximise crop yields it is thus important that control measures for *Pythium* be identified.

One solution to the lack of soil microbial buffering in hydroponic systems, is the artificial establishment of buffering microbial populations in the root zone, i.e., biological control. The theoretical ability for strict control of environmental factors such as soil moisture, water temperature, pH and nutritional status led Paulitz (1997) to believe that hydroponics was the field in which biocontrol was most likely to succeed. However, many commercial hydroponic facilities do not have the capacity for such strict environmental controls. Where optimum environmental conditions are not achieved yield losses in these relatively unbuffered systems can be significant. The research in this thesis thus looked at the four way interactions between the host plant, pathogen, biocontrol agent and environment. The ability of *Trichoderma* (Eco-T®) to buffer some of the negative influences of sub-optimal environmental conditions both in the presence and absence of pathogens was assessed. Host plant growth responses were observed and where possible explained.

In the following overview each of the above interactions shall be summarised in terms of the understandings developed out of this thesis and areas of future research shall then be highlighted.

7.2 Efficacy of Eco-T® in hydroponic systems.

7.2.1 Current Understanding

Although the biological control and growth stimulating abilities of *Trichoderma* have been well documented by numerous authors (e.g., Chet and Baker, 1981; Ousley *et al.*, 1993; Migheli *et al.* 1995; Lewis *et al.*, 1996; Lo *et al.*, 1996), little work has been done on the use of this fungal antagonist in hydroponic systems. Application rates and methods may vary according to the system used and it is not necessarily possible to directly transfer information developed from soil cultivation to hydroponics. It was found in the research for this thesis that application rates vary between open and closed systems of hydroponics. Open systems are more like field applications where *Trichoderma* conidia, added in formulation, may be leached below the root zone and lost from the system. In closed systems application rates should be lower because conidia

are not lost from the system and are instead re-circulated in the nutrient solution until adsorbed.

At high application rates Eco-T[®] was seen to reduce plant yields, and no significant growth stimulation was recorded. A lack of growth stimulation under hydroponic conditions provides some evidence for environmental stress buffering as a mechanism of growth stimulation. Under optimal conditions the plants are under no stress and so do not benefit from *Trichoderma* application. Possible reasons for growth inhibition include the secretion of growth hormones by *Trichoderma*. Such hormones at high levels could result in negative responses. A new explanation relating to the form of nitrogen in fertilizer applications and the interference in nitrogen cycling and nitrification bacteria populations by *Trichoderma* was proposed and further studied in Chapter 5.

7.2.2 Future research

Further trials need to be conducted on a wider range of crops, diseases and hydroponic systems, so as to enable separate registration of Eco-T[®] as a hydroponic formulation.

Research into the mechanisms behind growth stimulation, or inhibition, needs to be furthered. It is only through understanding these mechanisms that we can start to consistently achieve desired responses. This in turn will improve the chances for a commercial future for biological control. The controlled nature of the hydroponic environment can play a large role in aiding in this area of research.

7.3 Soil moisture effects

7.3.1 Current understanding

Pythium spp. cause greater reductions in yield in water stressed plants. In some crops where soil moisture (combined with other environmental factors) is at an optimum it is possible for the plant to out-grow the negative influences of the pathogen. In over- or under-watered plants, however, the stressed environment leaves them more susceptible to infection, and increased yield losses occur.

Trichoderma prefers moist conditions and is able to overcome the negative effects of *Pythium* resulting from over-watered growing media. In under-watered soils *Trichoderma* is less effective, although still achieving some control. Growth stimulation by Eco-T[®] increases with increasing soil moisture.

7.3.2 Future research

Screening of other *Trichoderma* isolates needs to be done to determine if isolates exhibit a differential response to soil moisture. Differentiation of isolates on this basis might allow for specialised formulations for use in unique circumstances where more moist or dry soils might be required or cannot be avoided (e.g., soils with high clay contents or gravel bed systems).

7.4 Water temperature and form of nitrogen effects

7.4.1 Current understanding

The independent and combined effects of root zone temperature and form of nitrogen on plant growth have been well documented. Kafkafi (2001) described how at high temperatures with high ammonium concentrations in the rhizosphere, root death resulted from ammonia toxicity. This was supported by the research in this thesis in

which it was found that reductions in plant yield with increasing temperature were far greater when a fertilizer with a high proportion of ammonium nitrogen ($\text{NH}_4\text{-N}$) was used. Where more nitrate nitrogen ($\text{NO}_3\text{-N}$) was used, temperature effects were seen to be less significant.

What has not been documented is the role which *Trichoderma* might play in the nitrogen cycle, specifically the nitrification of ammonium into nitrate. Where *Trichoderma* was added to plants receiving high levels of $\text{NH}_4\text{-N}$ at high temperatures, yield losses were seen to be even greater than in uninoculated controls. It was hypothesised that the addition of *Trichoderma* into the relatively sterile environment (provided by the Perlite medium) resulted in the *Trichoderma* becoming established to the exclusion of nitrification bacteria. This prevented the conversion of $\text{NH}_4\text{-N}$ to $\text{NO}_3\text{-N}$ and resulted in greater root death through ammonia toxicity.

Some progress was made towards proving the above hypothesis. An increase in the amount of *Trichoderma* added to a re-circulating hydroponic system resulted in an increase in the percent $\text{NH}_4\text{-N}$ (relative to total nitrogen) and a corresponding reduction in yield. In a separate experiment the increase in the percent $\text{NH}_4\text{-N}$ was correlated with a reduction in nitrifying bacteria populations. These responses were greater in perlite which is seen as a more sterile growing medium than pine bark.

7.4.2. Future research

More complex experiments using radioactive labelled $\text{NH}_4\text{-N}$ could be done to track the passage of $\text{NH}_4\text{-N}$ through the system. Other more accurate means could be used to quantify the change in population size of nitrifying bacteria within the rhizosphere of *Trichoderma* treated plants. Further work also needs to be done to establish the mechanisms by which nitrifying bacteria may be excluded. If these mechanisms were known then screening of potential biocontrol agents could be done to ensure minimal impact on nitrogen cycling. It is also possible that the cycling of other elements within the rhizosphere could be either negatively or positively affected by *Trichoderma*. These possibilities, and their effects on plant yield should be investigated.

7.5 pH effects

7.5.1 Current understanding

Trichoderma conidia do not germinate in pure liquid culture hydroponics at pHs above 3.0. However, germination occurs over a wide range of pHs when a growing medium is used. Reasons for this differential response are unknown but are probably related to the dilution of germination stimuli in liquid culture while in the presence of a growing medium such stimuli can accumulate in pockets between medium particles.

pH is the most promising factor on which we can differentiate between *Trichoderma* and *Pythium*. *Trichoderma* is typically favoured by more acidic pH levels while *Pythium* prefers pHs around 7.0. A balance needs to be found between the optimum pH for the plant and that at which *Pythium* disease will be decreased and *Trichoderma* activity increased. This optimum pH level is believed to be between 5.0 and 6.0.

7.5.2 Future research

The exact reasons for *Trichoderma* conidia not germinating in pure liquid culture at pHs above 3.0 need to be determined. If a trigger could be identified it could be used to establish *Trichoderma* formulations which would be effective in liquid culture systems. Other strains of *Trichoderma* need also to be screened for the ability to germinate in liquid environments at higher pHs.

pH effects need to be studied in relation to the complex interactions of temperature and form of nitrogen. It is known that more acidic pH levels favour the establishment of *Trichoderma*, while most bacteria have a preference for more alkaline conditions. The use of $\text{NH}_4\text{-N}$ results in acidification of the root zone, and it is possible that this could result in an environment even more suited to *Trichoderma*, and the greater exclusion of nitrification bacteria. This could further the problem of ammonia toxicity should fertilizers with a high $\text{NH}_4\text{-N}$ content be used.