BIOFUMIGATION OF *FUSARIUM* spp. the causal agent of head blight of wheat by volatiles from *Brassica* tissues

By

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DECLARATION

This thesis is my original work and has not been presented for award of a degree in any other University.

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This thesis has been submitted for examination with my approval as University supervisor.

PROF. E.M. GATHURU

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Date: 9/11/98

DEDICATION

This work is dedicated to my

parents; Dorka Adhiambo and Suleiman Otae.

"Their efforts were not in vain."

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ABSTRACT

The control of soil-borne pathogens is a major challenge facing agricultural production. *Fusarium* head blight pathogen, being soil-borne, has no currently available fungicide, resistant cultivar, bioprotectant or cultural practice which provides consistent economical control. Soil fumigation against this pathogen using Methyl bromide may soon be banned due to increasing environmental and health concerns, in particular regarding the stratospheric ozone depletion. Recent research findings show that *Brassicas* contain significant quantities of thioglucoside compounds called glucosinolates in their tissues, whose hydrolysis products (principally isothiocyanates) are biocidal and have the potential of being used as cheap source of soil biofumigants against the *Fusarium* species and other related pathogens.

This study was carried out with the broad objective of assessing the biofumigation potential of *Brassicas* against the *Fusarium* species (the causal organism of head blight of wheat). To accomplish this aim, the effects of volatile compounds released from the seed meal, shoot and root tissues of Mustards (*Brassica juncea* vars.$T_{1003\text{-}208}$; $T_{1003\text{-}195}$and R3243) and Rapeseed canola (*Brassica napus* var. Wester) on *Fusarium* conidia, chlamydospores and mycelial growth were assessed. In addition, the effects of volatiles from autoclaved seed meals were also examined. The conidial survival and inoculum potential was determined in the presence of volatiles produced by *Brassica* seed meals. Ultimately, the biofumigation effects of *Brassicas* was investigated in greenhouse conditions through different *Brassica* - wheat cropping sequences.

The volatile compounds released from *Brassicas* had detrimental effects on each stage in the life cycle of the *Fusarium* pathogen *in vitro*. These biotoxic compounds were shown to be volatile because the symmetry of *Fusarium* colony growth was not affected by the

proximity of the *Brassica* tissue. The seed meals released the most fungitoxic volatiles than the other tissues and Mustard tissues were generally more inhibitive than those of Rapeseed canola. The degree of inhibition varied significantly ($P \leq 0.01$) with the *Brassica* species, plant parts, rates of application, and the fungal growth phase. The *Fusarium* mycelial growth was most sensitive to these volatiles, whereas the chlamydospores were least sensitive. Moreover, the germinated conidia responded to these volatiles by the production of chlamydospores. The autoclaved Mustard seed meals evolved volatiles with less effects toward the *Fusarium* conidia, though these showed significant ($P \leq 0.01$) mycelial growth inhibition. The volatiles from Mustard seed meals significantly ($P \leq 0.01$) decreased the conidial inoculum potential as compared to the control treatments. In potted soils in the greenhouse, the Mustard varieties demonstrated greater biofumigation effects than the Rapeseed canola. The results obtained in this study indicate that the effectiveness of *Fusarium* suppression by *Brassica* crops will depend upon the species, type of *Brassica* tissue, and application rates, which seem to have a direct effect on the type and concentration of the isothiocyanates evolved.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

CHAPTER ONE

1.0 INTRODUCTION

The management of soil borne diseases is a continual challenge facing agriculture. The structural, physical and biological complexity of the soil environment in which soil borne pathogens interact with plant roots inherently limits the options available for disease control. The already proven control measures include soil fumigation, breeding for host plant resistance, clean tillage, crop rotations with non-hosts and organic amendments (Cook, 1980). The use of soil fumigants is frustrated by prohibitive costs involved and matters of risks to users and the environment (Fischer, 1993; Fernando *et al.*, 1997). Control through crop rotation or other cultural practices is not very feasible and field results from fungicide sprays are not encouraging (Reis and Kohli, 1993). Therefore breeding, although difficult, remains the most desirable strategy for a developing country like Kenya. However, host plant resistance carries the risks arising from the possibility of evolution of new virulences in the pathogen (Fischer, 1993). Despite great efforts to effectively combine these options with other control strategies, soil borne diseases continue to constrain crop production.

Diseases have been a major constraint to wheat production in Kenya since its introduction in the 1900s (Owuoche *et al.*, 1995). In addition, the Kenyan wheat farmer, suffers from several other production constraints like high cost of inputs (KARI, 1991). It is therefore imperative that the effects of diseases should be minimised at all costs. The major cereal fungal diseases that adversely affect wheat production in Kenya include the rusts, *Septoria* species and the take-all (*Gaeumannomyces graminis* var. *tritici*) (Cormack, 1973), all of which account for high yield losses. The soil-borne diseases like Fusarium

head blight (*Fusarium graminearum* schw. and *Fusarium culmorum* Smith) and nematodes (*Pratylenchus thornei*) have been reported to occur in Kenya's wheat growing zones (Otondo H. pers. Comm.). Therefore, the traditional cereal monoculture practiced in Kenya could lead to pathogen build up and subsequent significant yield losses.

The *Fusarium* head blight of wheat is one of the cereal root disease in Kenya which is currently neglected despite its imminent devastating effects on the wheat industry. The causal agent survives in the soil as chlamydospores where there exist certainly chlamydospore inducing factors (Ford *et al.*, 1969). These thick walled structures (Chlamydospores) are also found in infested plant debris from the previous season (Cook, 1980). The soilborne inocula gain entry into the plant crown approximately 2-3cm below the soil surface either through openings around emerging secondary crown roots or by infection of the newly emerging crown roots. From here, the pathogen progresses up the culm to produce the conidia on the surface of parasitised hosts (Ford *et al.*, 1969; Cook, 1980). These conidia serve as reproductive, dispersal units which may be washed by rain, wind or irrigation water. The conidia formed on parasitized host stems and later washed or tilled into the soil also convert into chlamydospores and thus constitute one source of these survival structures.

The complex ecological adaptability of this pathogen frustrates the control approaches employed for its management. Research on wheat rotations with non-host crops including grass-free leguminous pastures and *Brassica* 'break crops' have proved valuable in the suppression of fungal cereal diseases (Fischer, 1993; Angus *et al.*, 1994; Kirkegaard *et al.*, 1994;1996). This disease reduction phenomenon has been attributed to a denial of soil borne pathogens of a host or the biofumigation effects of these rotation crops (Angus *et al.*, 1994; Kirkegaard *et al.*, 1994; 1996).

Biofumigation is the suppression of soil borne pests and pathogens by volatile compounds, principally isothiocyanates, released in soil when naturally occurring glucosinolates (GSLs) in the tissues of *Brassica* plants are hydrolysed (Kirkegaard *et al.*, 1995; 1998). The glucosinolates are organic anions commonly found among the Cruciferous plants (Mojtahedi *et al.*, 1993) and are implicated in the biological activities of these plants. The glucosinolates are enzymatically hydrolysed to release a number of biologically active compounds, including the isothiocyanates (ITCs). The isothiocyanates are biocidal to fungal pathogens as they react with sulfhydryl (-SH) groups of certain amino acids and hence denatures the proteins and enzymes (Agrios, 1988). The biocidal ability of *Brassica* - derived isothiocyanates have been well documented (Walker *et al.*, 1937).

The isothiocyanates released from *Brassica* residues incorporated in the soils have shown some degree of suppressiveness against nematodes (*Heterodera rostochiensis and Meloidogyne chitwoodi*) (Ellenby, 1951, Mojtahedi *et al.*, 1991,1993), Fungi (Papavisas, 1966; Lewis and Papavisas, 1971, 1974; Chan and Close, 1987; Ramirez-Villapuda and Munnecke, 1987; Muehlchen *et al.*, 1990; Gamliel and Stapleton, 1993; and Smolinska *et al.*, 1997), Wireworms (Brown *et al.*, 1991) and to inhibit the germination of weed seeds (Jones, 1992). In one study, (Kirkegaard *et al.*, 1996), *in vitro* suppression of soil-borne fungal pathogens has been demonstrated. This finding was in agreement with an earlier report of Angus *et al* (1994), where they established that isothiocyanates released from *Brassica* roots inhibit the growth of take-all fungus [*Gaeumannomyces graminis*(Sacc.) (Arx and Oliver) var. *tritici*] of wheat (*Triticum aestivum* L.). The classic work of Smolinska *et al* (1997) unequivocally demonstrated the effects of isothiocyanates against the various stages in the life cycle of *Aphanomyces euteiches* f.sp *pisi*. It is against this background that

Brassica crops have been rendered more superior as a break crop in wheat fields than other crops owing to their biofumigation effects (Kirkegaard *et al.*, 1994).

With such overwhelming evidence notwithstanding, the biofumigation effects of *Brassica* crops has not been reported anywhere in Africa South of Sahara. There has been no attempt to screen candidate *Brassicas* for toxicity against soil-borne pathogens of economic importance in our agricultural systems.

With the exception of *Aphanomyces euteiches* Drechs. f.sp. *pisi* W.F. Pfender and D.J. Hagedorn, the toxicity of isothiocyanates to the growth stages of any pathogen has not been appropriately demonstrated. However, such investigations could be of importance in the development of a viable control strategy against the challenging soil-borne pathogens like *Fusarium* species. The long-term goal of this approach would be enhanced control of soil-borne pathogens with far reaching implications to agricultural production in Kenya;.

a) *Brassica* varieties with highest and potent levels of isothiocyanates (ITCs) could be identified and improved so that they could be used in rotation with wheat or maize,

b) Genes for high ITCs could be incorporated into other agronomically desirable varieties to improve their biocidal ability,

c) It could provide an impetus to open an industry to manufacture cheaper environment friendly biofumigants based on *Brassica* ITCs (which have biological degradability).

Therefore, the objectives of this study were;

ii) To determine the effect of volatiles evolved from different Brassicas and tissue types on the survival and germination of *Fusarium* conidia and chlamydospores,

iii) To assess the inoculum potential of *Fusarium* conidia in the presence of volatiles from different *Brassica* varieties and tissues,

iii). To establish which of the *Brassica* variety, tissue type and rate was more suppressive to *Fusarium* mycelial growth,

iv). To determine the impact of autoclaving on the most suppressive *Brassica* tissues on conidial germination and mycelial growth, and

v). To determine the biofumigation effects of different *Brassica* varieties on the *Fusarium* pathogen in potted soils in the greenhouse.

This research undertaking was justified by the pivotal role played by wheat in Kenya's economy. Wheat (*Triticum aestivum* L.) is one of the major food crops in Kenya whose consumption is substantially increasing (Annon, 1986). Currently, the demand for wheat flour and wheat products far outstrips the supply. This is because of Kenya's fast increasing population at a rate of about 4% annually, increasing incomes and an ever rising urban population (Hassan and Mwangi, 1992). In order to increase the wheat output in Kenya to match this worrying trend, new technologies are needed. These include improved varieties, appropriate fertilizer package, good crop protection in terms of diseases, insects and weeds. Yield losses from diseases are much higher than generally recognised and as such represent a major financial constraint in crop production.

Fusarium head blight of wheat is a soil-borne disease of economic importance. The pathogens produce deoxynivalenol and zearalenone in wheat kernels (Fernando *et al.*, 1997). The accumulation of these toxins increases the importance of this pathogen. The control of this pathogen is heavily dependent on the use of soil fumigation with synthetic organic compounds such as Methyl bromide and Metham sodium (Kirkegaard *et al.*, 1993). Increasing environmental and health concerns, in particular regarding the stratospheric ozone depletion caused by Methyl bromide is providing the impetus to investigate alternative control measures.

The other problem inherent in the use of Methyl bromide for soil fumigation include the high phytotoxicity of this compound (Kempton and Maw, 1972), necessitating the thorough removal of any unchanged residues from the soil prior to planting. The chemical breakdown of Methyl bromide in soil releases inorganic bromide which is phytotoxic to some plants and can also be taken up and accumulated in the foliage of many species (Kempton and Maw, 1972). A further setback in the use of soil fumigants is prohibitive costs which at present may only be feasible with high-value crops. Kirkegaard *et al.* (1993) reported that pest and disease control in Australia using synthetic methyl isothiocyanates (Metham sodium, vorlex, and dazomet) costs about $ 600/ha. Biofumigation with *Brassica* crops may provide effective control at a fraction of this cost.

Additional benefits to be gained from this control approach are many. The *Brassica* break crops in wheat rotations ensures maximum land and machinery utilization since they both require almost similar tillage practices and equipment (M. Mahasi, Pers. Comm.). *Brassica* break crops grown during wheat fallow periods provides an additional source of income to the farmers.

The *Brassica* crops also act as natural liming agents following soil acidification by Diammonium Phosphate (DAP) fertilizers applied in wheat fields which cause Aluminium (Al) and Manganese (Mn) toxicity and a deficiency of available phosphorus (P) and calcium (Ca). This may alleviate the problem of judicious applications of lime where most soils have been noted to respond but with varying degrees (Annon, 1986).

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Introduction

World food security depends on ample supply of three major cereals, namely; wheat, maize and rice (Ferrar, 1995), all of which account for 80% of global cereal production (Stubbs *et al.*, 1986). Of these three crops, wheat is of greatest importance, in terms of tonnage, if not in financial value.

Wheat is regarded as the world's most important staple food, and is consumed in a variety of ways (Stubbs *et al.*, 1986). Its most important use is in bakery industry where wheat flour is used in bread, biscuit and pastry products. In addition, wheat is used extensively in breakfast cereals, bulgur, couscous and macaroni products (Stubbs *et al.*, 1986). This continued reliance upon wheat requires that both the stability and level of production continues to increase in order to minimise malnutrition as much as possible.

The wheat production trends in Africa South of the Sahara is not very encouraging. The total wheat output in this region stands at 4.8 million tonnes occupying a total wheat area of 2.81 million ha. The deficit is met by a total importation of 2.57 million tonnes of wheat (Payne *et al.*, 1995). The Food and Agricultural Organization (FAO) of the United Nations (UN) estimates that the Sub-Saharan African countries will produce only 75% of their wheat requirements by the year 2000, only two years from now (Ndegwa, 1991). This means that Kenya will also be affected.

2.2 *Wheat production in Kenya*

Wheat farming began in Kenya in the early 1900s when European settlers, mainly in the highlands of the Rift Valley began producing wheat on large farms using highly mechanized production methods (Hassan *et al.*, 1993). However, since then wheat production has had many ups and downs (Harder, 1974). Currently, wheat is the second most important cereal crop in Kenya after maize (Kamidi, 1995). It has occupied in the recent years two thirds (about 141,234 ha) of cereal cropland with a total yield of about 220,500 tons (Kinyua *et al.*, 1989).

In general, wheat production has so far showed a slow growth rate of 0.09% while consumption of its products, spurred by population growth, urbanization and changing consumption habits has been increasing by 5.1% annually (Kamidi, 1995). This increasing demand has been met through imports. Thus the anticipated self-sufficiency in wheat production has consequently eluded this Nation, and has dropped from 132%, in 1961 - 1965 to 60% in 1990's (Byerlee and Morris, 1990, and Tanner and Mwangi, 1992). To bridge this production deficit, the country imports half of its domestic wheat requirements (Karanja and Pinto, 1994).

From the foregoing, it is an open secret that over the years, Kenya has gone from being self-sufficient in wheat production to being reliant on the world market to meet its consumption needs. The trend can only be reversed by increasing productivity in existing wheat areas and developing suitable technologies for potential expansion areas. This can be achieved by developing higher yielding and stable breeds, better crop management packages, and appropriate plant protection methods.

Wheat is grown in Kenya's five Rift Valley districts of Nakuru, Uasin Gishu, Trans-Nzoia, Laikipia and Nyandarua and Meru district (Kinyua *et al.*, 1989), and is

currently being extended to marginal rainfall areas (that is Narok and Kajiado districts) (Murage and Tanner, 1995). Due to the climatic conditions in the high potential wheat growing areas which include relatively heavy rainfall (750mm - 1500 mm) and warm weather during the critical period of wheat growth (Annon, 1960), the crop is, more than in other areas, subject to plant diseases.

2.3 *Wheat diseases in Kenya*

In Kenya a number of diseases affect cereals (Harder, 1974). These may be caused by fungi, bacteria, viruses, nematodes, mycoplasmas, parasitic flowering plants, animal pests or other abiotic factors in the environment (Wiese, 1977).

Among the fungal diseases, rusts (which thrive well in Kenya where there is no winter to kill them), *septoria* species and the take-all (*Gaeumannomyces graminis*) (Cormack, 1973) are considered to be the major threats to wheat farming. However, there are also many less obvious wheat diseases which take their annual toll. When added together their impact negatively affects Kenya's economy materially. These diseases include Fusarium head blight (Scab), Black point or kernel smudge (*Helminthosporium* spp.), powdery mildew (*Erysiphe graminis* f.sp. *tritici*) and many others (Wiese, 1977).

Fusarium head blight (FHB) fungi (*Fusarium graminearum* schw and *F. culmorum* Smith) have been regularly reported to occur in Kenya (Otondo H. Pers. Comm.) and it is just a matter of time before their population builds upto epidemic proportions because the pathogens are already present. Moreover, the occurrence of *Fusarium* spp. is favoured by high temperatures (25^0-30^0C) and high relative humidity (above 85%) (Inglis and Maloy, 1983; Tomasovic *et al.*, 1993), conditions which are

prevalent in Kenya's wheat growing zones. The situation is further compounded by the fact that prior to 1980, head blight resistance breeding was not a priority objective at CIMMYT (Bekele, 1989), implying that all of Kenya's advanced lines derived from CIMMYT wheat germplasm could show poor resistance. Therefore, the situation as it is today requires an appropriate in-depth study in readiness for any eventuality.

2.4 *Fusarium head blight (FHB)*

Fusarium head blight (FHB) is a fungal disease of wheat (*Triticum aestivum* L.) found in both temperate and semi-tropical regions, and is predominantly caused by *Fusarium graminearum* Schw. and *Fusarium culmorum* Smith. (Snijders, 1991). Besides head blight, *F. graminearum* and *F. culmorum* cause seedling blight, and root crown and foot rots (Nelson *et al.*, 1981). According to Cook (1968), these fungi are also responsible for early ripening of headed plants (White heads), and pinkish discolouration of stems inside the leaf sheaths. Both fungi also cause stalk rot and ear rot in Maize and infect other cereals such as barley, oats and *Triticale* (Booth, 1971). Heavy damage to wheat often occurs in areas of continuous cultivation or where wheat is planted after maize (Snijders, 1990). Damage from head blight is caused in the form of reduced kernel set and kernel weight, destroyed starch granules and storage proteins, and seed infection (Bechtel *et al.*, 1985), thereby lowering the baking quality and the general nutritional value (Mesterrhàzy and Bartók, 1996).

The Fusarium head blight fungi produce many secondary metabolites including the trichothecene mycotoxins, deoxynivalenol (Vomitoxin) and 3-acetyldeoxynivalenol (Wang and Miller, 1988). These toxins have a range of chronic and acute effects on humans and animals consuming infected grain (Marasas *et al.*, 1984 and Snijders, 1991). From experiments, it has been conclusively demonstrated that deoxynivalenol (DON) is transported from the chaff to the young kernel; the pathogen colonises the kernel later (Snijders, 1991).

The pathogen originates from seed, soil, and from inoculum on host residues, including those of maize, small grained cereals and certain grasses (Khonga and Sutton, 1988). This diversity in origin has greatly complicated the control strategies adopted against the FHB of wheat:

2.4.1 Breeding for FHB resistance

The development of wheat cultivars resistant or tolerant to head blight is the principal method of controlling the disease (Tomasovic *et al.*, 1993). The use of wheat cultivars resistant to Fusarium head blight limits yield loss under high *Fusarium* disease pressure (Snijders, 1990). According to Snijders (1991), the genetic variation to FHB resistance in wheat is quite large, quantitative, but complete resistance is non-existent.

The resistant wheat varieties show resistance not only to fungal penetration, but some lines also possess resistance to hyphal invasion of plant tissue, an active resistance expressed in the chaff (Snijders, 1991). In addition, the resistant lines show an inhibition of DON translocation from chaff to kernel and hence no colonization takes place (Snijders, 1992).

There are many advantages to the use of resistant varieties to control the disease including simplicity of use by the farmer and the low cost which at present relates only to the cost of varietal development (Gareth and Clifford, 1983). However this approach has been largely ineffective for most soil-borne pathogens (Cook, 1980), in part because of unreliable methods to test for resistance and in part because soil-borne pathogens are commonly unspecialised in their host range so that effective varietal resistance is unavailable.

2.4.2 Biological control

As a practice, biological control of cereal pathogens is very much in its infancy in so far as few of the known examples have reached commercial significance (Gareth and Clifford, 1983). Simply defined, it is the reduction of inoculum density of the disease - producing

activities of a pathogen or parasite by one or more organisms be they antagonistic or hyperparasitic (Gareth and Clifford, 1983).

The potential of biological control of root pathogens, as an alternative to soil fumigation has been reviewed (Cook, 1984). In this review, it was established that root health may also be maintained by biological seed treatments. Roots of crops can be protected from pathogens to a significant degree by non-pathogenic root-colonizing micro-organisms applied on the seed. In this respect, isolates of *Trichoderma harzianum* obtained from field soils have been found to be effective for the control of diseases of various crops (Ordentlich and Chet, 1989), caused by *Rhizoctonia solani*, *Pythium* and *Fusarium* species. By different application techniques, *T. harzianum* could establish in the soil for six months, acting as a mycoparasite (Ordentlich and Chet, 1989).

Other microbial antagonists already reported to be of value in integrated management of scab include *Paenibacillus macerans* and *Sporobolomyces roseus* (Stockwell, *et al.*, 1997). These bioprotectants have shown kernel weight improvements of 15% and 7% respectively, over the non-protected spikes. da Luz *et al.* (1997), also reported the use of *P. macerans*, *Pseudomonas putida* and *Trichoderma harzianum* strain T-22 as alternative or supplement to seed fungicides for the control of *Fusarium* species in cereals. Of all these bioprotectants, *P. macerans* in addition to showing *in vitro* antibiosis towards *Fusarium* species, already shows a promise as cereal seed protectants against seed-borne and soil-borne *Fusarium* species.

The major setback in this control strategy stems from the fact that these biological control agents have their activities dictated by various soil conditions such as soil moisture, soil pH and even temperature.

2.4.3 Chemical Control

Under *Fusarium* head blight epidemic conditions, fungicidal treatments can keep wheat crops healthy or suppress the infection sufficiently to keep toxin contamination below threshold values (Mesterrhàzy and Bartók, 1996).

Various chemicals have been used to control the FHB disease. Benomyl has been used effectively against FHB pathogen (Andenow, 1987). In Bavaria, Obst *et al*, (1992) demonstrated that an early-curative treatment of tebuconazole products significantly reduced Fusarium head blight and the mycotoxin contamination of the grain. A fungicide combination tebuconazole and Fenpropimorp was also effective against *Fusarium* species.

Leyva *et al.* (1992) reported that propicanazole, carbendazim and prochloraz in seed treatments decreased the disease incidence by 100% in the wheat varieties Salamanca S-75 and Temporalera M87 in Juchitepea, Mexico.

The *Fusarium* species can also be successfully controlled by other organo-mercury such as fuberidazole, thiabendazole and thiophanate-methyl seed treatments (Gareth and Clifford, 1983). Fortunately, many sprays used to control eyespot disease are also reasonably effective against *Fusarium*.

However, in a twist of fate, Milus and Parsons (1994) demonstrated that fungicides such as benomyl, chlorothalonil, fenbuconazole, flusilazole, tebuconazole, myclobutanil, potassium bicarbonate, propiconazole, thiabendazole, and triadimefon plus mancozeb, are not effective for controlling head blight or reducing DON levels in wheat, despite their effectiveness in controlling foliar wheat diseases. This renders the prospects for chemical control of head blight to be very remote. Moreover, the fungicidal efficacy depends on;

a). Host plant resistance, as a variety with average resistance can be protected much more effectively than highly susceptible genotypes (Mesterrhàzy and Bartók, 1996) and

b). High virulence levels, implying weaker fungicidal effect.

Therefore fungicidal efficacy is affected by both the resistance of the wheat varieties and the virulence of the fungal isolates.

2.4.4 Fumigation

The practice of soil fumigation since its inception many years ago has become an accepted agricultural practice in many parts of the world (Hemwall, 1962).

Fumigation refers to the application of a fumigant (a toxic gas or volatile substance) for disinfestation of an area (Agrios, 1988). Fumigation of soil with DD-MIT (Vorlex), a commercial mixture of Methyl isothiocyanate, dichloropropenes and dichloropropanes have been noted to reduce populations of *Fusarium* in soil and increase seed yields (Chew and Hall, 1984). Fumigation with chloropicrin or dazomet dramatically reduces the population of *Fusarium* species in fumigated plots (Hansen *et al.*, 1990).

In a ratio 100% methyl bromide (MB), 100% chloropicrin (CP), combinations of MB and CP, and dazomet significantly reduce populations of *Fusarium*, *Rhizoctonia*, *Pythium* species and total soilborne fungi (Enebak *et al.*, 1990).

This control measure has several added advantages. Fumigation of wheat fields with a mixture of chloropicrin and methyl bromide (2:1) causes changes in soil nutrients and microflora which results in increased vegetative growth, leading to increased grain yields (MacNish, 1986). Soil fumigation thus increases concentrations of mineral nitrogen in soil early in the season. Ammonium nitrogen in fumigated soil 49 days after treatment ranged from 18.2 to 26.5 ppm, compared with 1.4 - 2.2 ppm in untreated soil, while nitrogen ranged from 0.3 to 2.1 ppm and 1.5 to 7.9 ppm respectively (MacNish, 1986). Much of the growth increases appear to be due to this nitrogen and to a lesser extent the pathogen reduction.

However, the soil fumigation with synthetic organic pesticides such as methyl bromide and metham sodium has various hazardous environmental implications (Kirkegaard *et al.*, 1993). This concern has provided the impetus to investigate alternative control measures (Kirkegaard *et al.*, 1993), like biofumigation.

2.4.5 Biofumigation

Brassica species release biocidal compounds, principally isothiocyanates (ITCs) when glucosinolates in the tissues are hydrolysed during breakdown in the soil (*Angus et al.*, 1993). Biofumigation is a term which has been used to describe the suppression of pests or disease causing organisms by the ITCs released from *Brassica* green manure crops or rotation crops (Kirkegaard *et al.*, 1995). *Brassica* break crops in cereal rotations increases the yield of subsequent wheat crops by depriving soil-borne wheat pathogens of a host, and reducing infection of the subsequent wheat crop (Kirkegaard *et al.*, 1993). Recent field experiments have shown that wheat grown after Brassicas out yielded wheat grown after other break crops such as oats or linseed (Angus *et al.*, 1991; Kirkegaard *et al.*, 1993). In addition, yield of wheat following cropping of Indian mustard was significantly better than wheat following Canola. The differences appeared to be related to different levels of soil-borne diseases rather than residual soil water or nutrients. Angus *et al* (1991), speculated that improved growth may have resulted from suppression of soil-borne wheat pathogens by ITCs released from *Brassica* tissues. The improved growth of wheat following mustard compared to wheat following Canola may have resulted from higher levels or different forms of ITCs released from the mustard leading to more effective disease suppression (Kirkegaard *et al.*, 1993). This hypothesis was supported by the results of experiments investigating the suppression of

the take-all fungus (*Gaeumannomyces graminis* var. *tritici*) by *Brassica* root pieces *in vitro* (Angus *et al.*, 1993).

2.4.5.1 Glucosinolates

Glucosinolates are nitrogenous natural secondary plant products with the following attributes;

a). they occur in the plants as glycosides,

b). they exert deleterious effects on animals ingesting the glycoside rich plants. These effects range from acute toxicity often resulting in death to indirect effects caused by metabolites produced in the animal after ingestion (Conn, 1988).

Glucosinolates have been reported to co-occur in significant quantities with myrosinase enzyme (Thioglucosidase; EC 3.2.3.1) in all members of the family Cruciferae (Brassicaceae) so far examined (Kjaer, 1976 and Sang *et al.*, 1984). However, sporadic occurrences have been noticed in a number of families outside the Order Capparales, albeit with uncertain status of accompanying myrosinase activity, Chew(1988). According to Chew(1988), trace amounts of glucosinolate hydrolysis products (0.1 - 5µg/g wet tissue) in volatiles of some unrecorded sources like *Plantago major* suggest more widespread distribution of glucosinolates and myrosinase activity. The enzyme thioglucoside glucohydrolase (EC 3.2.3.1) is also called myrosinase (Macleod *et al.*, 1981). The work of Kirkegaard *et al.*, (1998) showed that it is spacially separated from the glucosinolates in intact plant tissues. The enzyme is released rapidly when the tissues are crushed, or when autolysis within the plant takes place (Larsen, 1981). Little evidence however, is available concerning how the glucosinolate-thioglucoside (glucohydrolase) system is held latent in plants until the tissues are disrupted (Chew, 1988).

2.4.5.2 *Glucosinolate profiles*

Upto about twenty different types of glucosinolates (GSLs) have been identified in crucifers (Kirkegaard *et al.*, 1998). These glucosinolates vary in structure depending on the type of organic side chain of the molecule (Table 1.). The three broad categories of glucosinolates are recognised as aliphatic, aromatic and indolyl, with considerable variations among them (Rosa *et al.* 1997).

The profiles of glucosinolates vary with *Brassica* species, plant parts, stage of plant development and the growth conditions (Fenwick *et al.*, 1983; Mithen, 1992; Kirkegaard and Sarwar, 1997; and Rosa *et al.*, 1997). According to Sang *et al.* (1984), there are high levels of 2 - phenylethyl glucosinolate ($CH_2^-CH\text{-}CH^-_2$) in the roots of Mustard (*Brassica napus* L.) but none is present in the seeds.

Mustard has high levels of allyl glucosinolates ($CH_2^-CH\text{-}CH^-_2$) in the roots and seeds but this form is absent in Rapeseed canola (Kirkegaard *et al.*, 1993). It is in this respect that the Rapeseed Canola (*Brassica napus* L.) is considered an improved variety with low erucic acid content in the oil and low glucosinolate in the meal (Adolphe, 1980). The combination of these two features makes Canola products (Margarine and Shortenings, and Salad and Cooking oil) more competitive with other oilseeds (Ward *et al.*, 1985).

Table 1:Glucosinolate types and their hydrolysis products

Glucosinolates type	Example	Hydrolysis Products
Aliphatic		
Sulphinated	3 - Methylsulphinylpropyl	ITC, nitrile
Alkenyl	2 - propenyl	ITC, nitrile
	3 - butenyl	ITC, nitrile
Hydroxy-alkenyl	2 - hydroxy- 3 - butenyl	Oxazolidine- 2 - thiones
Aromatic	benzyl	ITC, nitrile
	2 - phenylethyl	ITC, nitrile
Indolyl	3 - indoyl-methyl	Thiocyanate, Indole acetic acid, Indole- 3 - carbomil

Source Kirkegaard *et al.*, 1998.

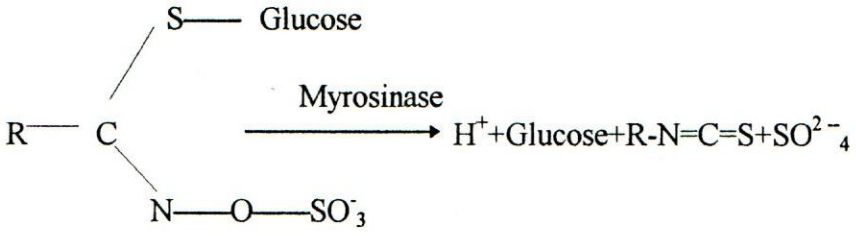
ITC-Isothiocyanate

Generally, the maximum glucosinolate concentration of above-ground plant parts coincides with the beginning of flowering (Fieldsend and Milford, 1994; Kirkegaard *et al.*, 1996), and thereafter declines while pod and seed glucosinolates levels increase.

Among the edaphic and biotic factors that may influence glucosinolate production in crucifers include insect attack, water stress, high sulphur and low nitrogen fertility in the soil (Kirkegaard *et al.*, 1998). All these factors have been reported to increase the glucosinolate concentration in crucifers (Rosa *et al.*, 1997).

2.4.5.3 *Hydrolysis of glucosinolates*

The hydrolysis of glucosinolates involves the enzymatic attack on the thioglucoside bonds (Chew, 1988), to produce a sulphate ion, glucose, an isothiocyanate and a nitrile or thiocyanate.



(GLUCOSINOLATE) (ITC)

This reaction occurs very rapidly when the glucosinolates contact the myrosinase enzyme which is normally partitioned separately in intact tissues (Brown *et al.*, 1991; Kirkegaard *et al.*, 1998). However, the type of isothiocyanates released are specific to the type of the parent glucosinolate present in the tissue (Kirkegaard *et al.*, 1993) (Table 1). For instance, aliphatic glucosinolates hydrolyse to form either isothiocyanates or nitriles, the latter being favoured by more acidic conditions. These volatile isothiocyanates or other related products are responsible for the flavour and pungency of cruciferous vegetables (Sang *et al.*, 1984). The isothiocyanates are generally considered the most toxic of the cruciferous hydrolysis products (Kirkegaard *et al.*, 1998), although nitriles, thiocyanates, oxazolidinethiones and the hydrolysis products of indolyl glucosinolates are also biocidal (Smolinska *et al.*, 1997).

CHAPTER THREE

3.0 MATERIALS AND METHODS

The materials and methodology used in these investigations followed closely those of Nash and Snyder (1962), Papavisas (1967), Cook (1968), Parke *et al.* (1991), Kirkegaard *et al.* (1995, 1996), and Smolinska *et al.* (1997).

3.1 Source and preparation of pathogens

The soil-borne *Fusarium* species (i.e. *Fusarium graminearum* and *Fusarium culmorum*) were isolated from roots of volunteer wheat crops (*Triticum aestivum*) from at least five locations (Table 2.) within the Njoro region, of Nakuru District in Kenya. Pieces of root about 0.5cm long were cut from symptom expressing plants and plated on potato dextrose agar (PDA) at 15^0C. The mycelium which grew from the ends of these pieces were isolated on fresh PDA and incubated at 25^0C. These formed the stock cultures which were stored in sterile water at 4^0C. Immediately prior to each experiment, plugs (diameter 6-8 mm) of actively growing mycelium were taken from the margins of fungal colonies grown on PDA and transferred to the centre of freshly poured sterile plates (85mm diameter) containing about 15ml of PDA. These plates were kept for no longer than 1 hr. at 25^0C on bench prior to the introduction of *Brassica* tissues.

3.1.1 Source and preparation of Brassica tissues

Field grown Rapeseed Canola (*Brassica napus* var. Wester) and Mustard (*Brassica juncea* vars. T1003-208; R3243; and T1003-195) plants were sampled from an experimental plot at the botanical garden, Egerton university, at flowering stage. These plants were pulled from the soil so that the main taproot and laterals were sampled. The soil was washed from the roots and the intact plants were frozen at -20^{0}C. After freezing, the plants were separated into roots and shoots (above ground material minus seed) and freeze-dried to preserve the intact glucosinolates and prevent hydrolysis by myrosinase enzyme during storage (Magrath *et al*., 1993 and Kirkegaard *et al.*, 1995). The root and shoot tissues were then grounded thoroughly into fine particles using a milling machine with a 1 mm sieve size to remove large fragments which were eventually discarded. These freeze-dried shoot and root preparations were stored in sealed bottles at 25^{0}C for 14 days prior to the experimentation (Plate 1). The seeds of the same varieties were obtained from the oil crops department, National Plant Breeding Station (NPBS), Njoro, Kenya. These seeds were crushed and the meal passed through a 1-mm sieve to remove large fragments of seed coat.

3.1.2 Source and preparation of greenhouse materials

The soil was collected from a single location at the Botany department's Botanical garden, Egerton university. These collected soils were steam sterilised in the autoclave at 82^{0}C for 1 hr.. They were then cooled and divided equally among 100 pots and left overnight prior to experimentation.

EGERTON UNIVERSITY LIBRARY

Table 2: Origin of the fungal isolate used in the experiments

Fungus	Isolate	Location*	Host	Year
Fusarium spp.	1	Matigari	Wheat	1997
Fusarium spp.	2	Egerton Univ. (Field 7)	Wheat	1997
Fusarium spp.	3	Mwigito	Wheat	1997
Fusarium spp.	4[a]	NPBS(Field 12)	Wheat	1997
Fusarium spp.	5	Likia	Wheat	1997

[a] Isolate used in the experimentations

* Location: Njoro - Latitude - 000 00'S

- Longitude = 036 00'E

Elevation = 2165M ASL.

NPBS - National Plant Breeding Centre, Njoro

Plate 1: Freeze-dried shoot(s) and Root (R) tissues of *Brassica*s after grinding and sieving.

3.2 EXPERIMENTAL PROCEDURES

3.2.1 *Experiment* 1. Effect of volatiles from *Brassica* tissues on conidial survival and germination

A pure culture of *Fusarium* species was maintained on PDA until it sporulated. The surface of the fungal culture was then rinsed twice with sterile distilled water to harvest the conidia.

The harvested conidia were enumerated with a haemacytometer and adjusted to a concentration of approximately 5×10^2 per ml.

Melted PDA was gently spread on sterile microscope slides to obtain a thin agar layer on the slide surface, then placed in sterile glass petri-dishes (90mm diameter) lined with sterile moistened filter paper (Smolinska *et al.*, 1997). A 100µl sample of conidia suspension was gently spread on each slide. An uncovered Aluminium dish containing either 1ml of sterile distilled water (control) or 500mg of *Brassica* tissues (Canola or Mustard) were placed in each petri dish. Water (1ml) was added to the tissues, petri-dishes covered and sealed with a double layer of parafilm.

After 24 hr. of incubation at 25^0C, all of the conidia on each slide were examined microscopically at a magnification of x100, and the percentage of germination was determined. This experiment was repeated three times with three replications per treatment.

3.2.2 *Experiment* 2: Effect of volatiles from *Brassica* tissues on chlamydospore survival and germination

The conidial suspension (2.0×10^6 per ml) was added to unsterilized moist soils. The inoculated soil was then allowed to dry slowly in the greenhouse.

After about 15days, a warm water-agar soil suspension was made from the dry inoculated soil (3g soil per 50ml 1.5% water agar) and pipetted quickly over the entire surface of sterile slides. These slides were observed under a microscope to confirm the presence of chlamydospores.

The slides were then placed in sterile glass petri-dishes (90mm diameter) lined with sterile moistened filter paper. An uncovered Aluminium dish containing either 1ml of sterile distilled water (control) or 500mg of *Brassica* tissues (Canola or Mustard) was placed in each petri-dish. After adding water (1ml) to the tissues, petri-dishes were covered and sealed with parafilm.

After 24 hr. of incubation at 25^0C, all of the chlamydospores on each slide were examined microscopically at a magnification of x100, and the percentage of germination was determined. The experiment was repeated once with two replications per treatment.

3.2.3 *Experiment* 3: Assessment of the inoculum potential in the presence of volatiles from *Brassica* tissues

The wheat seeds (var. K. Nyangumi) were placed in sterile distilled water for 24 hr.. Then, eighty imbibed seeds were immersed in conidia suspension (10^5 to 10^6 conidia per ml.) for 30 min. at 20^0C. (Smolinska *et al.*, 1997). The seeds were then removed and placed on milk filter papers (10 seeds per filter, with wet filter paper underneath). Milk filters with seeds were placed in beakers ($400cm^3$) containing 50ml of distilled water to maintain

humidity, plus an Aluminium weighing dish containing 10g of *Brassica* tissues (Canola or Mustard) or 10ml of water. Water (20ml) was added to the tissues and the beakers were sealed with a double layer of plastic film. Three replicates of this treatment were prepared.

After 24 hr. at 22-25^{0}C, the wheat seeds were removed, planted in plastic pots (soils used were autoclaved for 1 hr. at 121^{0}C, and autoclaved again after 24 hr. for 30 min.), and periodically watered. After 30 days at 20^{0}C, seedlings were removed, the roots washed, and the symptom severity rated on a 0-4 rating scale for root crown and foot rots (0= plant healthy; 1=roots and root crown slightly discoloured; 2= roots and root crown extensively discoloured and not shrunken; 3=roots and root crown extensively discoloured and shrunken; and 4 = roots and root crown partially or completely rotten or plant dead (Parke *et al.*, 1991).

Wet weights of the whole plants were obtained for each plant. The experiment was repeated once.

3.2.4 *Experiment* 4: Screening *Brassica* varieties and tissue types for isothiocyanates against *Fusarium* mycelial growth

The *Brassica* tissues were placed into small aluminium dishes at the rates of 0, 10, 50, 100, 200 and 500mg for all tissues (Kirkegaard, *et al.*, 1995, 1996). Duplicate plates of the fungus were prepared for each rate of *Brassica* tissues. The vessels containing the freeze-dried tissues were placed into the upturned lid of the plates while the inverted bottom containing the fungal plug on agar was held aside (this exercise was done in a laminar flow cabinet). Sterile water was then added to the tissues in the vessels at a rate of 6µl/mg of freeze-dried tissue to allow hydrolysis to commence (Kirkegaard, *et al.*, 1995). The inverted bottom of the plate with fungal plug was replaced to become the lid, and the plate was sealed

using two layers of parafilm so that only volatile hydrolysis products released from the tissues contacted the fungus.

Control plates were prepared containing sterile water only.

The plates were then incubated at 25^{0}C for 10 days. This was supposedly the time taken for the fungal colonies in the control plates to approach the edge of the plates. Every 24 hr., the diameter of each colony was measured and any unusual effects of the treatments on the fungal colonies (e.g. colour changes) were recorded. Following these measurements, treatments in which no hyphal growth occurred were opened in a laminar flow cabinet, the dish containing the *Brassica* tissue removed, and the plate aired for 10 min. and resealed. These plates were incubated for a further 10 days at 25^{0}C to determine whether the effects of the *Brassica* tissue was fungicidal (no regrowth) or fungistatic (regrowth occurs).

3.2.5. *Experiment* 5. Effect of volatiles released from autoclaved seed meals on conidial survival and germination; and mycelial growth.

Fusarium conidial survival and germination and mycelial growth were compared for sensitivity to volatiles released from raw (without autoclaving) and autoclaved (autoclaved for 15min at 121^{0}C) mustard seed meals. Mustard seed meals (Vars. T_{1003} - 195, and T_{1003}-208) were chosen as they had demonstrated the greatest suppression to all stages of the life cycle of *Fusarium* pathogen in the previous experiments. The experimental procedures were identical to those described in experiments 1 and 4, with the highly suppressive rates of 500mg of *Brassica* seed meals being used. The control plates were prepared having sterile distilled water only.

3.2.6 *Experiment* 6: Effect of isothiocyanates from various *Brassicas* on the population of soil-borne *Fusarium* inoculum in Wheat-*Brassica*; *Brassica*-Wheat; and *Brassica-Brassica* rotations

One hundred experimental pots containing steam disinfested soils were each inoculated by drenching with dilute microconidial suspension (2.0 x 10^6/ml) of *Fusarium* under controlled greenhouse conditions. The soils within the pots were then thoroughly mixed to disperse the inoculum.

Seven days after inoculation the experimental pots were divided into 5 lots of 20 pots each and treated according to split-plot factorial (Gomez and Gomez, 1984) design as follows:

LOT 1: All pots in this lot were planted with wheat (*Triticum aestivum* L. var K. Nyangumi) and were further divided into subgroups with different cropping regimes.

Group A_1: Wheat followed with wheat.

Group B_2: Wheat followed with Canola crop.

Group C_3: Wheat followed with mustard (var. T1003-208) crop.

Group D_4: Wheat followed with mustard (var. T1003-195) crop.

Group E_5: Wheat followed with mustard (Var. R3243) crop.

LOT 2: All pots in this lot had the Rapeseed Canola (*Brassica napus*) crops planted in them. They were further divided into subgroups of 4 pots each with different treatments as follows:

Group A_1: Canola crop followed with Canola crop.

Group B_2: Canola crop followed with Mustard (var. T1003-208) crop.

Group C_3: Canola crop followed with Mustard (var. T1003-195) crop.

Group D_4: Canola crop followed with Mustard (Var. R3243) crop.

Group E_5:Canola crop followed with wheat crop.

LOT 3: The pots in this category had the Mustard (*B. juncea* var. T1003-208) crop planted in them. They were further divided into subgroups of 4 pots each as follows:

Group A_1: Mustard (Var. T1003-208) crop followed by Mustard (Var. T1003-208) crop.

Group B_2: Mustard (Var. T1003-208) crop followed by Mustard (Var. T1003-195) crop.

Group C_3: Mustard (Var. T1003-208) crop followed by Mustard (Var. R3243) crop.

Group D_4: Mustard (Var. T1003-208) crop followed with Canola crop.

Group E_5: Mustard (Var. T1003-208) crop followed with a wheat crop.

Lot 4: All the pots in this category had the Mustard (*B. juncea* var. T1003-195) crops planted in them. They were further divided into subgroups of 4 pots each as follows:

Group A_1: Mustard (Var. T1003-195) crop followed by Mustard (Var. T1003-208) crop.

Group B_2: Mustard (Var. T1003-195) crop followed by Mustard (Var. T1003-195) crop.

Group C_3: Mustard (Var. T1003-195) crop followed by Mustard (Var. R3243) crop.

Group D_4: Mustard (Var. T1003-195) crop followed by Canola crop.

Group E_5: Mustard (Var. T1003-195) crop followed by a wheat crop.

LOT 5: All the pots in this lot had the Mustard (*B. juncea* var. R3243) crop planted in them. They were further divided into subgroups of 4 pots each as follows:

Group A_1: Mustard (Var. R3243) crop followed by Mustard (Var. T1003-208) crop.

Group B_2: Mustard (Var. R3243) crop followed by Mustard (Var. T1003-195) crop.

Group C_3: Mustard (Var. R3243) crop followed by Mustard (Var. R3243) crop.

Group D_4: Mustard (Var. R3243) crop followed by Canola crop.

Group E_5: Mustard (Var. R3243) crop followed by a wheat crop.

One and a half months after planting, soil samples were collected from 5 pots of first generation croppings in each lot. These soils were used to determine the populations of *Fusarium* species. Three 2.5 cm soil cores were taken from the surface 10 cm from each of the experimental pots and put on paper bags. The core samples were combined for each pot

within a lot, then a weighed amount of each of the five composite samples were diluted at the rates of 1:100 (wt/vol.) with 0.1% water agar.

One millilitre of agitated soil-water suspension was pipetted onto the surface of solidified Modified peptone-PCNB agar medium [Agar, 20.0g; Peptone, 5.0g; KH_2PO_4, 1.0g; $MgSO_4.7H_2O$, 0.5g; Oxygall, 1.0g; distilled water, 1000ml; Pentachloronitrobenzene (PCNB), 0.5g; Chlorotetracycline, 50.0g; and Streptomycin sulphate, 100.0mg.] contained in petri-plates (Papavisas, 1967). Two plates were prepared from each diluted sample, giving 16 dilution plates per lot. The suspensions were spread by rotating and tilting the plates until the whole surface of the plate was covered (Nash and Snyder, 1962). The plates were incubated for 5-7 days after which, the *Fusarium* colonies were identified and counted (Countable plates were those with 30-100 colonies in them) (Warcup, 1950). It was here assumed that each colony arose from individual propagule. Therefore by counting the number of colonies that developed from colony-forming units (CFUs) and by taking into account the dilution factors the concentration of fungal propagules in the original sample was determined as:

$$\text{Number of propagules/ml} = \text{Number of colonies on plate} \times \text{Dilution factor of sample}$$

One month and a half later, the soil samples were collected from 5 pots of second generation cropping and treatments in each lot and treated as explained above to determine the population of *Fusarium* propagules.

CHAPTER FOUR

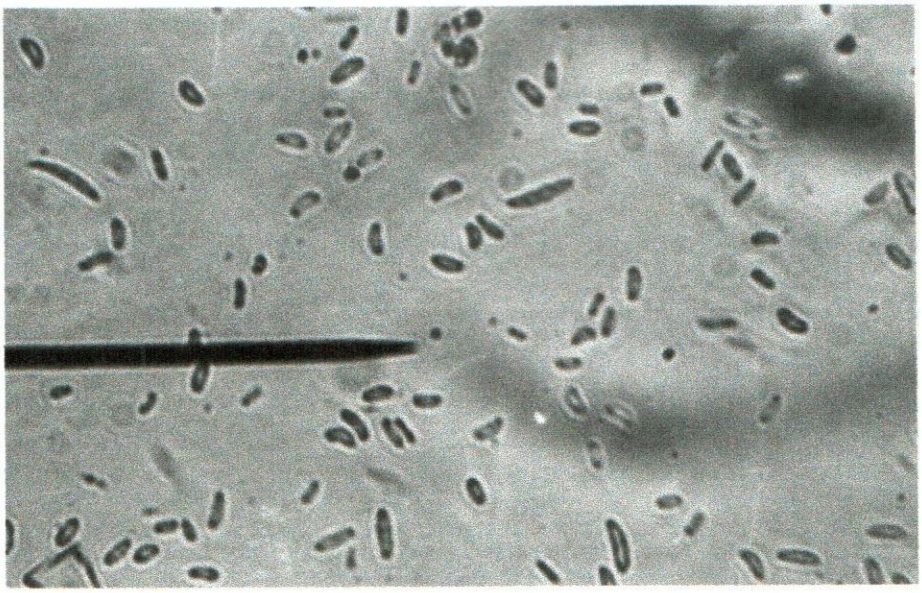
4.0 RESULTS

4.1 Effect of volatiles from Brassicas on conidia and chlamydospore survival and germiniation

The conidia produced by the *Fusarium* species used in these investigations were generally of two types, viz.; macroconidia and microconidia (Plate 2). In general, there was a delay in sporulation in most plates (12-15 days) than was expected. Cook (1980) reported abundant sporulation of *Fusarium* on acidified corn meal agar within 7 - 10 days upon incubation at a temperature of 20 - 25^{0}C with natural daylight. The delay in sporulation in these studies could be attributed to the medium (Potato dextrose agar) used to grow the cultures. Fisher *et al* (1982) stated that potato dexrose agar (PDA), being rich in carbohydrates, delays the formation of sporodochia, the typical fructifications of *Fusarium* species.

The isothiocyanates evolved from *Brassica* tissues and meals had various effects on *Fusarium* conidia and chlamydospore germination. These effects ranged from mild (7.5%) to complete (100%) inhibition of germination. The effects varied with the *Brassica* variety, plant part, the *Fusarium* inoculum type and size, and even the duration of exposure. The microconidia were most sensitive to these isothiocyanates and showed 0% germination after 24hr. exposure time. The chlamydospores on the other hand, were the least sensitive and showed fair germination rates (55%).

Overally, Mustard (*Brassica juncea* vars.$T_{1003\text{-}208}$ and $T_{1003\text{-}195}$) showed the greatest detrimental effects to conidial and chlamydospore survival and germination, whereas Rapeseed canola (*Brassica napus* Var. Wester) was least suppressive. The *Fusarium* inocula showed marked germination inhibitions when exposed to the hydrated mustard tissues as compared to those exposed to Rapeseed canola tissues. However, even the mustard varieties themselves demonstrated differential effects. Mustard varieties, $T_{1003\text{-}208}$ and $T_{1003\text{-}195}$) were more inhibitive based on the observed low inocula germination percentages (0-8%) when these were exposed to their hydrated tissues. The Mustard variety R3243, was however, not as inhibitive as the other Mustard varieties investigated in this study. These differences could be due to disparities in the glucosinolates hydrolysis products (isothiocyanates) evolved from those plant tissues. Kirkegaard *et al* (1996) reported that the fungal suppression rates obtained with *Brassica* tissues is a reflection of the isothiocyanate levels. The plant parts also showed marked differences in terms of percent inhibition rates (100% - 8%). The meals were most inhibitive (100%) at high concentrations of 500mg while shoot tissues were the least inhibitive (8%) (Plates 3 and 8).



(x400)

Plate 2: *Fusarium* macroconidia and microconidia in a sterile-distilled water suspension harvested from a sporulated culture.

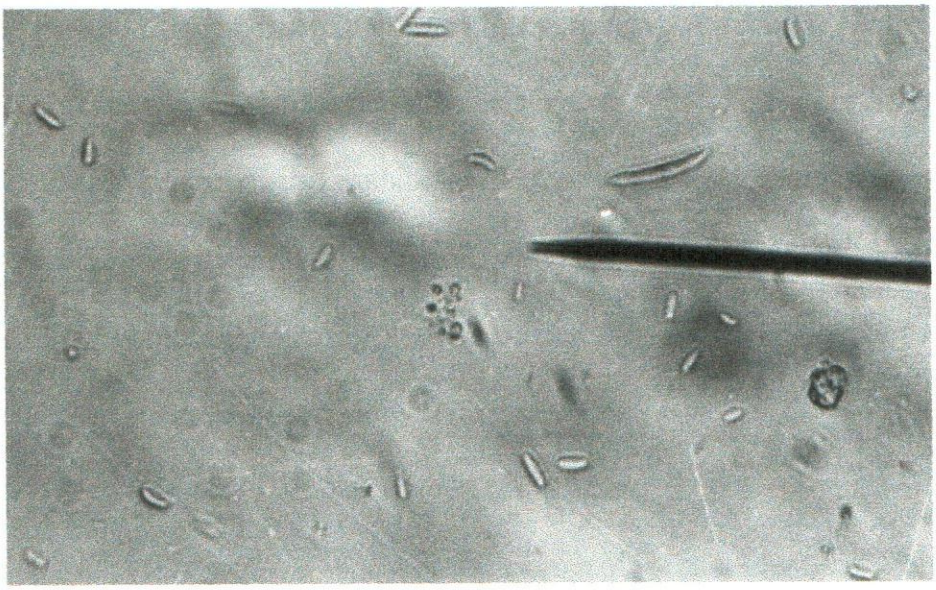


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Plate 3: *Fusarium* conidia on potato dextrose agar after exposure to isothiocyanates from *Brassica* seed meals (500mg), showing lack of germination.

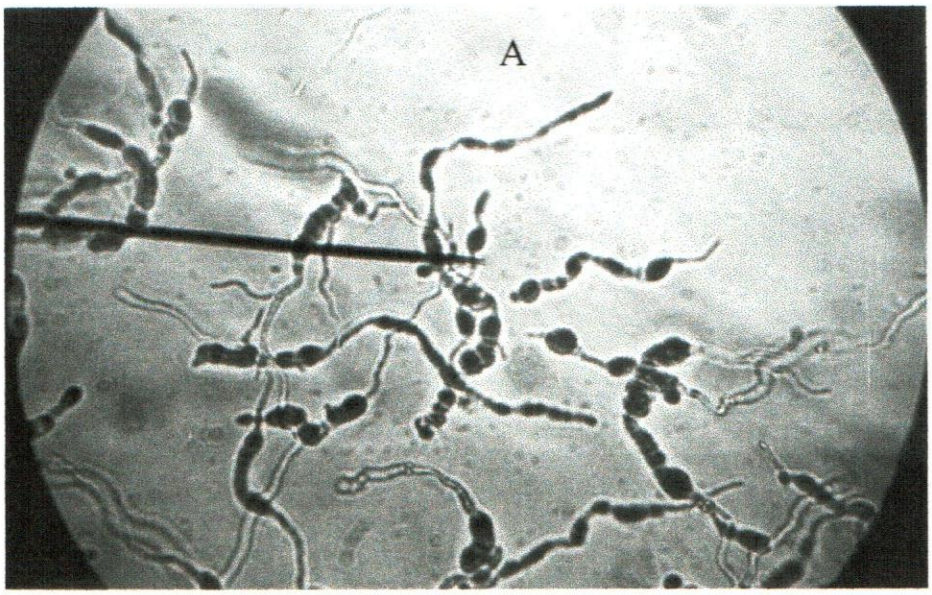
4.1.1 Effect of isothiocyanates from hydrated *Brassica* seed meals on conidia and chlamydospore survival and germination.

The isothiocyanates evolved from seed meals of *Brassica juncea* vars $T_{1003\text{-}208}$, $T_{1003\text{-}195}$) and R3243 completely inhibited the germination of *Fusarium* inocula (chlamydospores, macroconidia and micronidia) after 24 hrs of exposure (Plate 3). A further examination of the experimental set-ups (Experiments 1 and 2) after 72 hrs. revealed 0% germination under all *Brassica juncea* seed meals as opposed to those exposed to *Brassica napus* meals. The latter had visible germination at the range of 45-55% (Plates 4a and 4b). These observations could be attributed to different levels and types of isothiocyanates contained in the meals of *Brassica* species used. Kirkegaard *et al.* (1996) found out that *Brassica napus* seed meals contain moderate levels of phenylethyl isothiocyanates while *Brassica juncea* seed meals contain 2-propenyl isothiocyanates at higher levels. Therefore the high inhibition rates (100%) realised with *Brassica juncea* seed meals after 72 hrs exposure could be attributed to the higher fungicidal effects of 2-propenyl isothiocyanates than the 2-phenylethyl isothiocyanates found in *Brassica napus* seed meals.

The removal of hydrated *Brassica juncea* seed meals and airing the slides for about 10 min. after 24 hrs. exposure resulted in moderate (45%) germination for the conidia, whereas the chlamydospores showed no observable variation. The isothiocyanates evolved from the *Brassica*s were partly fungistatic in the short term (0-24hrs) based on the fact that removal and subsequent airing of the slides for these volatiles to escape resulted in some detectable conidial germination. The chlamydospores were, however, more resistant to these isothiocyanates and had higher germination rates (85%). This

resistance could be due to their thickwalled ultrastructure which could not easily be penetrated by the volatile isothiocyanates. The thickwalled structure (with a secondary wall) of *Fusarium* chlamydospores was demonstrated by Sitton and Cook (1981).

The introduction of the hydrated *Brassica juncea* seed meals, 20 hr. after the experimental set-up (when the conidia had germinated) resulted in hyphal growth stagnation and subsequent chlamydospore production (Plates 5 and 6). Some germinated hyphae converted into arthrospores (Plate 7). The chlamydospores and arthrospores are resistant structures (Ford *et al.*, 1969) formed when the protoplasmic material migrates and condense within a few cells in a hyphae (Ko and Lockwood, 1967) or a macroconidium. The *Fusarium* convert into these structures (chlamydospores and arthrospores) mostly under circumstances related to soil fungistasis (Ford *et al.*, 1969) to avoid mycolysis. Based on these findings, it is therefore probable that the *Brassica* derived isothiocyanates were fatal to the conidial germlings. The fungus was converting into spore forms which are resistant to lysis due to a stress-factor, the isothiocyanates. These survival structures do not germinate and are more resistant to inimical conditions which cannot support normal growth. They finally germinate when conditions become favourable. The results indicate that the *Brassica* derived isothiocyanates are causing hyphal lysis and therefore the fungus tries to evade this fatal condition by the formation of the resistant structures. This finding implies that the soil dressing with *Brassica* seed meals could reduce the *Fusarium* disease severity by curtailing the conidial germination and subsequent infection.



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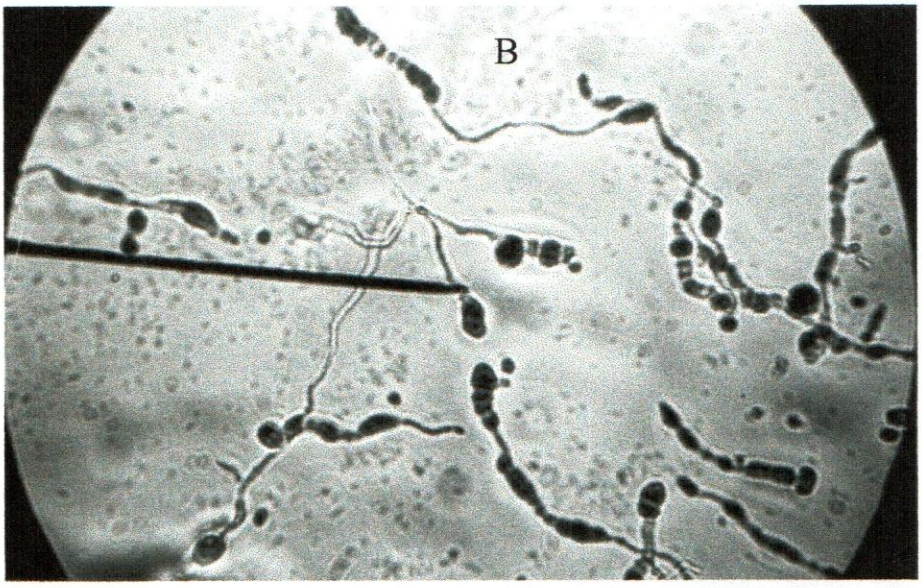

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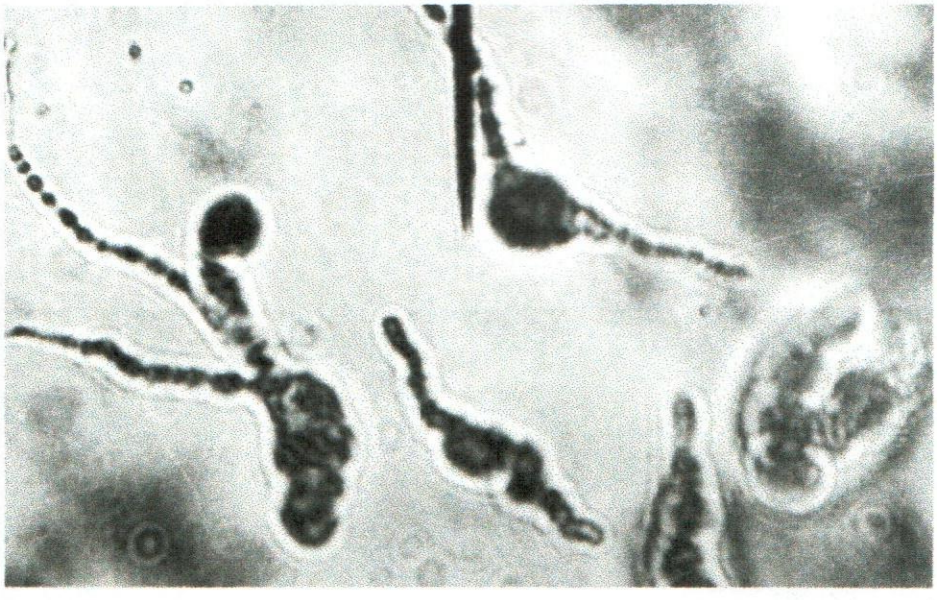
Plate 4a above and 4b, shows *Fusarium* conidial germination after 72 hr. exposure to isothiocyanates from hydrated *Brassica napus* var. Wester seed meals (500mg)

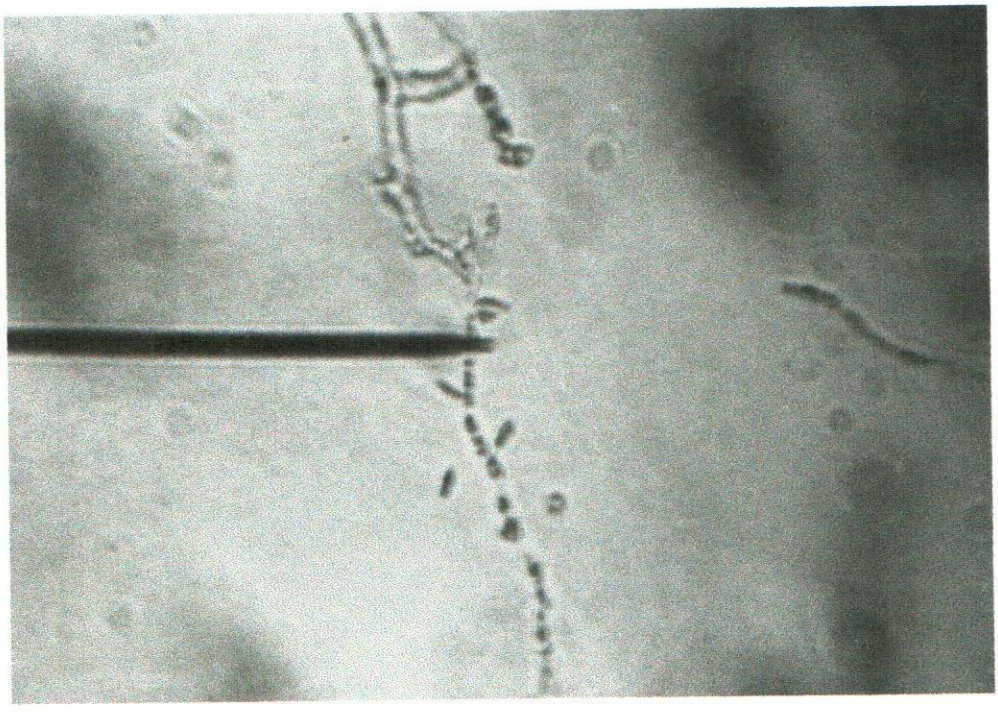


(x400)

Plate 5: Intercalay chlamydospores formed by 20 hr. old *Fusarium* germ-tubes upon the introduction of hydrated *Brassica juncea* seed meals(500mg)

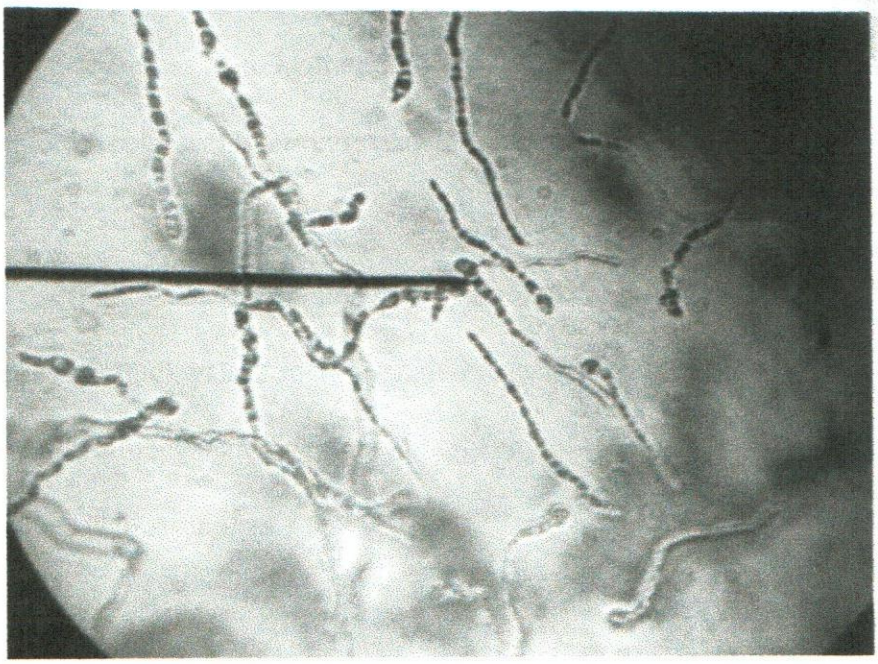
(x400)

Plate 6: Terminal chlamydospores produced by 20 hr. old *Fusarium* germ-tubes upon the introduction of hydrated *Brassica* seed meals (500mg) .



(x400)

Plate 7: *Fusarium* hypha forming arthrospores after the introduction of *Brassica juncea* meal tissues (500mg).



(x400)

Plate 8: *Fusarium* conidial germination in the presence of isothiocyanates from shoot tissues of *Brassica . juncea* (500mg).

4.1.2 Effect of isothiocyanates from hydrated *Brassica* shoot tissues on conidia and chlamydospore survival and germination.

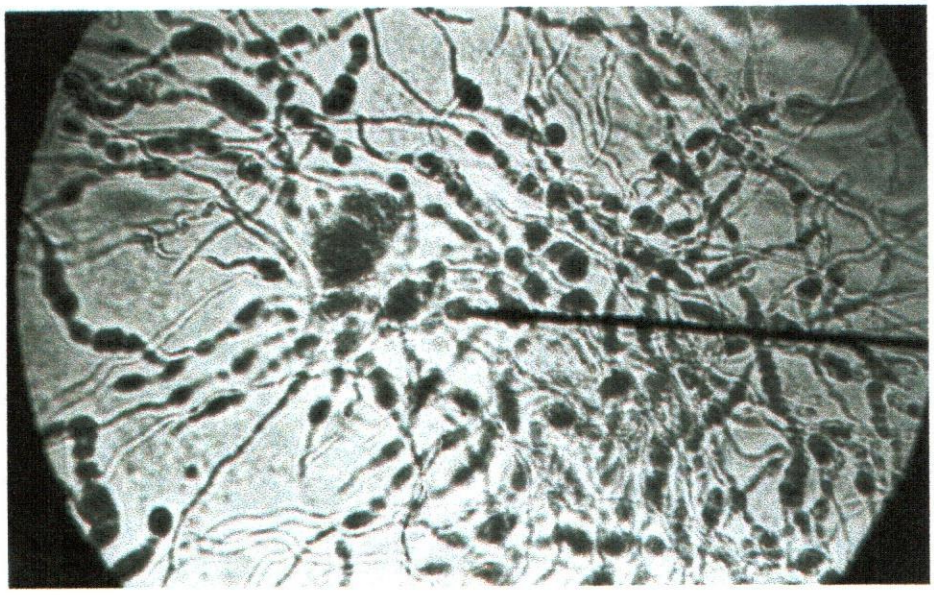
The hydrated shoot tissues of *Brassica juncea* evolved isothiocyanates which were less suppressive to the *Fusarium* inocula (chlamydospores and conidia) considered in this study (Plate 8). The germinated inocula showed characteristic pink coloured *Fusarium* colony after 72 hr. of incubation. However, the inocula (chlamydospores and conidia) exposed to isothiocyanates from hydrated *Brassica napus* shoot tissues had no visible growth, but microscopic examination revealed modest (55%) germination with short germ-tubes.

In all the treatments, the chlamydospores proved to be less sensitive. The chlamydospores are thickwalled survival structures and therefore were lesss permeable to *Brassica* derived isothiocyanates. The high longevity of these structures could be attributed to their wall structures.

4.1.3 Effect of isothiocyanates from hydrated *Brassica* root tissues on conidia and chlamydospore survival and germination.

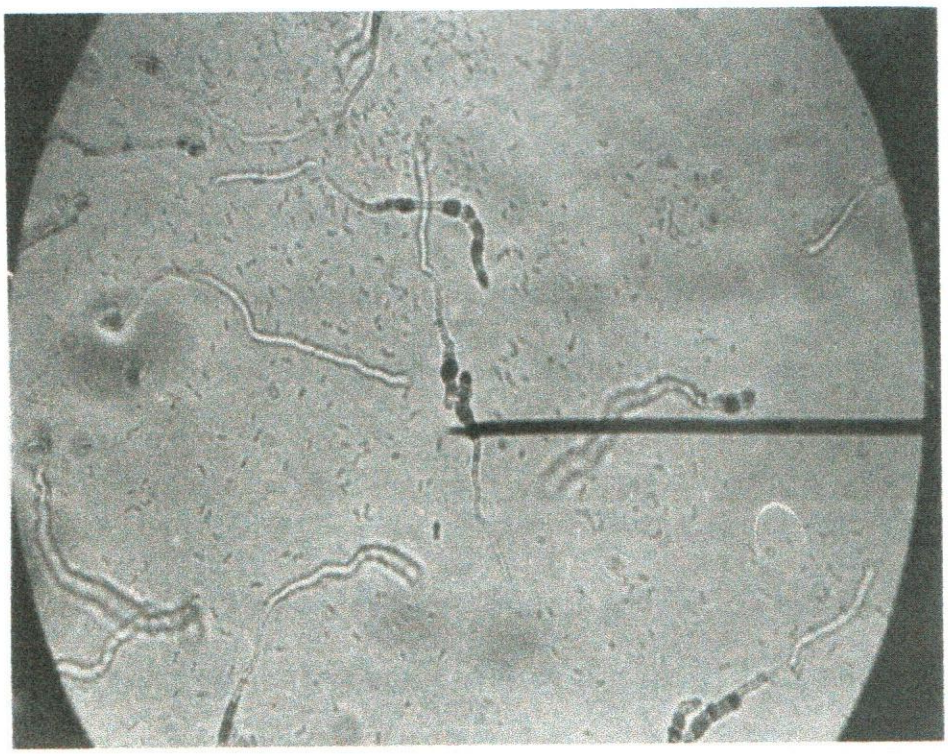
The isothiocyanates from *Brassica juncea* root tissues were the least suppressive (5-8%) in these investigations and were not different from the control treatments (Plate 9a). Nevertheless, after 24hrs exposure, the isothiocyanates evolved from *Brassica juncea* root tissues were more suppressive than those of *Brassica napus* roots.

Also, in all replicated trials in these experiments, the microconidia were the most suppressed (95-100%), macroconidia intermediate (45-50%), whereas the chlamydospores the least (0-8%) (9b). The strongest inhibition (95-100%) obtained with the microconidia could be attributed to their increased response to the fungistatic factors.



(x400)

Plate 9a: *Fusarium* conidia on potato dextrose agar not exposed to isothiocyanates from *Brassica* tissues. These were the controls; with distilled water only.



(x400)

Plate 9b: *Fusarium* conidia exposed to isothiocyanates from *Brassica* tissues. There was total lack of germination among the microconidia, whereas the macroconidia germinated.

4.2 *Conidial inoculum potential in the presence of volatiles from hydrated* Brassica *tissues.*

The isothiocyanates released from *Brassica juncea* seed meals were the most effective and significantly ($P \leq 0.01$) reduced the severity of root crown and foot rots of wheat plants grown from seeds inoculated with conidial suspension (Table 3). The root crown and foot rot ratings of treatments with seed meal hydrolysis products (isothiocyanates) from *Brassica juncea* were significantly lower than ratings of treatments with *Brassica napus* seed meals. The latter was not significantly different from the control treatments ($P \geq 0.01$) (Table 3).

In addition, the plant weights were lower in treatments with *Brassica napus* seed meals as compared with *Brassica juncea* vars. $T_{1003\text{-}208}$ and $T_{1003\text{-}195}$ seed meals (Table 3). These results imply that the isothiocyanates evolved from the seed meals effectively reduce the inoculum potential of the *Fusarium* pathogen. However, they are partly fungistatic, allowing some conidia to subsequently germinate and infect the wheat seedlings. The percentage germination of wheat seeds was high (on average 85%) without any differences among all the treatments. However, the high germination rates observed in this study contradicts other reports from elsewhere. Smith (1997) reported that soil amendment with *Brassica napus* leaves and stems suppressed the emergence of snap beans by 50%. The results obtained here therefore could be due to the low rates of application (10g/seeds) or the short exposure time (24hrs) to the *Brassica juncea* derived isothiocyanates. This implies that the isothiocyanates evolved from *Brassica juncea* seed meals have great potential as soil biofumigants when appropriate rates are applied to avoid any phytotoxic effects.

Table 3: Effect of isothiocyanates from hydrated *Brassica juncea and Brassica napus* seed meals on the inoculum potential of *Fusarium* conidia

Treatment[w]	Root/Crown Rot rating[x]	Plant weight (mg)[y]
Control (without meal)	2.7a	232.7c
M (Wester)	2.0ab	253.3c
M(R3243)	1.7bc	314.7ab
$M(T_{1003\text{-}208})$	1.1c	334.7a
$M(T_{1003\text{-}195})$	0.7d	350.7a
LSD	**0.78**	**62.8**

Means (N=15) followed by the same letter in a column are not significantly different (P≥0.01) using LSD.

[y]: Wet weight of the plant. Means (N=15) in same column followed by same letter are not statistically different (LSD; P=0.01)

M(Wester) = *Brassica napus* var. Wester seed meal

M (R3243) = *B. juncea* var. R3243 seed meal

$M(T_{1003\text{-}208})$ = *B. juncea* var. $T_{1003\text{-}208}$ seed meal

$M(T_{1003\text{-}195})$ = *B. juncea* var. $T_{1003\text{-}195}$ seed meal

4.3 *Effect of isothiocyanates from different* Brassica juncea *and* B. napus *varieties, tissue types, and tissue rates on* Fusarium *mycelial growth.*

The isothiocyanates released from hydrated *Brassica juncea* and *B. napus* tissues had varying detrimental effects on *Fusarium* mycelial growth as manifested by the colony diameter measurements taken daily for a period of 10 days.

Statistical analysis (using ANOVA) of the full data set resulted in significant ($P=0.01$) main effects, two-way and three-way interactions of all the factors considered (*Brassica* variety, plant part and tissue rates) on the degree of growth suppression. The varietal difference was significantly ($P\leq0.01$) affected by the plant part and tissue rates applied, and the tissue rate effect was also affected by the tissue types of the varieties tested. However, for ease of data interpretation, separate analysis were carried for the effect of each of the factors considered and the results are presented here.

4.3.1 Colony growth in the presence of hydrated *Brassica* seed meals.

Brassica juncea vars. $T_{1003\text{-}208}$ and $T_{1003\text{-}195}$ seed meals were the most suppressive and *Brassica napus* var. Wester one of the least. There were significant differences ($P\leq0.01$) in mean colony diameters measured for a period of 10 days after inoculation among the various *Brassica* varieties used (Table 4).

Table 4: *Fusarium* colony growth (mm) in response to isothiocyanates from hydrated meal, shoot and root tissues of different *Brassica juncea* and *B. napus* varieties.

Species	Plant part (500mg)		
	Meal	Shoot	Root
Control	50.0a	52.5a	47.5a
Brassica napus var. Wester	43.5a	28.2b	37.8bc
B. juncea var. R3243	31.8b	26.0b	35.1c
B. juncea var. $T_{1003\text{-}208}$	18.0c	40.0a	45.6a
B. juncea var. $T_{1003\text{-}195}$	17.2c	45.2a	35.1c

Means within a column followed by same letter are not significantly differently ($P \geq 0.01$) using Least Significant difference (LSD) test.

Brassica juncea vars. $T_{1003\text{-}208}$ and $T_{1003\text{-}195}$ seed meal treatments did not show any significant differences in growth suppression among themselves, but these differed significantly ($P \leq 0.01$) with *Brassica napus* var. Wester seed meal treatment. The ranking of the suppressive effects of the seed meals used in these investigations was *Brassica juncea* var. $T_{1003\text{-}195} \geq$ *B. juncea* var. $T_{1003\text{-}208} >$ *B. juncea* var. R3243 > *B. napus* var. Wester.

The seed meal rates also showed highly significant differences ($P=0.01$) in suppressiveness even among the same variety (Figs. 1, 2, 3 and 4). For instance, the *B. juncea* vars. $T_{1003\text{-}208}$ and $T_{1003\text{-}195}$ showed significant colony growth suppression at the treatment rates of 500mg - 100mg, than they did at 50mg and below. The latter case showed a significant difference from the control (Table 5 and Figs 1 and 2). The *Brassica*

napus var. Wester, as well as *Brassica juncea* var. R3243 seed meals did not show any significant differences with respect to tissue rates and were not statistically different from the control (Table 5 and Figs 3 and 4).

At the highest rates of seed meal (500mg), *B. juncea* vars. $T_{1003\text{-}208}$ and $T_{1003\text{-}195}$ proved to be fungicidal to the *Fusarium* mycelium (Plate 10), since the removal of the hydrated meal 10 days after the experimentation resulted in no colony regrowth (Plate 11). Introduction of the hydrated seed meals of these varieties 2 days after the fungal inoculation and subsequent mycelial germination on potato dextrose agar (PDA) caused mycelial growth stagnation and slight shrinkage in colony size (Plate 12.). This could be due to lysis of the germinated hyphae under the influence of isothiocyanates released by the *Brassica* tissues.

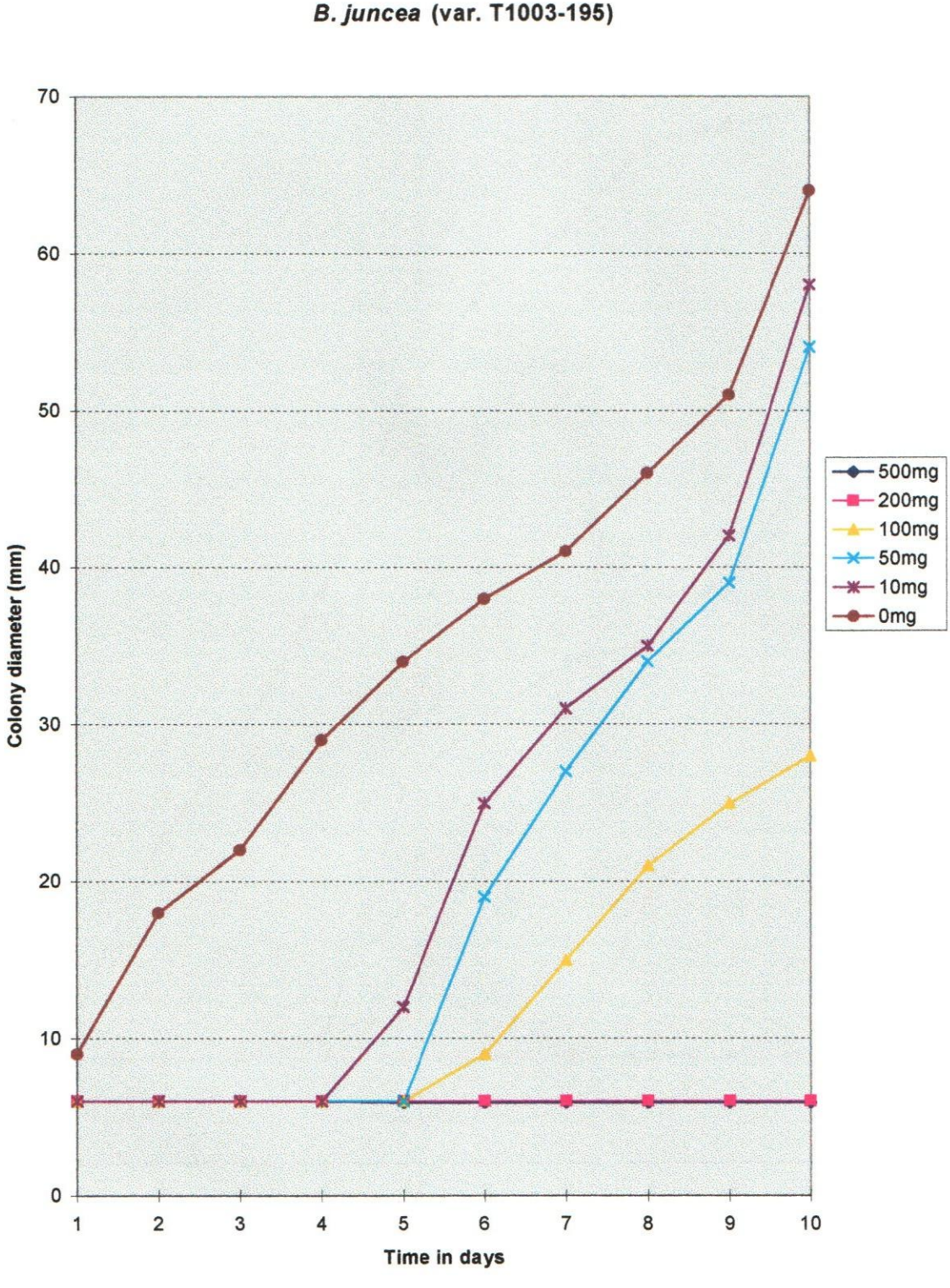


Figure 1: *Fusarium* colony growth (diameter) in the presence of isothiocyanates evolved from different concentrations of hydrated *Brassica juncea* var. T1003 - 195 seed meal. $LSD_{0.01}$=11.5

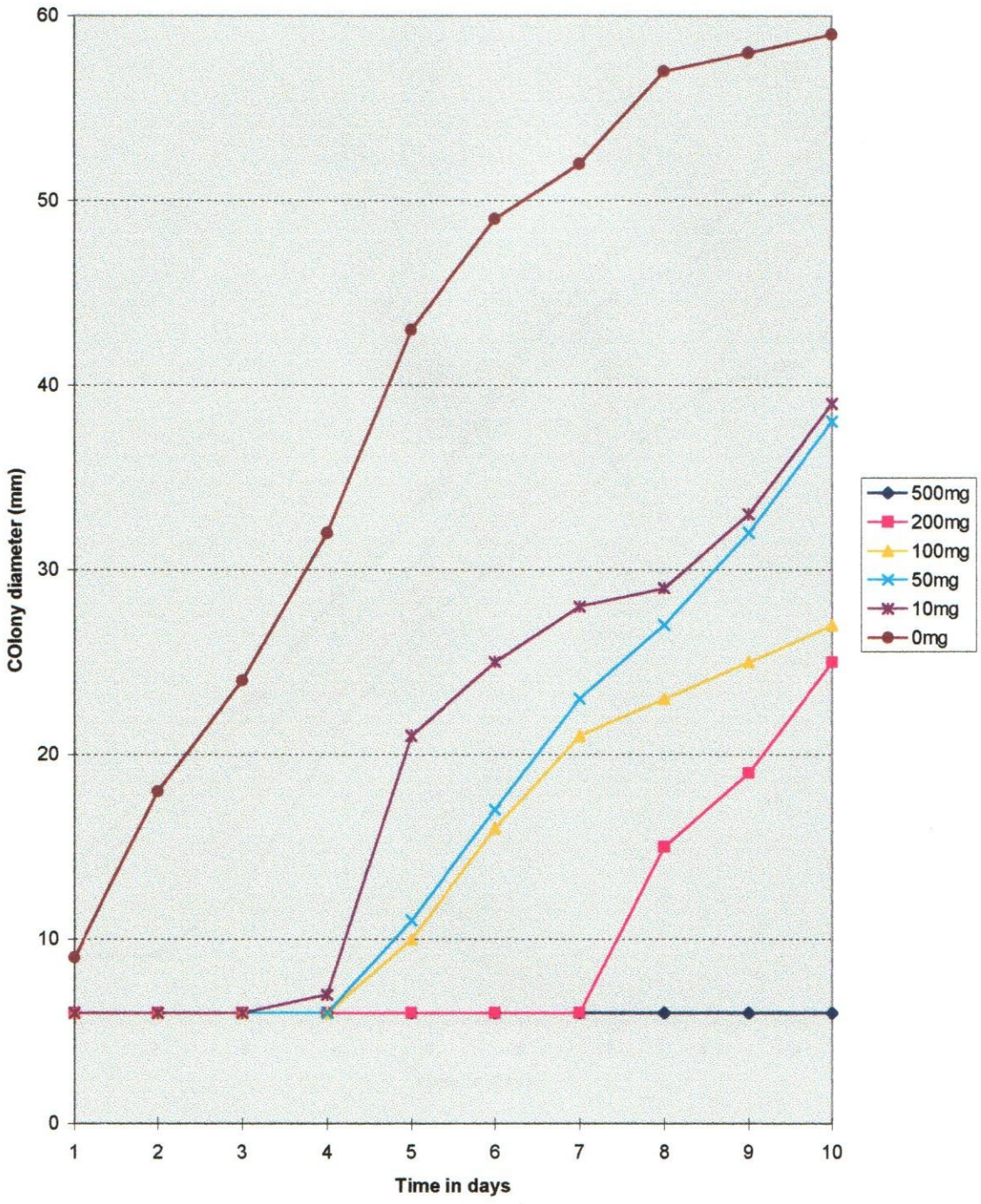


Figure 2: *Fusarium* colony growth (diameter) in the presence of isothiocyanates from different concentrations of hydrated *Brassica juncea* var. T1003-208 seed meal.

$LSD_{0.01}$=10.2

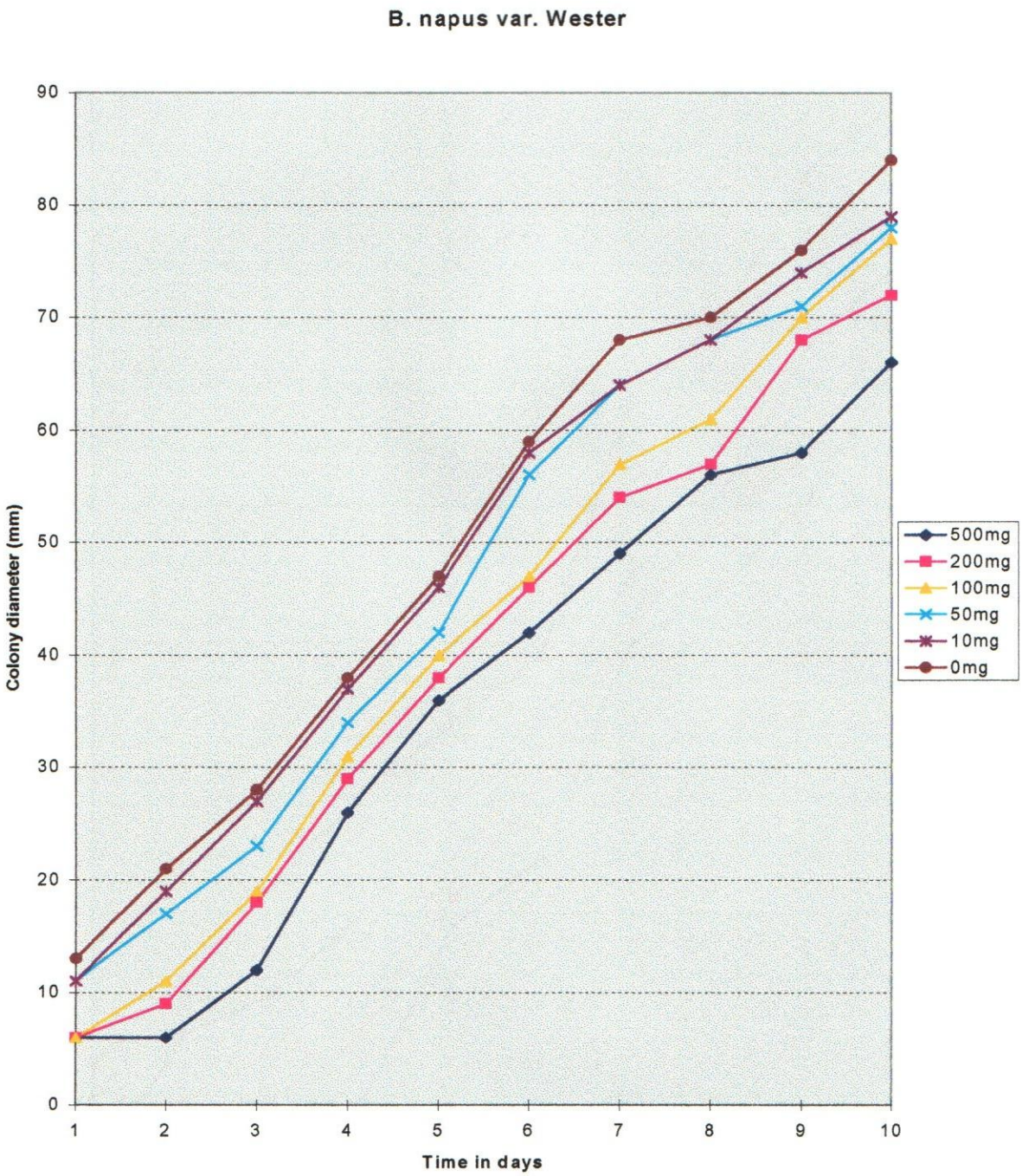


Figure 3:*Fusarium* colony growth (diameter) in the presence of isothiocyanates from different concentrations of hydrated *Brassica napus* var. Wester seed meal.

$LSD_{0.05}$=21.8

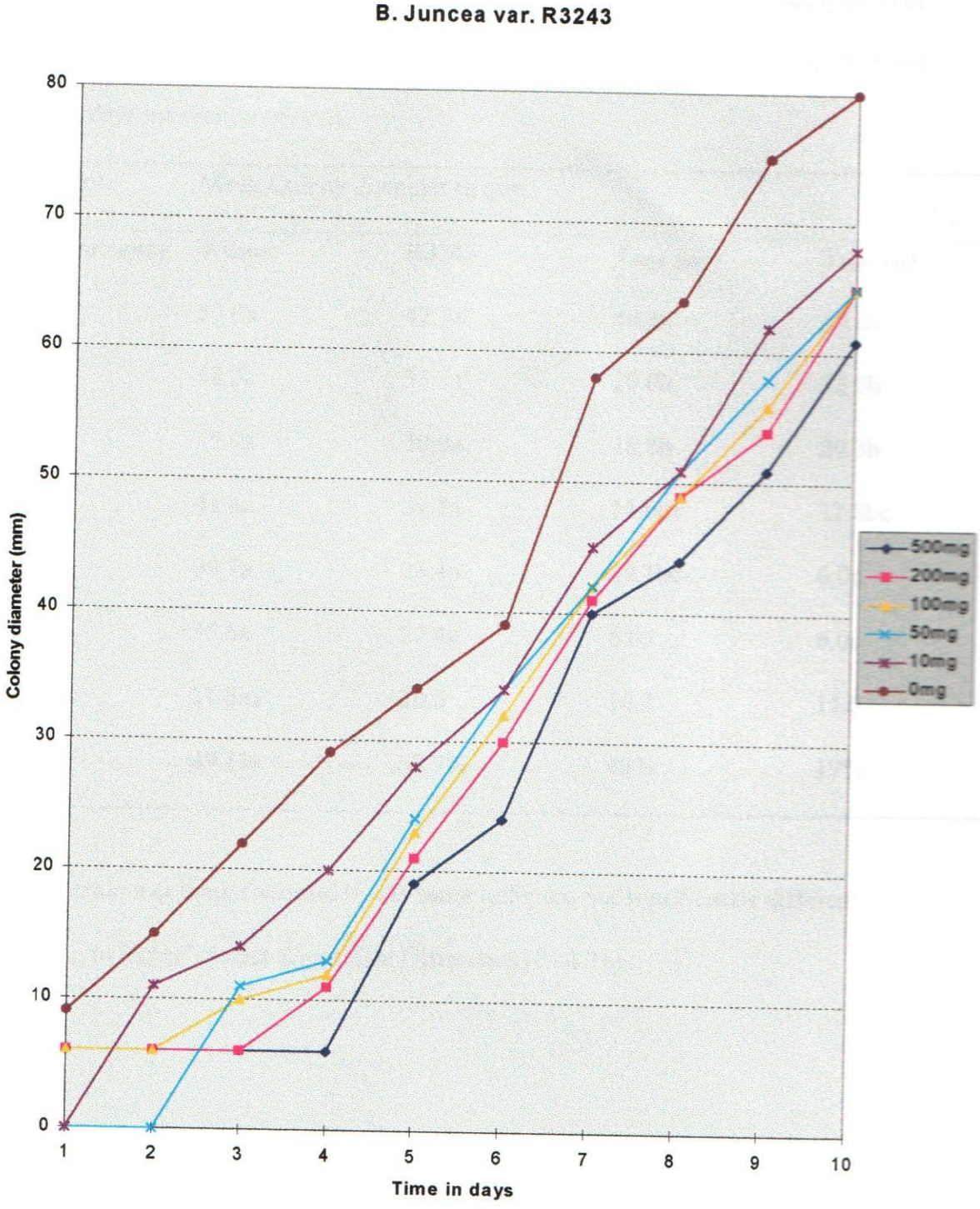


Figure 4: *Fusarium* colony growth (diameter) in the presence of isothiocyanates from different concentration of hydrated *Brassica juncea* var. R3243 seed meal.

$LSD_{0.05}$=20.5

Table 5: Effect of isothiocyanates from hydrated *Brassica* seed meals on the growth of *Fusarium* colonies on potato Dextrose Agar plates (Colony diameter readings obtained over ten days incubation period).

Treatment	Mean Colony diameter in mm.			
(Tissue rates)mg	Wester	R3243	$T_{1003\text{-}208}$	$T_{1003\text{-}195}$)
0	50.0a	42.5a	40.1a	35.2a
10	48.3a	33.1a	20.0b	22.7b
50	45.9a	30.0a	16.8b	20.3b
100	41.4a	29.3a	14.6bc	12.8bc
200	39.7a	28.4a	10.1bc	6.0c
500	35.6a	27.4a	6.0c	6.0c
LSD	**21.8ns**	**20.5**	**10.2**	**11.5**
Cv	**49.1%**	**58.9%**	**48%**	**17%**

Means within a column followed by the same letter are not significantly different according to Fisher's Least Significant Difference (P≥0.01).

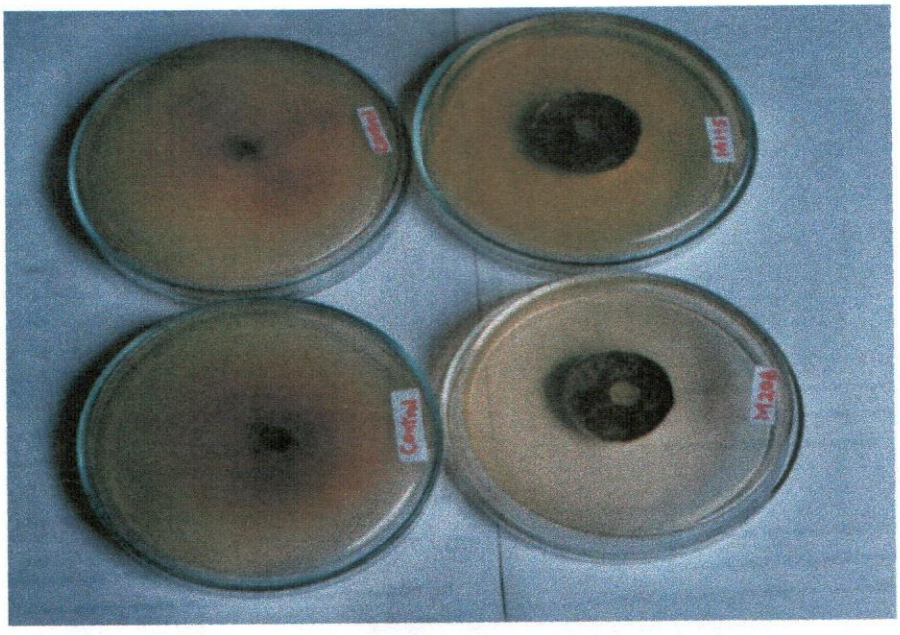


Plate 10 *Fusarium* mycelial plugs exposed to isothiocyanates from hydrated meals of *B. juncea* vars. $T_{1003\text{-}208}$ and $T_{1003\text{-}195}$ as compared with the controls (no meals). There was lack of mycelial growth even after 10 days of incubation

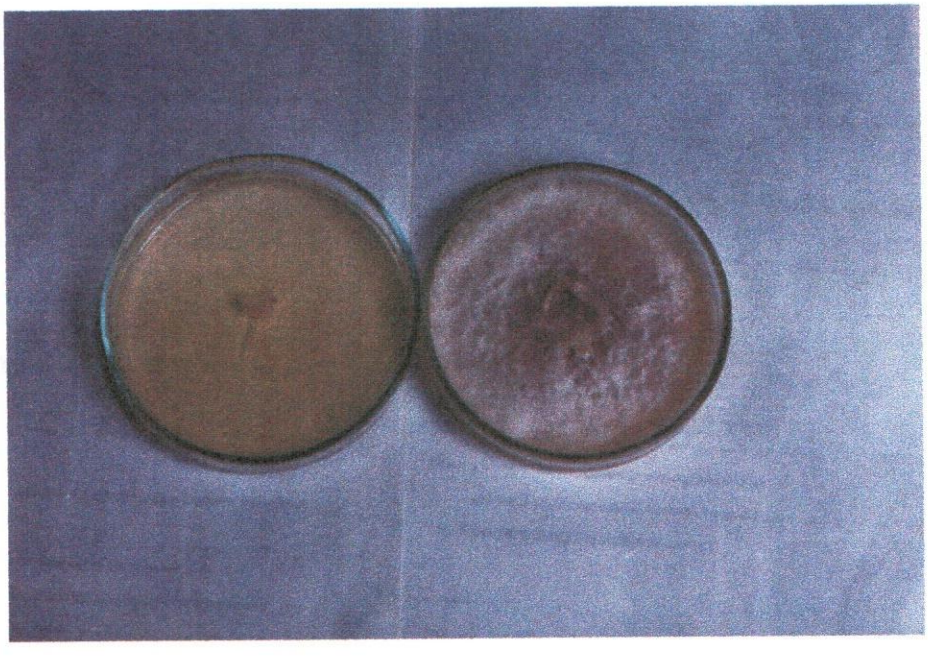


Plate 11 The left plate shows lack of *Fusarium* regrowth after the removal of the *Brassica juncea* seed meals, airing the plates for 10 mins. and incubating the culture for a further period of 10 days. On the right is the control plate

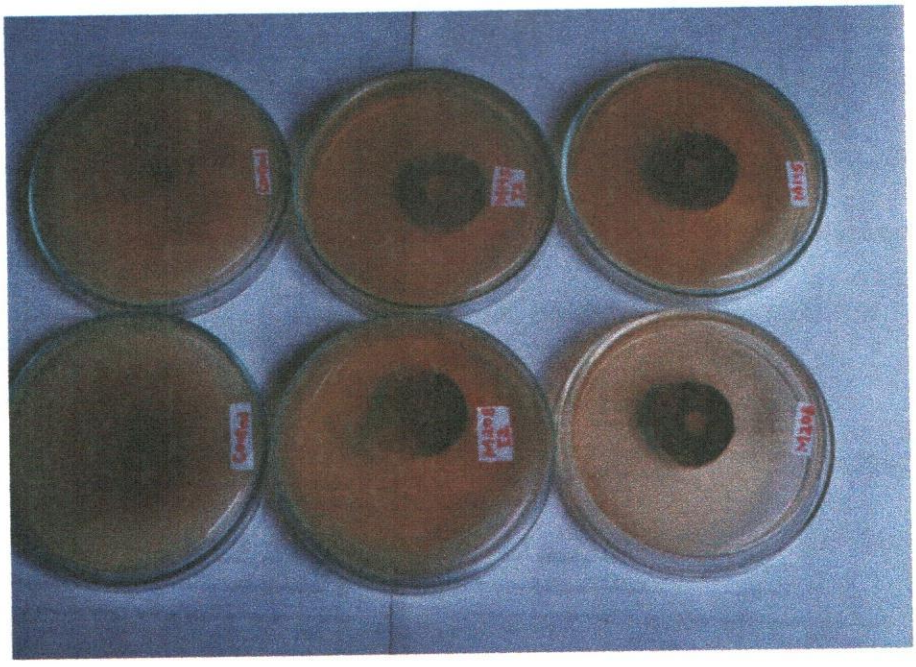


Plate 12: The column on the left shows *Fusarium* mycelial plug inoculated on potato dextrose agar and hydrated *Brassica juncea* seed meals introduced immediately; middle column shows when the meals were introduced 48hr. after inoculation while the column on the right represents the control plates (no meal). There was lack of growth in the left column plates and growth stagnation in the middle column plates.

4.3.2 Colony growth in response to hydrated *Brassica* shoot tissues

The suppression of *Fusarium* colony growth by isothiocyanates evolved from shoot tissue differed significantly ($P \leq 0.01$) with the *Brassica* variety and tissue application rates.

The *B. napus* var. Wester shoot tissues evolved suppressive (89%) isothiocyanates, and was not significantly different from *B. juncea* Var. R3243 shoot tissues. *B. juncea* vars. $T_{1003\text{-}208}$ and $T_{1003\text{-}195}$ on the other hand, were least suppressive (27%) and the colony diameter readings obtained under their hydrated shoot tissue isothiocyanates were not significantly different (Table 4.).

The shoot tissues also showed variations in suppressiveness depending on application rates (Fig 5 and 6). The shoot tissues of *B. napus* var. Wester showed the greatest suppression (89-63%) at application rates of 500-100mg without any significant differences ($P \geq 0.05$) in colony diameters obtained at this range (Table 6). However, application rates of 10-50mg showed least suppression (5-7%) which were not statistically different from the controls. This suppression was also associated with significant colony colour changes. The colour changed from pink (control) to white. Kirkegaard *et al* (1996) reported significant colour changes associated with *Bipolaris* colony suppression with *Brassica* - derived isothiocyanates. This colour change could be due to interference with the fungal pigment production pathway. Because of this observation, it is tempting to speculate that the isothiocyanates from *Brassica juncea* shoot tissues upsets the biochemical pathways of *Fusarium* pathogen and thereby interfering with its virulence. However, the biochemical aspect of this interference was not specifically investigated.

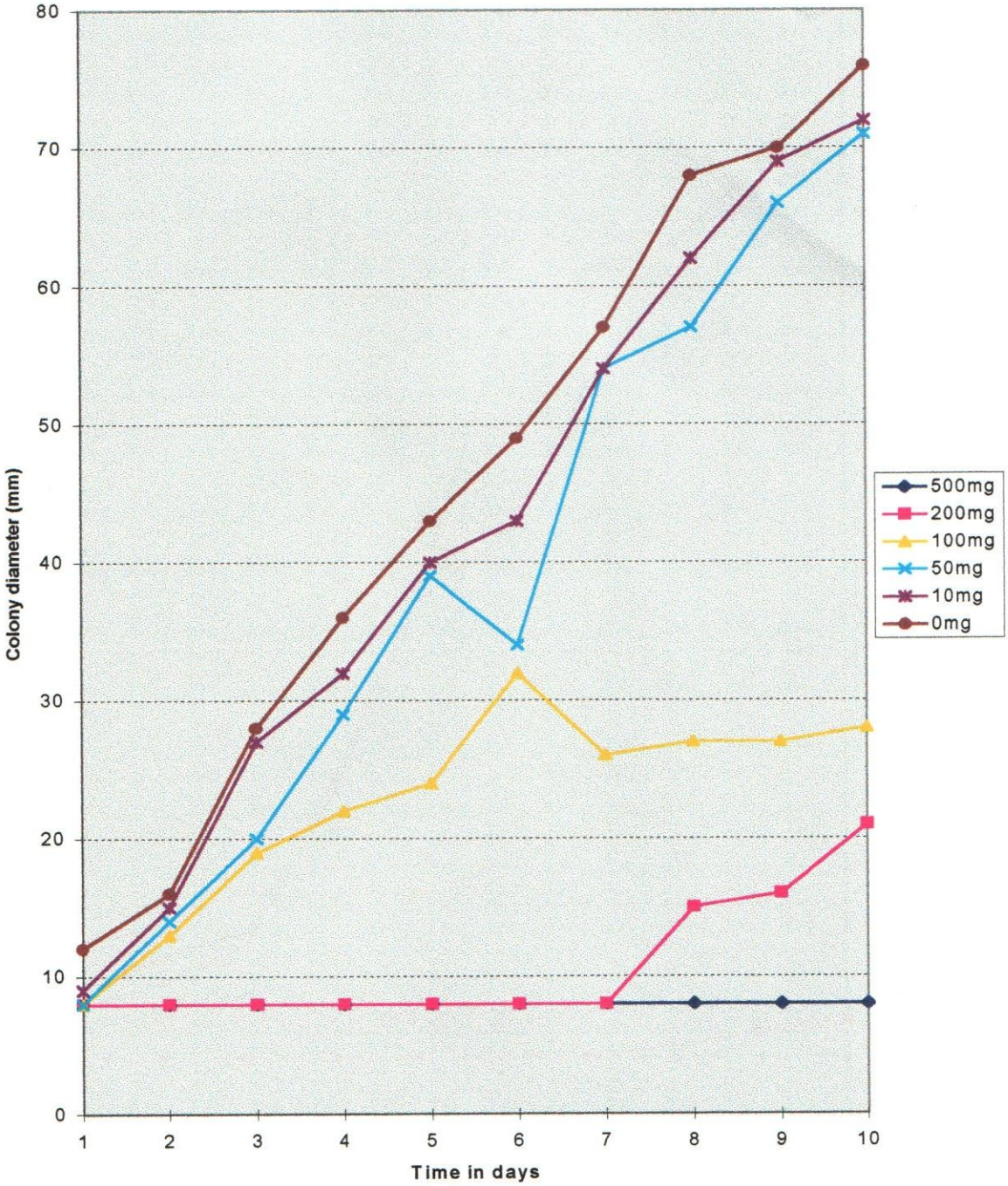


Figure 5: Fusarium colony growth (diameter) in the presence of isothiocyanates from different concentrations of hydrated *Brassica napus* Var. Wester shoot tissues.

$LSD_{0.01}$=14.4

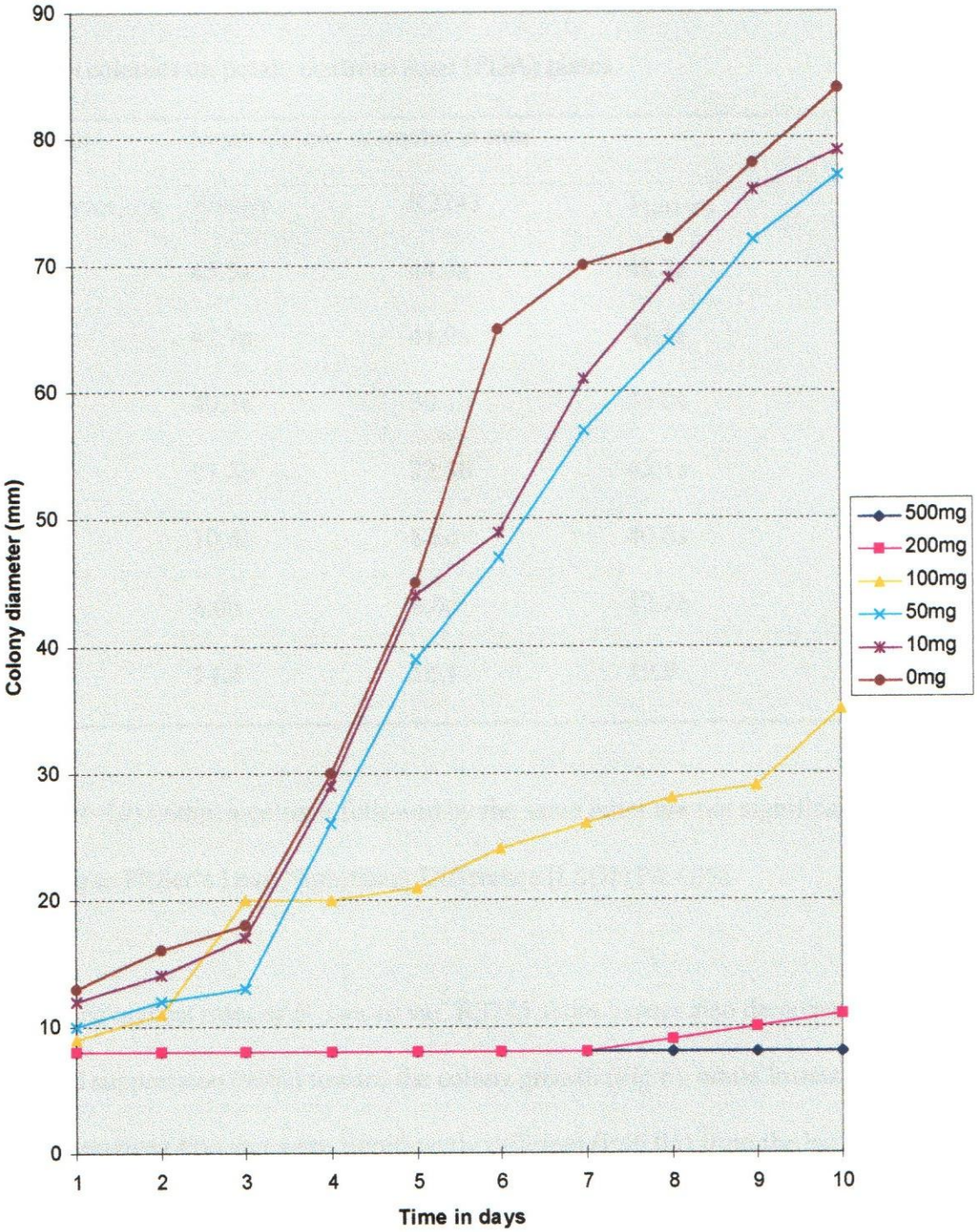


Figure 6: *Fusarium* colony growth (diameter) in the presence of isothiocyanates from different concentration of hydrated *Brassica juncea* var. R3243 shoot tissues.

$LSD_{0.01}$=10.1

Table 6:Effects of isothiocyanates from hydrated *Brassica* shoot tissues on the growth of *Fusarium* colonies on potato dextrose Agar (PDA) plates.

Treatment	Mean Colony diameter in mm.			
(Tissue rates)mg	Wester	R3243	$T_{1003\text{-}208}$	$T_{1003\text{-}195}$)
0	45.5a	44.9a	48.7a	52.5a
10	42.7a	41.9a	47.1a	51.3a
50	40.3a	30.1b	46.0a	46.6a
100	21.8b	22.3b	45.1a	46.0a
200	10.8b	8.6c	40.6a	37.3a
500	8.0b	8.0c	12.7b	37.2a
LSD	**14.4**	**10.1**	**19.9**	**21.6**

Means (N=10) within a column followed by the same letter are not significantly different according to Fisher's Least Significant Difference [LSD] ($P \geq -.05$).

The highest rates of *B. juncea* var. R3243 shoot tissues also demonstrated increased suppression (90%) toward the colony growth (Fig 6), while lowest rates were less suppressive (4%) and were significantly different ($P \leq 0.05$) from the highest rates (Table 6).

The shoot tissues of *B. juncea* var. 195 was the least *Fusarium* colony growth suppressor (0.5%) and did not show any significant difference between the control and all the other treatments.

4.3.3 Colony growth in the presence of hydrated *Brassica* root tissues

The hydrated *Brassica* root tissues of all the varieties tested ($T_{1003\text{-}208}$; R3243 and Wester) were least suppressive to *Fusarium* mycelial growth compared to all the other tissues (shoot and seed meals). In this respect, *B. juncea* var. 208 was the poorest colony growth inhibitor and differed significantly (P=0.05) from all the other varieties tested ($T_{1003\text{-}208}$;$T_{1003\text{-}195}$; R3243 and Wester) (Table 4).

However, all the *Brassica* tissue rates (10mg, 50mg, 100mg, 200mg and 500mg) tested in this study showed no statistical differences with the control (0mg) treatments (Table 7). Interestingly, the *Fusarium* colony growth was stimulated by *Brassica* root rates of 100 - 200 mg and hence growth was highest at this range among all the varieties tested ($T_{1003\text{-}208}$;$T_{1003\text{-}195}$; R3243 and Wester) (Table 7). In addition, the *B. juncea* root tissues caused a colour change from pink (control) to yellow. The observed increased growth could be due to the production of auxin precursor indole-3-acetonitrile from indoyl-methyl glucosinolates under the influence of chemical compounds produced by the fungus. The production of the auxin precusor (indole-3-acetonitrile) from glucosinolates was observed in the club root disease of crucifers (*Plasmodiophora brassicae*), in which there occur overgrowth symptoms and club formation (Chew, 1988).

Table 7: Effect of isothiocyanates from hydrated *Brassica* root tissues on the growth of *Fusarium* colonies on potato dextrose agar plates

Treatment[k]	Mean Colony diameter in mm.[m]			
(Tissue rates)mg	Wester	R3243	$T_{1003\text{-}208}$	$T_{1003\text{-}195}$)
0	36.4a	31.4a	47.1a	36.0a
10	35.2a	31.1a	40.4a	33.0a
50	35.8a	36.7a	39.8a	35.0a
100	41.7a	41.4a	52.5a	36.9a
200	41.7a	40.4a	53.1a	34.3a
500	35.9a	29.8a	40.8a	35.3a
LSD	**18.0**	**18.9**	**21.5**	**19.9**

[k] Freshly inoculated fungal plugs (8mm diameter) were exposed to volatile compounds from hydrated *Brassica* root tissues at the rates of 0 (control), 10mg, 50mg, 100mg, 200mg and 500mg

[m] Colony diameter readings made for a period of 10 days. Means within a column followed by the same letter are not significantly different according to Fisher's Least Significant Difference [LSD] ($P \geq -.05$).

4.4 *Impact of isothiocyanates evolved from autoclaved* Brassica juncea *seed meals on* Fusarium conidial *germination and mycelial growth.*

The autoclaved seed meals of *B. juncea* vars. $T_{1003\text{-}208}$ and $T_{1003\text{-}195}$ released ITCs which failed to show complete conidia and chlamydospore inhibition as did the raw (unautoclaved) meals. The hydrated autoclaved meals showed reduced conidial germination inhibition (65%) as compared to the control treatments (100%) after 24 hr. of exposure. However, on examination of the same after 10 days exposure, did not show any observable differences between the treatments and the control (Plate 13).

The *Fusarium* colony growth was significantly ($P \leq 0.01$) suppressed in the presence of autoclaved *B. juncea* vars. $T_{1003\text{-}208}$ and $T_{1003\text{-}195}$ seed meals (Plate 14). The results presented in Table 8 and Fig. 7 indicates that the hydrated autoclaved seed meals of these Mustard varieties evolved suppressive volatiles (isothiocyanates and nitriles) and the colony diameter under these treatments differed significantly ($P \leq 0.01$) from the controls. The *B. juncea* var. $T_{1003\text{-}208}$ and $T_{1003\text{-}195}$ autoclaved seed meals were the most suppressive (33-50%) and did not show any significant difference ($P \geq 0.01$) from raw (unautoclaved) seed meal. The inhibiton obtained with the autoclaved *Brassica juncea* seed meals could be due to increased production of nitriles as well as little amounts of isothiocyanates. Smolinska *et al* (1997) reported that nitriles are the dominant volatiles produced by autoclaved meals. Thermal degradation of glucosinolates during autoclaving enhances nitrile formation (MacLeod and Rossiter, 1986), whereas enzymatic hydrolysis promotes the production of isothiocyanates.

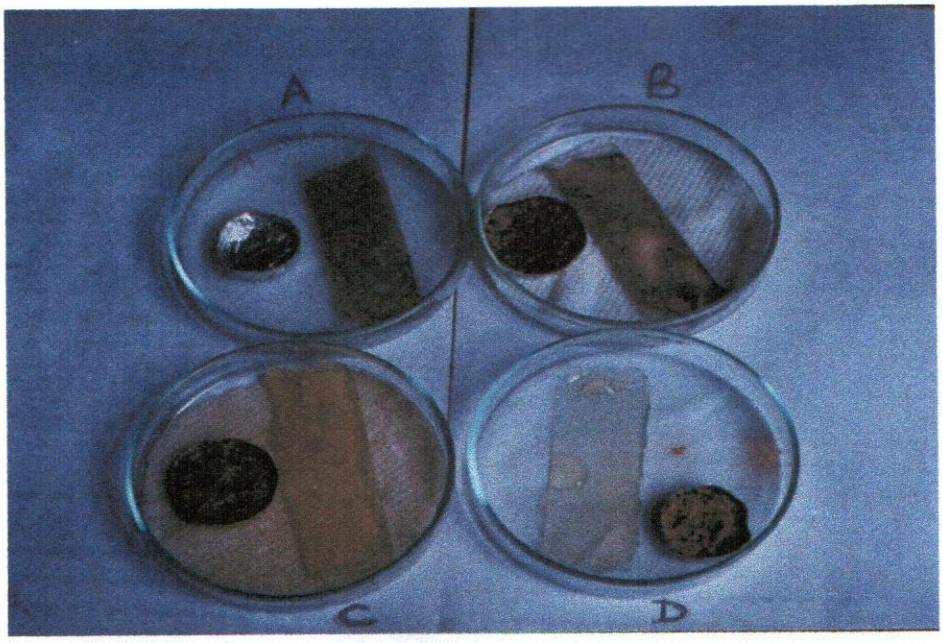


Plate 13: Effect of autoclaved *Brassica juncea* seed meals on *Fusarium* conidial germination;

A= Control (without meal)

B= Autoclaved meal of var. $T_{1003\text{-}208}$

C = Autoclaved meal of var. $T_{1003\text{-}195}$

D = Non-autoclaved meal

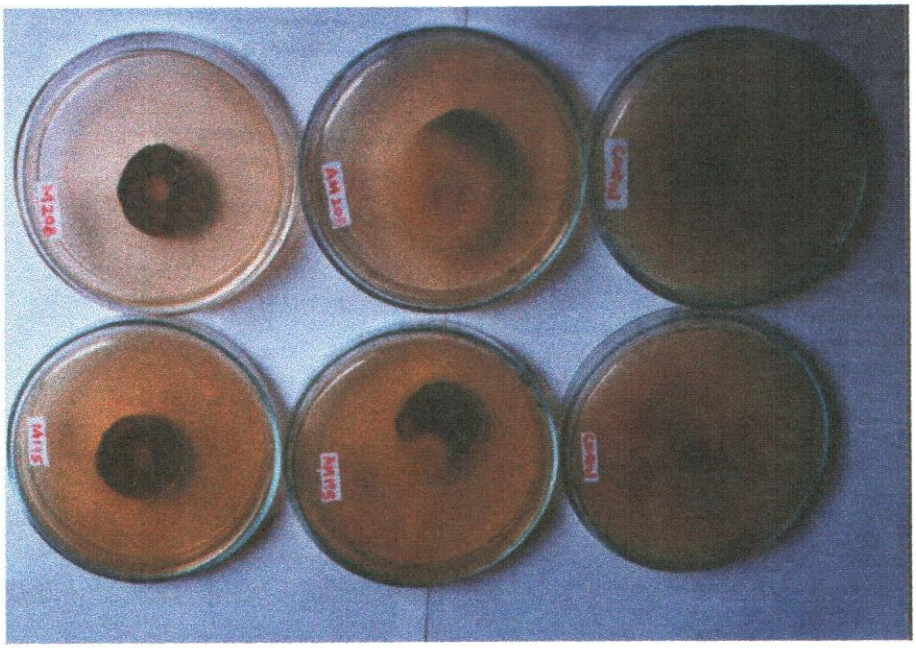


Plate 14: *Fusarium* colony growth in the presence of non-autoclaved meals (left column) autoclaved meals (middle column) and control (without meal) on the right column.

Table 8: A comparison of the effects of isothiocyanates evolved from autoclaved and non-autoclaved *Brassica juncea* seed meals on *Fusarium* colony growth.

Treatment[x]	Mean colony diameter [y] (mm)
Control	46.85a
AM ($T_{1003\text{-}208}$)	23.70b
AM ($T_{1003\text{-}195}$)	15.30bc
M(($T_{1003\text{-}208}$)	8.00c
M(($T_{1003\text{-}195}$)	7.00c
LSD	**9.16**

[x] Freshly inoculated fungal plugs (7-8mm) were incubated in the presence of hydrated autoclaved or unautoclaved seed meals, or none of these (control).

[y] An average of 10 replications. Means (N=50) in the same column followed by the same letter are not significantly different (Least Significant difference [LSD]; P≥0.01

AM = Autoclaved meal

M = Non-autoclaved meal

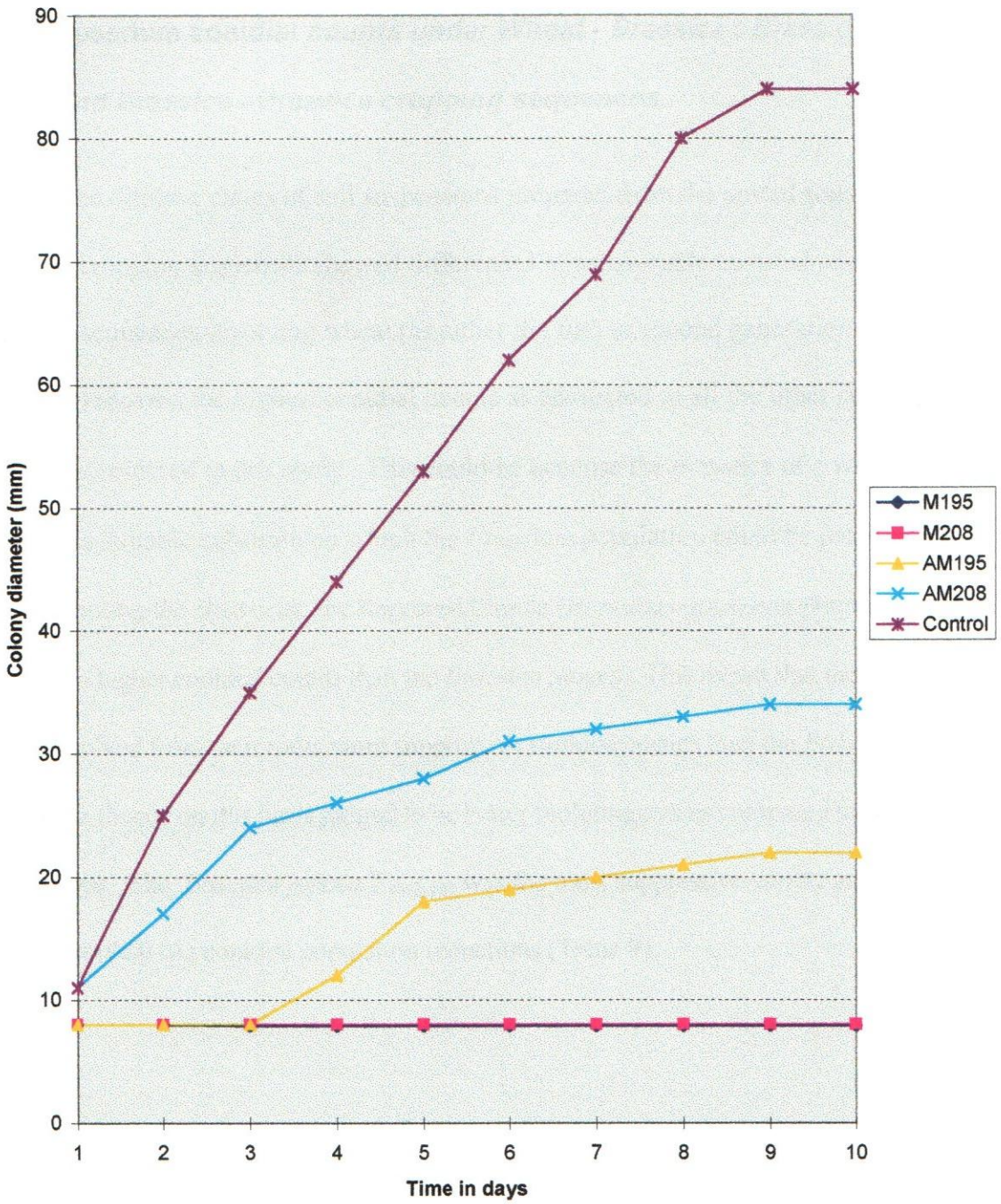


Figure 7: *Fusarium* colony diameter in the presence of isothiocyanates from autoclaved (AM) and non-autoclaved (M) hydrated *Brassica juncea* seed meals. $LSD_{0.01}$=9.16

4.5 Fusarium conidial *counts under Wheat - Brassica ; Brassica -Wheat; and Brassica - Brassica cropping sequences.*

The dilution plates of soil suspensions prepared from the potted soils under different cropping sequences showed differences in recoverable conidial counts. The cropping sequences involving wheat (as either the first or second generation cropping sequence) showed the highest conidial counts as compared to all the other cropping regimes considered in this study. This could be because the presence of a wheat crop provided a suitable substrate on which the *Fusarium* population could be perpetuated.

Among the *Brassicas*, the Rapeseed Canola (*Brassica napus*) cropping sequence resulted in higher conidial counts than the *Brassica juncea*. This meant that the *Brassica juncea* evolved from their roots more suppressive isothiocyanates than the *Brassica napus*. They were thence on this basis judged to be better biofumigants as compared to *Brassica napus* crops. The *Brassica juncea* $T_{1003\text{-}195}$ was the most suppressive variety and gave significant ($P \leq 0.01$) conidial population reductions (Table 9).

Table 9: *Fusarium* conidial counts under different cropping sequences.

	Conidial counts per ml[x]				
LOTS	A	B	C	D	E
LOT[1]	234.3^{aa}	165.8^{ab}	148.8^{ac}	147.8^{ad}	130.5^{ae}
LOT[2]	227.8^{ba}	200.8^{bb}	138.5^{bc}	134.5^{bd}	123.0^{be}
LOT[3]	158.3^{da}	155.3^{db}	153.3^{dc}	151.3^{dd}	124.8^{de}
LOT[4]	152.0^{ea}	141.3^{eb}	134.3^{ec}	101.5^{ed}	101.5^{ee}
LOT[5]	211.3^{ca}	174.8^{cb}	159.3^{cc}	147.8^{cd}	138.3^{ce}

[x] An average of four replications. The LSD values for comparing the group treatments (A-E) under the same lot are 24.4 conidia per ml at the 5% level of significance and 20.4 conidia per ml at the 1% level of significance. The letters in superscripts represents the cropping sequence, where;

a = Wheat (*Triticum aestivum* var. K. Nyangumi)

b = *Brassica napus* var. Wester

c = *Brassica juncea* var. R3243

d = *Brassica juncea* var. $T_{1003\text{-}208}$

e = *Brassica. juncea* var. $T_{1003\text{-}195}$

In terms of the conidial population reductions, the investigated *Brassica*s could assume the following ranking; *Brassica juncea* var. $T_{1003\text{-}195}$ > *B. juncea* var. $T_{1003\text{-}208}$ > *B. juncea* var. R3243 > *B. napus* var. Wester. This finding could be due to different levels of glucosinolates found in these *Brassica*s. The other factors which could directly affect the results include the soil physical and chemical properties which could influence the types and flow of glucosinolate hydrolysis products within the soil atmosphere. The sorption of isothiocyanates onto clay and organic matter reduces the effective concentration of these volatiles in the soil atmosphere (Kirkegaard *et al.*, 1996). Therefore the physical properties of the soil used in these investigations had an influence on the results obtained. The presence or absence of microbial myrosinase enzyme in the soil was of utmost importance. Chew(1988), reported that the glucosinolates exuded into the soil are converted into the biocidal isothiocyanates through hydrolysis by microbial myrosinase. The other factor which was important was soil pH. The pH determines the type of glucosinolate hydrolysis products. The edaphic and biotic factors that prevailed during the experimentation period are also important. The insect attack, water stress, high sulphur and low nitrogen fertility are among the factors already reported to increase glucosinolate concentrations and hence their hydrolysis products among the Cruciferous plants (Kirkegaard *et al.*, 1998).

CHAPTER FIVE

5.0 DISCUSSION

The isothiocyanates evolved from hydrated *Brassica juncea* and *Brassica napus* tissues had a detrimental effect on each stage in the life cycle of the *Fusarium graminearum* Schw. and *Fusarium culmorum* Smith. pathogen. These biotoxic effects depended on the *Brassica* species, type of *Brassica* tissue (whether from roots, shoots or seeds) and the susceptibility of fungal stage of development. These differences in inhibition could be attributed to the different levels of glucosinolates (GSLs) that could be found in the individual *Brassica* tissues and the toxicity of their hydrolysis products to the specific stages of the *Fusarium* life cycle.

5.1 *Suppression of germination of* Fusarium *conidia by isothiocyanates from Brassicas*

The *Fusarium* isolate used in these investigations (Table 2) showed a slight delay in sporulation and when conidiogenesis occurred, they were of a typical, non-uniform types. These observations could be due to the growth media (potato dextrose agar), which amongst other factors, dictates the growth and sporulation of the *Fusarium* spp. (Fisher *et al.*, 1982).

The isothiocyanates from non -autoclaved *Brassica juncea* seed meals completely suppressed the *Fusarium* conidial germination. In contrast, the isothiocyanates evolved from *Brassica napus* seed meals were partially suppressive and showed moderate germination (45%), despite prolonged periods of exposure. This increased levels of

suppression obtained with Mustard (*B. juncea*) seed meal tissues is consistent with a reported presence of large quantities of 2-propenyl (allyl) glucosinolates (Sang *et al.*, 1984; Kirkegaard *et al.*, 1996). This form is absent in Rapeseed canola (*Brassica napus*) seed meals.

In addition, the microconidia were more suppressed than the macroconidia. This is probably due to the greater need of exogenous energy needed by microconidia to germinate, and hence the strongest response to fungistatic factors (Steiner and Lockwood, 1969).

In the short term (after 24 hr. exposure), the isothiocyanates from Mustard seed meals were fungistatic but fungitoxic in the long term (after 72 hr. and above exposure time). The prolonged exposure of the conidia to these volatiles may have created fatal conditions through lysis of the conidia. However, lysis was not specifically demonstrated in these investigations. This *in vitro* antimicrobial activity of hydrolysis products of glucosinolates has also been shown to be effective against conidial germination of other fungal pathogens (Mari *et al.*, 1993).

The germinated conidia when exposed to these isothiocyanates created a stress to the germlings resulting in growth termination and the formation of survival structures (Plates 5, 6 and 7). Thus the fungus converts into a spore form which is resistant to lysis (Ford *et al.*, 1969) and serves as a survival mechanism (Nash *et al.*, 1961). These revelations brings into the fore the fact that isothiocyanates released from Mustard seed meals adversely affect the *Fusarium* pathogen prompting it to enter into a resting stage (by production of chlamydospores and arthrospores) which are resistant to mycolysis (Lockwood, 1960 and Nash *et al.*, 1961) as rapidly as possible. Ko and Lockwood (1967) reported that these structures are resistant to more inimical conditions than conidia and mycelium and eventually germinates when the conditions become favourable.

The shoot tissues of *Brassica juncea* released isothiocyanates with less suppressive effects to the *Fusarium* conidia as compared to isothiocyanates from *B. napus* shoot tissues. The root tissues, were however, least suppressive as compared to all the other tissues (Table 4). But a comparison among the root tissues themselves revealed that the *B. juncea* root tissues evolved more suppressive volatiles than those of *B. napus*.

The low rates of suppression observed for the shoot and root tissues as compared to the seed meal tissues was consistent with earlier reports by Kirkegaard *et al* (1996 and 1998), that the glucosinolate levels in the vegetative parts of the *Brassica* tissues declines with age and stage of development as the pod and seed levels increase.

The high rates of suppression obtained with Mustard roots (*B. juncea* var. $T_{1003\text{-}195}$ and R3243) could be attributed to the amounts of 2-propenyl isothiocyanates they evolve. Kirkegaard *et al.* (1996), did not detect this form of isothiocyanate in Rapeseed canola roots but instead detected low levels of phenylethyl isothiocyanates.

The Rapeseed canola (*B. napus*) shoot tissues demonstrated higher suppressiveness than those of Mustards. This finding contradicts an earlier finding by Kirkegaard *et al* (1996) where they reported the absence of both 2-propenyl isothiocyanates and 2 - phenylethyl isothiocyanates in Rapeseed canola shoot tissues. This discrepancy could be due to the edaphic and biotic factors that prevailed during the growth period of the *Brassicas* used in these investigations since the glucosinolate profiles is a function of all these factors (Rosa *et al.*, 1997). A second plausible reason could be that the canola (*B. napus* var. Wester) shoot tissues evolved other volatiles or isothiocyanates potentially suppressive to *Fusarium* species but were not measured by these workers. Sang *et al.* (1984), reported the presence of other volatiles which occur in *Brassicas* in low amounts. The amounts of those released could have been sufficient to cause the observed low levels of suppression.

5.2 *The Suppressive effects of isothiocyanates from* Brassicas *on* Fusarium *chlamydospores*

The most important propagule in the ecology of *Fusarium* species may be the thick-walled chlamydospores that serves as survival structures of the pathogen. The *Fusarium* propagules obtained from the soil by plate dilution method are actually chlamydospores (Baker and Snyder, 1970). Thus the chlamydospores play an important role in perpetuating the populations of this fungus in soil and initiating infection (Cook, 1968). The *Fusarium* macroconidia occurring in parasitized host stems and later washed or tilled into the soil convert into chlamydospores and thus constitute one source of these survival structures (Cook, 1980). Chlamydospores are also formed in colonized host tissues and presumably function as infective propagules while still in the host tissue or after being slowly freed in the soil by disintegration of the fragments. The results obtained in this study showed that the isothiocyanates evolved from *Brassica* tissues suppressed the chlamydospore germination. This observation compares well with that of Candole and Rothrock (1996). These workers reported the presence of a volatile factor in hairy-vetch which suppressed the chlamydospore germination. The seed meals produced the most toxic isothiocyanates to these structures, followed by shoot and root tissues in that order.

However, the response of the chlamydospores to these isothiocyanates was not as great as that observed with the conidia. The chlamydospores are known to be thick-walled (with a primary and secondary walls) (Sitton and Cook, 1981), an ultrastructure which may explain this longevity. A prolonged exposure of the chlamydospores to these isothiocyanates was more detrimental to these structures. Removal of these

isothiocyanates and airing the chlamydospores resulted in less observable germination. It is probable that some chlamydospores lysed in the presence of these volatiles.

5.3 *Reduction of* Fusarium *conidia inoculum potential by isothiocyanates from* Brassica *seed meals.*

The 24 hr. incubation of *Fusarium* conidia-infested wheat seeds in the atmosphere of isothiocyanates from the non-autoclaved seed meals decreased the disease severity (Table 10) by about 75% to 25% compared with the controls. *Brassica juncea* var. $T_{1003-208}$ and $T_{1003-195}$ seed meals evolved the most effective isothiocyanates while the *Brassica napus* var. Wester seed meals the least. These results indicate that the effect of isothiocyanates toward *Fusarium* conidia were fungistatic (after 24 hr. exposure), allowing some conidia to subsequently germinate and infect the wheat seedlings. This view was confirmed by recoverability of the *Fusarium* pathogen from treated seedlings. From the previous observation already explained at the beginning of this chapter, it could be postulated that the levels of infection realised in treated seedlings were initiated by the macroconidia and not microconidia.

Table 10: The *Fusarium* root rot disease severity reduction in the atmosphere of isothiocyanates from non-autoclaved seed meal tissues of *Brassicas*.

Treatment[a]	Disease severity as % of control[b]
M-Wester	75
M-R3243	62
M-$T_{1003\text{-}208}$	40
M-$T_{1003\text{-}195}$	25

[a] Imbibed wheat seeds (var. K. Nyangumi) were mixed with a conidial suspension (10^5 - 10^6/ml), then incubated for 24 hr. in the atmosphere of isothiocyanates compounds from non-autoclaved meals of *Brassicas* or not (controls). All the treatments were then planted separately in sterile (autoclaved for 1 hr. at 121^{0}C, and autoclaved again after 24 hr. for 30 mins)soils and allowed to grow for 30 days.

[b] Seedlings were uprooted, washed clean, and the disease incidence rated on a 0-4 rating scale for root and crown rot: 0 = plant healthy; 1=roots and root crown slightly discoloured; 2=roots and root crown extensively discoloured and not shrunken; 3=roots and root crown extensively discoloured and shrunken; and 4=roots and root crown partially or completely rotted or plant dead (Parke *et al.*, 1991). The disease severity was calculated as a percentage of the controls.

5.4 Fusarium *mycelial growth as affected by isothiocyanates from* Brassicas.

The *Fusarium* pathogens plated on potato Dextrose Agar (PDA) gave rise to a characteristic pink or orange coloured colony. Similar cultures have been reported by Wiese, (1977) and Cook, (1980). The experiments reported here demonstrated that the volatile compounds usually released from *Brassica* tissues were highly effective as *Fusarium* mycelial growth inhibitors. This finding was consistent with an earlier report by Mari *et al* (1993) where the isothiocyanates showed higher activity on mycelial growth inhibition of *Botrytis cinerea, Monilinia laxa, Penicillium expansum, Mucor piriformis,* and *Rhizopus stolonifer* than it did on conidial germination.

The results shown in Table 4 indicates that the suppression of *Fusarium* colony growth *in vitro* by *Brassica* isothiocyanates depended on *Brassica* species, application rates, and tissue types used. The glucosinolate hydrolysis products (isothiocyanates) evolved from *Brassica juncea* vars. $T_{1003\text{-}208}$ and $T_{1003\text{-}195}$ seed meals were more inhibitive to the *Fusarium* mycelial growth as compared to those of *Brassica napus* var. Wester. The isothiocyanates from Mustard meals were infact fungicidal at higher rates of application. The variation in mycelial suppression can be explained by previous reports on glucosinolate profiles in these *Brassicas* (Sang *et al.*, 1984; Kirkegaard *et al.*, 1996). These workers reported that Mustard (*B. juncea*) contain large quantities of 2-propenyl (allyl) glucosinolates in their meals (about 10 times higher than shoot), while the predominant glucosinolates in Rapeseed (*B. napus*) meals are phenylethyl (in moderate levels). It is therefore likely that the 2-propenyl hydrolysis products are more toxic to *Fusarium* species than the hydrolysis products of phenylethyl, or the observed differences in toxicity could be due to disparities in concentration levels between the two *Brassica* species. The effectiveness of 2-propenyl as a fungicide has been

demonstrated (Drobnica *et al.*, 1967), though the phenylethyl hydrolysis products was found to be more toxic than those of 2-propenyl to some fungi.

The introduction of seed meal hydrolysis products (isothiocyanates) after the *Fusarium* mycelial germination (48 hr. after inoculation) resulted in immediate ceasation of growth. These isothiocyanates were fungitoxic and may have caused the mycelial lysis.

The shoot tissues of Rapeseed canola (*B. napus*) evolved more suppressive isothiocyanates to the *Fusarium* mycelial growth as compared to those of Mustards (*B. juncea*) which were less suppressive. However, according to Kirkegaard *et al.* (1996), the Rapeseed canola shoot tissues contained no detectable levels of 2-propenyl and phenylethyl hydrolysis products. It is therefore probable that the Butenyl and pentenyl glucosinolates reported in *Brassica napus* shoot tissues (Fenwick *et al.*, 1983) release isothiocyanates or other related volatile products which could have played a part in the *Fusarium* mycelial suppression and the symptomatic colour change observed in this study. Moreover, the Rapeseed canola has undergone some genetic manipulations through which seed glucosinolate levels are compromised (bred out) and those of the vegetative parts improved (Adolphe, 1980). The low rates of inhibition observed in Mustard (*B. juncea* vars $T_{1003\text{-}208}$ and $T_{1003\text{-}195}$) shoot tissues as compared to their meal tissues is supported by a report of Sarwar and Kirkegaard (1997) where they stated that the glucosinolate concentrations of above ground plant parts declines with plant development and is very low in mature tissues.

The root tissues of *Brassica* evolved the least suppressive hydrolysis products (isothiocyanates). The Mustard root tissues were however, fairly suppressive as compared to those of Rapeseed canola. This finding is consistent with that of Angus *et al* (1994). These workers found out that the *Gaeumannomyces graminis* var. *tritici* was generally more inhibited by isothiocyanates from Mustard root tissues than Rapeseed canola root tissues.

Moreover, it has been postulated that there is some maintenance of root glucosinolate levels in Mustards as the plants approach maturity (Kirkegaard *et al.*, 1996).

5.5 *The effect of volatile compounds from autoclaved seed meals on Fusarium conidia and mycelial growth.*

The volatile compounds (isothiocyanates and nitriles) from autoclaved Mustard (*B. juncea*) seed meals have very little influence on *Fusarium* conidial germination. They only delayed the germination rates of these conidia. Autoclaving the meals denatured the glucosinolate hydrolysis enzyme-myrosinase, thereby inhibiting the enzymatic catalysis (Smolinska *et al.*, 1997), hence curtailing the production of isothiocyanates expected to play a role in these investigations. Smolinska *et al* (1997) reported a reduction in the concentration of volatile compounds (isothiocyanates) released from autoclaved meals, presumably due to myrosinase denaturation and the subsequent reduction in glucosinolate hydrolysis rates. Thermal degradation of glucosinolates during autoclaving enhances nitrile formation (Macleod and Rossiter, 1986). Thus the low suppression observed with autoclaved meal treatments could be due to low toxicity of the nitriles towards the *Fusarium* conidia *in vitro*, or their occurrence at low concentrations.

However, the *Fusarium* mycelial growth was still sensitive to autoclaved Mustard seed meals. This observation confirms the high rates of sensitivity of the mycelial growth to isothiocyanates from *Brassicas* already cited elsewhere in this report. It also supports an assertion that even though isothiocyanates are taken as the most toxic hydrolysis products of *Brassica* tissues, the nitriles and other volatile products of indolyl glucosinolates (Table 1) are also biocidal (Smolinska *et al.*, 1997; Kirkegaard *et al.*, 1998).

5.6 *The biofumigation effects of* Brassicas

The *Brassica* roots released biotoxic isothiocyanates. The Mustard roots were more effective than Rapeseed canola roots in inhibiting the *Fusarium* pathogen. This finding is in line with the results of Angus *et al.* (1994) who reported the suppression of the take-all fungus (*Gaeumannomyces graminis* var. *tritici*) by *Brassica* root pieces *in vitro*. The growth of this fungus was suppressed by the presence of Mustard and Rapeseed canola roots with suppression generally greater with Mustards.

The Mustard (*B. juncea*) has large quantitites of 2-propenyl glucosinolates in the roots while Rapeseed canola (*B. napus*) contain predominantly phenylethyl glucosinolates in the roots (Sang *et al.*, 1984; Kirkegaard *et al.*, 1996). The observed higher rates of biofumigation effects for Mustards could be due to the 2-propenyl hydrolysis products exuded into the soil from their roots. The results obtained here also indicate that the younger *Brassica* roots release more toxic isothiocyanates than the mature ones. However, it is noteworthy that several factors might have influenced the biofumigation effects of the *Brassica*s tested. The sorption of the exuded isothiocyanates onto clay particles and the organic matter in the soil may have reduced the soil atmosphere concentrations. Also, the availability or unavailability of microbial myrosinase in the soil was crucial

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

The hydrated *Brassica juncea* tissues release biocidal compounds with potential as soil biofumigants against *Fusarium graminearum* Schw. and *Fusarium culmorum* Smith. and other related soil-borne pathogens. The Mustard (*Brassica juncea*) tissues appeared to be more effective than the Rapeseed canola (*Brassica napus*) for inhibiting this fungus. These inhibitory compounds were volatiles, based on the observation that the growth of the *Fusarium* cultures on Potato dextrose Agar (PDA) was inhibited symmetrically irrespective of the positioning of the hydrated tissues inside the lid of the overturned plates.

The demonstrated differential toxicity toward the *Fusarium* pathogen by isothiocyanates from *Brassicas* confirm an earlier finding that the glucosinolate levels and hence their hydrolysis products vary with the *Brassica* species, and the plant parts. The Mustard (*B. juncea*) seed meals proved to be fungitoxic to *Fusarium* mycelial growth irrespective of whether added at the time of, or soon after inoculation. But it is probable that other *Brassica* tissues if applied at reasonable rates could have similar effects. This operation could require proper timing so that the application time coincides with a period of maximum glucosinolate levels in the plant tissues.

The Mustard (*Brassica juncea*) seed meals may have a potential as a seed dressing applied with the cereals at the time of sowing to provide some protection to the emerging seedlings from soil-borne pathogens during establishment. However, this method of application may require an appropriate placement method to alleviate any phytotoxic effects of *Brassica* on emerging seedlings. The phytotoxic effects of *Brassicas* has been reported in

wheat, Lettuce, Barnyard grass and Snap beans and hence should be taken into consideration before this technique is taken to practical farming.

Autoclaving the seed meals reduces the biocidal effects of these tissues. This is because autoclaving causes thermal degradation of the glucosinolate hydrolysis enzyme-myrosinase. The nitriles which dominate the volatile profiles in autoclaved meals are less toxic to the *Fusarium* conidia, but fairly inhibitive to the mycelial growth. It can generally be said that the mycelium is the most vulnerable stage of the *Fusarium* pathogen to the glucosinolate hydrolysis products. Thus the efficiency of these volatile compounds may be enhanced by amending the *Fusarium* infested soils with organic sources of carbon to prevent it from forming the resistant structures and therefore "escape".

In view of the results presented here and the reports reviewed in the introduction of the widespread antimicrobial activity of glucosinolate hydrolysis products, it is tempting to speculate that the isothiocyanates have a general biocidal effect. However, the action is clearly not universal in view of the fact that *Brassicas* themselves are attacked by numerous fungal pathogens and crucivorus pests.

The control of soil-borne pathogens presents one of the greatest challenges to sustainable agricultural production. No currently available fungicide, resistant cultivar, bioprotectant or cultural practice provides consistent, economical control to these pathogens. The use of fumigants such as Methyl bromide, though effective, is drawing condemnation from global environmental lobby groups and is soon likely to be banned. Therefore the use of environmental friendly botanical products such as isothiocyanates merits special attention as naturally occurring substances that can limit the effects of these pathogens. The *Brassicas* contain glucosinolates which are a raw material that can be made available at low costs and in large quantities as defatted meals or from other cruciferous materials.

However, Some important factors remain to be investigated before recommending the use of *Brassicas* as biofumigants in any agricultural system. The biological activity of the individual glucosinolate hydrolysis products on target organisms and their influence on disease processes should be investigated. Screening the already known *Brassicas* against target organisms should be undertaken as a matter of priority before embarking on *Brassica* breeding programmes to enhance their biofumigation effects. Under such studies, the matter of safety and phytotoxicity should also be carefully investigated.

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