STUDIES ON THE APPLICATION OF BIOCONTROL AGENTS FOR THE CONTROL OF SEEDLING DISEASES

By

Jeh-han Omarjee

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Faculty of Science and Agriculture

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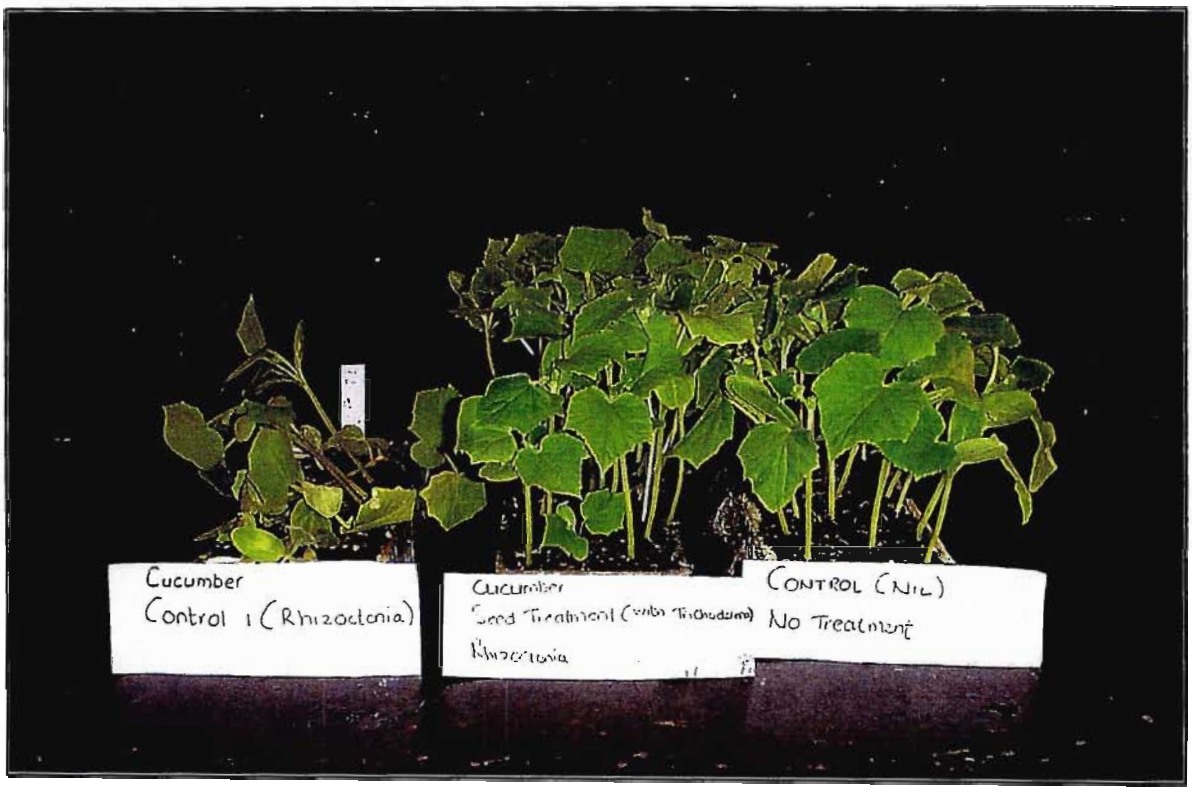
FRONTISPIECE – BIOCONTROL AGENT *Trichoderma harzianum* KMD



Comparison of *Trichoderma harzianum* KMD (commercial formulation) on cucumber seedlings with seed treatment (middle), Control–*Rhizoctonia solani* only (far left) and Control (Nil)- water only (far right).

ABSTRACT

The controlled environment of greenhouses, the high value of crops, and the limited number of registered fungicides offer a unique niche for the biological control of plant diseases. During the past ten years, over 80 biocontrol products have been marketed worldwide. A large percentage of these that have been developed in greenhouses could predominate over chemical pesticides in the same way that biological control of greenhouse insects predominated in the United Kingdom.

A review of the literature was undertaken to obtain information on biocontrol agents with specific reference to *Trichoderma* and *Gliocladium* spp. Literature on the application, types of formulations, limitations in formulation, registration and commercialization of these biocontrol agents were obtained.

Trichoderma harzianum Strain KMD has been used successfully as a biological control agent against several soil-borne plant pathogens. Biological control agents should possess several desirable characteristics, including, ease of preparation and application, stability during transport and storage, abundant production of viable propagules and good shelf-life.

A strain of *Trichoderma harzianum* KMD with potential biocontrol activity was used to determine the effect of culture conditions on spore shelf-life. The influence of four growing media were investigated on the spore ultrastructure and shelf-life, using a basal salts medium with C:N ratios of 3 and 14, and pH's of 4.0 and 7.0. Mycelial development and sporulation were positively affected by acidic conditions (pH 4.0). The effect of these culture parameters on viability and shelf-life were evaluated by counting colony forming units (c.f.u) before and after seven days of storage at 75% relative humidity (rH). The effect of carbon concentration on spore viability after seven days of storage was also determined by increasing concentrations of glucose while a constant C:N ratio of 3 or 14 at pH 4.0 was maintained at a 75% rH. Increasing carbon concentration and C:N ratios increased spore production times. Spore viability was greatest when harvested from a medium with a C:N of 14 at pH 4.0 even when storage time was increased to 45 days and rH was reduced to 12%. Ultrastructural studies showed that spores had two cell wall layers, with the outer being more electron-dense than the inner layer. This layer is the spore's first defense against adverse conditions. Spores obtained from this medium were larger, germinated better and had a longer shelf-life than

spores from C:N 3 medium, possibly because the two cell wall layers acted as a thicker barrier against adverse conditions. Increasing carbon concentration, while maintaining a constant C:N ratio of 3 or 14 at pH 4.0 slowed down spore production. Viability of spores were similar when introduced on media with variable carbon concentrations but fixed C:N ratios. The ultrastructural differences and shelf-life studies, confirmed empirical results from liquid fermentation studies, that the pH and C:N ratio of the medium upon which spores of *T. harzianum* KMD strain KMD were produced have critical effects on physical and chemical structure of the spores and viability. This, in turn, affects critical parameters for biocontrol agents spore germination and shelf-life.

Ultrastructural studies of mycoparasitism of *T. harzianum* KMD on a soil-borne pathogen, *Rhizoctonia solani* were investigated. The modes of antagonistic action by *Trichoderma* in biological control have not been fully elucidated. However several mechanisms have been described, such as mycoparasitism, antibiotics, production of inhibitors, which have been identified and shown to suppress soil-borne pathogens. Mycoparasitic activities of *T. harzianum* KMD against *R. solani* were studied using *in vitro* bioassays and Scanning electron microscopy (SEM). The fungal growth in dual cultures revealed that *T. harzianum* KMD made hyphal contact with the pathogen within four days of inoculation, leading to an inhibition of pathogen growth. SEM observations showed that *T. harzianum* KMD bound firmly to *R. solani* hyphae by coiling around the hyphae. Penetration of the pathogens hyphae occurred by the formation of hooks, haustoria and appressoria-like structures by *T. harzianum* KMD, followed by cell disruption. The pathogen's hyphae disintegrated and collapsed upon contact with *T. harzianum* KMD. It is hypothesized that the outcome of the interaction of antagonist and pathogen was most likely determined by initial hyphal contact that triggered a series of events in pathogen destruction.

An experimental trial was undertaken to evaluate various formulations if *T. harzianum* KMD and *Gliocladium virens* Strain MM1 for growth stimulation and biocontrol of *R. solani* and *Pythium* sp. on a variety of crops under greenhouse conditions using three application techniques at various dosages. Preparations of isolates of biocontrol agents *T. harzianum* KMD, *G. virens* MM1 and *Bacillus subtilis* Strain AW57 were evaluated for their efficacy in enhancing growth and preventing damping-off caused by *Pythium* sp. and *R. solani* on a variety of crops namely cabbage, cucumber, Namaqualand daisy and Eucalyptus. Percentage survival and plot weights were measured after 3-4 weeks of growth. The preparations that

were used included chlamydospores of biocontrol fungi in milled oats, powders containing conidia in an experimental compound, an oil base, and a commercial product. Formulations of bacteria were prepared with and without Nutristart. The evaluation of three delivery methods were used namely, a seed coating using an adhesive, Pelgel®, capping (a preparation is capped on the surface and incorporated into planting media) and as a drench (preparation drenched on seed at planting). Various dosage levels 0.25, 0.5, 1, 5 and 10g/l of each formulation was mixed with water and drenched on seed at planting.

Growth promotion of seedlings varied for the different formulations of different biocontrol organism. Overall, plot weight was significantly increased on all crops tested. Plant growth of seedlings was consistently increased by all conidial formulations of *T. harzianum* KMD and *G. virens* MM1. The best application technique that effectively delivered the biocontrol agents to the target was seed treatment followed by drenching and capping. Most formulations significantly increased plot weight on all seedlings ranging from 2000-5000% when compared to controls and percentage survival was comparable to the controls. In most instances it was recorded that all biocontrol organisms effectively enhanced growth of seedlings equally well irrespective of other main effects.

Most formulations of the different biocontrol organisms significantly reduced damping-off caused by *Pythium* sp. on eucalyptus and Namaqualand daisy. Formulations of *T. harzianum* KMD prepared with chlamydospores in milled oats and prepared with conidia effectively reduced damping-off on eucalyptus and Namaqualand daisy by 8-31% when compared to the controls. It was observed that biocontrol organisms *T. harzianum* KMD and *G. virens* MM1 effectively reduced damping-off better than *B. subtilis* AW57.

To effectively reduce damping-off caused by *Pythium* sp. seed treatment was the best application technique to deliver the biocontrol agent to the target. Biocontrol of damping-off caused by *R. solani* was achieved on all crops by all formulations of *T. harzianum* KMD, *G. virens* MM1 and *B. subtilis* AW57. Disease was reduced by 1000 fold with the application of biocontrol organisms when compared to disease controls. Conidial formulations performed better in reducing disease than formulations prepared with chlamydospores applied as a drench or a seed treatment. In most instances the best dosage to apply formulations were doses that ranged from 1-5g/l for both growth stimulation and biocontrol of soil-borne pathogens. Severe stunting of seedlings occurred at high dosages of 10g/l.

The compatibility of the biocontrol agent *T. harzianum* KMD with selected fungicides were determined on a variety of crops under greenhouse conditions. A commercial formulation of *T. harzianum* KMD was used for this investigation. An *in vitro* assay was used to determine the sensitivity of *T. harzianum* KMD to a range of rates of two fungicides, Benlate® and Previcur®. *Trichoderma harzianum* KMD was found least sensitive to both fungicides after 15 days of incubation at 25°C. The compatible mutants resulted in a lack of sporulation even when induced with UV light. Greenhouse trials were then carried out on cabbage, cucumber, Namaqualand daisy, eucalyptus and tomato. It was confirmed that *T. harzianum* KMD achieved better growth and biocontrol activity against *R. solani* and *Pythium* sp. when applied without fungicides to infested and non-infested composted pine bark. *Trichoderma harzianum* KMD was only compatible to fungicides when applied as a seed treatment prior to planting. As a disease integrated management programme, seed treatment application of *T. harzianum* KMD may be compatible with fungicides for control of damping-off of seedling diseases caused by *R. solani* and *Pythium* sp.

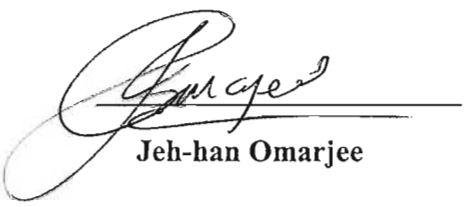
The effect of environmental stress (oxidative injury, cold and drought) on the growth enhancement of a variety of greenhouse crops by a commercial formulation of *T. harzianum* KMD was evaluated. In an absence of a disease colonization by *T. harzianum* KMD on maize and cucumber roots in rhizotron studies increased root area by 3104mm^2 and 1787, 48 mm^2 respectively. Oxidative stress was carried out by applying 0.05% NaOCℓ to cabbage, cucumber and tomato seeds. This stress did not reduce vigor of seedlings and hence the effect of subsequent treatment with *T. harzianum* KMD on stressed seeds was not determined. Treatments of imbibed but unemerged seeds of cucumber, tomato and white grain maize in cold temperatures (5-10°C night/day) for varying periods reduced subsequent growth. Seeds treated with cold stress and *T. harzianum* KMD did not display any growth enhancement. On cabbage, cucumber, tomato and white grain maize seeds sown in various media, which induced various levels of drought and water logging conditions, were not enhanced when seeds were coated with *T. harzianum* KMD. Overall, *T. harzianum* KMD did not enhance growth under stressed conditions of oxidative injury, cold and drought.

The results presented in this thesis shows that *T. harzianum* KMD has potential against soil-borne pathogens namely *Pythium* sp. and *R. solani* under greenhouse conditions. Applying conidial formulations of *T. harzianum* KMD using seed treatment and applying it at the correct dosage may increase the turnover of seedling production in nurseries. *Trichoderma*

harzianum KMD can replace toxic fungicides and fumigants under greenhouse conditions. More trials and research are needed on a wider variety of crops and diseases if growth promotion and biological control of *T. harzianum* KMD are to be fully exploited.

DECLARATION

I, Jeh-han Omarjee, declare that the research reported in this thesis, except where otherwise indicated, is my own original research. The thesis has not been submitted for any degree or examinations at any other university.



Jeh-han Omarjee

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FOREWORD

A research team, Biocontrol for Africa, at the University of Natal, Pietermaritzburg, has been researching and formulating potential biocontrol agents for soil-borne diseases in the period 1999-2002. All greenhouse research, laboratory and electron microscopy studies were conducted at the University of Natal, Pietermaritzburg, South Africa.

Biological control of plant pathogens is now an established sub-discipline in the science of plant pathology. Over the past 20 years, research in this area has increased dramatically. Within the past 10 years, biocontrol products have appeared on the commercial market, but these are still a small fraction of the total numbers and sales of chemical fungicides in the field. Of the commercial biocontrol products, over half have applications in nurseries or greenhouses, and many were specifically developed against the soil-borne pathogens, *Pythium* and *Rhizoctonia solani*, which are major greenhouse pathogens.

The world's total greenhouse area is 307, 000 ha, indicating plastic and glass, whereas the total land in outside cultivation in 1998 was 1.51 billion hectares. The use of biocontrol is more prevalent in greenhouses and protected structures than in field crops, even though greenhouses account for only 0.02% of the area used in agriculture. Biological control has also become a critical aspect of plant disease management in greenhouses. Pesticide registrations are being lost owing to concerns about their safety and environmental impacts. Fungicides, which are the predominant chemicals used for plant disease control, are of particular concern. Further, even chemical pesticides that are registered for use may be unavailable to growers because of pressures and concerns from the general public.

There are challenges when commercializing biocontrol agents. Each biocontrol agent, with each organism brings its own set of problems. Effective production and formulation protocols are usually proprietary, involving substantial investment to develop economic production and a formulation with adequate shelf-life, stability and titer. Even when all of these conditions are met, the formulated product may be incompatible with the grower's practice. Hence the above knowledge establishes a framework for research presented in this thesis.

Research conducted in this thesis is to obtain efficacy of biocontrol agents and solutions to problems facing biocontrol production systems in greenhouses.

The scope of this thesis is broad, spanning seven chapters.

1. Chapter 1 is a review of the literature on the formulation requirements of biocontrol agents, their mode of action and factors including shelf-life.
2. Chapter 2 reports the effect of culture conditions on spore shelf-life of the biocontrol agent, *T. harzianum* KMD.
3. Chapter 3 covers the mode of antagonistic action of *T. harzianum* KMD against *Rhizoctonia solani*.
4. Chapter 4 covers the evaluation of various formulations of *T. harzianum* KMD, *G. virens* Strain MM1 and *B. subtilis* AW57 for the growth enhancement and biocontrol activity on a wide variety of crops under greenhouse conditions, using different application methods at various dosages.
5. Chapter 5 encompasses the biocontrol activity of a selected formulation of *T. harzianum* KMD against a range of soil-borne pathogens with the use of selected fungicides.
6. Chapter 6 is on the effects of environmental stress on the biocontrol fungus, *T. harzianum* KMD.
7. Chapter 7 reviews the experimental results and conclusions are deduced as to how efficient the biocontrol agents are in providing disease control and growth stimulation. Each chapter is written as a discrete paper, resulting in some duplication of references between chapters.

CHAPTER 1

LITERATURE REVIEW OF FORMULATION OF BIOCONTROL PRODUCTS

1.1 GENERAL INTRODUCTION

This review deals with the formulation, application, shelf-life and storage and mechanisms of biocontrol activity of biocontrol fungi, namely *Trichoderma harzianum* Rifai and *Gliocladium virens* Miller, which have been shown to parasitize, inactivate, or compete with soil-borne plant pathogens.

Traditionally control of plant pathogens has been accomplished largely with the use of chemical pesticides. Biological control agents (BCA's) have received increased attention. *Trichoderma* spp. and *Gliocladium* spp. are capable of controlling several plant pathogenic fungi and have been subjected to extensive investigation for several years. Selected strains have been shown to suppress *Pythium* spp. (Chet *et al.*, 1982; Hadar *et al.*, 1984), *Sclerotium rolfsii* Sacc. and *Sclerotinia sclerotium* Lib.(Chet *et al.*, 1982), *Rhizoctonia solani* Kühn (Elad *et al.*, 1980; Harman *et al.*, 1981, Lewis & Papavizas, 1987), *Botrytis cinerea* Pers.:Fr. and *Fusarium* spp. (Cook & Baker, 1983; Sivan & Chet, 1986; 1989).

Authors of review articles of biocontrol agents have clearly referred to formulation being the most important aspect of biocontrol production. In a review by Burges (1998), it was stated that: "Formulation plays a vital role in the successful development of a commercially viable biocontrol agent. The advent of formulation technology has been crucial to solving these problems and in making an organism effective in practice. Beneficial organisms, which have been shown to be effective in the laboratory often fail at some stage in the field. Common causes of this failure include poor stability of the product during storage prior to application, too little active material actually reaching the field target, and, or rapid degradation of the active material on the target".

Burges (1998) defined that a formulation comprises methods or means of preserving organisms, which retain or enhance their activity once they are delivered to a targeted site. It

was also stated that: "A concentrate of an organism that has been formulated is termed a formulation".

Several reviews have shown that a formulated product does not necessarily serve all the requirements for use on all crops. On some crops, additives may be needed to achieve optimum application.

Xixuan *et al.* (1990) stated four critical components of biocontrol production systems:

- selection or development of a suitable biocontrol agent;
- development of a delivery system that permits full expression of the biocontrol ability of the strain;
- development of a fermentation process that gives rise to high levels of propagules;
- formulation of the end product which permits good storage

It has been pointed out by Xixuan *et al.* (1990) that formulation of *Trichoderma* and *Gliocladium* spp. involves incorporating these fungi into a suitable carrier. In the above author's review it was cited that the formulations of *Trichoderma* and *Gliocladium* spp. can be supplemented with additives to maximize survival in storage and application methods, shelf-life and storage requirements need to be determined and optimized prior to marketing. Biocontrol products are comprised of living, viable propagules, making them relatively sensitive to variable storage conditions. There are critical requirements for formulation, which are determined by the organism's features and of their environments. The mode of action of the biocontrol agent is important as it predicts the formulator's prime goal (Xixuan *et al.*, 1990).

1.2 FORMULATION OF BIOCONTROL FUNGI FOR SOIL-BORNE PATHOGENS

There are several processes to be followed in order for a biocontrol agent to be commercialized. The first process is the discovery of the biocontrol agent, which then proceeds through to efficacy trials, field-testing, toxicological and environmental tests. The final steps are registration and marketing.

Harman (2000) cited that: " Universities are best suited for initial processes and commercial companies are required for the final processes. Many of the innovative technological developments of biocontrol products have been developed by small to medium sized companies. As a result the processes, procedures, and equipment required for economical production of biocontrol agents are hoarded and highly proprietary. Hence much of the technology is unavailable to academic researchers except in the case of collaboration with biocontrol companies". The above statement is very true as not much research can be done without the help of other biocontrol companies. Related research may only be continued and further enhanced when biocontrol companies are willing to collaborate.

1.2.1 Commercial fungal biocontrol products

Liansky (1985) pointed out that: "The decisions to commercialize a biocontrol product are not only based on science but also on sound business principles". The above author states that: "Before scaling up for commercial production, a company must assess many factors, e.g., demand for the product, potential market size, and efficacy of the product compared to existing competing products". Other important factors suggested by Ricard (1979) are the amount and type of data required for registration e.g., field and greenhouse trials.

1.2.1.1 *Biocontrol products formulated with* Trichoderma *and* Gliocladium *spp.*

Fravel & Larkin (1996) concluded that alternate methods of plant disease control which have led to intense research of microbial products as biocontrol agents has resulted in the commercialization of a number of microbial products. It has been proved and reviewed by many scientists that biocontrol agents are directly associated with their target pathogens, host plants and environmental conditions. Many farmers regard biocontrol agents as having little potential to counteract plant diseases as other chemicals on the market do. This is probably due to the fact that farmers are provided with little knowledge regarding the requirements of the given product (Koch, 1998).

There are over 80 products for biocontrol of pathogens worldwide (Whipps & Davies, 2001). However, not all will be discussed in this section. Most of these formulated products include the fungi *Gliocladium* and *Trichoderma* spp. Some of these products are not registered as biocontrol agents, but are marketed as plant growth promoters, plant strengtheners, or soil

conditioners to avoid the toxicology or efficacy testing that would be required for plant protectants. This will then allow these products to get on to the market with ease.

Gliocladium virens is widely distributed in soil worldwide. This fungus was developed for control of *Pythium ultimum* and *Rhizoctonia solani* in soilless mixes (Lumsden & Locke, 1989). This fungus was formulated as an alginate prill and named GlioGard® by the company W.R. Grace Company. For the use of this product in greenhouses a granular fluid SoilGard® was developed and is presently being marketed by Thermo Trilogy Corp., Columbia, M.D. Lumsden *et al.* (1996a) and Howell & Stipanovic *et al.* (1995) demonstrated that the fungus produces two fungitoxic compounds, i.e., glioviren and gliotoxin compounds. SoilGard® has been reported to control damping-off caused by *Rhizoctonia solani* and *Pythium* spp. (Lumsden *et al.*, 1996a; Lumsden *et al.*, 1996b; Benson, 1997; Walter & Bruette, 1997). *Talaromyces flavus* is a mycoparasite of various phytopathogenic fungi and is a microbial active ingredient of the product PROTUS (Jefferies &Young, 1994).

Primastop® is a product that contains *Gliocladium catenulatum* Strain J1446. This product is used for applications in greenhouses and indoor use. The product received registration in July 1998 for 55 different crops. Primastop® is sold as a wettable powder that can be applied to soil, roots, or foliage to combat damping-off, seed rot, root rot, and wilt pathogens (Paulitz & Belanger, 2001).

The product SUPRESIVIT® that contains *Trichoderma harzianum* Rifai strain PV 5736-89 has been reported to stimulate plant growth and to control *Pythium ultimum* (Duskova, 1995). TRI 002, which includes the fungus *Trichoderma harzianum* Rifai strain KRL-AG2, is marketed in Europe as a plant growth stimulator as well as a plant-strengthening agent. The microbial active ingredient, *T. harzianum* Rifai strain KRL-AG2, is registered in the United States of America as a microbial pesticide (Harman *et al.*, 1996). ECOFIT®, *Trichoderma viride* Pers ex Gray, is marketed in India for control of various soil-borne pathogens on field crops and vegetables. Recommended application methods include seed treatment, soil drench and inoculation of farmyard manure, which is applied in the field after a period of incubation.

There are also several products, such as Promot®, which are sold as plant growth promoters rather than as biocontrol agents. Although not labelled for biocontrol, these products probably

provide some disease control. The fungus *Trichoderma* is most frequently used for control of plant pathogens (Fravel *et al.*, 1998). Twelve products (i.e., Biofungus®, Binab-T®, Rootshield®, Supresivit®, T-22G, T-22HB, Trichodex®, Trichopel®, Trichoseal®, Trichojet®, Trichodowels®, Trichoderma 2000) contain *Trichoderma* spp. to control a variety of pathogens (Fravel *et al.*, 1998). Formulations of *Trichoderma* vary considerably depending on their intended use.

For example, a combination of *T. viride* and *T. harzianum* is formulated for soil incorporation (Tricopel®), *Trichoderma*-colonized dowels for insertion into wood (Trichodowels®), and as a wettable powder for application to pruning wounds (Trichoseal®). Biofungus® is available as a granule, as a wettable powder, impregnated sticks, and as "crumbles" for mixing into soil (Fravel *et al.*, 1998).

T-22 is formulated as a granular formulation (Root Shield®) or as a water-soluble drench containing conidia (Plant Shield®). This product has been shown to reduce *Fusarium* crown and root rot of tomatoes grown in potting mix, containing T-22 and transplanted into the field (Datnoff *et al.*, 1995, Nemec *et al.*, 1996). Under greenhouse conditions, T-22 controlled the soil borne pathogen *R. solani* in pointsettia, geraniums, and *Cathoranthus*, and *Pythium* on geraniums, Impatiens and Petunias. T-22 is compatible to fungicides and must be applied as a preventative before disease occurs (Paulitz & Belanger, 2001).

1.2.2 Factors to consider for inoculation of biocontrol agents to soil

The mode of action of biocontrol micro-organisms is determined by the specific court of action, which plays an important role in effectively reducing the disease (Paau, 1998). Papavizas & Lewis (1981) states that: "*Trichoderma* and *Gliocladium* spp. are established biocontrol agents and relatively few researchers have undertaken quantitative studies to elucidate the survival, establishment and proliferation of these two antagonists in the plant rhizosphere".

Papavizas (1981) determined the effectiveness of *Trichoderma* and *Gliocladium* as seed treatments and proved their ability to colonize and multiply in the rhizosphere. Papavizas & Lewis (1981) hypothesized and proved that *Trichoderma* inoculum multiplies only at the site

of application and that it may suppress pathogens causing seed rot and seedling diseases, but not those that cause root diseases. This research also showed that *T. harzianum* did not establish in the rhizosphere of bean and pea seedlings grown from seed treated with conidia. The number of colony forming units (c.f.u.) recovered per agent of rhizosphere soil was found to be considerably less than the number of conidia added per individual seed. In addition, when conidia were added to the soil before planting, but not to seed, the rhizosphere populations never exceeded those recovered from non-rhizosphere soil, indicating no rhizosphere effect.

Several possible explanations for the decline of *Trichoderma* populations in the plant rhizosphere were proposed by Papavizas (1981). Papavizas (1981) suggested that this may be due to the lack of appropriate nutrients, the presence of toxic substances in the root exudates, and/or the presence of antagonistic or competing microorganisms at the rhizosphere or rhizoplane level.

It was cited by Paau (1998) that: "Many organisms used in commercial products for soil application are propagated in a rich medium, and later packaged as a concentrate with the organisms in a dormant or semi-dormant physiological state". Stathers *et al.* (2001) proved that dormant organisms could withstand relatively high temperatures and wide temperature fluctuations thereby facilitating transport and prolonged storage. However, in a dormant state, these organisms are not physiologically ready to compete with indigenous species in the soil that have had time to adapt to a specific ecological niche. Paau (1998) continued to review that many formulations specifically address this issue by including large amounts of carrier, selective food sources, suppressors for indigenous species, as well as buffers and other ingredients, which can transiently alter the micro-physiological environment of the soil. Paau (1998) then further stated that carriers temporarily provide a safe haven for the introduced species to establish itself in the rhizosphere.

Special considerations must be made for the development of soil-applied biocontrol agents. Van Elsas & Van Ouerbeek (1993) discuss this issue. These authors point out that there are a number of obstacles to develop soil inoculants. One of the obstacles is the extreme heterogenous nature of soil and the variability of the micro-physiological environment.

1.2.3 Types of formulations used to apply biocontrol agents to soil

A variety of formulations have been developed for the inoculation of biocontrol agents to soil. Formulations range from both liquid and solid. The main types currently used are products, which are dusts, granules, briquettes, suspensions, or liquids, i.e. oil or water based, and mixed emulsions.

1.2.3.1 *Liquid formulations and oil suspensions*

Fravel *et al.* (1998) demonstrated that a suspension of living organisms, can be applied to soil either as an in furrow or drip application, drench or as a spray. *Rhizobium* spp. are one good example. During early commercialization, agar cultures of *Rhizobium* spp. were diluted and sprayed into the soil. *Rhizobium* spp. are temperate organisms and are susceptible when transported or stored for long periods.

Johnston (1992) researched oil suspensions and illustrated that suspensions contain more viable and dormant organisms. These suspensions deliver organisms that are physiologically dormant and prevent the growth of contaminants. Fravel *et al.* (1998) proved that fungal spores namely, *Gliocladium*, *Trichoderma* and *Paecilomyces* are ideal for oil suspension formulations.

Biocontrol products such as BioJect Spot- Less®, which is used on turf and other crops, is formulated as a liquid formulation. The organism present in this type of formulation is *Psuedomonas aurefaciens.* Another biocontrol agent on the market that is formulated as a liquid suspension is Companion®. This formulation comprises *Bacillus subtilis* GBO3, B. *subtilis, B. licheniformis, B. megaterium.*

1.2.3.2 *Powder formulations*

Paau (1998) points out that organisms can be formulated into concentrated dry or wet powders for easy storage, transport and application. The powders are easily distinguished by the moisture content. The application methods vary for the incorporation of powders into soil.

Dry powder formulations

These formulations are made of lyophilized biomass. The biomass comprises of mostly spores and hyphal fragments (Paau, 1998). These formulations are usually manufactured with a wetter that allows for the absorption of water when applied to the soil. This is used to facilitate the rapid reactivation of the organism. It is clearly mentioned in a review article by Paau (1998) that lyophilization is a very expensive technique to use and is often not used in large-scale industries due to costs.

Biocontrol products such as Aspire®; BlightBan A506®; Kodiak® (several formulations) Messenger®; Mycostop®; Primastop®; RootShield®; Plant Shield®, T-22 Planter Box® and Serenade® are currently on the market and are marketed as dry powder formulations.

Moist powder formulations

Graham –Weiss *et al.* (1987) illustrated that organisms can be formulated directly in a moist powder. Bacteria, yeasts, protozoans and other micrscopic organisms can be formulated into a moist powder and are used for spraying and coating of seeds or direct application to soil. The above authors showed that suitable carriers are peat, vermiculite, sawdust and other materials that hold moisture and do not form hard aggregates. McCabe *et al.* (1994) proved that organisms formulated using this method are metabolically more active and respond better upon application to soil.

1.2.3.3 *Granules for soil applications*

Fravel *et al.* (1998) reviewed the granule formulation. These authors determined the suitability and use of these formulations. These authors cited that: "Granules are generally easier to handle and apply than powders. They are, however, more bulky and hence have higher material, storage and transport cost implications".

Burges (1998) further expanded categories of granules. Granules, which are non-sticky and do not form bigger aggregates, are often referred to as flowable granules. Most dry granules fall

into this category. Usually moist granule products are less free flowing. However, both types are suitable for broadcast and in-furrow application. Water-dispersable granules have been developed in such a way that they will disintegrate when they reach a certain moisture content. Such granules are traditionally used for spray applications.

Gliocladium roseum and *T. harzianum* have been formulated into granular formulations for the control of *R. solani* causing damping-off of peanut. Lignite is often used for these types of formulations. Fravel *et al.* (1998) demonstrated the technique of formulating *T. harzianum* and *G. roseum* in granules. These authors demonstrated that the fungi could be grown as granules for seven days. The granules are air dried before applying to *R.solani* infested soil. Lewis & Larkin (1997) showed that *G. virens*, *T. hamatum*, *T. harzianum* and *T. viride* formulated as granules significantly reduced damping-off in egg-plant caused by *R. solani*.

Biocontrol products that are on the market and formulated as granular formulations are AQ120 Biofungicide®; Actigard®; Actinovate®; Contans WG and Intercept WG®.
The products GlioGard® comprising of *G. virens* comprises an alginate granule formulation. This formulation is added directly to the soil or potting media, for the suppression of root and stem damping-off pathogens.

1.2.3.4 *Carriers*

Carriers are inert ingredients of formulation that can profoundly affect shelf-life and efficacy of the product (Kok *et al.*, 1996). Backman & Rodriguez-Kabana (1975) compared attapulgous clay and diatomaceous earth for their water-holding, water-retention, and physical properties after autoclaving. These authors showed that attapulgous clay granules swelled excessively in water and lost their integrity if stirred while moist. In contrast, diatomaceous earth granules did not swell significantly, withstood autoclaving, and remained intact after stirring. The diatomaceous earth was found to absorb up to 50% of its volume of liquid, making it suitable to absorb a molasses- base medium for delivery of *T. harzianum* to soil (Kelly, 1976; Ward, 1984; Fravel *et al.*, 1998).

Kok *et al.* (1996) demonstrated that manure pellets produced from processed swine faeces can be used as carrier material for *T. harzianum.* They showed that the antagonist could grow and sporulate with the processed manure powder as its sole source of carbon and nutrients. The

incorporation of conidia in pellets of the processed manure was shown to be feasible on a laboratory scale. Survival of the fungus in the pellets during storage was satisfactory. Population dynamics of *T. harzianum* were studied using a benomyl-resistance marker after introduction of conidia into soil. The antagonist could colonize and spread through a number of non-sterile soils and was able to establish a stable population over a period exceeding 125 days. Under sterile conditions, the propagation of *T. harzianum* in soil was much greater than under non-sterile conditions. The incorporation of antagonist conidia in pellets was found to be essential for the successful colonization of non-sterile soil (Savoy & Breitenbeck, 1998). In growth chamber experiments, application of *T. harzianum* via processed manure pellets reduced damping-off of sugar beet seedlings caused by *R. solani* in both artificially and naturally infested soil (Kok *et al.*, 1996).

1.2.3.5 *Formulations using seeds as vehicles*

Seed treatments involve the application of a biocontrol formulation to seed in a liquid or powder form (Papavizas, 1985). There are numerous examples in the literature where seed treated formulations have shown beneficial effects on crop production. For example, seed treatments with *Trichoderma* and *Gliocladium* have had beneficial effects on stands of cotton (Elad *et al.*, 1982a; Howell, 1982; Sivan & Chet, 1986). Stickers are added to most formulations to attach biocontrol agent propagules to seed. Pea and soybean seeds treated with *Psuedomonas putida* in a Pelgel sticker have been used to control *P. ultimum* (Paulitz *et al.*, 1992). A range of *T. harzianum* formulated seed treatments (i.e., Pelgel, Polyox N-10, ground particulate matter, Agro-Lig) have been successfully used to control *Pythium* spp. on cucumber (Fravel *et al.*, 1998). Seed treatments will be discussed further in 1.3.2.

1.3 APPLICATION OF BIOCONTROL FUNGI TO SOIL

1.3.1 Methods for delivery of biocontrol agents to soil

The development of effective delivery methods should be an important focus in any biocontrol research program. The delivery of biocontrol fungi must enable the organism to grow well upon application if they are to be effective against chemical pesticides and other microflora. In approaching the establishment of an effective delivery system, careful

consideration must be given to both the physiology of the fungus and the environment into which it will be applied. These aspects have been discussed and critically reviewed by Papavizas (1985), Harman (1990) and Taylor *et al.* (1990).

1.3.1.1 *Complexity of the soil environment*

Soil is a complex and competitive environment for microorganisms and it has received considerable attention in past years by Cook & Baker (1983); Griffin (1985); Baker & Scher (1987) and Harman & Lumsden (1990). Harman (1990) and Harman & Lumsden (1990) stated that: "Conditions required for successful biological control of soil-borne plant pathogens are not fully understood. Factors such as moisture, temperature, organic matter, pH, nutrient availability, and ionic balance vary within a single field". Clark (1967) points out that changes in soil factors have a significant ecological influence, not only on soil-borne pathogens, but also on the biocontrol fungus introduced into the soil.

Xixuan *et al.* (1990) mentioned that among the various soil microorganisms present, bacteria are usually the most abundant, with populations in the range of 10^{6}-10^{9} c.f.u per gram of soil and that fungal numbers are usually 10-fold less than those of bacteria. However, fungi may contribute more to the overall total soil biomass. Xixuan *et al.* (1990) also states that within micro-environments, a biocontrol fungus must compete with existing microorganisms for their share of nutrients. Energy-yielding substrates such as carbohydrates and amino acids are usually in short supply (Griffin, 1985).

1.3.1.2 *Improvement of the soil environment for inoculation of biocontrol agents*

Harman & Lumsden (1990) described that a basic concept for improving biological control in the soil micro-environment is to reduce the amount of competition imposed on the introduced biocontrol fungus. Previous attempts to do so by Harman & Lumsden (1990) include modification of soil pH, partial sterilization of soil and application of organic substrates. None of these approaches has successfully been used commercially in the control of soil-borne plant pathogens. Fravel *et al.* (1985); Lewis & Papavizas (1985); Lewis & Papavizas (1987) and Xixuan *et al.* (1990) showed that encapsulation and use of alginate pellets have also been investigated in an attempt to enhance the soil micro-environment for biocontrol fungi and have been relatively successful.

1.3.2 Seed treatments

Harman (1991) stated that: "Seed treatments with biocontrol fungi are being pursued to replace or supplement the use of chemical seed treatments for the control of soil-borne plant diseases". The above author explored these problems and how they may be overcome if the environment established by the seed treatment favours the biocontrol fungi and minimizes competition. Colonization of seeds by biocontrol fungi prior to planting permits the fungus to access the nutrients released by the seed, enabling them to compete with existing microflora and/or plant pathogens.

Xixuan *et al.* (1990) quoted from his review that: "Colonization enhances the ability of the biocontrol agent to utilize seed exudates, which are required for germination and initial growth and development of the seedlings. Some soil-borne pathogens such as *Pythium* exhibit an ability to colonize seeds more readily than the introduced biocontrol fungus. Therefore, it is important to alter the timing of microbial applications on biocontrol fungal-treated seeds to allow the biocontrol fungi to become effective".

Work cited by Harman & Taylor (1988) indicated that pH is a critical factor for germination and growth of biocontrol agents. For example, *Trichoderma* grows optimally at pH levels 4-5 whereas, the bacterium, *Enterobacter cloacae* grew poorly under fairly acidic conditions. In biological seed treatments using *Trichoderma*, low pH favours the fungal protectant and minimizes germination and growth of competitive microflora.

Selected toxicants also need to be considered. Selective toxicants are compounds that are toxic to one group of micro-organisms but have little or no effect on others. Smith *et al.* (1990) demonstrated that integration of selective toxicants, e.g. chemical pesticides, with the biocontrol agent in seed treatments, may extend the activity of the biocontrol fungus to achieve better biocontrol of plant pathogens. For example, metalaxyl is highly effective against pythiaceous fungi that are primary causes of seed rots in cold soils but is non-toxic to *Trichoderma*.

1.3.2.1 *Selected examples of seed treatment delivery systems*

- Solid matrix priming (SMP) of seeds

Bradford (1986) described that seed priming is a controlled hydration of seed to a level that permits pre-germinative metabolic activity to proceed without the actual emergence of the radicle. Harman & Taylor (1988) and Taylor *et al.* (1988) showed that solid matrix priming is a form of seed priming in which seeds are mixed with a solid material such as a ground leonardite shale (Agro-Lig) and water in known proportions.

Harman & Taylor (1988) showed that the efficacy of *Trichoderma* and *Enterobacter cloacae* on cucumber seeds in soil heavily infested with *P. ultimum* was markedly enhanced by SMP. Initial stands with seeds treated by *E. cloacae* increased from 0-70% as a consequence of SMP, and those treated with *T. harzianum* increased from 30-90%. Post-emergence damping-off was not effectively controlled, the overall performance of seeds treated with *T. harzianum* increased from 30% to 90%. Although post-emergence damping-off was not effectively controlled but the overall performance of seeds treated with *T. harzianum* was considerably better than treatment with the chemical fungicide, Thiram (Xixuan *et al.*, 1990). Harman & Taylor's (1988) research showed that *T. harzianum* treated tomato seeds performed as well as those treated with Thiram. However, the activity of *E. cloacae* dropped to nearly zero after SMP treatment due to acidic pH conditions.

- Dust or slurry treatments

Jeffs & Tuppen (1986) established that the simplest seed coating treatment method is the application of dry biocontrol fungal powders to seeds. They confirmed that these materials alone often do not adhere well to the seed surface resulting in poor loading, lack of uniformity, and dust problems. However, active biocontrol fungi may be dispersed or suspended in water and/or adhesives (i.e. stickers, glue, or binders) to form a slurry.

Work done by Xixuan *et al.* (1990) verified that application of protectants in slurries improves uniformity and overcomes other problems associated with dry powder application. Xixuan *et al.* (1990) also indicated that slurry seed treatment with *T. harzianum* is an effective method of controlling *Pythium* damping-off in snap beans. Slurry seed treatments with *T. harzianum* on cucumber seeds were found not to be as effective as that on snap beans. These results indicate that there are variations in host-pathogen–antagonist interactions between different crops.

Hadar *et al.* (1984) looked at improving application methods, which include the use of hard protective coatings or the application of *Trichoderma* in the form of a gel. The gel retains moisture and helps the fungus maintain viability until the seed is planted. For instance, root rot due to *Aphanomyces euteiches* Drechsler can be suppressed when pea seeds are treated with alginate gels of *Trichoderma*. The encapsulated fungus retains a viability of at least 90% for 6 months when stored at 5°C. Hard coatings may be superior to gels. When 20% (w/v) aqueous gel coatings of *Trichoderma* were compared with the hard coatings produced from *Trichoderma* suspended in 20% (w/v) aqueous solutions of polyvinyl alcohol, hard coating was found to be superior. Fewer spores and lower volumes of material were required to reach the same level of pea seed protection against *Pythium* rot. Pea seeds having a *Trichoderma* concentration of 10^4-10^5 propagules per seed were protected as well as those coated with 1.6 ppm Captan (Orthocide) (Anonymous, 2000).

- Liquid coating

Liquid coating technology was developed by Taylor *et al.* (1990) in Geneva, Switzerland for the application of biocontrol fungi to seeds. The above authors demonstrated that a mixture of aqueous binder, solid particulate, and biocontrol fungi could be sprayed onto seeds in a tumbling drum. The biocontrol fungus used was a strain of *T. harzianum.* Finely-ground carbonaceous material served as the solid particulate. A continuous uniform coating with a thickness of less than 0.1 mm was formed during the coating process. Seeds were then sown in a *Pythium*–infested soil in a laboratory bioassay. The efficacy of *Trichoderma* applied through the liquid coating process was greatly enhanced compared to application of the biocontrol fungus using a conventional slurry technique. This improvement was attributed partially due to the physical and chemical properties of the coating, which altered the timing favouring the biocontrol fungus. The liquid coating provides a physical barrier to the ingress of the pathogen. Studies showed that the ingress of *Pythium* was delayed by the liquid coating by about 4 hrs when compared with non-treated seeds or seeds with binder alone. This differential timing allows the *Trichoderma* to become active before pathogen invasion. Moreover, the solid particulate, which is a finely ground leonardite shale (Agro-Lig), has a pH of 4.1, which favors the germination of spores of this fungus (Harman & Taylor, 1980; Taylor *et al.*, 1990).

- Double coating

Another approach is the use of double coatings. Harman (1991) showed that double coating of a seed could change soil growth parameters to favour the biocontrol fungus. For instance, a seed can be coated once with a *Trichoderma* slurry, then again with a slurry containing solid particles of lignaceous shale. The shale layer physically separates the biocontrol fungus from competitive soil microflora. Since the shale has an acidic pH, *Trichoderma* growth is favored over soil bacteria that prefer growth conditions near pH 7.

1.3.3 Drenches and sprays

Moeity & Shatla (1981) and Marois *et al.* (1984) showed that there are several ways to amend soil with a biocontrol agent. The easiest and most direct approach is to drench soil with a suspension of agent propagules. Wilkinson *et al.* (1981) illustrated that depending upon the medium (e.g., natural soil or non-soil mix) in which control is being attempted, there are a few potential limitations to this approach, i.e. the inability to get a thorough infiltration of heavy-textured soils and the propagules of the agent may be filtered by the soil and hence diluted with increasing depth.

It has been shown by Smith *et al.* (1980) that in undisturbed soil cores, passive dispersal through the soil profile during infiltration occurs chiefly through channels and cracks and not through the pore system. Smith *et al.* (1980) suggested that it would be difficult to achieve an even distribution if the target propagules to be affected, or the plant part to be protected, are near the soil surface. Concentration of the agent in this zone may be an advantage, as stated by Smith *et al.* (1980). If, on the other hand, the target propagule or the plant surfaces to be protected are distributed throughout the soil profile, then the concentration of agent propagules in the surface horizon could be a disadvantage. A second limitation may be in adding the agent to the environment in a manner that gives it no competitive advantage against indigenous microflora for the available substrate. Agent propagules, independent of a protective carrier, may be subject to fungistasis and not germinate although they remain viable (Lockwood & Filinow, 1981). Also, exposed propagules may be more susceptible to predators and parasites (Stack *et al.*, 1988).

Hirte *et al.* (1994) proved that fungal spores applied to the soil surface in drenches with or without a minimal wetter, are mainly trapped in the upper 1-5cm by the filtration and absorptive powers of soil. In pots with established plants, penetration depended on soil composition. He also demonstrated that the concentration of conidia increased with depth in the potted medium with only conidia in the highest of three drench volumes penetrating extensively to the lowest levels. This difference is possibly partly related to the different water holding capacities of these soils.

Fungal spores are ideal candidates for oil suspension formulations. Aerial spores of fungi from species as *Gliocladium, Trichoderma*, and *Paecilomyces* can be easily collected from the top surface of solid-state (non-submerged) cultures by vacuum suction. These spores are relatively dry, low in free water and directly suspendable in oil without incurring significant costs for moisture removal (Paau, 1998).

1.3.4 Natural substrates colonized with biocontrol fungi

Numerous attempts have been made by Elad *et al.* (1981) to control several soil-borne pathogens by incorporating natural substrates, colonized by antagonists of the pathogens into the soil. The results of these attempts have varied according to substrate. Lowered disease severity, increased yield, and a decreased pathogen population (*R. solani*) resulted from incorporation of wheat bran colonized by *T. harzianum* in strawberry nursery and field plots.

Rhizoctonia solani on beans and carrots (Strashnow *et al.*, 1985), tomatoes and peanuts (Elad *et al.*, 1982b), potatoes (Elad *et al.*, 1980) and irises (Chet *et al.*, 1982) has been controlled, at least to some extent, by the incorporation of a *T. harzianum*-colonized wheat bran/peat mixture (Sivan *et al.*, 1983). Although wheat bran has been effective in many cases, it was ineffective in controlling *Rhizoctonia* damping-off of radish seedlings (Mihuta & Rowe, 1985). *Laetisaria arvalis* colonized on corn leaf meal or sugar beet pulp prevented an increase in *Pythium* spp. populations in non-sterile soil (Martin *et al.*, 1983). The non-colonized corn leaf meal stimulated *Pythium* populations. It would, therefore, seem very important to ensure a thorough colonization of the substrate by the biocontrol agent (Stack *et al.*, 1988).

In a comparison of natural carriers/substrates, it was reported by Moeity & Shatla (1981), that *T. harzianum*–colonization of barley grain was more effective than colonized wheat or bean

straw in controlling white rot of onion (*Sclerotium cepivorum* Berk). This clearly illustrates the importance of the substrate and carrier system in the ultimate performance of the biocontrol agent. The application procedure should not be evaluated just in terms of the mechanisms of delivery as subsequent agent performance may be a function of the substrate and carrier system utilized. Incorporation of composted hardwood bark into container media has given control of *Rhizoctonia* damping–off of radish seedlings (Nelson *et al.*, 1983). Deliberate and controlled infestation of hardwood bark with selected biocontrol agents may be an approach to pursue (Stack *et al.*, 1988).

1.3.5 Other methods of delivery

Alternative methods of delivery include root dips, direct wound applications and even injection of biocontrol agents into plant tissue. Successful control of *Verticillium* wilt of tomato has been obtained using a root dip of propagule suspensions of *T. viride* and *Penicillium chrysogenum* (Dutta, 1981). Applying *T. viride* (in glycerol) directly to wounds of 40 year old beach trees reduced the level of fungal decay by approximately 15% compared to the controls. For the control of Dutch Elm Disease (*Ophiostoma novo–ulmi* Brasier) direct injection of trees with a fluorescent, which was taken from a pseudomonad, has had some success (Scheffer, 1983). Control was dependent upon the introduction of the bacteria before pathogen colonization, and was also a function of the method of injection (Stack *et al.*, 1988).

1.4 STORAGE AND SHELF-LIFE OF FORMULATIONS CONTAINING BIOCONTROL FUNGI

Biocontrol agents should possess several desirable characteristics, including: ease of preparation and application, stability during transport and storage, abundant viable propagules and good shelf-life (Burges, 1998).

1.4.1 Formulation and viability

A biocontrol agent must be formulated and maintained in a viable or active form. There are a number of technical problems that arise when trying to formulate micro-organisms. Firstly proteins are easily denatured or inactivated by unfavourable environmental conditions. This

may result during the formulation process. Many Hyphomycete fungi produce chlamydospores, conidia, and mycelia that can be formulated and survive the harsh conditions. The key to preservation is to stop germination and reduce metabolism as much as possible. Moore *et al.* (1995) showed that drying of conidia produced on solid media and harvested wet can be accelerated by freeze-drying, vacuum drying and/or desiccants. Additives, notably sugar solutions, improve survival during drying. They serve as carriers in technical concentrates and wettable powders and are thought to act by reducing disruption of membranes by modifying the final moisture content of spores.

1.4.2 Storage of conidia in oil and water

Lewis & Larkin stated that: "Conidia generally mix readily in mineral oil because of their lipophilic surfaces". Survival of undried and pre-dried spores has been compared by Morley-Davies *et al* (1995). In their experiments they proved that pre-drying of spores was found to dramatically improve survival in oil at all temperatures tested. Silica gel pellets were also found to improve storage of undried conidia in one of three batches tested. The combined effect of pre-drying and using silica pellets sometimes exceeded the added effects of either treatment alone. However, pre-dried conidia exhibited a greater germination efficacy after storage as a powder with silica gel pellets than as an oil suspension. Matwele (1986) demonstrated that silica gel acts not only by drying but probably also by absorbing metabolites and preventing growth of contaminants.

Matwele (1986) concluded that: "Survival of spores in water was thought to be linked to a lowered metabolic rate, germination and possible anaerobiosis. Reduction of conidia survival with increasing temperature has been attributed to increased respiration and depletion of endogenous reserves". Savoy & Breitenbeck (1998) showed that various additives have been tested with an aim to depressing spore germination and contaminants. The results were found to be temperature and organism dependent. For example, additives such as maltose, sorbitol and polyethyleneglycol (PEG) were not found to benefit storage of *M. anisopliae* in water (Moore *et al.*, 1995), whereas, starch was found to be the best protectant. Starch, Ringers solution and glycerol improved survival at 4°C in comparison with deionized water, but not at 20°C.

1.4.3 Optimizing spore vigour

Hall & Paperiek (1982) showed that spore vigour is an important criterion when producing conidia on a large scale. The above author mentioned that poor production conditions may stress spores enough to limit shelf-life and impair formulation responses. Hallsworth & Magan (1994) & (1995) stated that media ingredients, light, temperature, humidity, duration of culture, agitation, and conditions during drying and harvest, all require attention. This proved by the above author where in three fungal species, media were modified to reduce content of low molecular polyols (glycerol and erythritol) or trehalose. The resulting conidia survived longer in storage at RH 75%, germinated more quickly and at relatively low water activity, and were more virulent.

1.4.4 Quality of biocontrol fungi

Lumsden *et al.* (1996a) cited in a review that: "As research on the mass production of biocontrol fungi develops, knowledge regarding both requirements of fungal biomass for maintenance of viability and methods of providing suitable storage conditions must be expanded. Biocontrol fungi usually pass through a series of processes during the course of commercial development. Downstream processes include harvesting of spores, desiccation, grinding, formulating packages, storage, and finally delivery". Xixuan *et al.* (1990) continued to demonstrate several factors that need to be considered to obtain a good biocontrol agent that can survive through harsh conditions during storage. They are as follows:

1.4.4.1 *Viability*

Xixuan *et al.* (1990) demonstrated that it is important that a significant proportion of the propagules are viable and able to germinate rapidly. High viability is related to economics because a low percentage of viable propagules equates to a wasted biocontrol product. Dead biomass and residual nutrients from the fermentation medium can act as a nutritional source for pathogens in soil, thereby increasing disease severity. Usually, viability testing is carried out at the end of fermentation to measure fermentation efficiency or after the biomass is dried to measure the propagule viability.

1.4.4.2 *Stability*

Lumsden *et al.* (1996) states that: "Stability is a major goal of present industrial research and development efforts in commercial development viability. The fermentor-produced biocontrol fungi must be stored and preserved for immediate future use". To prevent spoilage by microbial contamination, fungal biomass must be dried through rapid dehydration. Organisms, such as yeasts, have been found to be more stable in a dried state than in their active state (Becker & Rapoport, 1987). Therefore, the dried fungal biomass should have a longer shelf-life than non-dried materials. However, dehydration may be deleterious to many micro-organisms, including *Trichoderma*. The desiccation tolerance of yeast is well reviewed (Becker & Rapoport, 1987), but little has been done regarding filamentous fungi. After drying, the shelf-life of a biocontrol fungus is dependent on storage conditions, such as temperature and relative humidity. In addition, the physiological state and the type of propagule formed may substantially affect shelf- life.

1.4.4.3 *Vigour*

Xixuan *et al.* (1990) concluded that: "Vigour, as distinguished from viability, relates to the relative "strength" or "weakness" of spore germination and germ tube growth. Spores that germinate slowly and support weak germ tube growth generally are not effective as biocontrol agents, even though they may readily germinate in c.f.u". Spore vigour is a reflection of physiological condition and can be strongly influenced by the fermentation system and subsequent downstream processing. For example, varied fermentation conditions can influence the type and amount of nutrient reserves stored in spores. Weak, damaged, or partially dormant spores may require a longer time to germinate and consequently may not be able to compete with the resident micro-flora.

1.4.4.4 *Uniformity*

Quality of a biocontrol fungus must exhibit a high level of uniformity from batch to batch in production runs to ensure adequate efficacy. This is critical for both biocontrol practice and commercialization. To meet this goal, standardized procedures for the mass production of a biocontrol fungus must be established.

1.5 MECHANISMS OF *TRICHODERMA* AND *GLIOCLADIUM* SPP. FOR BIOCONTROL ACTIVITY

In a review, Hornby (1983) states that "a major problem that besets the subject of microbial antagonists in soil is that many of the mechanisms discussed are presumptive and ..... proof is difficult to come by". The mechanisms proposed during the last ten years to explain the biocontrol of plant pathogens by *Trichoderma* or *Gliocladium* are also presumptive. Suggested mechanisms for biocontrol by the two genera are antibiosis, lysis, competition, and mycoparasitism (Chet & Baker, 1980; Ayers & Adams, 1981; Papavizas, 1985). Bell *et al.* (1982) made several attempts to correlate *in vitro* antibiosis, by *Trichoderma* and *Gliocladium* against fungal pathogens, with *in situ* antibiosis in natural systems have usually failed. *In vitro* studies used to discern the mechanisms of action of biocontrol agents has greatly diminished, although research papers still appear on the subject.

Wright (1956) showed that several toxic metabolites are known to be produced *in vitro* by both *Trichoderma* and *Gliocladium*. Some evidence even suggests that such metabolites are produced in organically rich soil. However, Papavizas & Lewis (1983) proved that there is no direct evidence, which unequivocally confirms that these metabolites are produced in significant quantities in soil. It is evident that the intricacies of the soil and plant rhizosphere make it a difficult task to discover the precise mechanism (Papavizas, 1985).

Trichoderma spp. are common to many soil types and are often actively antagonistic to other fungi. Dennis & Webster (1971) showed that many isolates produce volatile and non-volatile antibiotics. From the literature it appears that the most effective antagonistic isolates belong to the species *T. harzianum* (Burges, 1998). However, when its activity was studied, no typical antibiotics could be detected (Hadar *et al.*, 1979; Chet & Baker, 1980; Harman *et al.*, 1980). Sivan *et al.* (1984) reported growing *Trichoderma sp.*, which is antagonistic to *P. aphanidermatum*, on a cellophane membrane placed on agar. By removing the membrane and inoculating the agar with *Pythium* they showed that the growth of the pathogen was partially inhibited by substances produced by antagonists.

In other cases, where antibiosis is not observed, but *Trichoderma* activity in soil is still significant, the possibility of competition (Alexander, 1982) between the biocontrol agent and the pathogen should be considered (Cook & Baker, 1983). In many other instances,

antagonistic *Trichoderma* spp. have been found to be mycoparasites (Hadar *et al.*, 1979; Chet & Baker, 1980; Harman *et al.*, 1980; Chet & Elad, 1982; Elad *et al.*, 1983).

Mycoparasitism is a complex process including several successive steps (Chet *et al.*, 1981). The initial detectable interaction shows that hyphae of the mycoparasite grow directly toward its host (Chet *et al.*, 1981). This phenomenon appears as chemotropic growth of the *Trichoderma* in response to some stimulus in the host's hyphae or toward a gradient of chemicals excreted by the host (Chet & Elad, 1983) (Figure 1.1).

Chemotactic responses in a host–parasite relationship have previously been found in lytic bacteria (Chet *et al.*, 1971) and nematode trapping fungi (Jansson & Nordbring-Hertz, 1979). When the mycoparasite reaches the host, its hyphae coil around host hyphae and are attached to it by means of hook-like structures (Figure 1.2) (Elad *et al.*, 1983). *Trichoderma hamatum* produces appressoria at the tips of short branches (Elad *et al.*, 1983). Following these interactions, the mycoparasite can penetrate the host mycelium, by partially degrading its cell wall (Elad *et al.*, 1983). Dennis & Webster (1971) studied the possible interaction of *Trichoderma* spp. with nylon threads with a diameter similar to that of *P. ultimum* hyphae. *Trichoderma* never coiled around the threads, suggesting that the coiling is not just randomly occurring contact. Moreover, the antagonism of *Trichoderma* has been found to be specific (Chet & Baker, 1980; Elad *et al.*, 1980; Sivan *et al.*, 1984; Chet, 1987).

Electron micrographs indicate that interactions of *Trichoderma* spp. with *S. rolfsii* and *R. solani* hyphae result in enzymatic digestion of hyphal cell walls (Figures 1.3 and 1.4). Extracellular fibrillar material, were found to be deposited between interacting cells.

In response to the invasion, the host produces a sheath matrix, which encapsulates the penetrating hyphae and the host cells then becomes empty of cytoplasm (Elad *et al.*, 1983; Chet,1987).

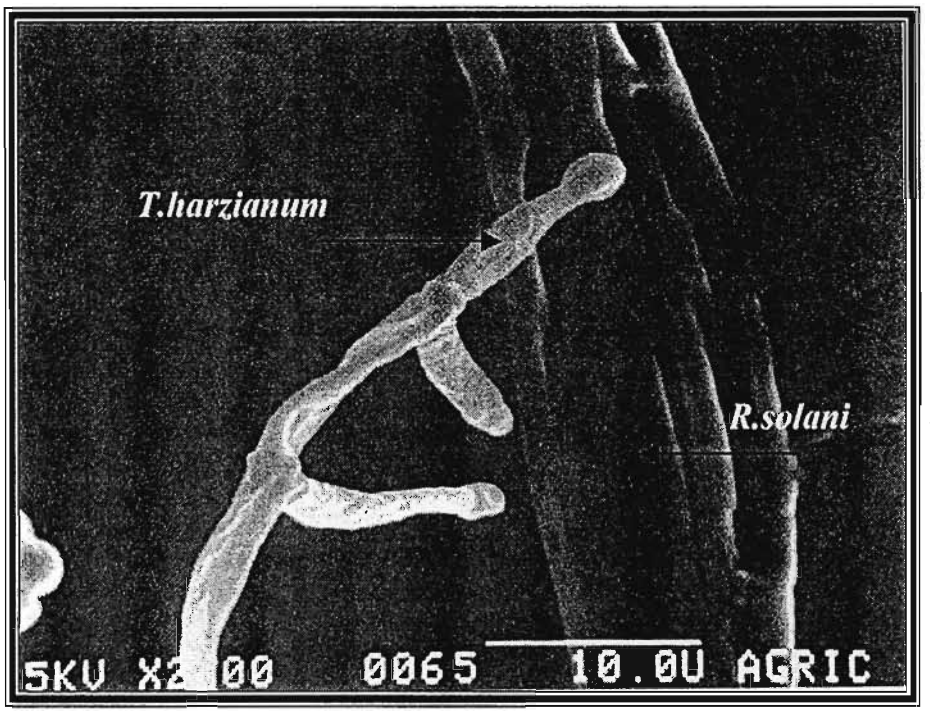

Figure 1.1. Chemotopic growth of *Trichoderma harzianum* toward *Rhizoctonia solani* (X3000) (Elad *et al.*,1983).

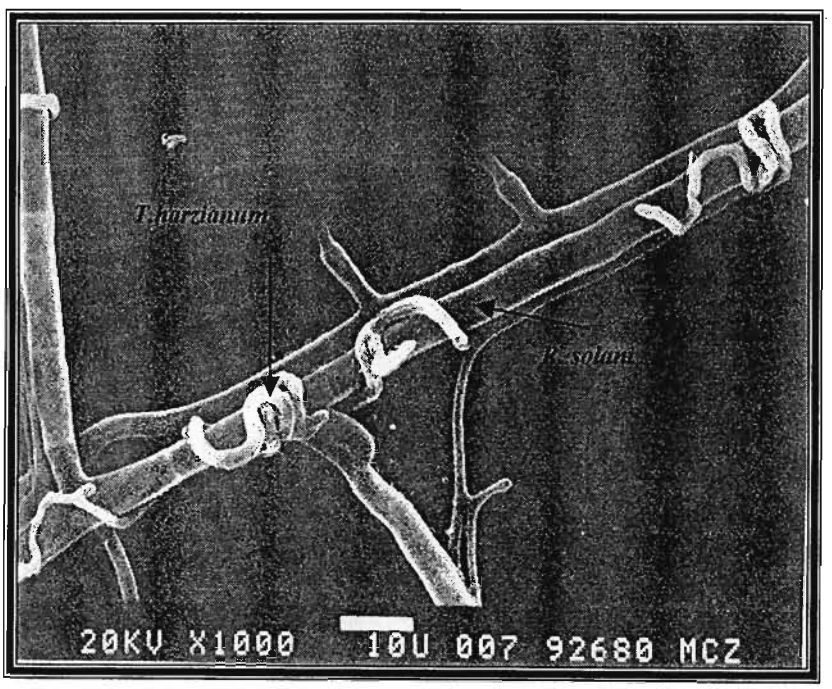

Figure 1.2. Scanning electron micrograph of *Trichoderma* coils around *Rhizoctonia solani* hypha (x1800) (Elad *et al.*,1983).

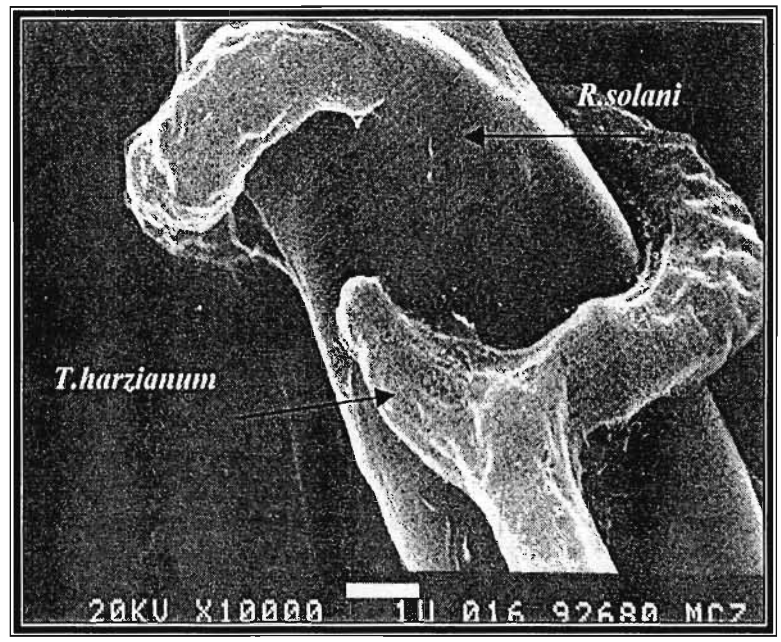

Figure 1.3. Hypha of *Trichoderma harzianum* coiling around and penetrating a hypha of *Rhizoctonia solani* (x8300) (Elad *et al.*, 1983).

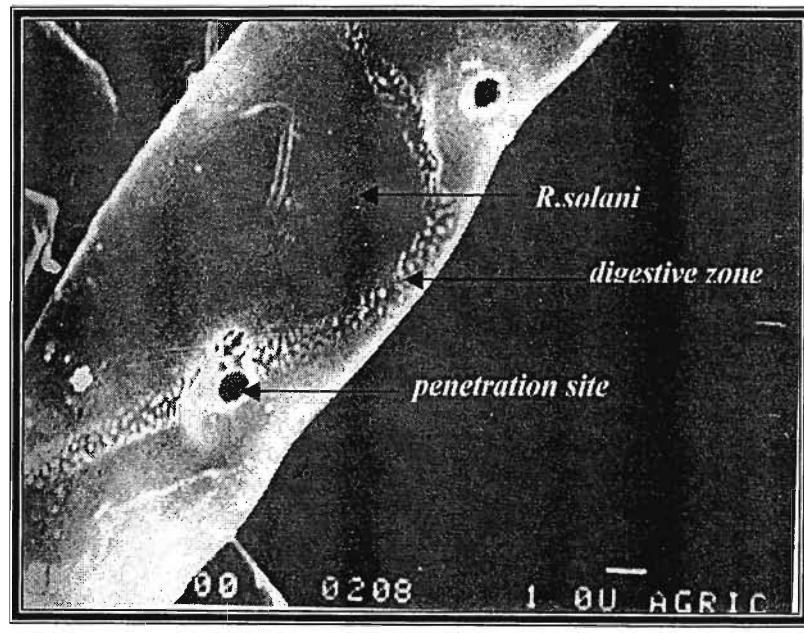

Figure 1.4. Hypha from *Sclerotinia rolfsii* from which a coiling hypha of *Trichoderma harzianum* was removed, showing digested zone with penetration site caused by the antagonist (x5500) (Elad *et al.*, 1983).

1.6 FUTURE PERSPECTIVES OF FUNGAL BIOCONTROL AGENTS: PROBLEMS THAT NEED TO BE SOLVED

From the preceding sections it is clear that much progress has been attributed to the development of fungal biocontrol agents. Butt *et al.* (2001) points out that progress still needs to be made in certain areas of biocontrol agents. It was cited by Butt *et al.* (2001) that: "Technical production, formulation and application systems need to be researched more in depth". Integration of biocontrol agents into cropping systems is also an important factor to consider. From my point of view socio-economic i.e. public perception and economic feasibility also needs to be considered. These issues will be discussed in this section.

Rzewnicki (2000) and Van Arsdall & Franz (2001) pointed out that there is a growing demand for sound, biologically-based pest management practices. Recent surveys by the above authors conclude that both conventional and organic growers indicate an interest in using biocontrol products, suggesting that the market potential of biocontrol products will increase in coming years.

Moore & Prior (1993) stated that the growth rate for biopesticides is expected to increase over the next 10 years, with fungal biocontrol agents probably having a substantial share of this market. This statement has been proved true. Many biocontrol products have been put on the market, i.e., biopesticides and insecticides have been replaced by biocontrol products. However Butt & Copping (2000) have raised issues about the socio-economic and political issues.

1.6.1 Time of action of biocontrol agents

For the development of more efficacious fungal biocontrol agents the speed or time of action of the fungal biocontrol agent is necessary. Criticisms have been made by the speed of action by most biocontrol agents (Harman, 2001). It has been stated by many reviewers that biocontrol agents act slowly and give limited protection to crops from pests and diseases. For effective pest control, strains should be more aggressive and work faster with less inoculum. Butt *et al.* (1998) & Amiri-Besheli *et al.* (2000) showed that enzymes and metabolites have

been identified which are important determinants of virulence or antagonism. In the case of disease control in the rhizosphere more attention needs to be given for effective control.

1.6.2 Survival of biocontrol agents through environmental conditions

To improve field efficacy of fungal biocontrol agents, formulations must be able to tolerate a range of climatic factors. These factors include, fluctuating temperatures, humidities, ultraviolet (UV) light, edaphic factors such as soil types and biotic factors such as antagonists and predators. The ability of these strains to proliferate and reduce disease is by physiological manipulation of the growth conditions, i.e., by using endogenous reserves stored in conidia and chlamydospores. These endogenous reserves can result in fast germination of these propagules (Butt *et al.*, 2001).

Costs of manufacturing a formulated biocontrol agent are largely an enormous problem for small to medium sized enterprises. Commercial products of biocontrol fungi need to be viable for up to 2-3 years when stored under commercial storage conditions and packaging. For cost-effective production of biocontrol products, conditions for the organisms need to be optimized to overcome problems resulting in loss of biocontrol without increasing production costs (Paulitz & Belanger, 2001).

Burges (1998) stated that when good biocontrol agents are recently formulated and applied to the target host or directly to the field, the outcomes are much higher when compared to formulations that were stored for a period before use. Formulations need to be further researched to overcome this problem as it is of limited use if a good biocontrol agent performs well only when applied freshly and not when applied after a period of storage. Perhaps some progress needs to be focused on more efficient additives that can prolong the shelf-life of organisms and be compatible with other organisms such as viruses nematodes and bacteria. This will result in farmers integrating biocontrol products with other products and pesticides.

1.6.3 Packaging

Another technical issue is the packaging of biocontrol agents. Packaging of biocontrol agents should be simple and easy to follow and should be similar to those used by farmers for other chemicals. This may increase the sales of these products. Farmers willing to use biocontrol

agents may be more comfortable handling products in the same way as normal chemicals (Menn & Hall, 1999).

1.6.4 Population dynamics

Inyang *et al.* (1999a & 1999b) demonstrated that the host plant can affect the efficacy of biocontrol agents through dilution of inoculum during growth and physical interference of the inoculum, i.e. trapping spores in epicuticular waxes and via exudates and allelochemicals. These interactions need to be fully investigated.

Leal-Bertioli *et al.* (2000) points out that the population dynamics of the added biocontrol agent need to determined. The relationships between entomogenous fungi and their hosts need to be studied in the field and to identify the vulnerable stages of the target. The above author also mentions that studies on the population dynamics could also provide invaluable information on fungal persistence in the environment, the fate of the inoculum and any genetic shift due to parasexual or sexual recombination.

1.6.5 Compounds released by biocontrol agents

Strasser *et al.* (2000) mentions that the compounds released by biocontrol agents are of a major concern as many people of the public believe that they are a health risk. This concern may decrease the demand of biocontrol products on the market. Strasser *et al.* (2000) suggested that further tests and investigations need to be done on the toxins released by these biocontrol agents. Strasser *et al.* (2000) poses questions that need to be answered: "Are they pathogenicity determinants? Do they help the survival of the biocontrol agent? Are they waste products? Also do they pose a risk to living organisms, and do they have any commercial value as pharmaceutical drugs?".

1.6.6 Integration of biocontrol agents with chemicals and pesticides

The use of other chemicals and pesticides with biocontrol agents is often a big question. This should be an essential question or task to be done when formulating or looking for a biocontrol agent. Many farmers often want to know whether biocontrol agents can be in the same tank mix as other chemicals. For example, entomogenous fungi may be harmful to

entmophilic nematodes, or fungicides used for disease control may kill entomogenous fungi. Industries should work in close relation with extension officers to resolve the problem (Butt *et al.*, 2001).

1.6.7 Spectrum of activity

Biocontrol agents seem to fall short when products fail to develop when temperatures, relative humidity and other environmental conditions fall outside its defined spectrum of activity (Paulitz & Belanger, 2001). This is particularly true in the field where crops represent their own set of varying environmental factors, antagonists and predators, which may alter the proliferation of the biocontrol agent in the field.

1.6.8 Adjuvants and additives

Many formulations prepared with biocontrol agents include additives or adjuvants that enhance the efficacy of the biocontrol agent. It has been demonstrated by Belanger & Menzies (1998) that these additives are fungicidal and are insecticidal and could contribute to the total effect of the biocontrol agent or may itself be the protectant against disease. These additives or adjuvants could contribute to the misleading of the true potential of the biocontrol agent. It is therefore important to conduct trials with proper controls to prove that additives or adjuvants do not contribute to the effect of the biocontrol agent.

1.6.9 Organic farming

In Europe, organic farming is expanding. In some countries organic farming consists of 8% of farms (Butt *et al.*, 2001). Funding of projects that integrate agronomic practices used by organic farmers can be considered with natural agents for increased productivity. Due to policies and regulations such as the European community (EC) 2078/92, which stands for "Agricultural production methods compatible with the requirements of the protection of the environment and the maintenance of the countryside" and EC regulation 2078/91 which stands for " Certified for organic food" has led farmers to convert to organic farming due to the premium prices for organic grown produce (Rzewnicki, 2000).

1.6.10 Cost - effective production of fungal biocontrol agents

Cultural conditions of biocontrol agents need to be identified without increasing production costs, to alleviate problems of loss of biocontrol. The production costs need to be decreased to allow competitive pricing with conventional pesticides. The biocontrol products must remain viable for up to 2-3 years under commercial storage conditions and maintain biocontrol efficacy. It has been stated by Butt *et al.* (2001) that at this present time, little progress has been made in this area, partly because the underlying mechanisms of attenuation have not been fully elucidated.

1.6.11 Application and after- care of organisms

Due to biocontrol agents being live and particulate many problems arise during application. The time of the application of biocontrol organisms is important. Many reviews have stated that attempts have been made to make products more user-friendly. This has gained momentum and will continue as a trend in the future. The after- care of the biocontrol agents has to be researched further.

Burges (1998) has reviewed some strong points that need to be considered on this subject. The above author has mentioned that information on the compatibilities of organisms with chemicals needs to be made more widely available and methods of application also require attention. Statements were also made by Moorehouse (1990) and Morley-Davies (1995) that sub-surface inoculation of soil leads to wastage when organisms lodge on the upper surface of soil particles, where they are exposed to sunlight and become desiccated.

Burges (1998) pointed out that inoculation could be carried out by drilling, which is convenient at seed planting, or by surface placement through direct incorporation by rotation or ploughing. Sprays, slurries and treated seed have also been used. Seed application effectively places organisms in direct contact with the infection court of the plant. Pre–treated seed are relatively user-friendly, because they need no formulation by the user. Subsurface inoculation can be integrated with many routine agricultural practices, including application of fertilizers.

Several experimental formulations are currently being investigated. Compounds can be added to formulations to give biocontrol organisms a selective advantage in soil. For example, an exotic nutrient source in the formulation could favour growth of the biocontrol agent. This could be a nutrient that the biocontrol organism requires naturally, or a trait that could be specifically introduced into the organism in the formulation. However, much of the work done on the different types of formulations does not attempt to involve costs and the amount of labour involved. Questions raised by Burges (1998) include,

- If certain carriers or humectants are needed, how many different compounds should be tried?
- What conclusions of each compound should be drawn?
- How often should the formulated materials be checked, not just for viability, but also for efficacy?
- What are the limits of shelf-life?

1.6.12 Conclusion

There are several challenges that scientists are confronted with in producing a good biocontrol agent. The challenges lie at the agricultural, environmental, and human and animal health levels. As science and technology are moving towards a more technological era, most of the challenges can be overcome.

Biocontrol of plant pathogens has not solved all problems. Perhaps during the technological era in the next few years significant advances will be reached. These solutions will only be achieved when scientists in various disciplines, i.e. engineering industry and government agencies collaborate.

Due to the upcoming technological era natural plant resistance and transgenic plants may be fully supported by the public and genetic engineering may result in more superior strains. There is still some scope for exploiting the natural resistance of plants to diseases and integrating this into an overall sustainable crop protection strategy. Transgenic plants are another opportunity to overcome diseases but are slandered by the public.

Communication between industries, research institutes and the grower need to be developed to promote a stronger extension service. This extension service should be strengthened by government bodies, which are responsible for biocontrol agents. Policies and procedures to reduce the product development time and costs need to be changed.

It should be pointed out that in order to achieve commercial success all trials and challenges must be overcome. The study of biocontrol thus needs to be a balanced one involving technical development, marketing research, and a close working relationship with regulatory bodies. This requires a very dynamic team of workers and provides exciting opportunities for people in this field. The challenges of biocontrol agents should not be viewed as problems that cannot be overcome but a challenge that will bring a hopeful future.

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CHAPTER 2

EFFECT OF CULTURE CONDITIONS ON SPORE SHELF-LIFE OF BIOCONTROL AGENT *TRICHODERMA HARZIANUM* KMD

J. Omarjee[1], M.D. Laing[1] and C.H. Hunter[2]

Disciplines of Plant Pathology[1], and Microbiology[2], SAES,

University of Natal, Private Bag X01, Scottsville, Pietermaritzburg, South Africa

Trichoderma harzianum has been used successfully as a biological control agent against several soil-borne plant pathogens. Biological control agents should possess several desirable characteristics for good shelf-life and stability during storage. The influence of pH, C:N ratio, carbon content and harvesting time on spore production and ultrastructure of the biocontrol agent *Trichoderma harzianum* KMD was evaluated. Spore viability and shelf-life of pelleted spores were also determined. Significant differences were found for pH, C:N ratio, carbon content and harvesting time of *Trichoderma harzianum* KMD. Mycelium development and sporulation were positively and significantly affected by acidic conditions (pH 4.0). Increasing carbon concentration and C:N ratios increased spore production times. Spore viability was significantly better when harvested from a medium with a C:N ratio of 14 at pH 4.0 even after 45 days of storage. These spores remained viable during a seven day period of storage at a rH of 12%. Ultrastructural studies showed that these spores had two distinct cell wall layers in the outer cell wall and large numbers of lipid bodies were present in the cytoplasm. This research showed that shelf-life of *Trichoderma harzianum* KMD spores can be manipulated by culture, as well as storage.

2.1 INTRODUCTION

Formulated biological control products should possess certain characteristics such as good market potential, being simple to prepare and apply, stability during transportation and storage, and abundant viable propagules with good shelf-life all at an acceptable cost (Churchill, 1982; Liansky, 1985; Powell & Faull, 1989). Jackson & Schister (1992) showed that liquid cultivation has been employed to produce spores from a number of biocontrol agents such *as Trichoderma* spp. Carbon concentration and the carbon to nitrogen (C:N) ratio

of the culture medium significantly influenced the number of spores produced as well as the morphology of cell wall structure and overall efficiency in controlling diseases. Papavizas & Lewis (1989) and Lewis *et al.* (1990) mentioned that preparations of *Trichoderma* sp. and *Gliocladium* sp. containing chlamydospores have been found to be more effective in preventing disease than preparations containing conidia only. Papavizas *et al.* (1984) studied the operating conditions during fermentation (i.e., aeration, pH, temperature) as well as media composition and found that these conditions may profoundly effect the quality and quantity of the spores produced.

An additional factor to consider in liquid fermentation is the rate of biomass production, which impacts on the cost of production as well as viability of spores and the likelihood of contamination. It is desirable to obtain the optimum amount of biomass in the shortest time. With isolates of *Trichoderma* and *Gliocladium*, satisfactory quantities of spores and biomass have been obtained in 6-7 days, but this time period is still long when compared with that for bacteria (2 days) (Papavizas *et al.*, 1984).

For biocontrol products to be effective, spores need to germinate readily and rapidly. Agosin *et al.* (1997) cited that: "Drying and storage of spore formulations challenge the cellular system to maintain cell viability". Germination processes and accompanying metabolic activities have been extensively studied by Weete (1980), but relatively little attention has been given to spore lipid content. Fungal spores contain variable quantities of lipids depending on the conditions under which they are formed. There are numerous reports correlating increased spore lipid content with enhanced germination. Lipid bodies, or globules, have been widely observed in spores of most fungal species examined to date using electron microscopy. The accumulation of lipids in fungal spores has been described as being a reserve energy source. This is supported by observations that lipid bodies disappear during germination (Weete, 1980). It has been demonstrated that lipids can serve as endogenous sources of energy for spore germination (Weete, 1980). Lipid accumulation in *Trichoderma* species was studied by Serrano-Carreon *et al.* (1992). These authors looked at the ability of these fungi to synthesize lipids on different carbon and nitrogen sources.

The aims of this study were to investigate the effect of several culture parameters on the viability and shelf-life of *Trichoderma harzianum* KMD spores. Mycelial growth and sporulation under defined liquid media with different pH, C:N ratios, and carbon

concentrations were assessed and ultrastructural studies and morphological differences of spores were also examined.

2.2 MATERIALS AND METHODS

2.2.1 Organisms

Trichoderma harzianum KMD, was originally isolated from soil obtained from Tala Valley, KwaZulu- Natal, South Africa (Machaba, 1998). The isolate was supplied by Plant Health Products[1]as a formulated product. The *Trichoderma* isolate was isolated directly from the formulation by plating onto V8 agar (Appendix A). The formulation was initially activated with 0.5 M HCℓ before plating out, to prevent contamination of bacteria. Cultures were maintained on silica gel (Davis & Serres, 1970) and periodically subcultured on V8 agar plates (Appendix A).

2.2.2 Inocula

Inocula used for the culture studies, were grown on V8 agar (Appendix A). Each plate was inoculated with a 3ml spore suspension containing approximately $1x10^7$ spores/ml. The inoculum was then spread, using the hockey stick spread plate technique, and then incubated at 28°C for three days until a dense sporulating mycelial matt formed. Spores were harvested by scraping them off the surface of the mycelial matt with a spatula and then suspending them in distilled water. The spore suspensions were then vortexed (Vortex, Thermolyte 16700 mixer) for three minutes. Spore counts were made using a Petroff-Hausser Counter (Thomas Scientific). Spore suspensions were then adjusted to approximately $6x10^6$ spores/ml by dilution.

2.2.3 Media and Culture conditions

Unless otherwise stated, a defined basal culture medium was used throughout this study (Appendix B). All experiments were repeated three times and each treatment consisted of three replicates. Analyses of data were conducted by analysis of variance (ANOVA) and were separated by Student Newmans Keul Test. Statistical analyses were conducted using the general linear procedure of SAS Version 6.08 (SAS, 1987).

(i) The evaluation of the effect of pH and C:N ratio on the kinetic parameters for spore production by T. harzianum KMD

This experiment was carried out using 250ml Erlenmeyer flasks containing 100ml basal culture medium. These flasks were agitated in an orbital shaker (Psychrotherm E.S.) at 200 rev/min. at 28°C for 92h –168h. The desired C:N ratios were obtained by adding 2.7 g/l , 12 g/l and 53 g/l glucose to the basal medium giving rise to C:N ratios of 3, 14 and 60 respectively. The pH of the media was adjusted to 7.0 or 4.0 with 50mM potassium phosphate and sodium tartrate buffers, respectively. For each culture 5ml samples were collected and analysed. A Petroff-Hausser chamber (Thomas Scientific) was used to quantify spore numbers by direct counting. Mycelial biomass was filtered through cheesecloth every 6h and dried at 60 ± 1°C for 24h in an oven (Gallenkamp) (Munoz & Agosin, 1993). Growth rate (cells h^{-1}), biomass yield ($Yx_{/s}$, g biomass/g carbon), spore yield ($Y_{P/s}$, spores/g biomass) and volumetric productivity (Q_P, spores/l culture medium/h cultivation) were calculated (Atkinson & Mavituna, 1991). Growth rate and biomass yield were calculated from biomass dry weights and the remaining parameters were determined by direct counting of spores using a Petroff-Hausser counting chamber.

(ii) The effect of pH and C:N ratio on shelf-life and ultrastructure of T. harzianum KMD spores

This experiment was set up as previously described in Section 2.2.3 (i). Culture media with C:N ratios of 3 and 14 at pH's of 4.0 or 7.0 were used. During cultivation, 1ml samples were removed 36, 60 and 92h and centrifuged (Eppendorf Centrifuge 5410) at x10,000g for 10 minutes and the supernatant discarded. The resulting spore pellet was resuspended in 1ml sterile distilled water and centrifuged again at 12,000g for 10 min, discarding the supernatant.

Fresh spore samples were processed for electron microscopy studies. Samples were fixed for 12h in 3% (v/v) gluteraldehyde in 0.2M cacodylate buffer (pH 7.4) and then washed three times in the same buffer and post-fixed in 1% (w/v) osmium tetroxide for 2 minutes. The

[1] Dr Mike Morris, Plant Health Products, P.O.Box 207, Nottingham Road, South Africa

samples were then dehydrated in a graded alcohol series and finally embedded in Epon 812 resin. Sections 80nm in width were cut with an LKBIII Ultramicrotome. Sections were then stained with uranyl acetate and an alkaline solution of lead citrate (Reynolds, 1963). Samples were examined and viewed using a Philips CM 120 BioTwin Transmission Electron Microscope at an acceleration voltage of 80kV.

To determine shelf-life, remaining culture media were filtered through compacted glass wool after 60h or 92h. The filtrate was centrifuged (Beckman, JA20) at x10,000g for 10 minutes and the supernatant discarded. The resulting spore pellet was resuspended and then adjusted to $6x10^6$ spores/ml using a Petroff–Hausser counting chamber. Colony forming units (c.f.u's) were determined on V8 agar plates (Appendix A) after storage and incubation at 25°C. Prior to storage the adjusted spore pellets were placed in a dessicator for 24h at 25°C. Pellet samples were then placed in a humidity chamber equilibrated to a rH value of 75% and maintained at 25°C. Saturated solutions of NaCℓ, were placed in the humidity chamber to obtain this rH value (Rockland, 1960). Spore samples were analysed to determine the number of viable spores after brief storage of an initial period of 7-8 days by c.f.u counts on V8 agar and incubated at 25°C. A long term storage of these spores were tested in later tests (Table 2.3).

(iii) The effect of carbon concentration on shelf-life of T. harzianum spores at pH 4.0

This experiment was designed as in Section 2.2.3 (i). A range of carbon concentrations were made up by preparing 3.0, 6.0, 12.0, 24.0, and 48.0 g/l of glucose while maintaining the C:N ratio at 3 or 14. A C:N ratio of 3 was obtained by adding 1, 2, 3, 8, 16g/l of NH_4SO_4, respectively. A C:N ratio of 14 was obtained by adding 0.214, 0.43, 0.86, 1.72, 3.42g/l of NH_4SO_4 to each culture medium respectively. pH was adjusted to 4.0 using a 50mM potassium phosphate buffer. Culture were inoculated with 2ml of *T. harzianum* KMD inoculum and incubated for 92h at 28°C. Culture media were filtered through compacted glass wool after maximum sporulation. Maximum sporulation was determined by removing 1ml samples every 6h and counting spore numbers using a Petroff-Hausser counting chamber. The filtrate was centrifuged (Eppendorf Centrifuge 5410) at x10,000g for 10 minutes and the supernatant discarded. The resulting spore pellet was resuspended in 1ml sterile distilled water and centrifuged again at 12,000g for 10 minutes. The supernatant was then discarded.

The spore suspension was adjusted to $6x10^6$ spores/ ml by direct count using a Petroff-Hausser chamber centrifuged at x12,000g to obtain a pellet which was then processed for storage. Appropriate storage conditions were obtained by placing the adjusted spore pellet ($6x10^6$ spores/ml) in a dessicator for 24h at 25°C. Pellet samples were then placed in a humidity chamber equilibrated to a value of 75% rH and maintained at 25°C. Spore samples were analysed after seven days of storage by direct c.f.u. counts on V8 agar plates (Appendix A) and incubated at 25°C for three days.

(iv) The effect of rH on viability of T. harzianum spores

This experiment was designed as in Section 2.2.3 (i). C:N ratios were obtained by increasing the initial glucose content from 2.7 g/l to 12 g/l giving rise to C:N ratios of 3 and 14, respectively. pH conditions of media were obtained by adjusting to pH 4.0 with 50mM potassium phosphate. Cultures were filtered through compacted glass wool after 60h. The biomass was dried and dry mass measured. The filtrate was centrifuged (Eppendorf Centrifuge 5410) at 10,000g for 10 minutes and the supernatant discarded. The resulting spore pellet was resuspended in 1ml sterile distilled water and centrifuged again at x12,000g for 10 minutes discarding the supernatant. The spore suspension was adjusted to $6x10^6$ spores/ml by direct counts using a Petroff-Hausser chamber and thereafter centrifuged again at x12,000 g for 20 minutes. Colony forming unit counts were determined at harvesting by plating out a dilution series onto V8 agar plates (Appendix A). The adjusted spore pellets were also processed for storage after seven days and 45 days at 25° C. Prior to storage the adjusted spore pellets were placed in a dessicator for 24h at 25°C. Pellet samples were then placed in humidity chambers equilibrated to rH values of 12%, 44% and 75% respectively, and maintained at 25°C. Saturated solutions of LiCℓ, Na_2CO_3 and NaCℓ, respectively were placed in different humidity chambers to obtain these rH values (Rockland, 1963). Spore samples were analysed after seven and 45 days by direct spore count using a counting chamber and c.f.u count on V8 agar (Appendix A) incubated at 25°C.

2.3 RESULTS

2.3.1 Evaluation of the effect of pH and C:N ratio on the kinetic parameters for spore production by *T. harzianum* KMD

Significant differences were found between most parameters tested. The pH and C:N ratio were found to have a profound effect on both growth and sporulation of *T. harzianum* KMD (Table 2.1)

Table 2.1 Effect of pH and C:N ratio on the kinetic parameters for spore production by *Trichoderma harzianum* KMD under different culture conditions

Culture condition*		Parameter♣				
PH♦	C:N	Growth rate (h^{-1})	Biomass Yield $Yx_{/s}$(g/g carbon)	Spore Yield $Y_{P/s}$ ($x10^9$ spores/g biomass)	Volumetric Productivity Q_P ($x10^8$ spores /g)	Maximum Sporulation Time (h)
4	3	0.1 ab	1.125 bc	8 ab	4 b	93
4	14	0.15 ab	2.29 a	10 a	5 a	96
7	3	0.07 c	0.56 c	4 c	3 b	94
7	14	0.9 a	1.88 ab	7 b	4 b	96
7	60	0.05 c	0.112 c	5 bc	2 c	168

*Kinetic parameters were determined in 250ml Erlenmeyer flasks containing 100ml of basal medium inoculated with *T. harzianum* KMD and agitated at 200 rev/min and incubated at 28°C. Spores were harvested at the time the maximum yield was reached as determined by direct count after every 6h intervals.

♣Values are the mean of three replicates; values within a column having the same alphabetical subscript are not significantly different at the 95% confidence interval. Means with the same letter are not significantly different according to the Student, Newman and Keuls comparison test.

♦ Media were adjusted to the required pH values with 50mM of potassium phosphate and sodium tartrate prior to autoclaving.

h^{-1}, growth rate; $Y_{x/s}$, biomass yield; $Y_{P/s}$, spore yield; Qp, volumetric productivity.

Spore yields and overall growth rates were found to be greater and statistically significant at pH 4.0 than pH 7.0. Cultures with C:N ratios of 14 at pH 4.0 achieved a significantly greater biomass and spore yields than those maintained at C:N ratios of 3 and 60 as well as those maintained at pH 7.0 For each pH tested the growth rate, biomass yield and volumetric productivity of the cultures were statistically greater at a C:N ratio of 14 than at 3. An

increase in C:N to 60, at pH 7.0, resulted in decreases in these parameters as well as in a significant delay in maximum sporulation time.

2.3.2 The effect of pH and C:N ratio on shelf-life and ultrastructure of *T. harzianum* KMD spores

The percentage viability of spores after a seven day storage period was greater and statistically significant at pH 4.0 than 7.0. Spores produced in C:N 14 at pH 4.0 and harvested after 92h were found to have a higher viability and was highly significant when compared to those maintained at C:N 3 as well those maintained at pH 7.0 before and after a seven day storage period. Time of cultivation of spores at 60h and 92h also had an effect on spore viability. Spores obtained from cultures that were harvested at 92h with a C:N 14 and at pH 4.0 were observed to have a higher viability and was significant than those harvested at 60h (Table 2.2).

Table 2.2. Effect of culture conditions on shelf-life of *Trichoderma harzianum* KMD spores.

Culture condition			Log viable spores/ ml after*			
PH	C:N ratio	Time♦ (h)	0 days♣	%Viable spores@ harvesting	7 days♠	%Viable Spores after 7 days
4.0	3	60	6.21± 0.01	27 ab	4.36 ± 0.06	0.38 b
		92	5.42 ± 0.02	4.38 c	4.38 ± 0.12	0.39 b
	14	60	5.71 ± 0.02	8.5 b	5.01± 0.07	1.70 ab
		92	6.64 ± 0.15	72 a	6.13 ± 0.18	22.4 a
7.0	3	60	6.11 ± 0.03	21.47 ab	2.46 ± 0.13	0.004 c
		92	5.90 ± 0.03	13.23 ab	2.48 ± 0.08	0.004 c
	14	60	5.43 ± 0.07	4.4 c	3.36 ± 0.03	0.038 bc
		92	5.36 ± 0.08	3.8 c	3.31 ± 0.09	0.034 bc

*For each sample, spore suspensions were prepared and then adjusted to (6.0 ± 0.6) x 10^6 spores/ ml and c.f.u.s/ml was then determined.

♣c.f.u.s were determined immediately after harvesting.

♠ c.f.u.s were determined after seven days storage at 25°C and 75% RH

Each value is the mean followed by the standard error of two experiments. Each treatment consisted of three replicates. Means with the same letter are not significantly different according to the Student, Newman and Keuls comparison test.

♦Time period at which spores were harvested from *T. harzianum* KMD cultures.

Ultrastructural differences between spores cultivated under various media conditions were evident (Plates 2.1, 2.2, 2.3 and 2.4). Differences were identified in the structure of cell walls, presence of lipid globules /bodies and the presence of extracellular mucilage layers.
Spores produced in the pH 4.0 media were found to have extracellular mucilage layers (Plate 2.1 and 2.2). Spores produced at C:N 3 and pH 4.0 after 36h exhibited little extracellular mucilage layers surrounding them (Plate 2.3). These spores had an outer wall (W2) layer, which varied in size with age from 36h to 92h. Spores obtained from culture media at pH 4.0 and C:N 3 at 36h revealed numerous mitochondria and ribosomes in the cytoplasm (Plate 2.1). Lipid bodies were also observed. The outer wall layer (W2) appeared thicker at 92h (0.107μ m) than at 60h (0.102μm) (Plate 2.1 and Plate 2.4). Numerous round mitochondria were present in spores harvested after 60h. Lipid bodies were found to be small and were present within the cytoplasm accompanied by large numbers of ribosomes. After 92h large lipid bodies were found together with few irregular shaped mitochondria. Spore diameter increased from 2.05μm at 60h to 3.38μm at 92h (Plate 2.1). Spore diameter and cell wall thickness was measured using an Image analyzer (analySIS® [SISP PRO, Version 3.0, Germany]. Spores obtained from C:N 14 at pH 4.0 (Plate 2.7) after 92h had two wall layers i.e., an inner wall layer (W1) and an outer wall layer (W2). Large numbers of lipid bodies were also present.

Spores obtained from media with a C:N 3 at pH 7 after 36h (Plate 2.2) had a thin outer wall layer (W2) (0.068μm). Few lipid bodies and mitochondria were present. Spores were found to be more oval in shape compared to spores obtained from the pH 4.0 media. After 60h spores exhibited a thicker cell wall (W2) of 0.079μm (Plate 2.5). Large numbers of vacuoles and ribosomes were present. Mitochondria were irregular in shape.

The outer wall (W2) layers increased to 0.108μm after 92h (Plate 2.6) These spores were oval in shape and not many lipid bodies were apparent in the cytoplasm.

Qualitatively, the most noticeable differences among all spores were the presence and size of lipid bodies and extracellular mucilage layers, which were most prominent under acidic conditions. The most striking differences between spores produced in acidic media were the formation of thick cell walls (W1 & W2) and large numbers of lipid bodies (Plate 2.7).

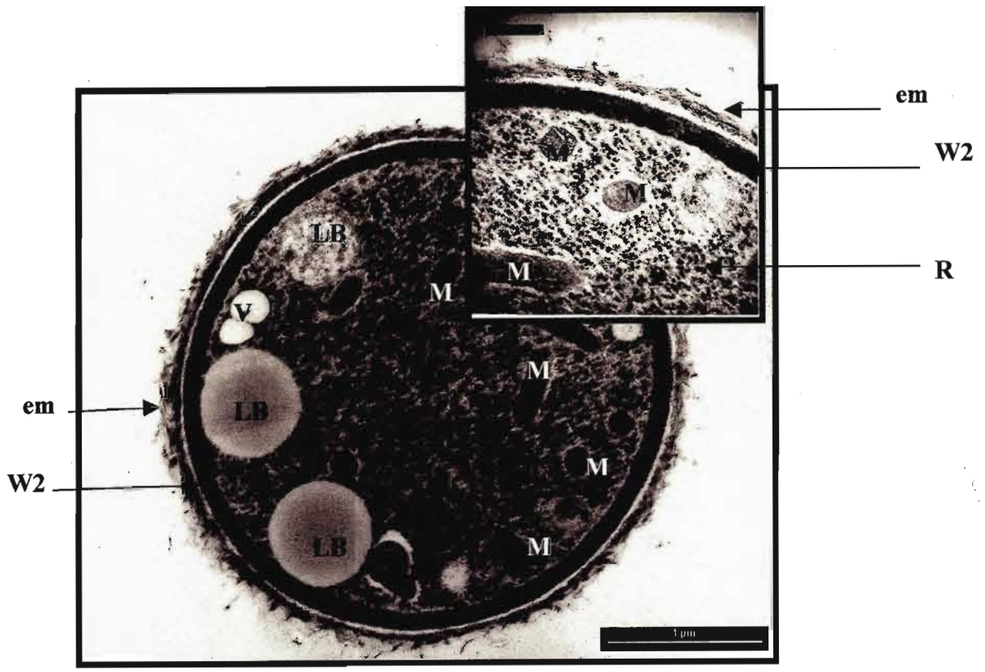


Plate 2.1

TEM micrographs of *Trichoderma harzianum* KMD spores in a liquid medium with a C:N ratio of 3 at pH 4.0. Spores were harvested at 92h. **W2**, outer wall layer; **M**, mitochondrion; **LB**, Lipid bodies/ globules; **mi**, membrane invagination; **em**, extracellular mucilage layer; **R**, ribosomes; **V**, vacuoles.

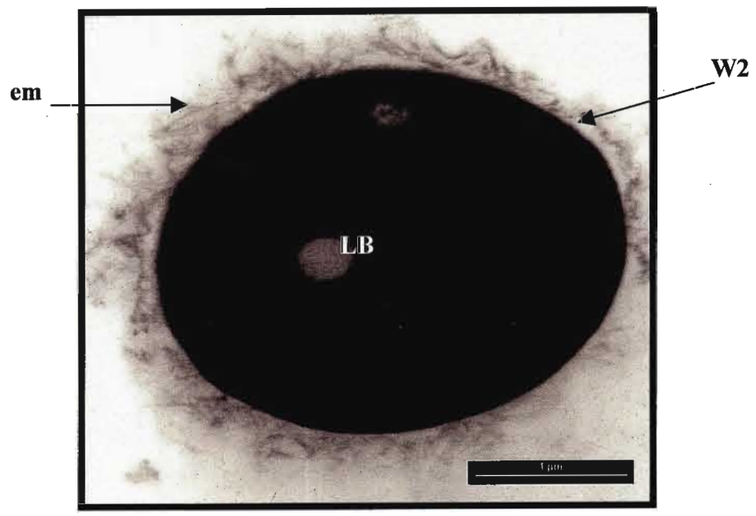


Plate 2.2

TEM micrographs of *Trichoderma harzianum* KMD spores in a liquid medium with a C:N ratio of 3 at pH 7.0. Spores were harvested at 36h. **W2**, outer wall layer; **LB**, Lipid bodies/globules; **em**, extracellular mucilage layer.

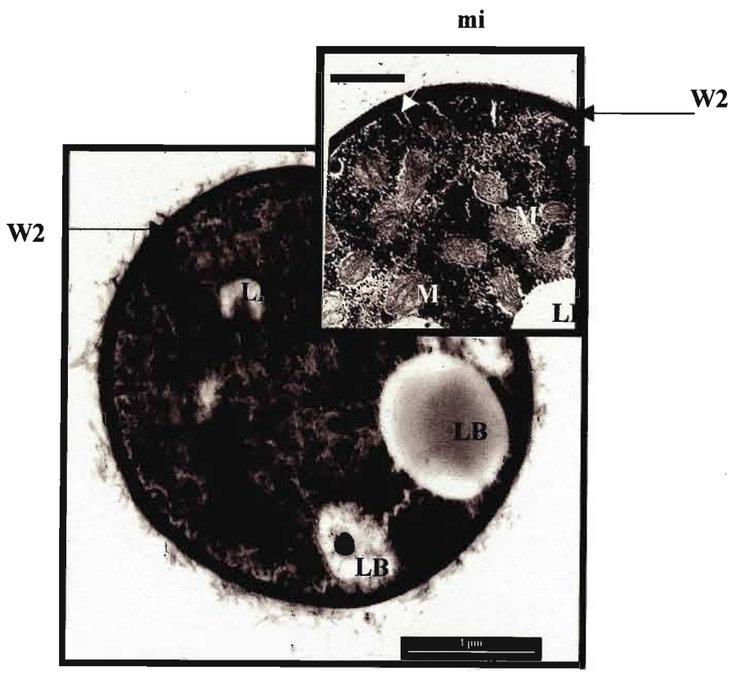


Plate 2.3

TEM micrographs of *Trichoderma harzianum* KMD spores in a liquid medium with a C:N ratio of 3 at pH 4.0. Spores were harvested at 36h. **W2**, outer wall layer; **M**, mitochondrion; **LB**, Lipid bodies/ globules; **mi**, membrane invagination; **em**, extracellular mucilage layer; **R**, ribosomes; **V**, vacuoles.

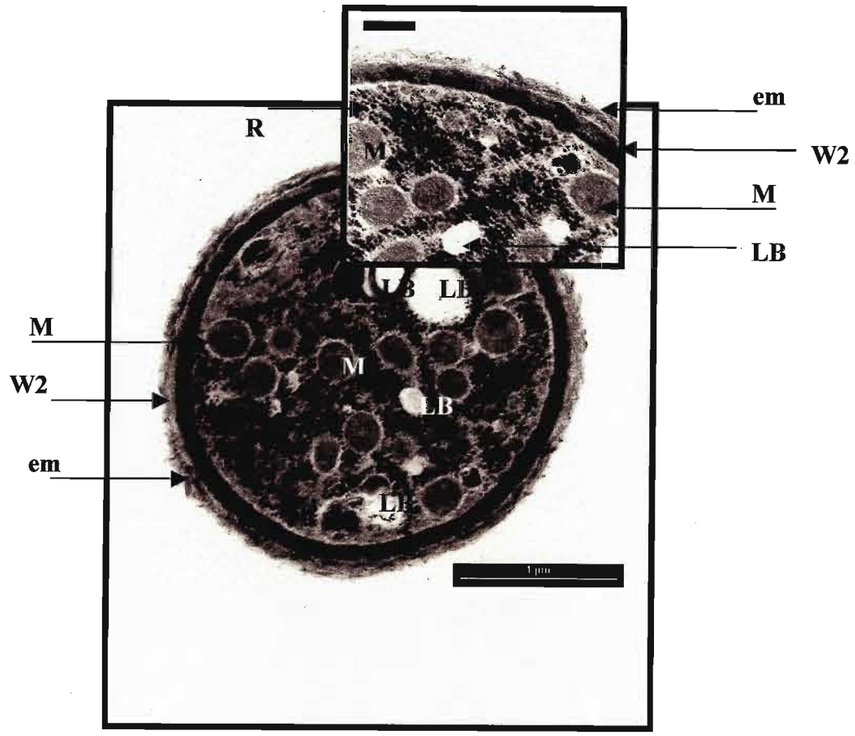


Plate 2.4

TEM micrographs of *Trichoderma harzianum* KMD spores in a liquid medium with a C:N ratio of 3 at pH 4.0. Spores were harvested at 60h. **W2**, outer wall layer; **M**, mitochondrion; **LB**, Lipid bodies/ globules; **mi**, membrane invagination; **em**, extracellular mucilage layer; **R**, ribosomes; **V**, vacuoles.

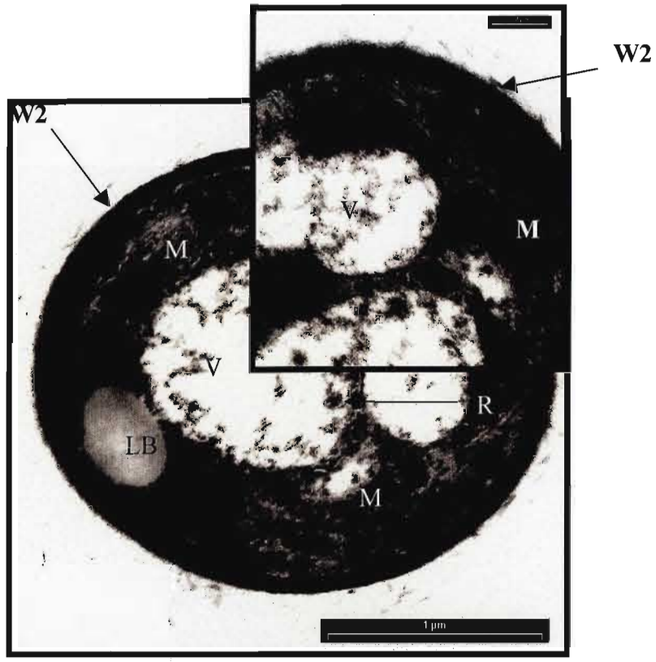


Plate 2.5

TEM micrographs of *Trichoderma harzianum* KMD spores in a liquid medium with a C:N ratio of 3 at pH 7.0. Spores were harvested at 60h. **W2**, outer wall layer; **M**, mitochondrion; **LB**, Lipid bodies/ globules; **mi**, membrane invagination; **em**, extracellular mucilage layer; **R**, ribosomes; **V**, vacuoles.

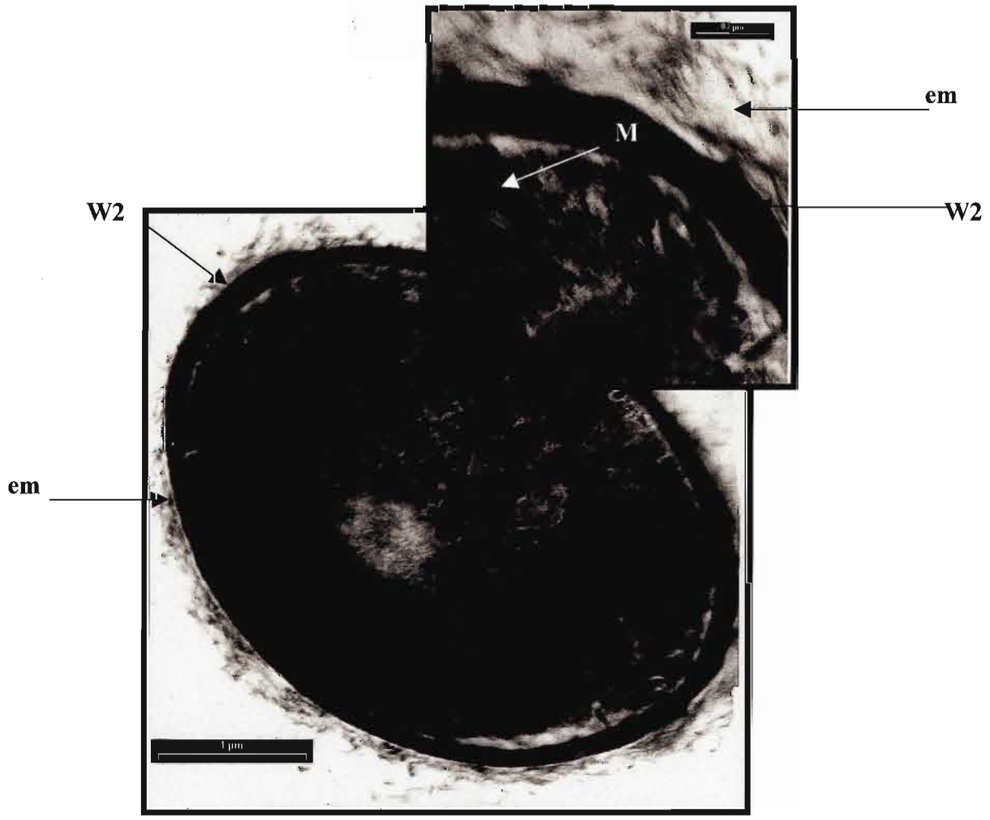
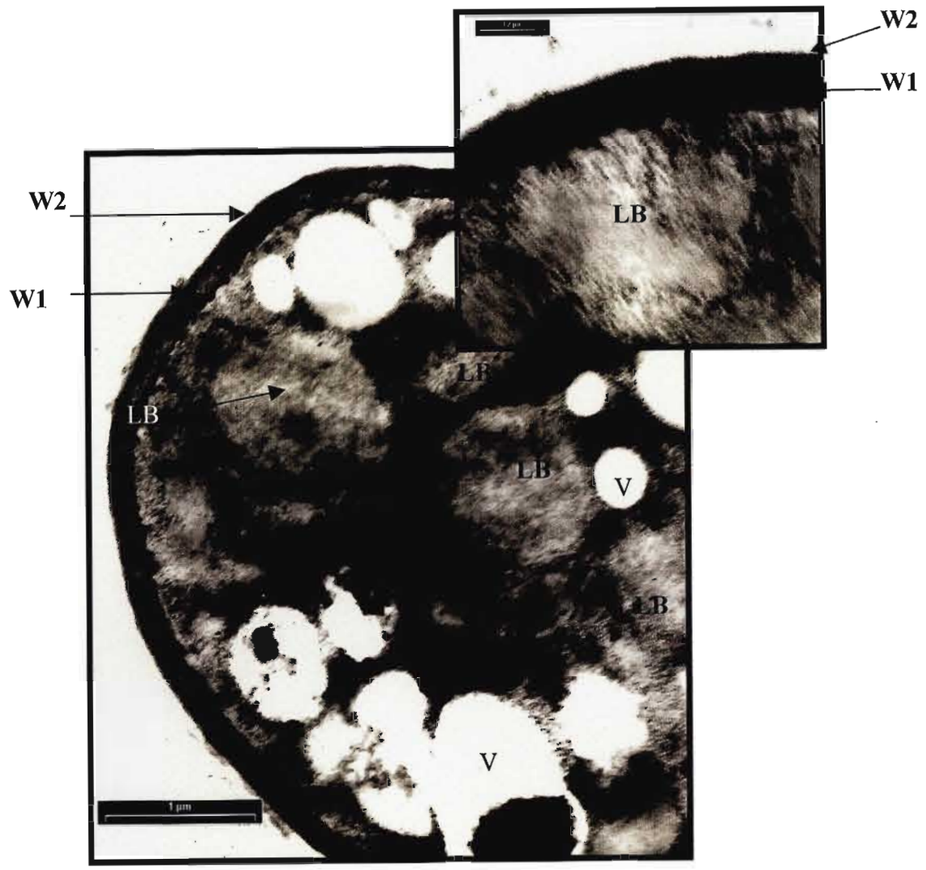


Plate 2.6

TEM micrographs of *Trichoderma harzianum* KMD spores in a liquid medium with a C:N ratio of 3 at pH 7.0. Spores were harvested at 92h. **W2**, outer wall layer; **M**, mitochondrion; **LB**, Lipid bodies/ globules; **mi**, membrane invagination; **em**, extracellular mucilage layer; **R**, ribosomes; **V**, vacuoles.

Plate 2.7

TEM micrographs of *Trichoderma harzianum* KMD spores in a liquid medium with a C:N ratio of 14 at pH 4.0. Spores were harvested at 92h. **W2**, outer wall layer; **M**, mitochondrion; **LB**, Lipid bodies/ globules; **mi**, membrane invagination; **em**, extracellular mucilage layer; **R**, ribosomes; **V**, vacuoles.



2.3.3 The effect of carbon concentration on shelf-life of *T. harzianum* spores at pH 4.0

To determine the effect of C:N ratio and carbon concentration on spore longevity, spores were cultured in media with increasing concentrations of glucose, while constant C:N ratios of 3 and 14 at pH 4.0 were maintained. These results are shown in Table 2.3, Figure 2.1, 2.2, 2.3.

Carbon concentration had a limited effect on spore shelf-life. Spore yield and biomass that were obtained from each culture media were found to be similar with an increase in glucose content and biomass (Figure 2.1 and Figure 2.3). The viability of spores after seven days storage was similar within media containing carbon concentrations at fixed C:N ratios (Table 2.3). Spores harvested at different C:N ratios recorded significant differences in spore shelf-life. It was evident that the C:N ratio had an effect on longevity of spores irrespective of carbon content. C:N 14 produced spores that were more viable and was not statistically significant than those produced at C:N 3 regardless of an increase of carbon content. Maximum sporulation time took longer as the carbon concentration increased (Figure 2.2).

Table 2.3. Effect of carbon concentration on shelf-life of *Trichoderma harzianum* KMD spores * at pH 4.0

Initial glucose concentration	Total Biomass (g)		Max. sporulation time (h)		LogViable spores after 7days	% viable after 7days	LogViable spores after 7days	% viable after 7days
g/l	C:N 3	C:N14	C:N 3	C:N 14	C:N 3	C:N 3	C:N 14	C:N14
3.0	0.1 c	0.25 c	90	92	2.2± 0.08 a	0.0002 a	5.1± 0.03 a	2.09 a
6.0	0.24bc	0.75 c	98	120	2.6± 0.02 a	0.0006 a	5.4± 0.08 a	4.18 a
12.0	0.99 b	1.29 b	108	138	3.2± 0.2 a	0.002 a	4.8± 0.03 ab	1.05 ab
24.0	1.39 a	2.86 a	120	162	2.7± 0.6 a	0.0007 a	5.3± 0.01 a	3.32 a
48.0	2.01 a	2.34 ab	160	168	2. ± 0.07 a	0.0009 a	4.7± 0.01 ab	0.8 ab

*A C:N of 3 or 14 was maintained, at pH 4.0, in all cultures tested. Spores were harvested at the time the maximum yield was reached, and processed for storage. After seven days at 75% rH, percentage viable spores were assessed as described in Table 2.2. Means with the same letter are not significantly different according to the Student, Newman and Keuls comparison test.

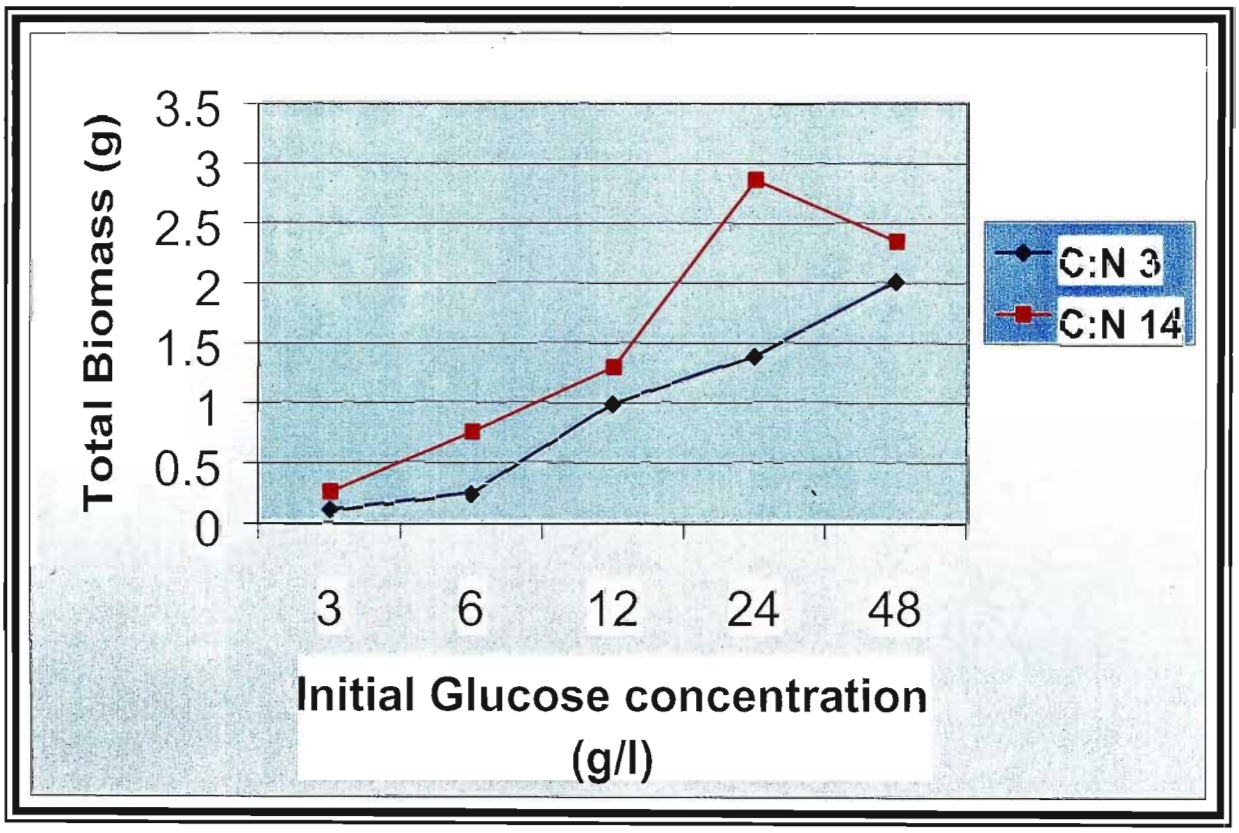


Figure 2.1 Effect of carbon concentration on total biomass of *Trichoderma harzianum* KMD spores.

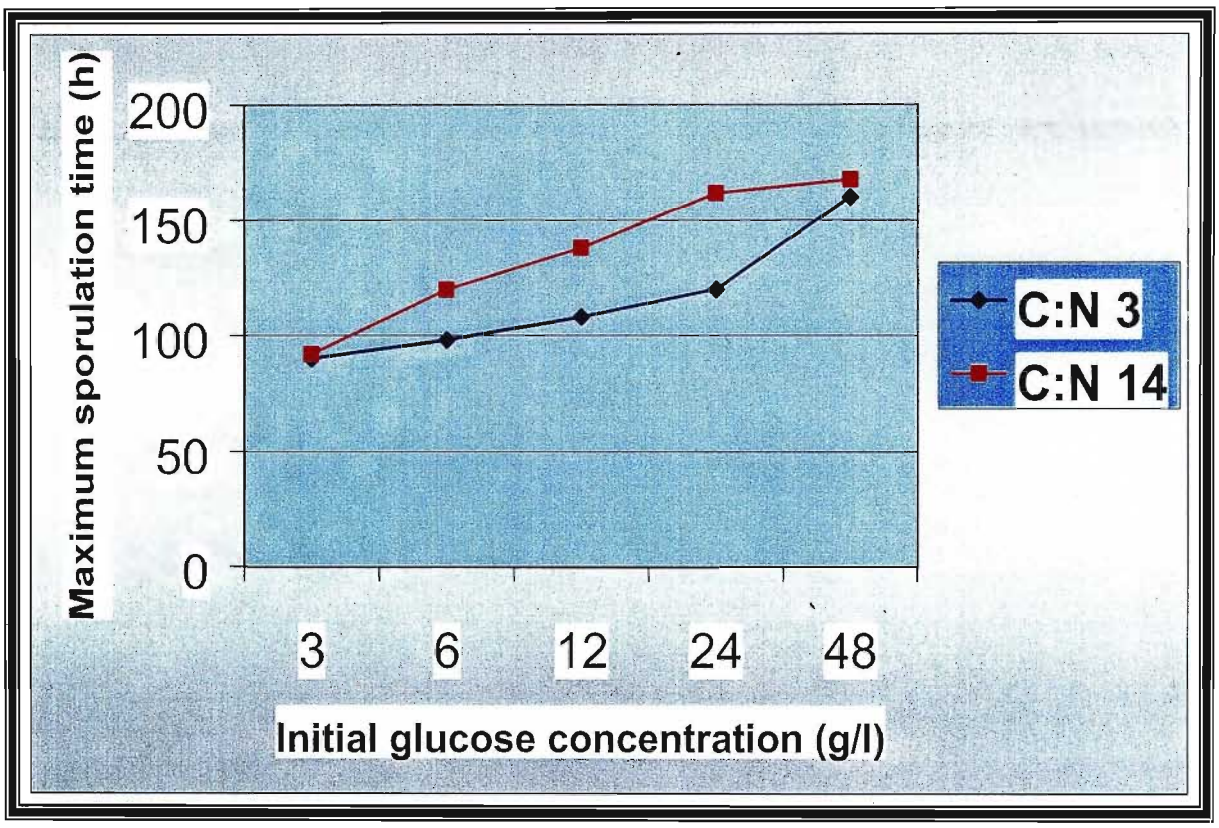


Figure 2.2 Effect of carbon concentration on maximum sporulation time of *Trichoderma harzianum* KMD spores.

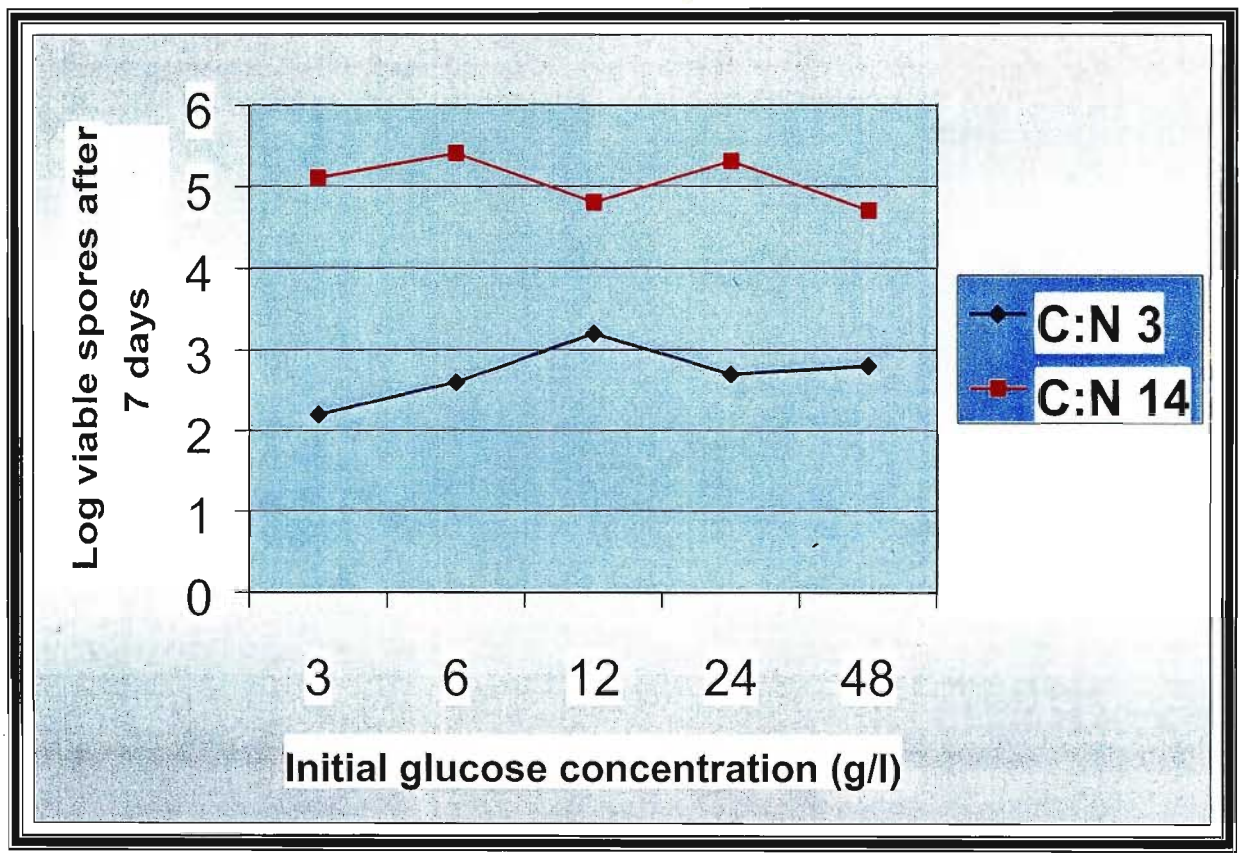


Figure 2.3 Effect of carbon concentration on viable of *Trichoderma harzianum* KMD spores after seven days of storage at 75% rH.

2.3.4 The effect of rH on viability of *T. harzianum* spores

The effect of rH on viability of *T. harzianum* KMD spores over a 45 day period is shown in Table 2.4 and Figure 2.4.

Table 2.4. Effect of relative humidity (rH) on shelf-life of *Trichoderma harzianum* KMD spores at different storage times* at pH 4.0 within a 45 day period

		Log viable spores/ml* after		
rH (%)	**C:N ratio**	**0 days**	**7 days**	**45 days**
75	3	6.1 ± 0.03 (20.9) b	5.6 ± 0.53 (6.6) ab	4.16 ± 0.09 (0.24) b
	14	6.2 ± 0.04 (20.9) b	5.1 ± 0.01 (2.09) ab	4.90 ± 0.03 (1.32) b
44	3	6.0 ± 0.02 (16.6) bc	5.60 ± 0.04 (6.6) ab	4.31 ± 0.08 (0.34) b
	14	6.5 ± 0.06 (52) a	6.13 ± 0.02 (22.48) a	5.08 ± 0.18 (2) b
12	3	6.1 ± 0.03 (20.9) b	5.60 ± 0.08 (6.6) ab	6.25 ± 0.12 (2,9) b
	14	6.1 ± 0.04 (20.9) b	6.15 ± 0.09 (23) a	6.30 ± 0.13 (33.32) a

**T. harzianum* spores were harvested after 60h cultivation in a defined medium at pH 4.0 and processed for storage at different relative humidities (12%, 44%, 75%).
Values in parentheses indicate % of viable spores (c.f.u.).
*Results are expressed as described in Table 2.2.
Each result is the mean followed by the standard error of two experiments. Each treatment consisted of three replicates. Means with the same letter are not significantly different according to the Student, Newman and Keuls comparison test.

This experiment was carried out at pH 4.0 as this pH provided the best results for both growth and sporulation. Spore viability declined significantly with storage at seven days and 45 days for all three rH levels tested. Spore viability from spores produced at a C:N 14 was significantly better than at C:N 3 after 45 days. When rH was reduced, spores had significant differences in shelf-life between C:N media. Spores grown at C:N 14 retained the greatest viability and was significantly different at 12% rH.

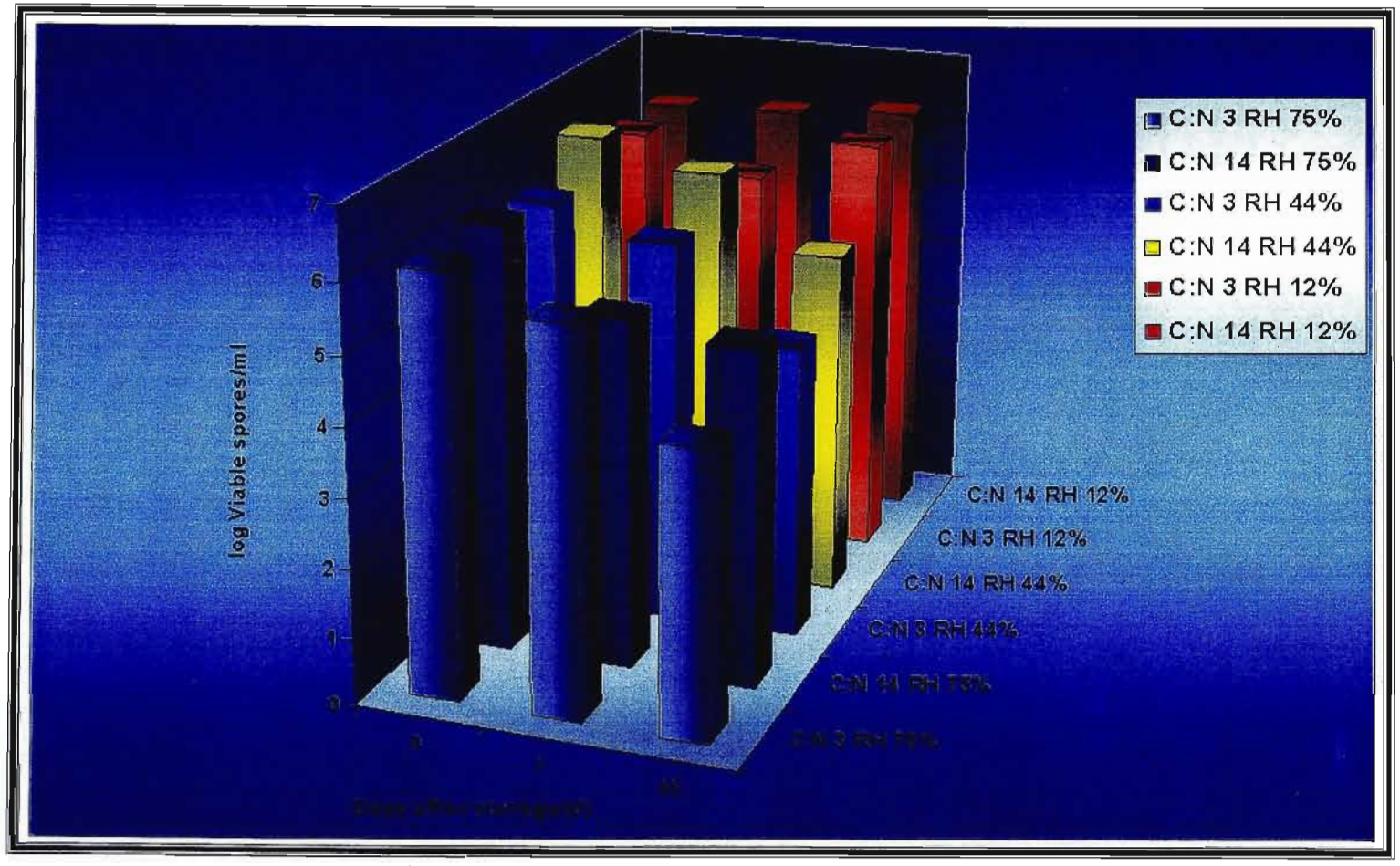


Figure 2.4. Histogram of relative humidity on shelf-life of *Trichoderma harzianum* KMD spores at different storage times at pH 4.0 after a 45 day period.

2.4 DISCUSSION

Trichoderma harzianum can be an effective biocontrol agent of several pathogenic fungi, e.g. *Rhizoctonia solani, Sclerotium rolfsi* and *Pythium ultimum* (Elad *et al.*, 1993). Conidia have been widely employed as the most effective means to apply *Trichoderma* in biocontrol programmes (Elad *et al.*, 1993). Spores must be durable, and viable to exert effectiveness in the field to control pathogens.

From the results of the experiments reported here, it was evident that pH, harvesting time and C:N ratio affect spore viability, numbers and shelf-life. Differences in ultrastructure and morphological differences of the spores produced were also observed. pH 4.0 was observed to be more suitable for cultivation of *T. harzianum* KMD due to higher and significant growth rates, biomass yields, spore yields and volumetric productivity (Table 2.1). C:N ratio also had a significant effect on both growth and sporulation of *T. harzianum* KMD (Table 2.1). In the three C:N ratios tested, C:N 3 appeared to have an excess of nitrogen while in C:N 14 there was a more suitable balance of glucose and nitrogen to be consumed simultaneously whereas at C:N ratio of 60, nitrogen levels appeared to be limiting.

A C:N ratio of 3 contained excess amounts of nitrogen, resulting in significantly lower growth rates and volumetric productivity compared to C:N 14. C:N 14 contained amounts of glucose and nitrogen that could have been consumed simultaneously and thus had higher growth rates, biomass yields, spore yields, and volumetric productivity. Due to C:N 60 recording low growth rates, biomass yields, spore yields and volumetric productivity, this C:N ratio was not persued in further investigations.

C:N ratios had a significant effect on maximum sporulation time with the maximum sporulation time increasing with an increasing C:N ratio (Table 2.1). The maximum sporulation times for C:N ratios of 3, 14 and 60 were 92, 92 and 168h respectively (Table 2.1).

The influence of four growing media were investigated on the spore ultrastructure and shelf-life of *T. harzianum* KMD spores (Table 2.2). Shelf-life of spores was affected by the time period at which spores were harvested from the cultures. Spores harvested after 92h at a C:N ratio of 14 at pH 4.0 were significantly more viable after seven days of storage at a rH of

75%. Similar results were recorded for spores cultured at C:N of 3 at pH 4.0. Ultrastructural studies showed that these spores were more viable due to the presence of thicker cell walls and the presence of many lipid globules which may have increased their shelf-life (Plate 2.1, 2.3, 2.4, and 2.7) (Munoz *et al.*, 1995).

Low C:N ratios in the medium favour protein synthesis and high ratios favour lipid accumulation (Weete, 1980). Spores cultivated from C:N ratios of 3 had large numbers of ribosomes and mitochondria and few lipid bodies, i.e., high protein synthesis. Large amounts of mitochondria and presence of ribosomes in spores cultured at C:N ratio of 3 at pH 4.0 (Plate 2.1, 2.3, 2.4) indicated high protein synthesis which may encourage, spores to assume a germination-like state with time (Munoz *et al.*, 1995).

The accumulation of lipids is another factor, which has increased shelf-life in spores. The most important nutritional parameters for lipid production by fungi are the C:N ratio (Weete, 1980).The accumulation of lipids as globules/bodies in fungal spores serve as reserve material, particularly for energy and this was supported by observation that the lipid bodies disappear during germination (Weete, 1980). The presence of lipid bodies enhance germination of spores thereby increasing their viability after storage (Weete, 1980). Large amounts of lipid bodies were found in spores cultured at C:N 14 at pH 4.0. (Plate 2.7) These spores were more viable than those cultured at pH 7.0 (Table 2.2). These findings could be due to the large numbers of lipid bodies present, hence serving as energy reserves during germination (Weete, 1980).

The presence of thick cell walls may have also contributed to spore's shelf-life. Spore walls of *T. harzianum* KMD are formed by two layers, the outer (W2) being notably more electron dense than the inner (W1) (Munoz & Agosin, 1993). W2 is spore specific (Rosen *et al.*, 1974). W2 is the spore's first barrier and therefore the first possible defense against adverse conditions (Agosin *et al.*, 1997). The presence of spore walls (W1&W2) and the thickness of spores harvested in C:N 14 and pH 4.0 after 92h could have acted as a stronger barrier against the adverse conditions in the rH chamber, hence its long shlelf-life. The two wall layers were only formed after 92h and were not present on spores at 60h of culturing.

Another factor to take into consideration was the presence of many extracellular mucilage layers found around all spores produced at C:N ratio of 3 and pH 4.0, protecting them from

dessication (Munoz *et al.*, 1995). Little or no mucilage layers were found in C:N ratio of 3 and 14 at pH 7.0. Large amounts of extramucilage layers were formed on spores of C:N ratio of 3 at pH 4.0 after 36h (Plate 2.3), C:N ratio of 3 at pH 4.0 after 60h (Plate 2.4) and C:N ratio of 3 at pH 4.0 after 92h (Plate 2.1) protecting these spores from dessication (Munoz *et al.*, 1995). Spores at pH 7.0 were observed to be less viable, more ovoid, with no production of two cell wall layers, fewer lipid bodies and extracellular mucilage layers.

As carbon concentration increased, the biomass significantly increased (Table 2.3). Spore yields from each culture medium within the C:N ratios were examined to be similar as glucose content increased with an increase in biomass, but did not support higher spore production yields. This suggests that some inhibitory compounds could be produced together with sporulation (Bodo *et al.*, 1985) or alternatively that the production of biomass is not so critically dependant upon C:N as is spore production.

Storage conditions showed a marked effect on shelf-life and spore viability being best under low rH (12%) (Table 2.4). Under these conditions, spores would dry rapidly, maintaining them in a nearly dehydrated state (Fahey *et al.*, 1978) as a consequence metabolic deterioration reactions would be retarded.

This research showed that the shelf-life of *T. harzianum* KMD spores can be manipulated by culture medium, as well as storage conditions. The results show that the culture system employed for the production of fungal spores can be determined by the quality and biocontrol efficacy of the resultant spores. pH, cultivation time and C:N ratios are important in obtaining the most effective spores of *T. harzianum* KMD for biocontrol propagules. The culture conditions used in this research hold promise for further research on biochemical processes involved in fungal spore tolerance to desiccation.

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CHAPTER 3

ULTRASTRUCTURE OF MYCOPARASITISM OF *TRICHODERMA HARZIANUM* KMD ON *RHIZOCTONIA SOLANI*

J. Omarjee[1], M.D. Laing[1] & C.H. Hunter[2]

Disciplines of Plant Pathology[1], and Microbiology[2], SAES,

University of Natal, Private Bag X01, Scottsville, Pietermaritzburg, South Africa

Mycoparasitic activities of *Trichoderma harzianum* KMD against *Rhizoctonia solani* were studied using *in vitro* bioassays and scanning electron microscopy (SEM). Macroscopic observations of fungal growth in dual cultures revealed that *T. harzianum* KMD made hyphal contact with the pathogen within four days of inoculation, leading to an inhibition of pathogen growth. SEM investigations demonstrated that *T. harzianum* KMD bound firmly to *R. solani* hyphae by coiling around the hyphae. Penetration of the hyphal cell wall by hooks, haustoria and appressoria-like structures were observed, usually followed by cell disruption. Contact of *T. harzianum* KMD with *R. solani* resulted in the disintegration and collapse of the pathogen hyphae. This was attributed to the production of lytic enzymes. It is hypothesized that the outcome of the interaction of antagonist and pathogen was most likely determined by initial hyphal contact that triggered a series of events in pathogen destruction.

3.1 INTRODUCTION

The modes of antagonistic action by *Trichoderma* in biological control have not been fully elucidated, however, several mechanisms have been described. Diffusible inhibitors (e.g., antibiotics and mycotoxins) have been identified and shown to suppress soil-borne pathogens (Harman & Hadar, 1983; Eveleigh, 1985; Chet, 1987; Lynch, 1987). Volatile inhibitors (e.g., alcohol, ketones and sesquiterpenes) are also produced by several isolates of *Trichoderma* spp. Some of these volatile metabolites may have stimulatory effects at low concentrations and inhibitory effects at higher concentrations. Chet (1987) showed that competition may play an important role in microbial interactions but it may be an independent phenomenon or it may be connected with other mechanisms. Mycoparasitism may also play a key role in the

antagonistic nature of *Trichoderma.* Mycoparasitism is a complex process that involves several successive steps. Elad *et al.* (1982) showed that *Trichoderma* hyphae may grow toward the target hyphae because of chemotrophism. Chet (1987) demonstrated that when the parasitic hyphae reach the host, galactose residues on their cell walls bind to a lectin on the host hyphal cell wall. Harman *et al.* (1981) showed that once binding has occurred, the parasitic hyphae tend to coil around the target host. Once recognition has occurred, *Trichoderma* will excrete extracellular enzymes (e.g. cellulases and chitinases) and may penetrate into the host, show rapid vacuolation, collapse, and subsequent lysis (Chet, 1987). Several mechanisms such as mycoparasitism, antibiosis, and competition may occur with any given strain.

Boland (1990) cited that: "Considerable attention has been paid to fungal antagonists such as isolates of *Trichoderma* and *Gliocladium* spp. because of their ability to attack pathogens at different stages of their development". Lumsden (1992) illustrated that a prerequisite for rational utilization of potential antagonists is an understanding of the mechanism underlying the antifungal activity. A study was therefore, undertaken to determine the mode of action of *Trichoderma harzianum* KMD against a target pathogen, *Rhizoctonia solani* using *in vitro* bioassays in conjunction with scanning electron microscopy.

3.2 MATERIALS AND METHODS

3.2.1 Fungal isolates

Trichoderma harzianum KMD used in this study, was originally isolated from soil obtained from Tala Valley, KwaZulu- Natal, South Africa (Machaba, 1998). The isolate was supplied by Plant Health Products[1] in a formulation. The *Trichoderma* isolate was isolated directly from the formulation by plating onto V8 medium (Appendix A). The formulation was initially activated with 0.5 M HCℓ before plating out, to prevent contamination by bacteria. Cultures were maintained on silica gel (Davis & Serres, 1970) and periodically subcultured on V8 medium (Appendix A). The *R. solani* isolate was obtained from C. Clark[2].

[1] Dr Mike Morris, Plant Health Products, P.O.Box 207, Nottingham Road, South Africa
[2] C. Clark, Discipline of Plant Pathology, School of Applied and Environmental Science, University of Natal, Scottsville, Private Bag X01, Pietermaritzburg, South Africa

3.2.2 Dual culture tests

To study hyphal interactions, mycelial agar plugs (4mm diameter, cut from the edge of a five day old mycelial mat on V8 agar) of the antagonist, *T. harzianum* KMD and the pathogen *R. solani,* were placed on opposite sides of 90mm diameter Petri dishes containing V8 medium. The dual culture test was replicated five times and incubated for nine days on a laboratory bench at ambient room temperature (25-26°C) under fluorescent light. Controls of the antagonist and pathogen were also prepared on V8 medium in Petri dishes and incubated for nine days at ambient room temperature. Mycelial plugs (4mm in diameter) were removed from zones of interaction on the agar plates, nine days post- inoculation, and samples were processed for SEM.

3.2.3 Scanning Electron Microscopy

Mycelial plug samples from interaction regions were fixed in 3% glutaldehyde in cacodylate buffer (0.1M; pH 7.0). After 6h of refrigeration at 4°C, the specimens were dehydrated in a graded alcohol-acetone series. Dehydrated samples were dried in a critical point drier (Hitachi HCP- 2 Critical Point Dryer CPD), mounted on copper stubs with double- sided sticky tape, and then sputter coated with gold- palladium in a sputter coater. The coated specimens were viewed under a SEM (Hitachi S-570 SEM) at 10 kv.

3.3 RESULTS

3.3.1 Fungal growth and interaction in dual cultures

All plates exhibited growth of both organisms after two days of incubation. *Trichoderma harzianum* KMD was green in colour, towards the outskirts of the colony, suggesting spore formation while *R. solani* hyphae were cream in colour. *Rhizoctonia solani* grew much faster than *T. harzianum* KMD, covering 75% of the plate by Day three. The first apparent hyphal contact between *R. solani* and *T. harzianum* KMD occurred within four days after inoculation (Plate 3.1). *Rhizoctonia solani* mycelium thickened and started to receed when *T. harzianum* KMD produced metabolites, which formed a zone of inhibition at the point of intersection between both organisms. In subsequent days, the *R. solani* colony was either overgrown or invaded by *T. harzianum* KMD leading to suppression in its growth (Plate 3.2). *Trichoderma*

harzianum KMD colonized *R. solani* hyphae and began to grow and sporulate on the pathogen's mycelium. After nine days incubation, *R. solani* hyphae collapsed and disintegrated resulting in complete suppression in its growth. Plates 3.1 and 3.2 show these effects of *T. harzianum* KMD on *R. solani* hyphae.

3.3.2 SEM observations on hyphal interface

Mycelial samples collected from the interaction region of dual cultures after inoculation were studied under SEM (Plates 3.3-3.5). *Rhizoctonia solani* was distinguished from *Trichoderma* by hyphal diameter, as it is broader than that of the antagonist (Plate 3.3). The hyphal diameter was evaluated by measuring the diameter of pure individual cultures of each organism (Plate 3.3).

Trichoderma harzianum KMD interacted with the pathogen as soon as their colonies established contact, and after invading deep into the pathogen colony. The interactions with the culture of *T. harzianum* KMD occurred in the form of coiling and/or penetration, which was an early event preceeding hyphal damage. *Trichoderma harzianum* KMD coiled several times around *R. solani* hyphae to obtain a grasp before penetration. Coiling occurred underneath, on top and around the pathogenic hyphae. Numerous short branches emanating from the main hyphae of *T. harzianum* KMD encircled the pathogen hyphae (Plate 3.4). Hyphae coiled at various sites and angles, forming loops around the pathogen (Plate 3.4). *Trichoderma hazianum* KMD hyphae also produced haustoria-like branches and hook-like structures for attachment and penetration particularly at the sites where it was in close proximity with pathogen hypha (Plate 3.5). Penetration of the hyphe of *R. solani* and degradation of its cell walls were apparent (Plate 3.5). The presence of hook- like structures were compared to a reference paper by Gupta *et al.* (1999). The structures were found to be similar as those found by Gupta *et al.* (1999) (Plate 3.5a & Plate 3.5b, Appendix D). *Trichoderma harzianum* KMD produced numerous small hook-like branches, which penetrated the pathogen hyphae (Plate 3.6), resulting in bursting and collapsing of the pathogen mycelia (Plate 3.7).

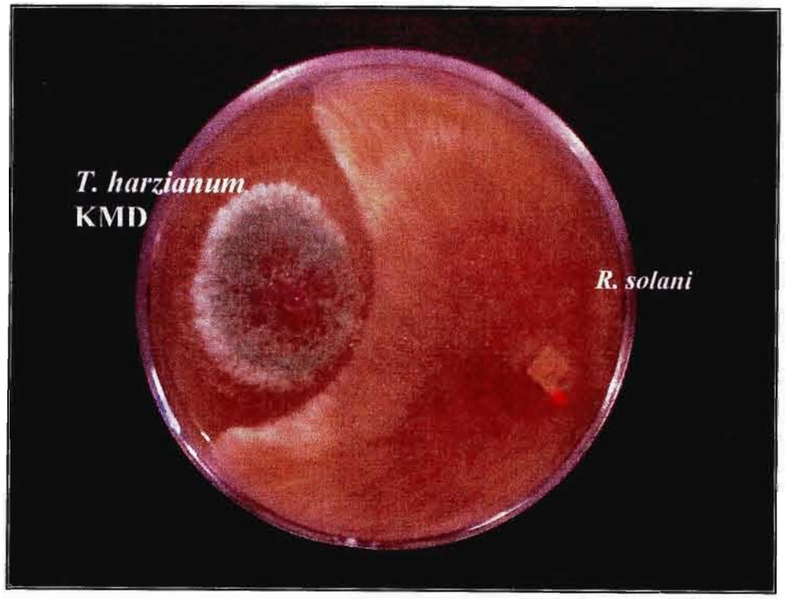

Plate 3.1

Dual cultures of *Trichoderma harzianum* KMD against *Rhizoctonia solani* on V8 medium incubated at room temperature for two days. Hyphal contact was established between the two fungi after two days.

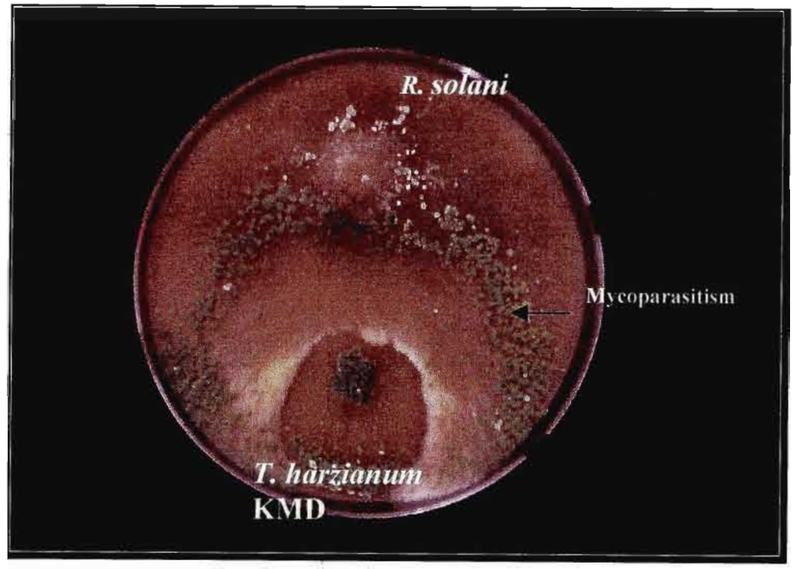

Plate 3.2

Dual cultures of *Trichoderma harzianum* KMD against *Rhizoctonia solani* on V8 medium incubated at room temperature after nine days. The *Trichoderma harzianum* KMD isolate had overgrown *Rhizoctonia solani* indicating probable mycoparasitism

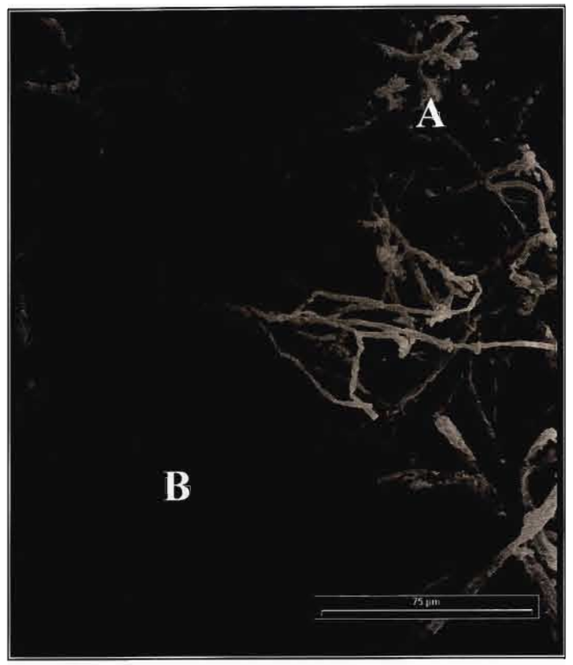

Plate 3.3

Scanning electron micrographs of *Trichoderma harzianum* KMD interactions with *Rhizoctonia solani,* showing the mycoparasitism of *T. harzianum* (A) and *R. solani* (B) and the difference in diameter of both fungi [Bar = 75µm].

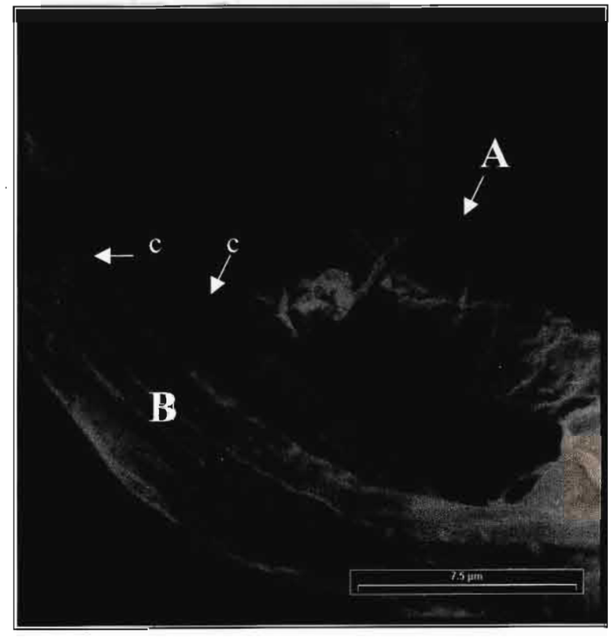

Plate3.4

T. harzianum KMD (A) coiling (c) around the hyphae of *R. solani* (B) at different angles. Short branches of *T. harzianum* (A) also encircle the hypha of *R. solani* (B) [Bar = 7.5µm].

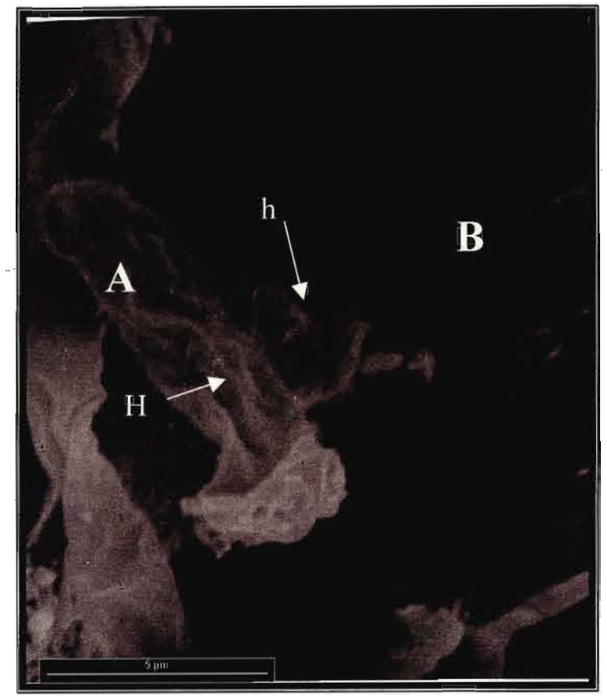

Plate 3.5

Hook- like structures (h) and haustorium - like branches (H) of *Trichoderma harzianum* KMD (A) attached to the hypha of *R. solani* (B) [Bar = 5µm]. (See Plate 3.5a and 3.5b, Appendix D)

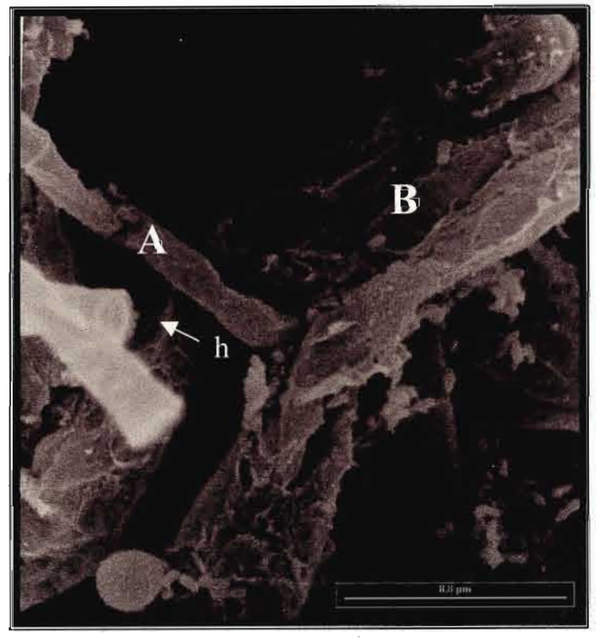

Plate 3.6

Hooks (h) of *Trichoderma harzianum* KMD (A) moving towards *Rhizoctonia solani* (B) resulting in disintegration of a hypha of *R. solani* [Bar = 8.8µm]. (See Plate 3.5a and 3.5b, Appendix D)

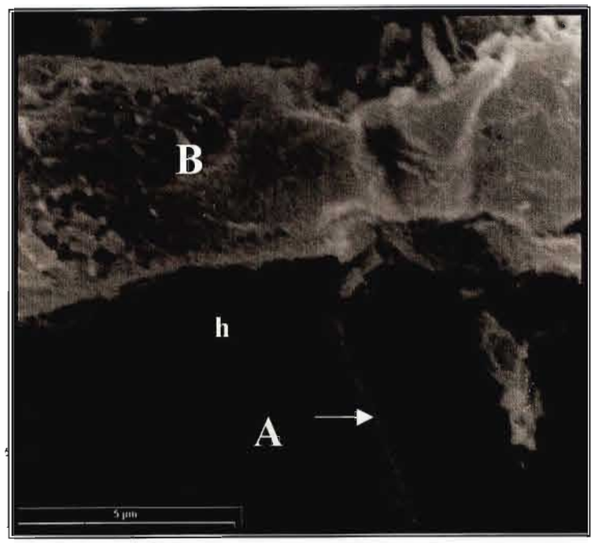

Plate 3.7

Loss of turgor and cell collapse of hyphae of *Rhizoctonia solani* (B). Hook- like structures (h) attaching to *Rhizoctonia solani*.[Bar = 5.0µm]. (See Plate 3.5a and 3.5b, Appendix D)

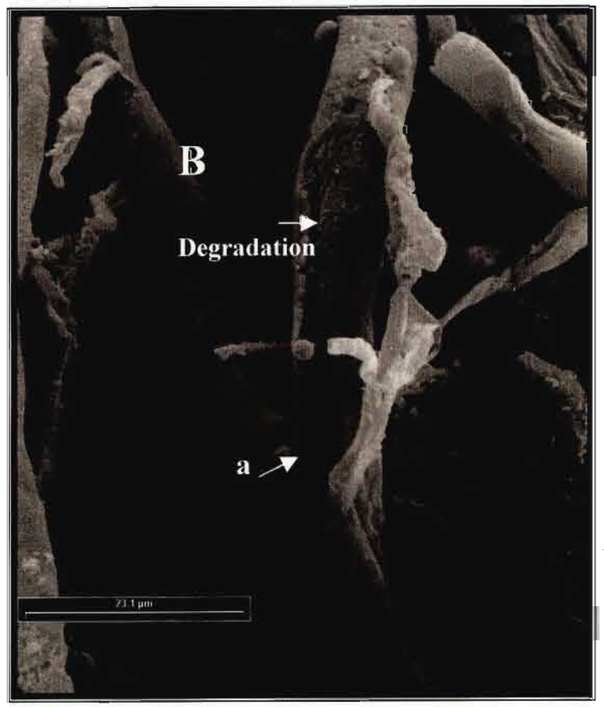

Plate 3.8

Penetration by *Trichoderma harzianum* KMD (A) and an appressorium-like structure (a), formed by *Rhizoctonia solani* (B). Degradation of *Rhizoctonia solani* resulted in cell collapse [Bar = 23.1µm]. (See Plate 3.8a, Appendix D)

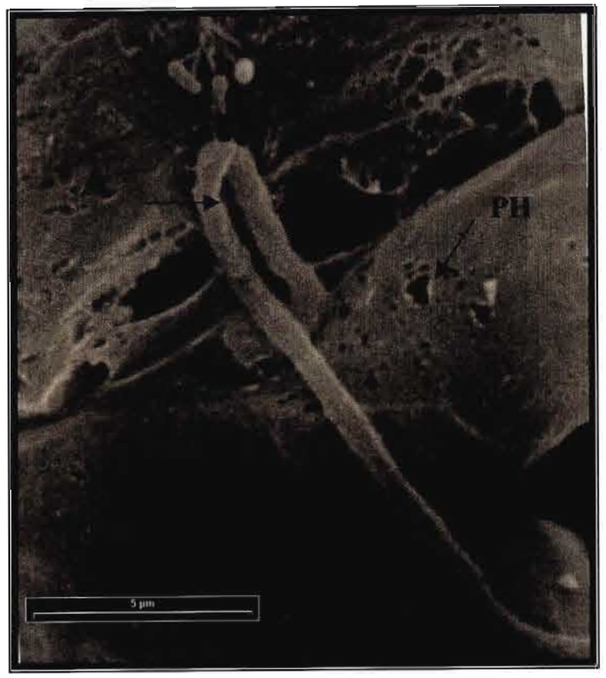

Plate 3.9

Trichoderma harzianum KMD (A) penetrating *Rhizoctonia solani* (B). Regions of hyphal degradation were indicated by the perforated holes found on the hyphae (PH) [Bar = 5µm].

Penetration and contact of these hook-like branches to the pathogen hyphae resulted in appressoria-like structures, at the site of penetration (Plate 3.8 & Plate 3.8a (Appendix D), which might have been due to the enhancement of wall lytic enzyme production by the intimate contact (Gupta *et al.*, 1999). Plate 3.8a (Appendix D) is a plate referenced from Gupta *et al.* (1999), which clearly compares the features represented in this work to those found by Gupta *et al.* (1999). Degradation of the cell wall of *R. solani* also ocurred (Plate 3.8 and 3.9). This is evident by the presence of perforated holes on the pathogen hyphae. Loss of turgor and cell collapse was also evident.

3.4 DISCUSSION

Overgrowth of *T. harzianum* KMD on the pathogen (Plates 3.1 and 3.2) was observed in colony interactions suggesting hyphal contact of antagonist and pathogen.The different types of interactions of *T. harzianum* KMD hyphae revealed in SEM observations, are a component of the mechanism, mycoparasitism. Mycoparasitism is a complex process that involves several successive steps. The first step is when *Trichoderma* hyphae grow toward the target pathogen because of chemotrophic stimuli (Elad *et al.*, 1982). This was evident in Plates 3.1 and 3.2. Upon reaching the hyphae of the target, galactose residues on the cell walls of the *Trichoderma* hyphae bind to a lectin on the host cell wall (Chet, 1987). Once binding occurs, the *Trichoderma* hyphae coil around the target host and form hook-like structures (Plates 3.5; Plate 3.5a & 3.5b (Appendix D) and Plate 3.6). Once recognition occurs, *Trichoderma* extracellular enzymes may penetrate into the host, through the formation of appressorial-like structures (Plate 3.8 and Plate 3.8 a (Appendix D) (Chet, 1987).

SEM observations showed all steps of mycoparasitism were present when *T. harzianum* KMD made hyphal contact with *R. solani.* Upon reaching the pathogen, *T.harzianum* KMD mycoparasitized the pathogen and arrested its growth by various modes of attack e.g., growing toward the pathogenic hyphae, coiling, and penetrating by hook-like structures, formation of haustoria and appressorial–like structures (Plates 3.3-3.9). These structures were found to similar as those referenced by Gupta *et al.* (1999) (Appendix D). Similar observations were made by Chet *et al.* (1981) and Elad (1983). The formation of appressorial-like structures at the site of penetration may have been due to the enhancement of wall lytic enzyme production by the intimate contact (Gupta *et al.*, 1999). Dennis & Webster (1971)

studied the possible interactions of *Trichoderma* spp. with nylon threads with a diameter similar to that of *Pythium ultimum* hyphae. *Trichoderma* never coiled around the threads, suggesting that the coiling is not just a randomly occurring contact. Moreover, the antagonism of *Trichoderma* was found to be specific (Elad *et al.*, 1980; Chet & Baker, 1981; Sivan *et al.*, 1984).

The problem of specificity in fungal-fungal interactions and the possible role of agglutinins in mycoparasitism have been studied. Lectin activity in a host-mycoparasite relationship was demonstrated with *R. solani* and *T. harzianum*. Attachment of Type O, but not A and B erythrocytes to hyphae occurred on *R.solani*, but not on its mycoparasite. A lectin, present in *R. solani* hyphae, binds to galactose residues on *Trichoderma* cell walls as well as to Type O erythrocytes. This agglutinin may play a role in prey recognition by the predator. Moreover since it does not distinguish among biological variants of the pathogen, it enables the *Trichoderma* species to attack various different *R.solani* isolates (Elad *et al.*, 1983). These agglutinins may have played a role in the hyphal interactions that had occurred between *T.harzianum* KMD and *R.solani* leading to its suppression of growth.

Observations made in SEM electron micrographs indicated the degradation of the pathogen's cell walls. This could be related to the production of enzymes e.g., β-1,3-glucanase, chitinase, lipase and proteases by *T. harzianum* KMD (Elad *et al.*, 1980). Elad & Misaghi (1985) demonstrated that the varying patterns and extent of antagonism, and the relationship susceptibility of the target pathogen to antagonism can be assigned to cell wall decomposition of the pathogen and production of wall lytic substances by the antagonist.

Inhibition of mycelial growth of *R. solani* occurred only after contact with the antagonist, suggesting that cell surface interaction between both fungi were crucial. This phenomenon has previously been reported by Elad *et al.* (1983) and Benhamou & Chet (1993). *Trichoderma harzianum* KMD appeared to cause perforations in *R. solani* hyphae just before penetration. This was attributed to lytic enzymes, which degraded the hyphal cell wall (Bell *et al.*, 1982). Damage to hyphae was evident immediately after contact with the antagonist hyphae, suggesting that the outcome of the interaction was most likely determined by initial contact, which triggered a series of events in pathogen degradation (Bell *et al.*, 1982). Involvement of genes in the production of lytic enzymes inhibitory substances in response to a signal generated by the antagonist on the hyphal contact the pathogen cannot be precluded (Bell *et*

al., 1982). This isolate of *T. harzianum* KMD may indeed excrete inhibitory substances, but evidence for its importance in biological control is yet, insufficient. Competition may play a role in this microbial interaction; it may be an independent phenomenon; or it may be connected with antibiosis or parasitism. However, further studies are still needed to elaborate and clarify these questions.

Mycoparasitism, as evidence by the interactions described in this paper, appears to be the mode of action by which this antagonist attacks other fungi. *Trichoderma harzianum* KMD may shift microbial equilibrium in soil by parasitising fungi. Its parasitic abilities enable it to serve as an efficient biocontrol agent of soil-borne pathogenic fungi (Ritchie *et al.*, 1976).

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CHAPTER 4

FACTORIAL TRIALS ON THE BIOCONTROL AND GROWTH STIMULATION OF *TRICHODERMA HARZIANUM* KMD AND *GLIOCLADIUM VIRENS* MM1 UNDER GREENHOUSE CONDITIONS

J. Omarjee[1], M.D. Laing[1] & C.H. Hunter[2]

Disciplines of Plant Pathology[1], and Microbiology[2], SAES,

University of Natal, Private Bag X01, Scottsville, Pietermaritzburg, South Africa

Preparations of isolates of biocontrol agents *Trichoderma harzianum* Strain KMD, *Gliocladium virens* Strain MM1 and *Bacillus subtilis* Strain AW57 were evaluated for their efficacy in enhancing growth and preventing damping-off caused by *Pythium* spp. and *Rhizoctonia solani* on, cabbage (*Brassica oleracea* var. *capitata* L.), cucumber (*Cucumis sativa* L.), Namaqualand daisy (*Dimophotheca hibrida* L.) and Eucalyptus (*Eucalyptus macarthuri*). Trials were held in greenhouse conditions with the growing medium, composted pine bark, artificially infested with pathogens. Each experiment was randomized and contained at least three replicates. The variables measured were percentage survival and plot weights after 3-4 weeks of growth. Preparations of formulations of biocontrol agents included milled oats containing chlamydospores of biocontrol fungi, powders containing conidia in an experimental compound, an oil base, and a commercial product. Bacterial formulations were prepared with and without Nutristart. Formulations were evaluated using three delivery methods: a seed coating (using an adhesive, Pelgel®), capping (preparation capped on surface and incorporated into planting media) and as a drench (preparation drenched on seed at planting). Formulations were drenched at various dosage levels: 0.25, 0.5, 1, 5 and10g/l of water. Formulations of the different biocontrol organisms resulted in variable effects on the growth promotion of seedlings. Overall, a significant increase was recorded in plot weight of all crops tested. All conidial formulations of *T. harzianum* KMD and *G. virens* MM1 consistently increased plant growth of seedlings. The best application technique that effectively delivered the biocontrol agents to the target was seed treatment followed by drenching and capping. On all seedlings most formulations significantly increased plot weight ranging from 2000-5000% when compared to controls. Percentage survival was comparable to the controls. In most instances all biocontrol organisms effectively enhanced growth of

seedlings equally well irrespective of other main effects. Damping-off caused by *Pythium* spp. on Eucalyptus and Namaqualand daisy seedlings was significantly reduced by most formulations of the different biocontrol organisms. *T. harzianum* KMD prepared as chlamydospores in milled oats, and with conidia effectively reduced damping-off on Eucalyptus and Namaqualand daisy by 8-31% when compared to the controls. *T. harzianum* KMD and *G. virens* MM1 more effectively reduced damping-off than *B.subtilis* AW57. Biocontrol of *R.solani* was achieved on all crops by all formulations of *T. harzianum* KMD, *G. virens* MM1and *B. subtilis* AW57. Biocontrol organisms reduced disease by 1000 fold when compared to disease controls. All conidial formulations performed better in reducing disease than formulations prepared with chlamydospores applied as a drench or a seed treatment. In most instances the best dosage to apply formulations were doses that ranged from 1-5g/l for both growth stimulation and biocontrol of soil-borne pathogens. Doses at 10g/l caused severe stunting of seedlings. These results have shown that *T. harzianum* KMD can be used as a biocontrol agent.

4.1 INTRODUCTION

Trichoderma and *Gliocladium* species are naturally occurring soil fungi, which have been studied extensively for their ability to act as biological control agents against soil-borne pathogens, especially, *Rhizoctonia* and *Pythium* species. These pathogens cause damping-off diseases in most greenhouse crops. Biocontrol studies have led to the observation that some isolates of *Trichoderma* and *Gliocladium* spp. may possess the additional benefit of direct plant growth enhancement (Lindsey & Baker, 1967; Baker *et al.*, 1984; Papavizas, 1985; Chang *et al.*, 1986; Chet & Inbar, 1994). Kliefeld & Chet. (1992) concluded that the increased plant growth caused by these biocontrol fungi appears to depend not only on the strain used, but also on how the inoculum is applied. In addition, *Trichoderma* strains increase some aspects of growth such as germination, weight, flowering of cucumber, lettuce, pepper, radish, tobacco, tomato, in potting compost or natural soil (Hadar *et al.*, 1979; Baker *et al.*, 1984; Chang *et al.*, 1986; Windham *et al.*, 1986; Lynch *et al.*, 1991). The responses depend on fungal isolate, as well as soil and environmental conditions. *Trichoderma* and *Gliocladium* spp. used for biocontrol studies are generally applied as conidial or chlamydospore preparations (Coley- Smith *et al.*, 1991; Mapelstone *et al.*, 1991; Papavizas, 1992). However, there, are several unique formulations of isolates of these fungi in which young, actively growing hyphae are added to soil or soilless mixes to suppress pathogens. Formulations of

these biocontrol fungi are usually supplemented with additives to maximize survival in storage and promote their effectiveness in biological control of soil-borne pathogens (Lewis & Papavizas, 1987).

Many *Bacillus* strains can suppress growth of plant pathogenic organisms by the production of peptide antibiotics (Liefert *et al.*, 1995). These peptide antibiotics are effective against other Gram-negative and Gram-positive bacteria, moulds and yeasts (Brock & Madigan, 1991). *Bacillus* spp. has been used for many years in attempts to control plant pathogens and increase plant growth (Turner and Backman, 1991; Holl & Chanway, 1992; Manero *et al.*, 1996; Kim *et al.*, 1997). *Bacillus* spp. strain L324-92 has been found to show a growth promoting benefit on turf grass when applied to foliage as a cell suspension (Mathre *et al.*, 1999). This strain was also shown to possess an *in vitro* antibiotic activity against all isolates of *Gaeumannomyces graminis* (Sacci) Arx and Oliver var. *tritici*, as well as species and anastomosis groups of *Rhizoctonia* and all species of *Pythium* tested (Kim *et al.*, 1997). Due to the high growth stimulation response on turf grass, *Bacillus* spp. strain L324-92 was awarded a license in 1998 for further developments and commercialization for use on turf grass (Mathre *et al.*, 1999).

Lumsden & Lewis (1989) stated that: "One of the major obstacles in the implementation of biocontrol technology is the need to develop appropriate formulations for the applications of the biocontrol agent to the ecosystem". Preparations formulated to contain dry fermentor biomass of biocontrol fungi, include dusts (Beagle-Ristaino & Papavizas, 1985), alginate pellets (Lewis & Papavizas, 1985) and vermiculite bran (Lewis *et al.*, 1989).

The objective of this investigation was to compare seven formulations of *Trichoderma harzianum* strain KMD, *Gliocladium virens* strain MM1 and *Bacillus subtilis* strain AW57 isolated from Kwa-Zulu Natal soils (Tala Valley) on the growth enhancement and biocontrol of *R. solani* and *Pythium* spp. on a range of crops under greenhouse conditions using three application methods at various dosages.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial cultures

Bacillus subtilis AW57 was provided by the Microbiology Department[1], University of Natal, as a concentrated spore suspension of 10^9c.f.u ml^{-1}. Bacterial cultures were maintained on Tryptic Soy Agar, incubated at 25- 28°C.

4.2.2 Pathogens

Cultures of *R. solani* and *Pythium* spp. that cause damping-off of greenhouse crops were obtained from C. Clark[2]. The isolates were maintained on V8 medium (Appendix A). Plates containing V8 medium were then inoculated with a 10mm by 10mm block of agar infested with *R. solani* or *Pythium* spp., and incubated at room temperature for two days, before adding to the potting medium.

4.2.3 Formulations of *T. harzianum* KMD and *G. virens* MM1

Formulations were obtained from Dr M. Morris[3], Plant Health Products. Formulation 1 and 2 of biocontrol fungi *T. harzianum* Strain KMD and an isolate of *G. virens* Strain MM1 included their respective chlamydospores suspensions in milled oats. Oats were boiled for three minutes, autoclaved at 121°C for one hour and spread on a tray. *Trichoderma harzianum* KMD conidia were taken from an agar plate containing water agar and inoculated to the boiled oats and left to incubate for approximately 10 days at 25°C. The inoculated mixture was then air dried and milled through a 0.4 mm sieve. Chlamydospores were found in the plant cells of the oat husk.

Formulation 3 contained a 1000 times more chlamydospores of *T. harzianum* KMD in milled oats, i.e., concentration were 10^8 chlamydospores per gram of formulation. This formulation was prepared as mentioned above.

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[1] Discipline of Plant Pathology, School of Applied and Environmental Science, University of Natal, Scottsville, Private Bag X01, Pietermaritzburg, South Africa.
[2] C. Clark, Discipline of Plant Pathology, School of Applied and Environmental Science, University of Natal, Scottsville, Private Bag X01, Pietermaritzburg, South Africa.
[3] Dr Mike Morris, Plant Health Products, P.O.Box 207, Nottingham Road, South Africa.

Formulation 4 and 5 of the biocontrol fungi *T. harzianum* KMD and *G. virens* MM1, contained conidial suspensions in a kaolin powder with mineral oil added to form a thick paste. The number of conidia per gram of formulation was 10^8 spores /g.

Formulation 6 was a commercial formulation of *T. harzianum* KMD obtained from Plant Health Products[1].The number of conidia per gram of formulation was 10^8 spores /g.

Formulation 7 contained a *T. harzianum* KMD conidial suspension in an experimental compound, which enhances spore dispersion. The number of conidia per gram of formulation was 10^8 spores/g.

Prior to each trial, the number of propagules per gram of formulation was determined by a serial 10-fold dilution. Total chlamydospores or conidia were counted of 1ml of appropriate dilutions (10^3-10^8) using a Petroff-Hausser Chamber (Thomas Scientific) and were plated on water agar. Plates were incubated at 25°C and colony forming units (c.f.u.) were counted. Number of chlamydospores or conidia per gram of formulation was 10^5 spores /g.

4.2.4 Application methods

4.2.4.1 *Drenching*

a) Drenching of fungal formulations

One gram of each formulation was added to one litre of tap water and mixed thoroughly. Untreated seeds of cabbage (*Brassica oleracea* var. *capitata* L.), cucumber (*Cucumis sativa* L.), Namaqualand daisy (*Dimophotheca hibrida* L.) and Eucalyptus (*Eucalyptus macarthuri* L. Deane & Maiden) were planted into three Speedling ® 24 trays, resulting in 24 seedlings per tray and 72 seedlings per treatment, filled with composted pine bark. Before seeds were covered, the shaken formulation mixture was dispensed in 3ml aliquots directly onto seeds in their respective trays. Speedling® 24 trays containing drenched seeds were not watered, and were left to establish themselves around the target area of the seed. At the first sign of germination, trays containing drenched seeds were placed in a greenhouse. Plants were grown under commercial conditions.

A study was also undertaken to evaluate the application of drenching of various dosages of different formulations of *T. harzianum* KMD and *G. virens* MM1 to enhance plant growth and to reduce damping-off of *R. solani* and *Pythium* spp. on all crops tested. Formulation dosages were applied as 0.25, 0.5, 1, 5 and 10g/l of formulation per 1L of tap water. The seeds were then left to imbibe overnight and were watered the following day. At the first sign of germination, trays containing drenched seeds were placed in a greenhouse. Plants were grown under commercial conditions.

b) Drenching of bacterial cultures with or without Nutristart

Two ml of a concentrated bacterial suspension ($2x10^9$) cells of *B. subtilis* AW57 were added separately to each of 12 conical flasks (250ml) containing 120 ml of sterilized Tryptic Soy Broth. These flasks were then placed in a water bath at 30°C for 18h at 150 rpm. The 18 hr cultures were then centrifuged at 12,000 rpm (Beckman Centrifuge JA20) for 15 min. The pellet was washed three times and resuspended in 20 ml of sterile Ringers Solution. One ml of this solution was then evaluated for the number of bacterial cells per ml by dilution plating and counting c.f.u.'s after incubation. Two ml of this mixture was used for drenching seeds. *Bacillus subtilis* AW57 with Nutristart was brewed using 0.6g of Nutristart product, obtained from Microbial Solutions[4]. The weighed product, 0.6g of Nutristart, was placed into 12 conical flasks (250ml). Volumes of 120 ml of distilled water were added to each flask before swirling to form a uniform mixture. These flasks were then autoclaved for 15 minutes at 121°C. Ten ml of the 18h brewed culture in tryptic soy broth was then added to the flasks containing the autoclaved Nutristart. The flasks containing the Nutristart and *B. subtilis* AW57 were then shaken in a water bath at 30°C for 18h at 150 rpm. This mixture was then evaluated for the c.f.u. present, by dilution plating. Two ml aliquots of this culture were then drenched on seeds in three Speedling® 24 trays at planting, resulting in 24 seedlings per tray and 72 seedlings per treatment. After seeds of all crops were drenched with bacterial cultures, seeds were allowed to imbibe overnight and were then watered the next day. At the first sign of germination, trays were moved to the greenhouse. Plants were grown under commercial conditions.

[4] Microbial Solutions (Pty) Ltd., P. O. Box 1180, Strubens Valley 1735, South Africa

4.2.4.2 *Capping*

Three Speedling® 24 trays were filled with composted pine bark and dibbled. Seeds were planted. One gram of each formulation was mixed with 1,000cm^3 of composted pine bark and applied as a cap. This resulted in 24 seeds per tray i.e., 72 seedlings per treatment. Seeds were allowed to imbibe overnight and were watered the next day. At the first sign of germination, Speedling® 24 trays were moved to the greenhouse and treated under normal commercial conditions. Bacterial cultures were not applied using capping as an application method.

4.2.4.3 *Seed treatment*

a) Bacterial cultures

Bacterial cultures that were prepared as mentioned above, i.e., in Tryptic Soy Broth and Nutristart, were added to a seed sticker, Pelgel®[5], a seed coat adhesive. Two grams of Pelgel®, were dissolved in 100ml of distilled water, stirred and allowed to stand for 1h, to allow the sticker to dissolve and form a uniform mixture. Twenty ml of 18h bacterial cultures were centrifuged and those cultures that were brewed up in Nutristart were then added to the sticker The sticker was then divided into 20ml aliquots and stirred. This resulted in a 1:1 sticker-bacterial suspension. The appropriate number of seeds needed for trials were suspended in the bacterial suspension-sticker. The seeds were left for 3h to allow the adhesion of bacteria to the seed coat. The treated seeds were then placed on filter paper and air-dried overnight. All seeds of all crops were treated with the same combination of the adhesive and *B. subtilis* AW57. One treated seed was planted in each cell containing composted pine bark of three Speedling® 24 trays resulting in a total of 24 seeds per tray i.e., 72 seedlings per treatment. Seeds were allowed to imbibe overnight and were watered the next day. At the first sign of germination trays were then placed under commercial conditions.

b) Fungal formulations

Four grams of Pelgel® was dissolved in 200 ml of distilled water, stirred and allowed to stand for 1h. One gram of each fungal formulation was added to a beaker containing 50 ml of sticker, Pelgel®. The mixture was then stirred. An appropriate number of seeds for trials were placed into the mixture and was allowed to stand for 1h. Seeds were then removed and air-dried. Each treated seed was planted in each cell containing composted pine bark of Speedling® 24 trays resulting in a total of 24 seeds per tray and 72 seedlings per treatment. Seeds were allowed to imbibe overnight and were watered the next day. At the first sign of germination trays were then placed under commercial conditions.

4.2.5 Pathogen inoculation

Initially a thin layer of composted pine bark was placed in Speedling® 24 trays. A 10mm square agar plug, infested with the pathogen was then placed on top this. The infested agar plug was covered completely with composted pine bark. A single seed was placed on top of the composted pine bark and either a drench, capping or seed treatment as previously described was applied.

4.2.6 Controls

Control treatments for growth enhancement

1. No antagonist or pathogen
2. Pelgel® only
3. Nutristart only
4. Nutristart and Pelgel®

Control treatments for biocontrol activity

1. No antagonist or pathogen
2. Pathogen only
3. Pelgel® and Pathogen
4. Nutristart only and Pathogen
5. Nutristart, Pelgel® and pathogen.

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[5] LiphaTech, Inc., Milwaukee, Wisconsin, U. S. A

Controls were replicated three times with one tray per replicate. All treatments were irrigated three times a day by microjet irrigation. Fertilizer was injected into the irrigation water. Soluble fertilizer [3. 1. 3(38)] Complete from Ocean Agriculture[6], applied at a rate of $1gl^{-1}$ to give approximately $33mgl^{-1}$ of Phosphorous and $100mgl^{-1}$ of Nitrogen and Potassium. Greenhouse temperatures were in the range of 20-30°C in greenhouses.

4.2.7 Crops evaluated

Cabbage cv. Glory of Enkuizen, Seed lot no. Y1011RR;
Cucumber cv. Cucumber Ashley, Seed lot no. Ay054YY;
African daisy cv. Namaqualand daisy, Seed lot no. 884-P14416;
Eucalyptus Seed lot no. M1697
Vegetable seeds were obtained from McDonalds Seeds[7] and Eucalyptus seeds were obtained from the Institute of Commercial Forestry Research (ICFR)[8].

4.2.8 Variables of seedlings

All seedlings of all crops were evaluated as follows:
1. Percentage survival after 4-6 weeks for cabbage, cucumber, Namaqualand daisy and after 10 weeks for Eucalyptus.
2. Plot weight. The dry weight of seedlings per plot was recorded by taking all surviving seedlings and measuring the total dry weight. Seedlings were harvested at their maturity at the base of the plant and placed in a brown paper bag. The plant material was dried in an oven at 55°C for two days. After drying, the contents of the bag were weighed and the plot weight calculated.

4.2.9 Statistical Analysis

Experiments were conducted once, with three replicates per treatment. The treatments were arranged in a randomised complete block design. Analysis of data by Analysis of Variance (ANOVA) and linear regression with a factorial treatment structure and interactions were performed. Treatment means were separated by the Student's Newman Keuls test. Regression

[6] Ocean Agriculture, P. O. Box 741 Mulders Drift 1747, South Africa
[7] McDonalds Seeds, 61 Boshoff Street, Box 238, Pietermaritzburg, South Africa.

analysis was performed on formulation dosages. Statistical analyses were conducted using the general linear model procedure of SAS Version 6.08 (SAS Institutional Inc, Cary, NC).

4.3 RESULTS

The following tables and figures reflect the results of greenhouse trials of formulations of biocontrol organisms and application techniques applied to a variety of crops at various dosages.

Figures 4.1-4.36 have various application techniques for each treatment, resulting in three bars i.e., drenching, capping and seed treatment which differ to the main treatment (the controls) resulting in one bar only. Main and interaction effects recorded in these trials are summarized in Table 4.13.

4.3.1 Growth promotion of seedlings by formulations of *T. harzianum* KMD, *G. virens* MM1 and *B. subtilis* AW57

Formulations of the different biocontrol organisms resulted in variable effects on the growth promotion of seedlings. Overall, a significant increase was recorded on plot weight of cabbage, cucumber, Namaqualand daisy and Eucalyptus seedlings when compared to controls (water only) (Tables 4.1-4.4). All of the various preparations of formulations tested, resulted in an increase in growth of all crops with respect to plot weight.

Significant differences (P=0.0001) between formulations were recorded in plot weight in cabbage seedlings. All formulations caused growth promotion of cabbage seedlings relative to the control (water only) (Figure 4.1). Significant differences (P=0.005) were recorded between formulations as reflected in percentage survival of cabbage seedlings (Tables 4.1 and Figure 4.2). However, most biocontrol formulations resulted in percentage survival rates comparable to controls (water only).

Preparations of formulations of *T. harzianum* KMD containing conidia in kaolin powder with oil, conidia in an experimental compound, commercially produced formulation, *G. virens* MM1 containing conidia in kaolin powder with oil and *B. subtilis* AW57, with and without

[8] Institute of Commercial Forestry Research (ICFR), University of Natal, Private Bag X01, Scottsville 3209, South Africa.

Table 4.1. Effect of formulations of *Trichoderma harzianum* KMD, *Gliocladium virens* MM1 and *Bacillus subtilis* AW57 on plant growth enhancement of cabbage after four weeks

Formulations	Application	Plot Weight (PW) (g)	%Control 1 (PW) (nil)	%Survival (%Surv)	% Control 1 (%Surv)(nil)
T. harzianum (10^5chlamydospores in milled oats)	Drenching	2.95 cdef	2158.01	86.00 abc	93.65
T. harzianum (10^5chlamydospores in milled oats)	Capping	1.90 ef	1392.10	96.00 ab	104.54
T. harzianum (10^5chlamydospores in milled oats)	Seed treatment	2.32 def	1697.15	98.67 a	107.45
T. harzianum (10^8chlamydospores in milled oats)	Capping	5.99 abc	4381.86	84.83 abc	92.38
T. harzianum (conidia in powder with oil)	Drenching	5.16 bcd	3774.69	87.45 abc	95.23
T. harzianum (conidia in powder with oil)	Capping	5.30 bcd	3877.10	91.83 abc	100.00
T. harzianum (conidia in powder with oil)	Seed treatment	8.56 a	6261.89	93.16 abc	101.45
T. harzianum (Commercial)	Drenching	3.50 a	2560.35	86.00 abc	93.65
T. harzianum (Commercial)	Capping	3.88 cde	2838.33	75.00 cd	81.67
T. harzianum (Commercial)	Seed treatment	7.63 ab	5581.57	82.00 abc	89.30
T. harzianum (conidia in an experimental compound)	Drenching	5.96 abc	4359.91	75.00 cd	81.67
T. harzianum (conidia in an experimental compound)	Capping	2.90 cdef	2123.63	86.00 abc	93.65
T. harzianum (conidia in an experimental compound)	Seed treatment	7.47 ab	5464.52	85.00 abc	92.56
G. virens (chlamydospores in milled oats)	Drenching	3.64 cde	2662.77	93.16 abc	101.45
G. virens (chlamydospores in milled oats)	Capping	1.67 ef	1221.65	94.50 abc	102.91
G. virens (chlamydospores in milled oats)	Seed treatment	2.38 def	1743.23	98.17 a	107.45
G. virens (conidia in powder with oil)	Drenching	8.513 a	6227.51	86.00 abc	93.65
G. virens (conidia in powder with oil)	Capping	7.38 ab	5398.68	76.3 bcd	83.09
G. virens (conidia in powder with oil)	Seed treatment	4.61 bcde	3372.35	62.5 d	68.06
B. subtilis AW57 +Nutristart	Drenching	7.05 ab	5161.67	77.6 a	84.50
B. subtilis AW57 +Nutristart	Seed treatment	6.08 ab	4453.55	82.1 a	89.40
B. subtilis AW57 (Washed)	Drenching	9.08 a	6645.21	84.6 a	92.13
B. subtilis AW57 (Washed)	Seed treatment	7.75 ab	5670.81	87.5 a	95.28
Control 1 (nil)	Nil	0.1367 f	100.00	91.83 abc	100.00
Control 2 (Nutristart and Pelgel®)	Seed treatment	0.1133 f	82.88	77.00 bcd	83.85
Control 3 (Nutristart only)	Drenched	0.1263 f	92.68	85.00 a	92.56
Control 4 (Pelgel®)	Seed treatment	0.1433 f	104.83	97.33 a	105.99
Effects		P-values		P-values	
Formulations		0.0001***		0.0053**	
Application		0.0001***		0.0001***	
Organism		0.4509 ^{NS}		0.0042***	
Formulation x Application		0.7166 ^{NS}		0.0006***	
Formulation x Organism		0.0261**		0.0211**	
Organism x Application		0.033**		0.0112**	
Formulation x Application x Organism		0.1707 ^{NS}		0.2623^{NS}	
		CV%=27.18		CV%=8.22	
		MSE=1.312		MSE=7.09	

1.NS = Not significant; **= significant at P ≤ 0.05; ***=significant at P ≤ 0.001

2. Means with the same letter are not significantly different (P= 0.05) according to Student, Newman and Keuls comparison test

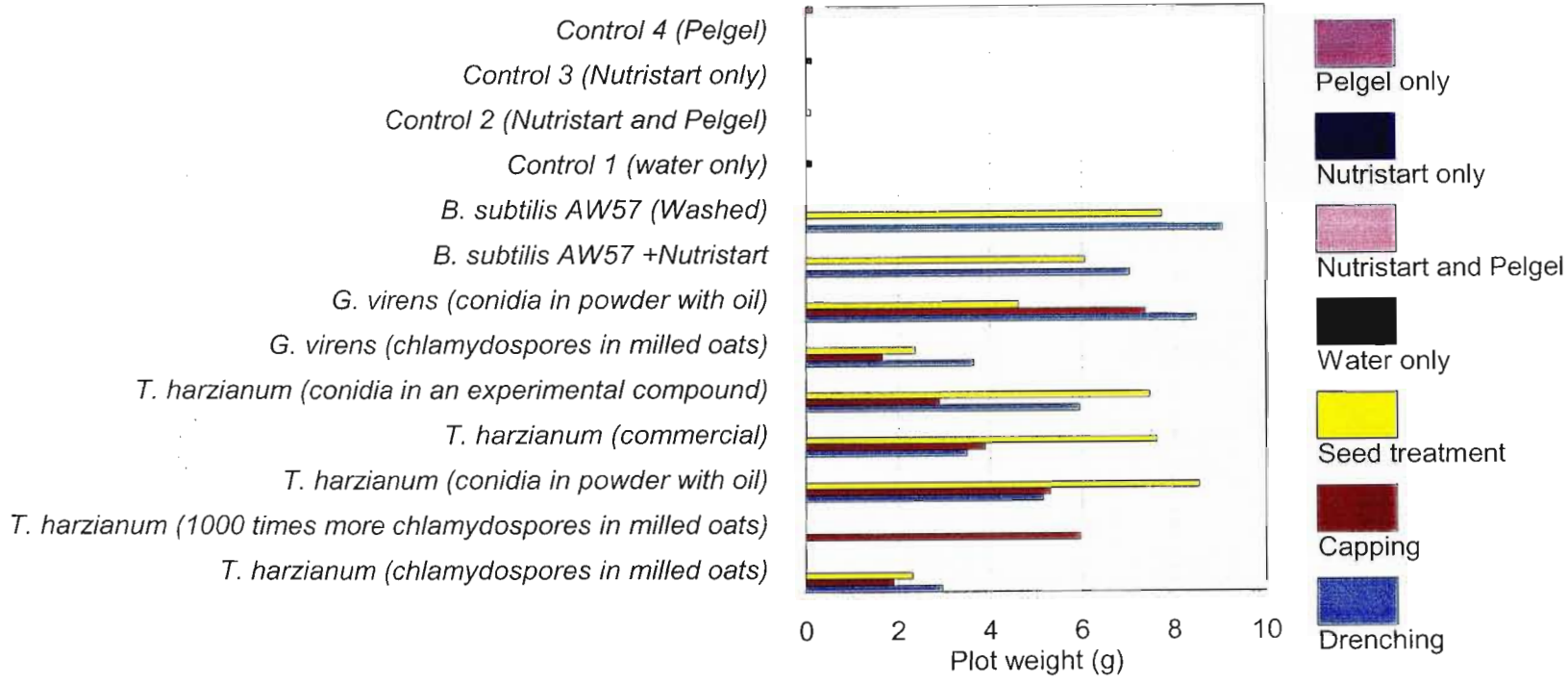

Figure 4.1 Effect of formulations and application methods of *Trichoderma harzianum* KMD, *Gliocladium virens* MM1 and *Bacillus subtilis* AW57 on growth enhancement of plot weight of cabbage after four weeks of growth.

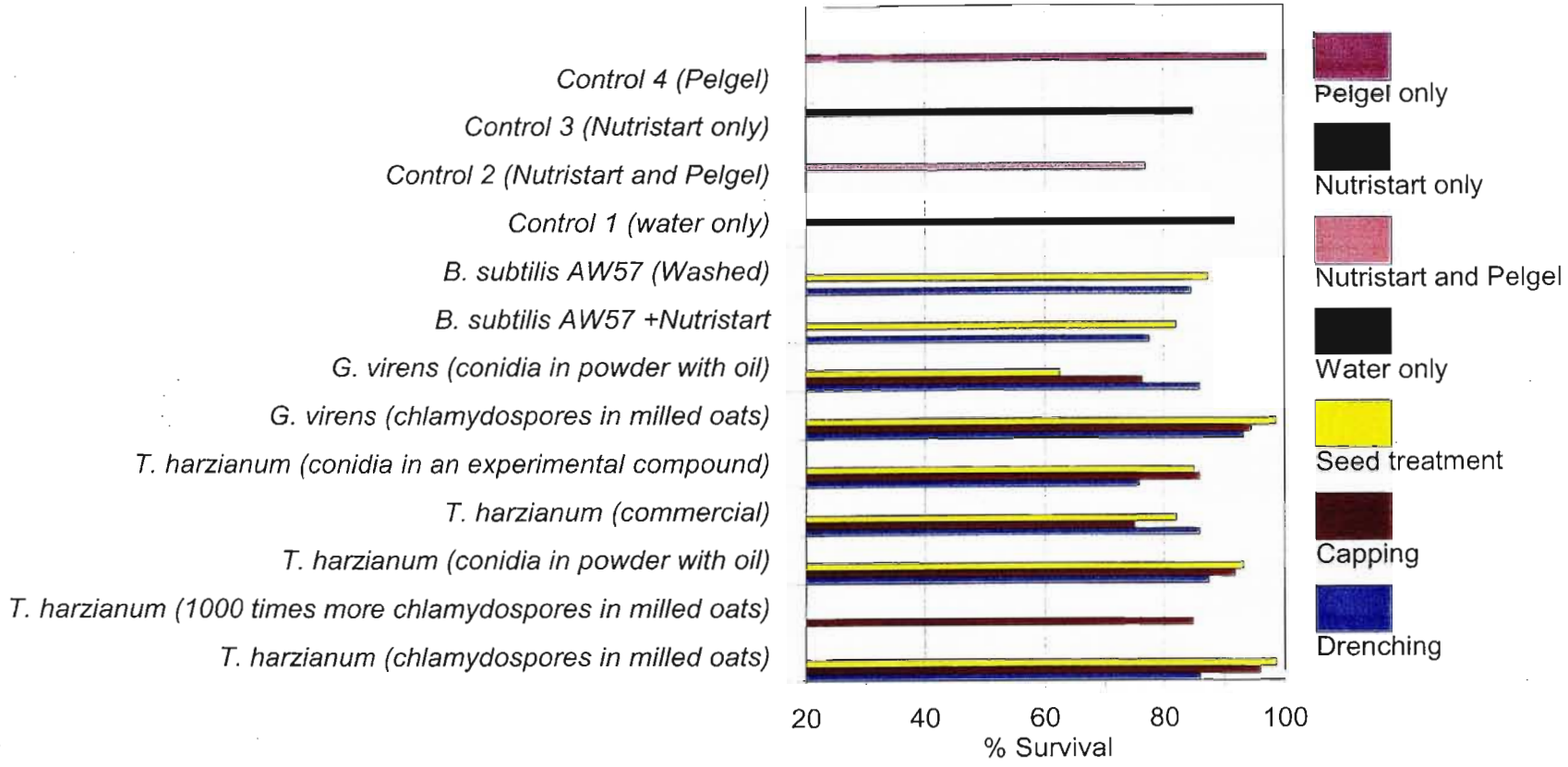

Figure 4.2 Effect of formulations and application methods of *Trichoderma harzianum* KMD, *Gliocladium virens* MM1 and *Bacillus subtilis* AW57 on growth enhancement of percentage survival of cabbage after four weeks of growth.

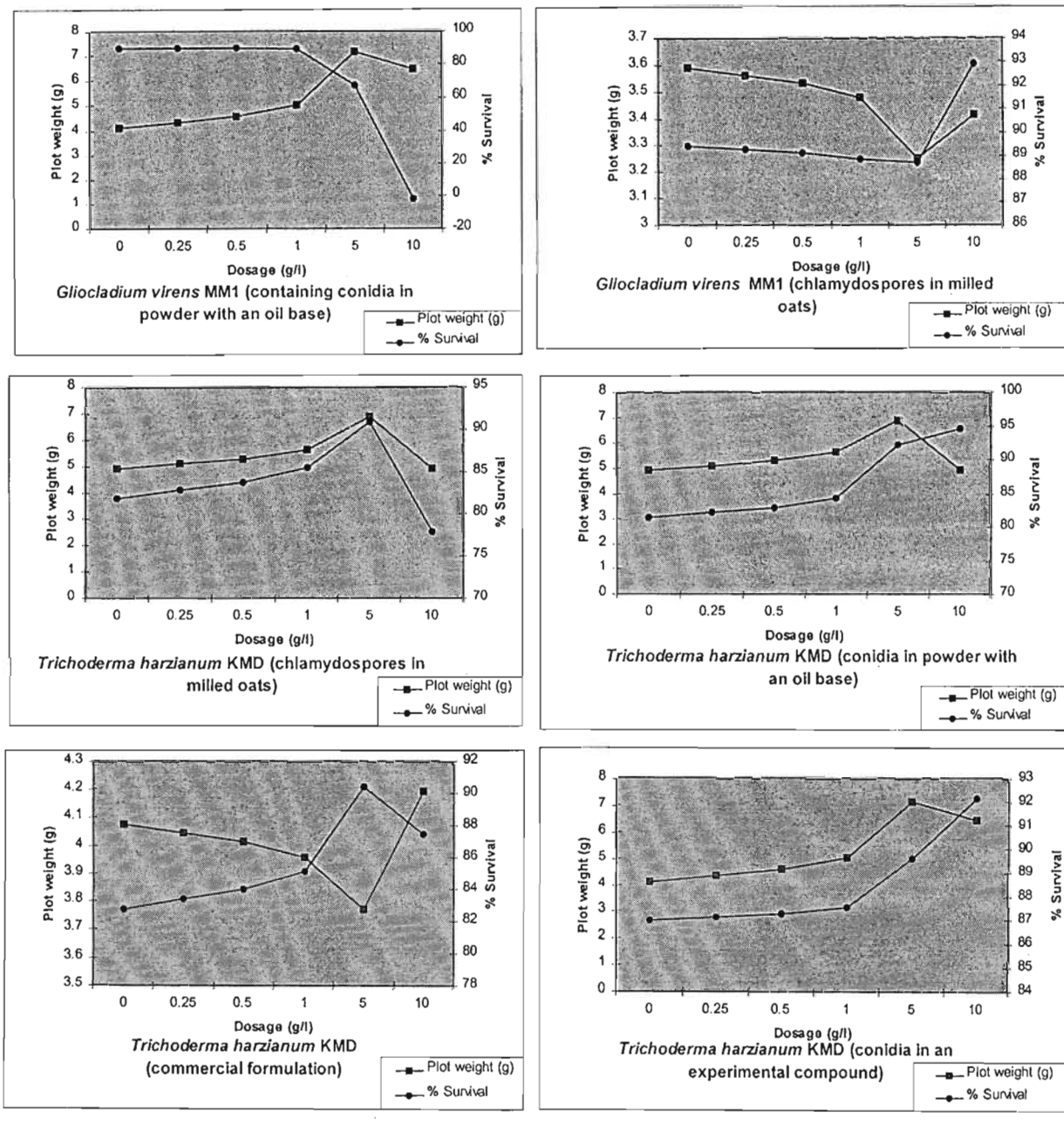

Figure 4.3 Dosage effects of six formulations applied as a drench on growth enhancement on plot weight and percentage survival of cabbage after four weeks of growth.

Table 4.2 Effect of formulations of *Trichoderma harzianum* KMD, *Gliocladium virens* MM1 and *Bacillus subtilis* AW57 on plant growth enhancement of cucumber after four weeks

Formulations	Application	Plot Weight (PW) (g)	%Control (PW) nil	%Survival (%Surv)	%Control (%Surv) (nil)
T. harzianum (10^5chlamydospores in milled oats)	Drenching	7.01 cde	277.07	83 .00a	98.0
T. harzianum (10^5chlamydospores in milled oats)	Capping	5.29 ef	209.09	97.30 a	114.9
T. harzianum (10^5chlamydospores in milled oats)	Seed treatment	3.29 fg	130.0	96.00 a	113.4
T. harzianum (10^8chlamydospores in milled oats)	Capping	6.73 de	266.00	89.00 a	105.1
T. harzianum (conidia in powder with oil)	Drenching	5.59 ef	220.4	93.33a	110.2
T. harzianum (conidia in powder with oil)	Capping	3.44 fg	135.9	91.83 a	108.5
T. harzianum (conidia in powder with oil)	Seed treatment	5.7 ef	225.2	93.33 a	110.2
T. harzianum (Commercial)	Drenching	9.43 bcd	372.7	93.33 a	110.2
T. harzianum (Commercial)	Capping	7.97 bcde	350	94.66 a	111.8
T. harzianum (Commercial)	Seed treatment	15.2 a	600.79	86.16 a	101.8
T. harzianum (conidia in an experimental compound)	Drenching	9.15 bcd	361.6	93.16 a	110.0
T. harzianum (conidia in an experimental compound)	Capping	9.47 bcd	374.30	87.50 a	103.4
T. harzianum (conidia in an experimental compound)	Seed treatment	10.5 b	415.01	72.00 b	85.0
G. virens (chlamydospores in milled oats)	Drenching	8.35 bcde	330.0	90.33 a	106.7
G. virens (chlamydospores in milled oats)	Capping	5.13 ef	202.76	91.83 a	108.5
G. virens (chlamydospores in milled oats)	Seed treatment	5.95 ef	235.17	87.33 a	103.2
G. virens (conidia in powder with oil)	Drenching	6.99 cde	276.28	94.66 a	111.8
G. virens (conidia in powder with oil)	Capping	10.0 bc	395.25	94.66 a	111.8
G. virens (conidia in powder with oil)	Seed treatment	10.5 b	415.0	97.30 a	114.9
B. subtilis AW57 +Nutristart	Drenching	5.28 ef	208.6	75.30 a	88.9
B. subtilis AW57 +Nutristart	Seed treatment	5.61 ef	221.7	77.60 a	91.7
*B. subtilis*AW57 (Washed)	Drenching	5.28 ef	208.6	84.60 a	99.9
B. subtilis AW57 (Washed)	Seed treatment	6.19 de	244.6	87.50 a	103.4
Control 1 (nil)	Nil	2.53 g	100	84.66 a	100.0
Control 2 (Nutristart and Pelgel®)	Seed treatment	2.56 g	101.1	90.67 a	107.0
Control 3 (Nutristart only)	Drenched	2.54 g	100.3	87.67 a	103.6
Control 4 (Pelgel®)	Seed treatment	2.62 g	103.5	100.00 a	118.1
Effects		P-values		P-values	
Formulations		0.0001***		0.9137^{NS}	
Application		0.0032**		0.0001***	
Organism		0.0001***		0.1322^{NS}	
Formulation x Application		0.0001***		0.2278^{NS}	
Formulation x Organism		0.0021**		0.284^{NS}	
Application x Organism		0.10241^{NS}		0.2876^{NS}	
Formulation x Application x Organism		0.0070**		0.1747^{NS}	
		CV%=17.94		CV%=6.72	
		MSE=1.378		MSE=6.111	

1.NS = Not significant; **= significant at P ≤ 0.05; ***=significant at P ≤ 0.001

2. Means with the same letter are not significantly different (P= 0.05) according to Student, Newman and Keuls comparison test

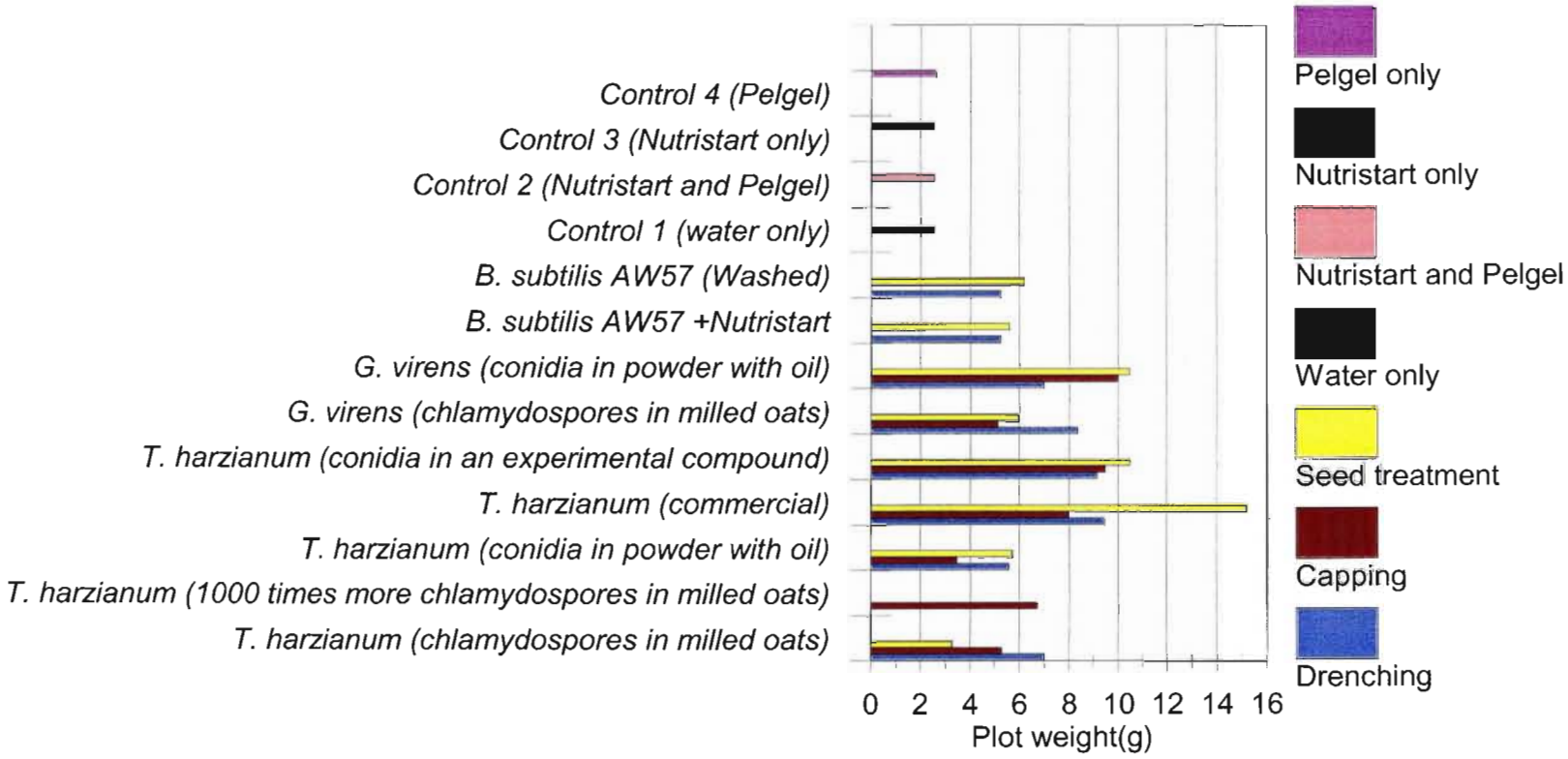

Figure 4.4 Effect of formulations and application methods of *Trichoderma harzianum* KMD, *Gliocladium virens* MM1 and *Bacillus subtilis* AW57 on growth enhancement of plot weight of cucumber after four weeks of growth.

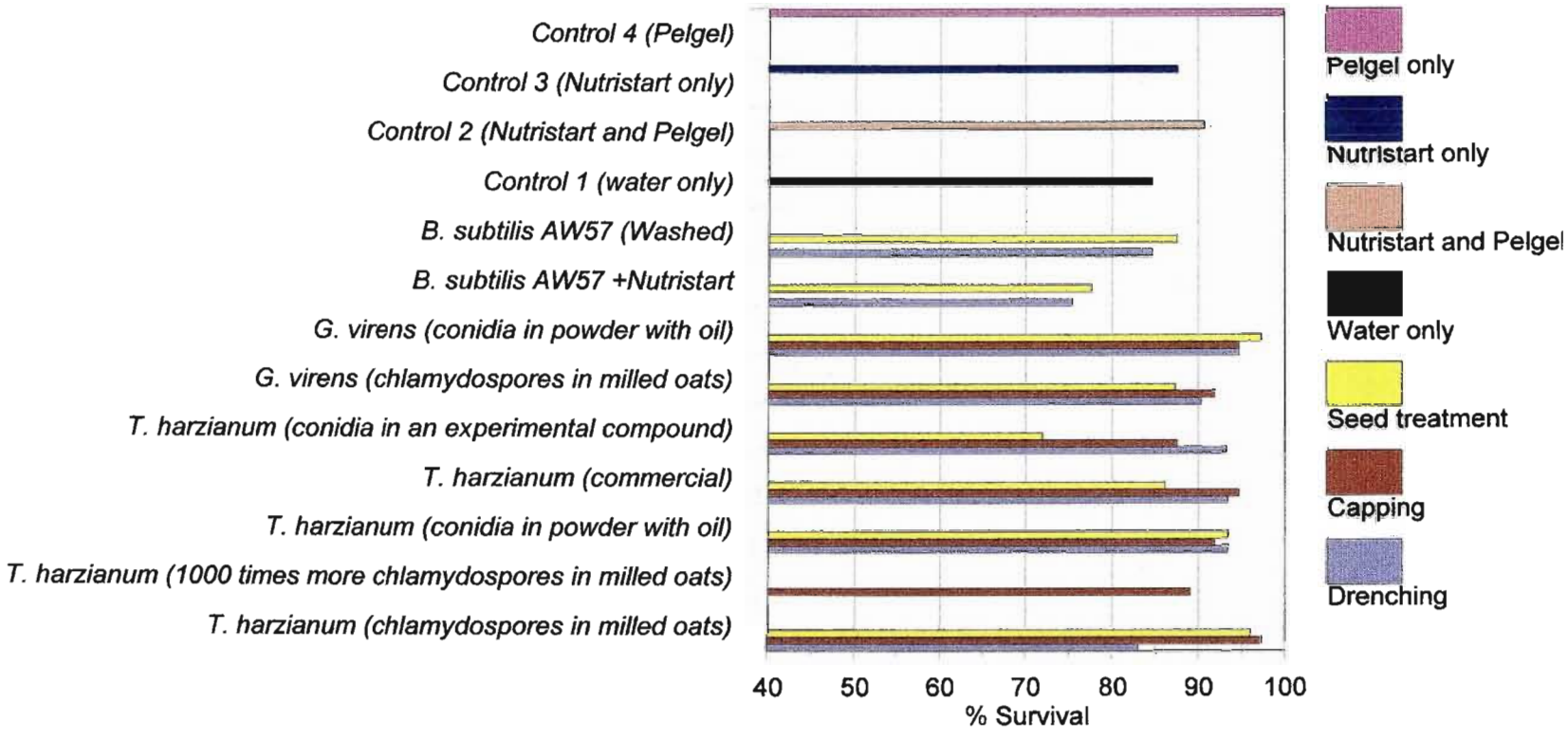

Figure 4.5 Effect of formulations and application methods of *Trichoderma harzianum* KMD, *Gliocladium virens* MM1 and *Bacillus subtilis* AW57 on growth enhancement of percentage survival of cucumber after four weeks of growth.

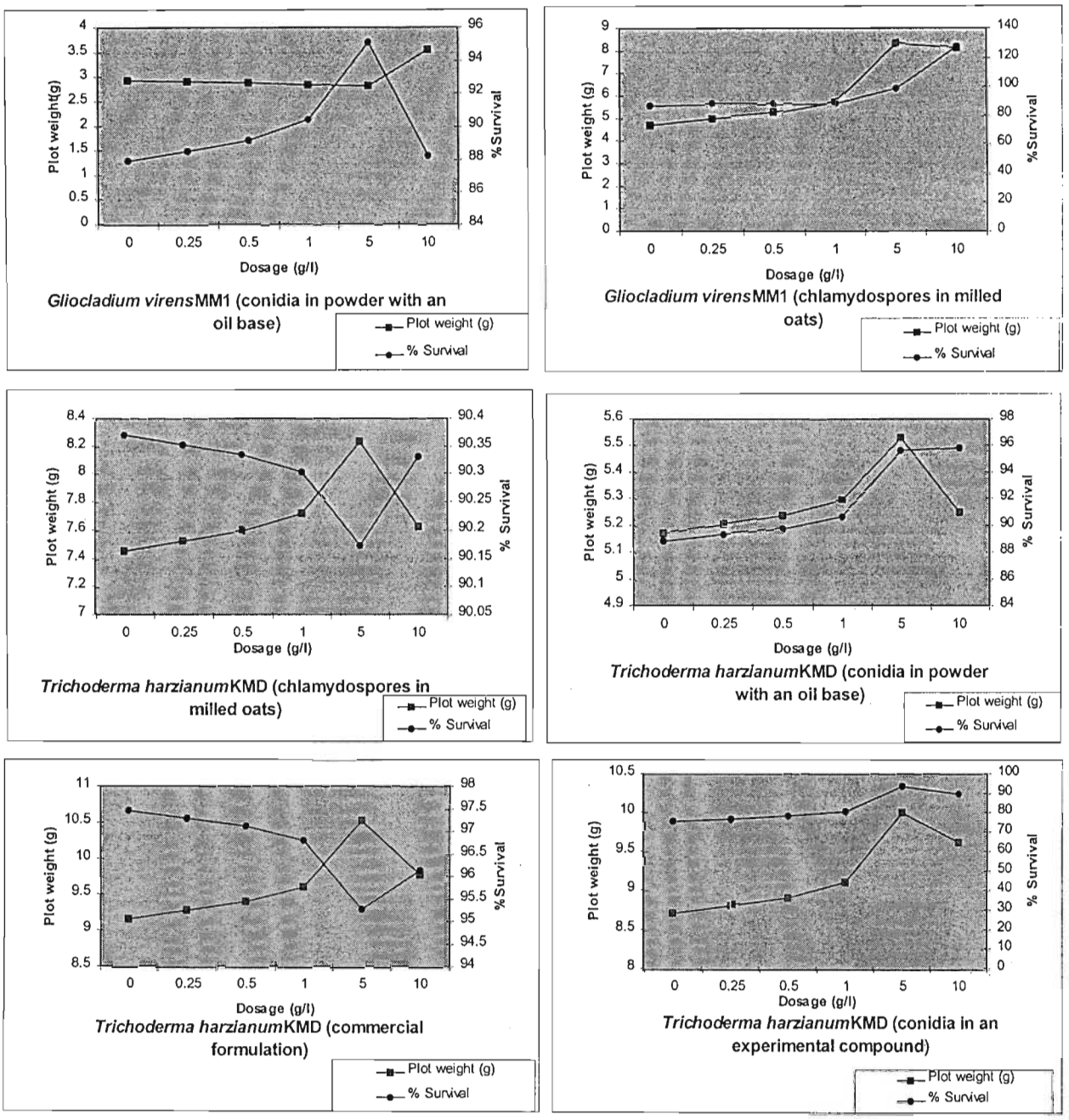

Figure 4.6 Dosage effects of six formulations applied as a drench on growth enhancement on plot weight and percentage survival of cucumber after four weeks of growth.

Table 4.3. Effect of formulations of *Trichoderma harzianum* KMD, *Gliocladium virens* MM1 and *Bacillus subtilis* AW57 on the plant growth enhancement on Namaqualand daisy after four weeks

Formulations	Application	Plot Weight (PW) (g)	%Control (PW) nil	%Survival (%Surv)	% Control (%Surv)(nil)
T. harzianum (10^5chlamydospores in milled oats)	Drenching	1.38 fgh	1091.55	80.33 abc	103.4
T. harzianum (10^5chlamydospores in milled oats)	Capping	0.65 gh	518.31	77.83 abc	100.2
T. harzianum (10^5chlamydospores in milled oats)	Seed treatment	0.39 h	307.81	38.83 d	50.0
T. harzianum (10^8chlamydospores in milled oats)	Capping	2.35 cdef	1860.06	90.3 a	116.3
T.harzianum (conidia in powder with oil)	Drenching	4.49 ab	3549.09	87.66 ab	112.9
T. harzianum (conidia in powder with oil)	Capping	4.37 ab	3449.09	83.33 abc	107.3
T. harzianum (conidia in powder with oil)	Seed treatment	2.42 cdef	1910.02	69.5 abc	89.5
T. harzianum (Commercial)	Drenching	1.59 efgh	1257.30	79.00 abc	101.7
T. harzianum (Commercial)	Capping	1.31 fgh	1036.31	55.33 c	71.2
T. harzianum (Commercial)	Seed treatment	4.74 a	3745.86	81.66 abc	105.2
T. harzianum (conidia in an experimental compound)	Drenching	2.64 cdef	2083.66	91.66 a	118.0
T. harzianum (conidia in an experimental compound)	Capping	2.08 defg	1644.04	59.33 bc	76.4
T. harzianum (conidia in an experimental compound)	Seed treatment	2.87 cde	2265.19	87.50 ab	112.7
G. virens (chlamydospores in milled oats)	Drenching	1.58 efgh	1247.04	70.83 abc	91.2
G. virens (chlamydospores in milled oats)	Capping	0.61 gh	481.45	78.83 abc	101.5
G. virens (chlamydospores in milled oats)	Seed treatment	1.16 fgh	917.92	89.00 ab	114.6
G. virens (conidia in powder with oil)	Drenching	3.34 bcd	2636.15	79.33 abc	102.2
G. virens (conidia in powder with oil)	Capping	3.60 abc	2846.09	77.66 abc	100.0
G. virens (conidia in powder with oil)	Seed treatment	3.2 bcd	2525.65	77.83 abc	100.2
B. subtilis AW57 +Nutristart	Drenching	1.24 fgh	985.00	90.30 a	116.3
B. subtilis AW57 +Nutristart	Seed treatment	1.34 fgh	1060.77	84.66 a	109.0
*B. subtilis*AW57 (Washed)	Drenching	1.34 fgh	1060.77	77.66 a	100.0
B. subtilis AW57 (Washed)	Seed treatment	1.44 fgh	1136.54	80.66 a	103.9
Control 1 (nil)	Nil	0.1267 h	100.00	77.66 abc	100.0
Control 2 (Nutristart and Pelgel®)	Seed treatment	0.1367 h	107.89	85.00 a	109.5
Control 3 (Nutristart only)	Drenched	0.13 h	102.60	77.83 abc	100.2
Control 4 (Pelgel®)	Seed treatment	0.15 h	118.39	81.66 abc	105.2
Effects		P-values		P-values	
Formulations		0.0001***		0.0968 ^{NS}	
Application		0.0020**		0.0665 ^{NS}	
Organism		0.8596 ^{NS}		0.1727^{NS}	
Formulation x Application		0.0001***		0.0311**	
Formulation x Organism		0.1064 ^{NS}		0.005**	
Application x Organism		0.0056**		0.002**	
Formulation x Application x Organism		0.1854 ^{NS}		0.0383**	
		CV%=26.52		CV%=13.79	
		MSE=0.625		MSE=10.57	

1.NS = Not significant; **= significant at P ≤ 0.05; ***=significant at P ≤ 0.001

2. Means with the same letter are not significantly different (P= 0.05) according to Student, Newman and Keuls comparison test

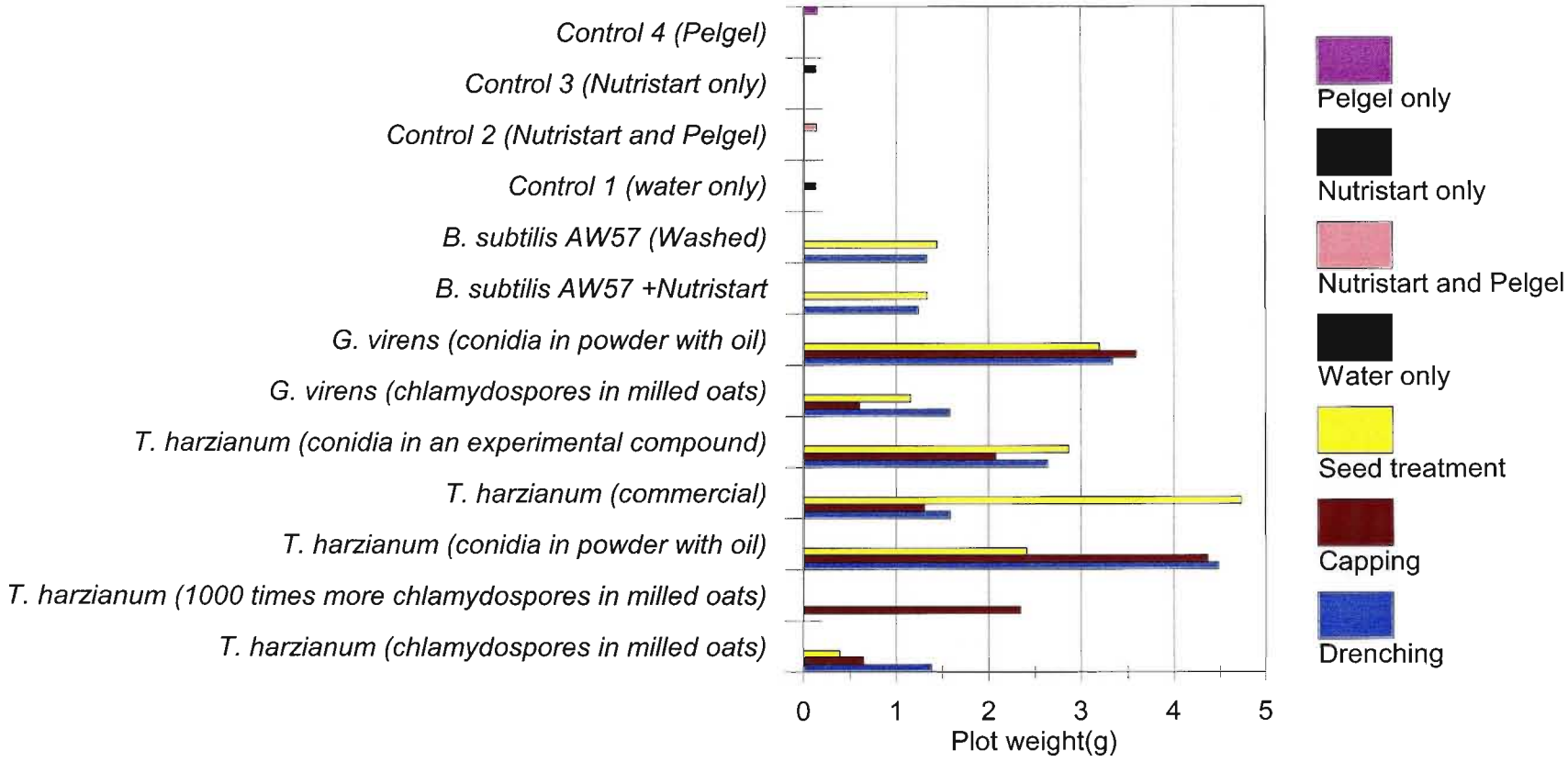

Figure 4.7 Effect of formulations and application methods of *Trichoderma harzianum* KMD, *Gliocladium virens* MM1 and *Bacillus subtilis* AW57 on growth enhancement of plot weight of Namaqualand daisy after four weeks of growth.

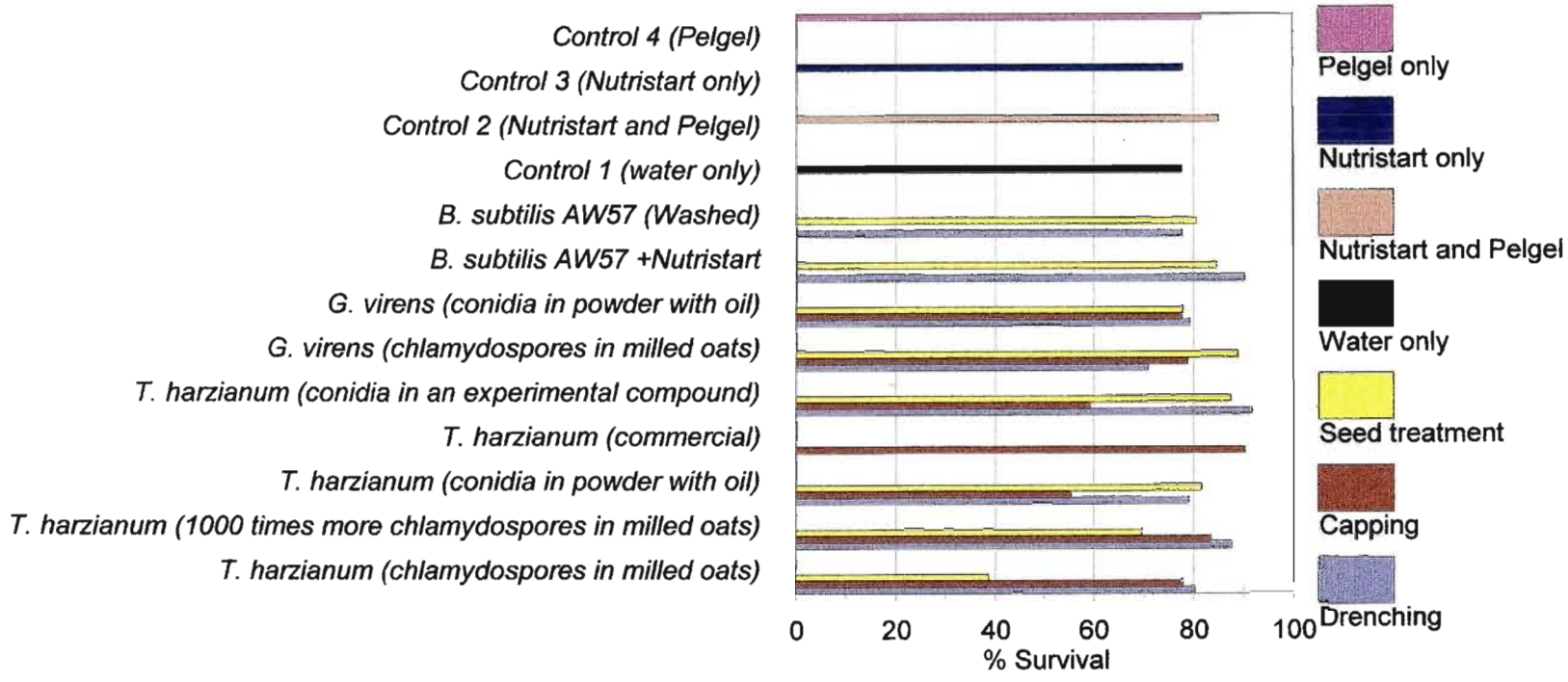

Figure 4.8 Effect of formulations and application methods of *Trichoderma harzianum* KMD, *Gliocladium virens* MM1 and *Bacillus subtilis* AW57 on growth enhancement of percentage survival of Namaqualand Daisy after four weeks of growth.

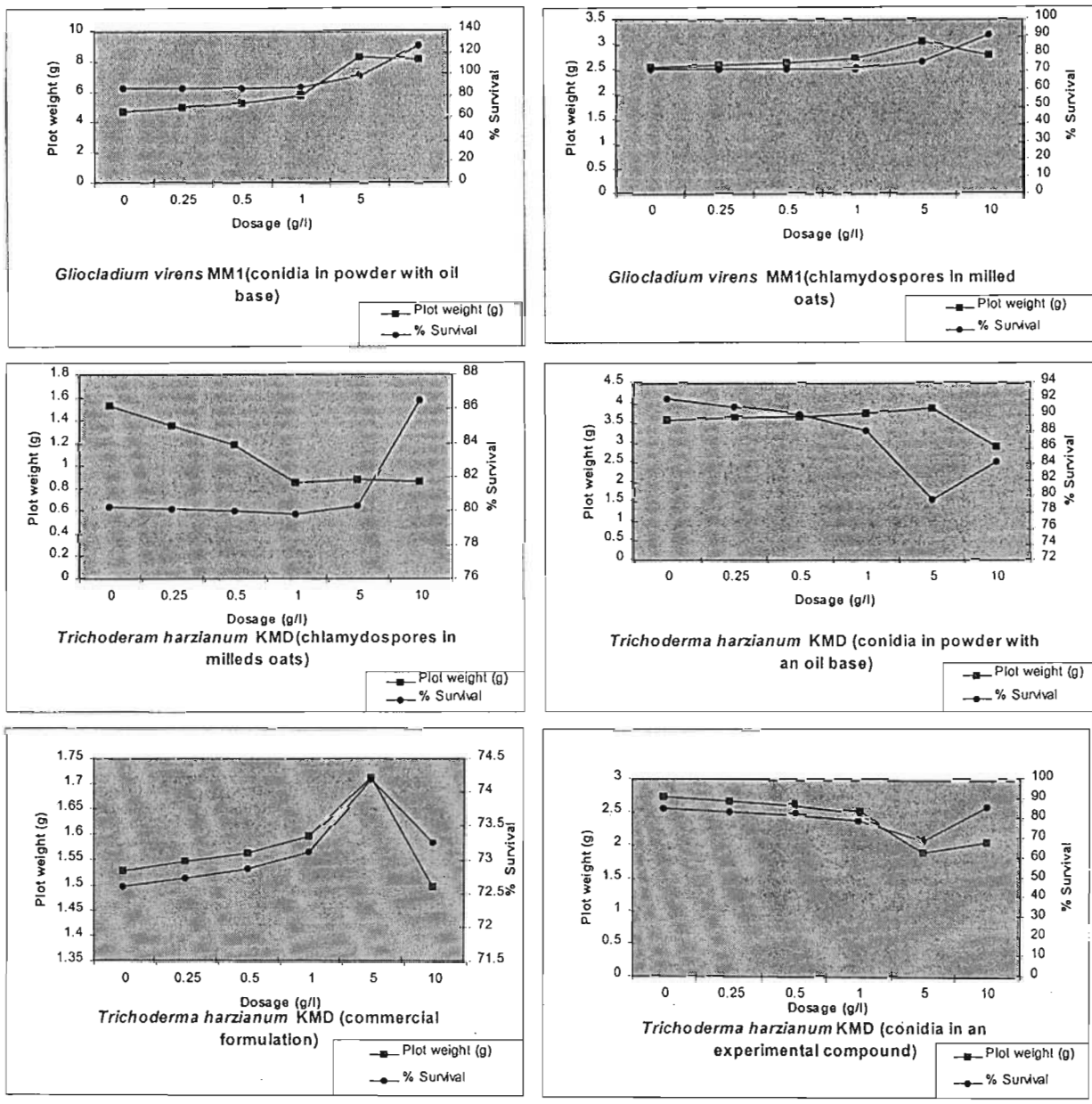

Figure 4.9 Dosage effects of six formulations applied as a drench on growth enhancement on plot weight and percentage survival of Namaqualand daisy after four weeks of growth.

Table 4.4. Effect of formulations of *Trichoderma harzianum* KMD, *Gliocladium virens* MM1 and *Bacillus subtilis* AW57 on plant growth enhancement of eucalyptus after four weeks

Formulations	Application	Plot Weight (PW) (g)	%Control (PW) nil	%Survival (%Surv)	% Control (%Surv)(nil)
T. harzianum (10^5chlamydospores in milled oats)	Drenching	1.70 b	351.97	96.00 ab	110.3
T. harzianum (10^5chlamydospores in milled oats)	Capping	0.49 b	102.90	76.00 bc	87.4
T. harzianum (10^5chlamydospores in milled oats)	Seed treatment	0.55 b	114.49	77.66 abc	89.3
T. harzianum (10^8 chlamydospores in milled oats)	Capping	2.45 b	507.87	85.60 abc	91.2
T. harzianum (conidia in powder with oil)	Drenching	8.48 a	1756.31	97.3 a	111.9
T. harzianum (conidia in powder with oil)	Capping	4.96 b	1028.36	49.86 d	57.3
T. harzianum (conidia in powder with oil)	Seed treatment	7.10 b	1469.98	96.00 ab	110.3
T. harzianum (Commercial)	Drenching	3.66 b	759.21	73.66 c	84.7
T. harzianum (Commercial)	Capping	8.20 b	1697.72	86.66 abc	99.6
T. harzianum (Commercial)	Seed treatment	5.96 b	1235.40	73.16 c	84.1
T. harzianum (conidia in an experimental compound)	Drenching	1.73 b	358.80	97.3 a	111.8
T. harzianum (conidia in an experimental compound)	Capping	2.80 b	579.71	79.33 abc	91.2
T. harzianum (conidia in an experimental compound)	Seed treatment	4.03 b	834.99	79.33 abc	91.2
G. virens (chlamydospores in milled oats)	Drenching	1.42 b	294.00	94.66 ab	108.8
G. virens (chlamydospores in milled oats)	Capping	3.23 b	669.36	86.00 abc	98.9
G. virens (chlamydospores in milled oats)	Seed treatment	2.43 b	503.73	96.00 ab	110.3
G. virens (conidia in powder with oil)	Drenching	3.16 b	655.69	97.33 a	111.9
G. virens (conidia in powder with oil)	Capping	1.33 b	275.36	59.83 bd	68.8
G. virens (conidia in powder with oil)	Seed treatment	3.10 b	641.82	93.16 abc	107.1
B. subtilis AW57 +Nutristart	Drenching	4.80 b	993.79	92.00 a	105.7
B. subtilis AW57 +Nutristart	Seed treatment	5.28 b	1093.17	96.00 a	110.3
B. subtilis AW57 (Washed)	Drenching	4.18 b	865.42	51.30 c	59.0
B. subtilis AW57 (Washed)	Seed treatment	5.12 b	1060.46	76.50 ab	87.9
Control 1 (nil)	Nil	0.48 b	100.00	87.00 abc	100.0
Control 2 (Nutristart and Pelgel®)	Seed treatment	0.53 b	110.35	80.50 abc	92.5
Control 3 (Nutristart only)	Drenched	0.48 b	100.83	86.00 abc	98.9
Control 4 (Pelgel®)	Seed treatment	0.51 b	105.59	91.83 abc	105.6
Effects		P-values		P-values	
Formulations		0.1223^{NS}		0.0153^{**}	
Application		0.2776^{NS}		0.101^{NS}	
Organism		0.6988^{NS}		0.002^{**}	
Formulation x Application		0.1503^{NS}		0.1480^{NS}	
Formulation x Organism		0.4387^{NS}		0.0001^{***}	
Application x Organism		0.9334^{NS}		0.0001^{***}	
Formulation x Application x Organism		0.9835^{NS}		0.0001^{***}	
		CV%=21.50		CV%=8.005	
		MSE=0.26		MSE=6.72	

1.NS = Not significant; **= significant at $P \leq 0.05$; ***=significant at $P \leq 0.001$

2. Means with he same letter are not significantly different (P= 0.05) according to Student, Newman and Keuls comparison test

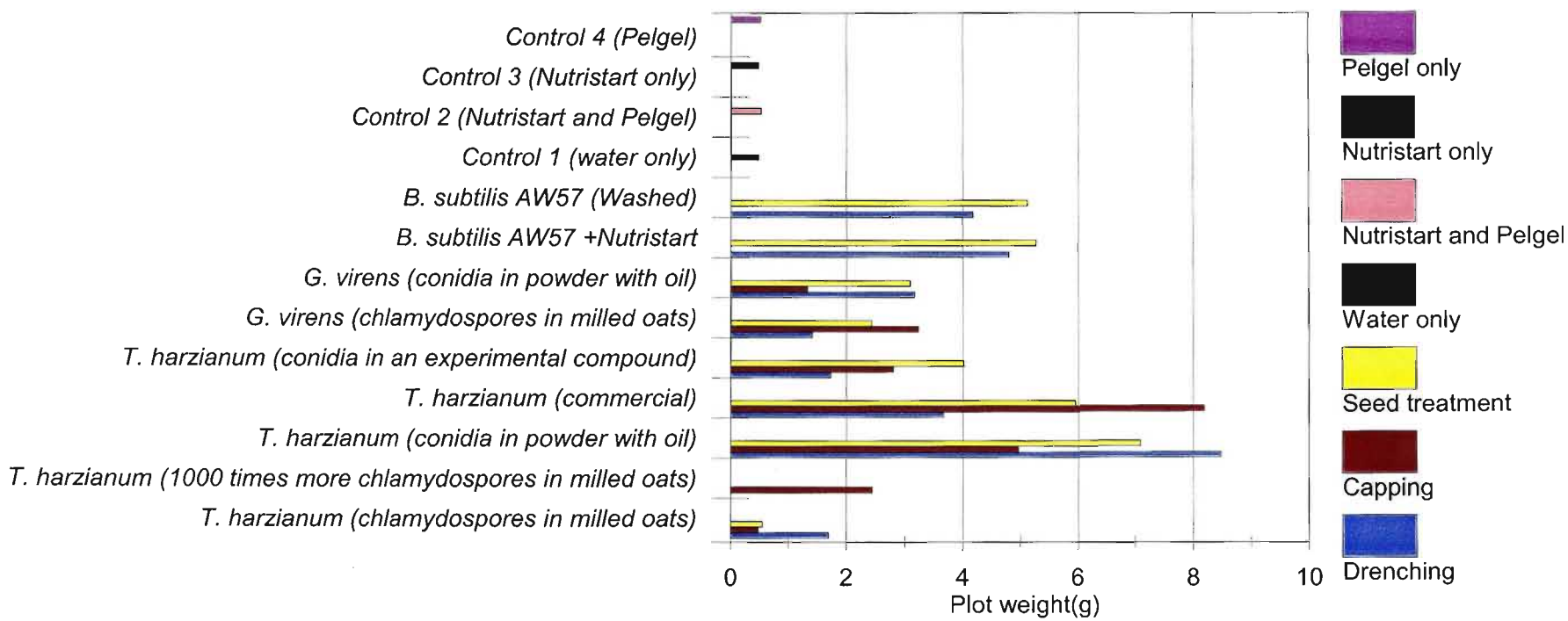

Figure 4.10 The effect of formulations and application methods of *Trichoderma harzianum* KMD, *Gliocladium virens* MM1 and *Bacillus subtilis* AW57 on growth enhancement of plot weight of eucalyptus after four weeks of growth.

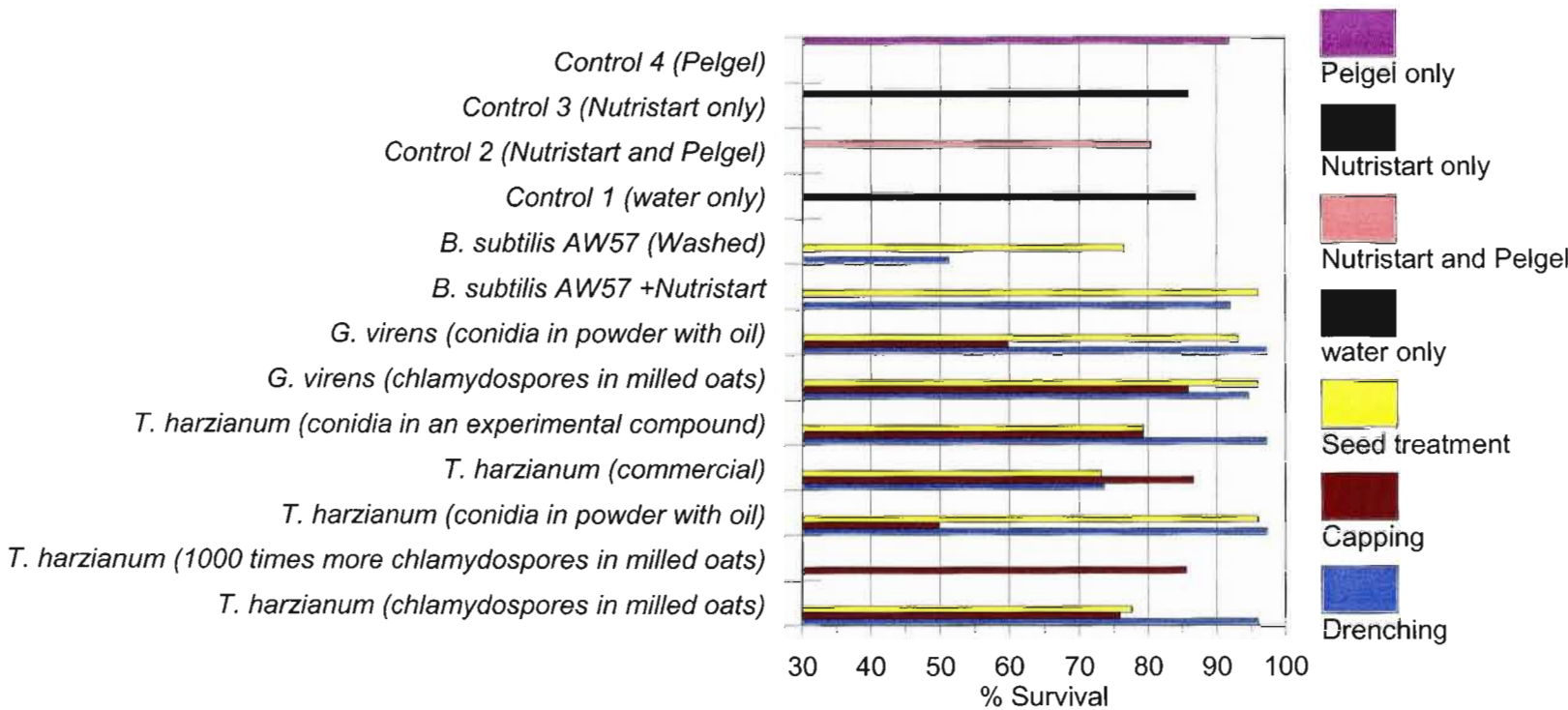

Figure 4.11 Effect of formulations and application methods of *Trichoderma harzianum* KMD, *Gliocladium virens* MM1 and *Bacillus subtilis* AW57 on growth enhancement of percentage survival of eucalyptus after four weeks of growth.

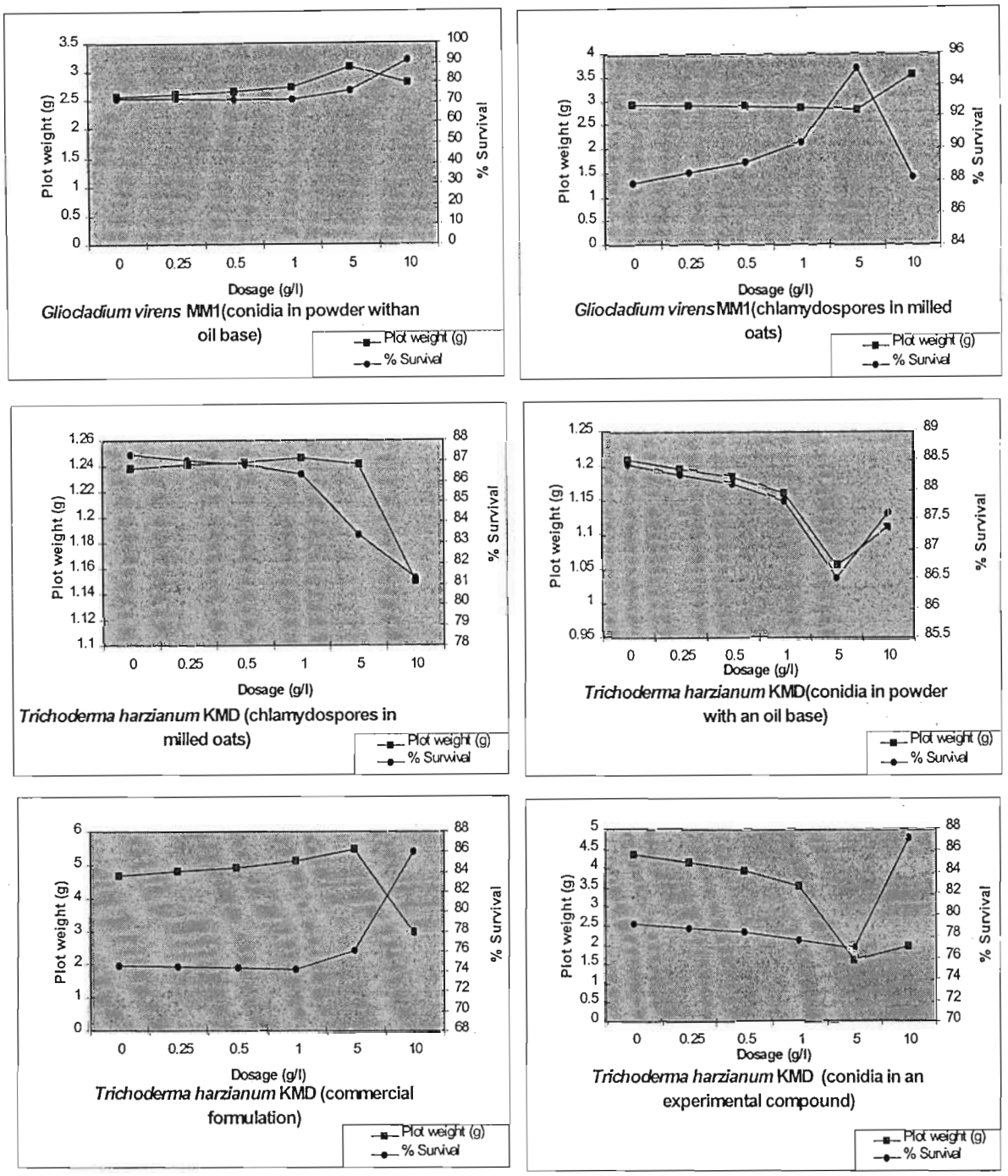

Figure 4.12 Dosage effects of six formulations applied as a drench on growth enhancement on plot weight and percentage survival of eucalyptus after four weeks of growth.

Table 4.5. Effect of formulations of *Trichoderma harzianum* KMD, *Gliocladium virens* MM1 and *Bacillus subtilis* AW57 on the control of damping-off of cabbage caused by *Pythium* sp after four weeks

Formulations	Application	Plot Weight (PW) (g)	%Control 2(PW / (*Pythium* sp.)	%Survival (%Surv)	%Control 2 (%Surv/*Pythium* sp.)
T. harzianum (10^5chlamydospores in milled oats)	Drenching	1.86 ab	92.09	96.00 ab	95.4
T. harzianum (10^5chlamydospores in milled oats)	Capping	2.07 ab	102.83	96.00 ab	106.5
T. harzianum (10^5chlamydospores in milled oats)	Seed treatment	2.10 ab	104.16	97.33 ab	107.9
T. harzianum (10^8chlamydospores in milled oats)	Capping	1.19 ab	59.19	94.67 ab	105.0
T.harzianum (conidia in powder with oil)	Drenching	1.56 ab	77.21	72.17 ab	80.0
T.harzianum (conidia in powder with oil)	Capping	2.04 ab	101.18	94.50 ab	104.8
T.harzianum (conidia in powder with oil)	Seed treatment	1.86 ab	92.09	86.00 ab	95.4
T. harzianum (Commercial)	Drenching	1.35 ab	66.96	62.50 ab	69.3
T. harzianum (Commercial)	Capping	1.62 ab	80.35	75.00 ab	83.2
T. harzianum (Commercial)	Seed treatment	1.11 ab	54.89	51.33 b	56.9
T. harzianum (conidia in an experimental compound)	Drenching	2.05 ab	101.51	84.67 ab	93.9
T. harzianum (conidia in an experimental compound)	Capping	1.83 ab	90.76	72.60 ab	80.5
T. harzianum (conidia in an experimental compound)	Seed treatment	1.56 ab	77.21	55.33 ab	94.7
G. virens (chlamydospores in milled oats)	Drenching	1.80 ab	89.44	83.50 ab	92.6
G. virens (chlamydospores in milled oats)	Capping	2.04 ab	101.34	94.67 ab	105.0
G. virens (chlamydospores in milled oats)	Seed treatment	1.98 ab	98.20	91.67 ab	101.7
G. virens (conidia in powder with oil)	Drenching	1.79 ab	88.78	83.00 ab	92.0
G. virens (conidia in powder with oil)	Capping	1.74 ab	86.14	80.33 ab	89.1
G. virens (conidia in powder with oil)	Seed treatment	2.16 a	107.13	100.00 a	110.9
B. subtilis AW57 +Nutristart	Drenching	1.68 ab	83.33	83.30 a	92.4
B. subtilis AW57 +Nutristart	Seed treatment	1.92 ab	95.23	91.83 a	101.8
B. subtilis AW57 (Washed)	Drenching	1.92 ab	95.23	93.30 a	103.5
B. subtilis AW57 (Washed)	Seed treatment	1.92 ab	95.23	91.83 a	101.8
Control 1 (nil)	Nil	2.10 ab	104.16	97.33 ab	107.9
Control 2 (*Pythium* sp. only)	Nil	2.02 ab	100.00	90.17 ab	100.0
Control 3 (Nutristart and Pelgel® and *Pythium* sp.)	Seed treatment	0.07 ab	3.57	29.00 b	32.2
Control 4 (Nutristart only and *Pythium* sp.)	Drenched	1.92 ab	95.23	81.83 ab	90.8
Control 5 (Pelgel® and *Pythium* sp.)	Seed treatment	1.95 ab	96.55	93.33 a	103.5
Effects		P-values		P-values	
Formulations		0.0004***		0.9713 ^{NS}	
Application		0.6296 ^{NS}		0.0004***	
Organism		0.9657 ^{NS}		0.0339**	
Formulation x Application		0.3583 ^{NS}		0.5345 ^{NS}	
Formulation x organism		0.5330 ^{NS}		0.3622 ^{NS}	
Application x organism		0.5811 ^{NS}		0.5822 ^{NS}	
Formulation x Application x organism		0.4539 ^{NS}		0.4311 ^{NS}	
		CV%=19.56		CV%=19.58	
		MSE=0.014		MSE=16.08	

1.NS = Not significant; **= significant at P ≤ 0.05; ***=significant at P ≤ 0.001

2. Means with the same letter are not significantly different (P= 0.05) according to Student, Newman and Keuls comparison test

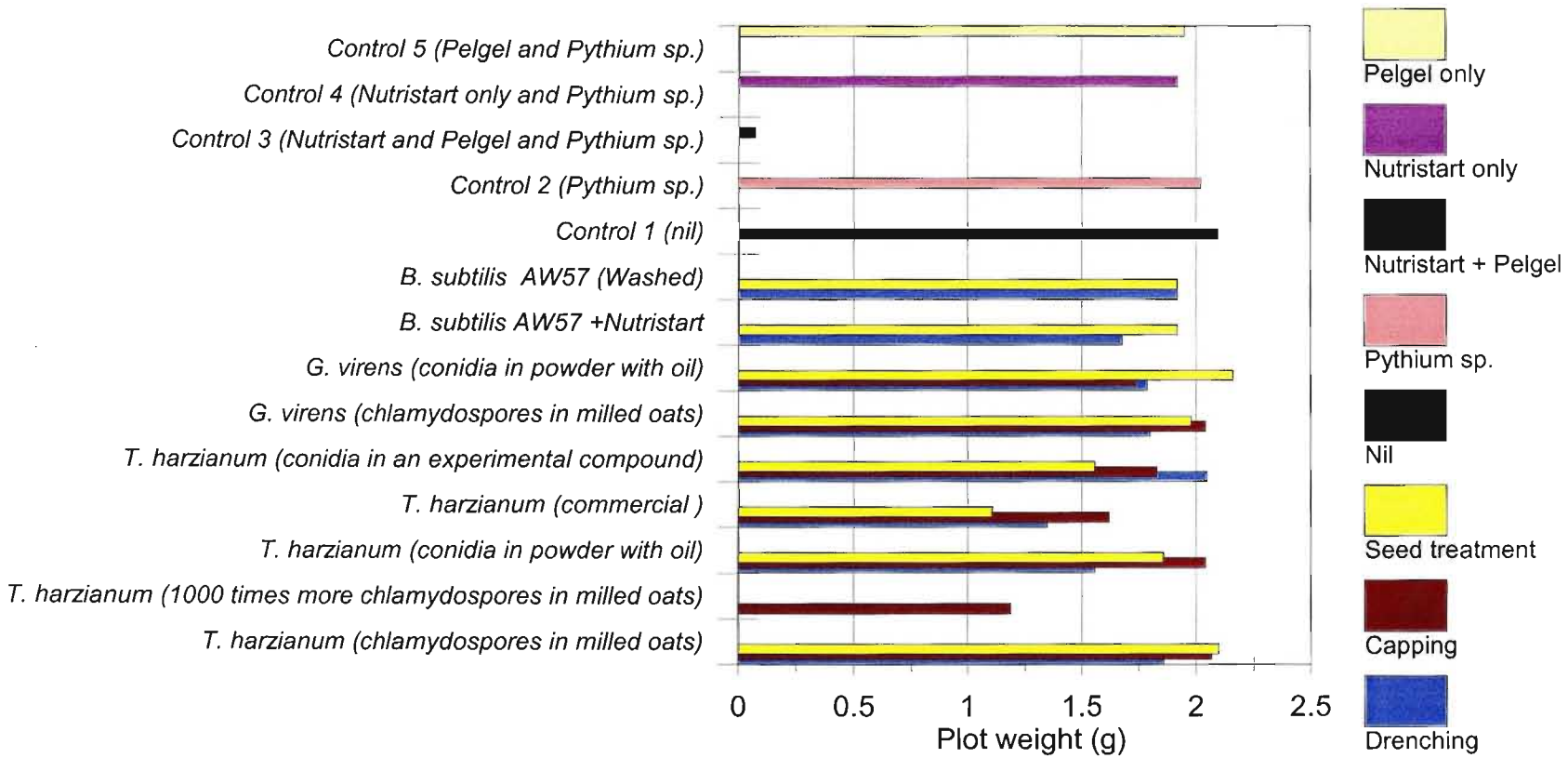

Figure 4.13 Effect of formulations and application methods of *Trichoderma harzianum* KMD, *Gliocladium virens* MM1 and *Bacillus subtilis* AW57 on the biocontrol of damping-off caused by *Pythium* spp. measuring plot weight of cabbage after four weeks of growth.

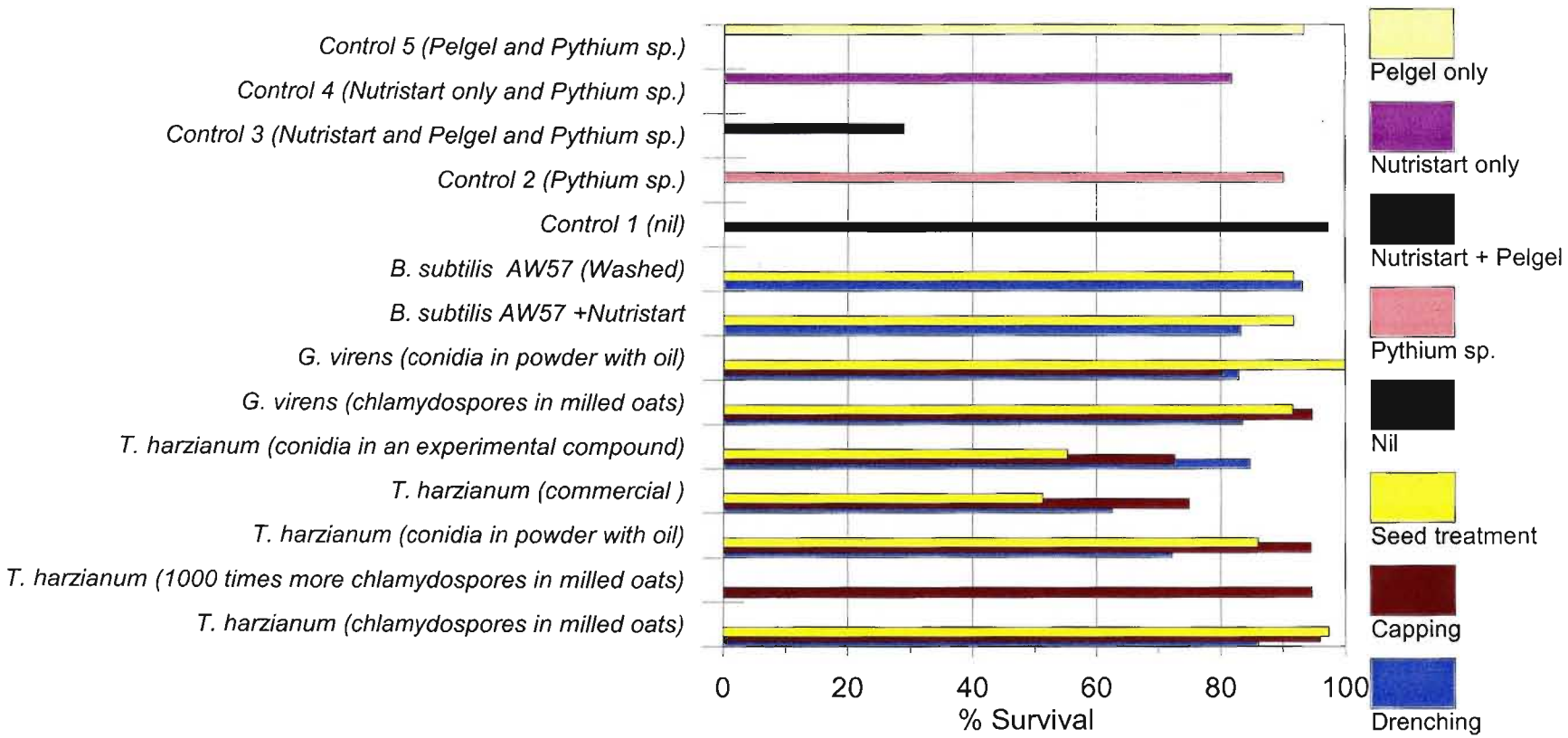

Figure 4.14 Effect of formulations and application methods of *Trichoderma harzianum* KMD, *Gliocladium virens* MM1 and *Bacillus subtilis* AW57 on the biocontrol of damping-off caused by *Pythium* spp. measuring percentage survival of cabbage after four weeks of growth.

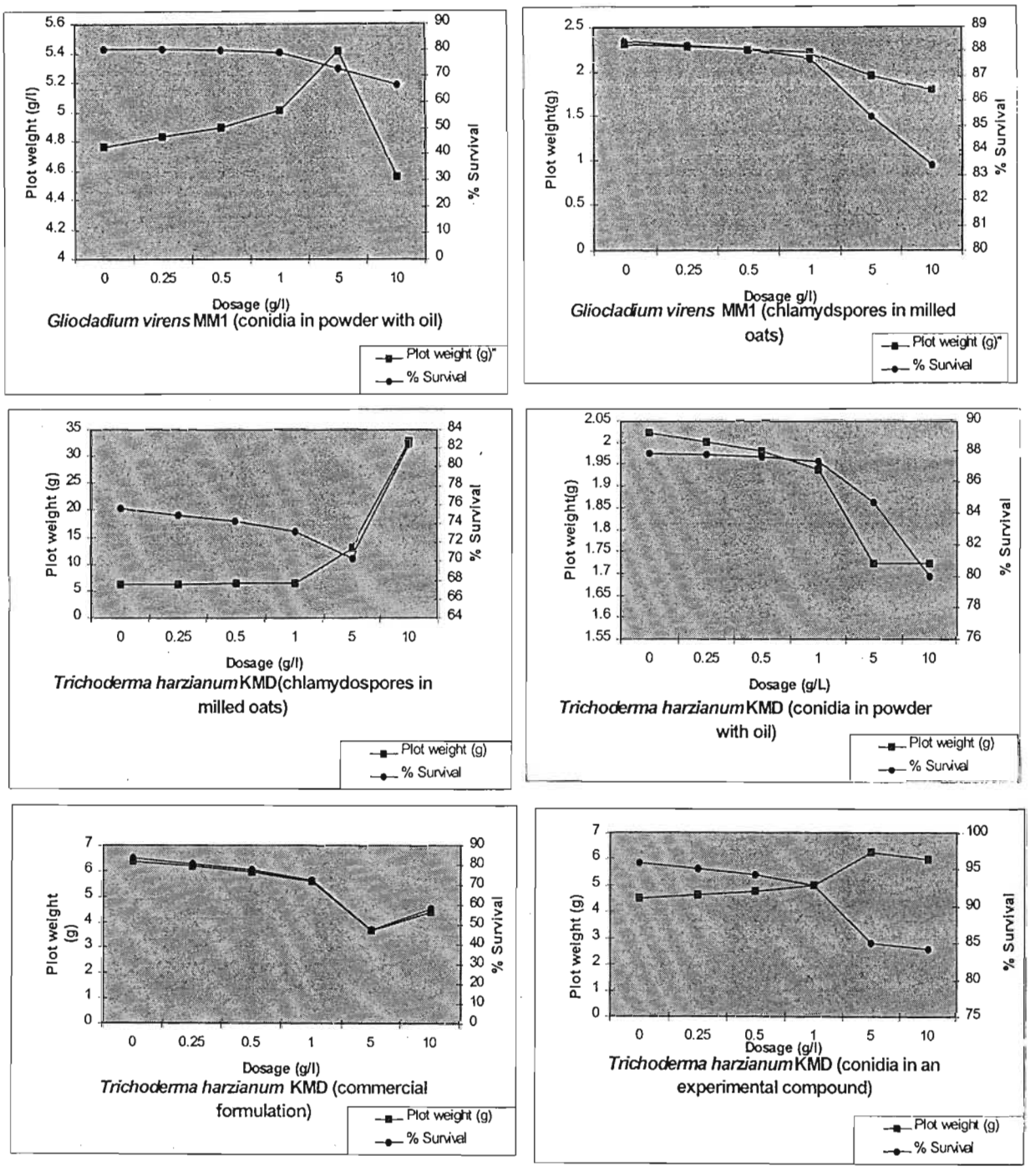

Figure 4.15 Dosage effects of six formulations applied as a drench on damping-off caused by *Pythium* spp. on plot weight and percentage survival of cabbage after four weeks of growth.

Table 4.6. Effect of formulations of *Trichoderma harzianum* KMD, *Gliocladium virens* MM1 and *Bacillus subtilis* AW57 on the control of damping-off of cucumber caused by *Pythium* sp. after four weeks.

Formulations	Application	Plot Weight (PW) (g)	%Control 2 (PW/ *Pythium* sp.)	%Survival (% Surv)	%Control 2 (%Surv /*Pythium* sp.)
T. harzianum (10^5chlamydospores in milled oats)	Drenching	1.82 a	89.82	84.50 a	135.6
T. harzianum (10^5chamydospores in milled oats)	Capping	2.04 a	100.49	94.50 a	151.6
T. harzianum (10^5chlamydospores in milled oats)	Seed treatment	2.13 a	104.93	98.67 a	158.3
T. harzianum (10^8chlamydospores in milled oats)	Capping	1.38 ab	67.98	83.00 a	133.2
T. harzianum (conidia in powder with oil)	Drenching	2.07 a	101.97	96.00 a	154.0
T. harzianum (conidia in powder with oil)	Capping	1.98 a	97.70	91.83 a	147.3
T. harzianum (conidia in powder with oil)	Seed treatment	1.68 a	82.60	77.67 a	124.6
T. harzianum (Commercial)	Drenching	2.01 a	98.85	93.00 a	149.2
T. harzianum (Commercial)	Capping	1.98 a	97.54	91.67 a	147.1
T. harzianum (Commercial)	Seed treatment	2.04 a	100.33	94.33 a	151.3
T. harzianum (conidia in an experimental compound)	Drenching	1.80 a	88.67	90.50 a	145.2
T. harzianum (conidia in an experimental compound)	Capping	1.96 a	96.39	86.50 a	138.8
T. harzianum (conidia in an experimental compound)	Seed treatment	1.86 a	91.46	64.00 b	102.7
G. virens (chlamydospores in milled oats)	Drenching	0.84 b	41.54	39.00 b	62.6
G. virens (chlamydospores in milled oats)	Capping	2.07 a	101.97	96.00 a	154.0
G. virens (chlamydospores in milled oats)	Seed treatment	1.88 a	92.77	87.33 a	140.1
G. virens (conidia in powder with oil)	Drenching	1.41 ab	69.62	65.33 ab	104.8
G. virens (conidia in powder with oil)	Capping	2.04 a	100.49	94.50 a	151.6
G. virens (conidia in powder with oil)	Seed treatment	1.92 a	94.74	89.00 a	142.8
B. subtilis AW57 +Nutristart	Drenching	2.08 a	102.46	51.50 c	82.6
B. subtilis AW57 +Nutristart	Seed treatment	1.34 ab	66.01	77.67 abc	124.6
B. subtilis AW57 (Washed)	Drenching	2.23 a	109.95	84.83 abc	136.1
B. subtilis AW57 (Washed)	Seed treatment	0.63 a	30.84	98.67 a	158.3
Control 1 (nil)	Nil	2.10 a	103.45	97.33 a	156.2
Control 2 (*Pythium* sp. only)	Nil	2.04 a	100.00	62.33 ab	100.0
Control 3 (Nutristart and Pelgel® and *Pythium* sp.)	Seed treatment	2.23 a	109.85	54.00 bc	86.6
Control 4 (Nutristart only and *Pythium* sp.)	Drenched	0.63 b	30.86	93.00 ab	149.2
Control 5 (Pelgel® and *Pythium* sp.)	Seed treatment	2.04 a	100.25	94.33 a	151.3
Effects		P-values		P-values	
Formulations		0.001***		0.0006***	
Application		0.001***		0.0011***	
Organism		0.0007***		0.0001***	
Formulation x Application		0.03**		0.050**	
Formulation x organism		0.05**		0.0001***	
Application x organism		0.0001***		0.0001***	
Formulation x Application x organism		0.3948^{NS}		0.3968^{NS}	
		CV%=11.37		CV%=11.37	
		MSE=0.0087		MSE=9.68	

1.NS = Not significant; **= significant at P ≤ 0.05; ***=significant at P ≤ 0.001

2. Means with the same letter are not significantly different (P= 0.05) according to Student, Newman and Keuls comparison test

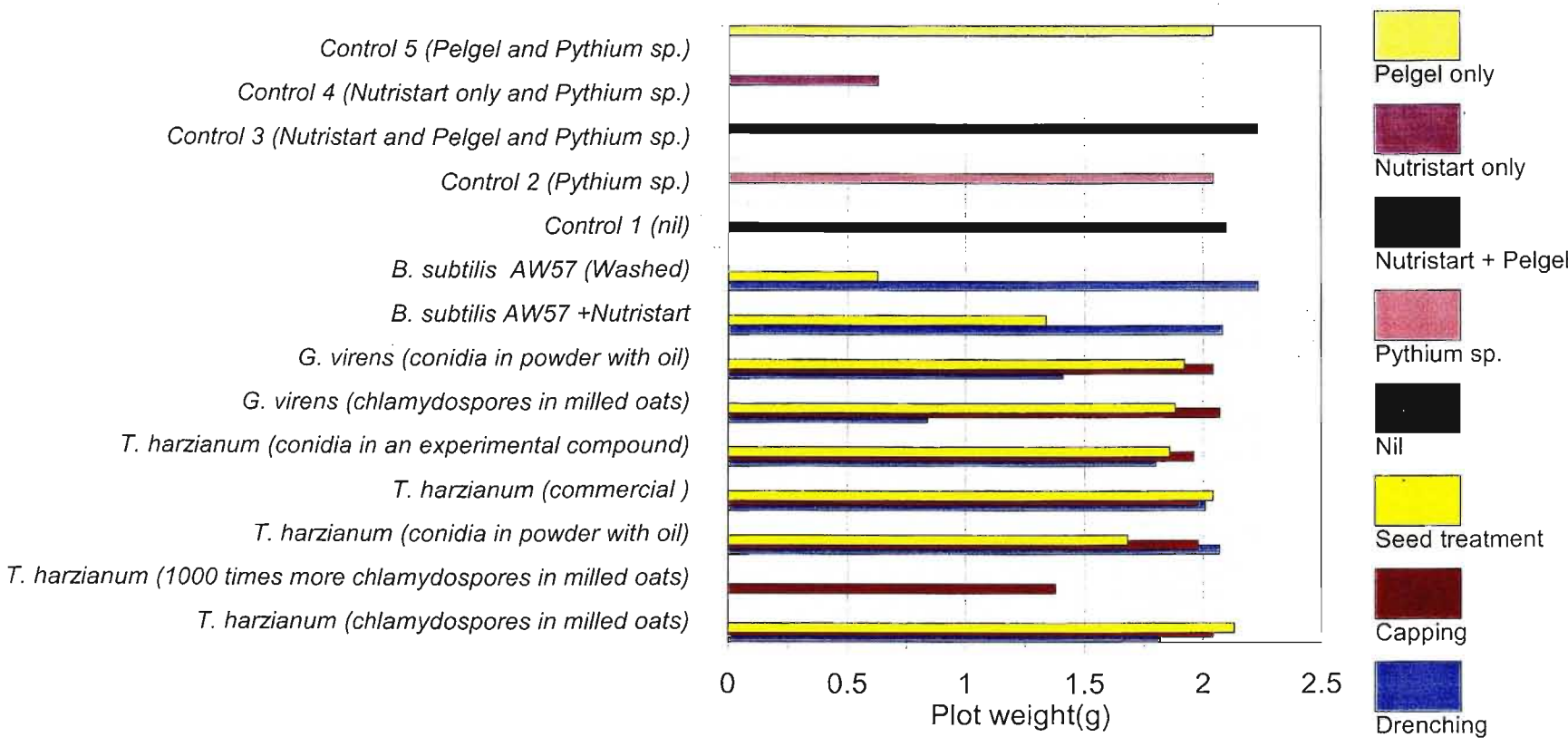

Figure 4.16 Effect of formulations and application methods of *Trichoderma harzianum* KMD, *Gliocladium virens* MM1 and *Bacillus subtilis* AW57 on the biocontrol of damping-off caused by *Pythium* spp. measuring plot weight of cucumber after four weeks of growth.

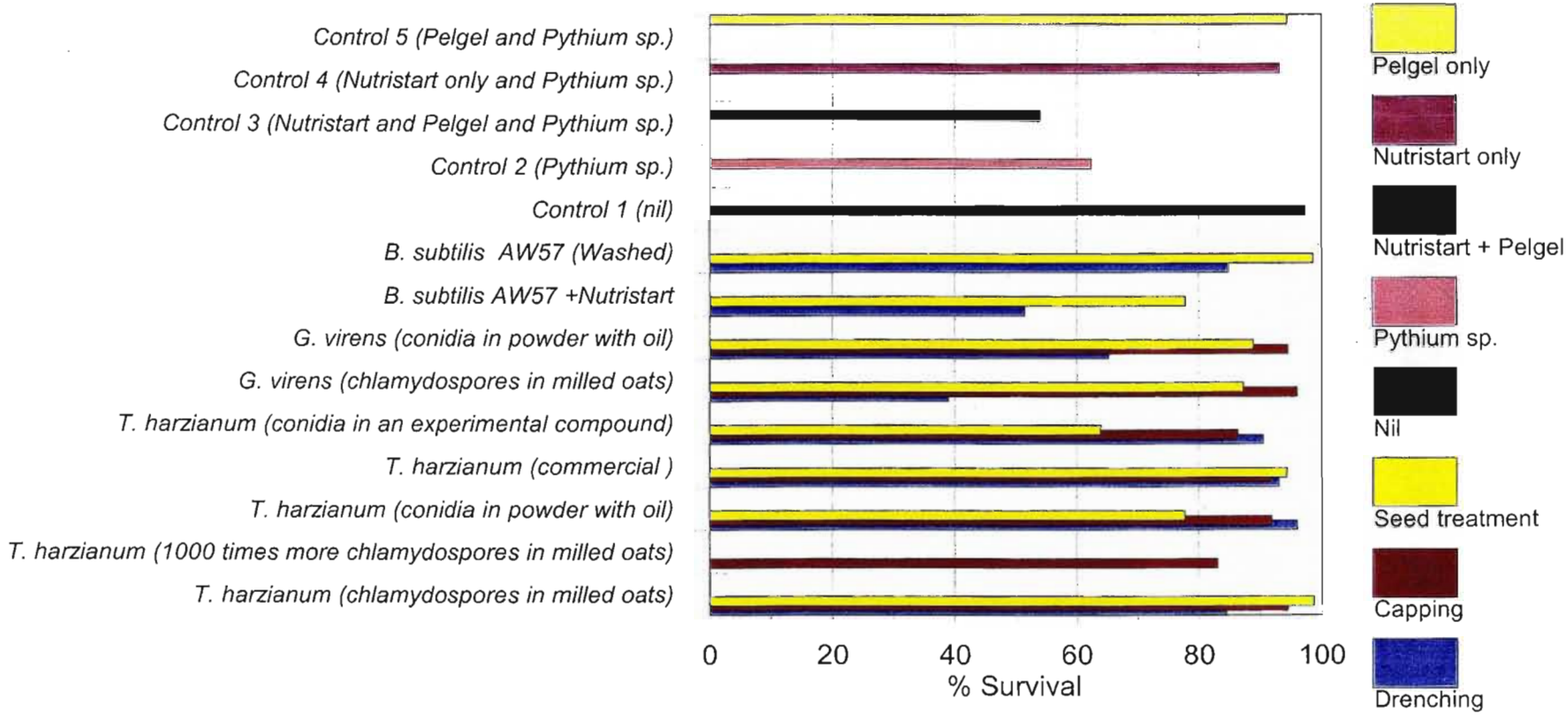

Figure 4.17 Effect of formulations and application methods of *Trichoderma harzianum* KMD, *Gliocladium virens* MM1 and *Bacillus subtilis* AW57 on the biocontrol of damping-off caused by *Pythium* spp. measuring percentage survival of cucumber after four weeks of growth.

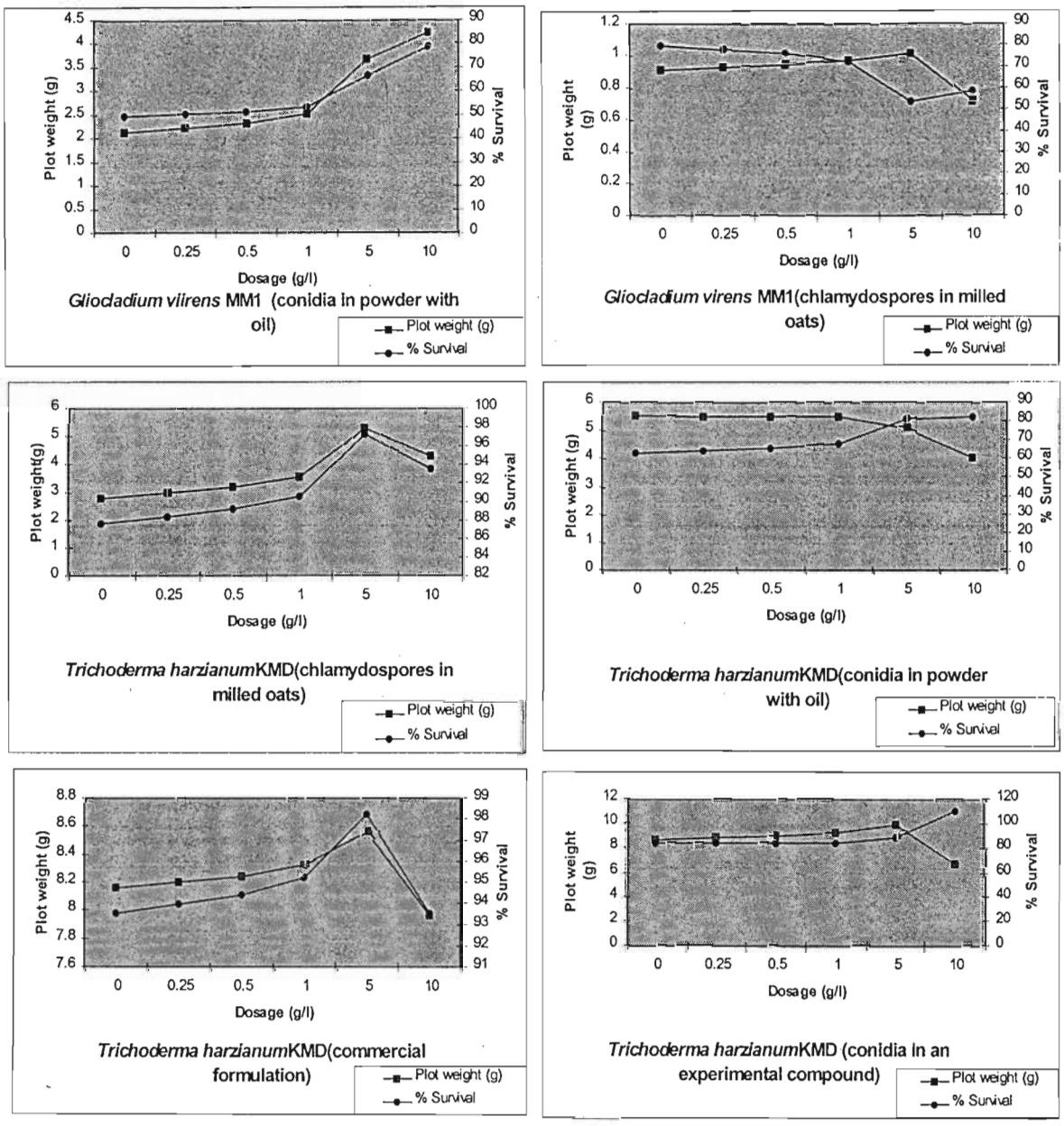

Figure 4.18 Dosage effects of six formulations applied as a drench on damping-off caused by *Pythium* spp. on plot weight and percentage survival of cucumber after four weeks of growth.

Table 4.7. Effect of formulations of *Trichoerma harzianum* KMD, *Gliocladium virens* MM1 and *Bacillus subtilis* AW57 on damping-off of Namaqualand daisy caused by *Pythium* sp. after four weeks

Formulations	Application	Plot Weight (PW) (g)	%Control 2 (PW/*Pythium* sp.)	%Survival	%Control 2 (%Surv/*Pythium* sp.)
T. harzianum (10^5chlamydospores in milled oats)	Drenching	1.56 ab	108.35	72.00 ab	108.3
T. harzianum (10^5chlamydospores in milled oats)	Capping	1.64 ab	114.38	76.00 ab	114.3
T. harzianum (10^5chlamydospores in milled oats)	Seed treatment	1.32 ab	91.65	61.00 ab	91.7
T. harzianum (10^8chlamydospores in milled oats)	Capping	1.32 ab	91.65	80.50 a	121.1
T.harzianum (conidia in powder with oil)	Drenching	1.73 a	120.62	80.33 a	120.8
T.harzianum (conidia in powder with oil)	Capping	1.80 a	125.06	83.33 a	125.3
T.harzianum (conidia in powder with oil)	Seed treatment	1.65 ab	114.85	76.33 ab	114.8
T. harzianum (Commercial)	Drenching	1.32 ab	91.65	61.00 ab	91.7
T. harzianum (Commercial)	Capping	1.89 a	131.55	87.50 a	131.6
T. harzianum (Commercial)	Seed treatment	1.86 a	129.23	86.00 a	129.3
T. harzianum (conidia in an experimental compound)	Drenching	1.74 a	120.88	89.66 a	142.3
T. harzianum (conidia in an experimental compound)	Capping	2.05 a	142.46	70.50 ab	106.0
T. harzianum (conidia in an experimental compound)	Seed treatment	1.52 ab	106.03	61.00 ab	91.7
G. virens (chlamydospores in milled oats)	Drenching	1.50 ab	104.18	69.33 ab	104.3
G. virens (chlamydospores in milled oats)	Capping	0.96 b	66.59	44.16 b	66.4
G. virens (chlamydospores in milled oats)	Seed treatment	1.62 ab	112.76	75.00 ab	112.8
G. virens (conidia in powder with oil)	Drenching	1.62 ab	112.53	74.83 ab	112.5
G. virens (conidia in powder with oil)	Capping	1.47 ab	102.09	67.83 ab	102.0
G. virens (conidia in powder with oil)	Seed treatment	1.35 ab	93.97	62.50 ab	94.0
B. subtilis AW57 +Nutristart®	Drenching	0.72 b	50.11	65.33 a	98.2
B. subtilis AW57 +Nutristart®	Seed treatment	0.96 b	66.82	73.67 a	110.8
B. subtilis AW57 (Washed)	Drenching	1.20 ab	83.52	75.00 a	112.8
B. subtilis AW57 (Washed)	Seed treatment	1.68 ab	116.93	81.67 a	122.8
Control 1 (nil)	Nil	1.89 a	131.78	87.66 a	131.8
Control 2 (*Pythium* only)	Nil	1.44 ab	100.00	66.50 ab	100.0
Control 3 (Nutristart and Pelgel® and *Pythium* sp.)	Seed treatment	1.74 ab	121.34	25.00 b	37.6
Control 4 (Nutristart only and *Pythium* sp.)	Drenched	1.06 ab	73.78	81.67 a	122.8
Control 5 (Pelgel® and *Pythium* sp.)	Seed treatment	1.13 ab	78.51	80.6 6a	121.3
Effects		P-values		P-values	
Formulations		0.0064**		0.0233**	
Application		0.9083 NS		0.0065**	
Organism		0.0236**		0.9168 NS	
Formulation x Application		0.0707 NS		0.5439 NS	
Formulation x organism		0.5512 NS		0.0709 NS	
Application x organism		0.0404**		0.0395**	
Formulation x Application x organism		0.0777 NS		0.0811 NS	
		CV%=16.01		CV%=16.06	
		MSE=0.0104		MSE=11.70	

1.NS = Not significant; **= significant at P ≤ 0.05; ***=significant at P ≤ 0.001

2.Means with the same letter are not significantly different (P= 0.05) according to Student, Newman and Keuls comparison test

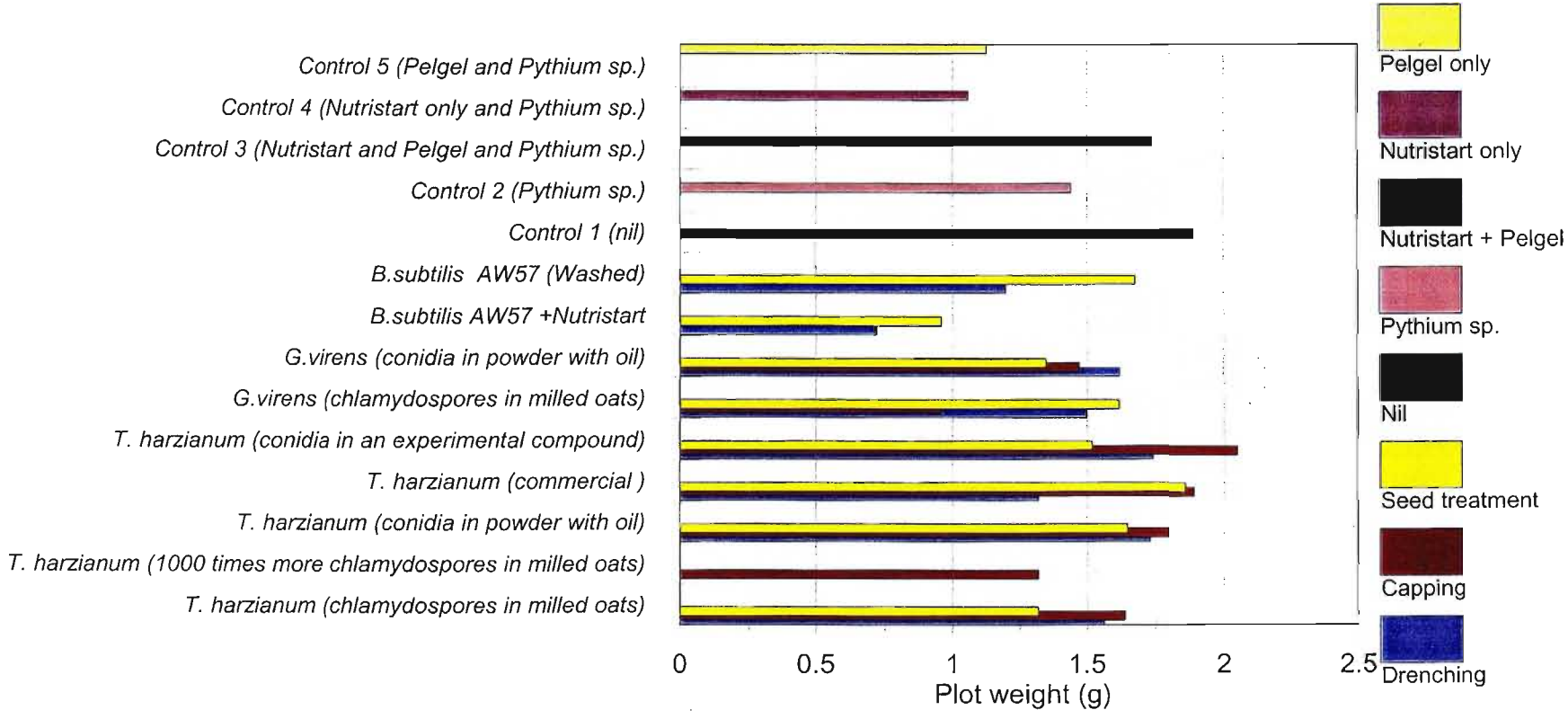

Figure 4.19 Effect of formulations and application methods of *Trichoderma harzianum* KMD, *Gliocladium virens* MM1 and *Bacillus subtilis* AW57 on the biocontrol of damping-off caused by *Pythium* spp. measuring plot weight of Namaqualand daisy after four weeks of growth.

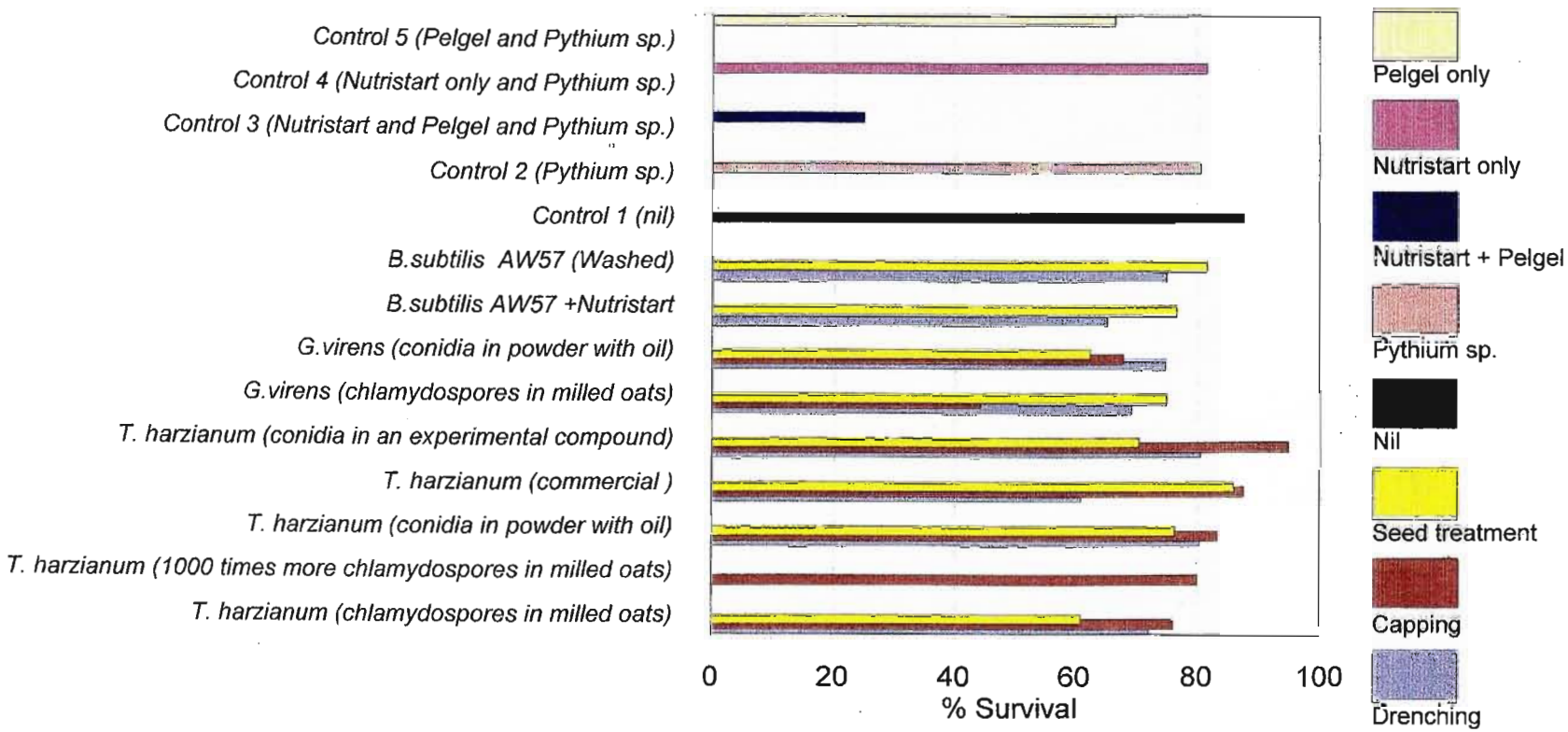

Figure 4.20 Effect of formulations and application methods of *Trichoderma harzianum* KMD, *Gliocladium virens* MM1 and *Bacillus subtilis* AW57 on the biocontrol of damping-off caused by *Pythium* spp. measuring percentage survival of Namaqualand daisy after four weeks of growth.

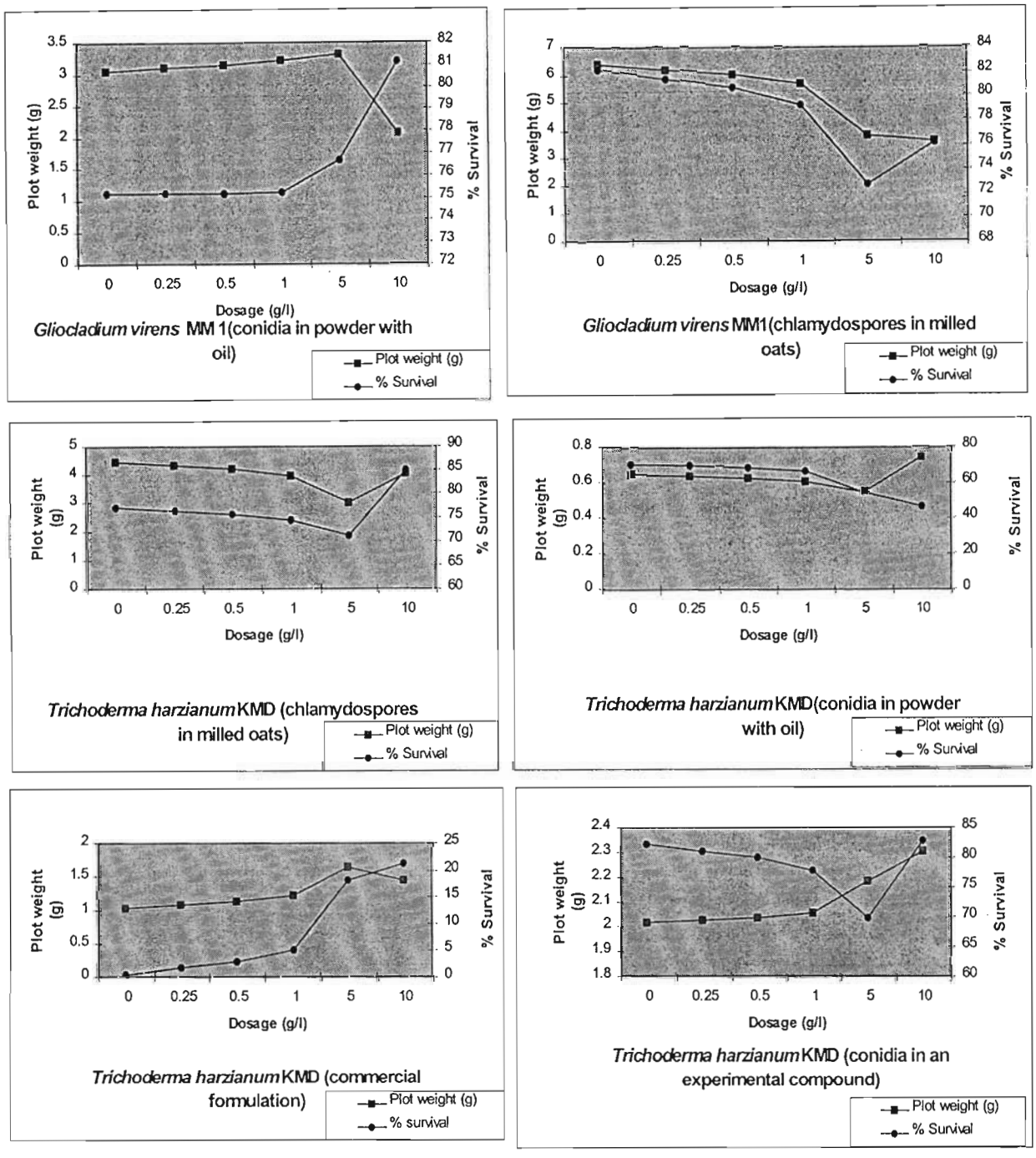

Figure 4.21 Dosage effects of six formulations applied as a drench on damping-off caused by *Pythium* spp. on plot weight and percentage survival of Namaqualand daisy after four weeks of growth.

Table 4.8. Effect of formulations of *Trichoderma harzianum* KMD, *Gliocladium virens* MM1 and *Bacillus subtilis* AW57 on damping-off of eucalyptyus caused by *Pythium* sp. after four weeks.

Formulations	Application	Plot Weight (PW) (g)	%Control 2 (PW/*Pythium* sp.)	%Survival (% Surv)	%Control 2 (% Surv//*Pythium* sp.)
T. harzianum (10^5chlamydospores in milled oats)	Drenching	2.16 a	130.91	100.00 a	107.1
T. harzianum (10^5chlamydospores in milled oats)	Capping	2.04 ab	123.64	94.50 ab	101.3
T. harzianum (10^5chlamydospores in milled oats)	Seed treatment	1.95 ab	117.98	90.167 ab	96.6
T. harzianum (10^8chlamydospores in milled oats)	Capping	1.62 abc	98.18	75.00 abc	80.4
T.harzianum (conidia in powder with oil)	Drenching	2.01 abc	121.82	75.00 abc	80.4
T.harzianum (conidia in powder with oil)	Capping	1.58 abc	95.56	73.00 abc	78.2
T.harzianum (conidia in powder with oil)	Seed treatment	1.58 abc	95.56	73.00 abc	78.2
T. harzianum (Commercial)	Drenching	1.83 ab	110.91	84.667 ab	90.7
T. harzianum (Commecial)	Capping	1.45 bc	87.68	67.00 bc	71.8
T. harzianum (Commercial)	Seed treatment	1.71 abc	103.84	79.33 abc	93.9
T. harzianum (conidia in an experimental compound)	Drenching	1.62 abc	98.18	87.66 abc	78.6
T. harzianum (conidia in an experimental compound)	Capping	1.90 ab	114.95	73.33 abc	99.6
T. harzianum (conidia in an experimental compound)	Seed treatment	1.58 abc	95.96	93.00 ab	93.8
G. virens (chlamydospores in milled oats)	Drenching	1.89 ab	114.55	87.50 ab	105.7
G. virens (chlamydospores in milled oats)	Capping	2.13 a	129.09	98.66 a	102.9
G. virens (chlamydospores in milled oats)	Seed treatment	2.07 a	125.45	96.00 a	47.9
G. virens (conidia in powder with oil)	Drenching	0.97 de	58.59	44.66 de	38.9
G. virens (conidia in powder with oil)	Capping	0.79 e	47.68	36.33 e	63.2
G. virens (conidia in powder with oil)	Seed treatment	1.27 cd	77.17	59.00 cd	86.5
Bacillus subtilis AW57 +Nutristart	Drenching	0.74 e	44.85	80.70 abc	105.72
Bacillus subtilis AW57 +Nutristart	Seed treatment	0.72 e	43.64	64.00 a	83.84
Bacillus subtilis AW57 (Washed)	Drenching	0.07 f	4.30	84.80 abc	111.09
Bacillus subtilis AW57 (Washed)	Seed treatment	0.07 f	4.36	83.20 a	109.00
Control 1 (nil)	Nil	1.89 ab	114.55	87.50 ab	93.8
Control 2 (*Pythium* sp. only)	Nil	1.65 abc	100.00	93.33 ab	100.0
Control 3 (Nutristart and Pelgel® and *Pythium* sp.)	Seed treatment	2.02 a	122.22	44.60 a	47.5
Control 4 (Nutristart only *Pythium* sp.)	Drenched	0.77 e	46.55	88.00 a	94.3
Control 5 (Pelgel® *Pythium* sp.)	Seed treatment	1.63 abc	98.91	76.33 abc	81.8
Effects		P-values		P-values	
Formulations		0.0001***		0.0001***	
Application		0.5591 NS		0.0001***	
Organism		0.0001***		0.5425 NS	
Formulation x Application		0.0625 NS		0.0002***	
Formulation x organism		0.0002***		0.0648 NS	
Application x organism		0.0835 NS		0.0506 NS	
Formulation x Application x organism		0.2640 NS		0.2640 NS	
		CV%=11.97		CV%=11.99	
		MSE=0.0084		MSE=9.39	

1.NS = Not significant; **= significant at P ≤ 0.05; ***=significant at P ≤ 0.001

2. Means with the same letter are not significantly different (P= 0.05) according to Student, Newman and Keuls comparison test

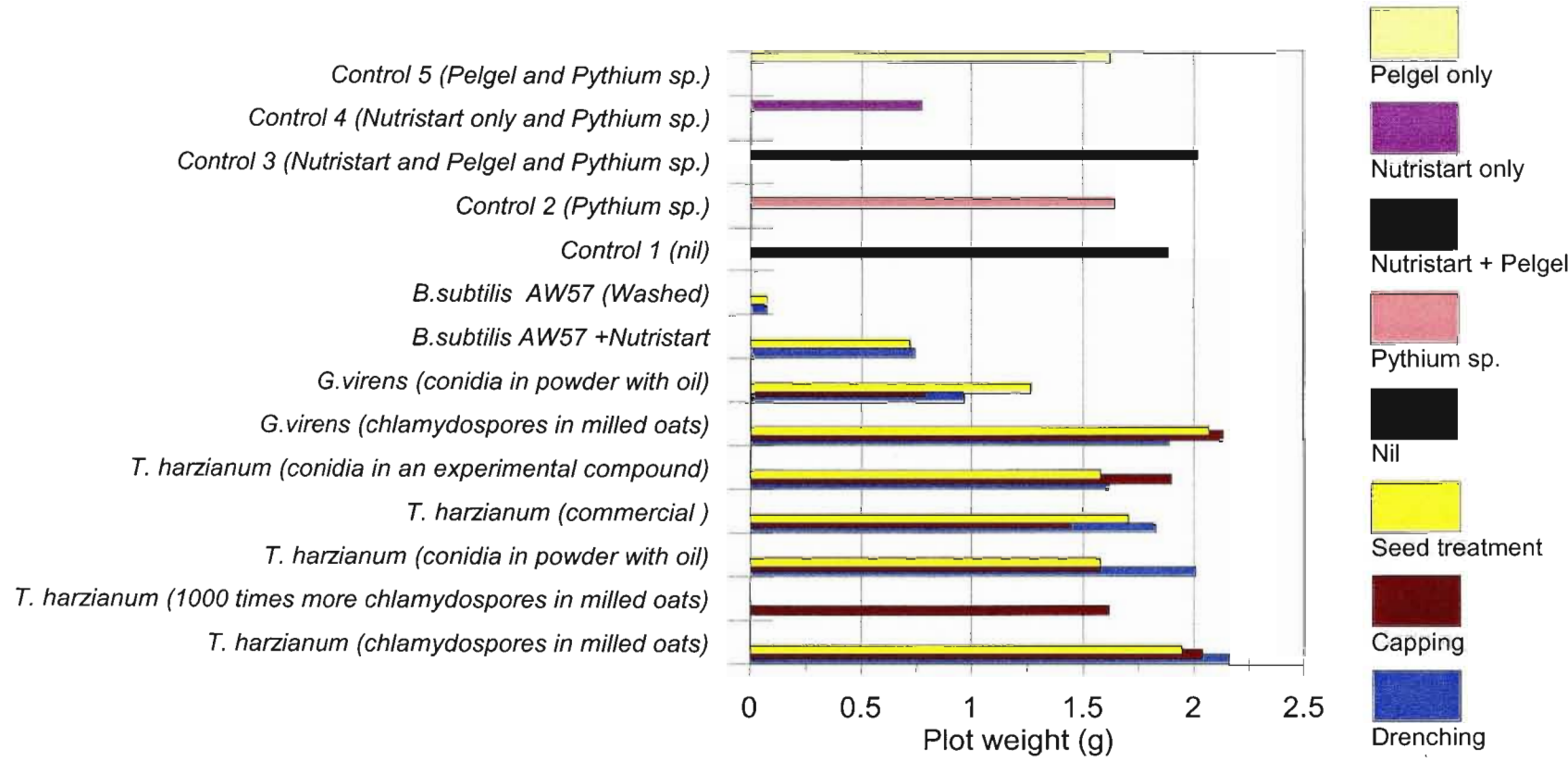

Figure 4.22 Effect of formulations and application methods of *Trichoderma harzianum* KMD, *Gliocladium virens* MM1 and *Bacillus subtilis* AW57 on the biocontrol of damping-off caused by *Pythium* spp. measuring plot weight of eucalyptus after four weeks of growth.

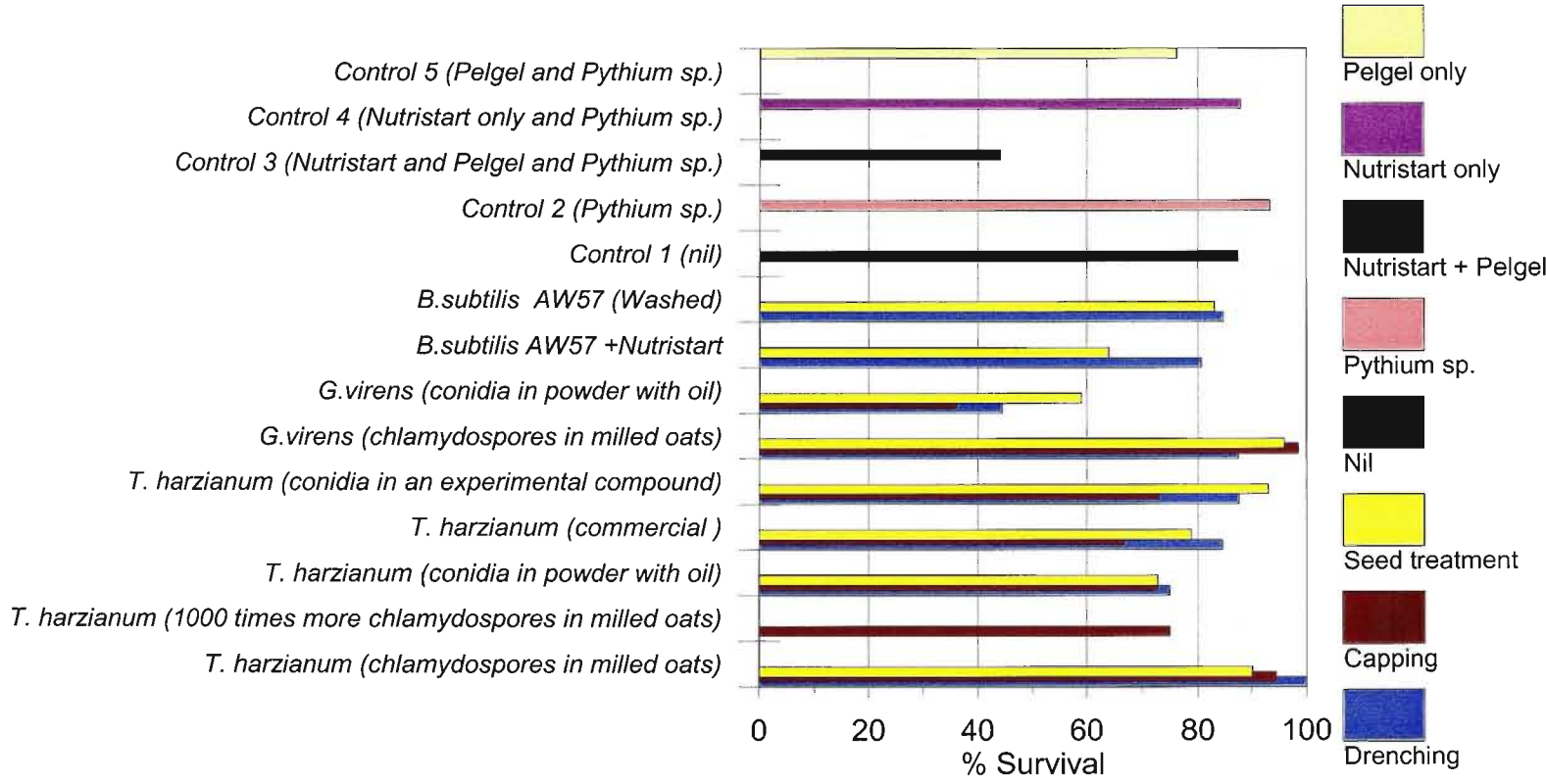

Figure 4.23 The effect of formulations and application methods of *Trichoderma harzianum* KMD, *Gliocladium virens* MM1 and *Bacillus subtilis* AW57 on the biocontrol of damping-off caused by *Pythium* spp. measuring percentage survival of eucalyptus after four weeks of growth.

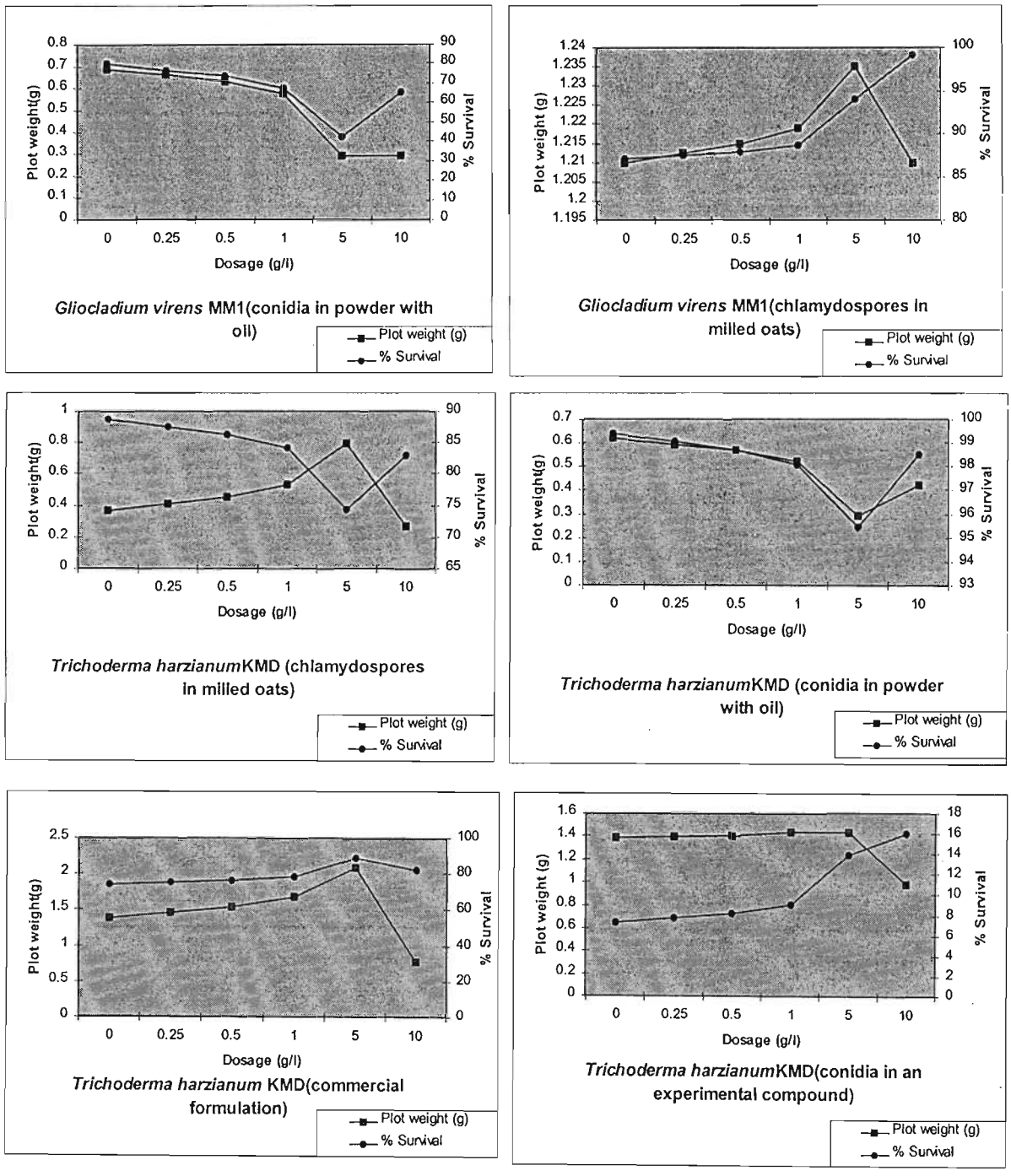

Figure 4.24 Dosage effects of six formulations applied as a drench on damping-off caused by *Pythium* spp. on plot weight and percentage survival of eucalyptus after four weeks of growth.

Table 4.9. Effect of formulations of *Trichoderma.harzianum* KMD, *Gliocladium virens* MM1 and *Bacillus subtilis* AW57 on damping-off of cabbage caused by *Rhizoctonia solani* after four weeks

Formulations	Application	Plot Weight (PW) (g)	%Control (PW/*R.solani*)	%Survival (%Surv)	%Control 2 (%Surv/*R.solani*)
T. harzianum (10^5chlamydospores in milled oats)	Drenching	0.33 f	68.23	72.00 abc	112.8
T. harzianum (10^5chlamydospores in milled oats)	Capping	1.17 ef	238.78	82.00 ab	128.5
T. harzianum (10^5chlamydospores in milled oats)	Seed treatment	0.83 ef	168.71	76.00 abc	119.1
T. harzianum (10^8chlamydospores in milled oats)	Capping	1.40 ef	285.71	60.66 bc	95.0
T. harzianum (conidia in powder with oil)	Drenching	5.93 b	1210.20	75.00 abc	117.5
T. harzianum (conidia in powder with oil)	Capping	5.69 a	1161.22	79.00 ab	123.8
T. harzianum (conidia in powder with oil)	Seed treatment	2.54 b	518.37	87.50 ab	137.1
T. harzianum (Commercial)	Drenching	2.54 cde	518.37	29.00 fg	45.4
T. harzianum (Commercial)	Capping	1.59 ef	323.82	27.83 fg	4360.0
T. harzianum (Commercial)	Seed treatment	4.11 c	838.10	47.16 cdef	73.9
T. harzianum (conidia in an experimental compound)	Drenching	4.12 c	841.43	77.66 ab	121.7
T. harzianum (conidia in an experimental compound)	Capping	3.37 cd	688.43	37.33 efg	58.5
T. harzianum (conidia in an experimental compound)	Seed treatment	3.96 c	807.49	33.16 fg	52.0
G. virens (chlamydospores in milled oats)	Drenching	0.27 f	55.78	73.66 abc	115.4
G. virens (chlamydospores in milled oats)	Capping	0.99 ef	201.37	90.33 ab	141.5
G. virens (chlamydospores in milled oats)	Seed treatment	1.08 ef	221.08	84.66 ab	132.6
G. virens (conidia in powder with oil)	Drenching	6.66 a	1766.67	62.33 bcde	97.7
G. virens (conidia in powder with oil)	Capping	2.13 def	434.69	39.00 defg	61.1
G. virens (conidia in powder with oil)	Seed treatment	1.28 ef	261.22	13.66 g	21.4
B. subtilis AW57 +Nutristart	Drenching	2.95 def	602.45	70.60 ab	110.6
B. subtilis AW57 +Nutristart	Seed treatment	3.41 def	695.51	80.30 ab	125.8
B. subtilis AW57 (Washed)	Drenching	2.47 def	504.49	77.60 ab	121.6
B. subtilis AW57 (Washed)	Seed treatment	0.31 f	63.67	75.00 ab	117.5
Control 1 (nil)	Nil	2.1 cde	428.5	94.667 a	148.3
Control 2 (*R. solani* only)	Nil	0.49 f	100.00	43.83 abcde	100.0
Control 3 (Nutristart and Pelgel® and *R. solani*)	Seed treatment	1.00 ef	204.08	33.00 c	51.7
Control 4 (Nutristart only and *R. solani*)	Drenched	1.08 ef	220.41	36.30 c	56.9
Control 5 (Pelgel® and *R. solani*)	Seed treatment	0.86 f	176.33	65.33 bcd	102.3
Effects		P-values		P-values	
Formulations		0.0001***		0.0001***	
Application		0.0001***		0.0001***	
Organism		0.0001***		0.0233 **	
Formulation x Application		0.0001***		0.0001***	
Formulation x organism		0.0001***		0.0002***	
Application x organism		0.0001***		0.0141**	
Formulation x Application x organism		0.0001***		0.0019**	
		CV%=25.00		CV%=17.91	
		MSE=0.771		MSE=10.82	

1.NS = Not significant; **= significant at P ≤ 0.05; ***=significant at P ≤ 0.001

2. Means with the same letter are not significantly different (P= 0.05) according to Student, Newman and Keuls comparison

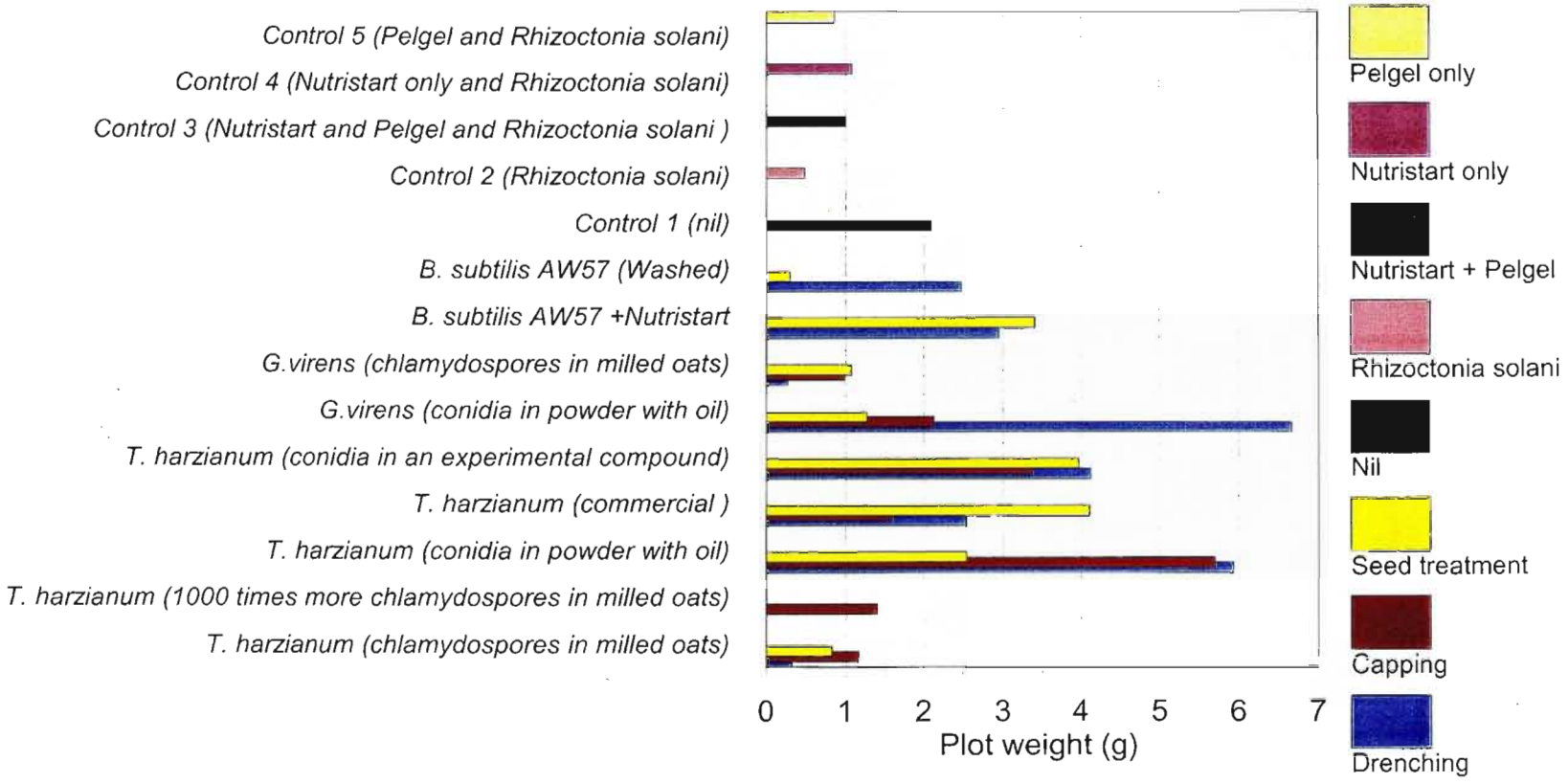

Figure 4.25 The effect of formulations and application methods of *Trichoderma harzianum* KMD, *Gliocladium virens* MM1 and *Bacillus subtilis* AW57 on the biocontrol of damping-off caused by *Rhizoctonia solani* measuring plot weight of cabbage after four weeks of growth.

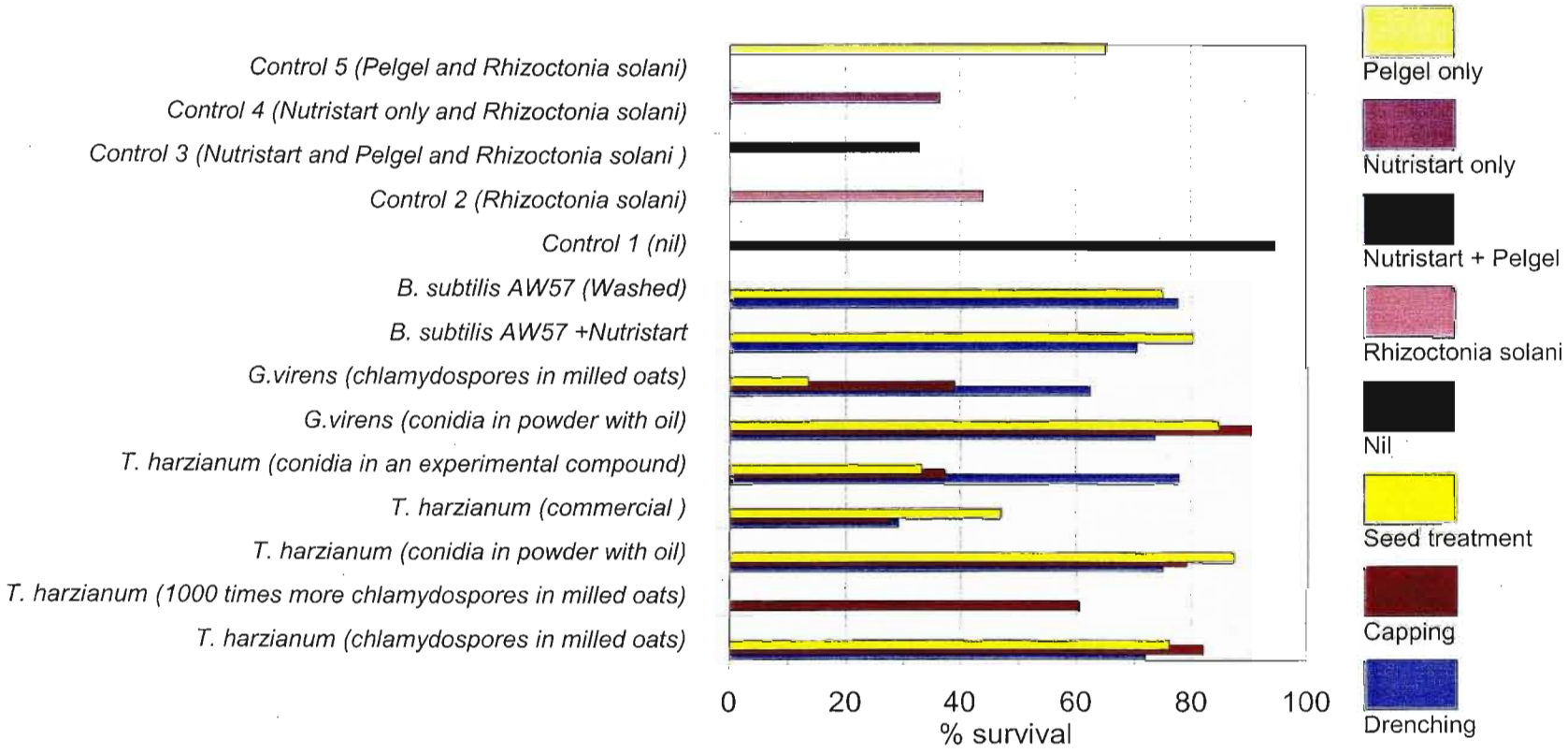

Figure 4.26 The effect of formulations and application methods of *Trichoderma harzianum* KMD, *Gliocladium virens* MM1 and *Bacillus subtilis* AW57 on the biocontrol of damping-off caused by *Rhizoctonia solani* measuring percentage survival of cabbage after four weeks of growth.

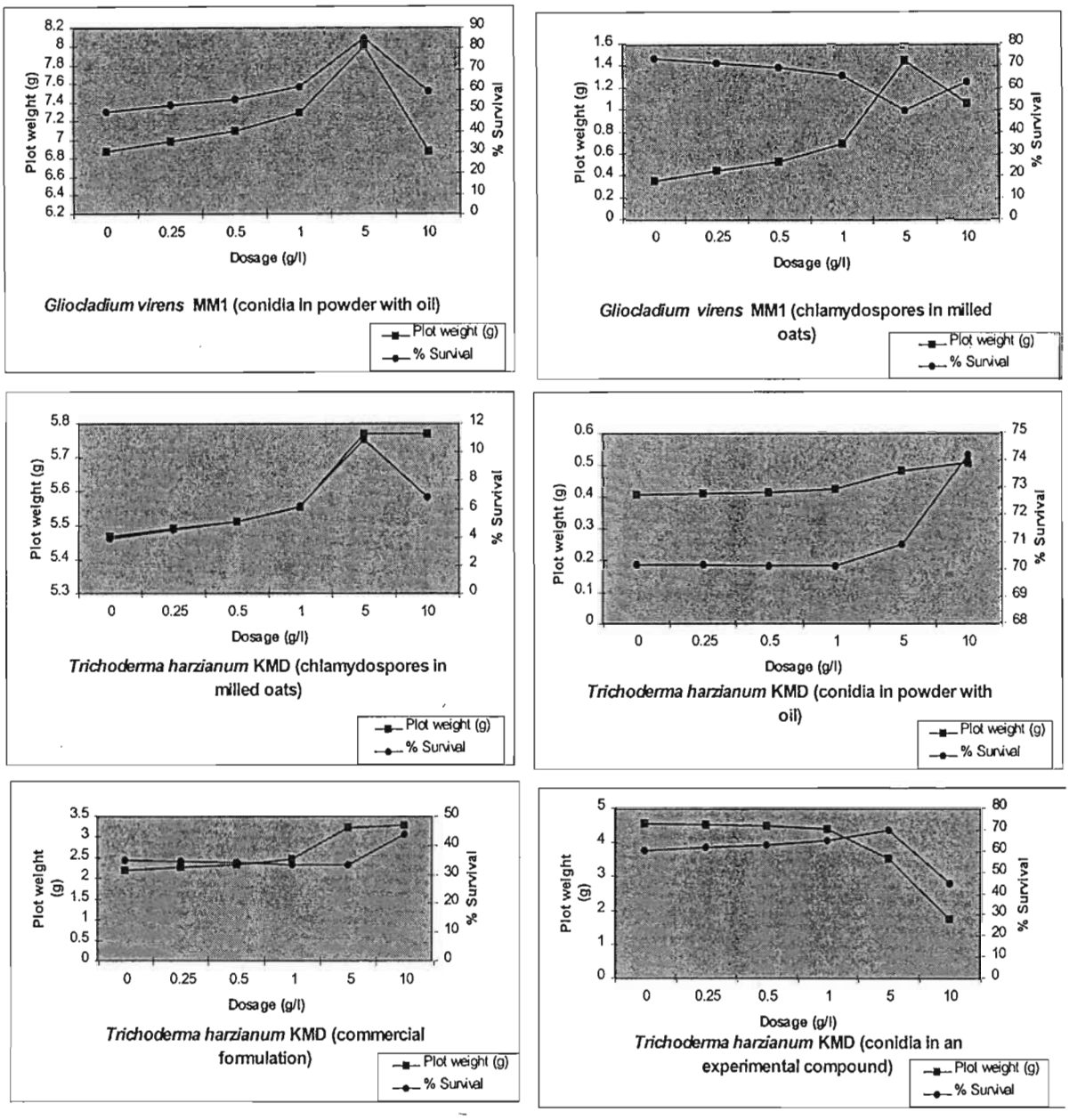

Figure 4.27 Dosage effects of six formulations applied as a drench on damping-off caused by *Rhizoctonia solani* on plot weight and percentage survival of cabbage after four weeks of growth

Table 4.10. Effect of formulations of *Trichoderma harzianum* KMD, *Gliocladium virens* MM1and *Bacillus subtilis* AW57 on damping-off on cucumber caused by *Rhizoctonia solani* after 4 weeks.

Formulations	Application	Plot Weight (PW)(g)	%Control 2 (PW/ *R.solani*)	%Survival (%Surv)	% Control 2 (% Surv/ *R.solani*)
T. harzianum (10^5chlamydospores in milled oats)	Drenching	0.16 b	134.19	50.10 bcde	180.0
T. harzianum (10^5chlamydospores in milled oats)	Capping	0.21 b	182.05	87.50 a	314.4
T. harzianum (10^5chlamydospores in milled oats)	Seed treatment	0.29 b	250.43	80.33 ab	288.6
T. harzianum (10^8chlamydospores in milled oats)	Capping	1.11 b	946.15	30.50 de	109.6
T. harzianum (conidia in powder with oil)	Drenching	2.55 ab	2176.92	49.67 bcde	178.5
T. harzianum (conidia in powder with oil)	Capping	3.82 ab	3264.96	56.67 abcd	203.6
T. harzianum (conidia in powder with oil)	Seed treatment	3.86 ab	3299.15	81.83 ab	294.0
T. harzianum (Commercial)	Drenching	1.84 ab	1572.65	34.67 cde	124.6
T. harzianum (Commercial)	Capping	0.88 b	749.57	19.17 e	68.9
T. harzianum (Commercial)	Seed treatment	1.66 ab	1421.37	63.83 abcd	229.4
T. harzianum (conidia in an experimental compound)	Drenching	1.79 ab	1527.35	79.17 ab	284.5
T. harzianum (conidia in an experimental compound)	Capping	2.12 ab	1811.97	41.67 de	149.7
T. harzianum (conidia in an experimental compound)	Seed treatment	5.11 a	4364.96	13.67 e	49.1
G. virens (chlamydospores in milled oats)	Drenching	1.20 ab	1028.21	29.17 de	104.8
G. virens (chlamydospores in milled oats)	Capping	0.43 b	364.96	80.50 ab	289.3
G. virens (chlamydospores in milled oats)	Seed treatment	0.86 b	735.04	70.67 abc	253.9
G. virens (conidia in powder with oil)	Drenching	2.51 ab	2147.86	63.67 abcd	228.8
G. virens (conidia in powder with oil)	Capping	2.13 ab	1820.51	63.67 abcd	228.8
G. virens (conidia in powder with oil)	Seed treatment	0.90 b	771.79	45.83 bcde	164.7
B. subtilis AW57 +Nutristart	Drenching	0.48 b	410.26	37.50 bc	134.7
B. subtilis AW57 +Nutristart	Seed treatment	0.04 b	30.77	30.30 bcd	108.9
B. subtilis AW57 (Washed)	Drenching	0.50 b	426.50	32.00 bcd	115.0
B. subtilis AW57 (Washed)	Seed treatment	0.11 b	92.31	17.16 cd	61.7
Control 1 (nil)	Nil	7.29	607.0	70.33 abc	252.7
Control 2 (*R. solani* only)	Nil	0.12 b	100.00	27.83 de	100.0
Control 3 (Nutristart and Pelgel® and *R. solani*)	Seed treatment	0.48 b	410.26	51.30 b	184.3
Control 4 (Nutristart only and *R. solani*)	Drenched	0.10 b	82.05	13.00 d	46.7
Control 5 (Pelgel® and *R. solani*)	Seed treatment	0.02 b	20.51	36.00 cde	129.4
Effects		P-values		P-values	
Formulations		0.1281 ^{NS}		0.0062 ^{NS}	
Application		0.001***		0.0001***	
Organism		0.1324 ^{NS}		0.0001***	
Formulation x Application		0.0013***		0.4138 ^{NS}	
Formulation x organism		0.0004***		0.0001***	
Application x organism		0.5047 ^{NS}		0.1017 ^{NS}	
Formulation x Application x organism		0.0148**		0.0291**	
		CV%=46.42		CV%=24.92	
		MSE=1.69		MSE=13.67	

1.NS = Not significant; **= significant at P ≤ 0.05; ***=significant at P ≤ 0.001

2. Means with the same letter are not significantly different (P= 0.05) according to Student, Newman and Keuls comparison test

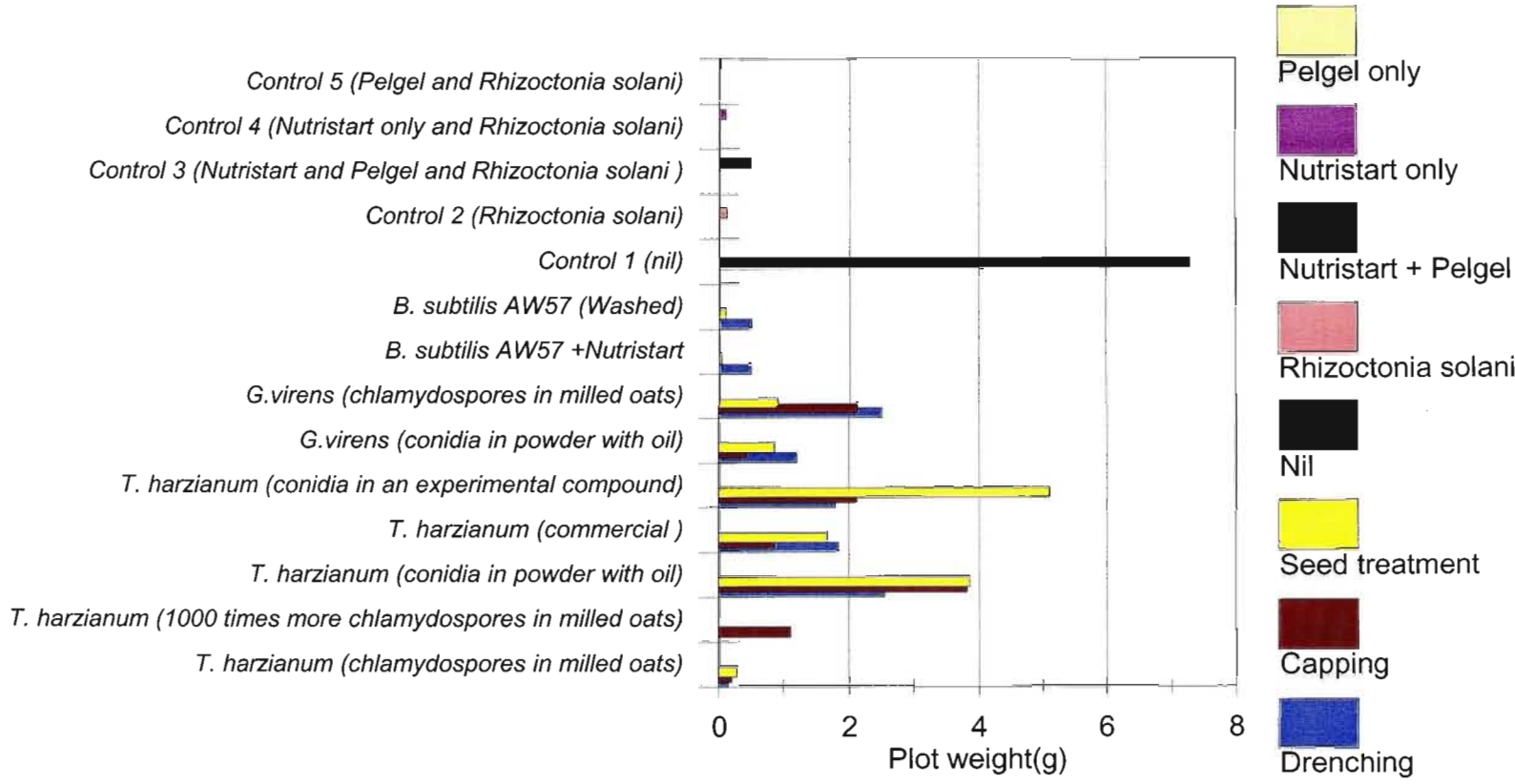

Figure 4.28 The effect of formulations and application methods of *Trichoderma harzianum* KMD, *Gliocladium virens* MM1 and *Bacillus subtilis* AW57 on the biocontrol of damping-off caused by *Rhizoctonia solani* measuring plot weight of cucumber after four weeks of growth.

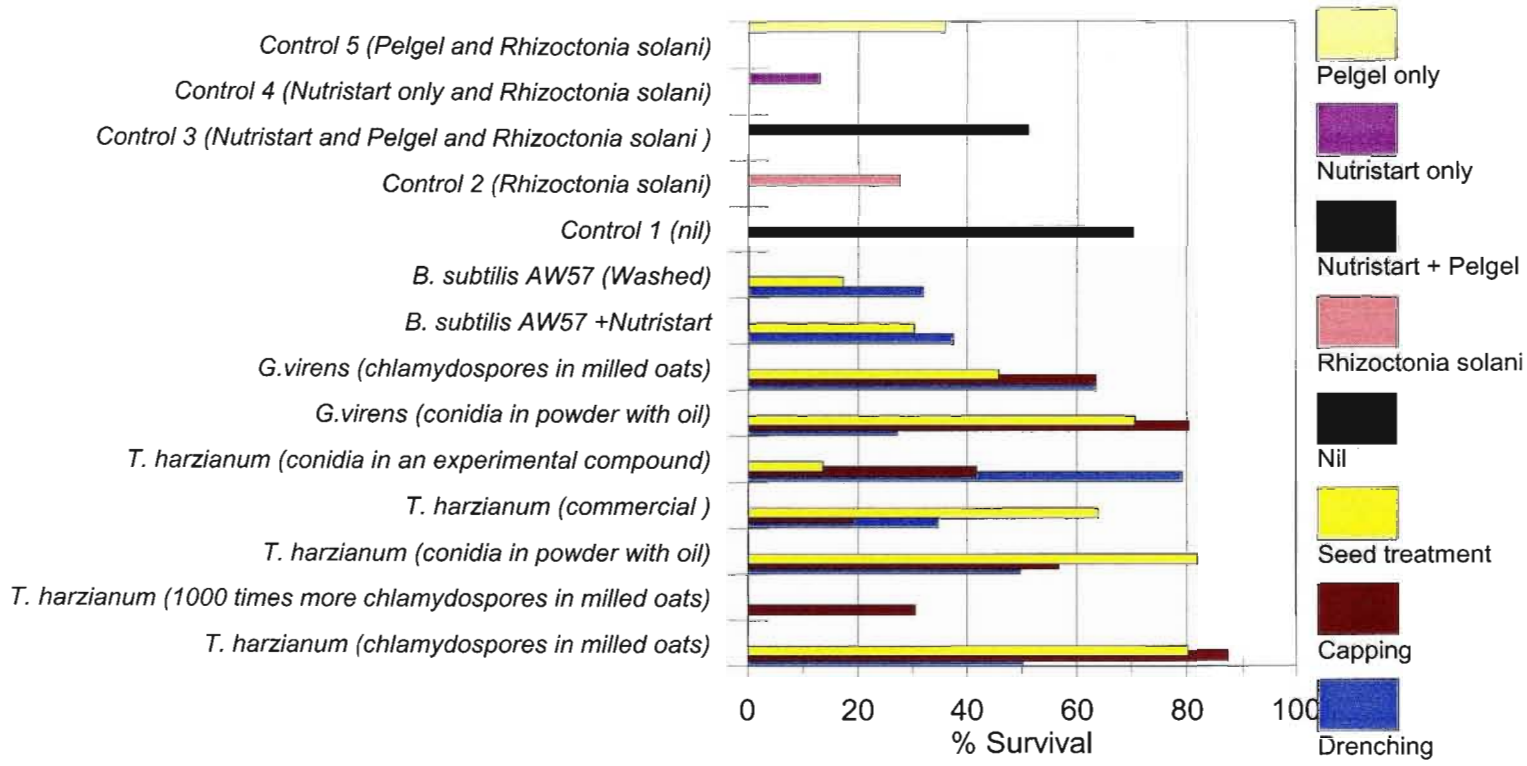

Figure 4.29 The effect of formulations and application methods of *Trichoderma harzianum* KMD, *Gliocladium virens* MM1 and *Bacillus subtilis* AW57 on the biocontrol of damping-off caused by *Rhizoctonia solani* measuring percentage survival of cucumber after four weeks of growth.

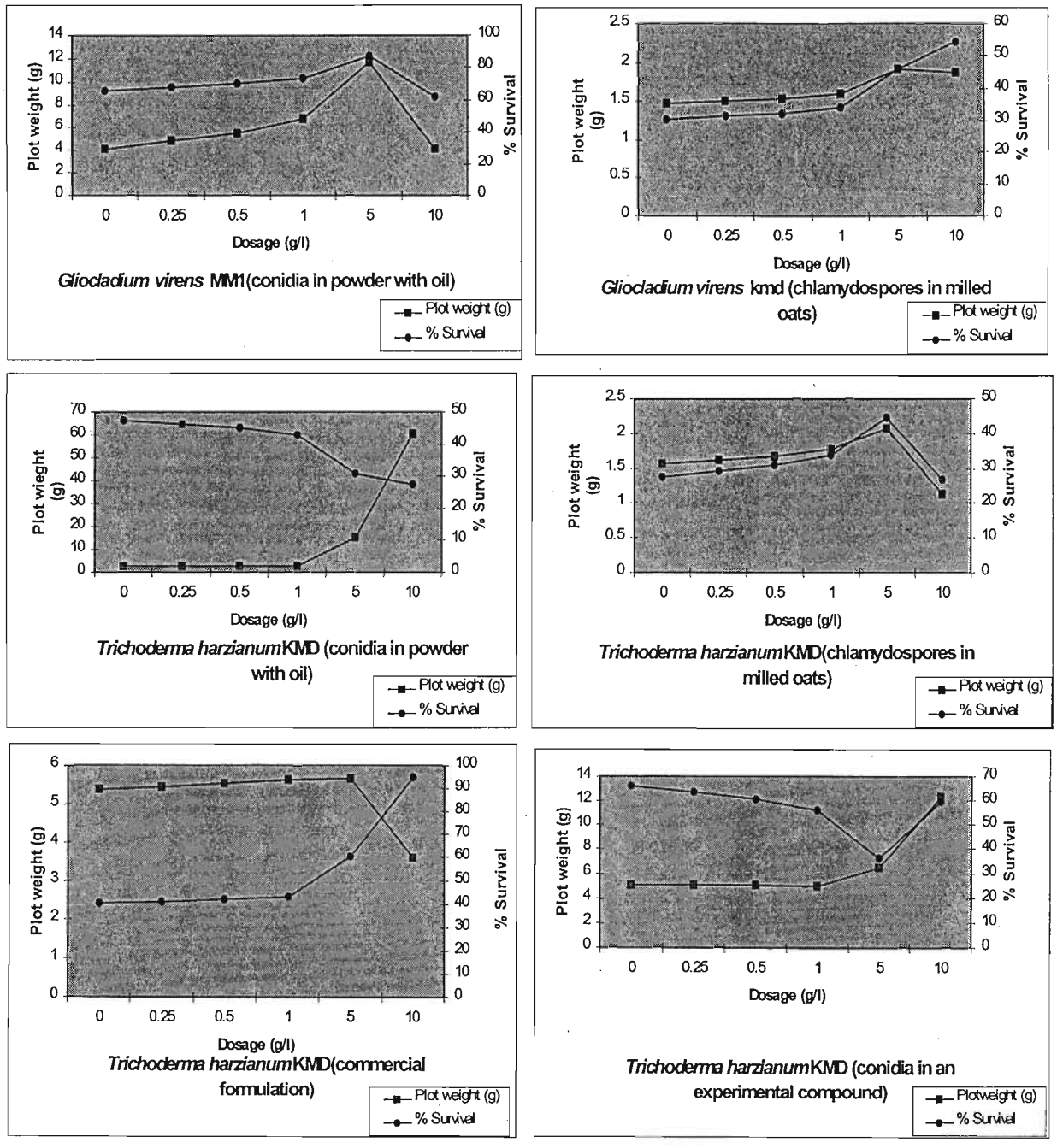

Figure 4.30 Dosage effects of six formulations applied as a drench on damping-off cause *Rhizoctonia solani* on plot weight and percentage survival of cucumber after four weeks of growth.

Table 4.11. Effect of formulations of *Trichoderma harzianum* KMD, *Gliocladium virens* MM1 and *Bacillus subtilis* AW57 on damping-off of Namaqualand Daisy caused by *Rhizoctonia solani*. after four weeks.

Formulations	Application	Plot Weight (PW)(g)	%Control 2 (PW/ *R.solani*)	%Survival (%Surv)	% Control 2 (%Surv/*R.solani*)
T.harzianum (10^5chlamydospores in milled oats)	Drenching	0.16 ab	21.77	72.00 ab	526.7
T.harzianum (10^5chlamydospores in milled oats)	Capping	0.21 ab	29.63	47.00 abcd	343.8
T.harzianum (10^5chlamydospores in milled oats)	Seed treatment	0.30 ab	41.00	39.00 abcd	285.3
T.harzianum (10^8chlamydospores in milled oats)	Capping	1.84 ab	255.33	68.00 ab	497.4
T.harzianum (conidia in powder with oil)	Drenching	2.55 a	353.70	65.33 ab	477.9
T.harzianum (conidia in powder with oil)	Capping	3.82 a	530.33	78.00 a	570.6
T.harzianum (conidia in powder with oil)	Seed treatment	3.86 a	536.10	61.00 abc	446.2
T.harzianum (Commercial)	Drenching	1.82 ab	253.33	39.00 abcde	285.3
T.harzianum (Commercial)	Capping	0.88 ab	121.77	30.33 bcde	221.9
T.harzianum (Commercial)	Seed treatment	1.66 ab	231.03	20.67 cde	151.2
T.harzianum (conidia in an experimental compound)	Drenching	2.11a	293.33	80.33 a	587.6
T.harzianum (conidia in an experimental compound)	Capping	5.10 a	709.00	43.00 abcde	314.6
T.harzianum (conidia in an experimental compound)	Seed treatment	1.11 ab	153.70	50.83 abcd	371.8
G. virens (chlamydospores in milled oats)	Drenching	1.20 ab	167.13	69.33 ab	507.2
G. virens (chlamydospores in milled oats)	Capping	0.42 b	59.00	33.33 bcde	243.8
G. virens (chlamydospores in milled oats)	Seed treatment	0.86 b	119.33	66.67 ab	487.7
G. virens (conidia in powder with oil)	Drenching	2.51 a	349.00	57.00 abc	417.0
G. virens (conidia in powder with oil)	Capping	2.13 a	295.67	30.50 bcde	223.1
G. virens (conidia in powder with oil)	Seed treatment	0.89 ab	123.33	5.33 e	39.0
B. subtilis AW57 +Nutristart	Drenching	0.86 ab	120.00	47.33 e	346.2
*B. subtilis*AW57 +Nutristart	Seed treatment	0.53 ab	73.33	96.00 a	702.3
B. subtilis AW57 (Washed)	Drenching	2.52 a	350.00	77.66 bc	568.1
*B. subtilis*AW57 (Washed)	Seed treatment	2.11 a	293.33	72.00 c	526.7
Control 1 (nil)	Nil	0.26 ab	36.67	66.7 ab	487.7
Control 2 (*R. solani* only)	Nil	0.72 ab	100.00	13.67 de	100.0
Control 3 (Nutristart and Pelgel® and *R. solani*)	Seed treatment	0.26 ab	35.67	52.66 de	385.2
Control 4 (Nutristart only and *R. solani*)	Drenched	0.16 ab	22.00	74.66 bc	546.2
Control 5 (Pelgel®and *R. solani*)	Seed treatment	0.12 ab	16.00	16.00 de	117.0
Effects		P-values		P-values	
Formulations		0.3358 ^{NS}		0.0023**	
Application		0.0006***		0.0001***	
Organism		0.5400 ^{NS}		0.0004***	
Formulation x Application		0.0323**		0.0003***	
Formulation x organism		0.346 ^{NS}		0.1141 ^{NS}	
Application x organism		0.3477 ^{NS}		0.13 ^{NS}	
Formulation x Application x organism		0.5866 ^{NS}		0.0125**	
		CV%=13.19		CV%=15.33	
		MSE=1.463		MSE=30.45	

1.NS = Not significant; **= significant at P ≤ 0.05; ***=significant at P ≤ 0.001

2. Means with the same letter are not significantly different (P= 0.05) according to Student, Newman and Keuls comparison test

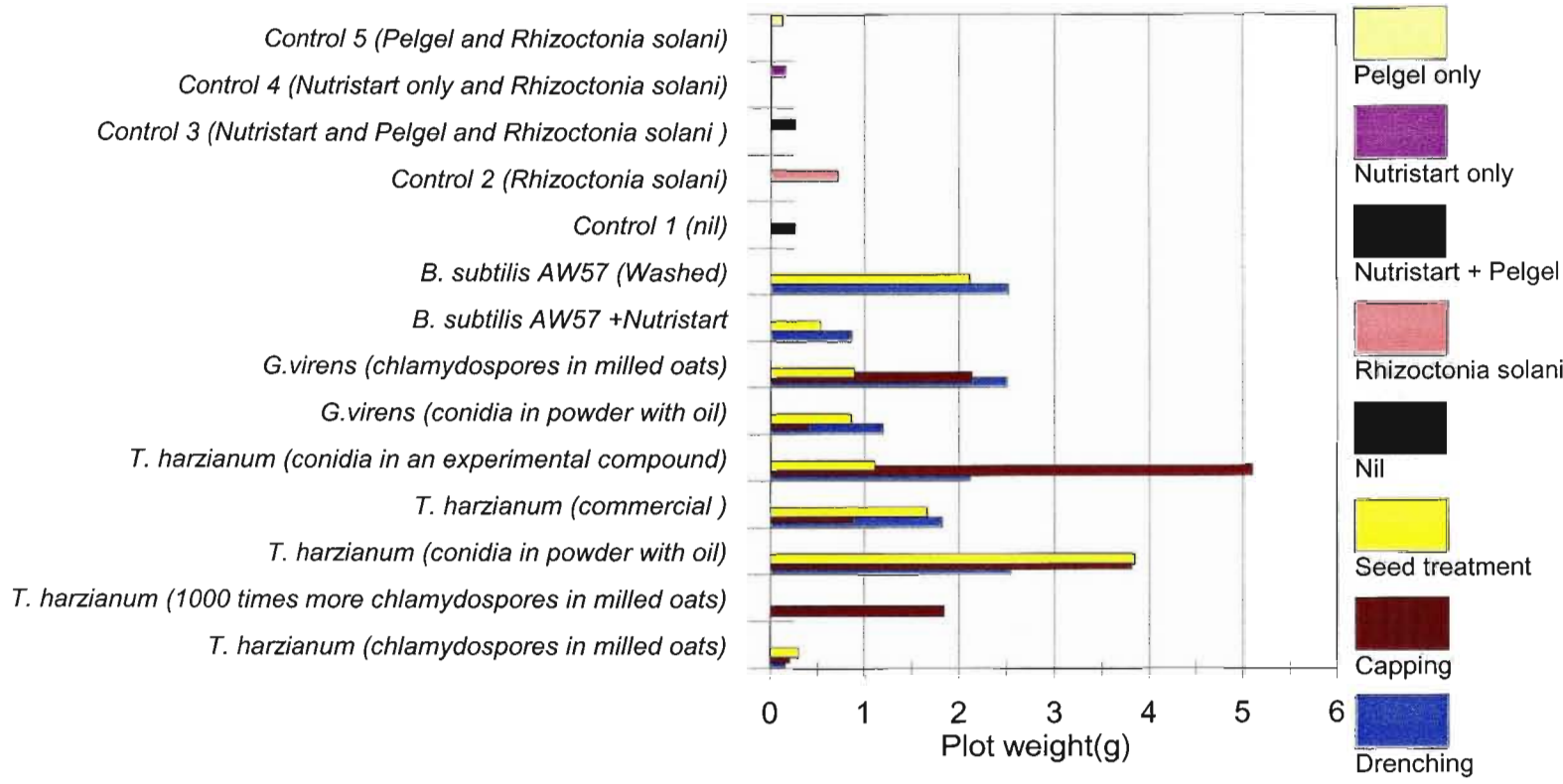

Figure 4.31 The effect of formulations and application methods of *Trichoderma harzianum* KMD, *Gliocladium virens* MM1 and *Bacillus subtilis* AW57 on the biocontrol of damping-off caused by *Rhizoctonia solani* measuring plot weight of Namaqualand daisy after four weeks of growth.

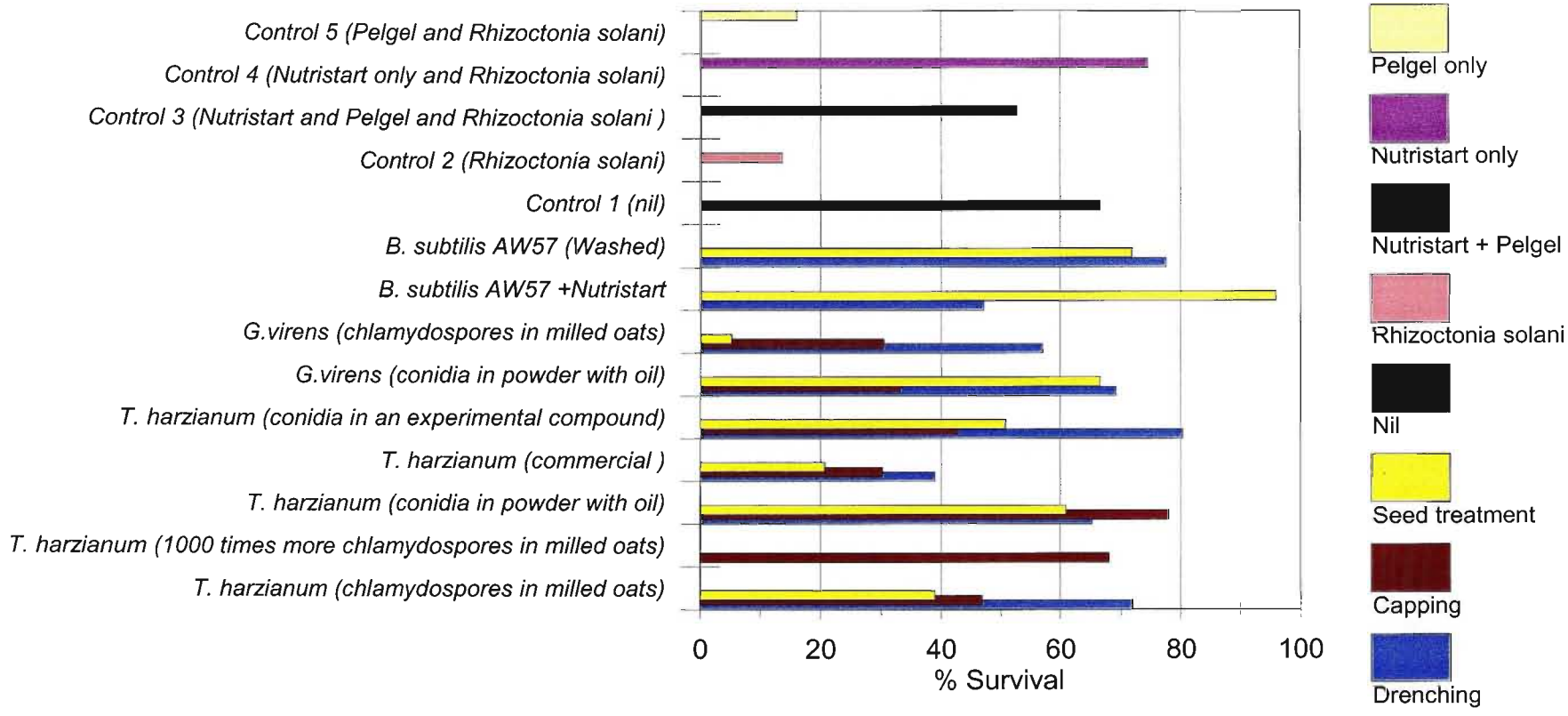

Figure 4.32 The effect of formulations and application methods of *Trichoderma harzianum* KMD, *Gliocladium virens* MM1 and *Bacillus subtilis* AW57 on the biocontrol of damping-off caused by *Rhizoctonia solani* measuring percentage survival of Namaqualand daisy after four weeks of growth.

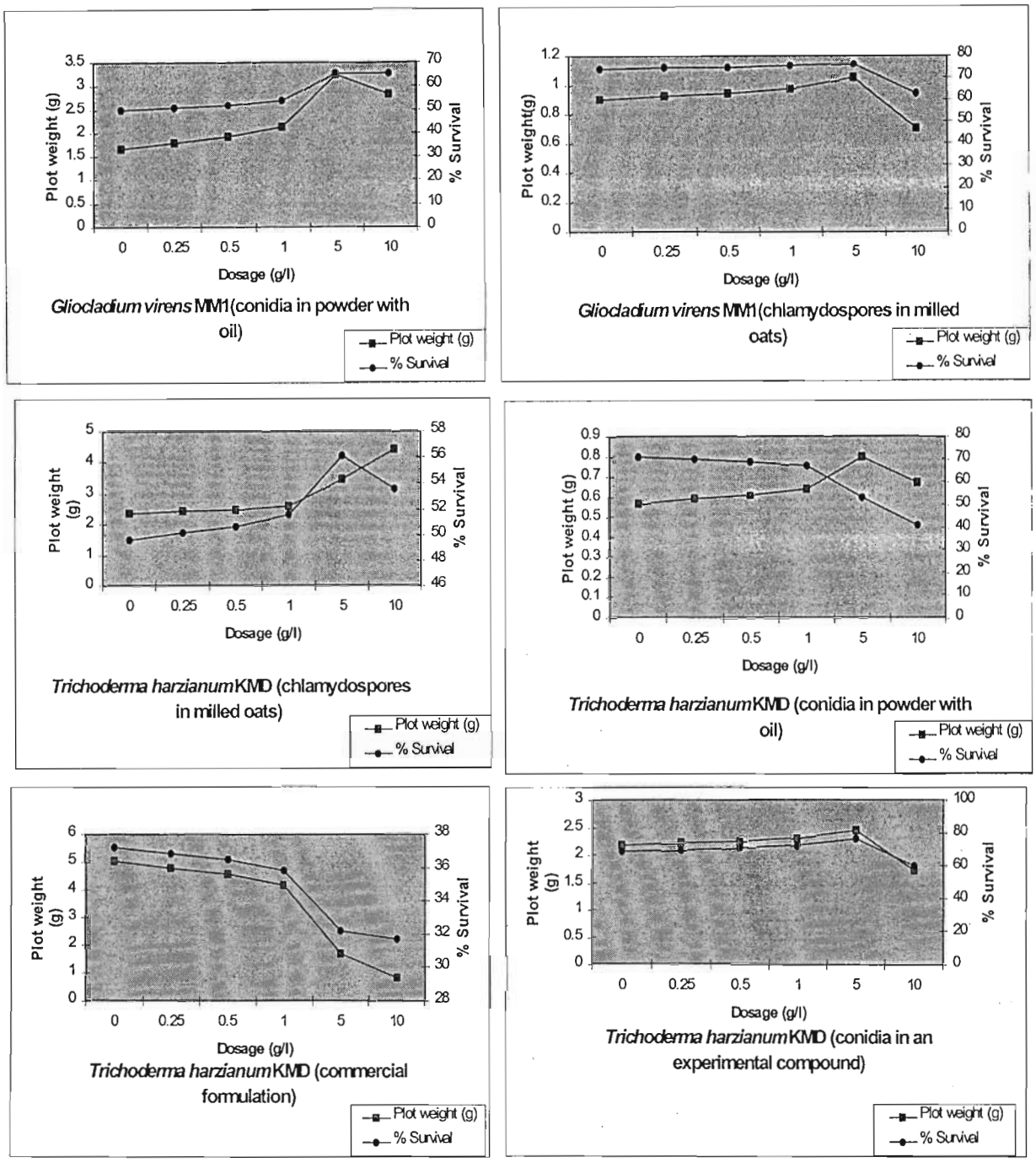

Figure 4.33 Dosage effects of six formulations applied as a drench on damping-off cause *Rhizoctonia solani* on plot weight and percentage survival of Namaqualand daisy after four weeks of growth.

Table 4.12 Effect of formulations of *Trichoderma harzianum* KMD, *Gliocladium virens* MM1 and *Bacillus subtilis* AW57 on damping-off of eucalyptus caused by *Rhizoctonia solani* after four weeks.

Formulations	Application	Plot Weight (PW)(g)	%Control 2 (TDW/ *R.solani*)	%Survival (%Surv)	% Control 2 %Surv/*R.solani*)
T. harzianum (10^5chlamydospores in milled oats)	Drenching	0.89 c	124.31	29.00 e	48.3
T. harzianum (10^5chlamydospores in milled oats)	Capping	0.59 cd	83.18	60.00 abcd	100.0
T. harzianum (10^5chlamydospores in milled oats)	Seed treatment	0.59 cd	82.25	39.00 cd	65.0
T. harzianum (10^8chlamydospores in milled oats)	Capping	0.50 cd	70.56	63.67 abcd	106.1
T. harzianum (conidia in powder with oil)	Drenching	2.91 a	408.43	44.33 bcd	73.9
T. harzianum (conidia in powder with oil)	Capping	0.75 cd	105.15	40.83 cd	68.1
T.harzianum (conidia in powder with oil)	Seed treatment	1.74 b	243.94	43.16 bcd	71.9
T. harzianum (Commercial)	Drenching	1.86 b	260.30	94.67 a	157.8
T. harzianum (Commercial)	Capping	0.56 cd	78.97	63.67 abcd	106.1
T. harzianum (Commercial)	Seed treatment	1.71 b	240.19	81.33 ab	135.6
T. harzianum (conidia in an experimental compound)	Drenching	1.85 b	259.36	93.00 a	155.0
T. harzianum (conidia in an experimental compound)	Capping	0.73 cd	102.76	70.83 ab	118.1
T. harzianum (conidia in an experimental compound)	Seed treatment	0.60 cd	84.58	70.33 ab	117.2
G. virens (chlamydospores in milled oats)	Drenching	0.29 d	40.19	37.66 cd	62.8
G. virens (chlamydospores in milled oats)	Capping	0.69 cd	96.27	33.33 cd	55.6
G. virens (chlamydospores in milled oats)	Seed treatment	0.43 cd	60.75	66.67 abcd	111.1
G. virens (conidia in powder with oil)	Drenching	0.27 d	37.85	90.00 a	150.0
G. virens (conidia in powder with oil)	Capping	0.72 cd	101.40	70.33 abc	117.2
G. virens (conidia in powder with oil)	Seed treatment	0.62 cd	87.38	81.00 ab	135.0
B. subtilis AW57 +Nutristart	Drenching	0.96 c	134.59	38.83 de	64.7
B. subtilis AW57 +Nutristart	Seed treatment	0.48 cd	67.29	44.33 bcde	73.9
B. subtilis AW57 (Washed)	Drenching	0.02 e	2.71	33.33 de	55.6
B. subtilis AW57 (Washed)	Seed treatment	2.21 a	309.55	55.50 cd	92.5
Control 1 (nil)	Nil	0.80 c	112.62	60.00 abcd	136.4
Control 2 *(R.solani* only)	Nil	0.71 c	100.00	81.83 abc	100.0
Control 3 (Nutristart and Pelgel® and *R.solani*)	Seed treatment	0.29 d	40.49	34.67 de	57.8
Control 4 (Nutristart only and *R.solani)*	Drenched	0.24 d	33.65	22.00 e	36.7
Control 5 (Pelgel® and *R.solani*)	Seed treatment	0.70 c	97.57	97.00 a	161.7
Effects		P-values		P-values	
Formulations		0.0001***		0.0001***	
Application		0.0001***		0.0001***	
Organism		0.0001***		0.1528^{NS}	
Formulation x Application		0.0001***		0.0005***	
Formulation x organism		0.0001***		0.0012**	
Application x organism		0.0001***		0.0362**	
Formulation x Application x organism		0.0001***		0.0062**	
		CV%=19.83		CV%=9.38	
		MSE=0.191		MSE=11.84	

1.NS = Not significant; **= significant at P ≤ 0.05; ***=significant at P ≤ 0.001

2. Means with the same letter are not significantly different (P= 0.05) according to Student, Newman and Keuls comparison test

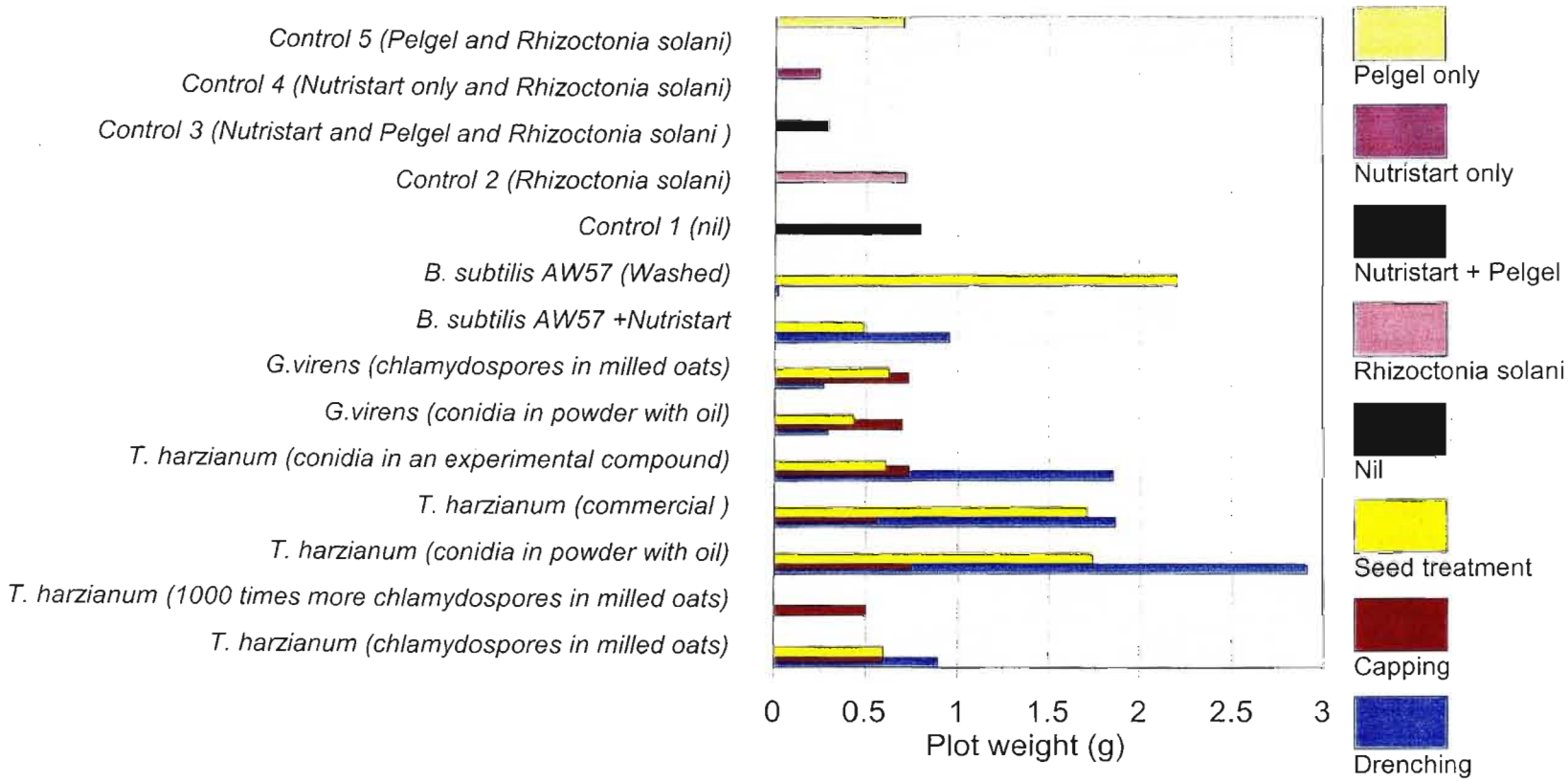

Figure 4.34 The effect of formulations and application methods of *Trichoderma harzianum* KMD, *Gliocladium virens* MM1 and *Bacillus subtilis* AW57 on the biocontrol of damping-off caused by *Rhizoctonia solani* measuring plot weight of eucalyptus after four weeks of growth.

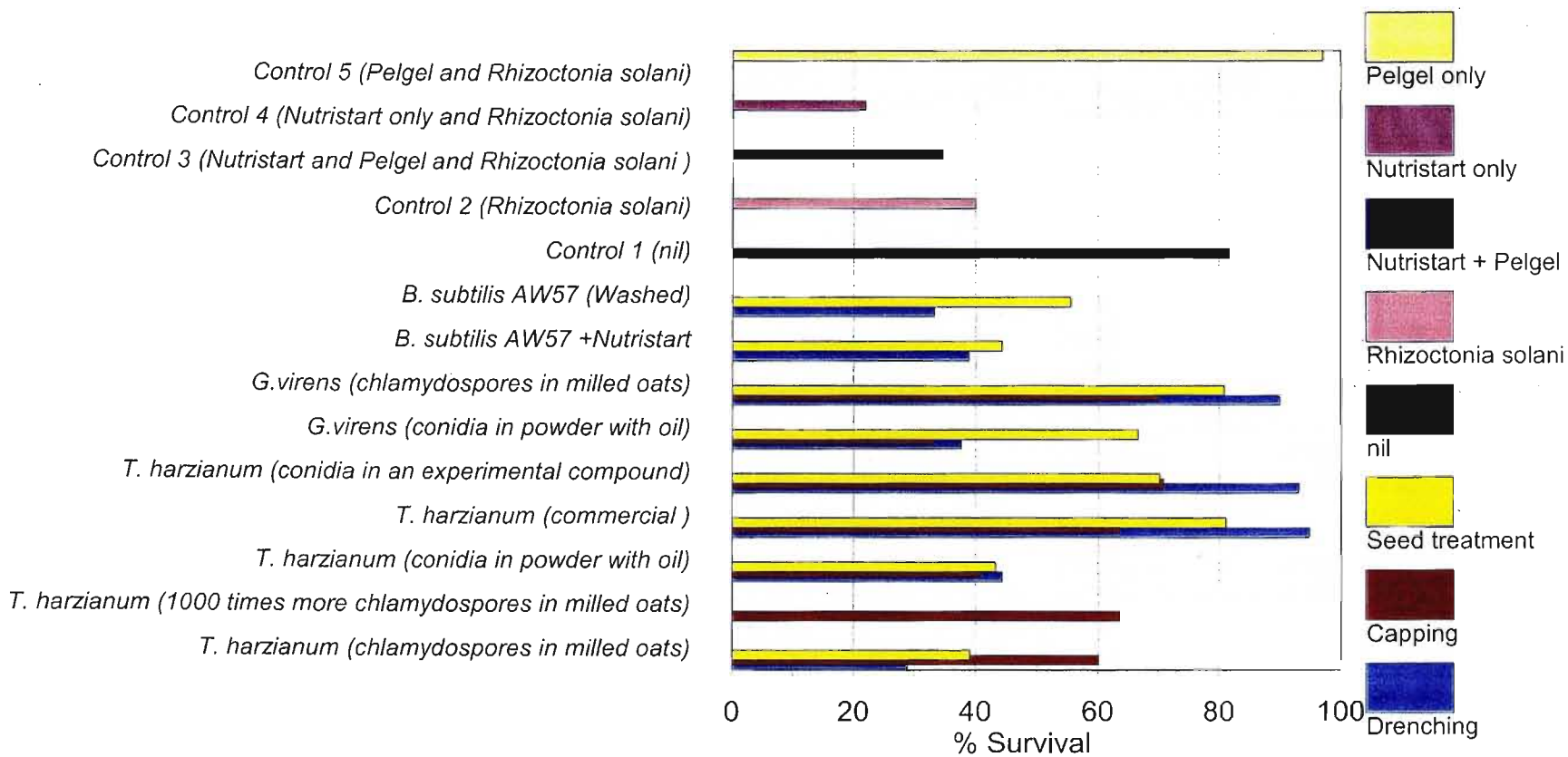

Figure 4.35 The effect of formulations and application methods of *Trichoderma harzianum* KMD, *Gliocladium virens* MM1 and *Bacillus subtilis* AW57 on the biocontrol of damping-off caused by *Rhizoctonia solani* measuring percentage survival of eucalyptus after four weeks of growth.

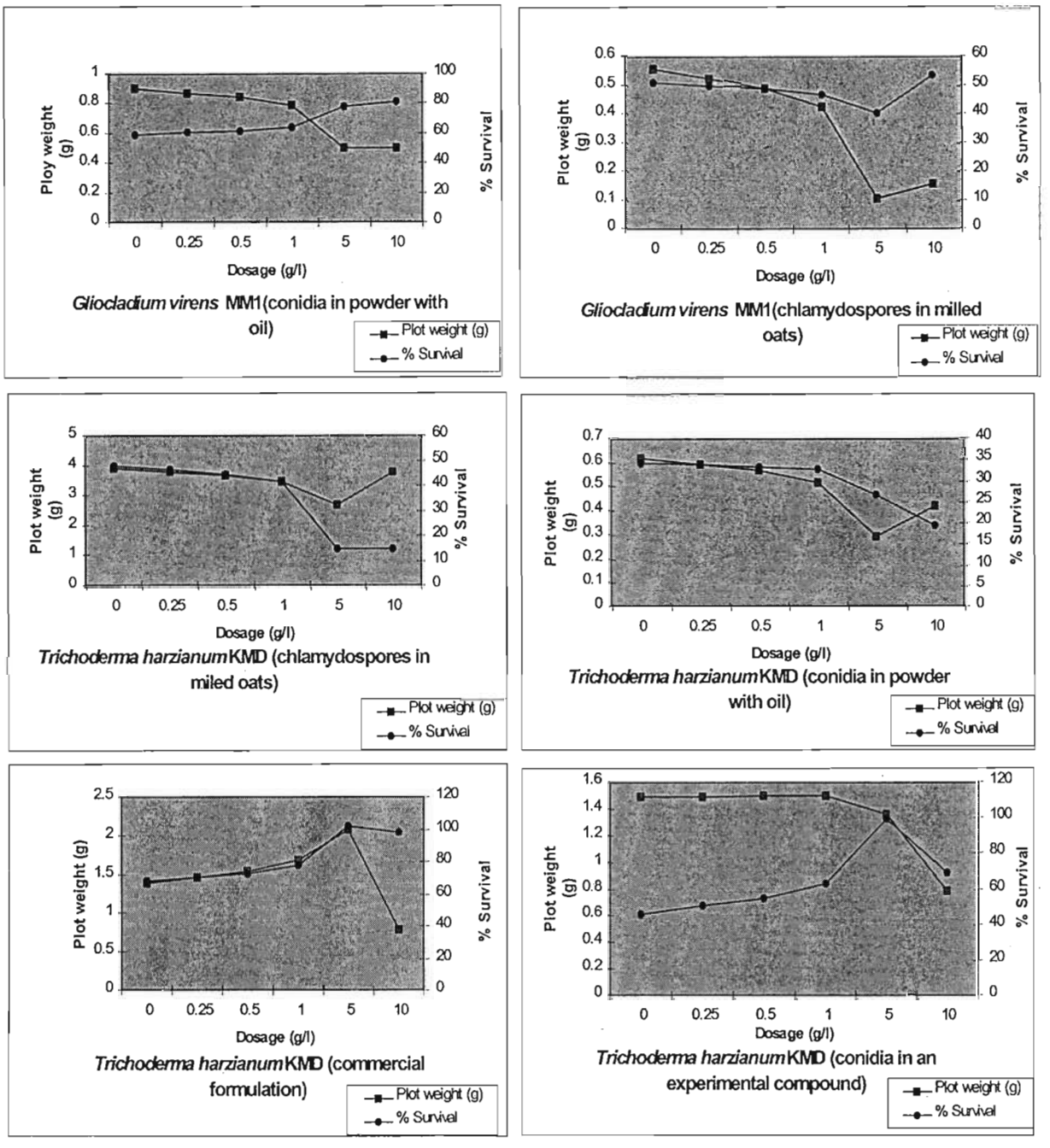

Figure 4.36 Dosage effects of six formulations applied as a drench on damping-off caused by *Rhizoctonia solani* on plot weight and percentage survival of eucalyptus after four weeks of growth.

Figure 4.37. Comparison of cucumber seedlings with *Trichoderma harzianum* KMD formulated with kaolin powder with oil drenched at different dosages. Growing media was artificially infested with *Pythium* sp. *Trichoderma harzianum* KMD drenched (from the far left) at 0.25g/l, 0.5g/l, 1g/l, 5g/l 10g/l, control (*Pythium* sp.) only and control (water only).

Table 4.13 Main and interaction effects of growth stimulation, biocontrol of *Pythium* spp. and *Rhizoctonia solani* trials by all formulations of biocontrol organsims under greenhouse conditions.

Main effects	Letter	Significance	Explanation
Organisms	A	significant	At least one organism performed better or worse than the other organisms
		Not significant	All organisms performed equally well or badly irrespective of the other main effects. No one treatment differs from any other, over all the treatments. There may still be significant differences in interaction effects
Formulation	B	significant	At least one formulation performed better or worse than the others
		Not significant	All formulations performed equally well or badly irrespective of the other main effects. No one treatment differs from any other, over all the treatments. There may still be significant differences in interaction effects
Application	C	significant	At least one application technique resulted in a better or worse response in enhancing plant growth and reducing disease than the other application techniques
		Not significant	All application techniques performed equally well or badly irrespective of the other main effects. No one treatment differs from any other, over all the treatments. There may still be significant differences in interaction effects
Interaction effects			
Organism*Formulation	AB	significant	At least one organism is significantly better or worse in its performance when coupled with one or more of the formulations.
		Not significant	None of the combinations of micro-organisms and formulations is better or worse than the others. The activity of the microbes is not affected by the formulations
Organism*Application	AC	significant	At least one organism is significantly better or worse in its performance when coupled with one or more of the application techniques
		Not significant	None of the combinations of micro-organisms and application is better or worse than the others. The activity of the microbes is not affected by the application techniques
Formulation*Application	BC	significant	At least one of the formulations is significantly better or worse depending on how it gets applied and the converse of that is that one of the applications really works well or badly only with some of the formulations
		Not significant	For each of the formulations irrespective on how it gets applied, the same effect is produced.
Organism*Formulation*Application	ABC	significant	Depending on the organism and how it is formulated and how it is applied, a significant result is produced
		Not significant	For any organism and any formulation, the result will not be affected by the application technique

Nutristart, significantly increased plot weight in cabbage seedlings ranging from 3000-5000% compared to the control (water only).

All formulations caused a significant increase (P=0.0001) on the plot weight of cucumber seedlings (Table 4.2). No significant increases were observed for percentage survival, as this parameter ranged from 80-90% for formulations and were observed to be comparable to the control (water only) (Table 4.2 and Figures 4.4-4.5). On cucumber seedlings, significant increases of plot weight were observed for all formulations tested, regardless of application methods used. Each formulation enhanced growth more than a 100 fold, irrespective of the application methods used.

On the growth of Namaqualand daisy and Eucalyptus seedlings, significant differences (P=0.0001) were recorded from the effect of different formulations on plot weight of seedlings. Increases in plot weight of both these crops were achieved when all formulations were applied when compared to the control (water only). However, the biocontrol agents resulted in only marginally significant differences for percentage survival on both crops (Table 4.3 and Figure 4.7-4.8, Figure 4.10 and 4.11). Increases of plant growth due to all formulations regardless of application method used were achieved when compared to the control (water only).

Significant differences on plot weight and percentage survival resulted due to the different application techniques (drenching, capping, seed treatment) employed in applying the formulations of different biocontrol organisms (Table 4.1-4.4). Tables 4.1-4.4 showed that each formulation resulted in better performances of seedling growth using the different application techniques. Significant differences were found between application techniques for plot weight of cabbage, cucumber and Namaqualand daisy seedlings. However, no significant differences between application techniques were recorded on Eucalyptus seedlings. This indicates that growth enhancement depends on the formulation type, crop, biocontrol organism tested and how it is applied in the field.

Major differences between application techniques were recorded on cabbage seedlings with regard to plot weight (Table 4.1, Figure 4.1 and Figure 4.2). The formulation of *T. harzianum* KMD containing conidia in kaolin powder mixed with oil caused an increase of 6161% with seed treatment against 3777% with capping against 3674% with drenching; *T. harzianum* KMD (commercial) caused a growth increase of 5481% with seed treatment against 2738% with capping against 2460% with drenching; *T. harzianum* KMD prepared with conidia in an experimental compound caused a growth increase of 2023% with capping; 5364% with seed

treatment and 4259% with drenching; *G. virens* MM1 prepared with conidia in kaolin powder with oil caused an increase of 6127% with drenching against 5298% with capping against 3272% with seed treatment. The remaining formulations were not found to be significantly different from each other. For example *B. subtilis* AW57 enhanced growth regardless of the application method used.

With respect to cucumber seedlings significant differences between application techniques were also recorded (Figure 4.4 and Figure 4.5). Significant increases in growth were stimulated by all formulations of biocontrol organisms when applied as either application technique (drench, capping or seed treatment). *Trichoderma harzianum* KMD prepared with conidia in kaolin powder mixed with oil, *G. virens* MM1 prepared with chlamydospores in milled oats and *B. subtilis* AW57 washed and with Nutristart, indicated no significant differences between applications, suggesting that growth stimulation was achieved regardless of application method used. However, with respect to the remaining formulations, significant differences were found between application techniques. *Trichoderma harzianum* KMD prepared with chlamydospores in milled oats recorded an increase of 167% with drenching against 109% with capping against 301% with seed treatment; *G. virens* MM1 prepared with; conidia in kaolin powder with oil recorded an increase of 1315% with seed treatment against 385% with capping against 176% with drenching. Pelgel® (Control 4) was observed to have a growth stimulatory effect on cucumber seedlings.

On Namaqualand daisy (Figure 4.7 and Figure 4.8), formulations of *T. harzianum* KMD prepared with conidia in kaolin powder with oil; *T. harzianum* KMD (commercial) and *G. virens* MM1 prepared with conidia in kaolin powder with oil recorded significant differences in plot weight between application techniques used. *Trichoderma harzianum* KMD prepared with conidia in kaolin powder mixed with oil performed better when applied as a drench (3449%) or as a cap (3349%) whilst *T. harzianum* KMD (commercial) increased growth when applied as a seed treatment (3645%). Percentage survival of cabbage seedlings as a result of the various application methods used were observed to be comparable to the controls (water only).

On Eucalyptus seedlings (Figures 4.10 and Figure 4.11), formulations using various application techniques resulted in no differences in plot weight and percentage survival of Eucalyptus seedlings. However, growth stimulation was initiated by all formulations tested

irrespective of application techniques used. Growth stimulation ranged from 100-1600% when compared to the control (water only). *Bacillus subtilis* AW57 performed best when applied as a seed treatment, which increased growth of Eucalyptus seedlings by 1000 fold when compared to the control (water only).

Significant differences were found (P=0.0001) between the responses of the four crops to the various organisms in plot weight and percentage survival of cabbage seedlings. *Bacillus subtilis* AW57 performed best in enhancing growth of cabbage seedlings from 4000-6000% followed by *T. harzianum* KMD and *G. virens* MM1, which resulted in an increase in growth by 2000-6000% on cabbage seedlings when compared to the control (water only). For cucumber and Namaqualand daisy seedlings, the different organisms used were significant for plot weight. For both these crops, *T. harzianum* KMD and *G. virens* MM1 performed best in increasing growth for plot weight. Significant increases in growth were also recorded with *T. harzianum* KMD causing an increase of 300-3000% and *G. virens* MM1 with an increase of 400-2000% when compared to the controls (water only).

Increases in growth response also differed according to the interaction of application technique and formulations applied. Response also differed as a result of the various organisms tested. A summary of the key results was constructed to clarify the difference between formulation, application and crops investigated. Comparisons based on plot weight are as follows:

On cabbage:

The interaction of formulation and application was found to be significant for percentage survival (P=0.0006) of cabbage seedlings.

- One formulation of *T. harzianum* KMD and *G. virens* MM1 prepared with chlamydospores in milled oats resulted in increases in plot weight when applied with any of the application techniques;
- Formulations of *T. harzianum* KMD prepared with 10^8 (1000 times more) chlamydospores in milled oats enhanced plot weight when applied as a capping by 4281% relative to the control (water only);

- *T. harzianum* KMD (commercial) and *T. harzianum* KMD prepared with conidia in kaolin powder with oil caused increases in plot weight when applied as a seed treatment or as a capping and
- *G. virens* MM1 performed best when applied as a drench and *B. subtilis* AW57 (washed and with Nutristart) increased growth regardless of application methods used.

On cucumber:

Significant differences were recorded for plot weight of cucumber (P=0.0001).

Formulations of *T. harzianum* KMD prepared with conidia in kaolin powder mixed with oil, *G. virens* MM1 prepared with chlamydospores in milled oats and both preparations of *B. subtilis* AW57 increased growth regardless of the application method used.

- *T. harzianum* KMD (chlamydospores in milled oats) and (conidia in an experimental compound) increased plot weight when applied as a drench or as a capping by 200-300%;
- *T. harzianum* KMD 10^8 times (1000 times more chlamydospores in milled oats) enhanced growth when applied as a cap by 200% and
- *T. harzianum* KMD (commercial) and *G. virens* MM1 prepared with conidia in kaolin powder with oil performed best when applied as a seed treatment and enhanced growth by 400-500 %.

On Namaqualand daisy:

- *T. harzianum* KMD prepared with chlamydospores in milled oats; prepared with conidia in an experimental compound; *G. virens* MM1 prepared with chlamydospores in milled oats; *G. virens* MM1prepared with conidia in kaolin powder with oil and *B. subtilis* AW57 all increased plot weight regardless of the application method used;
- *T. harzianum* KMD containing conidia in kaolin powder with oil performed well when applied as a drench or as a capping, with increases in plot weight as high as 3000 fold;
- *T. harzianum* KMD prepared with kaolin powder with oil performed well when applied as a drench and increased plot weight as high as 3000 fold and
- *T. harzianum* KMD prepared with 10^8 times more chlamydospores in milled oats increased plot weight by 1700 fold than the controls when applied as a capping.

On Eucalyptus:

- No significant differences were recorded between application methods. However, growth stimulation was achieved for all formulations and application methods when compared to the control (water only).

The above comparisons indicate that each formulation of each organism performed well when applied using drenching, capping or seed treatment, but varied for the different crops investigated.

The organisms tested (*T. harzianum* KMD, *G. virens* MM1 and *Bacillus subtilis* AW57) performed best when formulated as their specific formulation preparations. On most crops, it was evident that better growth stimulation was achieved when *T. harzianum* KMD and *G. virens* MM1 were applied using conidia as propagules rather than chlamydospores.

The interaction between formulation and organism was recorded to be significant (P=0.05) for all parameters of cabbage growth (Table 4.1). On cucumber seedlings, the interaction between formulations and organisms was significant for plot weight only. On Namaqualand daisy seedlings, the interaction between formulation and organisms was recorded to be highly significant (P=0.0005) with respect to percentage survival. On Eucalyptus seedlings the interaction between formulation and organism was recorded to be not significant as reflected in plot weight. However, significant differences were observed for percentage survival (P=0.0001).

With respect to the interaction between organisms and application technique it was found that this interaction varied for all parameters for each crop:

- On cabbage seedlings this interaction was significant (P=0.05) for percentage survival and plot weight.
- On cucumber seedlings percentage survival and plot weight were not significant for this interaction.
- On Namaqualand daisy significant differences were recorded for all parameters tested.

- On Eucalyptus seedlings, percentage survival was recorded to be significant. It is thus evident that the three different organisms performed best when applied as either a drench, seed treatment or as capping to enhance growth.

Growth responses also depended on the interaction of formulation x application x organisms (P=0.0001). Significant differences were recorded on percentage survival of Namaqualand daisy and Eucalyptus seedlings, and plot weight of cucumber seedlings. This interaction was recorded to be not significant for all parameters tested on cabbage seedlings.

4.3.1.1 Dosage levels of formulations of T. harzianum KMD and G. virens MM1 on growth promotion effects of seedlings under greenhouse conditions

All formulations that were applied as a drench were used to test the dosage effect on growth promotion, with the exception of formulations of *T. harzianum* KMD containing 10^8 chlamydospores in milled oats, which was applied as a capping only. *Bacillus subtilis* AW57 was applied as 2 ml per plant at planting. No dosage effects were recorded for this organism.

For all formulations tested, dosage resulted in a significant effect on growth promotion of seedlings (Figure 4.3; Figure 4.6; Figure 4.9; Figure 4.12). All formulations affected plot weight and percentage survival, as dosages increased to 10g/l.

Dose response curves for the relationship between dosage of each formulation on plot weight and percentage survival of each crop have been illustrated (Figure 4.3; Figure 4.6; Figure 4.9; Figure 4.12). Simple linear regression was performed to fit data, based on SAS, Inc. Cary (SAS, 1987).

On cabbage seedlings (Figure 4.3) strong linear relationships (r=0.74) existed between dosage and growth enhancement and it was estimated that about 1-5g/l are sufficient to effectively enhance growth. By varying the doses from 1-10g/l significant effects on percentage survival and plot weight were achieved. *Gliocladium virens* MM1 prepared with chlamydospores in milled oats and *T. harzianum* KMD prepared with conidia in an experimental compound exhibited a positive relationship when dosage was applied ranging from 1-10g/l on plot weight and percentage survival. The remaining formulations caused a negative relationship ranging from 5-10g/l on plot weight and percentage

survival. *Gliocladium virens* MM1 prepared with conidia with oil caused an inverse relationship between plot weight and percentage survival at 1-10g/l.

On cucumber seedlings (Figure 4.6), a strong linear relationship (r=0.95) existed between formulation dosage on plot weight and percentage survival. It was estimated that approximately 1-5g/l is sufficient for a positive influence on growth enhancement to occur. *Gliocladium virens* MM1 prepared with conidia in kaolin powder with oil resulted in a positive relationship between dosage ranging from 5-10 g/l on plot weight of cucumber seedlings. However, a negative relationship between dosages ranging from 5-10g/l on percentage survival of cucumber seedlings also occurred. This indicates that an inverse relationship was caused by dosage on plot weight and percentage survival of cucumber seedlings. *Gliocladium virens* MM1 prepared with chlamydospores in milled oats resulted in a positive relationship between dosage ranging from 1-10g/l on plot weight and percentage survival of cucumber seedlings. *Trichoderma harzianum* KMD prepared with chlamydospores in milled oats and *T. harzianum* KMD (commercial) resulted in an inverse relationship between plot weight and percentage survival of cucumber seedlings when applied at dosages of 5g/l/. *Trichoderma harzianum* KMD prepared with conidia in kaolin powder with oil and *T. harzianum* KMD prepared with conidia in an experimental compound caused a negative effect on plot weight when applied at 5-10g/l.

Strong linear relationships (r=0.82) existed between dosage of plot weight and percentage survival on Namaqualand daisy seedlings (Figure 4.9). Plot weight and percentage survival consistently enhanced growth when formulations of *G. virens* MM1 prepared with conidia in kaolin powder with oil and *G. virens* MM1 prepared with chlamydospores in milled oats were applied at dosages ranging from 0.25-10g/l. However, *T. harzianum* KMD prepared with chlamydospores in milled oats caused a negative relationship on plot weight when dosage ranged from 0.25-1g/l. Percentage survival was positively affected when *T. harzianum* KMD prepared with chlamydospores in milled oats was applied at dosages of 5-10g/l. Plot weight and percentage survival decreased when *T. harzianum* KMD prepared with powder in oil was applied at dosages ranging from 1-10g/l. The commercial formulations caused negative effects on both parameters when dosage ranged from 5-10g/l. Although all formulations were inconsistent in enhancing growth, it was estimated that 1g/l be is sufficient for growth enhancement.

On Eucalyptus seedlings, a strong linear relationship (r=0.70) existed between dosage of plot weight and percentage survival. It was estimated that 1-5g/l would be sufficient for growth enhancement, with the exception of *G. virens* MM1 prepared with conidia in kaolin powder with oil (Figure 4.12). Varying doses from 1-5g/l had significant effects on plot weight and percentage survival of Eucalyptus seedlings.

4.3.2 The effect of formulations of *T. harzianum* KMD, *G. virens* MM1 and *B.subtilis* AW57 on damping–off disease caused by *Pythium* spp. under greenhouse conditions

Damping-off caused by *Pythium* spp. on Eucalyptus and Namaqualand daisy seedlings were significantly (P=0.0001) reduced by most formulations of the various biocontrol organisms used (Tables 4.6 and Table 4.7). However, no significant differences on plot weight and percentage survival were recorded on cabbage and cucumber seedlings.

Comparing performances of the individual formulations of *T. harzianum* KMD, *G. virens* MM1 and *B. subtilis* AW57 in controlling damping-off of Namaqualand daisy, significant differences were observed for plot weight (P=0.0006). *Trichoderma harzianum* KMD prepared with 10^5 chlamydospores in milled oats reduced disease and increased plot weight by 8-14%; *T. harzianum* KMD prepared with conidia in powder with oil increased plot weight by 14-25%; *T. harzianum* KMD (commercial) increased plot weight by 29-31%; *T. harzianum* KMD prepared with conidia an experimental compound increased in plot weight by 20-42% and *G. virens* MM1 prepared with chlamydospores in milled oats increased plot weight by 4-12% when compared to diseased controls (Figure 4.19 and Figure 4.20).

On Eucalyptus seedlings, significant differences were recorded between formulations as reflected by plot weight and percentage survival. All formulations of *T. harzianum* KMD effectively reduced disease and increased plot weight by 30% when compared to disease controls. *Gliocladium virens* MM1 containing conidia in kaolin powder mixed with oil and *B. subtilis* AW57, which did not show any disease suppression.

The *Pythium* spp. isolate used appeared to be crop specific as it was not pathogenic on cabbage and cucumber seedlings.

On Namaqualand daisy significant differences between application techniques as reflected by percentage survival (P=0.05) in reducing damping-off of *Pythium* spp. were observed (Table 4.7, Figure 4.19 and Figure 4.20). No major differences were noted on plot weight when *T. harzianum* KMD prepared with conidia in an experimental compound was applied on Namaqualand daisy seedlings. This formulation caused a reduction in disease and enhanced growth by 20% with capping, 42% with drenching and 6% with seed treatment. Similar results were recorded when *T. harzianum* KMD prepared with conidia in kaolin powder with oil and the commercial formulation.

Trichoderma harzianum KMD prepared with conidia in kaolin powder with oil reduced disease and enhanced growth by 25% with capping, 20% with drenching and 14% with seed treatment. *Trichoderma harzianum* KMD (commercial) caused a reduction in disease and enhanced growth of Namaqualand daisy seedlings by 31% with capping and 29% with seed treatment. Nutristart and Pelgel® caused a reduction in disease and increases in growth when compared to controls, diseased control (*Pythium* spp. only) and water only.

On Eucalyptus seedlings (Table 4.8) significant differences were recorded between application techniques as reflected on percentage survival (Figure 4.23). When *G. virens* MM1 prepared with chlamydospores in milled oats was applied to Eucalyptus seedlings, a reduction in disease and healthy growth was promoted by 25% with seed treatment, 14% with drenching and 29% as a capping. *Trichoderma harzianum* KMD when formulated with conidia in powder (experimental compound) reduced disease and enhanced growth by 14% when applied as a capping.

On cabbage and cucumber seedlings (Table 4.5 and Table 4.6 respectively), significant differences were recorded between application techniques. However, no disease reduction was recorded when compared to diseased control (*Pythium* spp. only).

The specific biocontrol organisms, used were significant in the reduction of disease caused by *Pythium* spp. The effect of different organisms on the biocontrol of *Pythium* spp., caused significant differences on plot weight (P=0.05). All formulations of *T.*

harzianum KMD significantly reduced disease followed by *G. virens* MM1 and *B. subtilis* AW57 on all crops. *Bacillus subtilis* AW57 reduced disease by 16% and enhanced percentage survival rates by 22% on Namaqualand daisy seedlings when compared to the disease controls. *Trichoderma harzianum* KMD and *G. virens* MM1 reduced disease by 20-40% when compared to the diseased controls on Namaqualand daisy seedlings.

On cabbage seedlings, the different biocontrol organisms resulted in marginal differences on percentage survival (P=0.03). *Trichoderma harzianum* KMD enhanced growth by 4-6% when compared to the controls. *Gliocladium virens* MM1 enhanced growth and reduced disease by 5-10% when compared to disease controls. *Bacillus subtilis* AW57 reduced disease by 1-3% when compared to the diseased controls.

On cucumber seedlings, highly significant differences were recorded between differences of biocontrol organisms on damping-off of *Pythium* spp. *Trichoderma harzianum* KMD, *G. virens* MM1 and *B. subtilis* AW57 reduced disease on plot weight by 1-4%, 1% and 9% respectively when compared to disease controls. Significant differences were recorded on percentage survival of cucumber seedlings. The various biocontrol organisms resulted in significant differences on percentage survival. *Trichoderma harzianum* KMD increased percentage survival by 58%, *Gliocladium virens* MM1 by 54% and *B. subtilis* AW57 increased percentage survival by 58% when compared to disease controls.

On Eucalyptus seedlings, significant differences between organisms were recorded (P=0.0001) on plot weight. *Trichoderma harzianum* KMD and *G. virens* MM1 most effectively reduced disease ranging from 14-30% when compared to diseased controls

No significant effects were recorded on the interaction of formulations and application techniques on cabbage seedlings. Marginally significant differences on plot weight and percentage survival were recorded on the interaction of formulations and application techniques used on cucumber seedlings. For example, *B. subtilis* AW57 with and without Nutristart reduced disease by 2-9% when applied as a drench. *Gliocladium virens* MM1 applied with chlamydospores and conidia reduced disease percentage when applied as a capping. *Trichoderma harzianum* KMD containing chlamydospores in milled oats effectively reduced disease on plot weight and percentage survival by 4% and 58% respectively when applied as a capping or seed treatment.

Significant differences were recorded on the interaction of formulations between application techniques on plot weight of Namaqualand daisy. *Trichoderma harzianum* KMD prepared with chlamydospores reduced disease effectively by 8-14% when applied as either drenching or capping when compared to disease controls (Control 2). Major differences were noted when *G virens* MM1 prepared with chlamydospores in milled oats significantly reduced disease when applied as a drench and seed treatment.

Highly significant differences (P=0.0002) for the interaction of formulations and application techniques were recorded on percentage survival of Eucalyptus seedlings. For example, *T. harzianum* KMD and *G. virens* MM1, prepared with chlamydospores effectively reduced disease when applied as a drench and capping. *B. subtilis* AW57 reduced disease more effectively when applied as a drench.

No significant differences were recorded for the interaction of formulations and organisms on cabbage seedlings. However, significant differences for this interaction were noted on cucumber seedlings. Marginally significant differences were recorded on plot weight of cucumber seedlings. *Trichoderma harzianum* KMD prepared with 10^8 chlamydospores in milled oats effectively reduced disease better than the formulations prepared with 10^5 chlamydospores in milled oats. *Gliocladium virens* MM1, on the other hand reduced disease irrespective of formulation used. *Bacillus subtilis* AW57 reduced disease effectively when applied with Nutristart. Highly significant differences for this interaction (P=0.0001) on percentage survival of cucumber seedlings were recorded. *Bacillus subtilis* AW57 effectively reduced disease and enhanced growth when formulated without Nutristart.

Significant differences were noted for the interaction of formulations and organism on percentage survival of Namaqualand daisy seedlings. *Trichoderma harzianum* KMD performed best when all formulations of *T. harzianum* KMD significantly enhanced percentage survival by 8-42% when compared to the controls. *Gliocladium virens* MM1 and *B. subtilis* AW57 enhanced percentage survival by 2-12%, 10-22% respectively when compared to diseased controls (Control 2).

Highly significant differences were achieved for this interaction on plot weight percentage survival of Eucalyptus seedlings. *Trichoderma harzianum* KMD containing 10^5 chlamydospores increased plot weight and reduced disease by 17-30%. Compared to other formulations *T. harzianum* KMD performed best in reducing disease. *Gliocladium virens* MM1 effectively reduced disease by 14-29% when compared to the disease controls.

With respect to the interaction between organisms and application techniques, no significant differences were recorded on plot weight and percentage survival on cabbage seedlings. This interaction was highly significant (P=0.0001) for both plot weight and percentage survival. On cucumber, Namaqualand daisy and Eucalyptus seedlings *T. harzianum* KMD effectively reduced disease as a seed treatment or drenching for plot weight and percentage survival. Major differences were noted when *G. virens* MM1 effectively caused a reduction disease levels when applied as a capping. *Bacillus subtilis* AW57, on the other hand, effectively reduced disease when applied as a drench.

On Namaqualand daisy, *T. harzianum* KMD consistently resulted in a reduction in disease when applied as a capping. For example, *T. harzianum* KMD containing conidia in the experimental compound caused a reduction in disease and enhanced plot weight when applied as a drench. On Eucalyptus seedlings, *T. harzianum* KMD effectively enhanced plot weight when applied as a drench or as a seed treatment. *Gliocladium virens* MM1 was effective in reducing disease when applied as a capping.

The interaction of formulation, application and organism was not significant for Namaqualand daisy. No significant differences for this interaction were recorded on cabbage, cucumber and Eucalyptus seedlings.

4.3.2.1 Dosage levels of formulations of T. harzianum KMD, G. virens MM1 on biocontrol of Pythium spp., of seedlings under greenhouse conditions

All formulations applied as a drench were used to test the dosage effect on biocontrol of *Pythium* spp. with the exception of the formulations of *T. harzianum* KMD containing 10^8 times more chlamydospores in milled oats, which was applied as a cap. *B. subtilis* AW57 was applied of 2 ml per plant at planting. No test for dosage effects was recorded for this

organism. Dosage tested had a significant effect on the efficacy of all formulations on reduction in disease of seedlings (Figure 4.15; Figure 4.18; Figure 4.21; Figure 4.24). All formulations exhibited an effect on plot weight and percentage survival as dosages increased to 10g/l.

On cabbage seedlings, a strong linear relationship (r=0.73) existed between dosage and percentage survival or plot weight. It was estimated that approximately 1g/l is sufficient for effective control of *Pythium* spp. *Gliocladium virens* MM1 containing conidia in kaolin powder with oil exhibited a negative relationship between dosage ranging from 5-10 g/l. *Gliocladium virens* MM1 prepared with chlamydospores in milled oats and *T. harzianum* KMD prepared with conidia in kaolin powder with oil recorded a negative relationship for dosage of 1-10 g/l. *Trichoderma harzianum* KMD prepared with 10^5 chlamydospores exhibited a positive influence between dosages ranging from 5-10g/l. Inverse relationships existed between plot weight and percentage survival when *T. harzianum* KMD prepared with conidia in an experimental compound was applied at dosages 1-10g/l.

On cucumber seedlings, dose response curves (r=0.69) for the relationship between dose of formulations was evaluated and it was estimated that 5g/l was sufficient for control of *Pythium spp.* At higher dosages most formulations of *T. harzianum* KMD gave good control but caused stunting of seedlings at 10g/l (Figure 4.37).

On Namaqualand daisy (r=0.89) and Eucalyptus seedlings (r=0.61) a strong linear relationship was recorded. It was estimated that 1g/l was sufficient to effectively enhance growth establishment and reduce disease.

4.3.3 The effect of formulations of *T. harzianum* KMD, *G. virens* MM1 and *Bacillus subtilis* Aw57 on damping-off disease caused by *Rhizoctonia solani* under greenhouse conditions.

Biological control of damping –off disease, caused by *R. solani*, on cabbage, cucumber, Namaqualand daisy and Eucalyptus seedlings was achieved by applying formulations of *T. harzianum* KMD, *G. virens* MM1 and *B. subtilis* AW57 using three application techniques to infested composted pine bark (Tables 4.9- 4.12).

On cabbage seedlings (Table 4.9), significant differences were recorded between formulations as reflected on plot weight and percentage survival (P=0.0001). All formulations of biocontrol organisms reduced disease incidence and enhanced plot weight ranging from 100-1600% when compared to diseased control 2 (infested with *R. solani* only) (Figure 4.25 and Figure 4.26). Percentage survival increased by 10-41% (Figure 4.26) when compared to the diseased control (Control 2). Major differences were noted in disease reduction when formulation of *T. harzianum* KMD and *G. virens* MM1 prepared with conidia in kaolin powder with oil was applied. These formulations achieved an increase in plot weight by 1000 fold and percentage survival by 17-37% when compared to diseased control 2 (infested with *Rhizoctonia solani* only).

On cucumber seedlings (Table 4.10, Figure 4.28-4.29), no significant differences between formulations were reflected on percentage survival (P=0.05). Percentage survival was increased when formulations of *T. harzianum* KMD prepared with 10^5 chlamydospores increased plot weight by 100-200%; *T. harzianum* KMD prepared with conidia in kaolin powder with oil by 78-100%; *T. harzianum* KMD (commercial) by 24-129% (Figure 4.28); *T. harzianum* KMD containing conidia in an experimental compound by 49-184%, *G.virens* MM1 prepared with chlamydospores by 153-189%; *G. virens* MM1 containing conidia in kaolin powder with oil by 64-128% and *B. subtilis* AW57 by 8-34% when compared to diseased controls.

Formulations of *T. harzianum* KMD, *G. virens* MM1 and *B. subtilis* Aw57 caused a reduction in damping-off of *R. solani* on Namaqualand daisy (Table 4.11, Figure 4.31-2.32) as reflected on percentage survival (P=0.0001). Prominent disease control was achieved by 19-600% caused by most formulations as reflected on percentage survival (Figure 4.31 and Figure 4.32). Percentage survival was enhanced from 50%-600% when compared to disease controls (Control 2).

Significant differences were obtained between formulations for all parameters tested on Eucalyptus seedlings (Table 4.12, Figure 4.34-4.35). Plot weight was increased when treated with formulations of all biocontrol agents. All formulations enhanced growth and reduced disease, ranging from 24-300%. Percentage survival was comparable to controls.

Significant differences (P=0.0001) between application techniques were recorded on all crops in reducing disease. Major differences were noted for the formulation *T. harzianum* KMD prepared with conidia in kaolin powder with oil on cabbage seedlings (Table 4.9). This formulation applied as a capping enhanced plot weight by 1000%, and enhanced percentage survival by 37% on cabbage seedlings. *Trichoderma harzianum* KMD (commercial) caused a reduction in disease and enhanced plot weight by 700% with seed treatment against 418% with drenching and against 223% with capping. *Trichoderma harzianum* KMD prepared with conidia in an experimental compound reduced disease and enhanced plot weight by 707% with seed treatment against 741% with drenching and against 588% with seed treatment; *G. virens* MM1 prepared with chlamydospores in milled oats reduced disease and enhanced plot weight by 121% with seed treatment against 100% with capping. *Gliocladium virens* MM1 prepared with containing conidia in kaolin powder with oil reduced disease and enhanced plot weight by 1666% with drenching against 161% with seed treatment and against 334% with capping. *Bacillus subtilis* AW57 reduced disease and enhanced growth by 404%- 595% by either application method used. Percentage survival was also observed to be significant (P=0.0001) between application techniques. Percentage survival increased by 10-20% when compared to the disease control.

On cucumber seedlings, significant (P=0.05) differences were recorded on percentage survival as a result of application techniques (drench, seed treatment and capping) used (Table 4.10). *Trichoderma harzianum* KMD (commercial) increased plot weight by 129% with seed treatment against 24% with drenching; *T. harzianum* KMD prepared with conidia in the experimental compound enhanced percentage survival and reduced disease by 184% with drenching against 49% with capping. *Gliocladium virens* MM1 prepared with conidia in kaolin powder with oil had similar recordings of 128% with drenching against 64% with seed treatment and 128% with capping.

On Namaqualand daisy seedlings disease reduction was also obtained when formulations of biocontrol organisms were applied using drenching, capping and seed treatment. All formulations enhanced plot weight and percentage survival and reduced damping-off irrespective of application method used. *Trichoderma harzianum* KMD enhanced plot weight by 430% with capping, against 436% with seed treatment, against 253% with drenching; *T. harzianum* KMD (commercial) enhanced plot weight by 153% with

drenching, against 131% with seed treatment, against 21% with capping; *T. harzianum* KMD prepared with conidia in an experimental compound enhanced plot weight by 609% with capping, against 193% with drenching, against 153% with seed treatment; *G. virens* MM1 containing conidia in kaolin powder with oil enhanced plot weight by 249% with drenching, against 195% with capping, against 23% with seed treatment and *B. subtilis* enhanced plot weight by 120-250% with both drenching and seed treatment.

On Eucalyptus seedlings, significant differences between plot weight and percentage survival resulted from the various application techniques (drench, cap, seed treatment). *Trichoderma harzianum* KMD containing conidia in kaolin powder with oil, reduced disease and enhanced plot weight by 308% with drenching against 143% with seed treatment when compared to disease control 2 (infested with *R. solani* only); *T. harzianum* KMD (commercial) increased plot weight by 160% with drenching against 140% with seed treatment. *B. subtilis* AW57 (washed) increased growth of plants and reduced disease by 209% with seed treatment and *B. subtilis* AW57 with Nutristart increased plot weight by 34% when compared to the diseased control of *R. solani.*

ANOVA indicated significant effects as a result of the organism tested on all crops evaluated. On cucumber and Namaqualand daisy seedlings this effect was significant (P=0.0001) as reflected by percentage survival. *Trichoderma harzianum* KMD and *G. virens* MM1 and *B. subtilis* AW57 effectively reduced damping-off. Percentage survival of cabbage and cucumber seedlings were enhanced by all organisms tested. *Trichoderma harzianum* KMD and *G. virens* caused the most significant increases on percentage survival. *Trichoderma harzianum* KMD enhanced plot weight and reduced disease by 1000 fold. *Gliocladium virens* MM1 increased plot weight and reduced disease by 100 fold. *Bacillus subtilis* AW57 reduced disease by 500% when compared to the controls.

On cucumber seedlings, the various biocontrol organisms caused a significant difference on percentage survival. *Gliocladium virens* MM1 and *T. harzianum* KMD increased percentage survival by 100%, while *B. subtilis* AW57 increased percentage survival by 15% when compared to the controls.

On Namaqualand daisy seedlings, the various biocontrol organisms caused a significant difference on percentage survival. *Bacillus subtilis* AW57 increased percentage survival

by 600%. *Trichoderma harzianum* KMD and *G. virens* MM1 increased percentage survival by 400-500% when compared to the controls.

On Eucalyptus seedlings, the biocontrol organisms caused a significant difference on plot weight. *Trichoderma harzianum* KMD increased plot weight by 100-300% when compared to the controls.

ANOVA indicated that there were formulation and application effects in all four crops tested, indicating that formulations significantly reduced damping-off when applied as a drench, capping and seed treatment.

On cabbage seedlings, *G. virens* MM1 prepared with conidia in kaolin powder with oil increased plot weight by 1600% when applied as a drench. *Trichoderma harzianum* KMD prepared with conidia in kaolin powder with oil and the commercial formulation effectively reduced damping-off of cabbage seedlings when applied as a drench and seed treatment. Formulations containing 10^5 chlamydospores in milled oats increased plot weight and reduced disease by 138 % when applied as a capping and as a seed treatment. *Trichoderma harzianum* KMD prepared with conidia (experimental compound) reduced disease when applied as drench and as a seed treatment by 700%, but applied as capping reduced disease by 500%. *Bacillus subtilis* AW57 increased plot weight irrespective of application technique used.

On cucumber seedlings the interaction between formulations and application had a significant effect on plot weight (P=0.001). Formulations of *T. harzianum* KMD prepared with 10^5 chlamydospores in milled oats effectively reduced disease by 150% on cucumber seedlings when applied as a seed treatment when compared to the disease controls. *Trichoderma harzianum* KMD commercial and *G. virens* MM1 prepared with chlamydospores in milled oats effectively reduced disease when applied as a seed treatment or as a drench by 1000-2000 fold. *Gliocladium virens* MM1 prepared with conidia in kaolin powder with oil effectively reduced disease by 1000 fold when applied as drenching and capping when compared to the disease controls.

On Namaqualand daisy, significant differences were observed for the interaction of formulations and application techniques used, on plot weight (P=0.03) and percentage

survival (P=0.0003). *Trichoderma harzianum* KMD prepared with conidia in an experimental compound reduced disease by 600% when applied with capping and increased percentage survival by 487 % when applied as a drench, while *G. virens* MM1 prepared with chlamydospores reduced disease by 19-67% when applied as a drench and seed treatment and increased percentage survival by 400% when compared to the controls.

On Eucalyptus seedlings highly significant differences were recorded on plot weight (P=0.0001) and percentage survival (P=0.0005) for the interaction of formulations and application techniques used. *Trichoderma harzianum* KMD prepared with 10^5 chlamydospores in milled oats increased plot weight by 24% when applied as a drench when compared to the disease control. *Trichoderma harzianum* KMD applied with conidia in kaolin powder and commercial formulation reduced disease effectively when applied as a drench or as a seed treatment by 100 fold. *Trichoderma harzianum* KMD prepared with conidia in an experimental compound increased plot weight when applied as a drench and as a capping by 100 fold.

ANOVA indicated that there was an interaction effect of formulation and organisms on most crops. Highly significant differences (P=0.0001) were recorded for this interaction on cabbage seedlings. It was evident that *T. harzianum* KMD effectively reduced disease by 1000 fold when formulated with conidia in kaolin powder with oil. *Trichoderma harzianum* KMD prepared with 10^5 chlamydospores in milled oats was least effective in reducing disease. *Gliocladium virens* MM1 most effectively reduced disease when formulated as conidia in powder with oil and not with chlamydospores by 1000 fold. *Bacillus subtilis* AW57 performed the best when applied with Nutristart by increasing plot weight by 500% when compared to disease controls.

On cucumber seedlings, highly significant differences on plot weight (P=0.0004) and percentage survival (P=0.0001) were recorded by the interaction of formulation and organism. *Trichoderma harzianum* KMD performed the least in reducing disease on cucumber seedlings when formulated with 10^5 chlamydospores in milled oats and performed the best when formulated with conidia in a kaolin powder with oil. *Gliocladium virens* MM1 reduced disease and increased plot weight when formulated with conidia in kaolin powder with oil. Similar recordings were obtained for percentage survival.

On Namaqualand daisy seedlings no significant differences were obtained for the interaction of formulations and organisms.

On Eucalyptus seedlings highly significant differences were recorded for the above interaction on plot weight and percentage survival. *Trichoderma harzianum* KMD most effectively reduced damping-off caused by *R. solani*, when formulated with conidia in kaolin powder with oil by 100-300% when compared to disease controls.

ANOVA indicated significant effects of the interaction of application and organism. This interaction was observed to be significant for cabbage and Eucalyptus seedlings (P=0.0001). Each organism differed for each application for each crop in reducing damping-off of *R. solani.* On cabbage and Eucalyptus seedlings, *T. harzianum* KMD effectively reduced disease when applied as a drench and seed treatment. *Gliocladium virens* MM1 reduced disease when applied as a drench or capping.

Significant interaction effects (P=0.05) were observed for the formulation, application and organism effect on all crops evaluated.

4.3.3.1 Dosage levels of formulations of T. harzianum KMD, G. virens on biocontrol of Rhizoctonia solani, which causes damping-off of seedlings under greenhouse conditions

All formulations that were applied as a drench were used to test the dosage effect on biocontrol of *R. solani,* with exception to formulations of *T. harzianum* KMD containing 10^8 chlamydospores in milled oats, which was applied as a capping only. *Bacillus subtilis* AW57 was applied as 2 ml per plant at planting. No test for dosage effects was recorded for this organism.

For all formulations tested, dosage resulted in significant effects on reduction in disease of seedlings (Figure 4.27, Figure 4.30, Figure 4.33 Figure 4.36). All formulations exhibited an effect on plot weight and percentage survival as dosages increased to 10g/l.
Dose- response curves for the relationship between dose of formulations of each crop have been established (Figure 4.7, Figure 4.30, Figure 4.33 Figure 4.36)

On cabbage seedlings (Figure 4.27), a strong linear relationship (r=0.64) existed between dosage and biocontrol of *R. solani*. It was estimated that about 1- 5g/l was sufficient for the reduction of disease. Doses of 5g/l– 10g/l had negative responses on formulations *Gliocladium virens* MM1 prepared with conidia in kaolin powder with oil; *T. harzianum* KMD prepared with chlamydospores in milled oats; *T. harzianum* KMD prepared with conidia in kaolin powder with oil and *T. harzianum* KMD prepared with conidia in an experimental compound resulted in a negative relationship between dose ranging from 5-10g/l.

On cucumber seedlings (Figure 4.30) it was estimated that 1-5g/l is sufficient for the control of damping-off. *Gliocladium virens* MM1 prepared with conidia in kaolin powder with oil and *T. harzianum* KMD prepared with chlamydospores resulted in a negative relationship with dose ranging form 5-10g/l. Inverse relationships occurred when the formulation of *T. harzianum* KMD (commercial), between 5-10g/l. *Trichoderma harzianum* KMD prepared with conidia in an experimental compound caused an increase in plot weight and percentage survival at 5g/l.

On Namaqualand daisy and Eucalyptus seedlings it was estimated that 1-5g/l is sufficient to effectively control damping-off.

4.4 DISCUSSION

Inundative approaches to implement biocontrol of soil-borne plant pathogens vary greatly and range from seed treatment for field crops to application of antagonist-colonized grain for woody ornamentals grown in greenhouse potting mix (Hornby, 1990; Mehrotra *et al.*, 1997; Whipps, 1997). Controlled environmental conditions, increasing restrictions of the use of chemical pesticides, and the high commercial value of the commodities provide favourable circumstances for the use of biocontrol stategies in greenhouse production systems (Coley- Smith *et al.*, 1991; Naegley, 1997).

The results reported in this chapter are essential because they address these parameters with the use of formulations of biocontrol agents. These biocontrol agents have replaced various other treatments for the enhancement of growth and the control of the pathogens

Pythium spp. and *R. solani,* a major cause of damping-off diseases of greenhouse crops (Stephens *et al.*, 1982).

Mean dry weight per plot was not measured due to compensation effects. Information derived from single plant experiments may be valid for plants in the crop situation, but since plants in crops differ in behaviour from single isolated plants, the validity of the information has to be assessed in special field experiments. Plants in the crop situation share limited amounts of space, light, water, and nutrients. If one plant cannot utilize the available resources because of disease, its neighbours will do so. The crop is therefore more than the sum of the individual plants (Zadocks & Schein, 1979).

4.4.1 Plant growth enhancement of seedlings by formulations of *T. harzianum* KMD, *G. virens* MM1 and *B. subtilis* AW57 under greenhouse conditions

A summary of growth stimulation trials is reported in Table 4.14. Table 4.14 is a summary of the results presented in Tables 4.1-4.4, reflecting the significant or non-significant results from ANOVAs conducted.

Table 4.14 reflects the significant and non-significant results, incorporating the factor of crop and interaction of main effects from ANOVA's conducted. The control (water only) was effective in identifying the level of growth stimulation present in trials. Different levels of growth enhancement were achieved for each crop evaluated.

Factorial analysis revealed the relative contribution of various treatments, and the interaction effects between treatments. Statistical analysis determined that interaction effects are more important than main effects.

Explanation of the interaction effects (Table 4.13) and P-values of the respective interaction effects (Table 4.1-4.4) reveal that the first three interactions AxB, AxC and BxC, varied for plot weight and percentage survival on all four crops.

Cabbage seedlings

On plot weight of cabbage seedlings, significant differences were achieved for AxB and AxC. This means that the three main effects (A:organism; B: formulation; C: application techniques) were all affected by interaction effects, i.e., at least one organism was significantly better or worse in its performance when coupled with one or more formulations and at least one organism was significantly better or worse in its performance when coupled with one or more of the application techniques used. For example, the organism, *T. harzianum* KMD produced poor results when formulated with chlamydospores in milled oats. However, *T. harzianum* KMD performed well when formulated with all formulations prepared with conidia. *Trichoderma harzianum* KMD resulted in high plot weights when applied using drenching or a seed treatment. The interaction of BxC was not significant. This means that the same result was achieved for each formulation irrespective on how it was applied. The final interaction of AxBxC was not significant. This indicates that the efficacy of each organism and each formulation was not affected by the result and by its application technique (drenching, capping or seed treatment).

The three interactions, AxB, AxC and BxC, were significant on percentage survival of cabbage seedlings. This means that the three main effects (A: organism; B: formulation; C: application techniques) were all affected by interaction effects; i.e., when the treatments were combined in pairs, a positive or a negative result was achieved, acting additively in each paired combination. The final interaction AxBxC was not significant. This indicates that each organism and each formulation did not affect plot weight and percentage survival by application techniques (drenching, capping, seed treatment) used.

Cucumber seedlings

Significant differences were recorded for the interactions AxB and BxC on the plot weight of cucumber seedlings. This means that at least one organism (*T. harzianum* KMD, *G. virens* MM1 or *B. subtilis* AW57) was significantly better or worse in stimulating plant growth when coupled with one or more formulations. For example, *T. harzianum* KMD

Table 4.14 A summary of results in Tables 4.1-4.4

		Significant		Non- significant	
Effects and interactions	**Treatments/ comparisons**	**Plot weight**	**% Survival**	**Plot weight**	**% Survival**
Formulations	9 formulations vs. Controls	cabbage cucumber Namaqualand daisy Eucalyptus	cabbage Eucalyptus		cucumber Namaqualand daisy
Application	Differences in three application methods and controls	cabbage cucumber Namaqualand daisy	cabbage cucumber	Eucalyptus	Eucalyptus Namaqualand daisy
Organism	Differences between *T. harzianum* KMD, *G. virens MM1*, *B. subtilis* AW57	cucumber	cabbage Eucalyptus	Eucalyptus cabbage Namaqualand daisy	cucumber daisy
Formulation* Application	Interaction between formulations and three application methods	cucumber	cabbage Namaqualand daisy Eucalyptus	cabbage Namaqualand daisy Eucalyptus	cucumber Eucalyptus
Formulation *Organism	Interactions between 9 Formulation and three organisms	cabbage cucumber	cabbage Namaqualand daisy Eucalyptus	Eucalyptus Namaqualand daisy	cucumber
Application *Organism	Interaction between three application methods and organisms	cabbage Namaqualand daisy	cabbage Namaqualand daisy Eucalyptus	cucumber Eucalyptus	cucumber
Formulation* Application * Organism	Interaction between 9 formulations and three applications and three organisms	Cucumber	Namaqualand daisy Eucalyptus	cabbage Namaqualand daisy Eucalyptus	cabbage cucumber

performed well in increasing plot weights of cucumber seedlings when formulated with conidia rather than chlamydospores. The interaction of BxC indicated that at least one formulation was significantly better or worse in stimulating plant growth depending on how it is applied. The converse of this is that one of the application techniques really works well or badly in stimulating plant growth only with some of the formulations (Table 4.2) All conidial formulations of T. *harzianum* KMD and *G. virens* MM1 achieved a high plot weight when applied as a seed treatment, while capping was inconsistent and performed poorly with respect to other application techniques.

The interaction of AxC was not significant on plot weight. This indicates that none of the combinations of organisms and application techniques were better or worse than the other. The activity of the organisms was not affected by the application techniques used. The final interaction AxBxC was significant (P=0.007). This indicates that the two main effects, organisms and formulation, interacted synergistically with the various application techniques resulting in higher plot weights than one would expect from the primary effects by themselves, or combined with another treatment.

On percentage survival of cucumber seedlings, no significant differences were recorded for the first three interactions. This means that the three main effects (A: organism; B: formulation; C: application techniques) were all not affected by the interaction effects. This suggests that none of the combinations of organisms and formulations were better or worse than the others in increasing plot weight and the activity of organisms were not affected by the formulations evaluated. None of the combination of organisms and application techniques were better or worse than the other and the activity of the organism was also not affected by application technique. This result also suggests that for each of the formulation, irrespective on how it was applied, the same effect was produced. The final interaction was also not significant, i.e., each organism and each formulation, was not affected by the application technique used.

Namaqualand daisy seedlings

On plot weight of Namaqualand daisy, significant differences were achieved for the interactions of BxC and AxC. This means that the three main effects (A:organism; B: formulation; C: application techniques) were all affected by interaction effects. This shows that at least one organism was significantly better or worse in its performance

when coupled with one or more of the application techniques and that at least one of the formulations was significantly better or worse depending on how it was applied. The converse of this is that one of the application techniques really works well or poorly with some of the formulations. For example, *T. harzianum* KMD enhanced plot weight when applied as a seed treatment or as a drench. However, this resulted in poor plot weights when applied as a capping (Table 4.3). Capping gave poor results when used to apply all formulations prepared with chlamydospore formulations of *T. harzianum* KMD and *G. virens* MM1. On the other hand, seed treatment and drenching worked really well for all formulations prepared with conidia of *T. harzianum* KMD and *G. virens* MM1. The interaction of AxB was not significant, suggesting that none of the combinations of organisms and formulations were better or worse than the other. The activities of the organisms were not affected by the formulations evaluated.

On percentage survival, significant differences were achieved for the first three interactions AxB, AxC and BxC. This means that the three main effects (A: organism; B: formulation; C: application techniques) were all affected by interaction effects, i.e., at least one organism was significantly better or worse in its performance when coupled with one or more of the formulations. For example, *G. virens* MM1 and *T. harzianum* KMD increased plot weight by 2000 fold when coupled with formulations containing conidia in kaolin powder with oil (Table 4.3). The interaction AxC suggests that at least one organism was significantly better or worse in its performance when coupled with one or more of the application techniques. *Trichoderma harzianum* KMD, when applied as a capping, resulted in poor plot weights when compared to other techniques, while seed treatment and drenching worked well in enhancing plot weights of seedlings. The BxC interaction also indicates that at least one of the formulations was significantly better or worse in enhancing percent survival depending on how it is applied. For example, formulations prepared with chlamydospores in milled oats of *G. virens* MM1 and *T. harzianum* KMD enhanced percentage survival marginally by 14-18% when applied as a drench or as a seed treatment. The final interaction AxBxC was significant, suggesting that the two main effects, i.e., organism and formulation interacted synergistically with the application technique, resulting in higher plot weights and percentage survival rates than one would expect from the primary effects on their own, or combined with another treatment.

Eucalyptus seedlings

On plot weight of Eucalyptus seedlings, no significant differences were revealed for the first three interactions AxB, AxC and BxC. This means that the three main effects (A: organism; B: formulation; C: application techniques) were all unaffected by interaction effects, i.e., when the treatments were combined in pairs, they were neutral, acting additionally in each paired combination. This indicates that the activity of microbes were not affected by formulation and application techniques and that for each formulation irrespective how users apply it, the same effect will be produced. The final interaction of AxBxC was also not significant suggesting that any organism and any formulation, the result will not be affected by the application techniques.

However, on percentage survival, significant differences were revealed for the interactions, AxB and AxC. This suggests that at least one of the organisms were significantly better or worse in its performance when coupled with one or more of the formulations. This was evident in Table 4.4 were *B. subtilis* AW57 enhanced percentage survival when formulated with Nutristart, whilst *T. harzianum* KMD performed poorly when coupled with the formulation produced commercially. The interaction AxC was highly significant (P=0.0001). This means that at least one of the organisms were significantly better or worse in its performance when coupled with one or more of the application techniques. *Trichoderma harzianum* KMD and *G. virens* MM1 enhanced percentage survival when applied with a drench or a seed treatment Table 4.4. The interaction BxC was not significant suggesting that for each of the formulations tested, irrespective on how it gets applied, the same percentage survival rates were produced. The final interaction, AxBxC was highly significant (P=0.0001). This indicates that the two main effects (organism and formulation) interacted synergistically with the application techniques, resulting in a higher percentage survival than one would expect from the primary effects by themselves, or combined with another treatment.

The main effect, organism, was not significant on plot weight of cabbage seedlings. This suggests that all organisms, *T. harzianum* KMD, *G. virens* MM1 and *B. subtilis* AW57, performed equally well or poorly in enhancing plant growth, irrespective of the other main effects. No one treatment differed from any other, over all the treatments. This main effect was also highly significant (P=0.0042) on percentage survival of cabbage seedlings. This

was evident in Table 4.1 where *B. subtilis* AW57 performed worse at enhancing growth stimulation than the other organisms tested, whilst application of *T. harzianum* KMD and *G. virens* MM1 resulted in percentage survival rates that were comparable to the water control. This main effect was also highly significant (P=0.0001) on plot weight of cucumber seedlings, e.g., *Trichoderma harzianum* KMD enhanced plot weight ranging from 100-500% whilst *G. virens* MM1 only increased plot weight by 100-300% when compared to controls (Table 4.2). *Bacillus subtilis* AW57 performed poorly in increasing plot weights when compared to other organisms. The effect of organisms was not significant on percentage survival of cucumber seedlings, suggesting that all organisms performed equally well or poorly irrespective of other main effects and that no one treatment differed from any other, over all the treatments. This effect was also not significant on both plot weight and percentage survival of Namaqualand daisy and plot weight of Eucalyptus seedlings (Table 4.3 and 4.4). However, on percentage survival of Eucalyptus seedlings, organism effect was significant suggesting that at least one organisms performed better or worse than other organisms. Overall, the biocontrol organisms, *T. harzianum* KMD and *G. virens* MM1, were more effective than *B. subtilis* AW57 in most instances in enhancing growth stimulation. This could be related to durability in storage of these organisms. The survival time is much higher for fungi due to the production of chlamydospores and conidia. *Bacillus subtilis* AW57 on the other hand, is more prone to death and dessication if not formulated well (Burges, 1998). The organisms in these trials were not formulated with any additives or protectives but inoculated in a water based slurry. *Bacillus* spp. are more susceptible to fungistasis in the soil rhizosphere resulting in low growth stimulation activities (Burges, 1988). Chlamydospores of *T. harzianum* KMD also performed poorly as they enter a state of dormancy, which prevents their energy use from being switched on and off in strict response to moisture, temperature and acid conditions (Burges, 1998). These propagules are not recommended to the manufacturer for formulation as they have to be activated with 0.5M HCℓ, which activates these propagules to germinate and hence increases manufacturer and user costs. Conidia are better to use in formulations as they have a durable shelf-life and are more viable (Burges, 1998). These propagules enhance growth stimulation irrespective of the types of formulations used. Conidial propagules are thus recommended to the manufacturer for formulation of effective stimulation of plant growth. These propagules will cut down on costs and are more effective than chlamydospores.

The second main effect, formulation, was highly significant on plot weight (P=0.0001) and percentage survival (P=0.005) of cabbage seedlings. This means that at least one formulation performed better or worse in enhancing growth stimulation than the other formulations. For example, formulations prepared with chlamydospores in milled oats performed poorly in enhancing plot weight when compared to other formulations prepared with conidia. Conidial formulations enhanced plot weight ranging from 1000-3000 fold when compared to the control (water only). A similar trend was recorded on most crops evaluated, where conidial formulations performed best when compared to the formulations prepared with chlamydospores. This is probably related to chlamydospores having a longer lag period before germination than conidia (Burges, 1998). Chlamydospores have to be activated with 0.5M HCℓ before application. Activation of these propagules were not carried out in these experiments. When formulations containing 10^5 chlamydospores were compared to those containing a higher inoculum i.e., (10^8chlamydospores), higher plot weights and percentage survival rates were achieved. This could be related to the number of viable spores varying between formulations. It has been cited that values of between 10^5-10^7 spores/g are often quoted as the range needed for growth stimulation and biocontrol (Chang *et al.*, 1986; Windham *et al.*, 1986; Ahmad & Baker, 1988). In these experiments the addition of inoculum in formulations ranged from 10^7-10^8 spores/g, which is much higher than the recommended inoculum value. When viability tests were recorded it was observed that formulations of *T. harzianum* KMD and *G. virens* MM1 prepared with chlamydospores in milled oats had a viable inoculum of 10^5 spores/g. This could be another reason why these formulations did not enhance growth as much as the conidial formulations.

Formulation was not significant for percentage survival of cucumber seedlings and plot weight of Eucalyptus seedlings. This suggests that all formulations performed equally well or poorly on plot weight and percentage survival of cucumbers seedlings irrespective of other main effects. No one treatment under these parameters tested differed from any others, over all the treatments.

The third main effect, application techniques, was highly significant on plot weight and percentage survival (P=0.0001) of cabbage, cucumber and Namaqualand daisy seedlings. This means that at least one of the application techniques resulted in a better or worse

response in enhancing plant growth than other application techniques, e.g., seed treatment or drenching, in most instances resulted in a better response in enhancing plant growth than the other application techniques (Table 4.1-4.3). Capping on the other hand, resulted in a poorer response in most instances in enhancing plant growth than other application techniques.

Variation in responses of formulations to seed treatment also occurred and this was probably due to nutrition or number of conidia coated on the seed. Numbers of conidia were not constant in these trials. The variability of seed treatment in some formulations were perhaps due to differences in conidial survival of formulations. Further studies are needed in which various storage conditions and binding material are compared to conidia or other propagules coated onto seed.

Seed treatments performed better than drenching and capping. This is probably related to the lack of a dilution factor incorporated in this technique. For example, drenching was applied by diluting 1g of formulation to 1l tap water, i.e., less spore inoculum attached to each seed coat and rhizosphere. Capping was applied by diluting 1g of formulation in $1000cm^3$ of composted pine bark, resulting in less inoculum on the seed coat and in the rhizosphere.

In most instances, formulations prepared with conidia in kaolin powder with oil, experimental compound and the commercial formulation performed well when seed treated. The koalin and other carriers incorporated in the formulation may have served as an adhesive for the attachment of the inoculant to the seed.

Overall, these results confirm that conidial formulations enhanced growth stimulation. Formulations prepared with 10^5 chlamydospores recorded poor results. However, it has been cited that the formulation, and efficacy of a given organism and propagule type incorporated in formulation are of major importance in the implementation of biocontrol and growth enhancement (Lewis & Papvizas, 1985; Hebbar *et al.*, 1996, Mao *et al.*, 1996). Seed treatment and drenching resulted in better responses in growth stimulation of most crops, while capping resulted in the least response in growth stimulation.

Recommendations to the manufacturer are:

- Formulations of *T. harzianum* KMD and *G. virens* MM1 should be prepared with conidia with an initial inoculum of 10^8 spores/g in kaolin powder or with the experimental compound;
- Formulations should be applied as a drench or seed treatment.

It was found in these studies that all formulations containing conidial propagules prepared in kaolin powder with oil, in an experimental compound and the commercially produced product were easy to handle and easily dissolved in water.

When formulations prepared with 10^5 chlamydospores were compared to those prepared with 10^8 chlamydospores, it was evident that the formulations with the higher rated inoculum dose performed better in enhancing seedling growth of. Manufacturers formulating these propagules, should increase rates of inoculum effectively enhance growth.

4.4.2 Biocontrol of damping-off caused by *Pythium* spp. by *T. harzianum* KMD, *G. virens* MM1 and *B. subtilis* AW57.

These trials are summarized in Table 4.15. This table reflects the significant and non-significant results incorporating the factor of crop and the interaction of main effects from ANOVA's conducted. The controls (water only) and the disease control (*Pythium* spp. only) were effective in identifying the level of disease control in trials.
Factorial analysis revealed that the relative contributions of various treatments and the interaction effects between treatments. Statistical analysis determined that interaction effects are more important than main effects, and must be analyzed first (Zadoks & Schein, 1979).

Explanation of the interaction effects, shown in Table 4.13 and P-values of the interaction effects shown in Table 4.5-4.8 revealed that the first three interactions AxB, AxC and BxC varied for plot weight and percentage survival on all crops tested.

Cabbage seedlings

On plot weight and percentage survival, no significant differences were recorded for the first three interactions, AxB, AxC and BxC. This means that the three main effects (A: organism, B: formulation, C: application techniques) were all unaffected by interaction

effects, i.e., when the treatments were combined in pairs, they were neutral, acting additively in each paired combination. This also suggests that none of the combinations of organism and formulation, organism and application technique were better or worse than the others and that the activity of organisms or application techniques were not affected by formulations. Each formulation, irrespective on how it was applied, produced the same effect. However, the final interaction AxBxC was not significant. This indicates that the two main effects, i.e., organism and formulation, interacted to give a result that was not affected by the application technique.

Cucumber seedlings

On the plot weight and percentage survival, a significant difference was recorded on the first three interactions, AxB, AxC, BxC. This suggests that all three main effects are affected by interactions. This also means that at least one organism (*T. harzianum* KMD, *G.virens* MM1 and *B.subtilis* AW57) was significantly better or worse in the biocontrol of *Pythium* spp. when coupled with one or more of the formulations or application techniques. This also suggests that at least one of the formulations was significantly better or worse depending on how it gets applied and the converse of that is that one of the application techniques really works well or badly only with one of the formulations. For example, in Table 4.6, *T. harzianum* KMD prepared with 10^5chlamydospores was significantly better in its performance when prepared with milled oats, whilst *B. subtilis* AW57 effectively reduced disease when applied as a drench instead of seed treatment. However, the final interaction of AxBxC was not significant suggesting that any organism and any formulation, biological control will not be affected by the application techniques used.

Namaqualand daisy seedlings

No significant differences were recorded in plot weight with respect to interactions of BxC, AxC. This suggests that no organism was significantly better or worse in its performance when coupled with one or more of the application techniques e.g., *T. harzianum* KMD resulted in a significantly better control of *Pythium* spp. when applied as a drench or as capping (Table 4.7). The interaction of BxC was not significant, i.e., none of the formulations was not significantly better or worse in reducing disease depending on how it was applied.

Table 4.15 A summary of results in Tables 4.5-4.8

		Significant		Non-significant	
Effects and interactions	**Treatments/ comparisons**	**Plot weight**	**% Survival**	**Plot weight**	**% Survival**
Formulations	9 formulations vs. Controls	cucumber Namaqualand daisy Eucalyptus	cucumber Namaqualand daisy Eucalyptus	Cabbage	cabbage
Application	Differences 3 application methods and controls	cabbage cucumber	Cabbage cucumber Namaqualand daisy Eucalyptus	Namaqualand daisy Eucalyptus	
Organism	Differences between *T. harzianum* KMD, *G. virens*, *B. subtilis* AW57	cucumber Namaqualand daisy Eucalyptus	cabbage cucumber	Cabbage	Eucalyptus Namaqualand daisy
Formulation* Application	Interaction between formulations and three application methods	cucumber	cucumber Eucalyptus	Cabbage Eucalyptus Namaqualand daisy	cabbage Namaqualand daisy
Formulation *Organism	Interactions between 9 Formulation and three organisms	cucumber Eucalyptus	cucumber	Cabbage Namaqualand daisy	cabbage Namaqualand daisy Eucalyptus
Application *Organism	Interaction between three application methods and organisms	cucumber Namaqualand daisy Eucalyptus	cucumber Namaqualand daisy	Cabbage Eucalyptus	cabbage Eucalyptus
Formulation*Application* Organism	Interaction between 9 formulations and three applications and three organisms			Cabbage Cucumber Eucalyptus Namaqualand daisy	cabbage cucumber Eucalyptus Namaqualand daisy

The interaction of AxB was not significant on plot weight, i.e., none of the combinations of organisms and formulations were better or worse in disease control than others. The activity of the microbes was not affected by the formulations. However, the final interaction AxBxC, was marginally significant. This indicates that the two main effects (organism and formulation) interacted synergistically with the application techniques, resulting in greater levels of disease control than one would expect from the primary effects on their own, or combined with another treatment. On percentage survival, the interactions AxC was significant. This indicates that at least one organism was significantly better or worse in its performance when coupled with one or more application technique. For example, when drenching was coupled with the organism *T. harzianum* KMD a significantly better response in enhancing percentage survival and reducing disease was achieved. The interaction AxB was also significant on percentage survival, suggesting that at least one of the organisms was significantly better or worse in reducing disease when coupled with one or more of the formulations, e.g., For example *T. harzianum* KMD prepared as conidia in powder with oil effectively enhanced percentage survival and reduced disease on Namaquland daisy seedlings. The interaction BxC was not significant suggesting that for each the formulations, irrespective on how they were applied, the same effect was produced. The final interaction AxBxC was not significant.

Eucalyptus seedlings

The interaction of AxB and AxC was significant on plot weight of Eucalyptus seedlings. This means that the three main effects (A: organism, B: formulation, C: application techniques) were all affected by interaction effects, i.e., at least one organism was significantly better or worse in its performance in reducing disease when coupled with one or more of the formulations. For example, *T. harzianum* KMD and *G. virens* MM1 reduced disease and increased plot weight by 17-30% when prepared with chlamydospores in milled oats (Table 4.8). *Trichoderma harzianum* KMD effectively reduced disease when prepared with conidia but was comparable to the controls. *Bacillus subtilis* AW57 did not reduce disease when prepared with washed cells. The interaction of AxC was also significant and this suggests that at least one organism was significantly better or worse in its performance when coupled with one or more of the application techniques, e.g., *T. harzianum* KMD reduced disease when applied with chlamydospores in milled oats using drenching and capping, whilst *G. virens* MM1 reduced disease effectively when applied as chlamydospores in milled oats using drenching and capping.

The interaction BxC was not significant suggesting that for each formulation, irrespective on how it gets applied, the same effect was produced. The final interaction AxBxC was not significant. This indicates that the two main effects (organism and formulation) did not interact synergistically with application techniques and using any organism and any formulation, the result will not be affected by the application technique.

The interactions AxC and BxC, were significant on plot weight of Eucalyptus seedlings. This means that (A: organism, B: formulation, C: application techniques) were all affected by the interaction effects. For the interaction of AxB at least one organism was significantly better or worse in reducing disease when coupled with one or more of the formulations i.e., *T. harzianum* KMD and *G. virens* MM1 enhanced percentage survival and reduced disease when prepared with chlamydospores in milled oats and not with conidia (Table 4.8). For the interaction of AxC, this indicates that at least one organism was significantly better or worse in reducing disease when coupled with one or more application technique i.e., *Trichoderma. harzianum* KMD and *G. virens* MM1 enhanced percentage survival when prepared with chlamydospores in milled oats using drenching and capping (Table 4.8). Seed treatment gave a poor result in reducing disease. For the interaction of BxC, none of the formulations were significantly different. The final interaction was not significant on percentage survival, which suggested that a similar result would be obtained by any organism and any formulation with the relevant application technique used.

The main effect, organism was significant on percentage survival of cabbage seedlings, plot weight and percentage survival of cucumber seedlings, plot weight of Namaqualand daisy and Eucalyptus seedlings. This result indicates that at least one organism performed better or worse than other organisms. *Bacillus subtilis* AW57 effectively enhanced percentage survival and reduced disease on cabbage seedlings better than other organism. This could be related to the bacteria producing endospores tolerant to heat and desiccation. *Trichoderma harzianum* KMD and *G. virens* MM1 also reduced disease effectively. Chlamydospores produced by the biocontrol fungi effectively reduced disease. This result was expected as these propagules are durable and have a longer shelf-life (Burges, 1998). However, chlamydospores performed better in the biocontrol of *Pythium* spp. but not in growth stimulation. This could be related to the presence of organic acids

with *Pythium* sp. inoculation resulting in acidic conditions in the media, composted pine bark. The acid conditions cause chlamydospores to be activated and hence germinate and antagonize the pathogen (Burges, 1998).

The main effect, organisms, was not significant on plot weight of cabbage seedlings, percentage survival of Namaqualand daisy and Eucalyptus seedlings suggesting that all organisms performed equally well or poorly irrespective of other main effects. No one treatment differed from any other treatments. However, significant differences may still be present in the interaction effects.

All organisms formulated, effectively reduced damping-off caused by *Pythium* spp. in cucumber, Namaquland daisy and Eucalyptus seedlings. The development of biocontrol agents requires the elucidation of characteristics such as

(a) mechanism of action

(b) optimum rate(s) and

(c) concentration of antagonists applied to target areas

(d) carrier or preparation substrate

(e) methods of application (Habbar *et al.*, 1992).

Organisms tested in this study effectively reduced damping-off on most crops by either one of the characteristics mentioned above. *Trichoderma* and *Gliocladium* have antagonistic properties which involve parasitism and lysis of pathogens fungi and /or competition for limiting factors in the rhizosphere, mainly iron and carbon (Sivan & Chet, 1986). Concentrations of *B. subtilis* AW57 used for seed treatment and drench ranged from log 8-9 (10^8-10^9) c. f. u's/ml. These concentrations may be high enough to colonize plant roots and overcome pathogen activity.

Formulation, was significant on plot weight of cabbage seedlings, and plot weight and percentage survival of cucumber, Namaqualand daisy and Eucalyptus seedlings. This suggests that at least one formulation performed better or worse than others, e.g., conidial formulations of *T. harzianum* KMD and *G. virens* MM1 effectively enhanced percentage survival of cucumber seedlings better than chlamydospore formulations. This is possibly due to the presence of the oil component in formulations that gave a greater protection from environmental stresses (Burges, 1998). A similar result was obtained on percentage survival and plot weight of Namaqualand daisy seedlings. However, the converse of these

results occurred in Eucalyptus seedlings. This main effect was not significant on percentage survival of cabbage seedlings suggesting that all formulations performed equally well or poorly irrespective of the other main effects. Formulations of chlamydospores in milled oats effectively reduced disease but did not enhance growth stimulation. This could be related to the presence of milled oats serving as a food base for antagonists.

The development of practical and efficient delivery systems for biocontrol agents is equally as important as the identification of superior antagonists. Delivery systems must be economical, easy to use, and adaptable. Coated seeds with formulations have been widely tested as a potential delivery system for antagonists (Mihuta-Grimm & Rowe, 1986). Application techniques were significant on percentage survival of cabbage seedlings, plot weight and percentage survival of cucumber, percentage survival of Namqualand daisy and Eucalyptus seedlings. This suggests that at least one application technique resulted in a better or worse response in reducing disease than other application techniques. On cabbage seedlings, capping and seed treatment performed best in reducing disease. Drenching, on the other hand, consistently did not reduce disease on cabbage seedlings. However, in most instances drenching performed well when coupled with the formulation containing kaolin and those formulations prepared with the experimental compound. This could be related to the addition of kaolin or such substances, which improve the suspension of particles in the formulated product. Kaolin and like substances also assist in acting as an adhesive for spores to attach to the seed. On cucumber seedlings, drenching performed the least in enhancing percentage survival and plot weight of cucumber seedlings. On Namaqualand daisy seedlings capping performed the worst in reducing disease and plot weight and percentage survival when coupled with the formulations of *G. virens* MM1 in milled oats.

4.4.3 Biocontrol of damping-off caused by *R. solani* by *T. harzianum* KMD, *G. virens* MM1 and *B. subtilis* AW57.

These trials have been summarized in Table 4.16. This table reflects the significant and non-significant results incorporating the factor of crop and the interaction of main effects from ANOVA's. The controls (water only) and the disease control (*R. solani*) were effective in identifying the level of disease control.

Explanation of the interaction effects, shown in Table 4.13 and P-values of the interaction effects shown in Table 4.9-4.12, reveal that the first three interactions AxB, AxC and BxC varied for plot weight and percentage survival on all crops tested.

Cabbage seedlings

On plot weight and percentage survival, highly significant differences were recorded for, AxB, AxC and BxC. This means that the three main effects (A: organism, B: formulation, C: application techniques) were all affected by the interaction effects, i.e., when treatments were combined in pairs, a positive or negative result was produced in each paired combination. These interactions suggest that at least one organism (*T. harzianum* KMD, *G. virens* MM1 and *B. subtilis* AW57) was significantly better or worse in reducing disease caused by *R. solani* when coupled with one or more of the application techniques or formulations. For example *G. virens* MM1 did not reduce disease when applied as a drench. These interactions also suggest that at least one of the formulations was significantly better or worse depending on how it is applied. The converse is that one of the applications worked well or badly with only one of the formulations. This was evident in Table 4.9 were the chlamydospore formulations of *G. virens* MM1 and *T. harzianum* KMD were effective in reducing disease when applied as capping and not as a seed treatment or drench. *Trichoderma harzianum* KMD and *G. virens* MM1 effectively reduced damping-off of *R. solani* when coupled with formulations prepared with conidia in kaolin powder with oil by 1000% when compared to the disease control. Both these organisms were also effective in diseased control when applied using a drench or as a capping. The final interaction, AxBxC was significant for both parameters tested on cabbage seedlings. This suggests that the two main effects (organism and formulation) performed synergistically with the application techniques. Hence a significant result was produced depending on the organism and how it is applied and formulated, e.g., *T. harzianum* KMD prepared with conidia in kaolin powder with oil only effectively reduced disease when coupled with a formulation containing conidia in kaolin powder with oil and applied as a drench.

Cucumber seedlings

The first interactions, AxB, AxC, BxC was highly significant (P=0.0001) on plot weight. This suggests that all main effects were affected by interaction effects, i.e. at least one

organism (*T. harzianum* KMD, *G. virens* MM1 and *B.subtilis* AW57) was significantly better or worse in reducing disease when coupled with one or more of the formulations e.g., *T. harzianum* KMD performed poorly when prepared with chlamydospores in milled oats but performed better when prepared with 10^8 chlamydospores (1000 times more). The BxC interaction indicates that at least one of the formulations was significantly better or worse depending on how it is applied. For example, *T. harzianum* KMD prepared in a commercial formulation and *G. virens* MM1 prepared with chlamydospores in milled oats performed poorly in reducing disease when applied as a capping. The interaction AxC was not significant for plot weight and percentage survival. This indicates that none of the combinations of organisms and applications was better or worse than the others. The activities of organisms were not affected by application techniques. The final interaction AxBxC was significant on both plot weight and percentage survival. This indicates that the two main effects (organism and formulation) interacted synergistically with the different application techniques resulting in higher plot weights and percentage survival than one would expect from the primary effects by their own, or combined with another treatment.

The interaction AxB was significant on percentage survival. This suggests that at least one organism was significantly better or worse in its performance when coupled with one or more of the formulations. *Trichoderma harzianum* KMD performed well in increasing percentage survival prepared with conidia in powder with oil. The interactions BxC and AxC were not significant on percentage survival of cucumber seedlings. This indicates

Table 4.16 A summary of results in Tables 4.9-4.12

		Significant		Non- significant	
Effects and interactions	**Treatments/ comparisons**	**Plot weight**	**% Survival**	**Plot weight**	**% Survival**
Formulations	9 formulations vs Controls	cabbage Eucalyptus	cabbage Namaqualand daisy Eucalyptus	Cucumber Namaqualand daisy	cucumber
Application	Differences in three application methods and controls	cabbage cucumber Namaqualand daisy Eucalyptus	cabbage cucumber Namaqualand daisy Eucalyptus		
Organism	Differences between *T. harzianum* KMD, *G. virens*, *B. subtilis* AW57	cabbage Eucalyptus	cabbage cucumber Namaqualand daisy	Cucumber Namaqualand daisy	Eucalyptus
Formulation* application	Interaction between formulations and three application methods	cabbage cucumber Namaqualand daisy Eucalyptus	cabbage Namaqualand daisy Eucalyptus		cucumber
Formulation *Organism	Interactions between 9 Formulation and three organisms	cabbage cucumber Eucalyptus	cabbage cucumber Eucalyptus	Namaqualand daisy	Namaqualand daisy
Application *Organism	Interaction between three application methods and organisms	cabbage Eucalyptus	cabbage Eucalyptus	Cucumber Namaqualand daisy	cucumber Namaqualand daisy
Formulation * Application * organism	Interaction between 9 formulations and three applications and three organisms	cabbage cucumber Eucalyptus	cabbage cucumber Namaqualand daisy Eucalyptus	Namaqualand daisy	

that none of the combinations of organisms and formulation were better or worse than others and the activity of the organism was not affected by application techniques. The BxC interaction suggests that for each of the formulations irrespective on how they were applied, the same effect was produced.

Namaqualand daisy seedlings

The interactions BxC was significant on plot weight and percentage survival. This suggests that at least one of the formulations was significantly better or worse depending on how it is applied, while interactions of AxB and AxC were not significant. This indicates that none of the test combinations of organisms and formulations were better or worse than others. The activities of the organisms were not affected by formulations and application techniques. The final interaction AxBxC, was significant for percentage survival and not for plot weight. The significance of the interaction means, depending on the organism and how it gets applied, a significant result was produced. This interaction was found not significant on plot weight. This suggested that using any organism and any formulation, the result will not be affected by the application technique.

Eucalyptus seedlings

All three interactions AxB, AxC and BxC were significant on plot weight and percentage survival. This suggests that at least one organism was significantly better or worse in it performance when coupled with one or more of the application techniques and formulations. This also indicates that at least one of the formulations was significantly better or worse depending on how it is applied. *Trichoderma harzianum* KMD prepared with conidia in kaolin powder with oil, effectively reduced disease by 300% when compared to disease controls. The final interaction AxBxC was significant for both main effects (organism and formulation) performed synergistically in increasing plant yield when using any application technique, e.g., *T. harzianum* KMD performed well when formulated with 10^5 chlamydospores in milled oats applied as a capping.

Organism was significant on plot weight and percentage survival of cabbage seedlings. This suggests that at least one organism performed better or worse in effectively reducing disease than other organisms. *Trichoderma harzianum* KMD and *G. virens* MM1 performed best in reducing disease compared to *B. subtilis* AW57. *Trichoderma*

harzianum KMD and *G. virens* MM1 reduced disease and increased plot weight by 1000% and percentage survival by 17-18% when compared to the disease controls. Organism, was highly significant on percentage survival of cucumber seedlings. A similar trend was evident, were *T. harzianum* KMD and *G. virens* MM1 enhanced percentage survival more than *B. subtilis* AW57 by 100-200%. This main effect was not significant on plot weight of cucumber seedlings. This suggests that all organisms performed equally well in reducing disease irrespective of other main effects. No one treatment differed from any other over all treatments.

Similar significant differences of interactions were recorded on plot weight and percentage survival of Namaqualand seedlings. However *B. subtilis* AW57 performed best in reducing disease by 600% compared to other biocontrol agents. On plot weight of Eucalyptus seedlings, the organism effect was significantly different suggesting that at least one organism performed the best in reducing disease compared to the other biocontrol agents. For example, *T. harzianum* KMD effectively reduced disease better than *B.subtilis* AW57 and *G.virens* MM1 by 300%. On plot weight of Eucalyptus seedlings, organism was not significant suggesting that all organisms performed equally well irrespective of the other main effects. All biocontrol organisms responded differently in reducing disease of damping-off caused by *Pythium* spp. However *B.subtilis* AW57 was inferior to the other biocontrol organisms as *B. subtilis* were not formulated well and were applied as a water based slurry. These organisms were not formulated with a carrier for protection and desiccation.

According to scientific reports, *Trichoderma* and *Gliocladium* spp. can effectively control the following fungi: *S. rolfsii, R. solani, Pythium* spp., *Fusarium* spp., *Aspergillus niger* (Elad *et al.*, 1983, Chet & Henis, 1988) *Sclerotium cepivorum* (De Oliveira *et al.*, 1984). *Trichoderma and Gliocladium* successfully reduced disease when loaded into the root zone of seedlings in greenhouses. *Trichoderma* and *Gliocladium* thus forms a "protective layer". It was evident that *Trichoderma* and *Gliocladium* directly affects plants and can lie in their roots (Chet, 1987). Bacteria such as *B. subtilis* are popular for seed treatment but in these trials inconsistent results were achieved in reducing disease. This could be related to the fact that these organisms were not formulated with a suitable carrier to prevent desiccation and fungistasis in composted pine bark.

Formulations were significant for both plot weight and percentage survival of cabbage seedlings. This suggests that at least one formulation performed better or worse in reducing disease. Formulations prepared with *T. harzianum* KMD and *G. virens* MM1 with conidia in kaolin powder with oil performed the best in effectively reducing disease Table 4.9. This could be related to kaolin acting as an adhesive, which encourages seed treatment to be the best application technique. Formulations prepared with chlamydospores performed the worst in reducing disease as a result of the lack of germination. However, these formulations will be advantageous as the food base (milled oats), which will enable the antagonist to sustain itself much longer in the growing medium.

This main effect was also significant for plot weight and percentage survival of cucumber seedlings. *Trichoderma harzianum* KMD and *G. virens* MM1 prepared with conidia in kaolin powder with oil performed best in reducing disease while formulations prepared with 10^5 chlamydospores performed worst. Formulations with a higher inoculum of chlamydospores effectively reduced disease more effectively than those formulations prepared with a lower level of inoculum. On Namaqualand daisy seedlings, the second main effect, formulations were significantly different for both plot weight and percentage survival. Formulations containing conidia in an experimental compound and conidia in kaolin powder with oil performed best in reducing damping–off. On Eucalyptus seedlings, both parameters were significant for the difference of formulations. All formulations prepared with *T. harzianum* KMD conidia effectively reduced disease and performed best compared to other formulations.

The previous use of *Trichoderma* and *Gliocladium* for control of several diseases caused by soil-borne pathogens is well documented (Papavizas, 1985). These antagonists have been described as destructive mycoparasites as agents for controlling *Pythium* spp. and *R. solani*. It is also well documented that effectiveness of a biocontrol fungus depends on the type of propagule in the formulation. Formulations containing a low inoculum of chlamydospores of *T. harzianum* KMD were observed to be inferior than other formulations containing conidia (Table 4.9). This effect could be due to the amount of viable chlamydospores present in milled oats. This effect was evident on all crops. Another reason for the lack of disease control of these formulations was possibly due to milled oats which could have served as a food source for the fast growing pathogen, *R.*

solani, hence increasing inoculum potential and increasing disease (Mihuta-Grimm & Rowe, 1986).

Application techniques, were significant on both plot weight and percentage survival of cabbage seedlings. A similar result was noted on plot weight and percentage survival of cucumber seedlings, Namaqualand daisy and Eucalyptus seedlings.This suggests that at least one application technique resulted in a better or worse response in reducing disease than other application techniques. Seed treatment followed by drenching performed the best in reducing disease by 1000% and will be more cost-effective to the manufacturer. Seed treatment allows only a small amount of the formulation to be applied on the seed, causing effective disease control in the rhizosphere. Seed treatment, is an inexpensive, but effective way of delivering the antagonists to the pathogen, resulting in more profit for manufacturers. Coating seed with conidia has been widely tested as a potential delivery system for antagonists (Harman *et al.*, 1980; Chu & Wu, 1988; Elad *et al.*, 1981; Papavizas *et al.*, 1982; Ruppel *et al.*, 1983).

However, competition of soil biota is a great disadvantage of seed treatment. Koalin has no nutrients and thus cannot give the inoculants a good start, but may serve as a protective additive in the formulation. Another reason why *T. harzianum* KMD prepared with conidia in kaolin powder with oil has been successful, is because the presence of oil may have been used to regulate water availability in the formulation. In addition it may act as a binder to the seed and serve as nutrient for which it may be optimized for the chosen inoculant. It is also cost effective and conventional for easy use, compared to drenching and capping which require large amounts of formulation to be applied in the growing media, which could be prone to fungistasis by other micro-organisms in the rhizosphere. However these application techniques were not consistent for all formulations tested.

Variations in application techniques may be a result of fungal spores or bacterial cells applied to the composted pine bark surface and on seed with drenches, with or without a minimal wetter. Hence spores will be mainly trapped in the upper 1-5cm by filtration and absorptive powers of composted pine bark (Hirte *et al.*, 1994). Seed treatments also varied for each formulation for each crop. This could be related to the viable inoculum present on seed.

Variation of damping-off was observed for all crops and differences in degree of damping off were noted. In some crops where seedling stand was reduced by damping–off, the surviving plants may compensate through increased growth and yield due to the reduced competition. However, if some of the remaining seedlings have damaged root systems and stem damping-off they may develop into unthrifty plants. These unthrifty plants compete with thrifty, healthy free plants, thus lowering the ability of the latter to compensate. Suppression of *R. solani* by competition alone seems unlikely since plot weight and percentage survival were observed to be greater than the controls (Zadoks & Schein, 1979).

An effect of dose was shown by dose –response curves for the relationship between dose of formulations on plant growth. It was estimated that about 1-5 g/l was sufficient to effectively increase plot weight and percentage survival under greenhouse conditions. As dosage increased plot weight and percentage survival was correlated positively or negatively with the dose of some formulations applied.

Dosage rates of *T. harzianum* KMD and *G. virens* MM1 have differed to their formulation. The application rate at 10g/l was far too high and resulted in stunted growth of most seedlings. Massive overgrowth of *T. harzianum* KMD on seeds was observed (like a thick green carpet) within two days in the germination room at 25°C. It was noticed that percentage survival was low at 10g/l and plot weight was lower at this dose. This could have been caused by low levels of *T. harzianum* KMD parasitism of e seed (Askew, 1991).

A *Trichoderma viride* Rifai Strain causes fruit rot of citrus in South Africa (Eckert & Brown, 1988). We speculate that *Trichoderma* becomes a facultative parasite at high-extreme, inoculum potentials or it could be related to the mechanism of over systemic resistance that plays a role by the plant itself. It attacks both the seed and roots causing low stands and stunting. It is also possible that toxins produced by *Trichoderma* spp. could affect plant growth and disease control (Askew, 1991). Other workers have also shown that *Trichoderma* spp. Strains may be unstable and may lose biological activity. For example, in culture, the production of peptide antibiotics active against *Micrococcus luteus* declines rapidly (Brewer *et al.*, 1987) and the balance of secondary metabolites produced by *Trichoderma* spp. can vary (Taylor, 1986; Ghizalberti & Sivasithamparam,

1991). This type of effect may be involved in the switch from growth promotion to toxicity exhibited by strains 65 and strain 93, particularly as individual isolates can produce more than one secondary metabolite (Claydon *et al.*, 1987). The substrate used for the production of *Gliocladium virens*, a fungus closely related to *Trichoderma* spp. has also been shown to affect the metabolite "pool" produced (Howell & Stipanovic, 1984).

Variations in the control and the stunting effect with both organisms when applied at different rates were observed. It was also observed that as dose of formulations increased a negative response to plant growth was recorded. We suggest that the relative colonisation of seedling mix (composted pine bark) by each organism could be responsible for this phenomenon. Chet & Baker (1980) found that the minimal effective amount of *Trichoderma* was about $1x10^6$ c.f.u.'s/g soil. Thus, the crucial factor is not the application rate but rather the propagule concentration of the fungus (c.f.u.'s) resulting from its colonization of the media. An isolate that colonizes the media well and sporulates vigorously should have sufficient c.f.u.'s for effective disease control at even 1g/l. Higher application rates of this isolate would increase the inoculum levels excessively, thereby resulting in the stunted growth and low percentage survival rates observed. Conversely, isolates that resulted in relatively poor colonization of the growing media would only have sufficient cfu's for effective disease control at the higher application rates.

Controls of Pelgel® and Nutristart appear to have nutritive properties, which increased plot weight and percentage survival in both growth stimulation trials and biocontrol of *Pythium* spp. and *R. solani* but varied in the crops tested. In disease control trials these controls were severely affected as they served as a food source for the pathogens. We suggest that Nutristart not only acted as a nutrient boost for growth stimulation but acted as either a fertilizer that boosts the micro-organisms already present in the non-sterile composted pine bark, or as a food source for pathogens present in the growth medium.

Overall the data in this chapter indicate that control of *R. solani* and *Pythium* spp. with *T. harzianum* KMD, *G.virens* MM1 and *B. subtilis* AW57 under greenhouse conditions was variable. This was to be expected as for biological entities are influenced by many environmental, biological, and physical factors.

Under greenhouse conditions, all organisms inconsistently but often significantly increased yield and decreased disease. Improved methods of applying these biocontrol agents may produce better results. However, in this study the greenhouse trial results obtained with four plant species and two pathogens (which frequently co-exist), point to the high potential and reproducibility of biological control. All preparations of biocontrol agents improved growth (Table 4.1-4.4), which is an additional advantage over pesticides which frequently cause phytotoxicity.

The results also demonstrated that products using a wide variety of bases as carriers can be successfully formulated with conidia of biocontrol fungi. These formulations can be prepared without employing aseptic conditions, requiring minimal incubation to constrain contamination. They can be applied readily to planting media at low doses at the time of planting and can be easily used by farmers with low technology skills.

This study suggests that there is a need for *T. harzianum* KMD and *G. virens* MM1 to be tested under different environmental conditions using different inoculum delivery methods before they can be generally classed as plant growth promoting fungi or potential biocontrol agents. All biocontrol formulations effectively enhanced growth and biocontrol activity but varied for application method, formulation and crops evaluated. Variation in responses could be due to the variation in environmental, physical and biological factors.

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CHAPTER 5

COMPATIBILITY OF THE BIOCONTROL AGENT *TRICHODERMA HARZIANUM* KMD WITH SELECTED FUNGICIDES ON A VARIETY OF CROPS UNDER GREENHOUSE CONDITIONS

J. Omarjee[1], M.D. Laing[1] and C.H. Hunter[2]

Disciplines of Plant Pathology[1], and Microbiology[2], SAES,

University of Natal, Private Bag X01, Scottsville, Pietermaritzburg, South Africa

Trichoderma harzianum KMD is an effective biocontrol agent of *Pythium* sp. and *Rhizoctonia solani.* For this biocontrol agent to be integrated into an existing disease management programme, it must be compatible with commonly used fungicides. The sensitivity of *T. harzianum* KMD to a range of rates of two fungicides, Benlate® and Previcur®, was determined in an *in vitro* assay. Results indicated that *T. harzianum* KMD was least sensitive to both fungicides after 15 days of incubation at 25° C. Compatible mutants that resulted from the assay did not sporulate. Greenhouse trials on cabbage (*Brassica oleracea* var. *capitata* L.), cucumber (*Cucumis sativa* L.), Namaqualand daisy (*Dimorphotheca hibrida* L.), Eucalyptus (*Eucalyptus macarthuri* Deane and Maid) and tomato (*Lycopersicon esculentum* Mill.) confirmed that *T. harzianum* KMD achieved better growth and biocontrol activity against *R.solani* and *Pythium* sp. when applied without fungicide to infested and non-infested composted pine bark. *Trichoderma harzianum* KMD was compatible with fungicides when seeds were treated with *T. harzianum* KMD prior to planting. In a disease integrated management programme, seed treatment application of *T. harzianum* KMD would be compatible with fungicides for control of damping–off of seedling diseases caused by *R. solani* and *Pythium* sp.

5.1 INTRODUCTION

Integrated pest control is a flexible, multidimensional approach to a range of control components such as biological, cultural, and chemical strategies. These strategies are needed to hold diseases below damaging economic thresholds without damaging the agroecosystem (Andrews, 1983; Papavizas & Lewis, 1988). Different fungicides and soil fumigants are widely used for controlling soil-borne pathogens. As a result of the concern regarding the

toxicity of these compounds, there is a trend to reduce the amounts applied to soil. One of the most attractive ways of reducing amounts of fungicides is the integration of sublethal doses of chemicals with *Trichoderma* spp. (Chet, 1987). Davet *et al.* (1981) found that benomyl (Benlate ®) inhibited *Trichoderma* growth while Thiram® enhanced it. Papvizas *et al.* (1982) and Baker *et al.* (1984) found mutants resistant to benomyl and captan. The results that there is indicate the possibility of combining some appropriate chemicals with biocontrol agents. A positive effect of integrated control was also observed with *Pythium* spp. Application of a *Trichoderma harzianum* preparation combined with the fungicide Previcur® as a rooting mixture infested with *Pythium aphanidermatum* resulted in a synergistic effect, reducing disease incidence in gypsophila cuttings and was better than either treatment alone (Sivan *et al.* 1984). These results were similar to those obtained with cucumber (*Cucumis sativa* L.) grown in *Pythium* infested soil (Sivan *et al.*, 1984).

The impetus to use biocontrol agents in combination with reduced amounts of fungicides stems from the desire to reduce the use of fungicides and from developments of strains of biocontrol fungi resistant to certain fungicides (Papavizas, 1987). Integrating biological and chemical control seems a promising way to control pathogens with minimal interference with the biological equilibrium (Papavizas, 1973; Henis & Chet, 1975; Baker & Cook, 1982). Curl *et al.* (1977) obtained only a slight additive benefit to biological control of *R. solani* by adding pentochloronitrobenzene (PCNB) at doses of 1 and 10mg/g soil together with *Trichoderma* in sterilised soil (Hadar *et al.*1979). However, when PCNB was applied in small non-effective doses (1-2 ug/kg) to soil along together with a *Trichoderma* preparation (2g/kg) that the incidence of eggplant disease caused by *R. solani* declined from 40-13%, while *T. harzianum* alone, reduced disease incidence to only 26%. Similarly, Chet *et al.* (1979) reported a synergistic effect resulting from the interaction between *T. harzianum* and sublethal doses of PCNB when applied against *Sclerotinia rolfsii* Sacc. on peanuts (*Phaseolus vulgaris* L.).

The objectives of this study were to study the compatibity of the biocontrol agent *T. harzianum* KMD in a commercial formulation to determine its sensitivity to two fungicides, Benlate® and Previcur®, on a variety of crops, *in vitro* and greenhouse assays using three application methods as part of an integrated disease management programme.

5.2 MATERIALS AND METHODS

5.2.1 Formulation

The commercial formulation was obtained from Dr Mike Morris[1], Plant Health Products. The number of conidia per gram of formulation was 10^8 spores per gram of formulation.

5.2.2 Fungicides

One gram of Benlate®[2] and 1.2 ml of Previcur®[3] were added to one litre of tap water and shaken to obtain a homogenous mixture. Approximately 2.6 ml of the fungicide mixture was added to each planting cell of Speedling® 24 trays. These fungicides are compatable with each other and can be mixed as recommended by manufacturers.

5.2.3 Pathogens

Cultures of *R. solani* and *Pythium sp.* that cause damping-off of greenhouse crops were obtained from C. Clark[4]. The isolates were maintained on V8 medium (Appendix A). Plates containing V8 medium were then inoculated with a 10mm x 10mm block of agar infested with the pathogens. These plates were then incubated at room temperature for two days, before adding them to the potting medium.

5.2.4 *In vitro* assays testing the compatibity of *T. harzianum* KMD with varying doses of fungicides

5.2.4.1 *Organism*

The organism, *T. harzianum* KMD, was originally isolated from soil obtained from Tala Valley, KwaZulu-Natal, South Africa and was formulated to produce a commercial product due to its potential biocontrol activity (Machaba, 1998). For this assay, *T. harzianum* KMD was isolated directly from the formulation by plating out onto V8 medium (Appendix A) and incubating at 25° C for two days. The formulation was initially activated with 0.5M HC HCl before plating out, to prevent contamination by bacteria.

5.2.4.2 *Inoculation*

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[1] Dr Mike Morris, Plant Health Products, P.O.Box 207, Nottingham Road, South Africa.
[2] El du Pont de Nemours & Co. Wilmington, Delaware. 19898. U.S.A
[3] AgrEvo South Africa (Pty) Ltd, Reg No. 93/06650/07, P.O.Box 6065, Halfway House 1685, South Africa
[4] C. Clark, Disciplines of Plant Pathology, and Microbiology, School of Applied and Environmental Science, University of Natal, Scottsville, Private Bag x01, Pietermaritzburg, South Africa.

Agar plugs (10mm x 10mm) infested with *T. harzianum* KMD, *R. solani* and *Pythium* sp. were then placed onto V8 medium plates containing varying doses of 0.0025, 0.05, 0.1, and 1 gram w/v of Benlate® and 0.0025, 0.25, 0.1, 1.2 ml of Previcur®, respectively. Plates were incubated at 25°C for a period of 5 - 15 days and rated at two day intervals. Ratings were recorded by measuring the size of colonies at varying doses.

5.2.5 Greenhouse assays testing the compatibity of *T. harzianum* KMD on variety of crops

5.2.5.1 *Application methods*

a) Drenching of formulations and fungicides

A fungicide mixture containing 1.2 ml Previcur® and 1g Benlate® in 1 litre of water was dispensed into 2.6ml aliquots in three Speedling® 24 trays containing composted pine bark and were left to leach for three days. One gram of each formulation was added to 1litre of tap water and mixed thoroughly. Three millilitre aliquots of the formulation mixture were dispensed into each cell of three Speedling® 24 trays containing composted pine bark and fungicides. The trays were left to stand for an additional two days to allow the biocontrol agent to establish within the rhizosphere. The respective pathogens in (10mm x 10mm agar plugs) were placed in the composted pine bark and left to stand overnight. Untreated seeds of cabbage (*Brassica oleracea* var. *capitata* L.), cucumber (*Cucumis sativa* L.), Namaqualand daisy (*Dimophotheca hibrida* L.), Eucalyptus (*Eucalyptus macarthuri*) and tomato (*Lycopersicon esculentum* Mill.) were planted in three Speedling ® 24, resulting in 24 seedlings per tray i.e., 72 seedlings per treatment. Seeds were then watered and left to imbibe overnight and then watered again the next day. At the first sign of germination, trays were placed in a greenhouse, and treated under greenhouse conditions.

b) Capping of formulations and inoculation of fungicides

The fungicide mixtures containing Benlate® and Previcur® was dispensed in 2.6ml aliquots into each cell of three Speedling® 24 trays containing a thin layer of composted pine bark.

The trays were allowed to leach for three days. One gram of each formulation was mixed with 1000cm^3 of composted pine bark. The three Speedling ® 24 trays inoculated with the fungicides were then covered with a thin layer of composted pine bark, inoculated with the formulation. Trays were left for two days to allow the biocontrol fungus to establish itself within the rhizosphere. Pathogens on (10mm x 10mm agar plugs) were placed in the composted pine bark for 24 hours. Untreated seeds were planted into three Speedling® 24 trays, containing the fungicide treatments resulting in 24 seedlings per tray i.e., 72 seedlings per treatment. Seeds were then watered and left to imbibe for overnight and watered again the next day. At the first sign of germination, trays were placed in a greenhouse, and treated under greenhouse conditions.

c) Seed treatment with formulation and inoculation of fungicides

Fungicide mixtures containing Benlate® and Previcur® were dispensed in 2.6ml aliquots in each cell of three Speedling® 24 trays containing composted pine bark. Trays were allowed to leach for three days. Pathogens on (10mm x 10mm agar plugs) were inoculated into the treated composted pine bark and allowed to stand overnight.

Four grams of Pelgel®[5] were dissolved in 200ml of distilled water, stirred and allowed to stand for 1h. One gram of the formulation was added to four beakers containing 50ml of sticker, Pelgel®. The mixture was then stirred. An appropriate number of seeds for trials were placed into the mixture and allowed to stand for 1h. Seeds were then removed and airdried. Each treated seed was planted in a cell containing fungicide treated composted pine bark in three Speedling® 24 trays resulting in a total of 24 seeds per tray i.e., 72 seedlings per treatment. Seeds were allowed to imbibe overnight and were watered the next day. At the first sign of germination trays were placed in a greenhouse, and treated under greenhouse conditions.

5.2.6 All treatments

Control treatments:

1. Pathogen only
2. Neither antagonist nor pathogen

[5] LiphaTech, Inc., Milwaukee, Wisconsin, U. S. A

Treatments:

1. *T. harzianum* KMD only
2. Benlate® and Previcur® only
3. *T. harzianum* KMD and Benlate® and Previcur®
4. *T. harzianum* KMD and pathogens
5. Benlate® and Previcur® and pathogens
6. *T. harzianum* KMD and Benlate® and Previcur® and pathogens(*Pythium* sp.; *R.solani*)

Controls were replicated three times with one tray per replicate i.e., 72 seedlings per treatement. All treatments were irrigated three times a day by microjet irrigation. Fertilizer was injected into the irrigation water. Soluble fertilizer [3. 1. 3(38)] Complete from Ocean Agriculture[6], applied at a rate of $1gl^{-1}$ of approximately $33mgl^{-1}$ of P and $100mgl^{-1}$ to give N and K. Temperatures in the greenhouses ranged from 20-30°C.

5.2.7 Crops evaluated

Cabbage (*Brassica oleracea* var. *capitata* L.) cv. Glory of Enkuizen, Seed lot no. Y1011RR.

Cucumber (*Cucumis sativa* L.) cv. Cucumber Ashley, Seed lot no. Ay054YY

Namaqualand daisy (*Dimorphotheca hibrida* L.) cv. Namaqualand daisy, Seed lot no. 884-P14416

Eucalyptus (*Eucalyptus Macarthuri* Deane and Maid) Seed lot no. M1697

Tomato (*Lycopersicon esculentum* Mill.) cv. Heinz 1370, Seed lot no. YE 099YY

Vegetable and flower seeds were obtained from McDonalds Seeds[7] and Eucalyptus seeds were obtained from the Institute of Commercial Forestry Research (ICFR)[8].

5.2.8 Variables of seedlings

All seedlings of all crops were evaluated as follows:

1. Percentage survival after 4-6 weeks for cabbage, cucumber, Namaqualand daisy and tomato and 10 weeks for Eucalyptus.

2. Plot weight. The dry weight of seedlings was recorded by harvesting all surviving seedlings survived in each tray and measuring the total dry weight. Seedlings were harvested at maturity at the base of the plant and placed in a brown paper bag. Plant material was dried in an oven

[6] Ocean Agriculture, P. O. Box 741, Mulders Drift 1747, South Africa
[7] McDonalds Seeds, 61 Boshoff Street, Box 238, Pietermaritzburg, South Africa.
[8] Institute of Commercial Forestry Research (ICFR), University of Natal, Private Bag X01, Scottsville 3209, South Africa.

at 55°C for two days. After drying, the contents of the bag were weighed and the plot weight calculated.

3. Plant height was measured as the distance (mm) from the base of the plant to the extended longest leaf after four weeks of growth.

5.2.9 Statistical Analysis

Experiments were conducted once, with three replicates per treatment. The treatments were arranged in a randomized complete block design. Analysis of data was performed by Analysis of Variance (ANOVA) with a factorial treatment structure and interactions. Treatment means were separated by the Students Newman Keuls test. Statistical analyses were conducted using the general linear model procedure of SAS Version 6.08 (SAS,1987).

5.3 RESULTS

5.3.1 *In vitro* assays testing the compatibility of *T. harzianum* KMD with fungicides

When *T. harzianum* KMD was grown on non fungicidal-containing media, growth and sporulation was observed after an incubation period of 48h. Growth of the biocontrol fungus on fungicidal containing media occurred after 15 days when treated with all doses of the fungicide Benlate® (Table 5.1) and after 10-15 days for doses of Previcur®. The resulting growth of *T. harzianum* KMD after 10-15 days proved that *T. harzianum* KMD may be compatible with the fungicides. However, growth of *T. harzianum* KMD after this period failed to sporulate, even when induced by means of UV light. These colonies were slow growing and white in colour. The test pathogens, *Pythium* sp. and and *R. solani* were effectively killed by all doses of the fungicides.

Table 5.1. *In vitro* assays of commercial product, *T. harzianum* KMD, illustrating the compatibity of the biocontrol agent with selected fungicides, Benlate® and Previcur®, at varying doses

Organism	Fungicide	Dose	Incubation period @ 25°C (Days)		
			5	10	15
T. harzianum KMD	Benlate®	0	++	++	++
T. harzianum KMD	Benlate®	0.0025g	0	0	+
T. harzianum KMD	Benlate®	0.05g	0	0	+
T. harzianum KMD	Benlate®	0.1g	0	0	+
T. harzianum KMD	Benlate®	1g	0	0	+
T. harzianum KMD	Previcur®	0	++	++	++
T. harzianum KMD	Previcur®	0.0025ml	0	+	++
T. harzianum KMD	Previcur®	0.05ml	0	+	++
T. harzianum KMD	Previcur®	0.1ml	0	+	++
T. harzianum KMD	Previcur®	1.2ml	0	0	+

0 -no growth

+ - Poor growth (colonies less than 20mm in diameter)

++ - Good growth (colonies greater than 20 mm in diameter)

This experiment was carried out three times with each plate replicated three times and means were calculated from three trials

5.3.2 Effect of a commercial formulation, with selected fungicides on damping-off caused by *Pythium* sp.

Cabbage seedlings

Significant differences between treatments were observed for all parameters tested (Table 5.2 and Figure 5.1, 5.2, 5.3). The commercial formulation containing *T. harzianum* KMD increased plot weight when applied to non-infested composted pine bark by 51-78% when compared to the disease control (*Pythium* sp. only) and were comparable to the control (water only). The combination of fungicides with the formulated product, *T. harzianum* KMD applied to non-infested composted pine bark, resulted in an increase in plot weight by 26-162% when compared to the diseased control (*Pythium* sp. only).

Treatments applied with the commercial formulation, *T. harzianum* KMD to *Pythium* sp. infested composted pine bark reduced disease and further enhanced plot weight by 83-97% when compared to disease control (*Pythium* sp. only). The combination of the fungicides applied with the commercial formulation to *Pythium* infested composted pine bark resulted in

poor plot weights when compared to disease control. Treatments with fungicides alone had no adverse effects on plants and gave good plot weights but were not comparable to the control (water only). Fungicides applied in *Pythium* infested composted pine bark reduced disease and enhanced plot weight by 43% when compared to *Pythium* infested controls. Most treatments with the commercial formulation only, reduced disease and produced plot weights that were greater than those with the addition of fungicides only.

All treatments with the commercial product and the combination of fungicides recorded percentage survival rates that were comparable to the control (water only). Disease infested controls recorded poor survival rates indicating that *Pythium* sp. was pathogenic.

When *T. harzianum* KMD was applied with fungicides to *Pythium* sp., infested composted pine bark, plant height was consistently significant when compared to all control. Plot weight and percentage survival was not significant when compared to the *Pythium* sp. infested control. This suggests that the treatment of *T. harzianum* KMD applied with fungicides to *Pythium* sp. infested composted pine bark performed poorly for plot weight and percentage survival but increased plant height. This result could be related to the high cellulose activity of Benlate® and Previcur® resistant mutants of *T. harzianum* KMD. This characteristic may due to increased competitive saprophytic activity of the mutants so that they can utilize the cellulose in the mucigel of roots for a substrate resulting in the production of toxins or hormones toxic to the plant itself (Hornby, 1990). Seed treatment was the best application of *T.harzianum* KMD. This is due to the fact that seed treatment applies a high inoculum were it needs to germinate on the emerging root and it allows the fungus to largely escape long term exposure to the applied fungicides.

Table 5.2 Effect of a commercial formulation of *Trichoderma harzianum* KMD, with fungicides Benlate ® and Previcur® on damping-off caused by *Pythium* sp. of cabbage after four weeks.

Treatments	Application	Plot weight (g)	% Control 1 (*Pythium* sp. only)	% Survival	%Control 1 (*Pythium* sp.)	Plant height (mm)	% Control 1 (*Pythium* sp. only)
Trichoderma harzianum	Drenching	4.63 ab	178.84	79.16 a	143	154.a	192
Trichoderma harzianum	Capping	3.91 b	151.04	80.55 a	145	149 b	186
Trichoderma harzianum	Seed treatment	4.56 ab	176.06	76.39 a	138	139 b	173
Trichoderma harzianum + Benlate® + Previcur®	Drenching	6.79 a	262.24	81.94 a	148	149 b	186
Trichoderma harzianum + Benlate® + Previcur®	Capping	3.29 b	126.95	83.33 a	150	138 b	172
Trichoderma harzianum + Benlate® + Previcur®	Seed treatment	4.34 ab	167.72	84.70 a	152	151 a	188
Trichoderma harzianum + *Pythium* sp.	Drenching	4.80 ab	185.33	86.00 a	155	130 b	162
Trichoderma harzianum + *Pythium* sp.	Capping	4.75 ab	183.47	87.00 a	157	145 b	181
Trichoderma harzianum + *Pythium* sp.	Seed treatment	5.11 a	197.37	92.00 a	166	152 a	189
Trichoderma harzianum + Benlate® + Previcur®+ *Pythium* sp.	Drenching	2.33 b	89.88	77.78 a	140	66.6 d	83
Trichoderma harzianum + Benlate® + Previcur®+ *Pythium* sp.	Capping	1.68 b	64.86	83.33 a	150	93.3 c	116
Trichoderma harzianum + Benlate® + Previcur®+*Pythium* sp.	Seed treatment	3.43 b	132.51	80.55 a	145	11.3.3 b	141
Control 1 *Pythium* sp. only	Nil	2.59 b	100.00	55.55 b	100	80.3 cd	100
Control 2 (water only)	Nil	4.22 ab	163.09	83.33 a	150	144.0 b	179
Control 3 Benlate® + Previcur®+ *Pythium* sp.	Drenching	3.72 b	143.63	77.77 a	140	118 b	147
Control 4 Benlate® + Previcur®	Drenching	3.00 b	115.83	75.00 a	135	130b	171
Effects		**P-values**		**P-values**		**P-values**	
Treatments		0.0004***		0.0251**		0.0001***	
Applications		0.126^{NS}		0.325^{NS}		0.0001***	
Treatments*Applications		0.556^{NS}		0.112^{NS}		0.0001***	
		CV% = 22.6		%CV = 12.02		%CV = 8.743	
		MSE= 2.36		MSE =9.09		MSE =0.9214	

1. NS = Not significant; ** = significant at $P \leq 0.05$; *** = significant at $P \leq 0.001$
2. Means with the same letter are not significantly different ($p = 0.05$) according to Student, Newman and Keuls comparison test
3. Drench = 1g of formulation mixed with 1 litre of tap water
4. Capping = 1g of formulation mixed with 1 litre of composted pine bark
5. Seed treatment = application of formulations to seed with Pelgel®
6. Benlate® + Previcur® = 1g of Benlate® and 1.2 ml of Previcur® were added to 1 litre of tap water and thereafter drenched on seed at planting

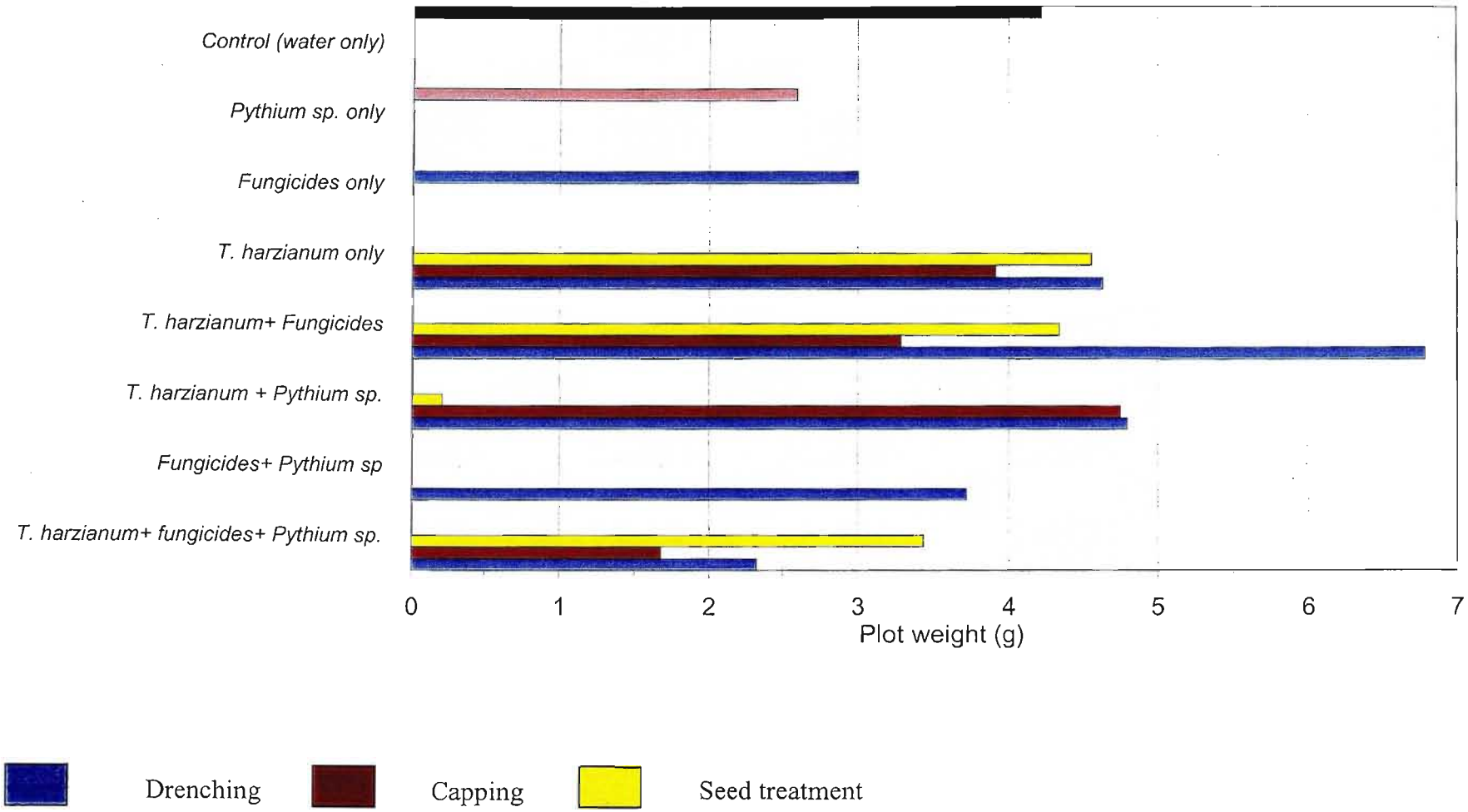

Figure 5.1 Effect of selected fungicides and a commercial formulation containing a biocontrol agent *Trichoderma harzianum* KMD on plot weight using three application methods on cabbage seedlings artificially diseased by *Pythium* sp. after four weeks of growth.

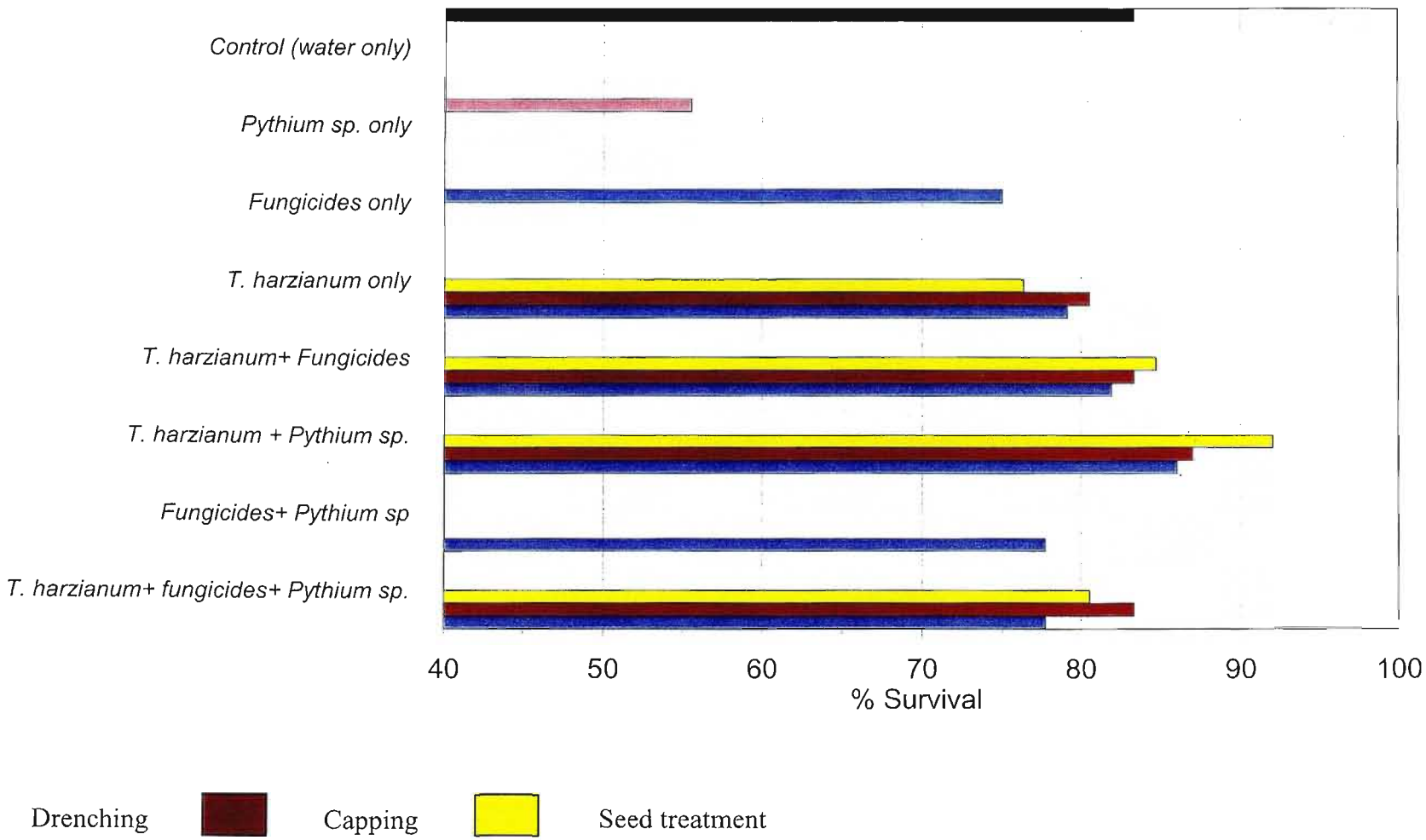

Figure 5.2 Effect of selected fungicides and a commercial formulation containing a biocontrol agent *Trichoderma harzianum* KMD on percentage survival using three application methods on cabbage seedlings artificially diseased by *Pythium* sp. after four weeks of growth.

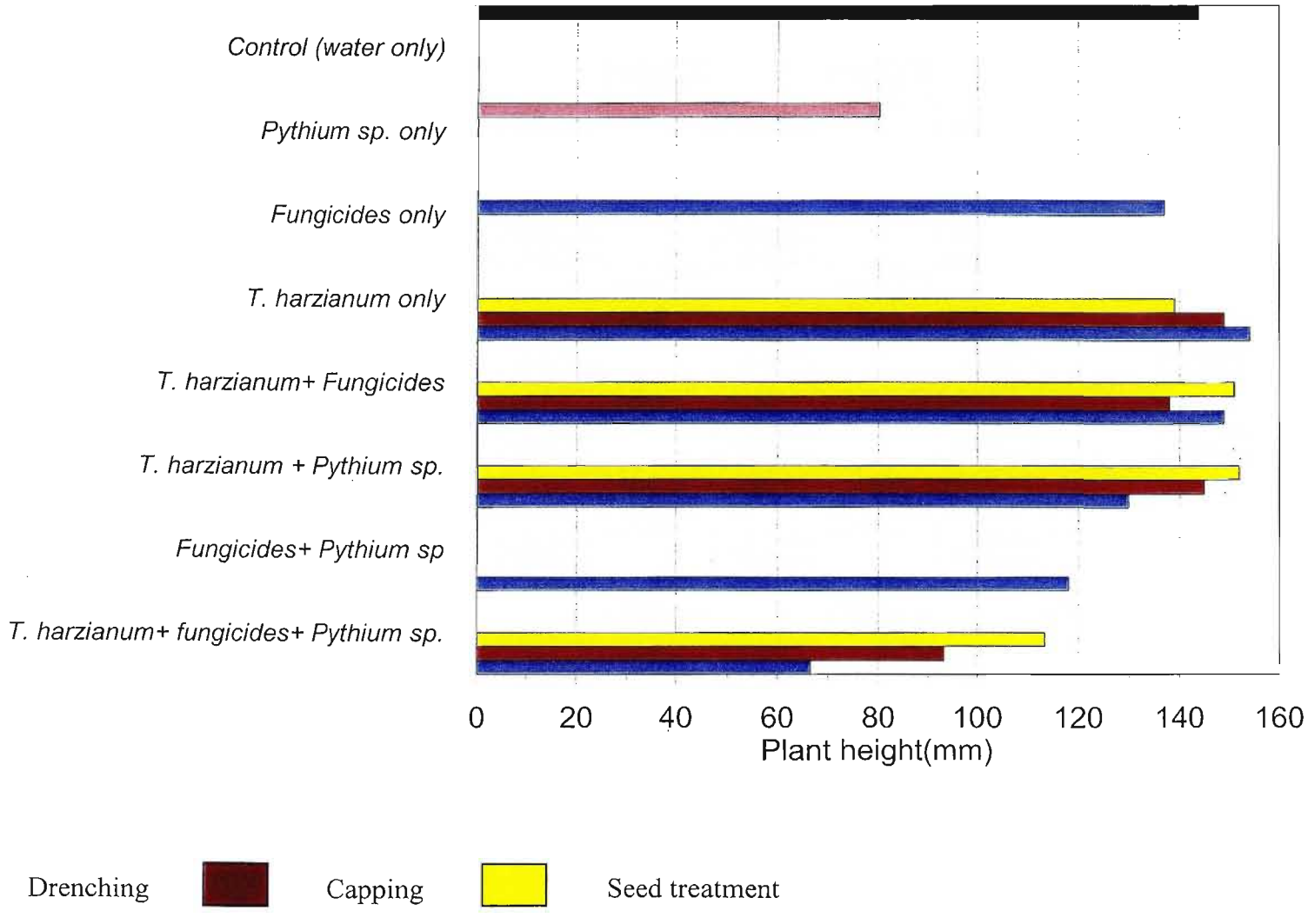

Figure 5.3 Effect of selected fungicides and a commercial formulation containing a biocontrol agent *Trichoderma harzianum* KMD on plant height using three application methods on cabbage seedlings artificially diseased by *Pythium* sp. after four weeks of growth.

Table 5.3 Effect of a commercial formulation of *Trichoderma harzianum* KMD, with fungicides Benlate ® and Previcur® on damping -off caused by *Pythium* sp of cucumber after four weeks

Treatments	**Application**	**Plot weight (g)**	**% Control 1 (*Pythium* sp. only)**	**% Survival**	**% Control 1 (*Pythium* sp. only)**	**Plant height (mm)**	**% Control 1 (*Pythium* spp. only)**
Trichoderma harzianum	Drenching	13.759 a	187.353	98.61a	161	304.7 a	289
Trichoderma harzianum	Capping	12.401 a	168.86	90.27 a	148	357.0 ab	339
Trichoderma harzianum	Seed treatment	13.279 a	180.82	94.43 a	155	431.0 b	409
Trichoderma harzianum + Benlate® + Previcur®	Drenching	13.032 a	177.45	100.0 a	164	312.0c	296
Trichoderma harzianum + Benlate® + Previcur®	Capping	13.224 a	180.07	88.9 a	145	348.0 c	330
Trichoderma harzianum + Benlate® + Previcur®	Seed treatment	14.472 a	197.06	91.6 a	150	516.0 a	490
Trichoderma harzianum + *Pythium* spp.	Drenching	14.160 a	192.81	92.00 a	151	320.0 a	304
Trichoderma harzianum + *Pythium* spp.	Capping	13.728 a	186.93	95.00 a	155	310.0 c	294
Trichoderma harzianum + *Pythium* spp.	Seed treatment	22.560 a	307.19	98.00 a	160	360.0 c	342
Trichoderma harzianum + Benlate® + Previcur®+ *Pythium* spp.	Drenching	11.376 a	154.90	66.67 bc	109	340.6 a	323
Trichoderma harzianum + Benlate® + Previcur®+ *Pythium* spp.	Capping	9.384 a	127.78	70.83 abc	116	303.3 a	288
Trichoderma harzianum + Benlate® + Previcur®+ *Pythium* spp.	Seed treatment	12.600 a	171.57	90.27 a	148	330.0 a	313
Control 1 *Pythium* sp. only	Nil	7.344 a	100.00	61.11 c	100	105.3 b	100
Control 2 (water only)	Nil	11.712 a	159.48	86.11 ab	141	320.0 a	304
Control 3 Benlate® + Previcur®+ *Pythium* spp.	Drenching	11.808 a	160.78	86.10 ab	141	300.0 a	285
Control 4 Benlate® + Previcur®	Drenching	16.944 a	230.72	81.9 a	134	443.0 b	421
Effects		**P-values**		**P-values**		**P-values**	
Treatments		0.4642 ^{NS}		0.0040***		0.0001***	
Applications		0.224 ^{NS}		0.05 **		0.10 ^{NS}	
Treatments*Applications		0.101 ^{NS}		0.036**		0.231 ^{NS}	
		CV%= 35.06		%CV = 11.125		%CV = 8.471	
		MSE=0.47		MSE =8.432		MSE =2.411	

1.NS = Not significant; * *= significant at P ≤ 0.05; *** = significant at P ≤0.001
2.Means with the same letter are not significantly different (p = 0.05) according to Student, Newman and Keuls comparison test
3.Drench = 1g of formulation mixed with 1 litre of tap water
4.Capping = 1g of formulation mixed with 1 litre of composted pine bark
5.Seed treatment = application of formulations to seed with Pelgel®
6.Benlate® + Previcur® = 1g of Benlate® and 1.2 ml of Previcur® were added to 1 litre of tap water and thereafter drenched on seed at planting

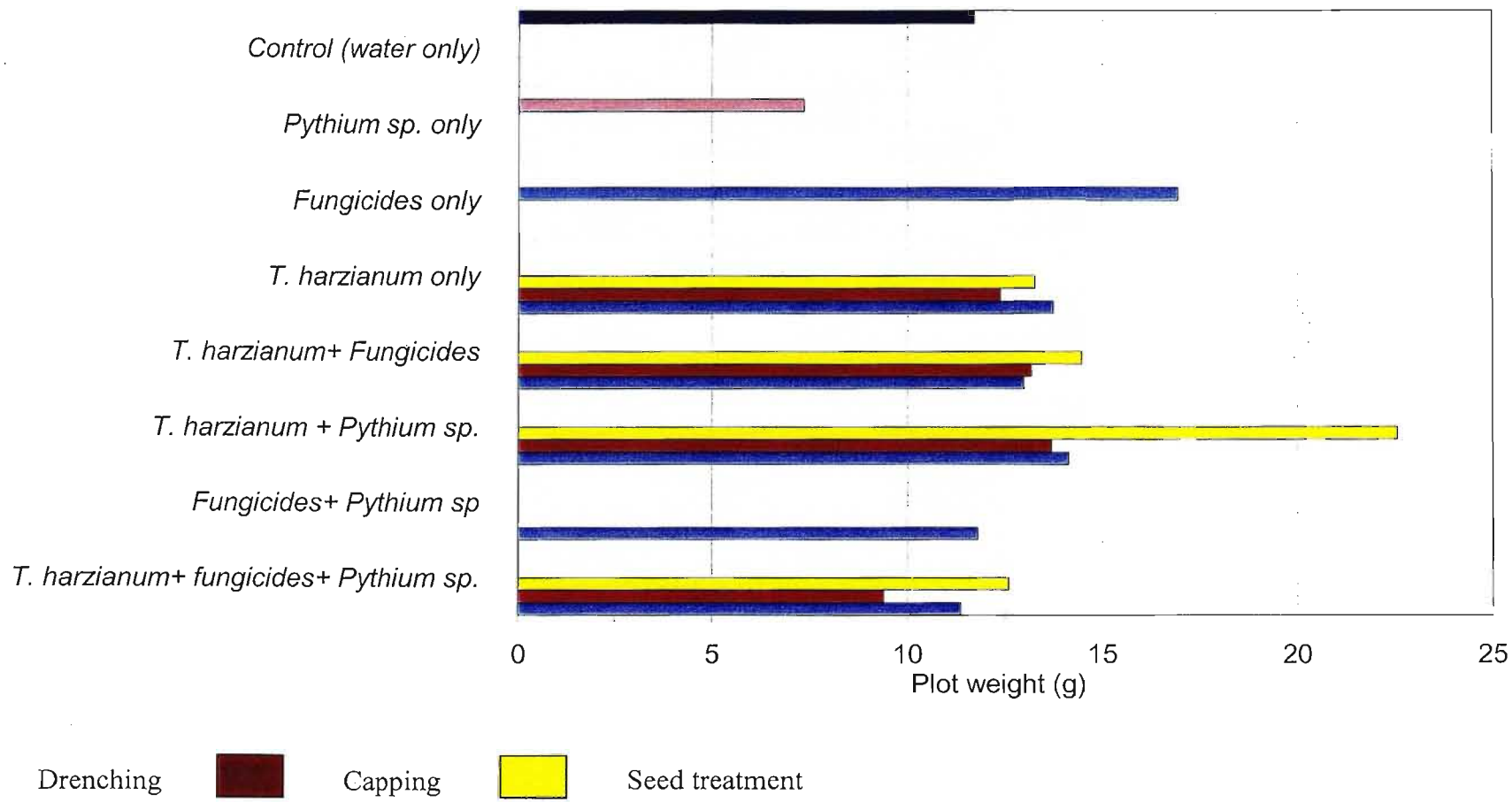

Figure 5.4 Effect of selected fungicides and a commercial formulation containing a biocontrol agent *Trichoderma harzianum* KMD on plot weight using three application methods on cucumber seedlings artificially diseased by *Pythium* sp. after four weeks of growth.

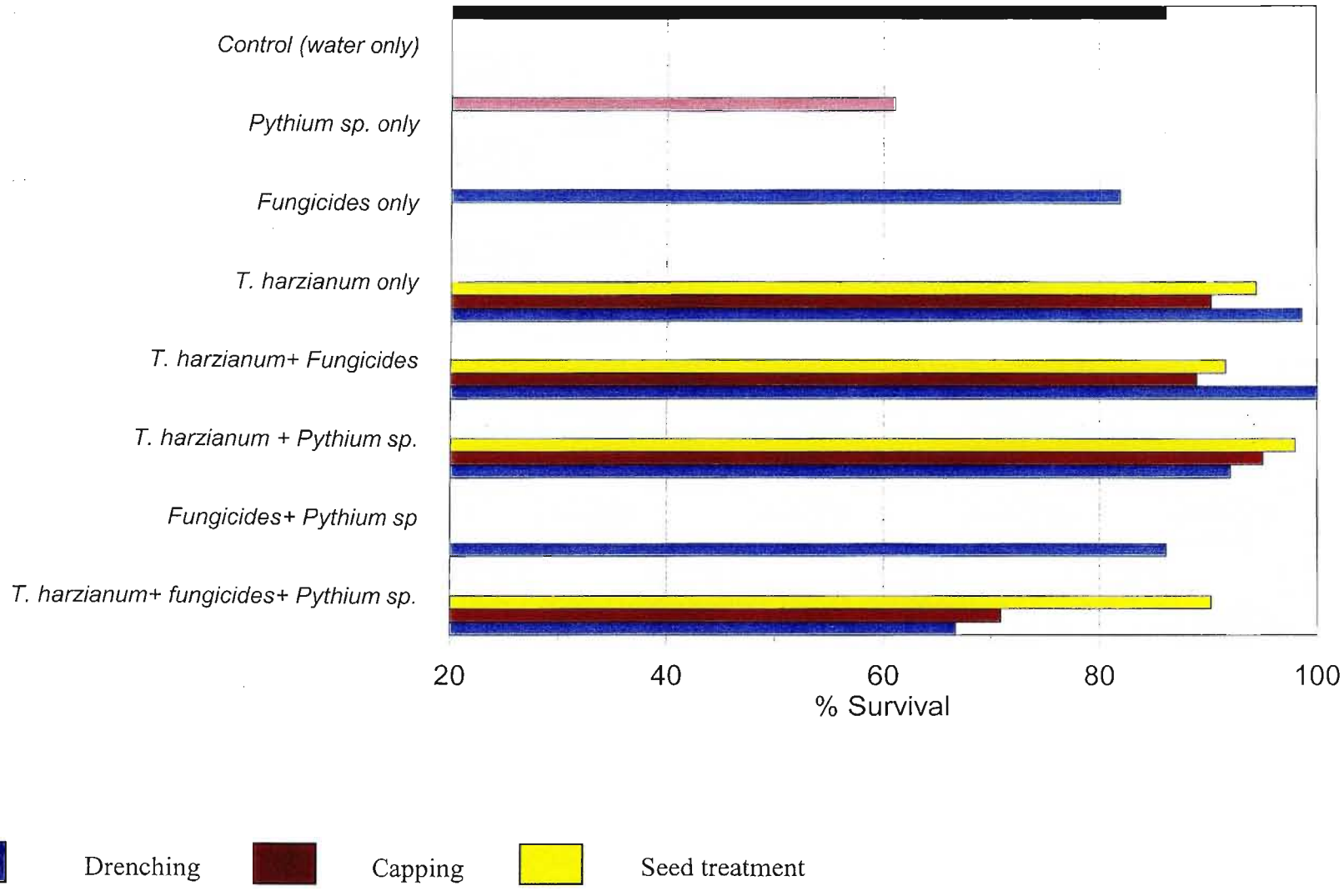

Figure 5.5 Effect of selected fungicides and a commercial formulation containing a biocontrol agent *Trichoderma harzianum* KMD on percentage survival using three application methods on cucumber seedlings artificially diseased by *Pythium* sp. after four weeks of growth.

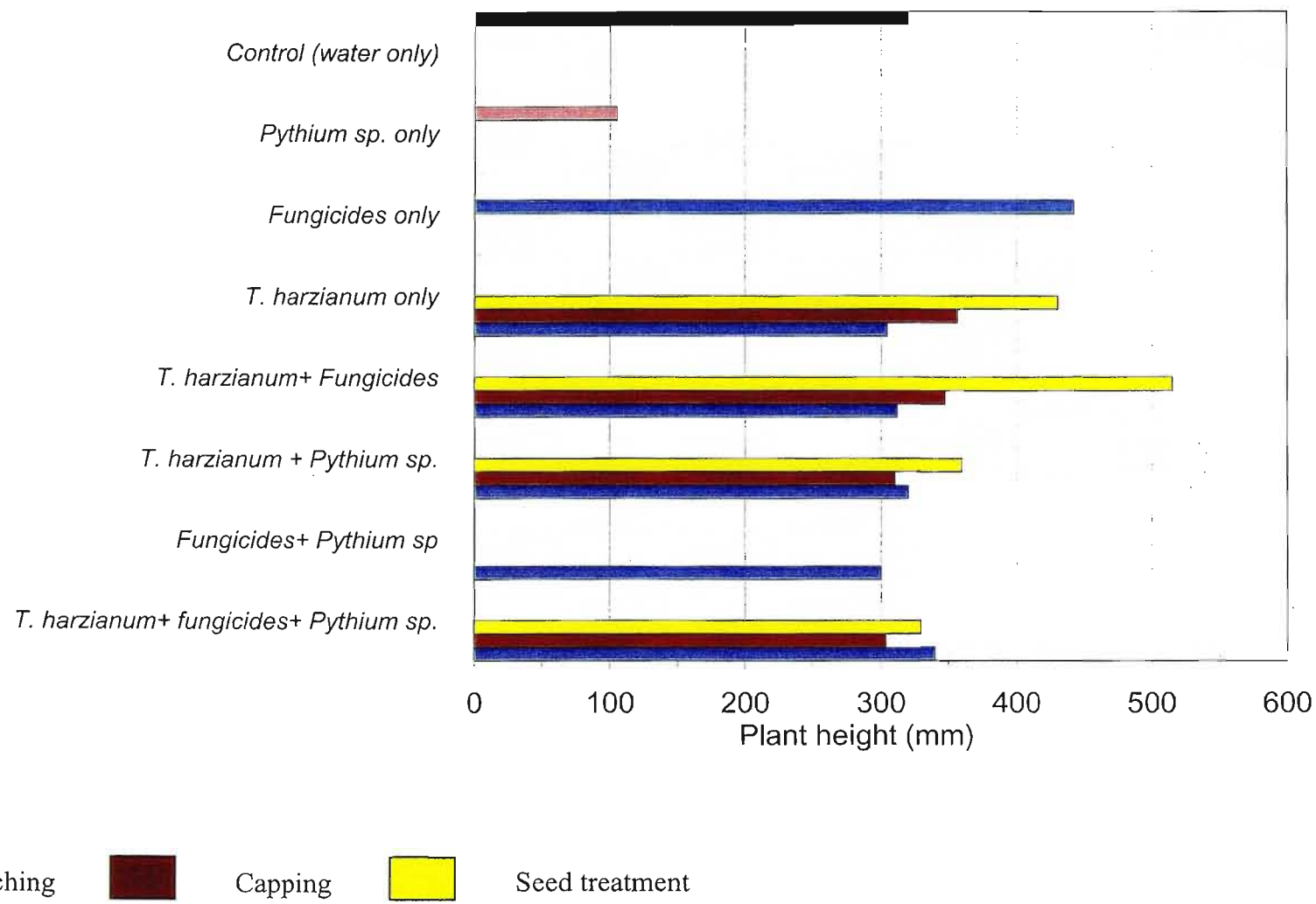

Figure 5.6 Effect of selected fungicides and a commercial formulation containing a biocontrol agent *Trichoderma harzianum* KMD on plant height using three application methods on cucumber seedlings artificially diseased by *Pythium* sp. after four weeks of growth.

Table5.4 Effect of a commercial formulation of *Trichoderma harzianum* KMD, with fungicides Benlate ® and Previcur® on damping-off caused by *Pythium* sp. of Namaqualand daisy after four weeks

Treatments	Application	Plot weight (g)	% Control 1 (*Pythium* sp. only)	% Survival	% Control 1 (*Pythium* sp. only)	Plant height (mm)	% Control 1 (*Pythium* sp. only)
Trichoderma harzianum	Drenching	2.4 ab	66.30	79.17 a	139	121.9 a	174
Trichoderma harzianum	Capping	1.68 b	46.41	77.78 b	137	128 a	183
Trichoderma harzianum	Seed treatment	3.12 ab	86.19	70.83 b	124	123 a	176
Trichoderma harzianum + Benlate® + Previcur®	Drenching	2.28 b	62.98	77.70 b	136	95.4 a	136
Trichoderma harzianum +Benlate® + Previcur®	Capping	1.44 b	39.78	75.00 b	132	122.6 a	175
Trichoderma harzianum + Benlate® + Previcur®	Seed treatment	4.41 ab	121.72	76.39 a	134	115.3 a	165
Trichoderma harzianum + *Pythium* sp.	Drenching	6.48 a	179.01	88.00 a	155	126 a	180
Trichoderma harzianum + *Pythium* sp.	Capping	6.98 a	192.93	89.00 a	156	112.3 a	160
Trichoderma harzianum + *Pythium* sp.	Seed treatment	6.48 a	179.01	90.00 a	158	136.0 a	194
Trichoderma harzianum + Benlate® + Previcur®+ *Pythium* sp.	Drenching	0.72 c	19.89	77.77 b	137	107.0 a	153
Trichoderma harzianum + Benlate® + Previcur®+ *Pythium* sp.	Capping	2.78 b	76.91	83.33 ab	146	99.3 a	142
Trichoderma harzianum + Benlate® + Previcur®+ *Pythium* sp.	Seed treatment	5.52 ab	152.49	83.33 ab	146	120.6 a	172
Control 1 *Pythium* sp. only	Nil	3.62 b	100.00	56.94 d	100	70.0 b	100
Control 2 (water only)	Nil	2.47 b	68.29	95.83 a	168	92.5 ab	132
Control 3 Benlate® + Previcur®+ *Pythium* sp.	Drenching	5.21 ab	143.87	84.72 ab	149	109.3 a	156
Control 4 Benlate® + Previcur®	Drenching	2.42 b	66.96	73.61 b	129	118.0 a	169
Effects		**P-values**		**P-values**		**P-values**	
Treatments		0.0001***		0.0001***		0.0076***	
Applications		0.023**		0.012**		0.236 ^{NS}	
Treatments*Applications		0.026**		0.035**		0.113 ^{NS}	
		CV%=12.68		%CV = 7.038		%CV = 12.836	
		MSE=1.36		MSE =5.5304		MSE =1.299	

1.NS = Not significant; * *= significant at P ≤ 0.05; *** = significant at P ≤0.001
2.Means with the same letter are not significantly different (p = 0.05) according to Student, Newman and Keuls comparison test
3.Drench = 1g of formulation mixed with 1 litre of tap water
4.Capping = 1g of formulation mixed with 1 litre of composted pine bark
5.Seed treatment = application of formulations to seed with Pelgel®
6.Benlate® + Previcur® = 1g of Benlate® and 1.2 ml of Previcur® were added to 1 litre of tap water and thereafter drenched on seed at planting

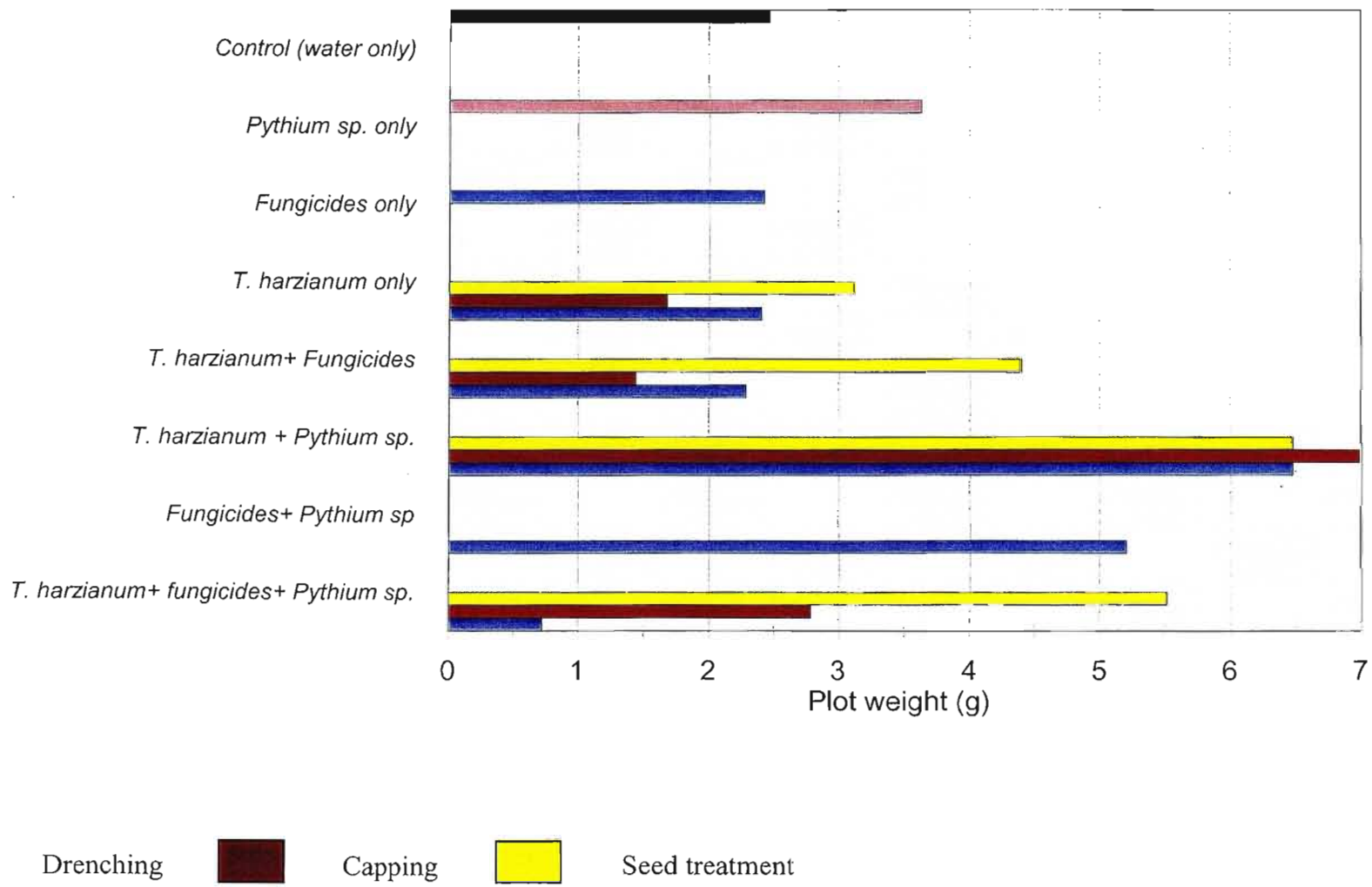

Figure 5.7 Effect of selected fungicides and a commercial formulation containing a biocontrol agent *Trichoderma harzianum* KMD on plot weight using three application methods on Namaqualand daisy seedlings artificially diseased by *Pythium* sp. after four weeks of growth.

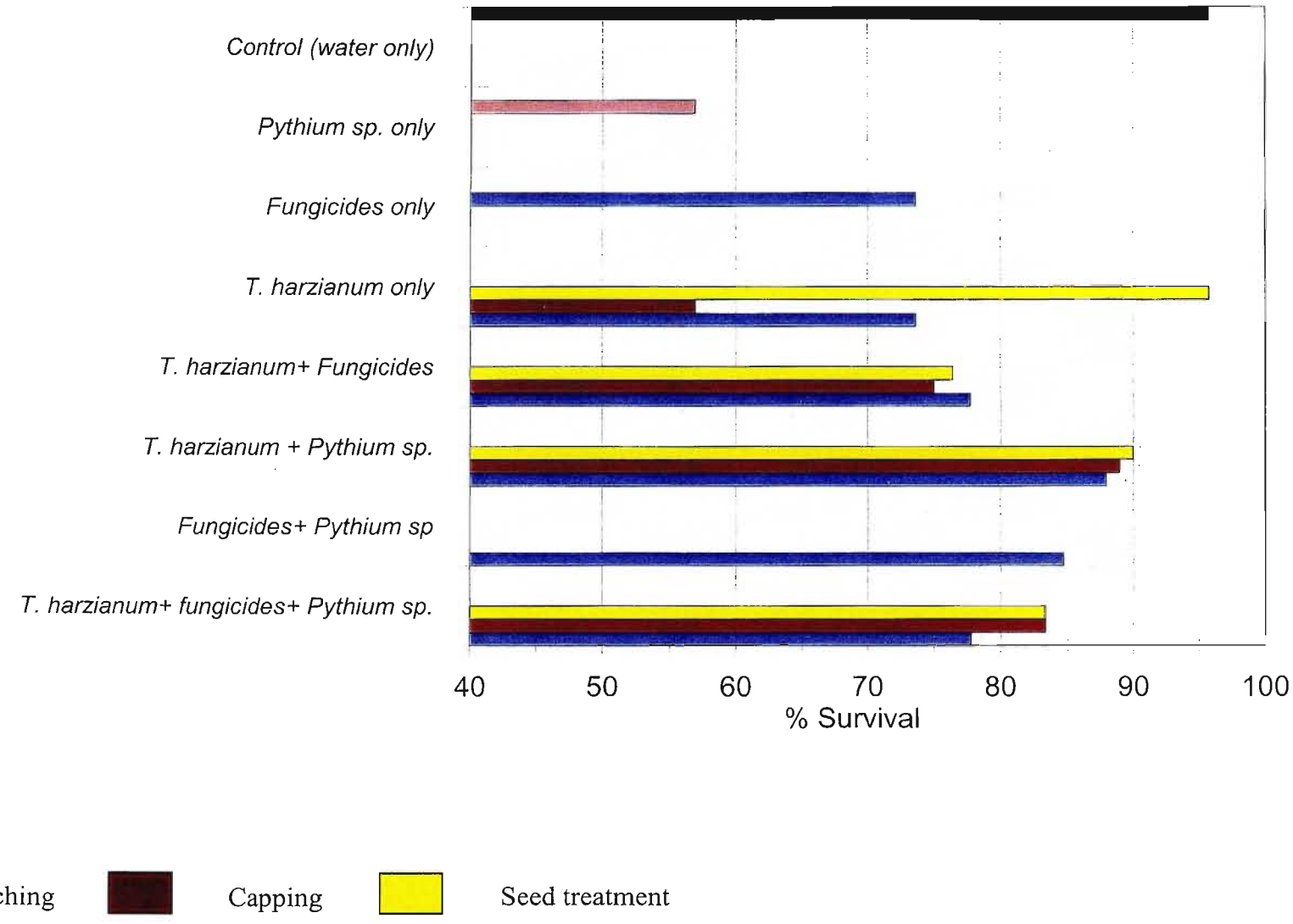

Figure 5.8 Effect of selected fungicides and a commercial formulation containing a biocontrol agent *Trichoderma harzianum* KMD on percentage survival using three application methods on Namaqualand daisy seedlings artificially diseased by *Pythium* sp. after four weeks of growth.

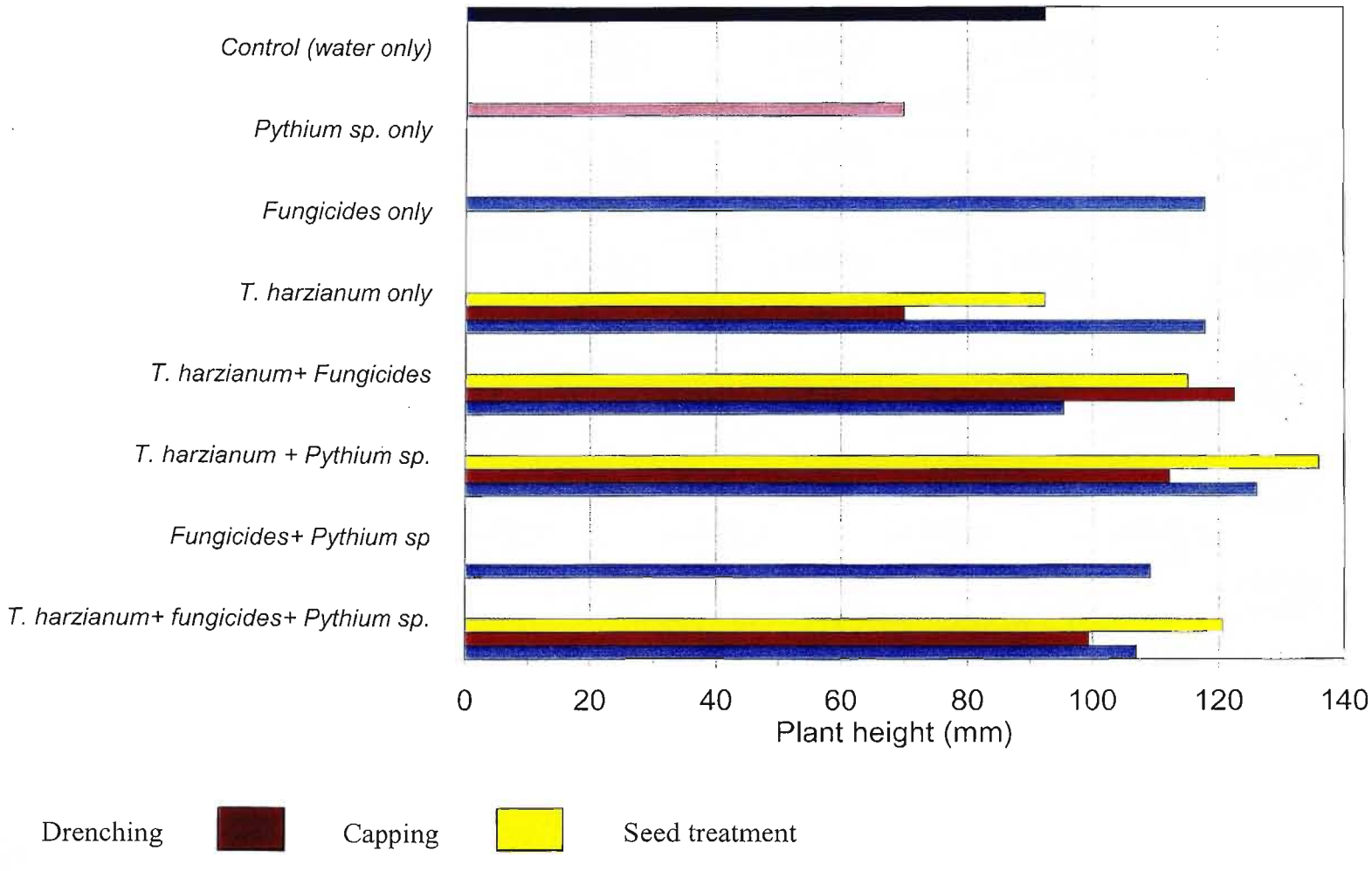

Figure 5.9 Effect of selected fungicides and a commercial formulation containing a biocontrol agent *Trichoderma harzianum* KMD on plant height using three application methods on Namaqualand daisy seedlings artificially diseased by *Pythium* sp. after four weeks of growth.

Table 5.5 Effect of a commercial formulation of *Trichoderma harzianum* KMD, with fungicides Benlate ® and Previcur® on damping-off caused by *Pythium* sp. of Eucalyptus after four weeks

Treatments	Application	Plot weight (g)	% Control 1 (*Pythium* sp. only)	% Survival	% Control 1 (*Pythium* sp. only)	Plant height (mm)	% Control 1 (*Pythium* sp. only)
Trichoderma harzianum	Drenching	3.60 b	94.99	84.7 a	156	126 a	201
Trichoderma harzianum	Capping	4.32 b	113.98	83.3 a	154	143 a	228
Trichoderma harzianum	Seed treatment	3.12 bc	82.32	81.9 a	151	147 a	235
Trichoderma harzianum + Benlate® + Previcur®	Drenching	6.48 ab	170.98	84.72 a	156	69.7 c	111
Trichoderma harzianum + Benlate® + Previcur®	Capping	11.35 a	299.53	83.33 a	154	104.6 b	167
Trichoderma harzianum + Benlate® + Previcur®	Seed treatment	3.36 bc	88.65	77.78 a	144	134.3 a	214
Trichoderma harzianum + Benlate® + Previcur®+ *Pythium* sp.	Drenching	1.44 c	37.99	76.39 ab	141	149.3 a	238
Trichoderma harzianum + Benlate® + Previcur®+ *Pythium* sp.	Capping	4.87 b	128.55	95.83 ab	177	130.0 a	207
Trichoderma harzianum + Benlate® + Previcur®+ *Pythium* sp.	Seed treatment	6.70 ab	176.87	98.61 a	182	126.6 a	202
Trichoderma harzianum + *Pythium* sp.	Drenching	6.14 ab	162.11	82.00 a	151	112.3 a	179
Trichoderma harzianum + *Pythium* sp.	Capping	5.06 ab	133.61	82.10 a	152	102.6 a	164
Trichoderma harzianum + *Pythium* sp.	Seed treatment	5.35 ab	141.21	84.00 a	155	123 a	196
Control 1 *Pythium* sp. only	Nil	3.79 bc	100.00	54.16 c	100	62.67 c	100
Control 2 (water only)	Nil	3.70 bc	97.52	87.50 ab	162	74.00 b	118
Control 3 Benlate® + Previcur®+ *Pythium* sp.	Drenching	6.19 ab	163.38	94.44 ab	174	130.3 a	208
Control 4 Benlate® + Previcur®	Drenching	3.60 bc	94.99	81.98 a	151	77.6 b	124
Effects		**P-values**		**P-values**		**P-values**	
Treatments		0.0001***		0.0006***		0.0007***	
Applications		0.0001***		0.026**		0.013**	
Treatments*Application		0.0001***		0.012**		0.231 NS	
		CV%= 27.89		%CV = 11.63		%CV = 17.39	
		MSE=2.45		MSE =9.622		MSE =1.979	

1.NS = Not significant; * *= significant at P ≤ 0.05; *** = significant at P ≤0.001
2.Means with the same letter are not significantly different (p = 0.05) according to Student, Newman and Keuls comparison test
3.Drench = 1g of formulation mixed with 1 litre of tap water
4.Capping = 1g of formulation mixed with 1 litre of composted pine bark
5.Seed treatment = application of formulations to seed with Pelgel®
6.Benlate® + Previcur® = 1g of Benlate® and 1ml of Previcur® were added to 1 litre of tap water and thereafter drenched on seed at planting

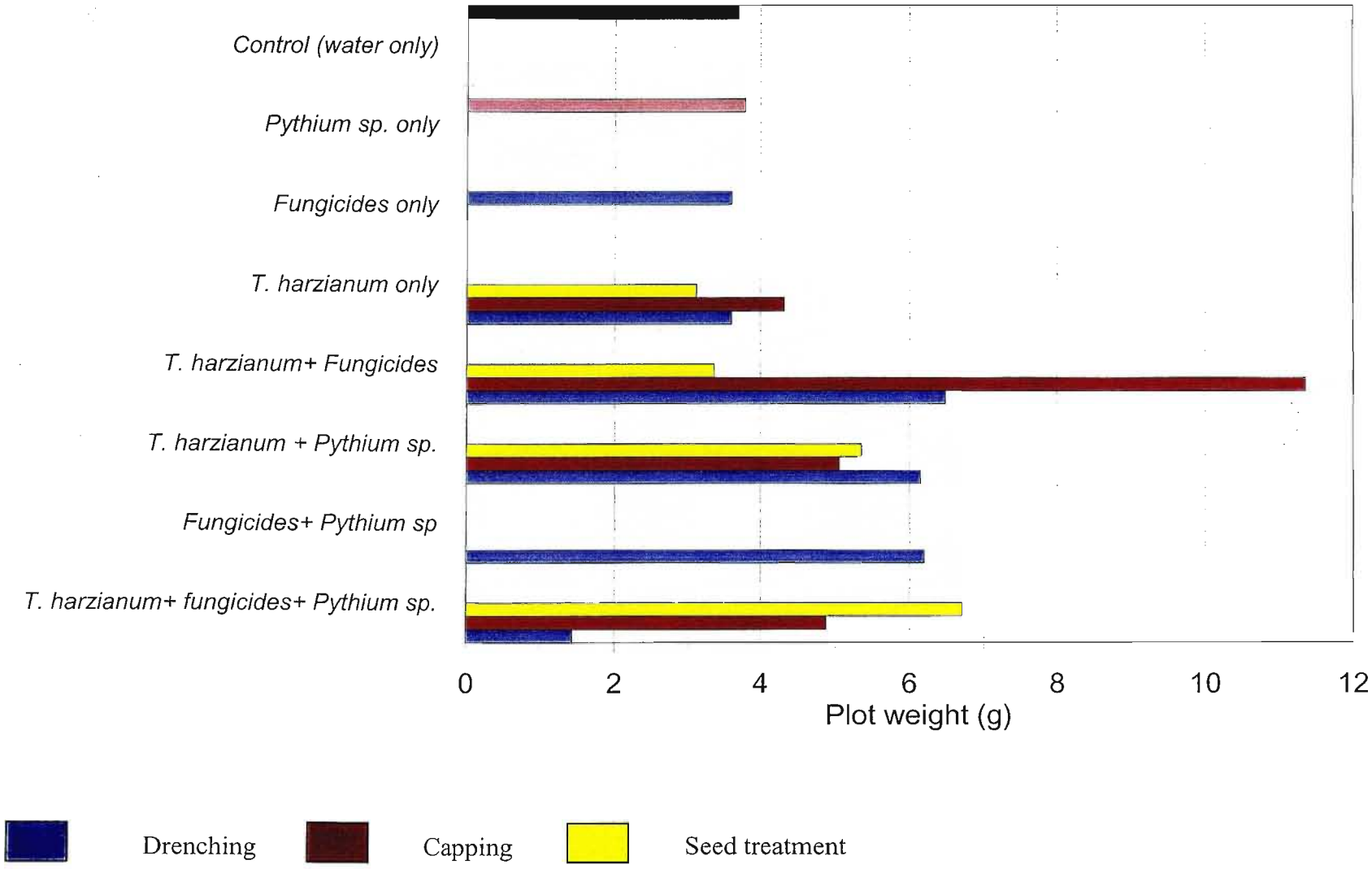

Figure 5.10 Effect of selected fungicides and a commercial formulation containing a biocontrol agent *Trichoderma harzianum* KMD on plot weight using three application methods on Eucalyptus artificially diseased by *Pythium* sp. after four weeks of growth.

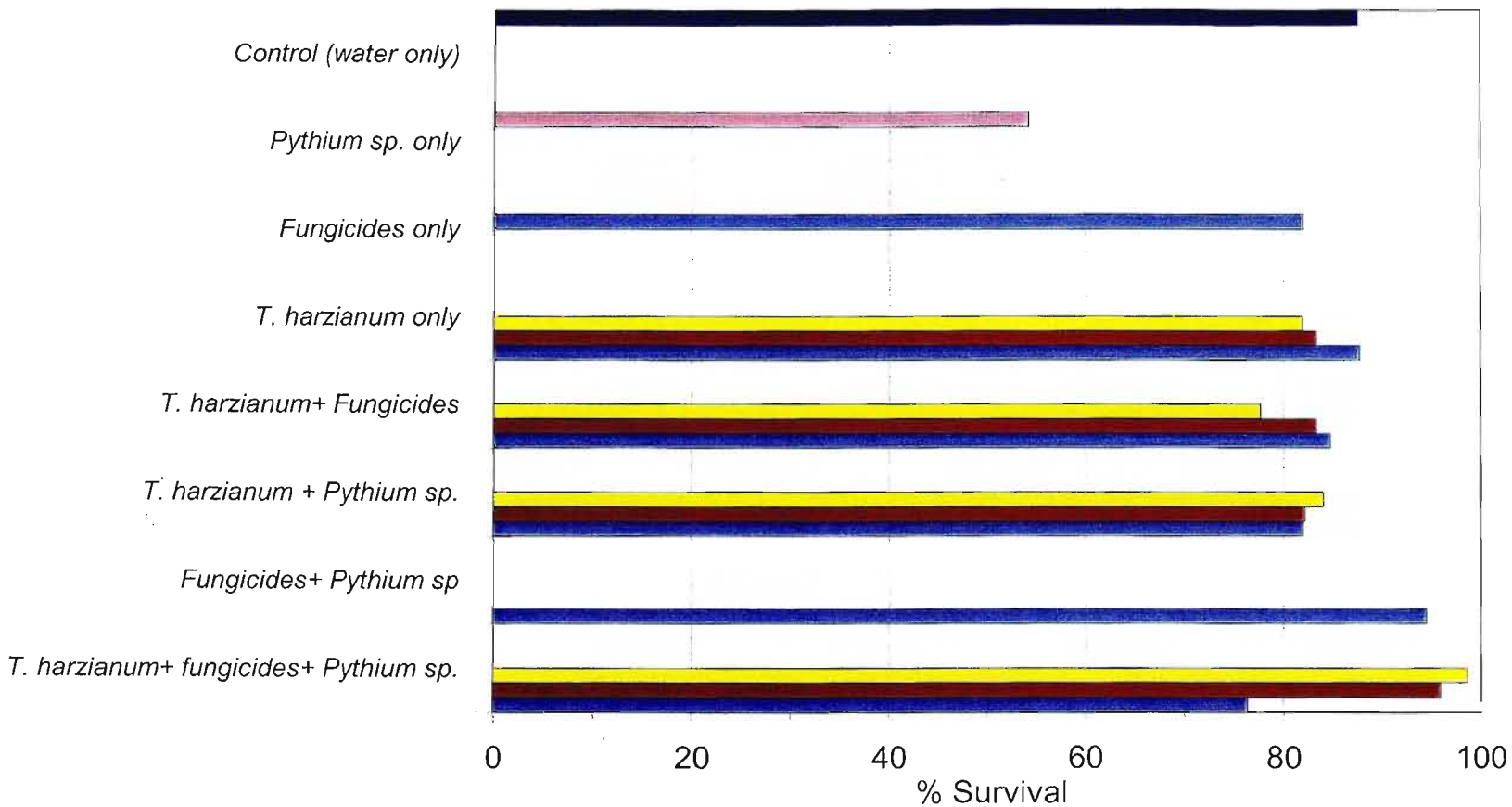

Figure 5.11 Effect of selected fungicides and a commercial formulation containing a biocontrol agent *Trichoderma harzianum* KMD on percentage survival using three application methods on Eucalyptus seedlings artificially diseased by *Pythium* sp. after four weeks of growth.

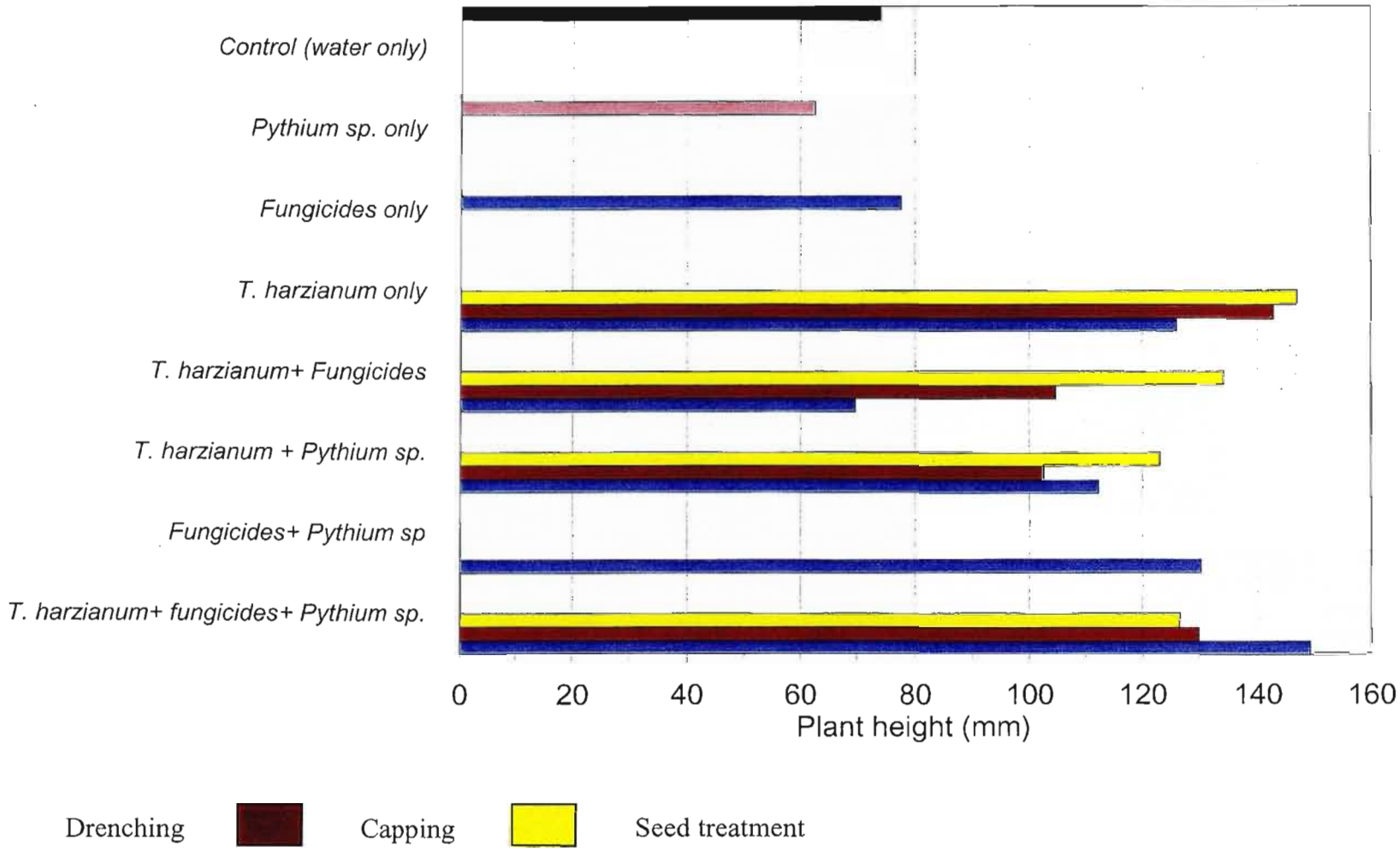

Figure 5.12 Effect of selected fungicides and a commercial formulation containing a biocontrol agent *Trichoderma harzianum* KMD on plant height using three application methods on Eucalyptus seedlings artificially diseased by *Pythium* sp. after four weeks of growth.

Table5.6 Effect of a commercial formulation of *Trichoderma harzianum* KMD, with fungicides Benlate® and Previcur® on damping –off caused by *Pythium* sp. of tomato after four weeks

Treatments	**Application**	**Plot weight (g)**	**% Control 1 (*Pythium* sp. only)**	**% Survival**	**% Control 1 (*Pythium* sp. only)**	**Plant height (mm)**	**% Control of 1 (*Pythium* sp. only)**
Trichoderma harzianum	Drenching	3.26 ab	63.87	86.11 a	187	259 b	199
Trichoderma harzianum	Capping	3.91 ab	76.56	93.06 a	203	254.5 b	196
Trichoderma harzianum	Seed treatment	3.89 ab	76.09	97.20 a	212	267 b	205
Trichoderma harzianum + Benlate® + Previcur®	Drenching	2.42 b	47.44	65.28 b	142	262.0 b	202
Trichoderma harzianum + Benlate® + Previcur®	Capping	3.50 ab	68.57	95.83 a	209	261.1 b	201
Trichoderma harzianum + Benlate® + Previcur®	Seed treatment	4.90 ab	95.81	90.27 ab	197	309.3 a	238
Trichoderma harzianum + *Pythium* sp.	Drenching	3.84 ab	75.15	88.00 a	192	339.5 a	261
Trichoderma harzianum + *Pythium* sp.	Capping	3.94 ab	77.03	89.23 a	194	342.1 a	263
Trichoderma harzianum + *Pythium* sp.	Seed treatment	3.60 ab	70.45	87.12 a	190	364.4 a	280
Trichoderma harzianum + Benlate® + Previcur®+ *Pythium* sp.	Drenching	3.84 ab	75.15	65.277 b	142	226.3 b	174
Trichoderma harzianum + Benlate® + Previcur®+ *Pythium* sp	Capping	5.50 ab	107.55	87.50 a	190	236.6 b	182
Trichoderma harzianum + Benlate® + Previcur®+ *Pythium* sp.	Seed treatment	2.16 ab	42.27	83.33 a	181	246.6 b	190
Control 1 *Pythium* sp. only	Nil	5.11 ab	100.04	45.83 c	100	130.0 c	100
Control 2 (water only)	Nil	2.16 ab	42.27	84.72 a	184	323.0 a	248
Control 3 Benlate® + Previcur®+ *Pythium* sp.	Drenching	7.37 a	144.19	79.10 a	172	243.3 b	187
Control 4 Benlate® + Previcur®	Drenching	3.60 ab	70.45	86.11 ab	187	207.2 c	159
Effects		**P-values**		**P-values**		**P-values**	
Treatments		0.0001***		0.0001***		0.0003***	
Applications		0.0001***		0.0001***		0.126 ^{NS}	
Treatments*Applications		0.0001***		0.0001***		0.265 ^{NS}	
		CV%=17.08		%CV =10.39		%CV = 13.728	
		MSE= 3.2		MSE =7.38		MSE =3.169	

1.NS = Not significant; ** = significant at P ≤ 0.05; *** = significant at P ≤0.001

2.Means with the same letter are not significantly different (p = 0.05) according to Student, Newman and Keuls comparison test

3.Drench = 1g of formulation mixed with 1 litre of tap water

4.Capping = 1g of formulation mixed with 1 litre of composted pine bark

5.Seed treatment = application of formulations to seed with Pelgel®

6.Benlate® + Previcur® = 1g of Benlate® and 1.2 ml of Previcur® were added to 1 litre of tap water and thereafter drenched on seed at planting

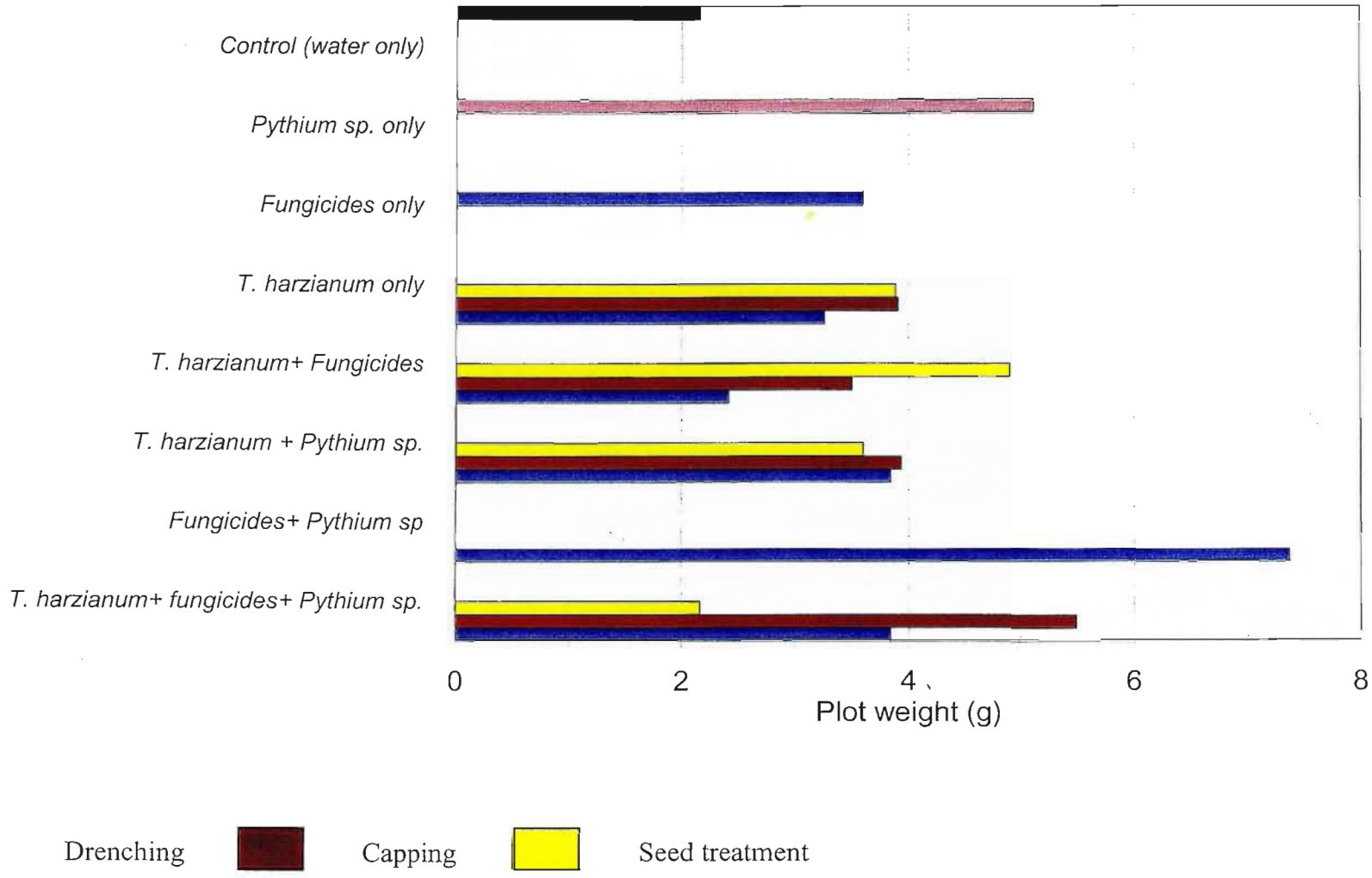

Figure 5.13 Effect of selected fungicides and a commercial formulation containing a biocontrol agent *Trichoderma harzianum* KMD on plot weight using three application methods on tomato seedlings artificially diseased by *Pythium* sp. after four weeks of growth.

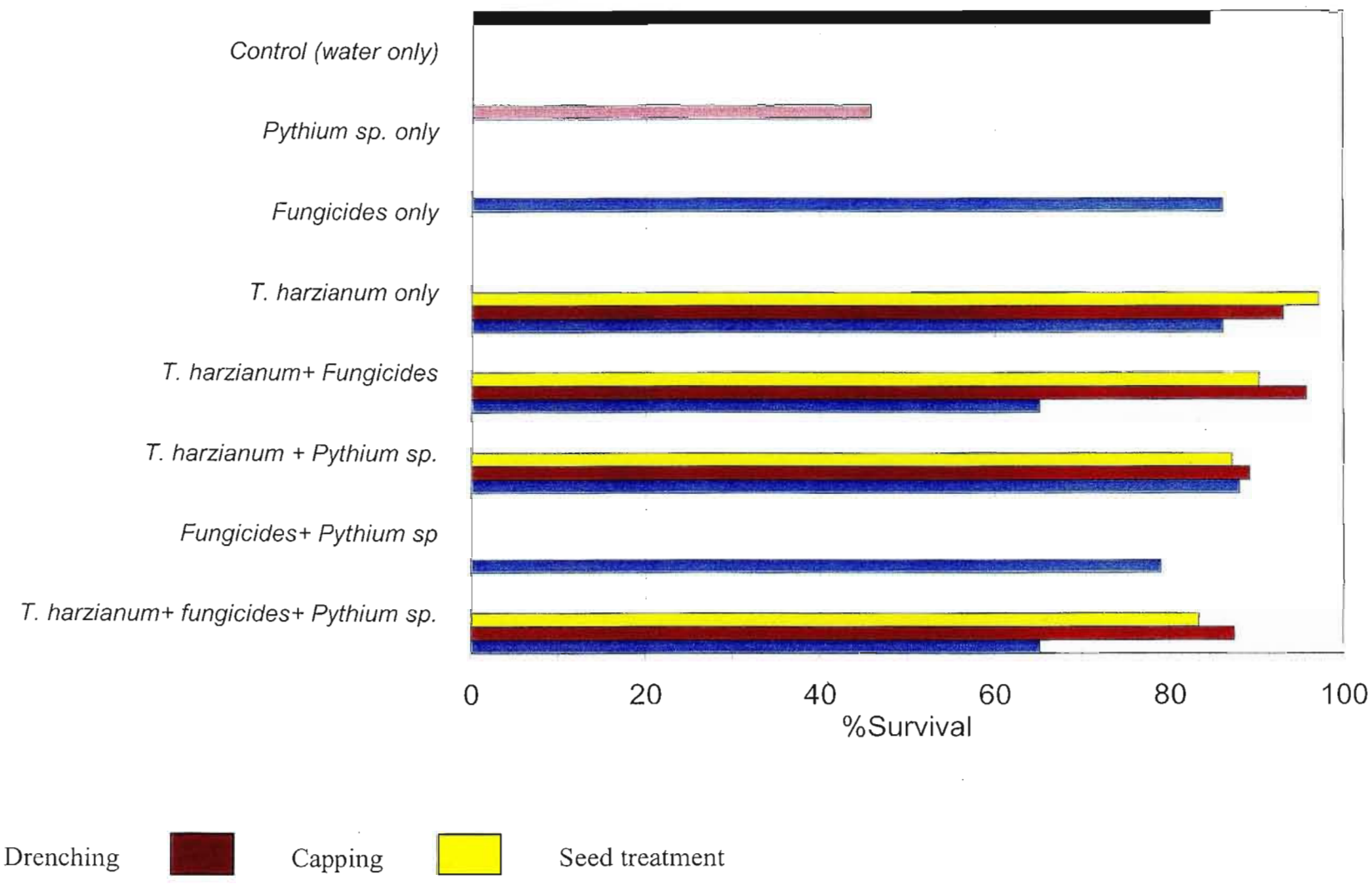

Figure 5.14 Effect of selected fungicides and a commercial formulation containing a biocontrol agent *Trichoderma harzianum* KMD on percentage survival using three application methods on tomato seedlings artificially diseased by *Pythium* sp. after four weeks of growth.

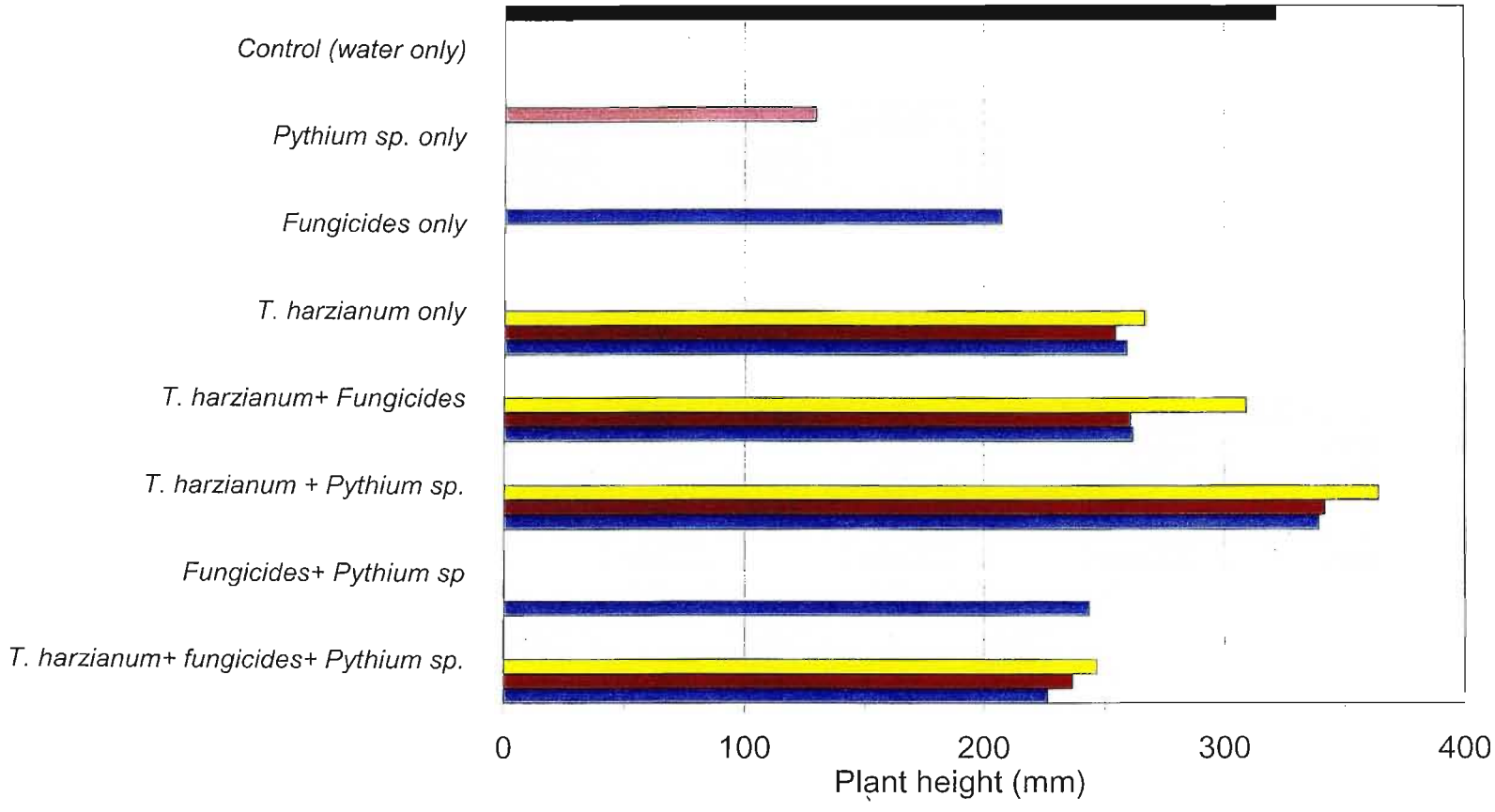

Figure 5.15 Effect of selected fungicides and a commercial formulation containing a biocontrol agent *Trichoderma harzianum* KMD on plant height using three application methods on tomato seedlings artificially diseased by *Pythium* sp. after four weeks of growth.

Table 5.7 Effect of a commercial formulation of *Trichoderma harzianum* KMD, with fungicides Benlate ® and Previcur® on damping-off of cabbage caused by *Rhizoctonia solani* after 4 weeks

Treatments	Application	Plot weight (g)	% Control 1 (*Rhizoctonia solani*)	% Survival	% Control 1 (*Rhizoctonia solani* only)	Plant height (mm)	% Control 1 (*Rhizoctonia solani* only)
Trichoderma harzianum	Drenching	4.32 a	166.67	81.94 a	642	149 a	497
Trichoderma harzianum	Capping	3.92 a	151.20	83.33 a	653	138 a	460
Trichoderma harzianum	Seed treatment	5.28 a	203.70	84.70 a	663	151 a	503
Trichoderma harzianum + Benlate® + Previcur®	Drenching	3.55 ab	137.04	79.16 a	620	154.0 a	513
Trichoderma harzianum + Benlate® + Previcur®	Capping	3.29 ab	126.85	80.55 a	631	139.0 a	463
Trichoderma harzianum + Benlate® + Previcur®	Seed treatment	4.34 a	167.59	76.39 a	598	139.0 a	463
Trichoderma harzianum + *Rhizoctonia solani*	Drenching	5.76 a	222.22	81.50 a	638	156.0 a	520
Trichoderma harzianum + *Rhizoctonia solani*	Capping	5.04 a	194.44	82.00 a	642	157.0 a	523
Trichoderma harianum + *Rhizoctonia solani*	Seed treatment	6.96 a	268.52	86.00 a	673	162.3 a	541
Trichoderma harzianum + Benlate® + Previcur®+ *Rhizoctonia solani*	Drenching	2.16 ab	83.33	62.50 b	489	84.1 b	280
Trichoderma harzianum + Benlate® + Previcur®+ *Rhizoctonia solani*	Capping	1.85 b	71.30	29.16 c	228	83.3 b	278
Trichoderma harzianum + Benlate® + Previcur®+ *Rhizoctonia solani*	Seed treatment	3.43 ab	132.41	30.55 c	239	150.3 a	501
Control 1 (*Rhizoctonia solani* only)	Nil	2.59 ab	100.00	12.77 d	100	30.0 b	100
Control 2 (water only)	Nil	4.32 a	166.67	79.167 a	620	133.77 a	446
Control 3 Benlate® + Previcur®+ *Rhizoctonia solani*	Drenching	3.60 ab	138.89	62.50 b	489	126.1 a	420
Control 4 Benlate® + Previcur®	Drenching	3.00 ab	115.74	83.33 a	653	135.0 a	450
Effects		**P-values**		**P-values**		**P-values**	
Treatments		0.0001***		0.0001***		0.0456 **	
Applications		0.025**		0.0001***		0.451 ^{NS}	
Treatments*Applications		0.061 ^{NS}		0.0001***		0.561 ^{NS}	
		CV%=22.60		%CV =16.05		%CV = 12.19	
		MSE=2.15		MSE =7.66		MSE =4.61	

1. NS = Not significant; ** = significant at P ≤ 0.05; *** = significant at P ≤0.001
2. Means with the same letter are not significantly different (p = 0.05) according to Student, Newman and Keuls comparison test
3. *Drench* = 1g of formulation mixed with 1 litre of tap water
4. Capping = 1g of formulation mixed with 1 litre of composted pine bark
5. Seed treatment = application of formulations to seed with Pelgel®
6. Benlate® + Previcur® = 1g of Benlate® and 1.2 ml of Previcur® were added to 1 litre of tap water and thereafter drenched on seed at planting

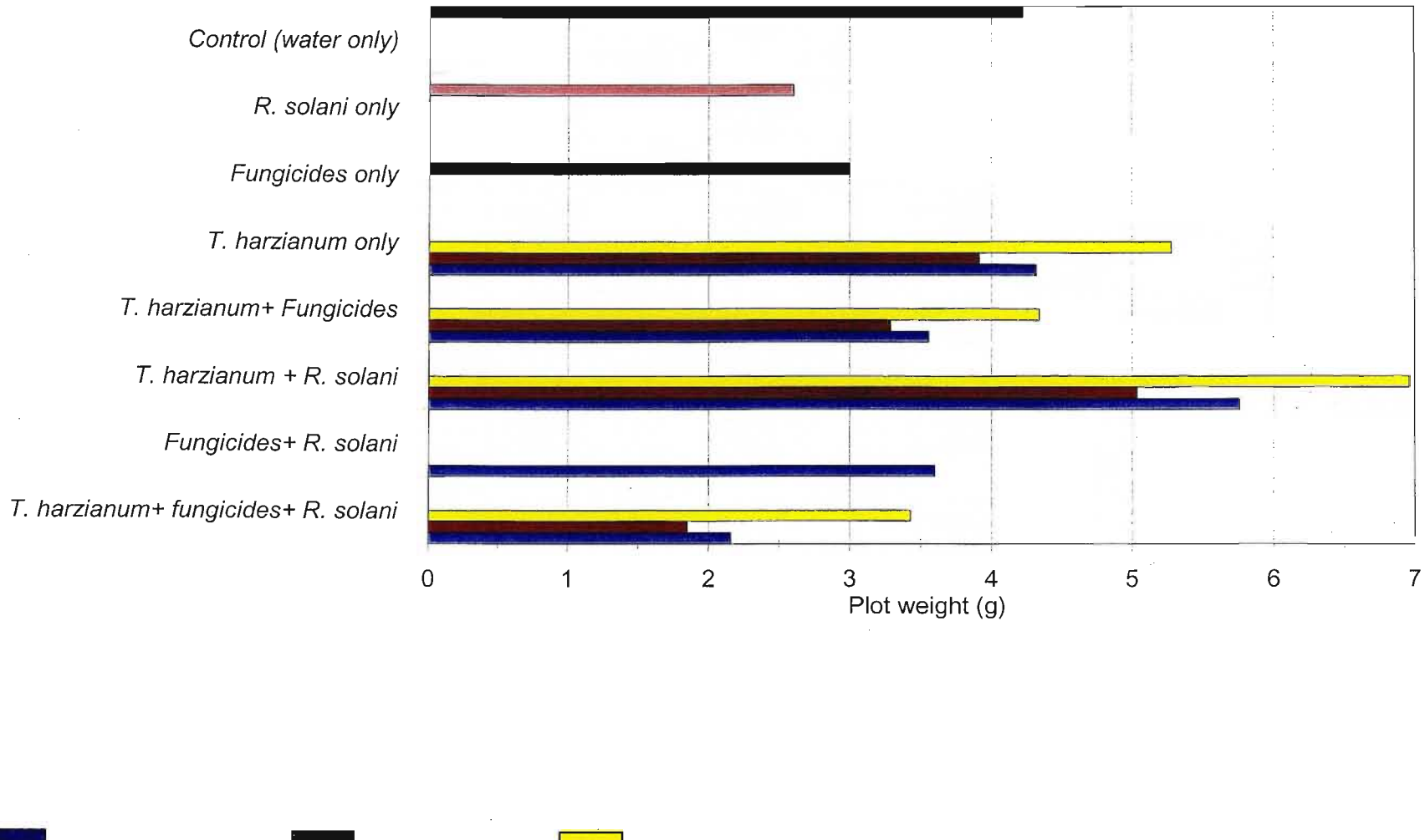

Figure 5.16 Effect of selected fungicides and a commercial formulation containing a biocontrol agent *Trichoderma harzianum* KMD on plot weight using three application methods on cabbage seedlings artificially diseased by R*hizoctonia. solani* after four weeks of growth.

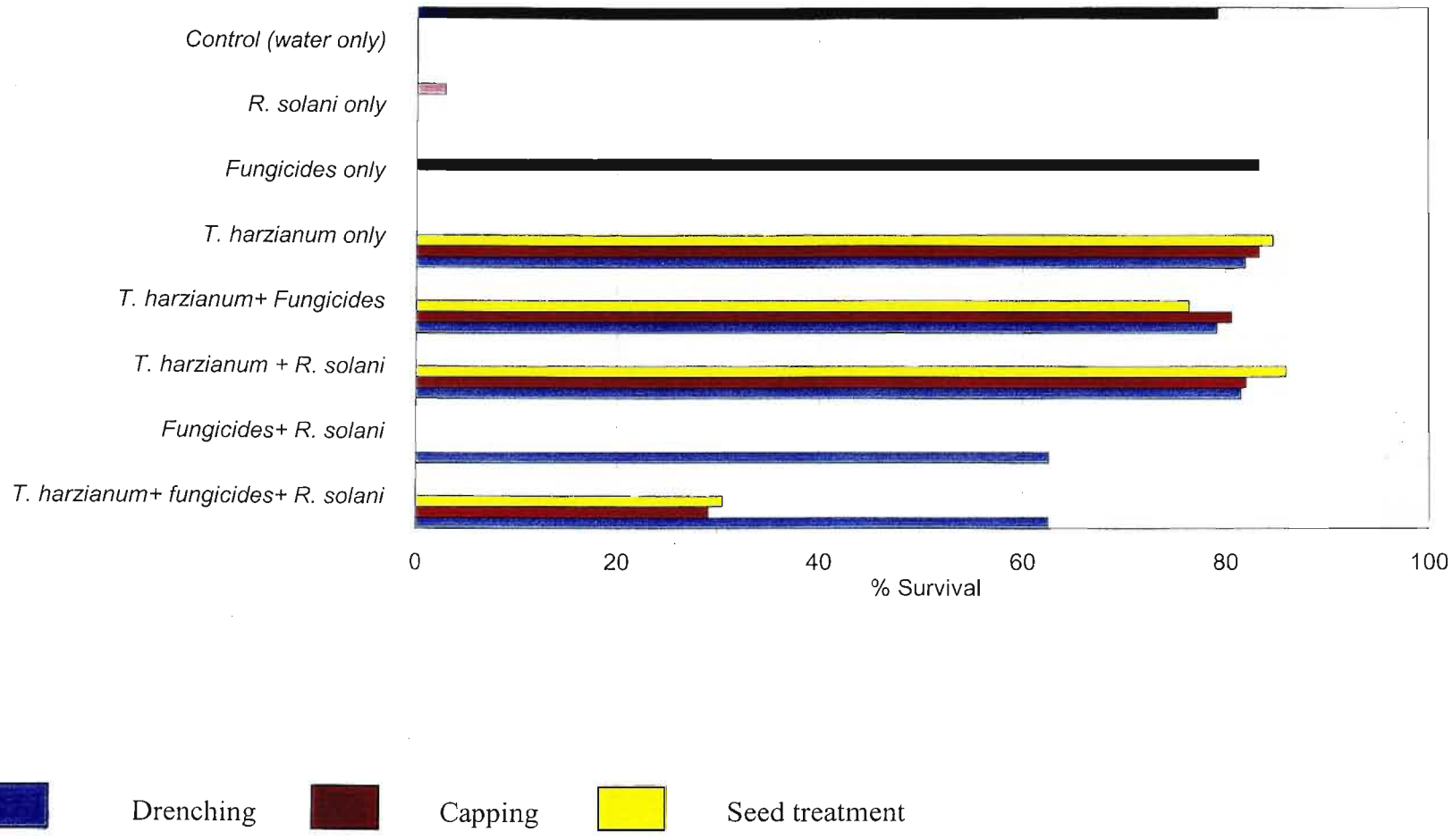

Figure 5.17 Effect of selected fungicides and a commercial formulation containing a biocontrol agent *Trichoderma harzianum* KMD on percentagesurvival using three application methods on cabbage seedlings artificially diseased by *Rhizoctonia solani* after four weeks of growth.

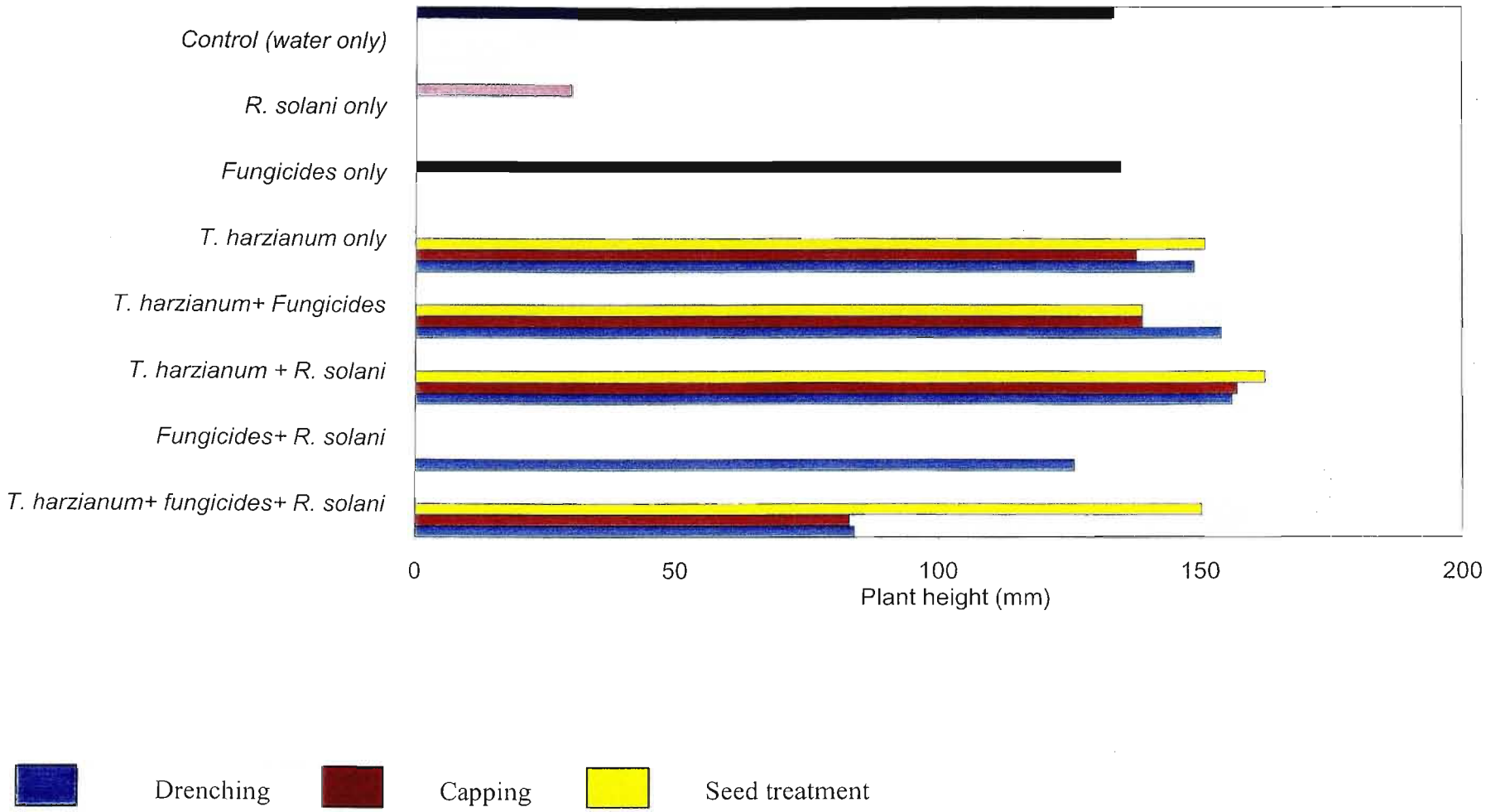

Figure 5.18 Effect of selected fungicides and a commercial formulation containing a biocontrol agent *Trichoderma harzianum* KMD on plant height using three application methods on cabbage seedlings artificially diseased by *Rhizoctonia solani* after four weeks of growth.

Table 5.8. Effect of a commercial formulation of *Trichoderma harzianum* KMD, with fungicides Benlate ® and Previcur® on damping-off caused by *Rhizoctonia solani* of cucumber after 4 weeks

Treatments	**Application**	**Plot weight (g)**	**% Control 1 (*Rhizoctonia solani*)**	**% Survival**	**% Control 1 (*Rhizoctonia solani* only**	**Plant height (mm)**	**% Control 1 (*Rhizoctonia solani* only**
Trichoderma harzianum	Drenching	20.16 a	274.66	100.0 a	1200	312 b	426
Trichoderma harzianum	Capping	14.88 a	202.72	88.9 a	1067	348 b	475
Trichoderma harzianum	Seed treatment	15.60 a	212.53	91.6 a	1100	516 a	704
Trichoderma harzianum + Benlate® + Previcur®	Drenching	13.03 a	177.55	98.61a	1184	304.7 b	416
Trichoderma harzianum + Benlate® + Previcur®	Capping	13.22 a	180.16	90.27 a	1084	357.0 b	487
Trichoderma harzianum + Benlate® + Previcur®	Seed treatment	14.47 a	197.17	94.43 a	1134	431.0 a	588
Trichoderma harzianum + *Rhizoctonia solani*	Drenching	13.44 a	183.11	98.00 a	1176	300.0 b	409
Trichoderma harzianum + *Rhizoctonia solani*	Capping	13.20 a	179.84	96.00 a	1152	320.0 b	437
Trichoderma harianum + *Rhizoctonia solani*	Seed treatment	12.96 a	176.57	97.00 a	1164	440.0 a	600
Trichoderma harzianum + Benlate® + Previcur®+ *Rhizoctonia solani*	Drenching	11.38 a	154.99	79.16 ab	950	259.5 b	354
Trichoderma harzianum + Benlate® + Previcur®+ *Rhizoctonia solani*	Capping	9.38 a	127.85	73.61 ab	884	249.6 b	341
Trichoderma harzianum + Benlate® + Previcur®+ *Rhizoctonia solani*	Seed treatment	12.60 a	171.66	76.39 ab	917	237.3 b	324
Control 1 (*Rhizoctonia solani* only)	Nil	7.34 a	100.05	8.33 c	100	73.300 c	100
Control 2 (water only)	Nil	11.71 a	159.56	98.610 a	1184	395.07 a	539
Control 3 Benlate® + Previcur®+ *Rhizoctonia solani*	Drenching	11.81 a	160.87	58.33 b	700	252.2 b	344
Control 4 Benlate® + Previcur®	Drenching	14.80 a	555.86	97.20 a	1167	399.4 a	545
Effects		**P-values**		**P-values**		**P-values**	
Treatments		0.612^{NS}		0.0001^{***}		0.0001^{***}	
Applications		0.782^{NS}		0.548^{NS}		0.442^{NS}	
Treatments*Applications		0.541^{NS}		0.111^{NS}		0.313^{NS}	
		CV%=35.06		%CV = 16.89		%CV =18.03	
		MSE= 4.68		MSE =11.06		MSE = 4.67	

1.NS = Not significant; ** = significant at P ≤ 0.05; *** = significant at P ≤0.001
2.Means with the same letter are not significantly different (p = 0.05) according to Student, Newman and Keuls comparison test
3.Drench = 1g of formulation mixed with 1 litre of tap water
4.Capping = 1g of formulation mixed with 1 litre of composted pine bark
5.Seed treatment = application of formulations to seed with Pelgel®
6.Benlate® + Previcur® = 1g of Benlate® and 1.2 ml of Previcur® were added to 1 litre of tap water and thereafter drenched on seed at planting

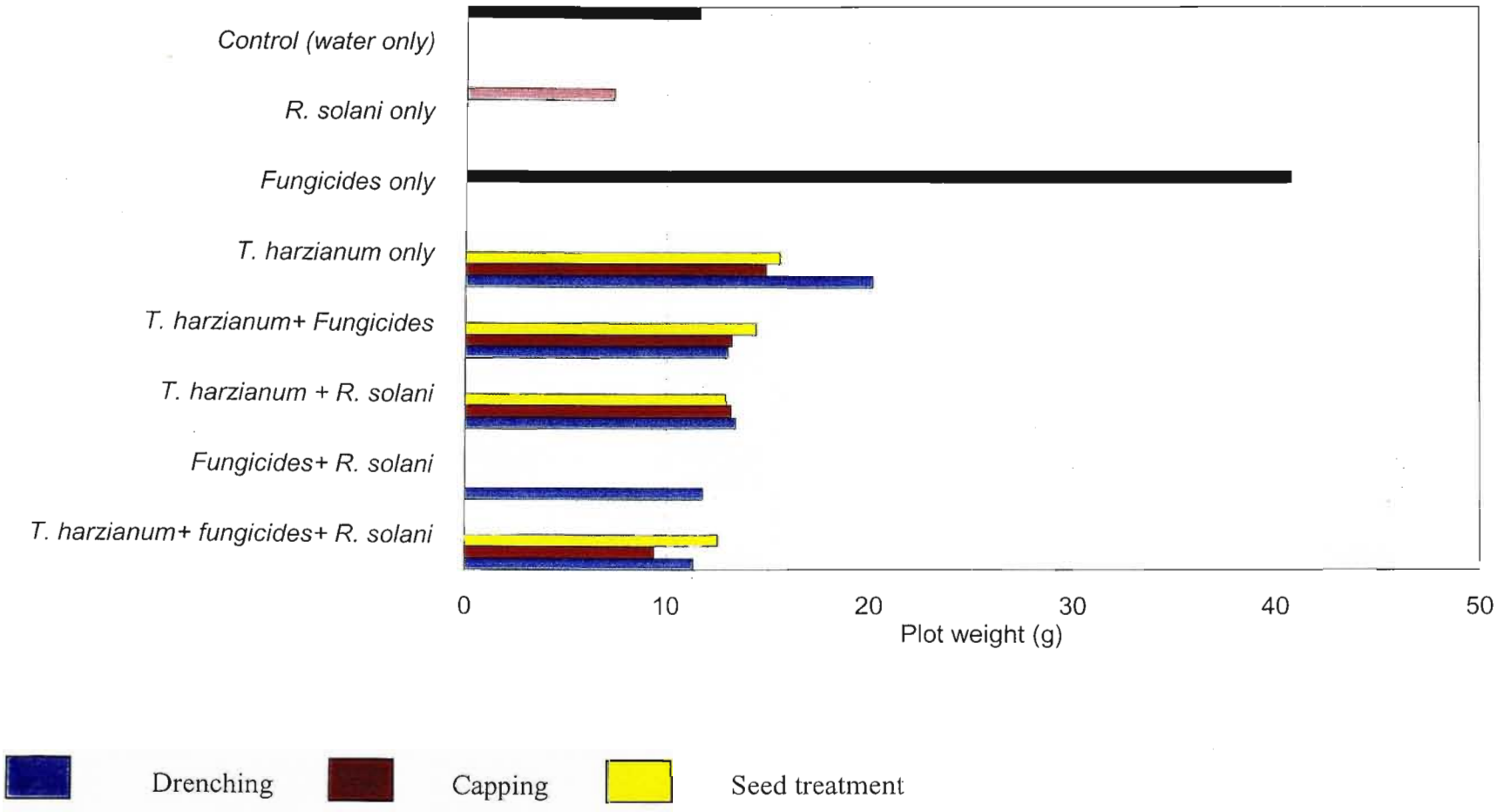

Figure 5.19 Effect of selected fungicides and a commercial formulation containing a biocontrol agent *Trichoderma harzianum* KMD on plot weight using three application methods on cucumber seedlings artificially diseased by *Rhizoctonia solani* after four weeks of growth.

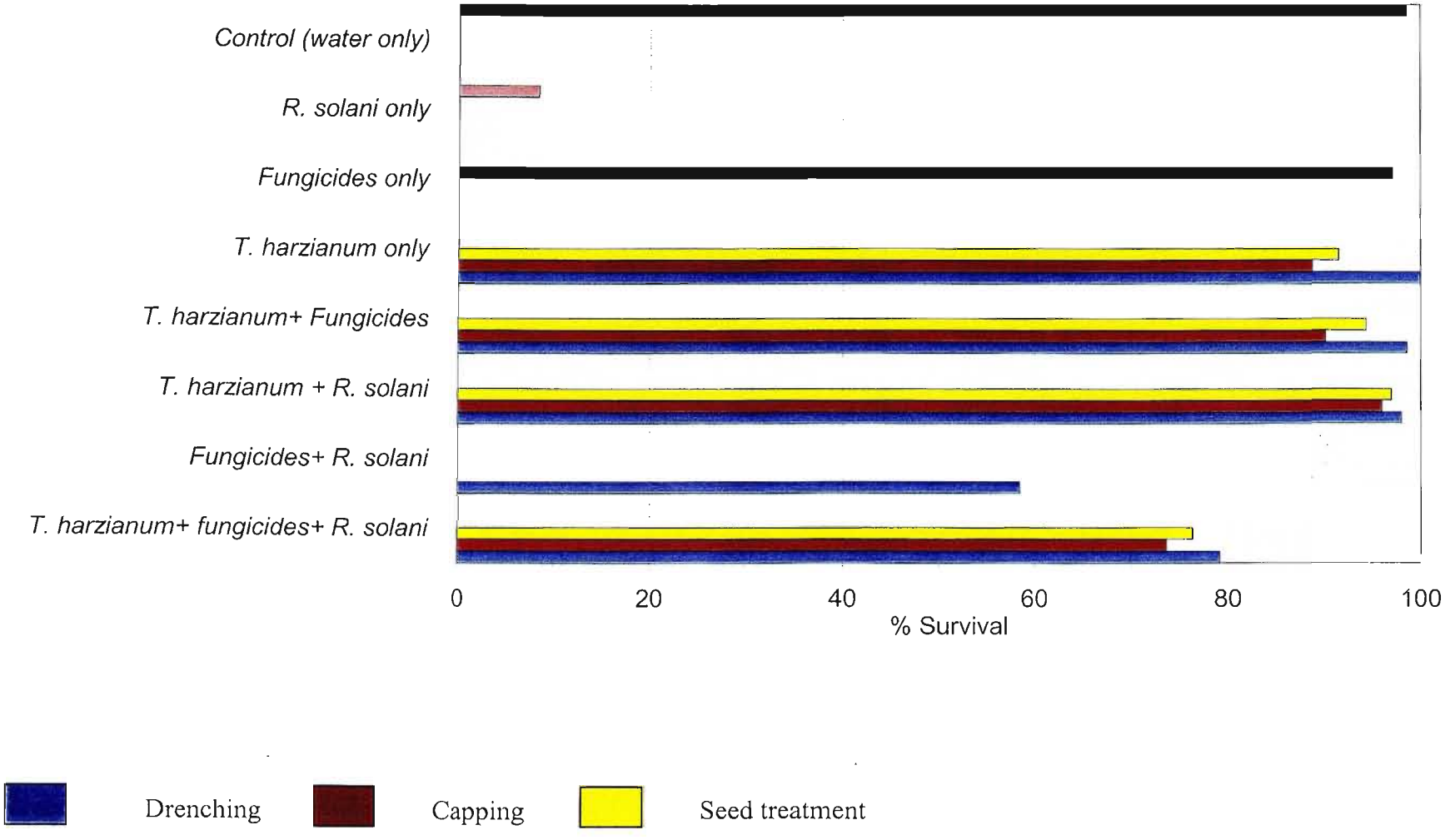

Figure 5.20 Effect of selected fungicides and a commercial formulation containing a biocontrol agent *Trichoderma harzianum* KMD on percentage survival using three application methods on cucumber seedlings artificially diseased by *Rhizoctonia solani* after four weeks of growth.

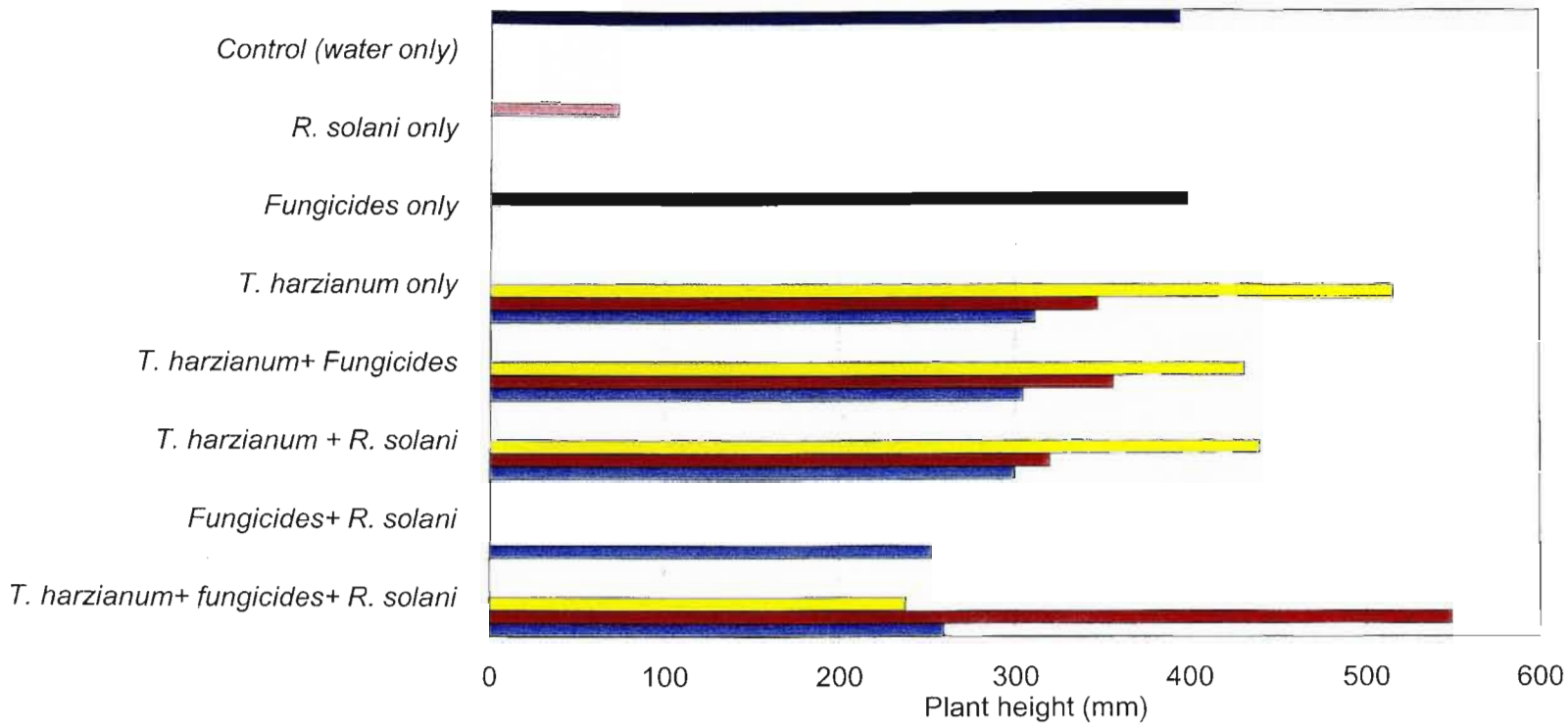

Figure 5.21 Effect of selected fungicides and a commercial formulation containing a biocontrol agent *Trichoderma harzianum* KMD on plant height using three application methods on cucumber seedlings artificially diseased by *Rhizoctonia solani* after four weeks of growth

Table 5.9 Effect of a commercial formulation, of *Trichoderma harzianum* KMD, with fungicides Benlate ® and Previcur® on damping-off caused by *Rhizoctonia solani* of Namaqualand daisy after 4 weeks

Treatments	Application	Plot weight (g)	% Control 1 (*Rhizoctonia solani)*	% Survival	% Control 1 (*Rhizoctonia solani* only	Plant height (mm)	% Control 1 (*Rhizoctonia solani* only)
Trichoderma harzianum	Drenching	3.12 abc	86.19	77.7 a	136	94 a	71
Trichoderma harzianum	Capping	1.92 a	53.04	75.00 a	132	122.6 a	93
Trichoderma harzianum	Seed treatment	5.76 a	159.12	76.39 a	134	115.3 a	87
Trichoderma harzianum + Benlate® + Previcur®	Drenching	2.28 bcd	62.98	79.17 a	139	121.9 a	92
Trichoderma harzianum + Benlate® + Previcur®	Capping	1.44 cd	39.78	77.78 a	137	128 a	97
Trichoderma harzianum + Benlate® + Previcur®	Seed treatment	4.41 a	121.72	70.83 a	124	123 a	93
Trichoderma harzianum + Rhizoctonia solani	Drenching	2.88 a	79.56	77.8 a	137	130.9 a	99
Trichoderma harzianum + Rhizoctonia solani	Capping	2.64 a	72.93	79.00 a	139	135.5 a	102
Trichoderma harianum + Rhizoctonia solani	Seed treatment	3.60 abc	99.45	77.00 a	135	134.6 a	102
Trichoderma harzianum + Benlate® + Previcur®+ *Rhizoctonia solani*	Drenching	0.82 d	22.54	61.11 a	107	120.8 a	91
Trichoderma harzianum + Benlate® + Previcur®+ *Rhizoctonia solani*	Capping	2.78 bcd	76.91	63.89 a	112	104.6 a	79
Trichoderma harzianum + Benlate® + Previcur®+ *Rhizoctonia solani*	Seed treatment	5.59 a	154.48	61.11 a	107	109.8 a	83
Control 1 (*Rhizoctonia solani* only)	Nil	3.62 abc	100.00	56.94 a	100	132.2 a	100
Control 2 (water only)	Nil	2.47 bcd	68.29	68.05 a	120	120.9 a	91
Control 3 Benlate® + Previcur®+ *Rhizoctonia solani*	Drenching	5.21 a	143.87	62.50 a	110	119.3 a	90
Control 4 Benlate® + Previcur®	Drenching	2.42 bcd	66.96	72.22 a	127	120 a	91
Effects		**P-values**		**P-values**		**P-values**	
Treatments		0.0001***		0.8903 ^{NS}		0.1041 ^{NS}	
Applications		0.0001***		0.202 ^{NS}		0.118 ^{NS}	
Treatments*Applications		0.0001***		0.403 ^{NS}		0.575 ^{NS}	
		CV%= 12.68		%CV =17.05		%CV = 8.719	
		MSE= 2.54		MSE =10.52		MSE =1.02	

1.NS = Not significant; * *= significant at P ≤ 0.05; *** = significant at P ≤0.001

2.Means with the same letter are not significantly different (p = 0.05) according to Student, Newman and Keuls comparison test

3.Drench = 1g of formulation mixed with 1 litre of tap water

4.Capping = 1g of formulation mixed with 1 litre of composted pine bark

5.Seed treatment = application of formulations to seed with Pelgel®

6.Benlate® + Previcur® = 1g of Benlate® and 1.2 ml of Previcur® were added to 1 litre of tap water and thereafter drenched on seed at planting

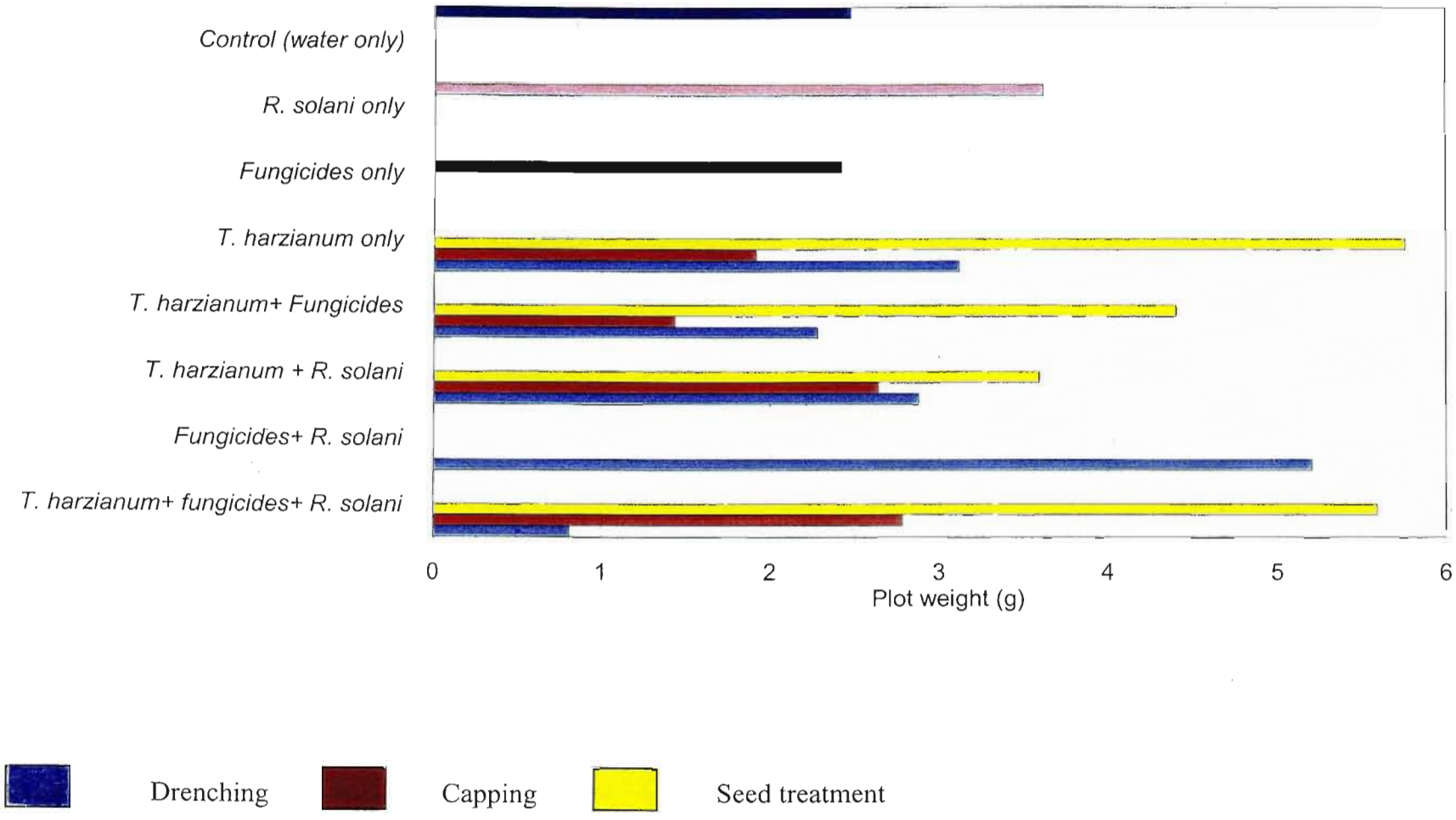

Figure 5.22 Effect of selected fungicides and a commercial formulation containing a biocontrol agent *Trichoderma harzianum* KMD on plot weight using three application methods on Namaqualand daisy seedlings artificially diseased by *Rhizoctonia solani* after four weeks of growth.

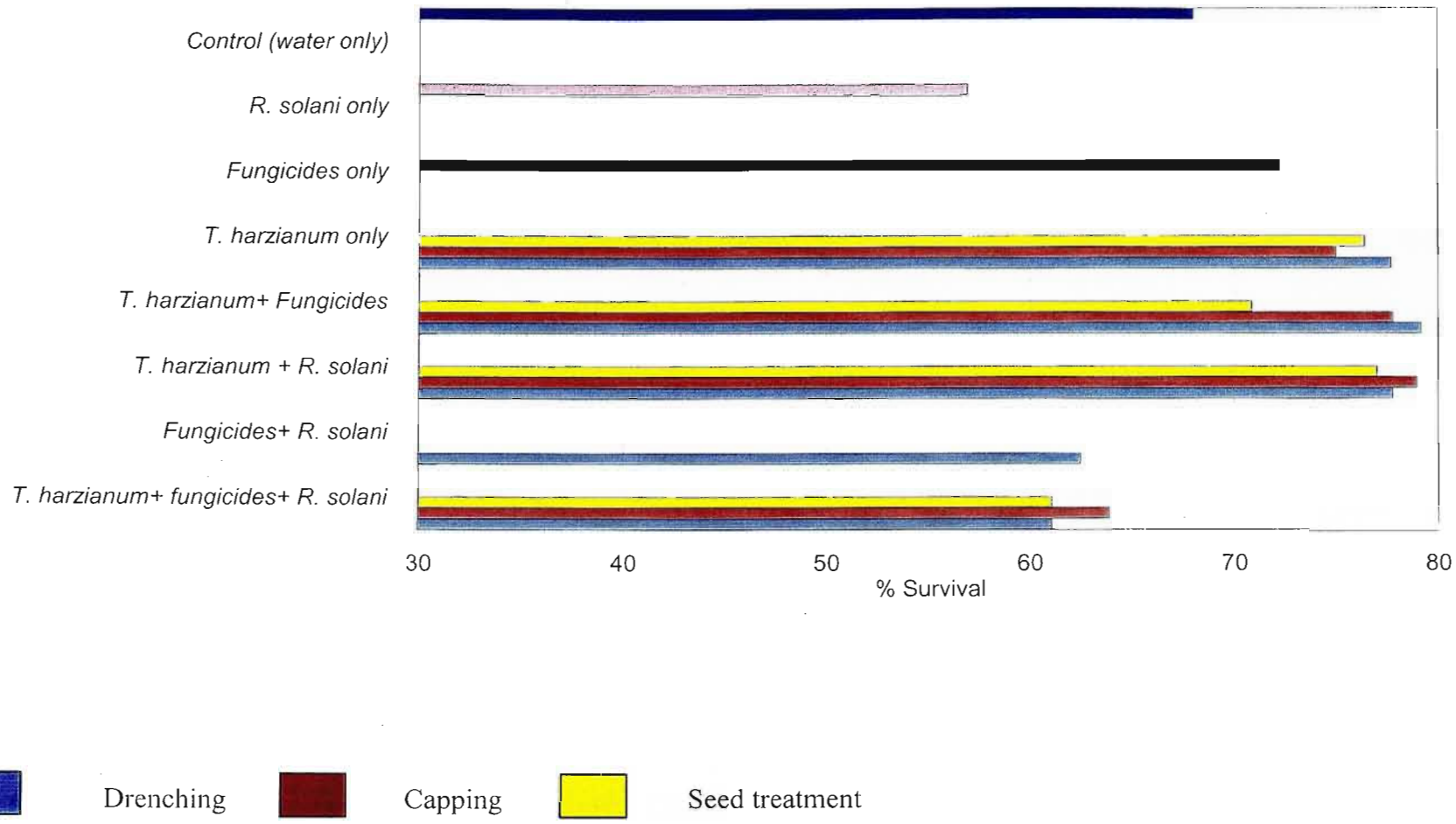

Figure 5.23 Effect of selected fungicides and a commercial formulation containing a biocontrol agent *Trichoderma harzianum* KMD on percentage survival using three application methods on Namaqualand daisy seedlings artificially diseased by *Rhizoctonia solani* after four weeks of growth.

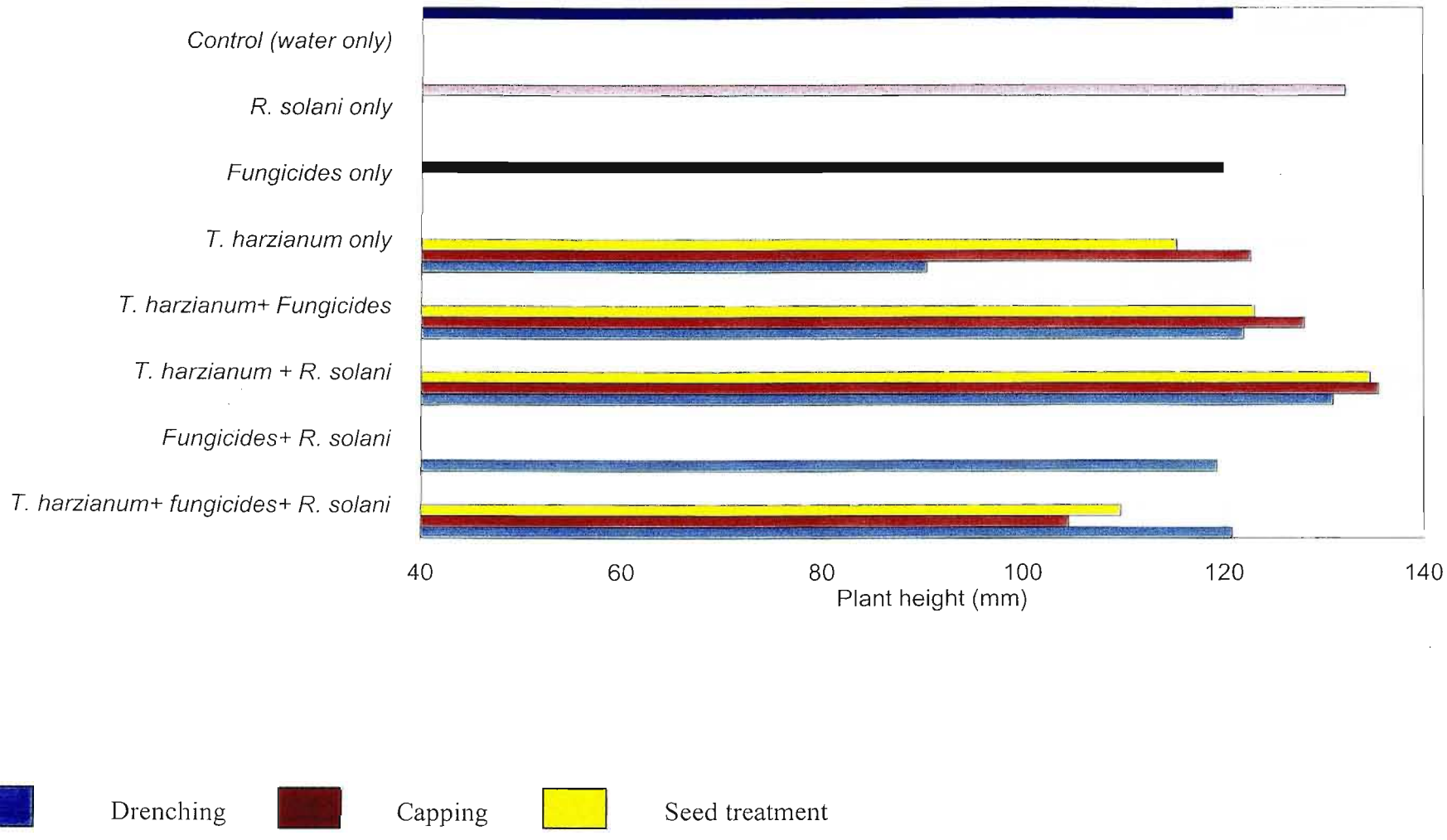

Figure 5.24 Effect of selected fungicides and a commercial formulation containing a biocontrol agent *Trichoderma harzianum* KMD on plant height using three application methods on Namaqualand daisy seedlings artificially diseased by *Rhizoctonia solani* after four weeks of growth

Table 5.10 Effect of a commercial formulation of *Trichoderma harzianum* KMD, with fungicides Benlate ® and Previcur® on damping-off caused by *Rhizoctonia solani* of Eucalyptus after 4 weeks

Treatments	Application	Plot weight (g)	% Control 1 (*Rhizoctonia solani* only)	% Survival	% Control 1 (*Rhizoctonia solani* only)	Plant height (mm)	% Control 1 (*Rhizoctonia solani* only)
Trichoderma harzianum	Drenching	3.60 c	94.99	84.72 a	109	69.7 b	100.29
Trichoderma harzianum	Capping	4.32 a	113.98	83.33 a	107	104.6 b	150.50
Trichoderma harzianum	Seed treatment	4.32 a	113.98	77.78 a	100	134.3 a	193.24
Trichoderma harzianum + Benlate® + Previcur®	Drenching	3.05 c	80.42	84.70 a	109	126.0 a	181.29
Trichoderma harzianum + Benlate® + Previcur®	Capping	3.53 c	93.09	83.30 a	107	143.0 a	205.76
Trichoderma harzianum + Benlate® + Previcur®	Seed treatment	3.36 c	88.65	81.90 a	105	147.0 a	211.51
Trichoderma harzianum + *Rhizoctonia solani*	Drenching	4.32 a	113.98	88.00 a	113	143.0 a	205.76
Trichoderma harzianum + *Rhizoctonia solani*	Capping	5.04 a	132.98	85.00 a	109	150.0 a	215.83
Trichoderma harianum + *Rhizoctonia solani*	Seed treatment	4.80 a	126.65	86.00 a	111	162.3 a	233.53
Trichoderma harzianum + Benlate® + Previcur®+ *Rhizoctonia solani*	Drenching	1.57 d	41.35	69.443 a	89	71.50 b	102.88
Trichoderma harzianum + Benlate® + Previcur® +*Rhizoctonia solani*	Capping	4.87 b	128.55	66.67 a	86	72.67 b	104.56
Trichoderma harzianum + Benlate® + Previcur®+ *Rhizoctonia solani*	Seed treatment	6.70 a	176.68	80.55 a	104	80.17 b	115.35
Control 1 (*Rhizoctonia solani* only)	Nil	3.79 c	100.05	77.77 a	100	69.5 b	100.00
Control 2 (water only)	Nil	3.70 c	97.52	80.55 a	104	158.4 a	227.91
Control 3 Benlate® + Previcur®+ *Rhizoctonia solani*	Drenching	6.19a	163.38	61.11 a	79	97.40 b	140.14
Control 4 Benlate® + Previcur®	Drenching	3.60 c	94.99	75.00 a	96	133.0 a	191.37
Effects		**P-values**		**P-values**		**P-values**	
Treatments		0.0001***		0.375 NS		0.0001***	
Applications		0.0001***		0.136 NS		0.056 NS	
Treatments*Applications		0.0001***		0.159 NS		0.012**	
		CV%= 27.89		%CV = 10.22		%CV = 14.51	
		MSE= 1.59		MSE =7.38		MSE =1.32	

1.NS = Not significant; ** = significant at P ≤ 0.05; *** = significant at P ≤0.001
2.Means with the same letter are not significantly different (p = 0.05) according to Student, Newman and Keuls comparison test
3.Drench = 1g of formulation mixed with 1 litre of tap water
4.Capping = 1g of formulation mixed with 1 litre of composted pine bark
5.Seed treatment = application of formulations to seed with Pelgel®
6.Benlate® + Previcur® = 1g of Benlate® and 1.2 ml of Previcur® were added to 1 litre of tap water and thereafter drenched on seed at planting

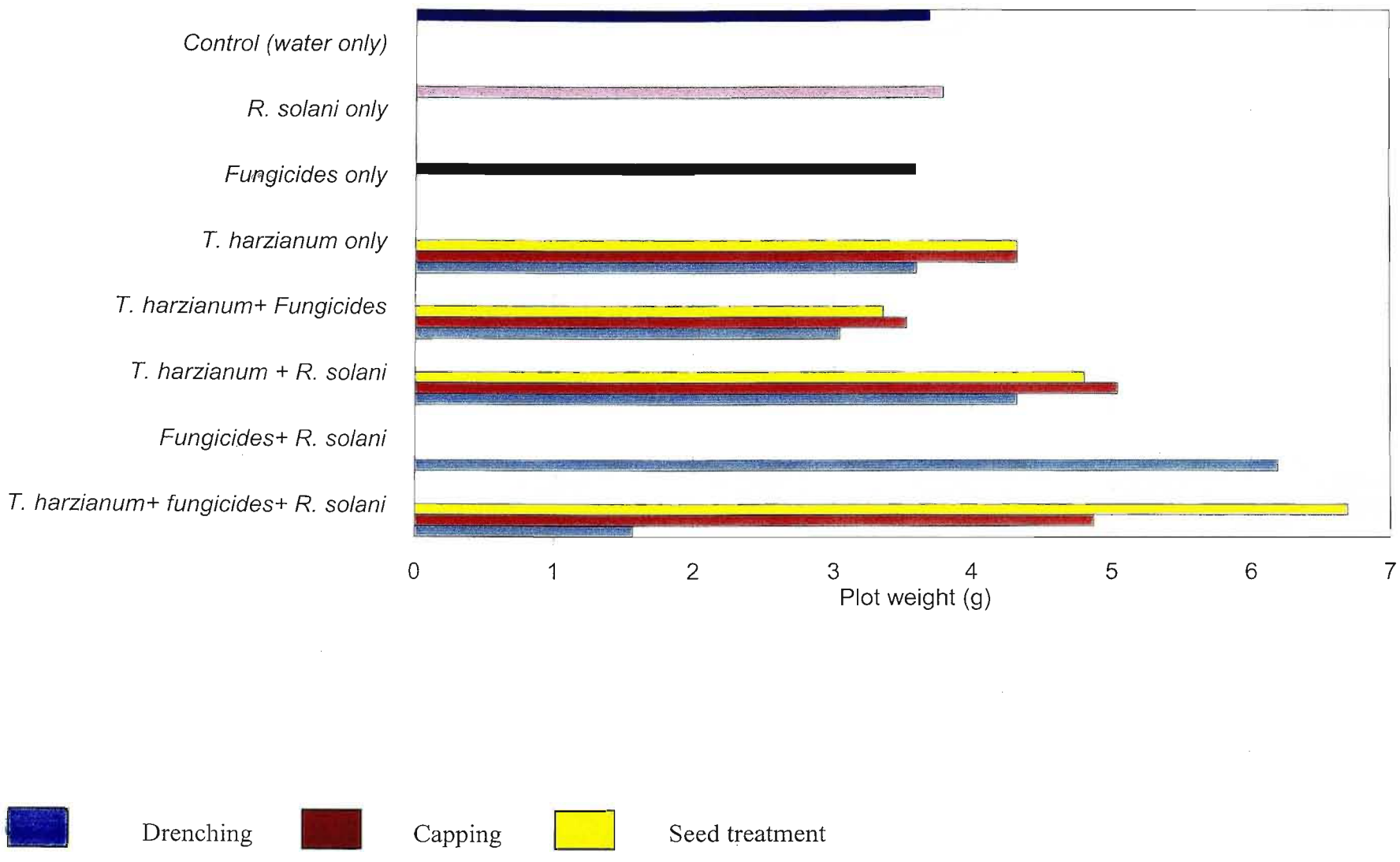

Figure 5.25 Effect of selected fungicides and a commercial formulation containing a biocontrol agent *Trichoderma harzianum* KMD on plot weight using three application methods on Eucalyptus seedlings artificially diseased by *Rhizoctonia solani* after four weeks of growth.

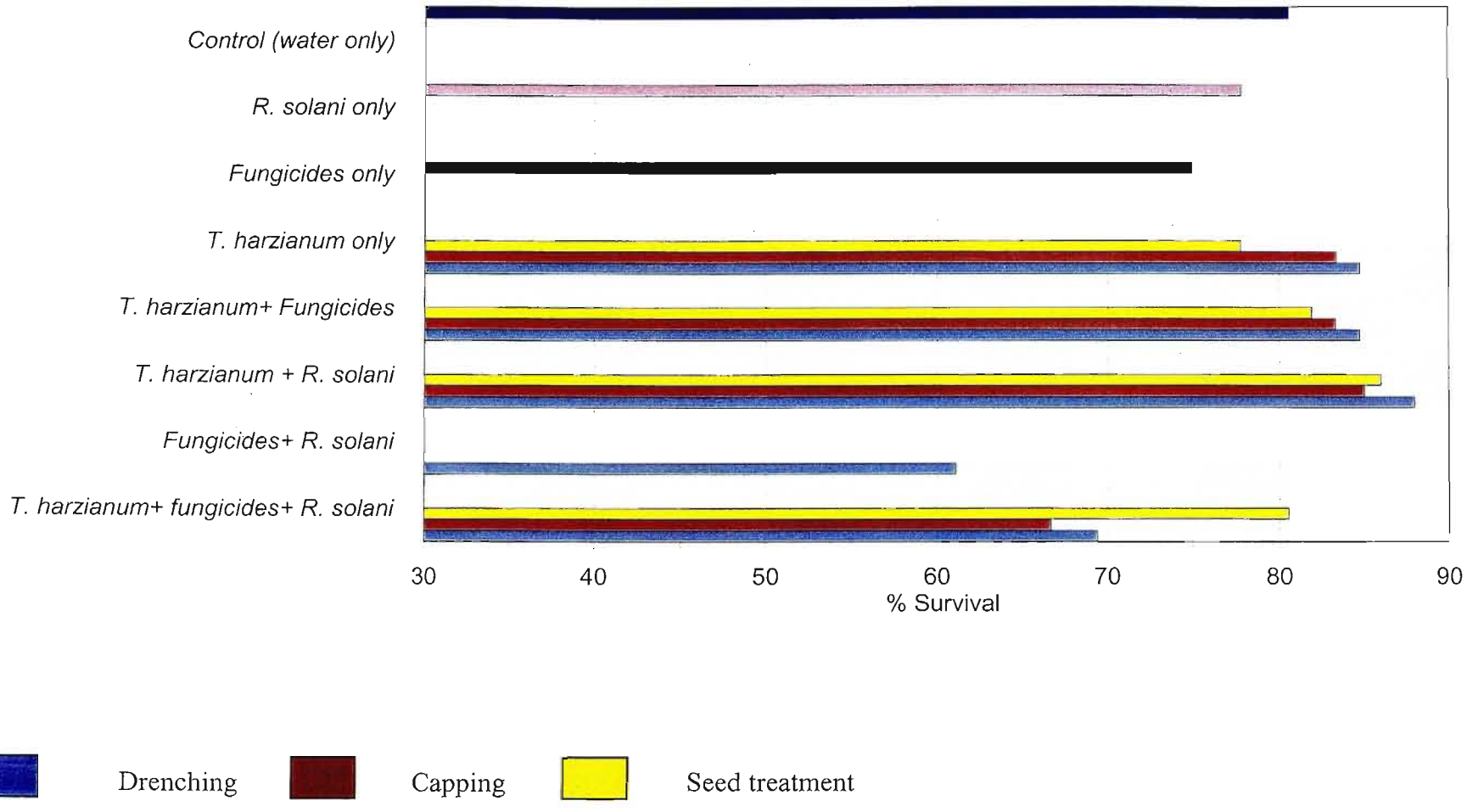

Figure 5.26 Effect of selected fungicides and a commercial formulation containing a biocontrol agent *Trichoderma harzianum* KMD on percentage survival using three application methods on Eucalyptus seedlings artificially diseased by *Rhizoctonia solani* after four weeks of growth.

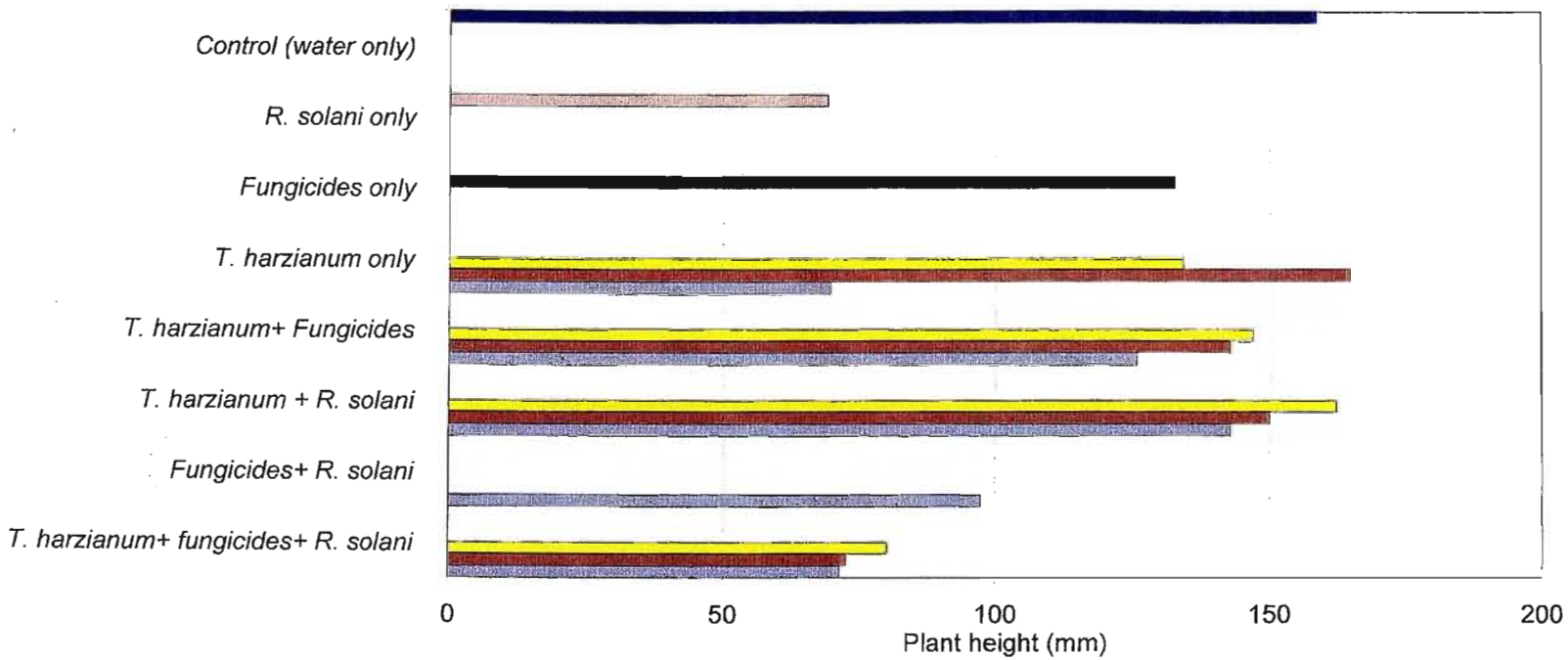

Figure 5.27 Effect of selected fungicides and a commercial formulation containing a biocontrol agent *Trichoderma harzianum* KMD on plant height using three application methods on Eucalyptus seedlings artificially diseased by *Rhizoctonia solani* after four weeks of growth

Table 5.11 Effect of a commercial formulation of *Trichoderma harzianum* KMD, with fungicides Benlate® and Previcur® on damping-off caused by *Rhizoctonia solani* of tomato after four weeks

Treatments	Application	Plot weight (g)	% Control 1 (*Rhizoctonia solani* only	% Survival	% Control 1 (*Rhizoctonia solani* only)	Plant height (mm)	% Control 1 (*Rhizoctonia solani* only)
Trichoderma harzianum	Drenching	3.84 a	75.15	65.28 bc	89	262.0 a	163
Trichoderma harzianum	Capping	3.60 a	70.45	95.83 a	130	261.1 a	162
Trichoderma harzianum	Seed treatment	6.24 a	122.11	90.27 a	123	309.3 a	192
Trichoderma harzianum + Benlate® + Previcur®	Drenching	2.42 c	47.44	86.11 a	117	259 a	161
Trichoderma harzianum + Benlate® + Previcur®	Capping	3.50 bc	68.57	93.06 a	126	254.5 a	158
Trichoderma harzianum + Benlate® + Previcur®	Seed treatment	4.90 b	95.81	97.20 a	132	267 a	166
Trichoderma harzianum + *Rhizoctonia solani*	Drenching	3.96 a	77.50	88.00 a	120	260 a	162
Trichoderma harzianum + *Rhizoctonia solani*	Capping	4.01 a	78.43	89.00 a	121	250.0 a	156
Trichoderma harianum + *Rhizoctonia solani*	Seed treatment	4.03 a	78.90	87.00 a	118	270.0 a	168
Trichoderma harzianum + Benlate® + Previcur®+ *Rhizoctonia solani*	Drenching	4.03 bc	78.90	79.16 abc	108	186.1 a	116
Trichoderma harzianum + Benlate® + Previcur®+ *Rhizoctonia solani*	Capping	5.50 b	107.55	77.78 abc	106	159.0 a	98
Trichoderma harzianum + *Benlate® + Previcur®+ Rhizoctonia solani*	Seed treatment	7.42 a	145.13	83.33 ab	113	161.6 a	101
Control 1(*Rhizoctonia solani* only)		5.11 b	100.00	73.610 bc	100	160.70 a	100
Control 2 (water only)	Nil	2.26 c	44.15	97.220 a	132	241.20 a	150
Control 3 Benlate® + Previcur®+ *Rhizoctonia solani*	Drenching	7.37 a	144.19	83.33 ab	113	190.0 a	118
Control 4 Benlate® + Previcur®	Drenching	3.60 bc	70.45	94.40 a	128	268.0 a	167
Effects		**P-values**		**P-values**		**P-values**	
Treatments		0.0019***		0.0048***		0.1124^{NS}	
Applications		0.0051***		0.046^{NS}		0.542^{NS}	
Treatments*Applications		0.0001***		0.023^{NS}		0.784^{NS}	
		CV%= 17.0		%CV = 10.37		%CV = 24.70	
		MSE= 3.56		MSE = 8.23		MSE =4.78	

1.NS = Not significant; **= significant at P ≤ 0.05; *** = significant at P ≤0.001
2.Means with the same letter are not significantly different (p = 0.05) according to Student, Newman and Keuls comparison test
3.Drench = 1g of formulation mixed with 1 litre of tap water
4.Capping = 1g of formulation mixed with 1 litre of composted pine bark
5.Seed treatment = application of formulations to seed Pelgel®
6.Benlate® + Previcur® = 1g of Benlate® and 1.2 ml of Previcur® were added to 1 litre of tap water and thereafter drenched on seed at planting

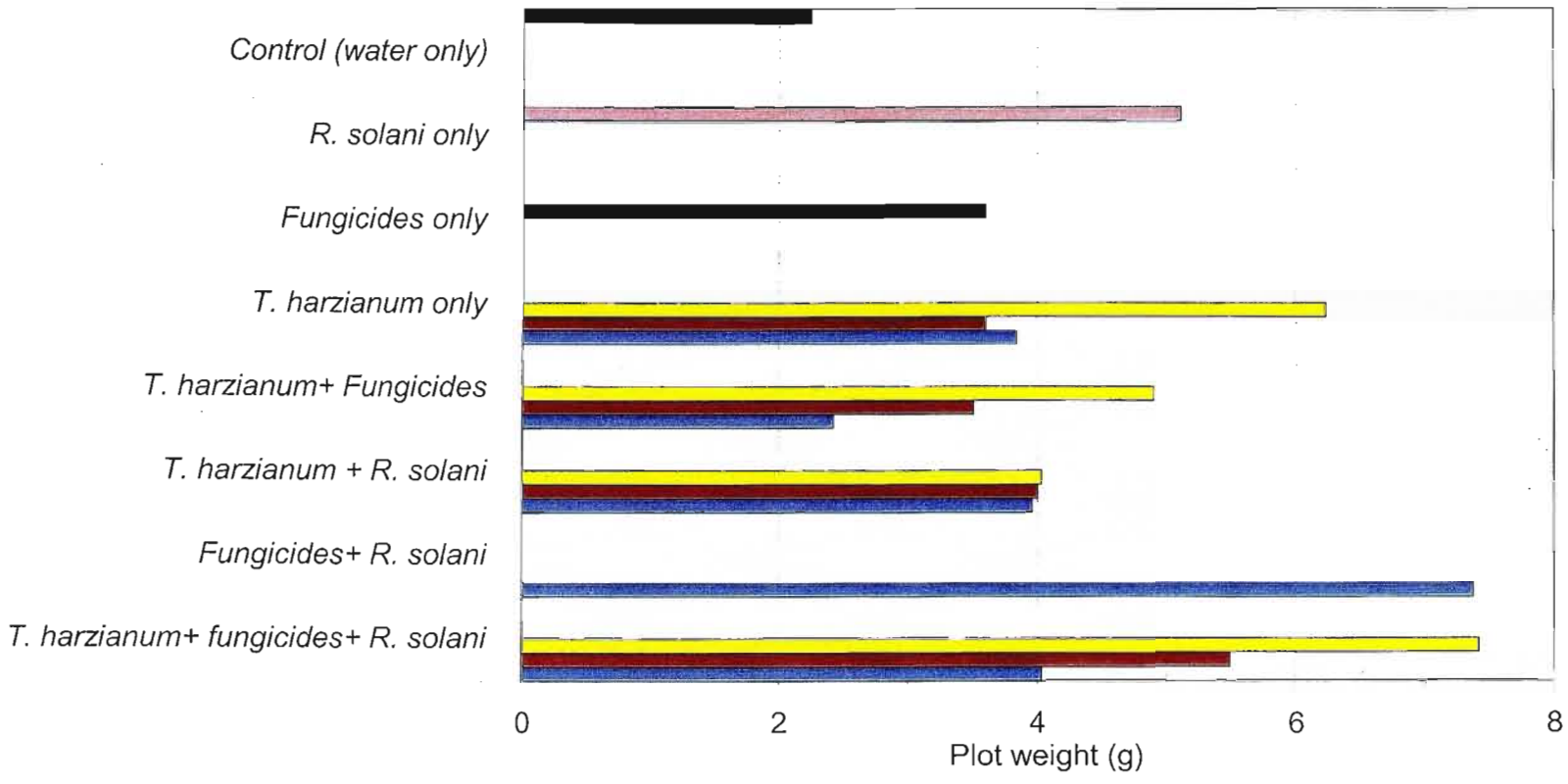

Figure 5.28 Effect of selected fungicides and a commercial formulation containing a biocontrol agent *Trichoderma harzianum* KMD on plot weight using three application methods on tomato seedlings artificially diseased by *Rhizoctonia solani* after four weeks of growth.

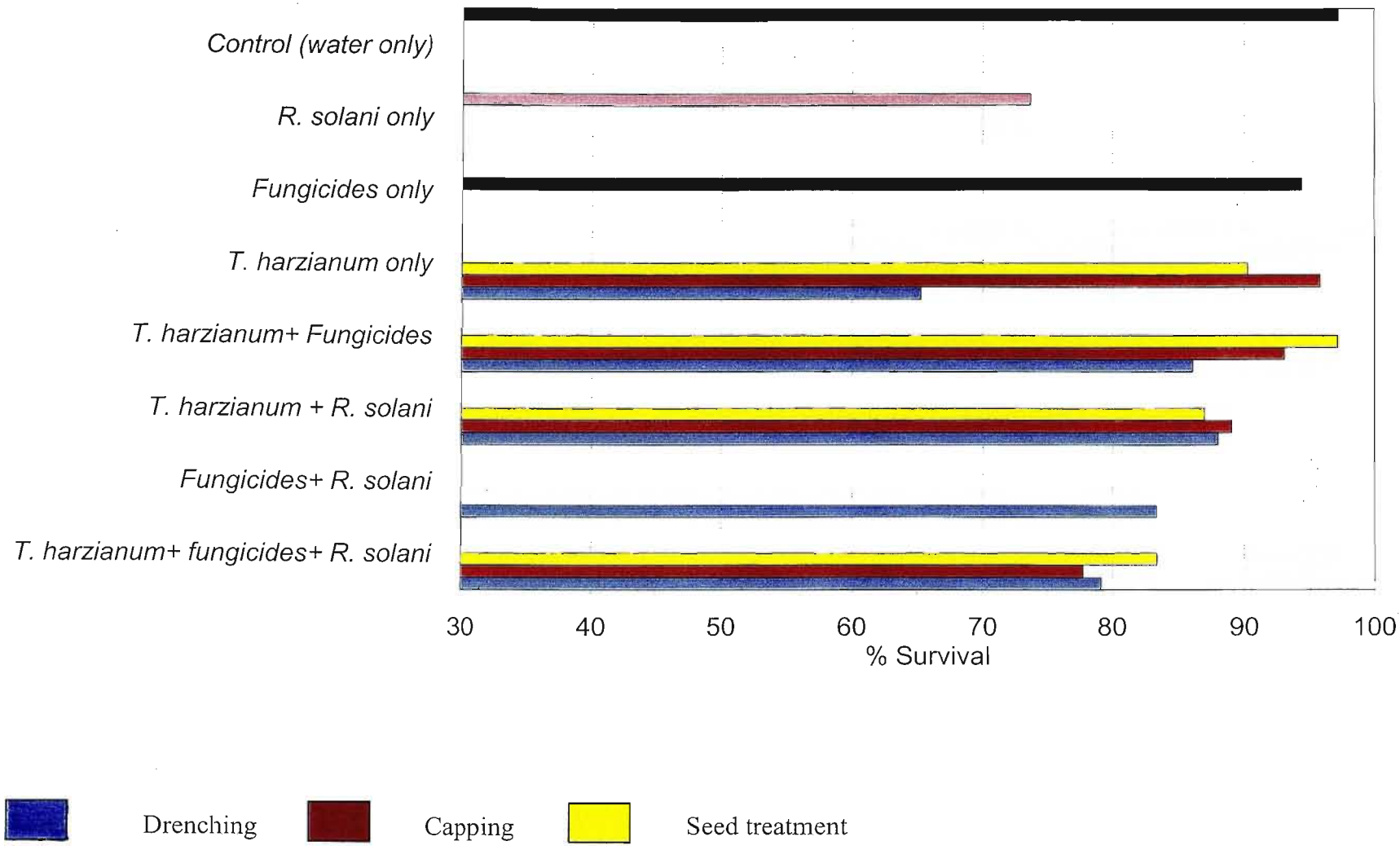

Figure 5.29 Effect of selected fungicides and a commercial formulation containing a biocontrol agent *Trichoderma harzianum* KMD on percentage survival using three application methods on tomato seedlings artificially diseased by *Rhizoctonia solani* after four weeks of growth.

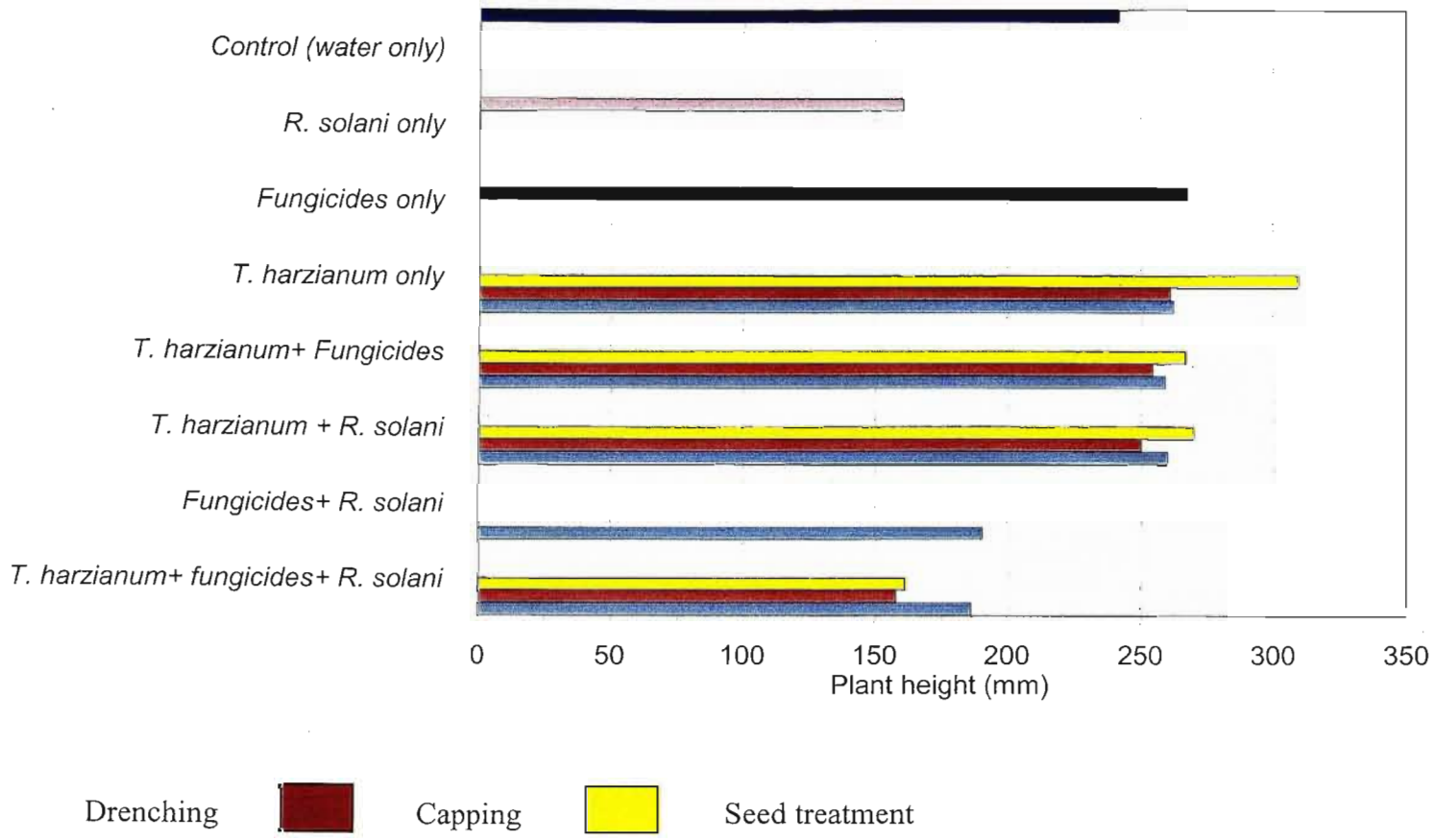

Figure 5.30 Effect of selected fungicides and a commercial formulation containing a biocontrol agent *Trichoderma harzianum* KMD on plant height using three application methods on tomato seedlings artificially diseased by *Rhizoctonia solani* after four weeks of growth.

Plant height ranged from 130-150 mm when compared to the diseased control (*Pythium* sp. only) which recorded to be 80.3 mm in height. All treatments with *T. harzianum* KMD only and treatments with the combination of *T. harzianum* KMD applied to non-infested composted pine bark with fungicides produced taller plants.

No significant differences were observed between application techniques for plot weight and percentage survival. Significant differences were recorded between application techniques for plant height. There were major differences noted for treatments with the commercial formulation only applied to infested and non-infested controls. This formulation increased plant height to 92% with drenching, against 86% with capping and 73% with seed treatment when compared to disease infested control (*Pythium* sp. only).

Treatments applied with the commercial formulation to *Pythium* infested composted pine bark also resulted in major differences between application techniques. Increases in plant height applied as a seed treatment increased to 89% against 81% with capping and against 62% with drenching when compared to the diseased control (*Pythium* sp. only). Similar trends were observed for treatments applied with the commercial formulation and fungicides to non-infested composted pine bark. Treatments of formulation and fungicides applied to *Pythium* sp. infested composted pine bark, resulted in seed treatment being the best application to increase plant height. Mean plant height increased by 41% against 16% with capping when compared to the diseased control (*Pythium* sp. only).

There were no significant interaction differences between treatments and application techniques for plot weight and percentage survival. However, this interaction recorded significant differences for plant height.

Cucumber seedlings

No significant differences were recorded between treatments for plot weight (Table 5.3 Figure 5.4, 5.5, 5.6). However when treatments were compared to the disease infested control (*Pythium* sp. only), a reduction in disease and an increase in plot weight ranging from 27% to 130% was observed when compared to all the controls.

Significant differences between treatments for percentage survival of seedlings and plant height were observed. Treatments applied with the commercial formulation only resulted in an increase in percentage survival ranging from 48% -61% when compared to the diseased control (*Pythium* sp. only). Percentage survival rates were comparable to the control (water only). Treatments applied with the commercial product, *T. harzianum* KMD, to *Pythium* sp. infested composted pine bark resulted in healthy percentage survival rates ranging from 51-60% when compared to the diseased control (*Pythium* sp. only). When the commercial product was applied with fungicides to non-infested composted pine bark, percentage survival increased by 45-61% when compared to the diseased controls. Treatments applied with the commercial formulation and fungicides to *Pythium* sp. infested composted pine bark resulted in lower percentage survival rates when compared to other treatments. Fungicides alone applied to non-infested and infested-composted pine bark resulted in percentage survival rates that were comparable to the control (water only).

Highly significant differences were observed for plant height. Treatments applied with only the commercial formulation to non-infested composted pine bark were observed to have high plant heights ranging from (189-309%) when compared to *Pythium* sp. infested controls. Similar recordings were observed for those treatments with the application of the formulation and fungicides to non-infested composted pine bark. Treatments applied with the commercial product only to *Pythium* sp. infested-composted pine bark resulted in plant height greater (194% -242%) than that of the disease control. Similar recordings were observed for those treatments with the application of the formulation and fungicides to *Pythium* sp. infested composted pine bark. Fungicides only applied to infested and non-infested composted pine bark also recorded an increase in plant height ranging from 185-321% when compared to the diseased control (*Pythium* sp. only).

Significant differences (P=0.05) between application techniques were observed for percentage survival. Treatments applied with the commercial product and fungicides to *Pythium* sp. infested composted pine bark resulted in marginal differences between application techniques. Seed treatments exhibited an increase in percentage survival by 48% against 16% with capping and 9%with drenching when compared to the diseased control (*Pythium* sp. only). No significant differences between application techniques were observed for other treatments.

Significant differences were observed for the interaction of treatments and application methods for percentage survival. However, no significant differences were observed for other parameters tested.

Namaqualand daisy seedlings

Highly significant differences were observed between treatments for all parameters tested on Namaqualand daisy (Table 5.4, Figure 5.7, 5.8, 5.9). With respect to plot weight, treatments that are applied with the commercial formulation of *T. harzianum* KMD to *Pythium* sp. infested composted pine bark reduced disease by 79-92% compared to the diseased control (*Pythium* sp. only). The commercial formulation alone had a poor response on plot weight when compared to other treatments but was comparable to the control (water only). Treatments of the commercial formulation applied with fungicides to non-infested and infested composted pine bark had a better response in plot weight when seeds of Namaqualand daisy were treated. Fungicides only, applied to infested composted pine bark increased plot weight by 43% while fungicides applied to non-infested composted pine bark had no increase in plot weight and were comparable to the water control.

Significant differences were observed between application techniques for plot weight. Major differences between treatments applied with the commercial formulation and with fungicides to infested composted pine bark caused an increase in plot weight by 52% applied with a seed treatment. Treatments applied with the commercial formulation with fungicides to non-infested composted pine bark, resulted in differences between application techniques. Increases in plot weight by 21% with seed treatment were recorded when compared to controls, *Pythium* sp. only and the water control.

Significant differences were recorded between treatments for percentage survival. Treatments of the commercial formulation only, applied to *Pythium* sp. infested composted pine bark reduced disease and gave healthy plant survival rates ranging from 55-58% when compared to the diseased control (*Pythium* sp. only). Treatments of the commercial formulation applied with fungicides to *Pythium* sp.infested and non-infested composted pine bark recorded percentage survival rates ranged from 37-46% which were comparable to treatments applied with fungicides, to *Pythium* sp. infested composted pine bark.

Significant differences were recorded for plant height. All treatments were significantly taller than the control (*Pythium* sp. only) and controls (water only). Plants treated with fungicides only were also considerably taller by 56-69% when compared to both diseased and water controls.

Significant differences were recorded between application techniques for plot weight and percentage survival. Major differences were observed in treatments applied with the commercial formulation and fungicides to non-infested composted pine bark. Seed treatment gave healthy percentage survival rates, which increased against the diseased control by 34% against 36% with drenching against 32% with capping. Differences were also observed for application techniques when the commercial formulation was applied alone. Drenching gave a healthy percentage survival greater than the diseased control by 39% against 32% with capping when compared to disease controls (*Pythium* sp. only). No differences were recorded between application techniques on treatments applied with the commercial formulation and fungicides to infested composted pine bark. No significant differences were observed between application techniques for plant height measured.

The interaction between treatments and applications resulted in significant differences for plot weight and percentage survival when compared to the diseased and water controls.

Eucalyptus seedlings

Significant differences between treatments were recorded for all parameters tested (Table 5.5 Figure 5.10, 5.11, 5.12). Treatments applied with the commercial formulation to *Pythium* sp. infested composted pine bark reduced disease and increased plot weight by 33-62% when compared to the diseased control. The commercial formulation applied alone to non-infested composted pine bark recorded poor plot weights when compared to the diseased and water controls. Treatments with *T. harzianum* KMD and fungicides to non-infested composted pine bark increased plot weight to 70-199% when compared to the diseased controls and water only. Treatments with the combination of fungicides and the commercial formulation applied to infested composted pine bark also reduced disease and increased plot weight by 28-76% when compared to the diseased control (*Pythium* sp. only). Fungicides applied to infested composted pine bark also increased plot weight and reduced disease by 63% when compared to *Pythium* sp. only.

All treatments had a higher percentage survival when compared to the diseased control (*Pythium* sp. only). Percentage survival ranged from 41-50% when compared to the diseased control. No significant differences were observed between treatments applied with fungicides only. Similar recordings were obtained for plant height. Most treatments were significantly taller than the diseased and water controls.

Significant differences were observed for plot weight, percentage survival and plant height with respect to application techniques. Differences in application techniques were recorded for treatments applied with *T. harzianum* KMD with fungicides to infested and non-infested composted pine bark. Treatments applied with the formulation and fungicides to infested composted pine bark resulted in differences between application techniques. For these treatments plot weight increased when applied as a seed treatment by 76% against 28% with capping when compared to water control. Treatments applied with formulation and fungicides to non-infested composted pine bark increased plot weight to 199% with capping against 70% with drenching when compared to the control (*Pythium* sp. only).

Significant differences were also observed for percentage survival. Significant differences were observed for treatments applied with the combination of formulation and fungicides to infested composted pine bark. Significant differences were also observed on plant height when compared to controls, *Pythium* sp. control and water control.

The interaction between treatments and application was significant (p=0.05) for percentage survival of Eucalyptus and not for plot weight and plant height.

Tomato seedlings

Highly significant differences were observed for plot weight (P=0.001) for all interactions recorded (Table 5.6 Figure 5.13, 5.14, 5.15). Major differences for plot weight were noted for treatments applied with the commercial formulation and fungicides to infested and non-infested composted pine bark. For most treatments tested, increases in disease and a decrease in plot weight were recorded when compared to the controls (*Pythium* sp. only).

Most treatments recorded a high percentage survival compared to the diseased controls. Treatments with the commercial product only, resulted in an increase of 87-112% percentage

survival while treatments with the commercial formulation applied with fungicides to non-infested composted pine bark gave a healthy increase in percentage survival by 42-109% when compared to the control (*Pythium* sp. only). Treatments with the addition of the commercial formulation only, applied to *Pythium* infested composted pine bark gave percentage survivals ranging from 90-94%, while treatments with the commercial formulation applied with fungicides to non-infested composted pine bark gave percentage survival rates ranging from 42-90% when compared to the diseased controls and were comparable to the water control.

Most treatments measured were significantly different for plant height when compared to the *Pythium* sp. control. Treatments applied with the commercial formulation to infested composted pine bark were comparable in height to the water control.

There were significant differences between application techniques for plot weight and percentage survival. There were major differences for treatments applied with the commercial formulation and with fungicides to infested composted pine bark and in non-infested composted pine bark. For example, treatments with the commercial formulation applied with fungicides only to infested composted pine bark increased percentage survival by 40% with capping against 81% with seed treatment against 42% with drenching when compared with controls (*Pythium* sp. only). Similar recordings were obtained for this treatment to non-infested composted pine bark. No significant differences were recorded for mean plant height.

Significant differences were recorded for the interaction treatments and application for plot weight and percentage survival.

5.3.3 Effect of a commercial formulation with selected fungicides on damping-off caused by *Rhizoctonia solani*

Cabbage seedlings

Significant differences between treatments were recorded for all parameters tested (Table 5.7 Figure 5.16, 5.17, 5.18). Highly significant differences were recorded between plot weights of treatments. Treatments with the commercial formulations applied to *R. solani* infested composted pine bark, caused a reduction in damping-off and increased plot weight by 94%-

168% when compared to the diseased control (*R. solani* only). However treatments of the commercial formulation applied with fungicides to infested composted pine bark caused poor plot weights. It is noteworthy that fungicides alone, applied to infested and non-infested composted pine bark gave comparable plot weights to the diseased controls.

Highly significant differences were recorded between all treatments for percentage survival. Major differences were noted for treatments applied with formulation and fungicides to infested composted pine bark. Increases in plot weight for this treatment ranged from 128-389% when compared to the diseased controls. Most of the treatments gave percentage survival rates that were comparable to the water control and were significantly better than the diseased control.

Significant differences were recorded for plant height between treatments. Major differences were noted between treatments of the commercial formulation applied with fungicides to infested composted pine bark. Other treatments were comparable to the water control. *Rhizoctonia solani* infested plants were unhealthy with symptoms of damping-off after 2-4 weeks of growth were evident.

Application techniques were significant for plot weight, percentage survival and plant height. For example, major differences were observed when the commercial formulation was applied with fungicides to *R. solani* infested composted pine bark. Application with seed treatment increased plot weight by 32% while drenching and capping had no significant effect on plot weight.

Application techniques were recorded highly significant for percentage survival. Major differences were noted with treatments of the commercial formulation applied with fungicides to infested composted pine bark. This treatment applied as a drench enhanced survival by 389% when compared to capping which increased percentage survival by 128% and 139% with seed treatment when compared to the diseased control, *R. solani* only. Application techniques resulted in a similar result to other treatments. Significant differences were also recorded between application methods for plant height for this treatment. Seed treatment gave a better result by increasing plant height by 400% against 178% with capping and 180% drenching when compared to the diseased controls.

The interaction of main effects, treatments and application techniques showed significant results for all parameters tested.

Cucumber seedlings

Highly significant differences for percentage survival and plant height were noted (Table 5.8 Figure 5.19, 5.20, 5.21). Major differences were noted between most treatments when compared to fungicides only and the diseased controls. All treatments applied to infested composted pine bark enhanced percentage survival ranging from 700-1000 % when compared to the disease control and were comparable to the water control. All treatments resulted in significant increases in plant height, were significantly taller than the disease controls. No significant differences were noted between application techniques.

Namaqualand daisy seedlings

The two main effects were recorded to be highly significant on plot weight (Table 5.9 Figure 5.22, 5.23, 5.24). No significant differences were recorded for the main effects on percentage survival and plant height. Treatments applied with fungicides only, to infested composted pine bark increased plot weight by 43% when compared to the diseased control. Treatments of the combination of the commercial formulation with fungicides and treatments applied with the commercial formulation only to non-infested composted pine bark increased plot weight by 21% and 59%, respectively. All other treatments had no significant effect on plot weight.

Application techniques recorded significant effects on plot weight. Seed treatments of all treatments increased plot weight better than other application techniques. For example, treatments with the commercial formulation applied with fungicides to infested composted pine bark increased plot weight when applied as a seed treatment. Similar recordings were observed for this treatment applied to non-infested composted pine bark.

The interaction of the main effects was significant for plot weights but was not significant for other parameters tested.

Eucalyptus seedlings

Significant differences were recorded between treatments for most parameters tested (Table 5.10 Figure 5.25, 5.26, 5.27). Major differences were noted for treatments applied with commercial formulation with fungicides to infested composted pine bark, which increased plot weight by 28-76% when compared to the diseased control. Major differences were also noted between application techniques for this treatment. Differences in this treatment were noted for plant height when compared to the diseased control. Treatments applied with the commercial formulation alone to infested composted pine bark also increased plot weight by 13-32% when compared to the controls. Fungicides applied alone to infested composted pine bark increased plot weight by 63% when compared to the diseased control, *R. solani*

The interaction of main effects treatments and application methods used were found to be significant for plot weight and plant height.

Tomato seedlings

Significant differences were recorded between treatments for plot weight and percentage survival (Table 5.11 Figure 5.28, 5.29, 5.30). Major differences were noted for treatments applied with the commercial formulation with fungicides to infested composted pine bark when compared to the diseased control, *R. solani.* For example, the commercial formulation applied alone to non-infested composted pine bark, gave an increase in plot weight by 22% whilst other treatments increased plot weight by 45% when *T. harzianum* KMD was applied with fungicides to infested composted pine bark. Reduction in disease and increases in plot weight for this treatment ranged from 7-45% when compared to the diseased control. Plot weight decreased when *T. harzianum* KMD was applied with fungicides to non-infested composted pine bark. Differences were noted between application techniques for plot weight and percentage survival.

The interactions between the main effects were only significant for plot weight and were not significant for percentage survival and plant height.

5.4 DISCUSSION

In vitro assays showed that *T. harzianum* KMD was highly sensitive to Benlate® and Previcur® after 10 days of incubation at 25° C and became less sensitive after 15 days of incubation at 25° C. Application of fungicides with *T. harzianum* KMD resulted in resistant mutants of *T. harzianum* KMD which failed to sporulate. However, the greenhouse trials were less extreme, with no combination of fungicides completely suppressing the activity of *T. harzianum* KMD in the composted pine bark.

Farmers are both risk and cost averse, and will not implement practices which are difficult to perform or expensive to implement, unless they provide rapid, clearly visible, and substantial savings. Fungicide applications were used before the advent of biocontrol agents. Hence in this research fungicides were applied first. This would determine the effect of the application of *T. harzianum* KMD in direct contact with the fungicides used, as it would portray a more realistic picture of what would happen under normal field conditions.

5.4.1 Effect of a commercial formulation of *Trichoderma harzianum* KMD applied with selected fungicides on *Pythium* sp.

Greenhouse results are a more realistic assessment of the compatibility of *T. harzianum* KMD with the various fungicides, since it is unlikely that the level of direct contact between the fungus and fungicide observed in the *in vitro* assay would occur in the greenhouse environment, given the strong buffering capacity of composted pine bark. However, the *in vitro* results allow us to better explain the trends detected in the greenhouse trials.

Table 5.12 shows significant and non-significant results as a matrix of treatments and parameters, which were tested on all crops against *Pythium* sp.

Cabbage seedlings

When *T. harzianum* KMD was applied alone to non-infested composted pine bark, significant differences were recorded on one or more of the parameters tested when compared to the controls water only, fungicides only; *Pythium* sp. only and fungicides applied to *Pythium* sp. infested composted pine bark (Table 5.12). Significant differences were recorded between application techniques suggesting that at least one application technique performed better or

worse than the other two. For example, capping performed the worst and drenching and seed treatment performed the best in effectively delivering *T. harzianum* KMD to composted pine bark.

When *T. harzianum* KMD was applied with fungicides to non-infested composted pine bark significant differences were recorded for plant height when compared to the control (water only). Percentage survival and plot weight were not significant for this treatment when compared to the control (water only), suggesting that percentage survival and plot weight were found comparable to the control (water only). This result indicates that the application of *T. harzianum* KMD and fungicides to non-infested composted pine bark performed equally or better to the control (water only). When *T. harzianum* KMD and fungicides were applied to non-infested composted pine bark, significant differences for plot weight and plant height were recorded when compared to the control (fungicides only). This indicates that this treatment performed better than the fungicides applied alone. This result could be related to the presence of *T. harzianum* KMD. *Trichoderma harzianum* KMD may have triggered a reaction that produces growth stimulatory hormones (auxins or gibberellins), which increases plant growth.

When *T. harzianum* KMD was applied as a seed treatment to *Pythium* sp infested composted pine bark, significant differences on plot weight and plant height were recorded when compared to the control (fungicides only). This indicates that this combination is a better treatment that the application of fungicides alone. Significant differences were also recorded on all parameters when compared to the diseased control (*Pythium* sp. only). This result indicates that *T. harzianum* KMD is a good biocontrol agent against *Pythium* sp. on cabbage seedlings. When this treatment was compared to the control (fungicides applied to *Pythium sp.* infested composted pine bark), plot weight and plant height recorded significant differences suggesting that *T. harzianum* KMD performed better than the application of fungicides to *Pythium* sp. infested composted pine bark.

Table 5.12. Shows significant and non-significant results as a matrix of treatments and parameters which were tested on all crops against *Pythium* sp.

	Controls	Water only			Fungicide only			*Pythium* sp. only			Fungicide and *Pythium* sp.		
		PW	**PS**	**PH**	**PW**	**PS**	**PH**	**PW**	**PS**	**PH**	**PW**	**PS**	**PH**
Cabbage	T only	NS	NS	S	S	NS	S	NS	S	S	NS	NS	S
	T + F only	NS	NS	S	S	NS	S	S	S	S	S	NS	S
	T + Py only	NS	NS	S	S	NS	S	S	S	S	S	NS	S
	T+ F + Py only	NS	NS	S	NS	NS	S	NS	S	S	NS	NS	S
Cucumber	T only	NS	NS	S	NS	NS	S	NS	S	S	NS	S	S
	T + F only	NS	NS	S	NS	NS	S	NS	S	S	NS	S	S
	T + Py only	NS	NS	S	NS	NS	S	NS	S	S	NS	S	S
	T+ F + Py only	NS	S	S	NS	S	S	NS	S	S	NS	S	NS
Namaqualand daisy	T only	NS	S	NS	NS	S	NS	NS	S	S	NS	NS	NS
	T + F only	S	S	S	NS	NS	NS	NS	S	S	NS	NS	NS
	T + Py only	S	NS	S	S	S	NS	S	S	S	NS	NS	NS
	T+ F + Py only	S	S	S	S	NS	NS	S	S	S	S	NS	NS
Eucalyptus	T only	NS	NS	S	NS	NS	S	NS	S	S	NS	NS	NS
	T + F only	S	NS	S	S	NS	S	S	S	S	NS	NS	S
	T + Py only	S	NS	S	NS	S	S	NS	S	S	S	NS	NS
	T+ F + Py only	NS	NS	S	NS	NS	S	NS	S	S	NS	NS	NS
Tomato	T only	NS	NS	S	NS	S	S	NS	NS	S	NS	NS	NS
	T + F only	NS	S	S	NS	S	S	NS	S	S	NS	S	NS
	T + Py only	NS	NS	NS	NS	NS	S	NS	S	S	NS	NS	NS
	T+ F + Py only	NS	S	S	NS	S	S	NS	S	S	NS	S	NS

T only = *T. harzianum* KMD only; T + F only = *T. harzianum* KMD applied with fungicides to non-infested composted pine bark; T+ Py only = *T. harzianum* KMD applied to *Pythium* sp. infested composted pine bark; T+F+ Py only = *T. harzianum* KMD applied to *Pythium* sp. infested composted pine bark with fungicides.
PW= plot weight; PS = percentage survival; PH= plant height
S = Significant; NS non-significant

The interaction between treatments and applied techniques was significant for plant height of cabbage seedlings. This indicates that at least one treatment was significantly better or worse in its performance when applied with one or more of the applications.

Overall, *T. harzianum* KMD applied with fungicides to *Pythium* sp. infested composted pine bark performed poorly when applied as a capping and best as a seed treatment or drench. This treatment did not reduce disease for plot weight and percentage survival. However when *T. harzianum* KMD was applied with fungicides to non-infested composted pine bark, growth stimulation occurred. A negative effect occurred on plot weight and percentage survival when *T. harzianum* KMD was applied with fungicides to *Pythium* sp. infested composted pine bark. This phenomenon needs to be investigated further. Thus *T. harzianum* KMD is an integrated scheme with fungicides does not reduce disease as effective as *T. harzianum* KMD applied alone. It is recommended to the cabbage farmer or grower to apply *T. harzianum* KMD without fungicides to reduce disease and increase plot weight and percentage survival.

Cucumber seedlings

Trichoderma harzianum KMD applied alone to non-infested composted pine bark, similarto its performance on cabbage seedlings, when compared to all controls (Table 5.12). This suggests that *T. harzianum* KMD is a good growth stimulatory agent for cucumber seedlings. Significant differences were recorded on plant height when *T. harzianum* KMD was applied with fungicides applied to non-infested composted pine bark when compared to the control (water only) and (fungicides only). No significant differences were noted for plot weight and percentage survival. This suggests that the application of *T. harzianum* KMD with fungicides performed equally or better than the control (water only) and (fungicides only).

When *T. harzianum* KMD was applied with fungicides to *Pythium* sp. infested composted pine bark, percentage survival was consistently significant when compared to all controls. In most instances plot weight and plant height was not significant when compared to all controls. This indicates that if *T. harzianum* KMD is applied with fungicides to infested composted pine bark, plot weight and plant height will be negatively affected.

Recommendations to the farmer or grower is that by applying *T. harzianum* KMD with fungicides to diseased composted pine bark, a reduction in growth of cucumber seedlings will occur.

Namaqualand daisy seedlings

When *T. harzianum* KMD was applied alone to non-infested composted pine bark, significant differences were recorded on percentage survival and plot weight when compared to the control (water only) (Table 5.12). This result indicates that *T. harzianum* KMD applied alone stimulates or enhances percentage survival and plant height better than untreated seeds or seedlings.

However when *T. harzianum* KMD was applied alone to non-infested composted pine bark and was compared to the control (fungicides only) it was evident that plot weight and plant height was not affected but percentage survival was.

Treatments of *T. harzianum* KMD applied to non-infested composted pine bark with fungicides performed well in enhancing plant growth when compared to the control (water only) and performed better than the diseased control (*Pythium* sp. only). This combination was found non-significant when compared to the application of fungicides to infested and non-infested composted pine bark. This suggests that the combination of *T. harzianum* KMD applied with fungicides to non-infested composted pine bark performed equally well as fungicides applied alone.

Significant differences in plot weight and plant height were recorded when *T. harzianum* KMD was applied to *Pythium* sp. infested composted pine bark and was compared to the control (water only). This indicates that *T. harzianum* KMD suppressed disease and enhanced growth equally well as the control (water only).

When *T. harzianum* KMD was applied with fungicides to *Pythium* sp. infested composted pine bark significant diffrernces were recorded on plot weight and perenatage survival. This indicates that *T. harzianum* KMD applied as either a drench, seed treatment or as capping suppressed disease and enhanced growth better than the application of fungicides only. *Trichoderma harzianum* KMD effectively reduced disease when compared to the *Pythium* sp. infested control, however when compared to the fungicides applied to *Pythium* sp. infested control, no significant differences were recorded suggesting that *T. harzianum* KMD applied to non-infested composted pine bark performed equally well as those fungicides applied alone. The combination of *T. harzianum* KMD applied as a seed treatment or drench with

fungicides to *Pythium* sp. infested composted pine bark recorded significant differences when compared to the control (water only) and *Pythium* sp. infested controls, indicating that disease was effectively suppressed. However when this treatment was compared to the controls (fungicide only), significant differences were recorded on plot weight. Similar trends were found when compared to the fungicides and *Pythium* sp. infested controls. Hence both treatments performed equally well.

Overall, the combination of *T. harzianum* KMD applied with fungicides to *Pythium* sp. infested composted pine bark performed equally well as the application of fungicides only to *Pythium* sp. infested composted pine bark. If the farmer or grower wants to suppress disease or stimulate plant growth of Namaqualand daisy seedlings, *T. harzianum* KMD would have to be applied alone. Application of fungicides and *T. harzianum* KMD does not enhance or stimulate growth any better than the applications of fungicides only.

Eucalyptus seedlings

Significant differences were recorded for plant height when *T. harzianum* KMD was applied alone and compared to the control (water only) (Table 5.12). Plot weight and percentage survival were comparable when compared to the control (water only) suggesting that *T. harzianum* KMD may be an effective growth stimulant. The combination of *T. harzianum* KMD applied with fungicides significantly enhanced plot weight and plant height when compared to the control (water only) and (fungicide only). This indicates that *T. harzianum* KMD applied with fungicides performed better than the control (water only) and the application of fungicides only. This treatment recorded significant differences for all parameters when compared to *Pythium* sp. control, suggesting that effective diseased suppression was accomplished. However when this treatment was compared to the control (fungicides applied to *Pythium* sp. infested composted pine bark) no significant differences were recorded for plot weight and percentage survival suggesting that the combination of *T. harzianum* KMD applied with fungicides performed equally well when compared to the control (fungicides applied to *Pythium* sp. infested composted pine bark). In most instances treatments of *T. harzianum* KMD applied to *Pythium* sp. infested composted pine bark reduced disease whn compared to all the controls. Results of treatments of *T. harzianum* KMD applied with fungicides to *Pythium* sp. infested composted pine bark was inconsistent since no major enhances in growth or disease suppression were recorded when compared to all controls.Recommendations to the farmer or grower are that when *T. harzianum* KMD is

applied with fungicides to *Pythium* sp. infested composted pine bark, disease will not be effectively reduced, however the application of *T. harzianum* KMD applied as a seed treatment to *Pythium* sp. infested composted pine bark does reduce disease and enhances growth better that the application of fungicides applied alone.

Tomato seedlings

Trichoderma harzianum KMD applied alone gave comparable results when compared to the control (water only). Treatments consisting of *T. harzianum* KMD with fungicides to non-infested composted pine bark recorded significant differences when compared to the controls (water only; and the application of fungicides to *Pythium* sp. infested composted pine bark) (Table 5.12). This result suggests that the combination of *T. harzianum* KMD applied with fungicides, performed better than the application of fungicides and the diseased control (*Pythium* sp. only).

However when *T. harzianum* KMD was applied to *Pythium* sp. infested composted pine bark, no significant differences were observed when compared to the control (water only). Significant differences were recorded when compared to *Pythium* sp. infested control. This indicates that *T. harzianum* KMD does enhance growth but works equally well as the control (water only and suppresses disease when compared to the *Pythium* sp. infested control. This treatment worked equally well as the application of fungicides only to infested and non-infested composted pine bark.

The combination of *T. harzianum* KMD applied with fungicides to *Pythium* sp. infested composted pine bark was found significant when compared to the control (water only) and (the application of fungicides to *Pythium* sp. infested composted pine bark). This indicates that this treatment resulted in a better growth response and disease suppression compared to the controls (water only, *Pythium* sp. only and fungicides applied to *Pythium* sp. infested composted pine bark). However when this treatment was compared to the control (fungicides applied to *Pythium* sp. infested composted pine bark), no significant differences were found for most parameters tested. This indicated that this treatment performed equally well or better than the control. This also suggests that *T. harzianum* KMD could have been eliminated from the combination due to the toxicity of fungicides present in the *Pythium* sp. infested composted pine bark. Recommendations to the farmer or grower are that *T. harzianum* KMD does enhance growth and reduce disease best when applied alone. However if *T. harzianum*

KMD was applied as an integrated scheme with fungicides, a similar result will be recorded as the application of fungicides only.

5.4.2 Effect of a commercial formulation of *Trichoderma harzianum* KMD applied with selected fungicides on *Rhizoctonia solani*

Table 5.13 shows significant and non-significant results as a matrix of treatments and parameters which were tested on all crops against *R. solani.*

Cabbage seedlings

Trichoderma harzianum KMD applied alone to non-infested composted pine bark gave comparable results when compared to the control (water only). Significant differences on plot weight were recorded when *T. harzianum* KMD was applied to non-infested composted pine bark and compared to the control (fungicides only). No significant differences were recorded on percentage survival and plant height. This indicates that *T. harzianum* KMD performed equally well to the application of fungicides. For percentage survival and plant height the application of *T. harzianum* KMD with fungicides to non-infested composted pine bark, performed equally well as the control (water only). Significant differences were recorded on plot weight suggesting that *T. harzianum* KMD applied with fungicides enhances growth. Hence *T. harzianum* KMD is a good growth stimulant applied with fungicides. When this treatment was compared to the control (fungicides only), no significant differences were recorded suggesting that that treatments performed equally well.

On percentage survival and plant height, *T. harzianum* KMD applied to *R. solani* infested composted pine bark resulted in no significant differences when compared to the control (water only). This indicates that *T. harzianum* KMD is a good biocontrol agent as it reduces disease. When this treatment was compared to the fungicide control no significant differences were recorded suggesting that *T. harzianum* KMD performed similary tos fungicides applied alone.The application of *T. harzianum* KMD with fungicides to *R. solani* infested composted pine bark recorded no significant differences on percentage survival and plant height when compared to the control (water only), suggesting that *T. harzianum* KMD applied with fungicides to *R. solani* infested composted pine bark worked equally well as the control (water only). When this treatment was compared to the fungicide control no significant differences were recorded for plot weight and plant height. This suggests that these treatments

performed equally well for plot weight and plant height. However when compared to the control (fungicides applied to *R. solani* infested composted pine bark) no significant differences were recorded for plot weight and plant height. This indicates that both treatments performed equally well in reducing disease.In most instances *T. harzianum* KMD worked equally well to those applied with fungicides, however one must keep in mind that *T. harzianum* KMD was killed by fungicides in *in vitro* tests. Hence the combination of *T. harzianum* KMD applied with fungicides to *R. solani* infested composted pine bark may not play an active role in disease suppression.

Cucumber seedlings

Trichoderma harzianum KMD applied to *R. solani* infested composted pine bark reduced disease effectively when compared to the *R. solani* diseased control (Table 5.13). However when *T. harzianum* KMD was applied to *R. solani* infested composted pine bark with fungicides no significant differences were recorded on plot weight and percentage survival when compared to the control (water only) and fungicides control. This suggests that *T. harzianum* KMD performed equally well to the application of fungicides only. However there is no proof that *T. harzianum* KMD may be playing an active role. Hence further research needs to be persued with respect to this finding.

Namaqualand daisy seedlings

No significant differences were recorded on most parameters when compared to all the control. No significant differences were recorded when *T. harzianum* KMD was applied with and without fungicides to *R. solani* infested composted pine bark (Table 5.13).

Eucalyptus seedlings

Trichoderma harzianum KMD applied to *R. solani* infested composted pine bark, reduced disease on plot weight and plant height when compared to *R. solani* infested composted pine bark. No significant differences on plot weight and percentage survival were recorded when this treatment was compared to the application of fungicides to *R. solani* infested composted pine bark (Table 5.13). It was evident that *T. harzianum* KMD effectively reduced disease more effectively and consistently when applied alone.

Tomato seedlings

Trichoderma harzianum KMD applied with or without fungicides effectively reduced disease when compared to the disease control (*R. solani*) and performed equally well to the fungicide control and water control (Table 5.13).

Overall, biocontrol activity against *Pythium* sp. and *R. solani* showed that *T. harzianum* KMD was more effective when applied alone to infested and non-infested composted pine bark. The application of fungicides with *T. harzianum* KMD resulted in inconsistent reduction in disease and caused poor responses to plant growth. This result indicates that the lack of sporulation from *in vitro* assays was related to the presence of fungicides, which may have resulted in inconsistent disease reduction. The lack of sporulation may have prevented the proliferation of *T. harzianum* KMD in the rhizosphere and reduced germination capability of the initial spore inoculum. Subsequently spores did not germinate or sporulate in the composted pine bark, hence reducing spore count and antagonistic action against the pathogens. Prior to these studies the mycelium of resistant mutants of *T. harzianum* KMD lacking sporulation, were not tested *in vitro* to determine the antagonistic action against the pathogens. Hence, no evidence was present to support the fact that the lack of sporulation could be related to *T. harzianum* KMD's lack of antagonistic action or the resistant mycelium of the mutants of *T. harzianum* KMD were effective or not against the pathogens.

Khattabi *et al.* (2001) has demonstrated that *T. harzianum* does reduce its antagonistic ability when fungicides such as benomyl were added to a soil-less mix. The fungus was affected with all three doses of benomyl tested. Some species of *Trichoderma* have the ability to be compatable with fungicides. Kaur & Mukhopadhyay (1992) have shown that *T. harzianum* responeded well to the integration of fungicides to prevent " Chickpea wilt complex." There are several reports regarding successful disease control in crops by integration of biological and chemical methods (Henis *et al.*, 1978; McLean & Stewart, 2000; McLean, 2001). Elad *et al.*, (1980) reported the combined use of *T. harzianum* and PCNB against *Rhizoctonia* damping-of. Kraft & Papavizas (1983) showed the highest field seed yields from a pea cultivar susuceptibel to *P. ultimum* after seed treatment with metalaxyl and *T. harzianum*.

Table 5.13. Shows significant and non-significant results as a matrix of treatments and parameters which were tested on all crops against *Rhizoctonia solani*

	Controls	Water only			Fungicide only			*R.solani* only			Fungicide and *R.solani*		
		PW	PS	PH	PW	PS	PH	PW	PS	PH	PW	PS	PH
Cabbage	T only	NS	NS	NS	S	NS	NS	NS	S	S	NS	S	NS
	T + F only	S	NS	NS	NS	NS	NS	NS	S	S	NS	S	NS
	T + Rs only	NS	NS	NS	S	S	NS	NS	S	S	NS	S	NS
	T+ F + Rs only	S	NS	NS	NS	S	NS	S	S	S	NS	S	NS
Cucumber	T only	NS	NS	S	NS	NS	S	NS	S	S	NS	S	S
	T + F only	NS	NS	S	NS	NS	S	NS	S	S	NS	S	S
	T + Rs only	NS	NS	NS	NS	NS	NS	NS	S	S	NS	S	S
	T+ F + Rs only	NS	NS	S	NS	NS	S	NS	S	S	NS	NS	NS
Namaqualand daisy	T only	S	NS	NS	S	NS	NS	S	NS	NS	NS	NS	NS
	T + F only	S	NS	NS	S	NS	NS	S	NS	NS	S	NS	NS
	T + Rs only	S	NS	NS	S	NS	NS	S	NS	NS	NS	NS	NS
	T+ F + Rs only	S	NS	NS	S	NS	NS	S	NS	NS	S	NS	NS
Eucalyptus	T only	S	NS	S	S	NS	S	S	NS	S	NS	NS	S
	T + F only	NS	NS	NS	NS	NS	NS	NS	NS	S	S	NS	S
	T + Rs only	S	NS	NS	S	NS	NS	S	NS	S	NS	NS	S
	T+ F + Rs only	S	NS	S	S	NS	S	S	NS	NS	S	NS	NS
Tomato	T only	S	S	NS	S	S	NS	S	NS	NS	NS	NS	NS
	T + F only	S	NS	NS	S	NS	NS	S	NS	NS	S	NS	NS
	T + Rs only	S	NS	NS	S	NS	NS	S	NS	NS	NS	NS	NS
	T+ F + Rs only	S	S	NS	S	S	NS	S	NS	NS	S	NS	NS

T only = *T. harzianum* KMD only; T + F only = *T. harzianum* KMD applied with fungicides to non-infested composted pine bark; T+ Rs only = *T. harzianum* KMD applied to *R.solani* infested composted pine bark; T+F+ Rs only = *T. harzianum* KMD applied to *R.solani* infested composted pine bark with fungicides.
PW= plot weight; PS = percentage survival; PH= plant height
S = Significant; NS non-significant

Variation of disease control with and without fungicides, of *T.harzianum* KMD where observed for most crops. In some instances where seedling stand was reduced by damping-off, the surviving plants may compensate through increase in growth and yield due to the reduced competition. However if some of the remaining seedlings have damaged root systems and stem damping-off they may develop into unthrifty plants. These unthrifty plants compete with thrifty, undiseased plants, thus lowering the ability of the latter to compensate (Zadoks & Schein, 1979). Although further trials are needed to determine the sensitivity of *T. harzianum* KMD to repeated fungicide application, the preliminary results indicate that integrated control of damping-off may be possible. *T. harzianum* KMD could be applied at planting with fungicides with seed treatments providing effective control against damping –off but it may be advisable to let the fungicide leach for three days before planting and that the *T. harzianum* KMD coated seeds is not placed in direct contact of fungicides. This integration of fungicides and biological control agent, *T. harzianum* KMD may allow the number of fungicide sprays to be reduced while still providing control of damping- off of seedlings.

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CHAPTER 6

GROWTH ENHANCEMENT OF A VARIETY OF GREENHOUSE CROPS BY A FORMULATION OF *TRICHODERMA HARZIANUM* KMD: EFFECT OF ENVIRONMENTAL STRESS

J. Omarjee[1], M.D. Laing[1] and C.H. Hunter[2]

Disciplines of Plant Pathology[1], and Microbiology[2], SAES

University of Natal, Private Bag X01, Scottsville, Pietermaritzburg, South Africa

Trichoderma harzianum strain KMD is a biocontrol fungus, which has the potential to reduce soil-borne diseases and increase growth of seedlings. Root growth was markedly enhanced by colonization with *T. harzianum* KMD in rhizotron studies. In the absence of a disease, colonization by *T. harzianum* KMD on maize (*Zea mays* L.) and cucumber (*Cucumis sativa* L.) roots in rhizotron studies increased root area by 3104.52 mm^2 and 1787.48 mm^2, respectively. Seeds of cabbage (*Brassica oleracea* var. *capitata* L.), cucumber and tomato (*Lycopersicon esculentum* Mill.) that were subjected to oxidative stress with 0.05% NaOCℓ, did not reduce vigor. The effect of subsequent treatments with *T. harzianum* KMD on stressed seeds was not determined. Treatments of imbibed but unemerged seeds of cucumber, tomato and white grain maize in cold temperatures (5-10°C night/day) for varying periods reduced subsequent growth. Seeds that were subjected to cold stress and treated with *T. harzianum* KMD did not display enhanced growth. Seeds of cabbage, cucumber, tomato and white grain maize sown in various media which induced various levels of drought and water-logging conditions were not enhanced by *T. harzianum* KMD application.

6.1 INTRODUCTION

The fungus, *Trichoderma harzianum,* has been studied as a biocontrol organism against a wide range of soil-borne pathogens for many years. In addition, root colonization with this fungus has been reported to promote plant growth (Chang *et al.*, 1986; Windham *et al.*, 1986, 1989; Kliefeld & Chet, 1992; 1989), but the mechanism of this growth promotion is unknown. The growth promoting effect could be a valuable property of the fungus when used with crops that have weak seedling growth (Björkman *et al.*, 1998).

Most beneficial fungi that have been commercialised have not been used due to the inconsistency of colonization of crop root systems. Roots are colonized by naturally occurring strains of *Trichoderma*. These strains of *Trichoderma* may be localised depending on the host plant, growing conditions and the competition of microbes (Deacon, 1994). Due to this variability, development of an effective biocontrol agent is difficult. Few strains of *Trichoderma* can effectively colonize root surfaces (Harman *et al.*, 1989).

In the growth of seedlings under greenhouse conditions several problems may arise and have detrimental effects on plant growth. One such problem is the lack of water due to inconsistent irrigation, which may result in a lack of emergence and seedling vigor (Parera *et al.*, 1996). Production would be easier if seedling vigor could be increased so that plants grow uniformly. The growth enhancing effect of *T. harzianum* application, combined with the protection from root diseases, may increase vigor and solve many problems.

This investigation describes the nature of the increase in growth and the effect of a selected formulation of *T. harzianum* KMD on growth reduction associated with three stress factors, commonly experienced by seedlings: oxidative injury, low temperatures and drought. These experiments were designed to determine whether *T. harzianum* KMD application overcomes the negative effects of oxidation injury, cold and drought, by enhancing plant growth and vigor.

6.2 MATERIALS AND METHODS

6.2.1 Formulation of *T. harzianum* KMD

A commercial formulation containing conidia of *T. harzianum* KMD was obtained from Dr. Mike Morris[1], Plant Health Products. The number of conidia per gram of formulation was 10^8 spores/g.

6.2.2 Crops evaluated

Cabbage (*Brassica oleracea* var. *capitata* L.) cv. Glory of Enkhuizen, Seed lot no.:Y1011RR.
Cucumber (*Cucumis sativa* L.) cv. Cucumber Ashley, Seed lot no.: AY054YY.
Tomato (*Lycopersicon esculentum* Mill.) cv. Heinz 1370, Seed lot no.: YE099YY
White Grain Maize (*Zea Mays* L.) cv. Kalahari Early Pearl, Seed lot no.: AY018YY.

[1] Dr Mike Morris, Plant Health Products, P.O.Box 207, Nottingham Road, South Africa.

All seeds were obtained from McDonalds Seeds[2].

6.2.3 Plant growth

Oxidation injury and cold trials

Oxidation injury and cold treatments trials were carried out under greenhouse conditions. Crops that were evaluated for oxidation injury trials were cabbage, cucumber, and tomato. For cold trials, tomato, maize and cucumber were evaluated. All seedlings were grown for 21 days in composted pine bark (seedling potting mix)

All treatments were irrigated three times a day by microjet irrigation. Fertilizer was injected into the irrigation water. Soluble fertilizer [3. 1. 3 (38)] Complete from Ocean Agriculture[3], was applied at a rate of $1gl^{-1}$ to give approximately $33mgl^{-1}$ of P and $100mgl^{-1}$ of N and K. Temperatures were in the range of 20-30°C in greenhouses. The growing medium used, was a grade of a composted pine bark. Seeds were planted in Speedling® 24 trays, 24 plants in a tray. Each tray represented one replicate and was replicated three times, resulting in 72 seedlings per treatment.

Drought trials

Crops that were evaluated for drought trials were cabbage, tomato, maize and cucumber. Drought trials were initiated in glasshouses. Treatments were irrigated by using a watering can with 1g of soluble fertilizer [3. 1. 3(38)] Complete from Ocean Agriculture per litre of tap water, once a day. Temperatures ranged from 20-30°C. Plant growth in drought trials were assigned in plastic pots (150 mm in height, 150 mm in diameter) from PC Plastics[4]. Pots were filled with approximately 500ml of a mixture of coir peat and coarse potting mix, grades of composted pinebark, which resulted in a number of media with a range of drainage characteristics (Appendix C) as described in Handreck and Black (1994). These were selected for their range in air filled porosity and water holding capacities. Five seeds were sown in each pot and after germination of seeds plants were thinned to three plants per pot. All plants were grown for 21days. Each pot represented one replicate and each treatment was replicated three times, resulting in three plants per plot, and nine plants per treatment. The experiment was repeated three times.

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[2] McDonalds Seeds, 61 Boshoff Street, Box 238, Pietermaritzburg, South Africa
[3] Ocean Agriculture, P. O. Box 741, Mulders Drift 1747, South Africa
[4] PC Plastics CC, Durban, South Africa.

Effect of colonization of T. harzianum KMD on root system architecture using Rhizotrons

Plants for analysing root system architecture were grown in perspex boxes (Rhizotrons), 20mm thick, 20mm wide, and 200mm deep, that held 500ml of pasteurised coarse sand. Sand was pasteurised for one hour at 50 lb/ in^2. Perspex boxes were placed in a growth chamber supplemented with 500$\mu mol^{-1}.s^{-1}$ metal halide lights, in a relative humidity of 60%, with a day temperature of 24°C. Rhizotrons were covered with aluminium foil to prevent sunlight from affecting the root system. Plants were watered with 1g/l of soluble fertilizer [3. 1.3(38)] Complete from Ocean Agriculture. Plants were watered once a day. Cucumbers and maize were used as an example of dictotyledenous and monocotyledenous plants, respectively This trial was replicated three times. Plants were grown for 21days.

6.2.4 *Trichoderma harzianum* KMD: Seed treatment

Four grams of Pelgel®[5] were dissolved in 200ml of distilled water, stirred and allowed to stand for 1h. One gram of the fungal formulation was added to a beaker containing 50ml of sticker, Pelgel®. The mixture was then stirred. An appropriate number of seeds for each trial was placed into the mixture and allowed to stand for 1h. Seeds were then removed and air-dried.

6.2.5 Growth measurements

Several measurements of plant growth were used to determine the nature of growth enhancement. All measurements except where stated were made after 21days of growth.

Oxidation injury trials

(i) Plant height was measured as the distance from the soil surface to the longest extended leaf.

(ii) Plot weight of shoots was measured by cutting the shoots at the base of the stem and drying them at 80° C in a forced air-dried oven for 48h.

(iii) Percentage germination was measured after seven days of growth. This was calculated by counting the number of seedlings that emerged after seven days.

[5] LiphaTech, Inc., Milwaukee, Wisconsin, U. S. A

Cold treatment trials

(i) Plot weights of shoots were measured by cutting the shoots at the base of the stem and drying the shoots at 80°C in a forced air-dried oven for 48h.

(ii) Total dry weight of roots was measured by removing the rooting system and carefully rinsing off excess composted pine bark. Roots were then collected per tray and dried at 80°C in a forced air-dried oven for 48h.

(iii) Plant height was measured as the distance from the soil surface to the longest extended leaf.

(iv) Percentage germination was measured after 14 days of growth. This was calculated by counting the number of seedlings that emerged after 14 days.

For cold trials, root and shoot dry mass, percentage germination and plant height were measured for each of three Speedling® 24 trays containing 24 plants, resulting in 72 plants per treatment. In cold trials root dry mass was measured because cold tends to affect the rooting system more severely than shoot physiology. Each tray represented one replicate and was replicated three times, resulting in 72 seedlings per treatment.

Drought trials

(i) Plot weights of shoots were measured by cutting the shoots at the base of the stem and drying the shoots at 80°C in a forced air-dried oven for 48h.

(ii) Plant height was measured as the distance from the soil surface to the longest extended leaf.

Root system architecture

The root system architecture was analysed by removing intact root systems from the rhizotrons. Coarse sand was gently washed off the roots. Roots were photographed while in their original orientation. Image analysis was then used (analySIS® [SIS PRO, Version 3.0, Germany] to determine the area (mm^2) of the rooting system.

6.2.6 Oxidative injury trials

In experiments testing the oxidative effects of hypochlorite, seeds were treated for 15min at room temperature in 0.05% NaOCℓ, rinsed three times in tap water, and air-dried (Parera & Cantliffe, 1991). Seeds were treated with *T. harzianum* KMD as a seed treatment. Each treatment consisted of three Speedling 24® trays of 24 plants, each treatment resulting in 72

plants. Plant height was measured after 21days of growth to permit non-destructive growth measurements. Treatments consisted of:

1. Water only
2. NaOCℓ, only
3. NaOCℓ, and *Trichoderma harzianum* KMD
4. *Trichoderma harzianum* KMD only

The experiment was repeated three times.

6.2.7 Cold treatment

For applying cold treatment, seeds, treated with *T. hazianum* KMD, were sown in Speedling® 24 trays containing composted pine bark (seedling potting mix) with each treatment consisting of three Speedling® 24 trays resulting in 24 plants per tray and 72 seedlings per treatment. The seeds were allowed to imbibe for 24h at 20 -25°C. They were then transferred to a growth chamber at a diurnal temperature cycle of 12h light and 12h of dark each at 5-10°C. No lights were used because the seeds did not emerge during the cold period. After the cold treatment, the Speedling® 24 trays were returned to the greenhouse and grown under greenhouse conditions as described above. To provide all the plants with the same greenhouse conditions, planting was staggered so that all cold-treated plants were returned to the greenhouse at the same time as the unchilled controls, which had imbibed for 24h. Plants were harvested at 21 days of growth, but not those used in the cold treatment. Four different cold exposure times were used i.e., 1, 3, 5 and 7 days. A control and two cold treatments were analysed at a time due to limited growth chamber space. The experiment was repeated three times.

6.2.8 Drought trials

The experiment was set up as previously described in Section 6.2.3. (ii) and Appendix C.

6.2.9 Statistical analysis

Treatments were arranged in a randomised complete block design. Analysis of data by Analysis of Variance (ANOVA) with a factorial treatment structure and interactions was carried out. Treatment means were separated by the Students Newman Keuls test. Statistical analyses were conducted using the general linear model procedure of SAS Version 6.08 (SAS, 1987).

6.3 RESULTS

6.3.1 Oxidative injury trials

Treatments of cabbage, cucumber and tomato with dilute NaOCℓ to cause oxidative injury resulted in no reduction in vigor of plants (Table 6.1-6.3). Seeds of all crops treated with NaOCℓ only, resulted in plot weight, plant height and percentage germination comparable to those seeds treated with *T. harzianum* KMD only, water only and those of seeds treated with both *T. harzianum* KMD and NaOCℓ. Application of *T. harzianum* KMD did not affect growth of all plants any better than those subjected to stress (NaOCℓ only). NaOCℓ and *T. harzianum* KMD did not significantly change plant growth compared to untreated seedlings. However, marginal differences were noted for plant height of cabbage seedlings.

6.3.2 Cold stress trials

Cucumber seedlings

Trichoderma harzianum KMD enhanced plot weight of cucumber seedlings exposed to cold for a period of 1day by 14.41% (Table 6.4) when compared to untreated seeds exposed to cold for 1day. *Trichoderma harzianum* KMD did not alleviate stress after seeds were exposed to cold for 3, 5 and 7days. This could be attributed to *T. harzianum* KMD not growing well at <15°C, hence no growth enhancement resulted after substantial exposure to cold treatment.

The effect of cold on plants was minor and there was not much evidence of stress after 1 day. It was evident that seeds treated with *T. harzianum* KMD only and not exposed to cold resulted in much higher plant growth.

Table 6.1.Growth response of cabbage to *Trichoderma harzianum* KMD colonization following oxidation stress treatment of seeds with sodium hypochlorite (NaOCℓ).

Treatments	Dry weight (g)	Control (water only)	% Germination rate	Control (water only)	Plant height (mm)	Control (water only)
Sodium hypochlorite (NaOCℓ) only	0.99 a	116	62.5 a	95 a	77.5 b	91
Sodium hypochlorite (NaOCℓ) only + *T. harzianum*	0.570 a	70	59.72 a	91 a	87.9 b	104
T. harzianum only	0.6 a	88	55.55 a	85 a	114 a	135
Control 1 (Water only)	0.87a	100	65.28 a	100 a	84.3 b	100
Treatments	P-values 0.2723^{NS} %CV = 29.14 MSE= 0.0155		P-values 0.4105^{NS} %CV = 11.37 MSE = 6.909		P-values 0.0272 ** %CV = 13.27 MSE = 1.207	

Table 6.2.Growth response of cucumber to *Trichoderma harzianum* KMD colonization following oxidation stress treatment of seeds with sodium hypochlorite (NaOCℓ).

Treatments	Dry weight (g)	Control (water only)	% Germination rate	Control (water only)	Plant height (mm)	Control (water only)
Sodium hypochlorite (NaOCℓ) only	7.5 a	80	95.83 a	98	373.2 a	95
Sodium hypochlorite (NaOCℓ) only + *T. harzianum*	7.74 a	85	93.05 a	95	375.8 a	96
T. harzianum only	8.07 a	82	100 a	102	372.9 a	95
Control 1 (Water only)	9.48 a	100	97.21 a	100	389.3 a	100
Treatments	P-values 0.1538^{NS} %CV = 11.58 MSE= 0.0410		P-values 0.4337^{NS} %CV = 5.13 MSE = 4.9608		P-values 0.5433^{NS} %CV = 4.07 MSE = 1.539	

Table 6.3.Growth response of tomato to *Trichoderma harzianum* KMD colonization following oxidation stress treatment of seeds with sodium hypochlorite (NaOCℓ).

Treatments	Dry weight (g)	Control (water only)	% Germination rate	Control (water only)	Plant height (mm)	Control (water only)
Sodium hypochlorite (NaOCℓ) only	2.9 a	118	98.61 a	100	265.6 a	109
Sodium hypochlorite (NaOCℓ) only + *T. harzianum*	2.41 a	96	97.22 a	98	277.8 a	114
T. harzianum only	2.26 a	93	94.44 a	95	268.9 a	110
Control 1 (Water only)	2.52 a	100	98.61 a	100	242.9 a	100
Treatments	P-values 0.5183^{NS} %CV = 20.98 MSE= 0.0229		P-values 0.7813^{NS} %CV = 5.803 MSE = 5.642		P-values 0.0779^{NS} %CV = 5.361 MSE = 1.414	

1.NS = Not significant; *= significant at P ≤ 0.05; ***=significant at P ≤ 0.001
2. Means with the same letter are not significantly different (P= 0.05) according to Student, Newman and Keuls comparison
3. Seeds treated for 15 min with 0.05% NaOCL before seed imbibition
4. Control (water only) represents the comparison of treatments to the control, water only and presented as a percentage.

Application of *T. harzianum* KMD to seedlings did not indicate any growth enhancement when compared to the controls, which were exposed to cold stress.

With respect to total dry weight of roots, treatments subjected to all intensities of cold resulted in low root weights, indicating that cold did affect root growth and caused stress on the plant. However, *T. harzianum* KMD did not counteract the stress caused by cold on root dry weights. Root dry weights of controls exposed to cold were significant than those exposed to cold and treated with *T. harzianum* KMD. No significant differences were found between treatments and period of cold for plant height and percentage germination of cucumber seedlings. Marginal differences were noted for the interaction of the period of cold treatments, but there were no interactions between *T. harzianum* KMD and cold.

Tomato seedlings

Significant differences on plot weight of tomato seedlings (Table 6.5) were noted between treatments of cold, and treatments of cold with the application of *T. harzianum* KMD. *Trichoderma harzianum* KMD enhanced vigor of seedlings exposed to cold for a period of 1, 3, and 5days by 3-73% when compared to untreated seeds exposed to cold for similar periods. Seeds treated with *T. harzianum* KMD exposed to cold for 7days did not exhibit any changes in plant growth.

Seeds treated *with T. harzianum* KMD only and water only were much healthier and resulted in an increase in plant vigor when compared to seedlings exposed to cold stress. No significant differences were noted between periods of cold on plot weight of tomato seedlings nor between treatments and periods of cold on total dry weights of roots.

Significant differences between treatments were recorded for plant height of tomato seedlings. It was evident that *T. harzianum* KMD restored vigor, by increasing plant height of seedlings exposed to all intensities of cold and were comparable to untreated seeds. No significant differences were recorded between treatments and periods of cold for percentage germination. Interactions between treatments and periods of cold were significant for plant height and plot weight measured.

Table.6.4 . Growth response of cucumber seedlings to *Trichoderma harzianum* KMD colonization following cold treatment of 1, 3, 5, 7 days.

Treatments	Period (days)	Plot weight of shoots (g)	Control (Water only)	Total dry weight of roots (g)	Control (Water only)	Plant height (mm)	Control (Water only	% Germination	Control (Water only)
Cold	1d	6.96 bc	79.28	0.05b	50.00	360.0a	95.74	93.06ab	101.52
Cold	3d	8.4 c	71.43	0.05b	59.26	349.5a	89.34	94.44ab	95.77
Cold	5d	7.44 b	69.63	0.05b	48.48	421.7a	107.84	95.83ab	95.83
Cold	7d	6 b c	71.03	0.05b	75.00	314.9a	87.56	87.50ab	87.50
Cold +*Trichoderma*	1d	8.4 bc	93.69	0.03b	35.71	390.6a	103.88	84.72b	92.42
Cold +*Trichoderma*	3d	6.72bc	56.46	0.05b	59.26	311.8a	79.69	90.28b	91.55
Cold +*Trichoderma*	5d	6 c	55.56	0.06b	54.55	354.6a	90.69	81.94b	81.94
Cold +*Trichoderma*	7d	5.04cc	57.94	0.04b	55.00	520.0a	144.58	80.55b	80.55
***Trichoderma* only**	1d	10.08 a	112.61	0.08a	82.14	297.8a	79.20	76.39ab	83.33
***Trichoderma* only**	3d	10.56 a	89.12	0.07a	77.78	384.6a	98.30	91.67ab	92.96
***Trichoderma* only**	5d	6.24 c	58.52	0.11a	100.00	337.4a	86.28	88.89ab	88.89
***Trichoderma* only**	7d	6.96 ab	81.31	0.07a	100.00	428.0a	119.00	97.22ab	97.22
Nil (water only)	1d	8.88 b	100.00	0.09a	100.00	376.0a	100.00	91.67a	100.00
Nil (water only)	3d	11.76 a	100.00	0.09a	100.00	391.2a	100.00	98.61a	100.00
Nil (water only)	5d	10.8 a	100.00	0.11a	100.00	391.0a	100.00	100.00a	100.00
Nil (water only)	7d	8.64 a	100.00	0.07a	100.00	359.7a	100.00	100.00a	100.00
Effects		**P-values**		**P-values**		**P-values**		**P-values**	
Treat		0.0001***		0.0013**		0.3814 ^{NS}		0.0224**	
Period		0.0004***		0.1325 ^{NS}		0.1071 ^{NS}		0.2250 ^{NS}	
Treat*Period		0.0504**		0.9424 ^{NS}		0.0015 ^{NS}		0.3533 ^{NS}	
		%CV= 17.54		**%CV**= 43.80		**%CV**= 14.14		**%CV**= 9.74	
		MSE= 1.23		**MSE**= 0.029		**MSE**= 5.295		**MSE**= 8.797	

1.NS = Not significant; **= significant at $P \leq 0.05$; ***=significant at $P \leq 0.001$

2. Means with the same letter are not significantly different (P= 0.05) according to Student, Newman and Keuls comparison

3. Seeds were treated for 1, 3, 5, 7 days at 5/10°C after seed imbibition

4. Control (Water only) represents the comparison of treatments against the water control and presented as a percentage.

Table. 6.5. Growth response of tomato seedlings to *Trichoderma harzianum* KMD colonization following cold treatment of 1, 3, 5, 7 days.

Treatments	Period (days)	Plot weight of shoots (g)	Control (Water only)	Total dry weight of roots (g)	Control (Water only	Plant height (mm)	Control (Water only)	% Germination	Control (Water only)
Cold	1d	1.68bc	75.86	0.02a	176.99	25.05b	91.92	93.06a	94.37
Cold	3d	1.44c	56.67	0.00a	22.33	23.10b	93.02	100.00a	107.46
Cold	5d	2.16ab	107.69	0.02a	210.97	23.33b	105.50	98.61a	98.61
Cold	7d	1.68c	72.41	0.00a	42.14	23.47b	90.96	91.67a	100.00
Cold +*Trichoderma*	1d	3.36a	148.28	0.01a	117.99	26.26a	96.34	95.83a	97.18
Cold +*Trichoderma*	3d	1.44c	60.00	0.02a	175.33	25.15a	101.28	97.22a	104.47
Cold +*Trichoderma*	5d	2.4a	115.38	0.01a	126.58	25.18a	113.84	97.22a	97.22
Cold +*Trichoderma*	7d	1.4c	65.52	0.01a	67.36	24.99a	96.85	95.83a	104.55
***Trichoderma* only**	1d	2.4ab	103.45	0.02a	176.99	28.30a	103.83	98.61a	100.00
***Trichoderma* only**	3d	3.12a	126.67	0.01a	133.33	28.98a	116.71	100.00a	107.46
***Trichoderma* only**	5d	2.4a	111.54	0.01a	93.25	23.15a	104.67	98.61a	98.61
***Trichoderma* only**	7d	2.64a	113.79	0.01a	93.18	25.30a	98.06	95.83a	104.54
Nil (water only)	1d	2.4ab	100.00	0.01a	100.00	27.26ab	100.00	98.61a	100.00
Nil (water only)	3d	2.4ab	100.00	0.01a	100.00	24.83ab	100.00	93.06a	100.00
Nil (water only)	5d	2.16ab	100.00	0.01a	100.00	22.12ab	100.00	100.00a	100.00
Nil (water only)	7d	2.4ab	100.00	0.01a	100.00	25.80ab	100.00	91.67a	100.00
Effects		**P-values**		**P-values**		**P-values**		**P-values**	
Treat		0.0068***		0.1113 ^{NS}		0.3814 ^{NS}		0.0224 ^{NS}	
Period		0.0844 **		0.1325 ^{NS}		0.1071 ^{NS}		0.2250 ^{NS}	
Treat*Period		0.0067***		0.9424 ^{NS}		0.1515 ^{NS}		0.3533 ^{NS}	
		%CV=24.63		**%CV**=43.80		**%CV**=14.14		**%CV**= 9.745	
		MSE= 0.598		**MSE**= 0.0292		**MSE**=5.295		**MSE**= 8.797	

1.NS = Not significant; **= significant at P ≤ 0.05; ***=significant at P ≤ 0.001

2. Means with the same letter are not significantly different (P= 0.05) according to Student, Newman and Keuls comparison

3. Seeds were treated for 1, 3, 5, 7 days at 5/10°C after seed imbibition

4. Control (Water only) represents the comparison of treatments against the water control and presented as a percentage.

Table.6.6 . Growth response of maize seedlings to *Trichoderma harzianum* KMD colonization following cold treatment of 1, 3, 5, 7 days.

Treatments	Period (days)	Plot weight of shoots (g)	Control (Water only)	Total dry weight of roots (g)	Control (Water only	Plant height (mm)	Control (Water only)	% Germination	Control (Water only)
Cold	1d	13.44ab	116.55	0.25a	77.55	577.6a	96.18	95.83b	97.18
Cold	3d	10.08ab	114.41	0.20a	92.19	555.7a	101.43	98.61b	101.43
Cold	5d	11.52ab	107.52	0.22a	147.73	545.5a	93.19	100.00b	112.50
Cold	7d	10.08ab	106.78	0.29a	187.23	596.0a	106.71	100.00b	114.29
Cold +*Trichoderma*	1d	12.48ab	107.59	0.27a	82.65	557.9a	92.91	100.00b	101.41
Cold +*Trichoderma*	3d	10.8ab	121.62	0.19a	90.63	565.8a	103.27	100.00a	102.86
Cold +*Trichoderma*	5d	11.52ab	109.02	0.23a	159.09	563.3a	96.24	100.00a	112.50
Cold +*Trichoderma*	7d	12.72ab	133.90	0.25a	157.45	563.4a	100.87	100.00a	114.29
***Trichoderma* only**	1d	13.44a	116.55	0.32a	97.96	564.2a	93.94	100.00a	101.41
***Trichoderma* only**	3d	12.96a	145.95	0.23a	107.81	592.3a	108.11	98.61b	101.43
***Trichoderma* only**	5d	10.8ab	102.26	0.24a	161.36	588.7a	100.57	87.50b	98.44
***Trichoderma* only**	7d	11.04a	116.10	0.23a	148.94	593.3a	106.24	88.89b	101.59
Nil (water only)	1d	11.52b	100.00	0.33a	100.00	600.5a	100.00	98.61b	100.00
Nil (water only)	3d	8.88b	100.00	0.21a	100.00	547.9a	100.00	97.22b	100.00
Nil (water only)	5d	10.56b	100.00	0.15a	100.00	585.3a	100.00	88.89b	100.00
Nil (water only)	7d	9.36b	100.00	0.16a	100.00	558.5a	100.00	87.50b	100.00
Effects **Treat** **Period** **Treat*Period**		**P-values** 0.0457** 0.0213** 0.0587** **% CV**= 15.40 **MSE**= 1.52		**P-values** 0.3281 ^{NS} 0.0033 ^{NS} 0.1730 ^{NS} **% CV**= 24.70 **MSE**= 0.058		**P-values** 0.5979 ^{NS} 0.8859 ^{NS} 0.6858 ^{NS} **%CV**= 7.062 **MSE**= 4.04		**P-values** 0.0103** 0.0769 ^{NS} 0.1714 ^{NS} **%CV**=5.92 **MSE**= 5.70	

1..NS = Not significant; **= significant at P ≤ 0.05; ***=significant at P ≤ 0.001
2. Means with the same letter are not significantly different (P= 0.05) according to Student, Newman and Keuls comparison
3. Seeds were treated for 1, 3, 5, 7 days at 5/10°C after seed imbibition
4. Control (Water only) represents the comparison of treatments against the water control and presented as a percentage.

Table 6.7. Growth response of cabbage seedlings to *Trichoderma harzianum* KMD following growth in different ratios of media, resulting in drought and water- logging conditions after four weeks of growth.

Ratios of Media	Treatments	Plot weight (g)	Plant height (mm)
100% Koir	*T. harzianum KMD*	0.786 a	138.0 a
75% CPM + 25% Koir	*T. harzianum KMD*	0.560 ab	118 ab
50%CPM + 50% Koir	*T. harzianum KMD*	0.280 bc	88.5 abc
25% CPM + 75% Koir	*T. harzianum KMD*	0.222 c	86.0 abc
100% CPM	*T. harzianum KMD*	0.783 a	120 abc
SPM	*T. harzianum KMD*	0.743 a	136.3 abc
100% Koir	Not treated	0.580 abc	120.6 abc
75% CPM + 25% Koir	Not treated	0.776 a	138.3 ab
50%CPM + 50% Koir	Not treated	0.62 ab	118.3 abc
25% CPM + 75% Koir	Not treated	0.35 c	87.0 bc
100% CPM	Not treated	0.226 c	85.3 bc
SPM	Not treated	0.681 ab	126.3 abc
Effects		P-values	P-values
Treatments		0.9158 ^{NS}	0.6224 ^{NS}
Media		0.0001***	0.0001***
Treatments*Media		0.4417 ^{NS}	0.7674 ^{NS}
		CV%=28.43	CV%=16.46
		MSE=0.759	MSE=0.65

Table 6.8 Growth response of cucumber seedlings to *Trichoderma harzianum* KMD following growth in different ratios of media, resulting in drought and water- logging conditions after four weeks of growth.

Ratios of Media	Treatments	Plot weight (g)	Plant height (mm)
100% Koir	*T. harzianum KMD*	1.893 abc	225.0 a
75% CPM + 25% Koir	*T. harzianum KMD*	0.821 bcd	246.3 a
50%CPM + 50% Koir	*T. harzianum KMD*	0.900 bcd	155 ab
25% CPM + 75% Koir	*T. harzianum KMD*	0.243 abc	130 ab
100% CPM	*T. harzianum KMD*	1.21 abc	203 ab
SPM	*T. harzianum KMD*	0.73 bcd	183 ab
100% Koir	Not treated	1.59 abc	238 a
75% CPM + 25% Koir	Not treated	1.48 ab	259.67 a
50%CPM + 50% Koir	Not treated	1.34 abc	165 b
25% CPM + 75% Koir	Not treated	0.51 bcd	135 b
100% CPM	Not treated	1.48 ab	2.85 a
SPM	Not treated	1.30 bc	209 ab
Effects		P-values	P-values
Treatments		0.05*	0.0139*
Media		0.0001***	0.0001***
Treatments*Media		0.086 ^{NS}	0.0706 ^{NS}
		CV%=13.69	CV%=12.73
		MSE=0.654	MSE=0.841

NS = Not significant, * *= significant at P≤ 0.05,, *** = significant at P≤0.001
Means with the same letter are not significantly different (P= 0.05) according to Student, Newman and Keuls comparison test.
CPM = coarse potting mix, SGM = Seedlings Growers mix (composted pine bark)

Table 6.9. Growth response of tomato seedlings to *Trichoderma harzianum* KMD following growth in different ratios of media, resulting in drought and water- logging conditions after four weeks of growth.

Ratios of Media	Treatments	Plot weight (g)	Plant height (mm)
100% Coir	*T. harzianum* KMD	0.206 b	125 ab
75% CPM + 25% Coir	*T. harzianum* KMD	0.576 a	144 ab
50%CPM + 50% Coir	*T. harzianum* KMD	0.85 a	200 a
25% CPM + 75% Coir	*T. harzianum* KMD	0.8 a	88 c
100% CPM	*T. harzianum* KMD	0.653 a	245 a
SPM	*T. harzianum* KMD	0.236 b	152 ab
100% Coir	Not treated	0.163 b	147 ab
75% CPM + 25% Coir	Not treated	0.382 b	152 ab
50%CPM + 50% Coir	Not treated	0.383 b	243 a
25% CPM + 75% Coir	Not treated	1.01 a	110 ab
100% CPM	Not treated	0.81 a	250 a
SPM	Not treated	0.236 b	158 a
Effects		P-values	P-values
Treatments		0.0569**	0.0007***
Media		0.0001***	0.0001***
Treatments*Media		0.0088**	0.999 NS
		CV%=42.85	CV%=11.53
		MSE=0.92	MSE=0.91

Table 6.10. Growth response of maize seedlings to *Trichoderma harzianum* KMD following growth in different ratios of media, resulting in drought and water- logging conditions after four weeks of growth.

Ratios of Media	Treatments	Plot weight (g)	Plant height (mm)
100% Coir	*T. harzianum* KMD	2.09 a	600 ab
75% CPM + 25% Coir	*T. harzianum* KMD	2.10 a	500 a
50%CPM + 50% Coir	*T. harzianum* KMD	1.770 a	385 ab
25% CPM + 75% Coir	*T. harzianum* KMD	2.43 a	388 ab
100% CPM	*T. harzianum* KMD	1.816 a	498 ab
SPM	*T. harzianum* KMD	1.62 a	482 ab
100% Coir	Not treated	2.43 a	700 a
75% CPM + 25% Coir	Not treated	1.81 a	550 ab
50%CPM + 50% Coir	Not treated	2.37 a	485 ab
25% CPM + 75% Coir	Not treated	1.43 a	385 ab
100% CPM	Not treated	1.71 a	500 ab
SPM	Not treated	1.82 a	400 ab
Effects		P-values	P-values
Treatments		0.4729 NS	0.0765 NS
Media		0.0219*	0.0001***
Treatments*Media		0.8901 NS	0.1039 NS
		CV%=35.96	CV%=15.59
		MSE=2.43	MSE=0.702

NS = Not significant, *= significant at P≤ 0.05, ** = significant at P≤0.01, *** = P≤0.001
Means with the same letter are not significantly different (P= 0.05) according to Student, Newman and Keuls comparison test.
CPM = coarse potting mix, SGM = Seedlings Growers mix (composted pine bark)

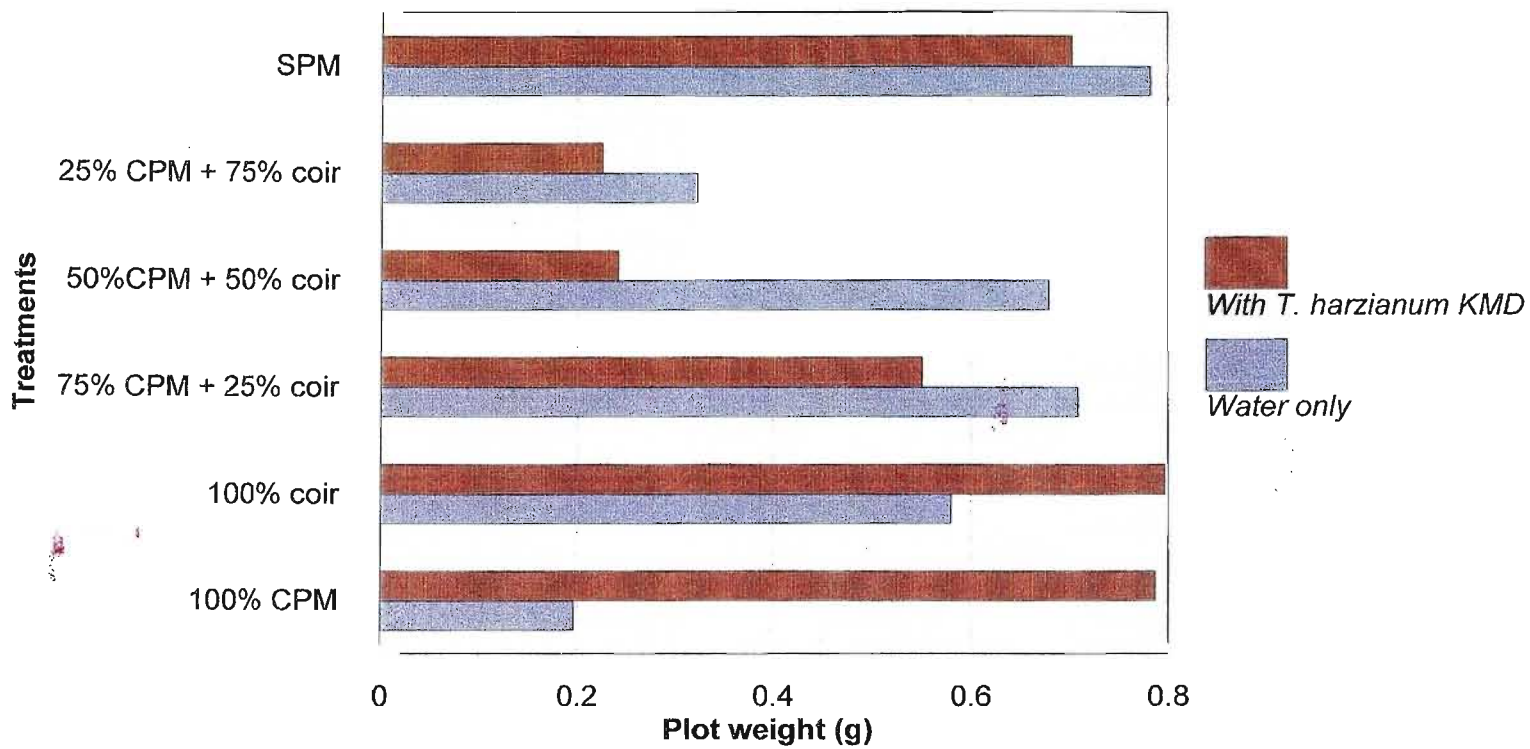

Figure 6.1.Plot weight of cabbage seedling shoots to *Trichoderma harzianum* KMD following growth in different ratios of media, resulting in drought and water- logging conditions after four weeks of growth.

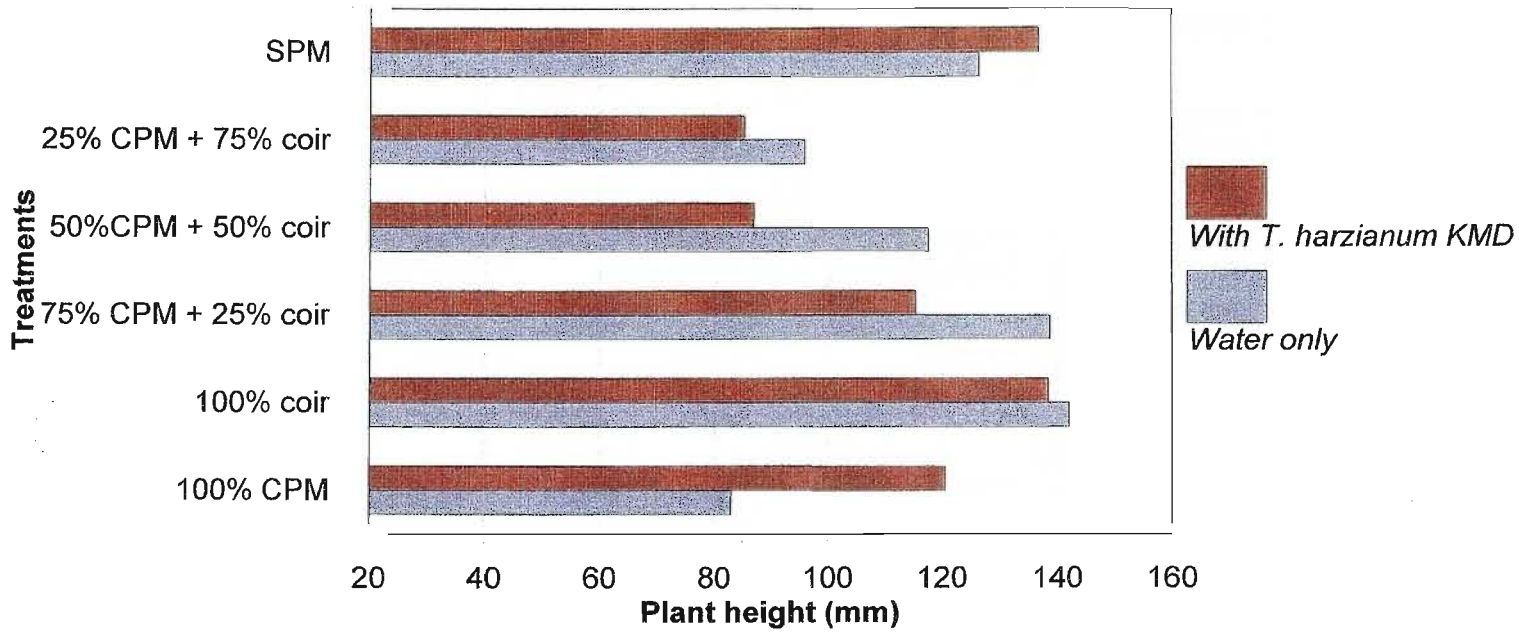

Figure 6.2. Plant height of cabbage seedlings to *Trichoderma harzianum* KMD following growth in different ratios of media, resulting in drought and water- logging conditions after four weeks of growth.

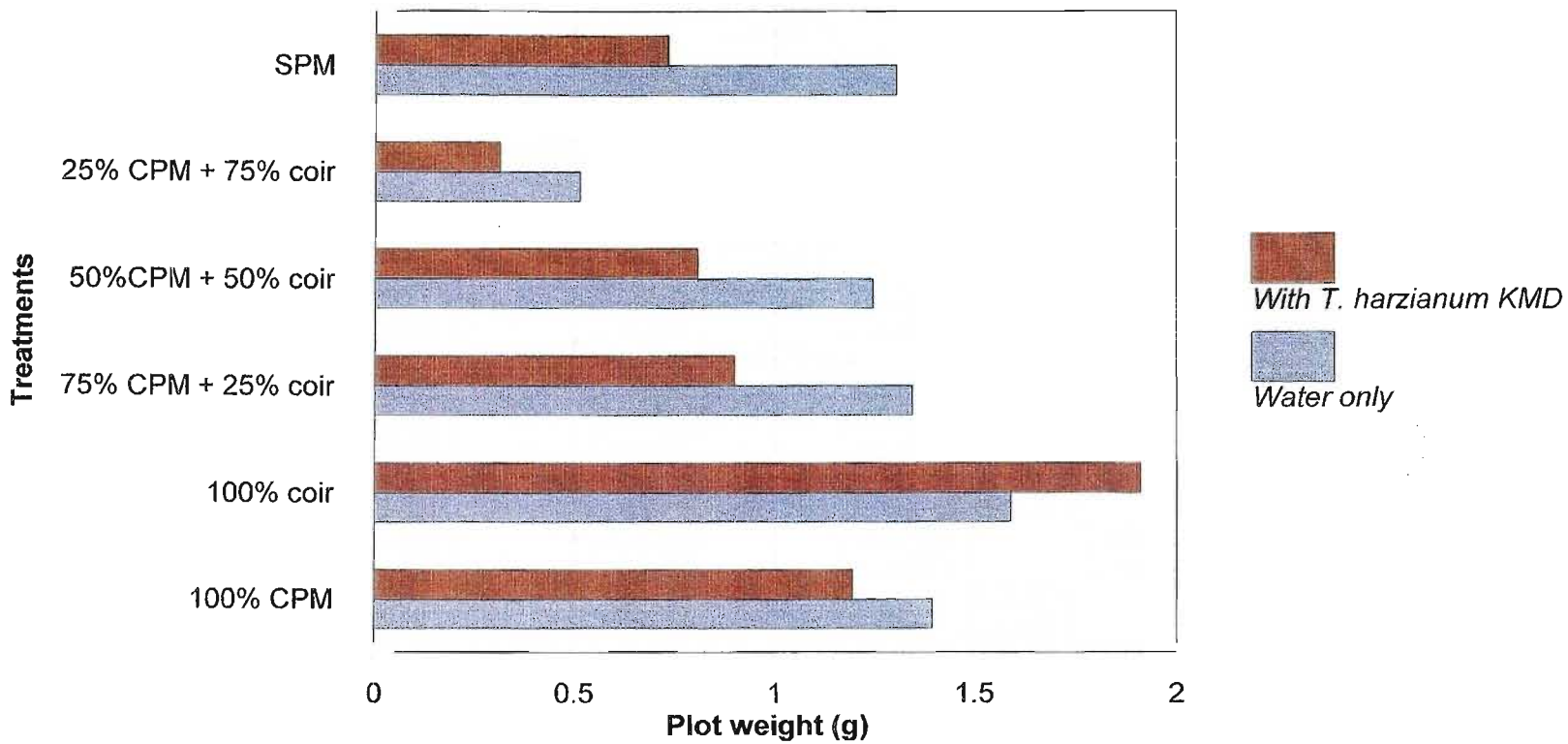

Figure 6.3. Plot weight of cucumber seedling shoots to *Trichoderma harzianum* KMD following growth in different ratios of media, resulting in drought and water- logging conditions after four weeks of growth.

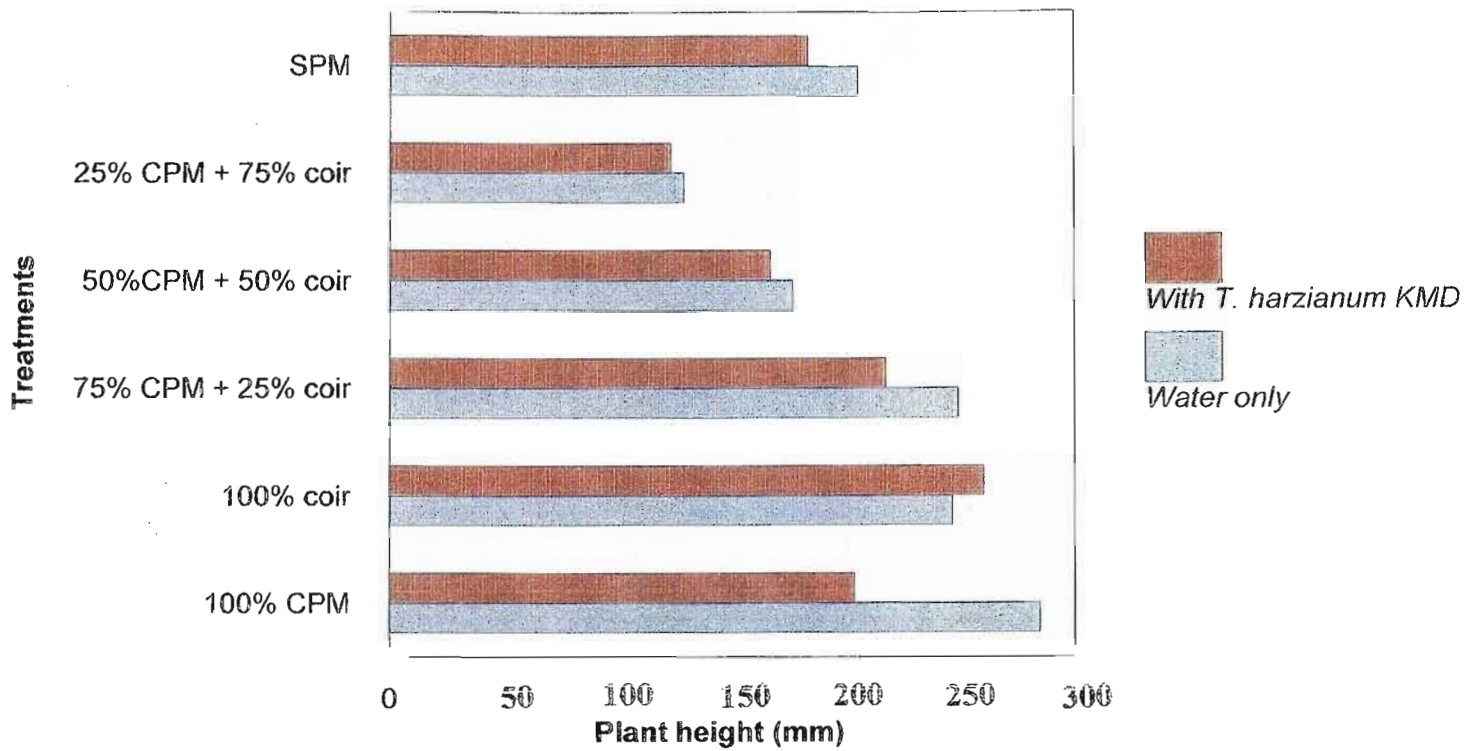

Figure 6.4.Plant height of cucumber seedlings to *Trichoderma harzianum* KMD following growth in different ratios of media, resulting in drought and water- logging conditions after four weeks of growth.

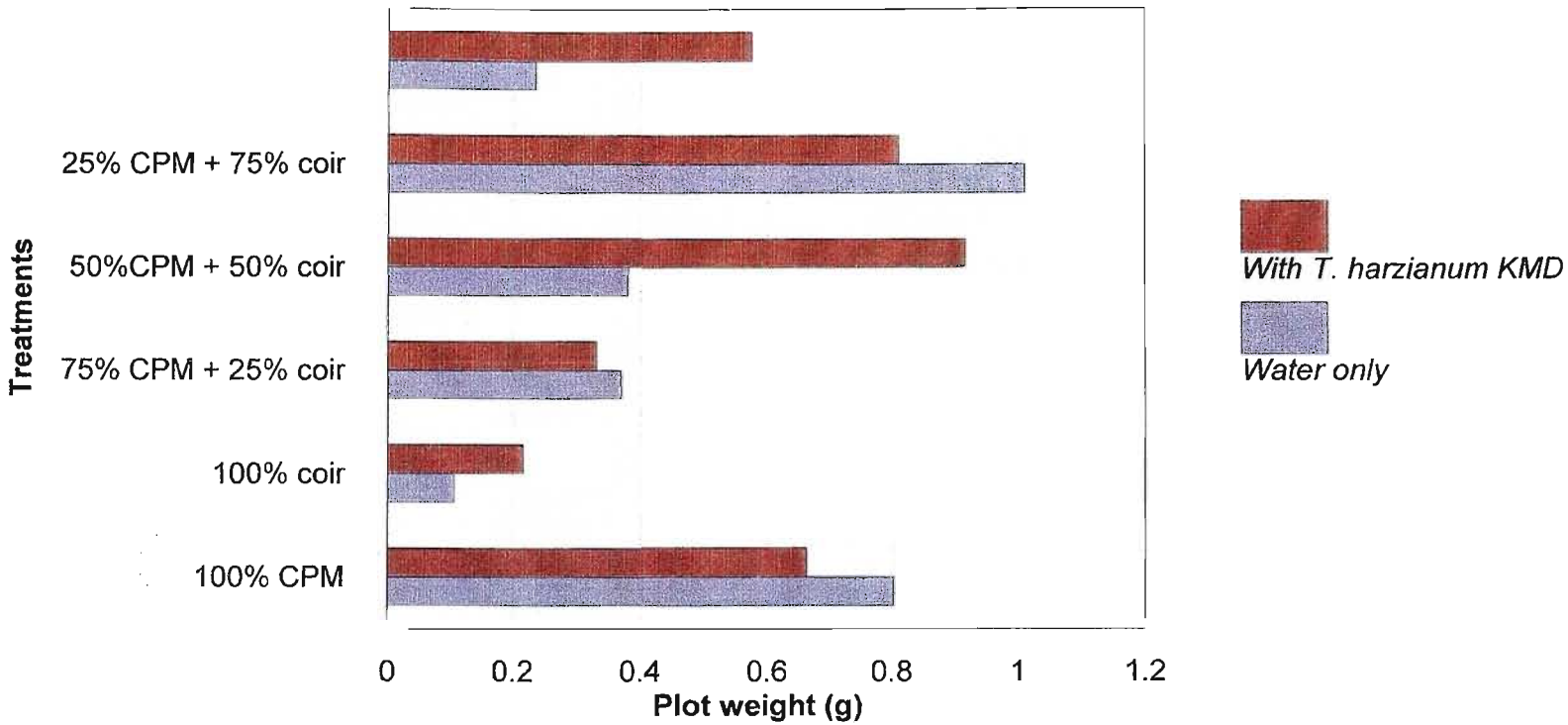

Figure 6.5. Plot weight of tomato seedling shoots to *Trichoderma harzianum* KMD following growth in different ratios of media, resulting in drought and water- logging conditions after four weeks of growth.

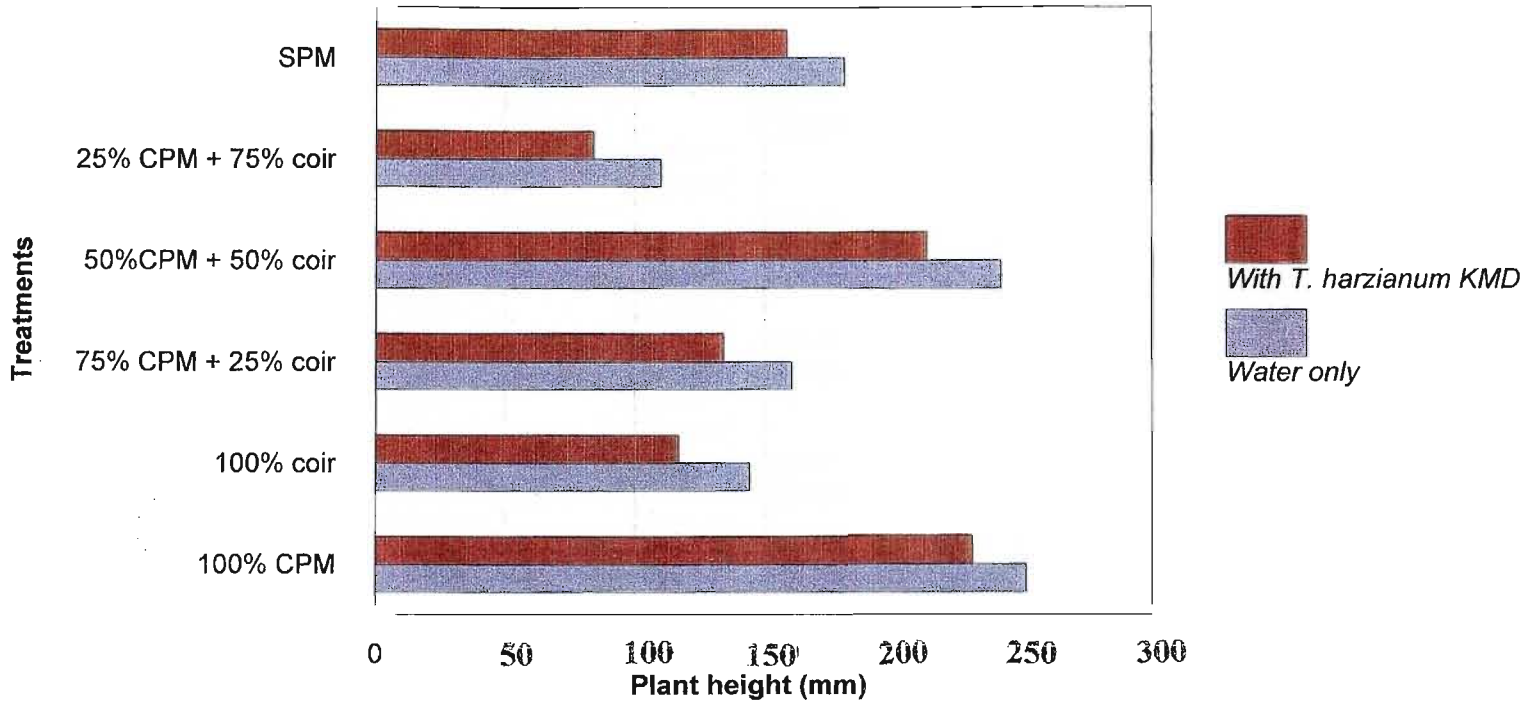

Figure 6.6. Plant height of tomato seedlings to *Trichoderma harzianum* KMD following growth in different ratios of media, resulting in drought and water- logging conditions after four weeks of growth.

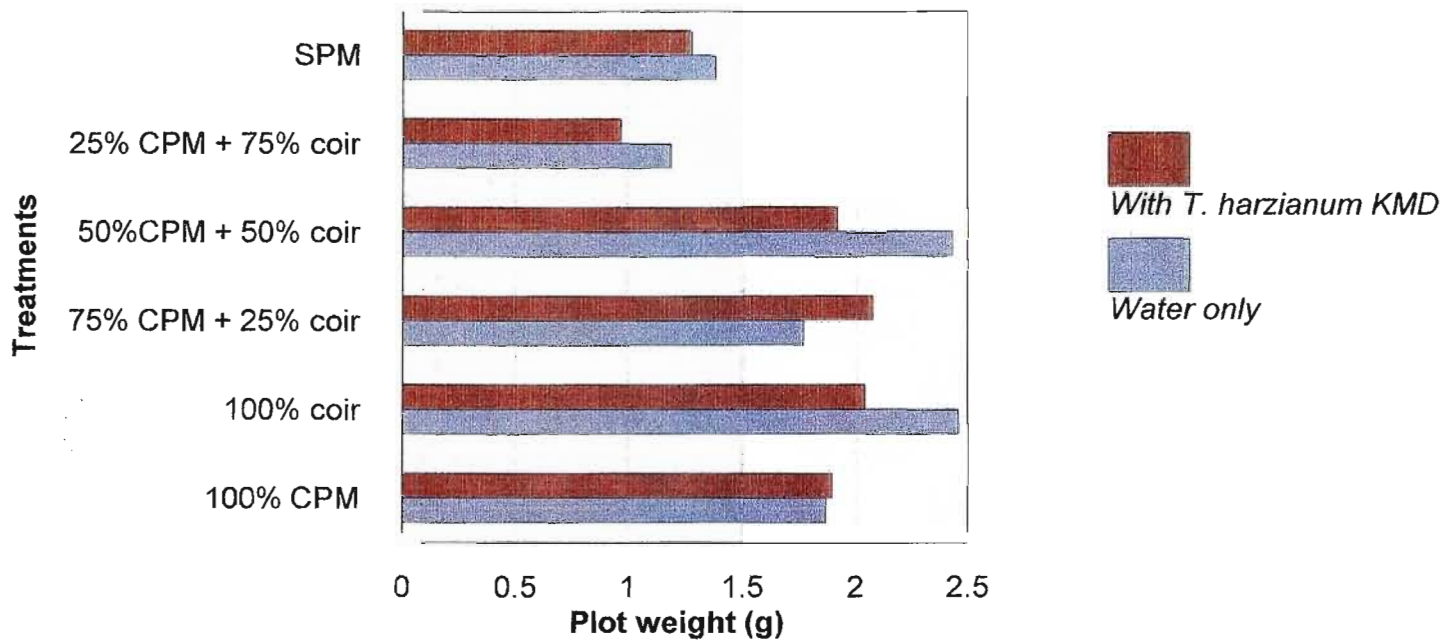

Figure 6.7. Plot weight of maize seedling shoots to *Trichoderma harzianum* KMD following growth in different ratios of media, resulting in drought and water- logging conditions after four weeks of growth.

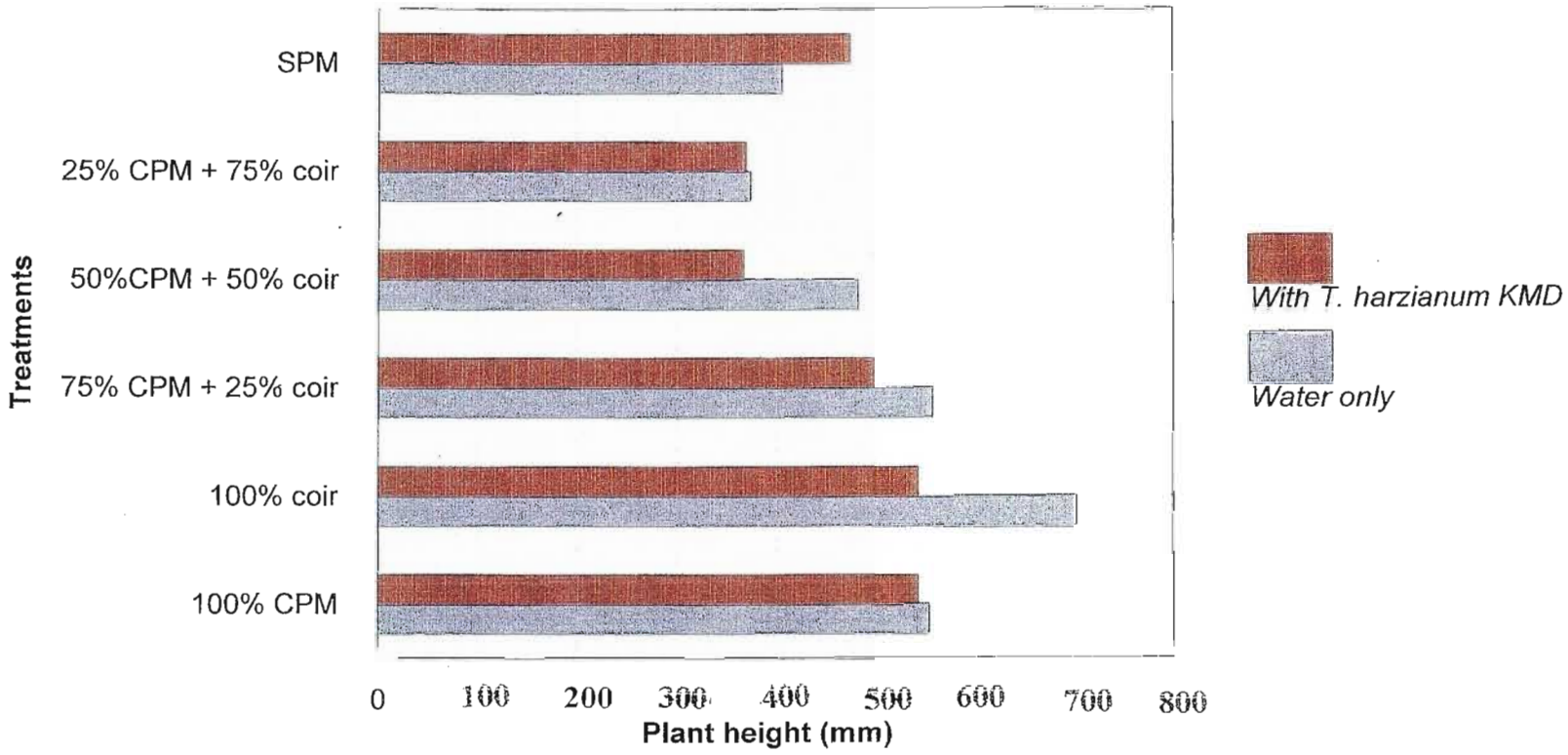

Figure 6.8. Plant height of maize seedlings to *Trichoderma harzianum* KMD following growth in different ratios of media, resulting in drought and water- logging conditions after four weeks of growth.

Maize seedlings

Significant differences between treatments and periods of cold were recorded for plot weight of maize seedlings (Table 6.6). All seeds treated with cold only and treatments with the application of *T. harzianum* KMD and cold were comparable and no reduction in vigor was caused by cold stress. Significant differences were noted between treatments of cold exposure for total dry weight of roots. As the cold period increased, root dry weights decreased indicating that cold stress had an adverse effect on roots. *Trichoderma harzianum* KMD did not alleviate cold stress on plants, since *T. harzianum* KMD produced similar root dry weights to the control for all periods of cold only. No significant differences were recorded between treatments for plant height and percentage germination nor significant differences were recorded between periods of cold exposure on maize seedlings.

6.3.3 Drought trials

Cabbage seedlings

A potting medium containing 100% coir peat resulted in high water retention and water–logging conditions on cabbage seedlings. Application of *T. harzianum* KMD to seeds and sown in this medium increased plot weights of cabbage seedlings when compared to seeds that were untreated and grown in a similar medium. Plant height was enhanced significantly when compared to the controls (Table 6.7, Figure 6.1- 6.2).

Media containing 75% coarse potting mix and 25% coir peat reduced water-logging and allowed for some drainage of water. This medium had an air filled porosity of 35.3% (Appendix C) hence an increase in water drainage. For this medium the application of *T. harzianum* KMD resulted in poor plot weights compared to the untreated controls.

The medium containing 50% coarse potting mix and 50% coir peat allowed for an air filled porosity of 19.5% and a water holding capacity of 33.28% (Appendix C). Seeds grown in this medium and treated with *T. harzianum* KMD showed reduced plot weight when compared with untreated controls and sown in a similar medium.

The medium containing 25% coarse potting mix and 75% coir peat, had a water holding capacity of 56.3% (Appendix C), hence a high water content. For this treatment, *T. harzianum* KMD did not enhance plot weight when compared to the untreated seeds.

The 100% coarse potting medium had low water holding capacity and a high air filled porosity resulting in drought stress for the plant. The application of *T. harzianum* KMD for this treatment did counteract stress and thus did not increase the vigor of plants for this treatment.

Cucumber seedlings

Media prepared with a 100% coir peat had a water holding capacity of 68.7% and a low air filled porosity of 10.9%. The medium allowed a high percentage of water to be present and low oxygen content. When seeds were treated with *T. harzianum* KMD and sown in the above medium, *T. harzianum* KMD enhanced growth and seedling vigor when compared to the untreated control (Table 6.8, Figure 6.3-6.4).

Media prepared with a 75% coarse potting mix (Appendix C), 25% coir peat and, resulted in an air filled porosity of 35.3%, 19.5% and a water holding capacity of 46.6% and 49.6%, respectively. Seeds treated with *T. harzianum* KMD sown in 50% coarse potting mix, 50% coir peat medium did not enhance plot weight when compared to the untreated controls.

Media prepared with a 100% coarse potting mix, resulted in a water holding capacity 33.2% and air filled porosity of 46.5% resulted in drought conditions. Seeds treated with *T. harzianum* KMD and sown in this medium resulted in poor plot weights. Plant height was significantly enhanced when seeds were applied with *T. harzianum* KMD and sown in most media.

Tomato seedlings

In most instances, media with a high water holding capacity containing, *T. harzianum* KMD enhanced plant height of tomato seedlings when compared to the untreated controls. Percentage germination and plot weight was enhanced when seeds were applied with *T. harzianum* KMD and sown in media with 75 % and 50% coir peat compared to the controls (Table 6.9, Figure 6.5-6.6).

Maize seedlings

No significant differences between treatments were recorded on plot weight of maize seedlings. Significant differences were recorded on plant height of seedlings. All seeds treated

with *T. harzianum* KMD resulted in taller and healthier plants than those untreated and stressed (Table 6.10, Figure 6.7-6.8).

6.3.4 Root system architecture- Rhizotron studies

Cucumber and maize seedlings treated with *T. harzianum* KMD resulted in an increase in root area than the untreated controls. In the absence of a disease, colonization by *T. harzianum* KMD on maize and cucumber roots in rhizotron studies increased root area by 3104.52 mm^2 and 1787.48 mm^2, respectively. Root areas of treated seeds increased by 1000 mm^2 when compared to the controls. These root areas were larger than those that were untreated. More branching was seen of rooting systems of treated *T. harzianum* KMD seedlings.

6.4 DISCUSSION

Trichoderma harzianum KMD did not effectively enhance the growth of seedlings and the vigor of seedlings was not restored after cold treatment. This strain of *Trichoderma* did not alleviate stress at the three stress physiological sites in this study.

6.4.1 Oxidation injury trials

All crops exhibited high vigor, even after being treated with hypochlorite. Future studies need to be carried out by exposing seeds for a longer period to hypochlorite treatment. Results were consistent with seedlings treated with hypochlorite resulting in healthy plant growth. Reasons for the failure of hypochlorite treatment of seeds could be attributed to the length of exposure of seeds to the chemical, as seeds were only exposed for 15min. This window of exposure may not be long enough for the peroxidation of membranes, lipids and proteins within the seed to cause injury. If hypochlorite treatment caused stress on plants, one would have expected *T. harzianum* KMD application to overcome the negative effects of hypochlorite, and increased plant growth by producing growth regulating hormones e.g., auxins and gibberellins. If *T. harzianum* KMD enhanced plant growth after the negative effects of hypochlorite injury this would provide a clue to the mechanism of growth enhancement by *Trichoderma*. There are many injurious oxidative reactions caused by hypochlorite (Abdul-Baki, 1979; Sauer & Burroughs, 1986; Schraufstaetter *et al.*, 1990), but of most interest is the peroxidation of membrane lipids; a reaction that can be prevented by metabolic activity of the fungus. If *T. harzianum* KMD enhanced plant growth during hypochlorite injury, one of the alternatives would be by producing growth regulator hormones which may accelerate cell elongation in the root tips (Winham *et al.*, 1986) A second alternative is that *T. harzianum*

KMD could suppress minor pathogens through competition and mycoparasitism (Bjőkman *et al.*, 1998).

6.4.2 Cold stress

Cold treatment effectively reduced root and shoot dry weight for most crops. In most instances, the growth of stressed plants was not enhanced by *T. harzianum* KMD. Cold stress was applied in a way that causes injury primarily by accelerated respiration that would occur before active colonization of *T. harzianum* KMD (Stewart *et al.*, 1990). Thus cold reduces the amount of seed reserves available for growth but in this instance seed reserves were not reduced. Little difference was noted between 1 to 7 days of cold. It was evident that *T. harzianum* KMD performed better in enhancing growth when applied to unstressed plants. There were no interactions between cold treatment and *T. harzianum* KMD. However, tomato seedlings were enhanced by *T. harzianum* KMD colonization. This could be attributed to the proposed hypothesis of the production of an independent auxin, which enhances growth rate (Blanchard & Björkman, 1996).

6.4.3 Drought

Trichoderma harzianum KMD colonization on tomato, cabbage and maize was not consistent on drought stressed plants. In some instances *T. harzianum* KMD did not enhance plant growth by producing growth regulating hormones to reverse any mechanisms related to drought stress. However, *T. harzianum* KMD enhanced plot weight and plant height of cucumber in more water-logged conditions. It was evident that *T. harzianum* KMD enhanced plant growth more in plants that were grown in media containing a high water content.

6.4.4 Root system architecture- Rhizotron studies

Root architecture showed that application of *T. harzianum* KMD to cucumber and maize roots consistently enhanced growth of roots by increasing area. The increased area resulted in increased branching of roots that are the primary site of nutrient uptake. The colonized seedlings were able to begin taking up substantial amounts of mineral nutrients in the soil sooner than uncolonized seedlings (Björkman *et al.*, 1998).

Another mechanism of growth enhancement of *T. harzianum* KMD could be the displacement of other components of the rhizosphere microflora, which can result in stronger root growth. It is difficult to rule out this possibility because all crop root growth depends on an

appropriate rhizosphere flora. Although the composition of this flora is not well defined, altering its composition and abundance is likely to affect root growth by affecting its growth supporting functions (Björkman *et al.*, 1998).

The strain of *T. harzianum* used in this study can control the most important soil-borne pathogens, including *Pythium* sp. and *R. solani* (Chapter 2). This ability indicates that this strain could be rhizosphere competent. Growth enhancement could have occurred primarily as a consequence of root protection against pathogenic or other deleterious micro-flora (Björkman *et al.*, 1998).

Overall, growth of seedlings was not enhanced by *T. harzianum* KMD even after stress treatments of cold, drought and oxidation injury. If enhancement of plant growth by *T. harzianum* KMD had occurred on stressed plants this would indicate that *T. harzianum* KMD application reversed or limited the damage by producing growth regulating hormones. No interactions occurred between stress and *T. harzianum* KMD colonization suggesting that mechanisms are independent. These results indicate that further studies are needed, to determine the effect of *T. harzianum* KMD application on plant vigor.

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CHAPTER 7

GENERAL OVERVIEW

In the development of a successful inoculant product, the identification of an organism with the desired beneficial action is often not difficult. To develop biocontrol organisms, one must be able to produce a commercially viable product. Formulation of live organisms in commercial products, however, continues to be a challenging and often success-limiting step. Formulated organisms are suspended in a suitable carrier, which is supplemented by additives to maximize survival, optimise application to the target and protect the organisms after application. Burges (1998) stated that: "In contrast to chemical active ingredients, they are particulate and live or proteinous in nature, making them relatively sensitive to storage conditions and the environment".

No other research had actively pursued the development of a commercial product in South Africa. Basic methods and ideas were borrowed from successful research in other countries. Much of our research has therefore verified facts about the particular strains of formulated biocontrol organisms that were used in this study. Our research confirmed the following:

1. All formulations of all biocontrol organisms are effective growth stimulation agents on a variety of crops.
2. All formulations of both *T. harzianum* KMD and *G. virens* MM1 effectively reduced disease caused by *Rhizoctonia solani.* In most instances biocontrol of *Pythium* spp. was achieved by chlamydospore formulations of *T. harzianum* KMD and *G. virens* MM1 on specific crops.
3. *Trichoderma harzianum* KMD was not compatible with commonly used fungicides except when seed treated.
4. *Trichoderma harzianum* KMD can be mass produced in culture conditions of C: N 14 at pH 4. Spores have a longer shelf-life and are more viable at this culture condition.
5. *Trichoderma harzianum* KMD possesses a mechanism, mycoparasitism, which arrests the growth of a soil-borne pathogen, *R. solani*, hence making it an effective biocontrol agent.
6. *Trichoderma harzianum* KMD increases plant growth by increasing root area of plants. The results show that *T. harzianum* KMD has no, or little effect on alleviating stress.

Individual phenomena and facts established about biocontrol, growth stimulation, shelf-life and compatibility with fungicides in each study are discussed and conducted within that study. These facts are necessary for R & D to register a commercial *Trichoderma* formulation.

Trichoderma and other biocontrol organism research continues at this university. My in depth knowledge and experience accumulated over the past two years will be useful to my successors. The remainder of my conclusions will take the form of practical recommendations and comments to the manufacturer or students for future research.

7.1 Growth stimulation and disease control of formulations of *Trichoderma harzianum* KMD, *Gliocladium. virens* MM1 and *Bacillus subtilis* AW57.

Our research has found that all three biocontrol organisms effectively enhanced plant growth. Compared to controls, increases in yield as high as 1000-5000% were observed. In our growth stimulation trials, all formulations of biocontrol organisms *T. harzianum* KMD, *G. virens* MM1 and *B. subtilis* AW57, stimulated plant growth equally well, although growth stimulation differed from one crop to another.

- Formulations prepared with conidia in kaolin powder with oil, experimental compound and the commercial formulations performed the best whilst formulations of chlamydospores performed the worst. *Bacillus subtilis* AW57 on the other hand also increased plant growth consistently with both formulations as Nutristart and as washed cells. By maximizing these biocontrol organisms under greenhouse conditions, they could aid in improving seedling production and increase plant growth.
- For effective growth stimulation, recommendations to the manufacturer would be to formulate *T. harzianum* KMD and *G. virens* MM1 with conidia, rather than chlamydospores. Conidia are more cost effective as they donot need to be activated with 0.5M HCL before application.
- Biological control of soil-borne *Pythium* spp. by these biocontrol organisms was inconsistent. This could be related to the lack of pathogenicity of the pathogen during the course of the study or the biocontrol organism being specific on certain crops. Formulations of chlamydospores performed better in reducing disease than those containing conidia.

- Biological control of *R. solani* was effective in most instances by *T. harzianum* KMD and *G. virens* MM1 on all crops. All formulations of conidia performed well whilst chlamydospores did not.

- Future success can be achieved if *Trichoderma* was successfully inoculated into the root zone of seedlings in the nursery before transplanting them to infected fields. Growing the seedlings in trays ("Speedling") enables the grower to transfer them with their surrounding soil mixed with *Trichoderma*, thus forming a "protective layer."

- Formulations that were prepared with a lower inoculum of chlamydospores per gram of formulation were inferior to those containing a higher inoculum of chlamydospores.

Hence recommendations to the manufacturer:

- Prepare formulations that contain conidia rather than chlamydospores. Activation of chlamydospores will cost the manufacturer extra money and the consumer time.
- Formulations of *T. harzianum* KMD and *G. virens* MM1 need to be prepared with a higher inoculum of 10^8-10^9 spores/gram of formulation.
- Formulations prepared with conidia in kaolin powder with oil, commercial formulation were much easier to use and apply, whilst formulations prepared in an experimental compound were difficult and impractical to use. The experimental compound formulation was difficult to suspend in water.
- Preparing formulations of *B. subtilis* AW57, is time consuming, but effective in disease control and enhancing growth. New and innovative ways are needed to formulate these organisms in powder, which will be easy to use.

1. Application methods

- Use of seed treatment and drenching for *in vivo* screening of *T. harzianum* KMD and *G.virens* MM1, was the most reliable method of application for growth stimulation and biocontrol.
- The drench application was more effective when formulations were prepared with conidia with 10^8 spores per gram of formulation.
- Seed treatment was the best application to apply organisms to soil and holds potential for future experiments. This method allows more of the biocontrol agent to colonize without

being already in contact with the pathogen. Seed treatment prevented seed decay and early damping-off over the first two weeks.

- Capping can be improved in greenhouse trials. This application method can be employed by inoculating the soil area around the seed with the formulation.
- Further investigations are also necessary to compare *T. harzianum* KMD *and G.virens* MM1 efficacy in various sticker solutions.

2. Dosage

- Drenching of formulations at dosages between 1-5g/l was more effective in both growth stimulation and biocontrol of all crops.
- 10g/l of each formulation caused severe stunting of seedlings. This suggests that *T. harzianum* KMD and *G. virens* MM1 produce metabolites that inhibit growth of plants when applied as large inocula.
- Consumers should be aware of the dose to be used in the field. These recommendations should be illustrated on packaging.
- Future studies are required with respect to dosage. Overdose results in stunting of seedlings. The mechanism responsible for this has to be solved to make this biocontrol agent more user-friendly for growers. This problem may cause the biocontrol agent to lose its potential on the market.

7.2 Compatibility of *T. harzianum* KMD with selected fungicides under greenhouse conditions.

- *Trichoderma harzianum* KMD was affected when in contact with fungicides by resulting in a lack of sporulation.
- However seed treatment was the best application method to deliver the biocontrol agent with the presence of fungicides under greenhouse conditions.
- Future studies are needed to determine whether the biocontrol activity and growth stimulation of *T. harzianum* KMD is affected when a lack of sporulation occurs.
- Future requirements are needed to determine whether *T. harzianum* KMD can be a part of an integrated system.

Since this local strain of *Trichoderma* when applied with a foodbase or as a seed coating can survive for long periods of time and even propagate in soil (Harman *et al.*, 1980), its

combination with chemical, cultural, or physical methods (Katan *et al.*, 1976; Chet *et al.*, 1982) can achieve a long term controlling effect on soilborne plant pathogenic fungi.

No biocontrol agent and no fungicide can totally suppress all the propagules of the pathogen under practical conditions. But, in many cases, this strain of *Trichoderma* could successfully replace common fungicides. Moreover, while many chemicals are degraded after a short time, it has been cited that many scientists have found *Trichoderma* to survive and even propagate in the soil. Future studies need to be done on the integration of resistant isolates of this strain of *Trichoderma* with low doses of fungicides, which could lead to a synergistic effect resulting from suppression of competitive soil micro-flora.

7.3 Effects of culture conditions on *Trichoderma harzianum* KMD

- *Trichoderma harzianum* KMD grew well under acidic conditions.
- Maximum sporulation was reached after 60-92h when cultured in liquid media containing C:N 14 at pH 4.
- This culture condition is the most suitable to use as spores obtained from this culture condition were more viable and survived adverse storage conditions.
- These culture parameters are useful to the manufacturer as this opens an interesting area to bulk up live and particulate organisms and to keep them viable through transport and storage.
- However, future studies are needed to determine the difference between liquid and solid fermentation on the production of *T. harzianum* KMD.
- Future research is needed to determine the costing of both fermentations. Which fermentation produces more viable spores? Which fermentation is less time consuming?

7.4 Possible mechanisms of *Trichoderma harzianum* KMD

- *Trichoderma harzianum* KMD is an effective mycoparasite.
- However, one cannot rule out the presence of other mechanisms that may play a role in arresting soil-borne pathogens. This isolate of *Trichoderma* may indeed excrete inhibitory substances, but evidence of their importance in biological control is, as yet, insufficient. Competition may play a role in this microbial interaction; it may be an independent

phenomena; or it may be connected with antibiosis or parasitism. However, further studies are needed to elaborate and clarify this question.

- Production of antibiotics and enzymes by *T. harzianum* KMD etc. need to be researched further.
- Future studies need to be elaborated on genetic engineering, which may provide the gene coding for lytic functions. Cloning of these genes into bacteria or yeasts will enable the manipulation of gene expression in the new host. The isolated genes may then be reintroduced into the lytic antagonist, to produce a super-active biocontrol agent. This future work depends on thorough physiological-ecological research, which will reveal the essential mechanisms enabling a specific isolate of *Trichoderma* to be an efficient and successful biocontrol agent.

7.5 Effect of environmental stress on *Trichoderma harzianum* KMD

- *Trichoderma harzianum* KMD increases plant growth by increasing the root area of plants.
- The effect of stress, cold, drought and oxidative injury on plants were not alleviated by this isolate of *Trichoderma* in our trials.
- Future trials need to be done, by increasing the level of stress on *T. harzianum* KMD. This would determine the potential of *T. harzianum* KMD under stressed conditions.

7.6 Nursery trials

- Quality control of growing media is necessary to support bacterial and fungal growth.
- Tray sterilisation must be performed to prevent any source of bacterial or fungal contaminations.
- Heating and cooling devices are essential for temperature control in the greenhouses and plastic covered tunnels. They must be installed or other control facilities found.
- Controlled environmental conditions, such as plastic tunnels, greenhouses and nurseries, where temperatures and moisture levels can be predicted and the right isolate chosen, will undoubtedly improve the chances for successful biological control.

Several challenges need to be resolved if the full potential of a biocontrol agent is to be exploited for commercial purposes. The challenge of developing consistent benefits and product delivery needs to be met. Formulations need not only maintain viability but must possess the ability to sustain growth promotion and biological control potentials of micro-organisms. The formulations must be developed with a simple delivery system that allows easy application by small-scale farmers and seed companies with existing equipment and application practices. Fungal fermentations systems must also be optimized and the quality of their output controlled with respect to inoculum density and biological activity.

7.7 A need for field experiments

Many of our promising results in biological control and growth stimulation were achieved in composted pine bark under a controlled environment in the greenhouse. These results will not be of great importance if they are not tested in the field. Field trials are essential if these potential biocontrol agents are intended for large scale production for commercial purposes. The product must be tested in the field in different areas, and the environmental limits on the biological activity must be determined. Survival and dispersal of the organisms in the environment must be closely monitored.

7.8 Overall conclusion

Trichoderma harzianum KMD shows promise as a biocontrol agent against soil-borne pathogens i.e., *Pythium* sp. and *R. solani*. This biocontrol agent may also serve as part of a replacement strategy for toxic fungicides and fumigants under greenhouse conditions. Growth stimulation was significantly enhanced when *T. harzianum* KMD was applied to seedlings when the correct use of formulation, application and dosage were tested. These criteria showed how successful this biocontrol agent is when the above criteria were manipulated.

Such formulations used in this research required production methods, which produced more viable spores with a longer shelf-life. Manufacturers need to apply culture conditions, which showed the best method to culture more viable and long shelf-life spores.

The application of *T. harzianum* KMD protects plants from soil-borne diseases just as well as fungicides, resulting in improved yields.

Trichoderma harzianum KMD does not fall within the defined spectrum of a good growth stimulatory agent under stressful environmental conditions e.g. cold, drought and oxidative injury. A thorough forecast of the interactions between environmental conditions and mechanisms of growth stimulation and disease control needs to be studied.

Trichoderma harzianum KMD possesses a mechanism of action, mycoparasitism. Other mechanisms need to be researched e.g. production of chitinases, antibiotics and competition etc. It is only when mechanisms are completely understood that *T. harzianum* KMD can be applied in future research to obtain better and more consistent results.

7.9 Proposed future research priorities

- More scientific efficacy trials

Given the guarded but hopeful prognosis for the future of biological control in greenhouses, how can technology be moved from the laboratory to the commercial grower? More scientific efficacy trials with proper replication and statistical analysis are needed under commercial or near commercial conditions to obtain registration.

- Integration of biological control agents for insect and disease control in the greenhouse

Greenhouse trials should include integration of biocontrol agents for insect and disease control, given the predominance of insect biocontrol. Biocontrol registrations now request data on the interaction of pesticides with beneficial insects. Growers need to know whether *T. harzianum* KMD formulations are compatible with their current pest management strategies.

- Epidemiology and ecology of pathogens

More studies are needed on the epidemiolology and ecology of pathogens in the greenhouse, which may be different from the field. How is the pathogen introduced and how does it spread? What is the relationship between population density and damage?

How can the environment be manipulated to favour our biocontrol agent, *T. harzianum* KMD.

- Challenges of production and formulations of biocontrol agents

Rhodes, (1993) and Bok *et al.* (1996) states that: "The challenge of production and formulation of biocontrol agents remains, with each organism bringing its own set of problems". Effective production and formulation protocols are usually propriety, involving substantial investment to develop economic production and a formulation with adequate, shelf-life, stability, and titer. Even when all these conditions are met, the formulated product may be incompatible with the grower's practice. The mycoherbicide "Collego" produced by Ecogen, Inc. in the mid- 1980's, for example, was sold as a dry powder formulation that was stable for 2 years. However, actual application of the product was so laborious for the grower that sales plummeted and Ecogen, Inc. eventually stopped manufacture (Cross & Polonenko, 1996). Formulation difficulties may also explain the lack of *Pseudomonas* products, either for soil-borne pathogens or foliar pathogens, despite extensive basic research in this group. Based on experience, we estimate that production and formulation represent at least 50% of the costs of research and commercialisation of a biofungicide. Plant pathologists are generally ill prepared to shoulder these business arrangements (Paulitz & Bélanger, 2001).

Before *T. harzianum* KMD is marketed, it must be thoroughly tested by growers, whose comments, critiques, and suggestions for improvement, however drastic, will be crucial in avoiding unsuspected problems and preventing failures.

- Use of adjuvants/ stickers

Adjuvants, either added to the diluted product or incorporated into the formulation, are used as spreaders, anti-desiccants or stickers to improve the efficacy of the biofungicides. However, many of these adjuvants have fungicidal and/ or insecticidal properties which alone will often account for most of the reported activity of the mixed product (Bélanger & Jarvis, 1994). This trend toward activity from unknown adjuvants could lead to a

general depreciation of our biocontrol agent if growers cannot be convinced of the added benefits of the biocontrol agent. This relates to the properties of the active ingredients.

- Production and application of biocontrol agents

To improve on nature by intensive production, by eliminating deaths in storage, and by employing formulation to improve application and protect biocontrol agents, one needs to answer the following questions: Do organisms growing in nature and on nature's own substrates, perform differently to artificial, formulated products? This question needs to be asked as both better and poorer performances is informative. Although production yield can be optimized and organisms can be grown in apparent peak condition, this peak condition needs to be established, what is really needed to achieve it, and how it can best be preserved. Quicker action of products used inundatively, and an unexpected constitutive dormancy that might lead to better storage, are two features particularly to be sought. Experimental application still in the natural medium, nature's carrier, could be compared with formulated products (Burges, 1998).

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Appendix A

V8 agar medium plates were made as the following: 270 ml V8 juice, 15g of agar, 2g Calcium carbonate ($CaCO_3$) to 1 litre of distilled water. Autoclaved at 121°C at 1 Kpa for 15 minutes

Appendix B

The basal culture medium contained (g/l): $(NH_4)_2 SO_4$, 1.7; glucose, 2.7; and 50ml of mineral solution.

The mineral solution contained (g/l): $Mg\ SO_4.7H_2O$, 12.3; $KC\ell$, 10; $FeC\ell_2.4H_2O$, 0.1; $MnSO_4.H_2O$, 0.1; $ZnSO_4.7H_2O$, 0.1; thiamine hydrochloride, 0.05.

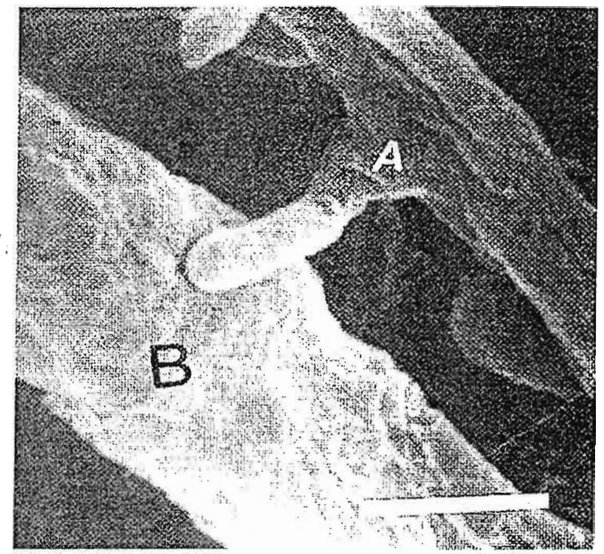
Appendix C

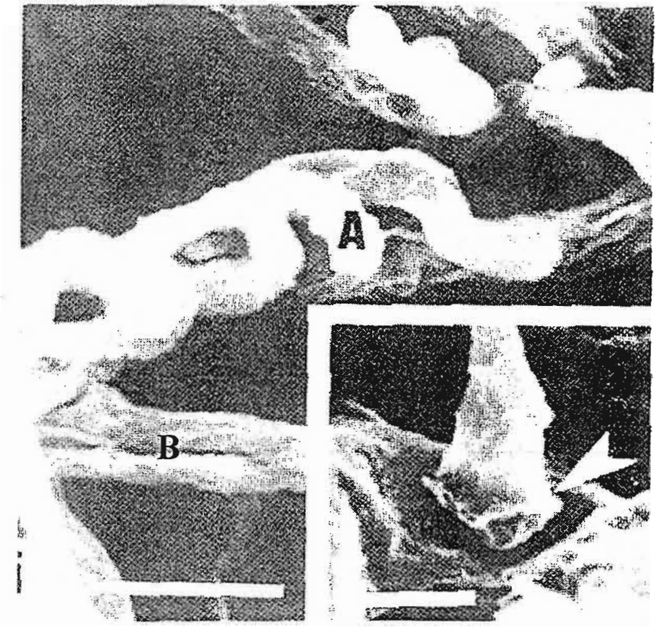
%Ratios of media	Water holding capacity	Air filled porosity
100% CPM + 0% coir	33.2%	46.5
90% CPM + 10% coir	34.7%	41.85
80% CPM + 20% coir	37.5%	37.7
75% CPM + 25% coir	46.6%	34.87
60% CPM + 40% coir	48.4%	27.9
50% CPM + 50% coir	49.6%	23.25
40% CPM + 60% coir	55.3%	16.9
25% CPM + 75% coir	56.3%	11.62
0% CPM + 100% coir	68.7%	10.9
100% SPM	51.6%	29.6

CPM = coarse potting mix

SPM = Seedling potting mix

Water holding capacity = % in a medium after drainage i.e. before actual drying out begins.





Appendix D

Plate 3.5a

Trichoderma viride TV-3 (A) was observed to be penetrating pathogen *Laetisaria arvalis* (B) hypha by producing hook-like structures (Gupta *et al.*, 1999).

Plate 3.5b

The main hyphae of isolates *Trichoderma harzianum* Th-2 (A) produced numerous characteristic small hook-like branches (inset) which penetrating the pathogen hyphae *Laetisaria arvalis* (B) (Gupta *et al.*, 1999).

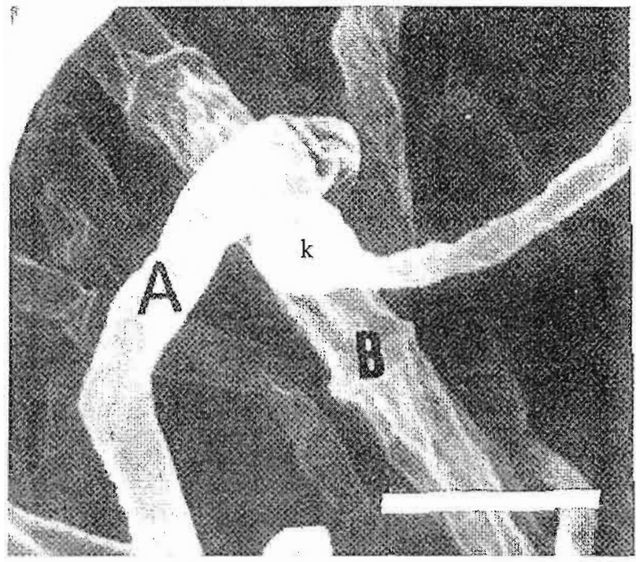


Plate 3.8a

Trichoderma longibrachiatum (A) produced appressorium-like structures at its hyphal tips and helped to grasp the pathogen hyphae *Laetisaria arvalis* (B) with the help of appressoria (k) (Gupta *et al.*, 1999).

EFFECTS OF CULTURE CONDITIONS ON THE SPORE ULTRASTRUCTURE OF A BIOCONTROL AGENT, *TRICHODERMA HARZIANUM* kmd

J. Omarjee[1], V. Bandu[3], M.D. Laing[1] and C.H. Hunter[2]

Disciplines of Plant Pathology[1], and Microbiology[2], SAES
[3] Center for Electron Microscopy.
University of Natal, Private Bag X01, Scottsville, Pietermaritzburg, South Africa

Trichoderma harzianum has been used successfully as a biological control agent against several soil–borne plant pathogens[1]. Biological control agents should possess several desirable characteristics, including: ease of preparation and application, stability during transport and storage, abundant production of viable propagules and good shelf-life. The influence of four growing media were investigated on the spore ultrastructure of a biocontrol agent *Trichoderma harzianum* strain kmd, using a basal salts medium with C:N ratios of 3 and 14, and pH's of 4.0 and 7.0. The effect of these culture parameters on viability and shelf-life were evaluated by counting colony forming units (C.F.U) before and after seven days of storage. Spore ultrastructural differences were carried out by embedding spores in Epon and sections were cut using a LKBIII ultramicrotome followed by examination in a Philips CM 120 BioTWIN transmission electron microscope at an accelerating voltage of 80KV.

Spores grown in media with a C:N ratio of 3 and 14 at pH 7.0 had an outer cell wall (W2), little mucilage and no lipid globules produced. These spores had poor viability and shelf-life before and after storage. However, spores grown in a medium with a C:N ratio of 3 and a pH of 4.0 (Fig.1) had a thick electron dense outer cell wall layer (W2) and mucilage layers. The presence of an outer cell wall (W2) and mucilage production in spores resulted in better survival of dessication and adverse conditions. The presence of lipid globules in these spores were associated with improved viability. This could be due to lipid globules serving as endogenous energy reserves for germination. Spores obtained from media with a C:N ratio of 14 at pH 4.0 (Fig.2) showed the development of two cell wall layers (W1 & W2), the outer being more electron dense than the inner. The outer wall is specific to *T. harzianum* (W2)[2]. This layer is the spore's first barrier and the first defense against adverse conditions. Spores obtained from this medium were larger, had germinated better and had a longer shelf-life than spores from C:N 3 medium, possibly because the two wall layers acted as a thicker barrier against adverse conditions.

The ultrastructural differences confirmed empirical results from liquid fermentation studies, that the pH and C:N ratio of the medium upon which spores of *T. harzianum* are produced have critical effects on physical and chemical structure of the spores. This, in turn, affects critical parameters for a biocontrol agent's spore germination and shelf-life.

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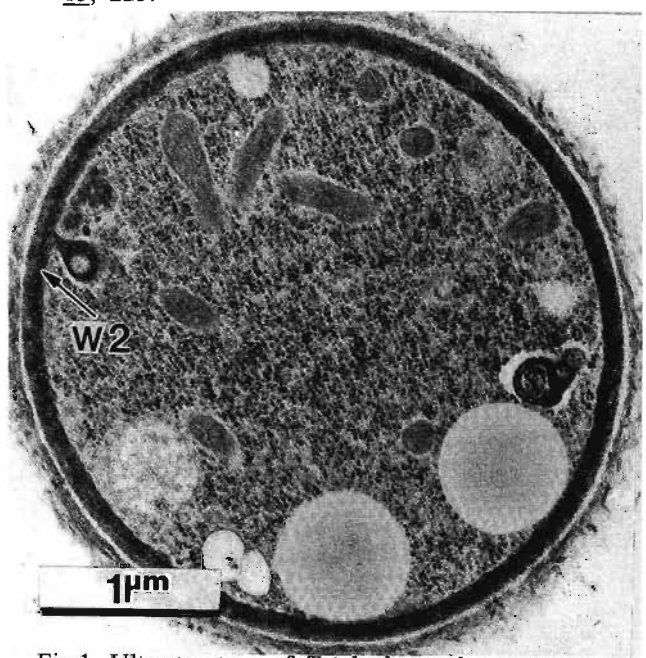

Fig.1. Ultrastructure of *Trichoderma harzianum* spore produced in liquid medium with C:N ratio of 3.0 at pH4.0.

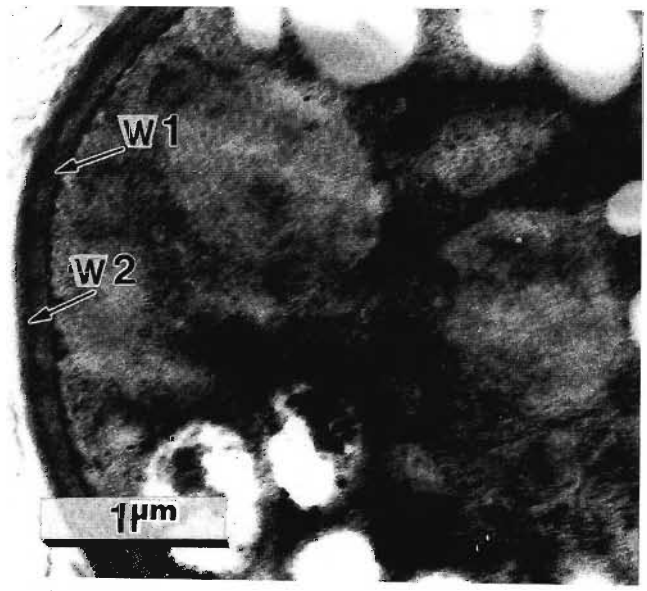

Fig.2. Ultrastructure of *Trichoderma harzianum* spore produced in liquid medium with C:N ratio of 14.0 at pH4.0.

bunches. This was not the case for the berry cheek, which yielded *B. cinerea* at low incidences from pea size to véraison for both cultivars. The fungicides significantly reduced infection in cheeks of Merlot, which showed a higher infection level at this site at harvest. These findings indicated that incipient infections can cause both mid- or late season bunch rot following a period of fungal latency in the rachises, laterals or pedicels, and not in berry cheeks and style ends. Owing to the efficacy of the fungicides in reducing high *B. cinerea* inoculum levels on and in vegetative parts of bunches, management strategies should concentrate on the pre-véraison stage, and on effective coverage of the inner bunch parts.

Biocontrol efficacy of selected *Bacillus* spp. against *Rhizoctonia solani* in different crops under greenhouse conditions

B.P. Kubheka, M.D. Laing and C.H. Hunter
Microbiology and Plant Pathology discipline, School of Applied Environmental Sciences, University of Natal, Private Bag X01, Scottsville 3209.

In plantae greenhouse trials were carried out to evaluate the biocontrol efficacy of selected *Bacillus* spp. isolates against the causal organism of damping-off, *Rhizoctonia solani*. Isolates were initially chosen from *in vitro* bioassays in which antifungal activity against *Rhizoctonia solani* was demonstrated. Three seed types, maize, cucumber, and wheat were coated with 255×10^6, 54×10^6 and 65×10^6 cfu/seed, respectively, of selected *Bacillus* spp. isolates and plated in speedling trays. Agar plugs colonized with *R. solani* were used to introduce the pathogen into the infection court. Plant dry weight and percentage germination were rated after 6 weeks and the first week, respectively, and compared to the controls. Four isolates out of 21 showed significant levels of disease suppression ($P = 0.0001$) when compared to diseased controls. The isolate that showed greatest antifungal activity protected 75% of the seedlings in a speedling tray. It was, therefore, concluded that the four isolates have antifungal activity.

Proactive measures in nurseries for control of black goo decline in grapevines

P.H. Fourie and F. Halleen
Disease Management Division, ARC-Infruitec-Nietvoorbij, Private Bag X5026, Stellenbosch 7599.

Black goo decline of grapevine (also known as Petri disease) is associated with decline and dieback of young grapevines that were subjected to stress. A major means of spread of the causal organisms (*Phaeomoniella chlamydospora* and *Phaeoacremonium* spp.) is via infected propagation material. Since no curative control measures are known, proactive measures in grapevine nurseries must therefore be taken to control this disease. Richter 110 rootstock material was harvested from a naturally infected mother block and 5000 cuttings were separated into separate treatments as follows: 1 h drench in suspensions of benomyl, phosphoric acid, two *Trichoderma* formulations, a *Bacillus subtilis* formulation, water, or hot water treated (HWT; 30 min at 50°C followed by 30 min in cold water). Cabernet Sauvignon was bench-grafted onto treated rootstocks and callusing was induced (10 days at 28°C).

Percentage callus and shoot length were rated after the callus period. Callused graftlings were planted in two commercial field nurseries and in a greenhouse (in sterile potting mix in 1 litre plastic bags). Vines were uprooted 8 months later and percentage take, root and shoot mass were determined. Prior to planting and at uprooting, small xylem segments (1×0.5 mm) were aseptically isolated 5 cm from the basal end of rootstocks onto potato dextrose agar amended with 250 mg/l chloramphenicol. Inoculated plates were incubated at 23°C for 21 days and fungal cultures identified. Incidence of natural *P. chlamydospora* and *Phaeoacremonium* infection was expressed as the mean percentage of isolated segments (5 segments isolated per vine, 25 vines per repetition, 4 repeats) yielding these fungi. The remaining nursery and greenhouse vines (433) received HWT and the fungal incidence was determined. None of the treatments affected callus or initial shoot growth negatively, nor was percentage take, root or shoot mass of vines from the field nurseries and greenhouse significantly affected. *Phaeomoniella chlamydospora* and *Phaeoacremonium* incidence in callused graftlings, of which the rootstocks received chemical or biological treatments (incidence varied from 1.3 to 2.6%), was statistically similar to that of the water treatment (1.98%). A markedly lower incidence was observed in the HWT graftlings (0.22%). Significantly less *P. chlamydospora* and *Phaeoacremonium* was isolated at lifting from HWT vines (2.5%) compared to that isolated from water (6.0%) and the chemical and biological treatments (incidence varied from 5.0 to 9.6%). HWT of the dormant, lifted vines grafted on HWT rootstocks reduced *P. chlamydospora* and *Phaeoacremonium* incidence from 2.5% to undetectable levels, while HWT of vines from the chemical and biological treatments reduced its incidence from a mean of 7.2% to 0.8% (incidence varied from 0 to 1.7%). These results clearly indicate that HWT did not eradicate *P. chlamydospora* or *Phaeoacremonium* spp., but was effective in reducing the incidence of these pathogens. HWT of propagation material and dormant vines in combination with chemical and/or biological treatments will be studied to optimize the proactive control of this pathogen in grapevine nurseries.

Effects of culture conditions on the spore shelf-life of a biocontrol agent, *Trichoderma harzianum* strain kmd

J. Omarjee[1], V. Bandu[3], M.D. Laing[1] and C.H. Hunter[2]
Disciplines of Plant Pathology[1] and Microbiology[2], School of Applied Environmental Sciences Centre for Electron Microscopy[3], University of Natal, Private Bag X01, Scottsville 3209, South Africa.

Trichoderma harzianum has been used successfully as a biological control agent against several soil-borne plant pathogens. The influence of four growing media was investigated on the spore ultrastructure of *T. harzianum* strain kmd, using a basal salts medium with C:N ratios of 3 and 14, and pH of 4.0 and 7.0. The effect of these culture parameters on viability and shelf-life was evaluated by counting colony-forming units (cfu) before and after seven days of storage at 75% relative humidity (RH). The effect of carbon concentration on spore viability after seven days of storage was also determined by increasing concentrations of glucose while a constant C:N ratio of 3 or 14 at pH 4 was maintained at 75% RH. Spores grown in media with C: N ratios of 3 and 14 at pH 7.0 had an outer cell wall, little mucilage and produced no lipid globules. These spores had poor viability and shelf-life before and after storage. However, spores grown in a medium with a C:N ratio of 3 and a pH of 4.0 had a thick electron-dense outer cell wall layer and produced mucilage layers. The presence of an outer cell wall and mucilage production in spores resulted in resistance to desiccation and survival under adverse conditions. The presence of lipid globules in these spores was associated with improved viability, probably owing to lipid globules serving as endogenous energy reserves for germination. Spores obtained from media with a C:N ratio of 14 at pH 4.0 developed with two cell wall layers, with the outer layer being more electron dense than the inner layer. The outer cell wall layer is specific to *T. harzianum*. This layer is the spore's first defence against adverse conditions. Spores obtained from this medium were larger, germinated better and had a longer shelf-life than spores from C:N 3 medium. This could be because the two cell wall layers acted as a thicker barrier against adverse conditions. Increasing carbon concentration, while maintaining a constant C:N ratio of 3 or 14 at pH 4, slowed down spore production. Viability of spores was similar on media with variable carbon concentrations but fixed C: N ratios. The ultrastructural differences and shelf-life studies confirmed empirical results from liquid fermentation studies. Here it was found that the pH and C:N ratio of the medium upon which spores of *T. harzianum* strain kmd are produced have critical effects on the physical and chemical structure of the spores and viability. This, in turn, affects critical parameters for a biocontrol agent's spore germination and shelf-life.

Incidence of *Fusarium* spp., section *Liseola*, and associated mycotoxins from maize grain silos

E. Smit and B.C. Flett
ARC-Grain Crops Institute, Private Bag X1251, Potchefstroom 2520, South Africa.

Fusarium spp. in section *Liseola* are known to produce the mycotoxins fumonisin and moniliformin. These fungi are commonly associated with ear, stalk and root rot of maize worldwide. *Fusarium verticillioides* (= *F. moniliforme*) and *F. subglutinans* are most frequently isolated from maize kernels, particularly symptomless kernels. Owing to the high incidence of these fungi in staple food and animal feeds, concerns have been raised regarding toxigenicity and its association with human and animal diseases. Maize grain silos (47) were sampled during the 1999/2000 season to determine the incidence of *Fusarium* spp. and fumonisin concentrations. These silos were mainly situated in the Free State and North-West provinces. Yellow and white maize samples from each silo were handled separately. Two hundred kernels of each sample were surface sterilized and plated onto Rose Bengal glycerine-urea medium to determine frequencies of *F. verticillioides* and *F. subglutinans*. The fumonisin concentration (ppm) of each sample was determined using a

and treatment in most packhouses in South Africa is not less than four hours. This implies that the decay pathogen is often established in the fruit before treatment. For any antagonist to be of benefit to the grower, it therefore has to provide curative protection The screening assay used in this study indicated that the antagonists OPL2A and OPF2 have the potential to provide such protection. They are therefore potentially useful biological agents for control of green mould either on their own or when integrated with other measures.

Alternative control of green mould caused by *Penicillium digitatum* Sacc. on Valencia oranges

I. Paul, J. Obagwu and L. Korsten

Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria, 0002 South Africa. E-mail: ipaul@bioagric.up.ac.za

Green mould, caused by *Penicillium digitatum* Sacc., is one of the most common post-harvest diseases of Valencia sweet oranges. The pathogen is responsible for a high percentage of post-harvest losses worldwide. Although fungicides are currently used to control this fungus, the possibility of resistance and therefore reduced efficacy of the chemical is of major concern. In addition, public resistance to the use of fungicides emphasizes the need to find alternative methods to control post-harvest diseases of citrus. The aim of this study was to explore the possibilities of using alternative agents to control citrus green mould. Six trials were conducted at Letaba Estate, Mpumalanga Province, using a total of 18 000 fruits. Valencia oranges were artificially wounded, inoculated with *P. digitatum* at concentrations ranging between 1×10^3 spores/ml and 1×10^5 spores/ml. Fruit were subsequently treated with alternative agents, such as natural antagonists *Bacillus subtilis* and *Candida saitoana* as well as the patented preparations Biocure and Bioactive, used either on their own or in combination. After treatment, fruit were incubated either at room temperature for two weeks or, to simulate export conditions, at 11°C for four weeks. Biocure-*Candida saitoana*, Bioactive-*Candida saitoana*, Biocure-*Bacillus subtilis* and Bioactive-*Bacillus subtilis* exerted more than 80% control against *P. digitatum*, with Bioactive-*Candida* giving up to 96% control. Overall, the agents were more effective at 11°C than at 25°C. None of the treatments gave total control. This, however, leaves possibilities for integrated control, where chemicals are applied, but at reduced rates and in conjunction with alternative agents. In conclusion, although there are still many questions that need to be answered, these trials clearly show the potential for control of citrus green mould using alternative products.

Successful biocontrol of post-harvest decay on plums and nectarines

L. Williamson and M. Groenewald

ARC-Infruitec-Nietvoorbij, Private Bag X5013, Stellenbosch, 7500 South Africa. E-mail: linda@infruit.agric.za

After the successful biological control of decay in pome fruit in 1997, success can now be reported for the biocontrol of stone fruit. Laboratory-scale biocontrol tests were conducted with new yeast isolates, *Cryptococcus* and *Rhodotorula* spp., on Laetitia and Sapphire plums, and Fiesta Red, Flamekist and Zaigina nectarines. Tests were conducted in open wounds, using the fruit as the substrates, and counter-inoculated with *Botrytis cinerea* or *Penicillium expansum*. Decay incidence was significantly reduced in the open-wound tests, with control of *B. cinerea* being as high as 78.3% and control of *P. expansum* as high as 74.2% after 5–7 days decay development at 20°C.

Biocontrol of damping-off caused by *Rhizoctonia* and *Pythium* sp. with formulations of *Trichoderma harzianum* and *Gliocladium virens*

J. Omarjee, C.H. Hunter and M.D. Laing

Discipline of Plant Pathology, School of Applied Environmental Sciences, University of Natal, Private Bag X01, Scottsville, Pietermaritzburg, 3209 South Africa. E-mail: laing@nu.ac.za

Preparations of isolates of biocontrol fungi *Trichoderma harzianum* and *Gliocladium virens* were evaluated for their efficacy in preventing damping-off caused by *Rhizoctonia* and *Pythium* sp. A variety of crops was evaluated: cabbage (*Brassica oleracea var. capitata* Alef.); cucumber (*Cucumis sativus* L.); daisy (*Bellis perennis* L.) and *Eucalyptus macarthuri* Deane and Maiden. Trials were conducted in greenhouse conditions with the growing medium, composted pine bark, artificially infested with the pathogens. Each experiment was randomized and contained at least three replicates. The variables observed were percentage germination and dry weights after 3–4 weeks. Preparations included milled oat containing chlamydospores of biocontrol fungi; powders containing conidia in an experimental compound, an oil base, and a koalin base Formulations were evaluated with three delivery methods: a seed coating using an adhesive, Pelgel; broadcast (preparation broadcast on the surface and incorporated into infested soil) and drench (preparation drenched on surface at planting). Drenching of formulations was done at various dosage levels of 0.25, 0.5, 1, 5 and 10 g/l of water. All formulations significantly ($P < 0.05$) reduced disease caused by *Rhizoctonia* and *Pythium* sp. but were not 100% effective. Formulations containing chlamydospores in milled oat, koalin and in an oil base reduced disease from approximately 70% to 5%. No stimulation of plant growth was observed. The most effective delivery method was drenching. Performance of each dosage level varied for each crop. The strains and formulations of *Trichoderma harzianum* and *Gliocladium virens* used in the trial were effective biocontrol agents. Future research will entail more experiments on a wider variety of crops to confirm the biocontrol shown against *Rhizoctonia* and *Pythium* sp.

Evaluation of Biostart®, a *Bacillus*-based plant probiotic as a plant growth stimulant on containerized seedlings

K.S. Yobo and M.D. Laing

Discipline of Plant Pathology, School of Applied Environmental Sciences, University of Natal, Private Bag X01, Scottsville, Pietermaritzburg, 3209 South Africa. E-mail: yobok@nu.ac.za

Numerous microorganisms produce beneficial effects on plant development when applied to seeds or incorporated into the soil. Research efforts world-wide over the past two decades have renewed commercial interest in plant growth promoting rhizobacteria (PGPR). The effect on plant growth of seven probiotic *Bacillus* spp. and Biostart® 2000 (a combination of three of the seven species) was studied. Growth stimulation trials in tunnels were carried out using four crops, i.e., lettuce, tomato, sorghum and beans. Seed treatment and seed treatment plus drenching with or without NutriStart-AC were evaluated. All *Bacillus* spp. used stimulated plant growth. Growth stimulation was more pronounced with a 4% NutriStart-AC supplement. Growth stimulation was best in lettuce, with Biostart® 2000. There was an increase of 466% compared to the dry biomass of the water control lettuce seedlings. The lowest responses were recorded in sorghum and beans. Three tomato cultivars, i.e., Roma, Floradade and Rodade, and a pepper cultivar, Tha were evaluated for growth stimulation by applying Biostart® as seed treatment and seedling drench. The highest growth stimulation, 96% was obtained using *B. licheniformis* on Roma as a seedling drench Growth responses were better in Roma and Floradade cultivars than in the Rodade cultivar. Pepper plants drenched with Biostart® *Bacillus* spp supplemented weekly with a 4% NutriStart-AC suspension, showed increased fruit yield. Using *B. subtilis*, a 533% increase of fruit yield was recorded when seedlings were supplemented weekly with a 4% NutriStart-AC suspension. Similar results were recorded using an unidentified *Bacillus* strain CM-33 (433%) and *B. licheniformis* (333%). The results presented here have some practical applications to seedling growers in South Africa, especially in growth promotion. Applying Biostart® probiotic *Bacillus* spp. may increase the turnover of seedlings in nurseries. Further research is needed if the growth promotion potential of probiotic *Bacillus* spp. is to be fully exploited.

Transfection of plant pathogenic fungi with mycoviruses

N. Moleleki, O. Preisig, M.J. Wingfield and B.D. Wingfield

Department of Genetics, and Tree Pathology Cooperative Programme, Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, 0002 South Africa.

Sphaeropsis sapinea is an important pathogen of *Pinus* spp. in South Africa. The pathogen exists as a symptomless endophyte in cones of healthy pine trees. It is only after the onset of stress conditions such as hail, drought or wounding by cambiophagus insects that disease symptoms appear. Disease symptoms caused by *S. sapinea* include shoot blight, canker and tip-dieback. Two mycoviruses, SsRV1 and SsRV2 have been found to co-infect this pathogen. The infection of *S. sapinea* by these mycoviruses is not associated with any observable phenotypic traits. As part of our effort to develop a biological control strategy for this fungus, mycoviruses have been sought in other fungi. A promising mycovirus has been discovered in *Diaporthe ambigua*. Different isolates of this fungus display differences in pathogenicity. Hypovirulent isolates of *D. ambigua* have been shown to harbour this virus, known as DaRV. We have sequenced and cloned the cDNAs of the RNA genomes of these three viruses as a first step towards their use in the biological control of