Preface

This study was conducted at the Department of Chemistry, Biotechnology and Food Science (IKBM), Norwegian University of Life Sciences (UMB) during November 2009 to November 2010. My supervisors were Professor Dr Arne Tronsmo and PhD student Md. Hafizur Rahman, Department of IKBM, UMB.

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Abstract

This thesis has been focused on methods to control diseases caused by *Botrytis cinerea*. *B. cinerea* causes grey mould disease of strawberry and chickpea, as well as many other plants. The fungal isolates used were isolated from chickpea leaf (Gazipur, Bangladesh) or obtained from the Norwegian culture collections of Bioforsk (Ås) and IKBM (UMB). Both morphological and molecular characterization helped to identify the fungal isolates as *Botrytis cinerea* (*B. cinerea* 101 and *B. cinerea*-BD), *Trichoderma atroviride*, *T. asperellum Alternaria brassicicola,* and *Mucor piriformis*. The identity of one fungal isolate, which was obtained from the culture collection of Bioforsk under the name *Microdochium majus,* could not be confirmed in this study. Growth rate and morphology of the *Trichoderma* species and *B. cinerea* strains were affected by temperature. The optimum temperature for *B. cinerea* was found to be 25^0C and 25-30^0C for *T. atroviride* and *T. asperellum*. *B. cinerea* strains could grow at 5^0C and 30^0C, but their growth was very slow at those temperatures. *T. asperellum* could grow at 37^0C. Both *B. cinerea* strains germinated well in a pH range from 3 to 8.5 and the optimum pH of *B. cinerea*101 was 5.

Assessment of antagonistic activity and sensitivity to fungicides of *T. atroviride* and *T. asperellum* were investigated using *in vitro* tests and it was found that the antagonistic properties of both *Trichoderma* isolates were more effective in reducing radial growth of *B. cinerea,* as well as other test fungi, in high nutrient media (PDA) than in low nutrient media (SNA). In most cases *T. atroviride* showed more antagonistic activity than *T. asperellum.* Water-soluble inhibitory metabolites produced by both *Trichoderma* isolates showed fungicidal and fungistatic activity. *T. atroviride* was highly sensitive to the fungicides Amistar and Signum. *T. asperellum* was not sensitive to low concentrations of Amistar and Signum. *T. atroviride* and *T. asperellum* were moderately sensitive to Switch. Teldor stimulated conidia germination of both *Trichoderma* isolates.

Four fungicides and unhydrolyzed chitosan were evaluated to investigate their antifungal activity against *B. cinerea* 101 and other test pathogens in synthetic media. Switch and Signum successfully inhibited conidia germination of *B. cinerea* 101. Though Teldor did not inhibit conidia germination, it was very effective in inhibiting germ tube elongation at low concentration. Amistar was less effective against *B. cinerea* 101. Almost all of the fungicides were effective against the other test pathogens as well. Among the tested fungicides, Signum was the most effective fungicide against all of the test pathogens. Even 1/10 of the recommended dose of Signum completely inhibited conidia germination of all test fungal

pathogens. Unhydrolyzed chitosan showed dose response effect against *B. cinerea* and 0.25% (vol/vol) unhydrolyzed chitosan completely inhibited conidia germination of *B. cinerea.* It was also effective against *A. brassicicola.* Hydrolysis did not improve the antifungal activity of chitosan against *B. cinerea* 101 in this study.

Synergistic interactions of fungicides and chitosan on inhibition of conidia germination of the test fungi were studied in synthetic media and on detached strawberry flowers, with the aim to investigate if the effective concentrations of fungicides could be reduced when combined with chitosan. Combinations of chitosan and fungicides showed synergistic effect in reducing the germination and growth of *B. cinerea* 101, *B. cinerea*-BD and *M. majus* in synthetic media, and also on the disease infection rate, disease severity and sporulation of *B. cinerea* 101 in strawberry flowers. Combination of chitosan and Teldor showed better synergism than the other treatment combinations of chitosan and other fungicides on inhibition of conidia germination of *B. cinerea* 101 and *B. cinerea*-BD in synthetic media. Combination of chitosan with Teldor also showed better inhibition of *B. cinerea* 101 on detached strawberry flowers than combination of chitosan with other fungicides.

Contents

1 Introduction

1.1 Background

Many fungal species are the most important causes of crop diseases and responsible for billions of dollars worth of damage each year. Among them *Botrytis* species are the most ubiquitous plant pathogens and saprophytes (Jarvis, 1977). *Botrytis* species are found in all countries of the world, including subtropical countries like Bangladesh and temperate countries such as Norway. Strawberry (*Fragaria x ananassa Duch*) is an important fruit crop in the Nordic countries. It is also famous in Norway due to its great flavor and taste. In spite of extreme cold weather, strawberries are grown in all parts of Norway. Unfortunately strawberry is very susceptible to *Botrytis cinerea*. Chickpea (*Cicer arietinum*) is the most important pulse crop in Bangladesh and a valuable source of protein. Like strawberry, the main constraint of chickpea production is *B. cinerea*. Grey mould which is caused by *B. cinerea* is economically important disease of strawberry (Maas, 1987) and chickpea (Haware, 1998a). Synthetic fungicides are successfully used to control grey mould disease throughout the world. But continuous use of synthetic fungicide creates the problem of developing fungicide resistant strains of *B. cinerea.* Moreover increasing public concern towards fungicide residues in the environment and foodstuffs insists on reducing the use of fungicides in agriculture and emphasizes finding alternative methods for controlling grey mould disease of strawberry and chickpea. In this chapter biology, epidemiology and control of *B. cinerea* as well as few other plant pathogenic fungi are described briefly.

1.2 *Botrytis cinerea*

1.2.1 Taxonomy

Kingdom: Fungi, phylum: Ascomycotina, class: Ascomycetes, order: Helotiales, family: Sclerotiniaceae, genus: *Botryotinia*. There are many botrytis species which have asexual state and some other have sexual stage, so it poses confusion about the taxonomy of this genus (Ormrod and Jarvis, 1994). Mutation, aneuploidy and heterokariosis can play a vital role for genetic variation within the species. Recently it is believed, there are genetic variation for the sexual reproduction within the species which was ignored in the early 1930s. Many scientists used the term ‘Botrytis of the cinerea type’ (Bessey, 1950, Scheffer, 1997)

1.2.2 Morphology

Botrytis cinerea is morphologically characterized by the light brown, septate, and erect conidiophores with slightly enlarged tips bearing small pointed sterigmata bearing 1-2 celled, hyaline, and oval or globose conidia forming clusters. The conidia cover the ultimate branches and are produced synchronously. Conidia look like a branch of grapes at the top of conidiophores resemble ashes. Conidia on PDA was measured to 4-16×4-10 µm, but when conidia was collected from infected chickpea, was measured 4-25×4-18 µm (Pande et al., 2001). When *B. cinerea* is cultured on potato dextrose ager (PDA), the primary culture look like white, cottony appearance, which turns light grey with age. Initially the hyphea look like thin, hyaline, and 8-16 µm wide which gradually becomes brown and septate with age. In unfavorable environment such as cold temperature *B. cinerea* can produce resting structure known as sclerotia which germinate asexually by producing conidiophores bearing conidia. Apothecia which sexually produce ascospores, may also be produced on sclerotia (Jarvis, 1977), But still now sexual stage of *B. cinerea* has not been reported in strawberry field (Strømeng et al., 2009).

1.2.3 Disease cycle of *Botrytis cinerea*

Inoculum production and dispersal

It is assumed that inoculum of *B. cinerea* is always present in the field and that production, liberation and dispersal of inoculums is a continuous process (Jarvis, 1980). It is not always the case in all crops. In strawberry field, conidiophores and conidia are produced from mycelia and sclerotia in necrotic plant tissues and debris during spring (Strømeng et al., 2009). Many factors influence the propagule number in the air: inoculum source, environmental condition for propagule production and dispersal at the source site (Holz et al., 2007). Each part of the fungus thallus can serve as a dispersal unit. Condia and other propagules can disperse by wind, rain and insect (Holz et al., 2007).

Attachment of Conidia

Two steps are needed for the attachment of conidia to the host tissue. The first step starts by the hydration of conidia, which involves weak adhesive forces resulting from hydrophobic interactions between host and conidial surfaces (Doss et al., 1993).The second step start several hours after inoculation when conidia germinate. Extracellular matrix material covers the tip of the fungal germ tube which helps the conidia to stick with the host surface (Doss et al., 1995), resulting in stronger binding than the first step.

Germination

Germination of conidia depends on many factors that can influence the germination rate. It is found that if free surface water is available and there is high relative humidity (> 93% RH), conidia can germinate and penetrate the host epidermis easily(Williamson et al., 1995). It has been found from several studies that gaseous compounds may speed up conidial germination. Exogenous ethylene can stimulate germination of conidia on a hydrophobic surface but the germ tube length remains unaffected (Kepczynski and Kepczynska, 1977). In the ripening stage or senescence stage, fruits produce a lot of ethylene which influences the elongation of germ tubes and penetrates into the host tissue as ethylene may weaken the host at the same time (van Kan, 2003).

Appressoria

Appresorria are fungal attachment organs that mediate penetration through host surface and usually are conceived as swollen terminal compartment (Tenberge, 2007). The swelling of the hyphal tips of germ tubes act as an appressorium like structure for penetration of host tissue(Cole et al., 1996).

Penetration of the host surface

Botrytis cinerea can enter into host tissue by active penetration or by passive ingress. By enzymatic (cutinolytic) activity the fungus makes the host surface weaker which facilitates the penetration through the intact host surfaces (Salinas and Verhoeff, 1995). In passive ingress, *B. cinerea* can enter into the host surface through wound sites, or at sites previously infected by other pathogens, or can inter the substomatal cavity via an open stoma (van Kan, 2003).

Killing the host

It was reported that prior to invasion by hyphae, *B. cinerea* needs to kill host cells. *B. cinerea* secretes some diffusible factors into its environments, which may be proteins or low molecular weight compounds that have a direct or indirect phytotoxic activity(Clark and Lorbeer, 1976). Phytotoxic compounds can kill the host cell and facilitates *B. cinerea* for successful infection (Govrin and Levine, 2000).

Primary lesions formation

The development of primary necrotic lesions occurs when neighboring host tissue shows defense mechanism in response to the death of an invaded cell. Whether cell death caused by a necrotroph, such as *B. cinerea*, is equivalent to cell death during a hypersensitive response

to a biotropic pathogen is still unclear (Lamb and Dixon, 1997). The primary necrotic lesion is the result of host defense response in which the fungus is effectively restricted.

Disease expansion and tissue maceration

For disease expansion *B. cinerea* macerates the plant tissue and makes it to a fungal substrate. It secretes some toxic substances to kill the neighboring cells to expanse of primary lesions. In order to expanse from the primary lesion into the neighboring tissue, *B. cinerea* must actively degrade plant cells. The pathogen comes in contact the with host cell wall for degradation that facilitates the entry of the pathogen and it provides nutrients for growth (Ten Have et al., 2002). After penetration of the cuticle the *B. cinerea* degrade the middle lamella of the epidermal cell which is made of pectin substances by producing pectin degrading enzymes which is needed for primary infection (van Kan, 2003).

1.2.4 Economical importance

As *B. cinerea* is found everywhere in the world therefore it is called a ubiquitous pathogen. It has over 100 hosts listed in New Zealand (Pennycook, 1989) and all over the world it has over 230 hosts (Jarvis, 1977). *B. cinerea* can live either pathogenically or saprophytically and for this unique characteristic it is the most interesting fungal pathogens. The virulence strains of *B. cinerea* cause serious diseases on a wide range of nursery plants, vegetables, orchard crops, ornamental flowers and fruits within the field, and especially on glasshouse grown crops. It can also be a serious problem during transit, storage and in the market place. Many herbaceous annuals and perennials plants, including houseplants, trees, shrubs, and vegetable and small fruit plants can be affected by the disease caused by *B. cinerea*. All plant parts such as flowers, leaves, buds, shoots, seedlings, stems and fruits can be damaged by grey mould.

As a herbaceous perennial species with periodical dying of the above-ground plant parts (in winter) strawberry is well adopted to outlive Norwegian winter in inland and in coastal regions (Strømeng, 2008). All strawberry cultivars are susceptible to *B. cinerea* and differences in susceptibility depend on cultivar (Legard et al., 2000, Daugaard, 2000). One of the most frequently grown cultivars in Norway, 'Korona', is indeed favoured by the consumers but also quite susceptible to *B. cinerea* (Daugaard, 2000). Grey mould caused by *B. cinerea* is a most important disease all over the world wherever strawberries are grown (Maas, 1987). All the plant parts are susceptible to the pathogen including the leaves, flowers, fruits and crown (Sutton, 1990). Senescent strawberry leaves can play an important role as an inoculum source in the pathogen's disease cycle (Braun and Sutton, 1987). It is also reported

that overwintering strawberry plant debris is the most important source of conidial inoculumn of *B. cinerea* in the spring in strawberry fields in Norway (Strømeng et al., 2009). If the dispersal conidia arrive during the blossom time of strawberry flower, a quiescent infection frequently occurs and the disease expression occurs during fruit maturation (Powelson, 1960). Depending on the cultivar, *B. cinerea* causes up to 15% pre-harvest fruit losses of susceptible strawberry cultivars (Legard and Chandler, 2000, Legard et al., 2000). The average strawberry post harvest losses were found to be 28% in Iran (Salami et al., 2010).

Chickpea (*Cicer arietinum* L) is the third most important pulse worldwide and in Bangladesh it is a traditional crop grown in cooler temperatures after the rainy season (Davidson et al., 2007). In Bangladesh Pulses such as chickpea are valuable protein sources, where protein-energy malnutrition is a great problem (Bellamy, 1996). *B. cinerea* which cause grey mould disease of chickpea is the main production constraint in several chickpea growing areas of the world, where cool and humid weather exists in the cropping season. Botrytis grey mould (BGM, *Botrytis cinerea* Pers.ex. Fr.) is considered the second most important foliar disease after ascochyta blight (*Ascochyta rabiei* (Pass) Lab) and is existing in South Asia (northern and eastern India, Pakistan, Nepal, Bangladesh, and Myanmar), Australia and the America (Haware, 1998b). Many scientists reported the first occurrence of Botrytis grey mould (BGM) from India (Shaw and Ajrekar, 1915, Bulter, 1997). In Argentina the first epidemic of BGM resulted in a crop loss of 95% (Carranza, 1965). Subsequently, it has been reported from many chickpea growing countries that several BGM epidemics caused almost complete yield loss. It is the major concern in India, Bangladesh, Nepal, Pakistan, Australia and Argentina (Haware and McDonald, 1992) where yield losses of up to 100% were reported under conductive conditions. It has been also reported about BGM from Canada, Chile, Colombia, Hungary, Mexico, Myanmar, Spain, Turkey, the USA, and Vietnam (Nene et al., 1984). In India during the 1978-79 crop seasons, this disease reached epidemic proportions, destroying about 20,000 ha of chickpeas (Grewal and Laha, 1983). In Nepal BGM attacks the chickpea every year, that reduce average yield losses of 15% (Joshi, 1992). In 1981 BGM was first found in Bangladesh and in 1988 the disease outbreaks as an epidemic and it destroyed almost all the crop (Bakr et al., 1993). Recently, among other foliar diseases BGM is the most devastating one in Bangladesh (Bakr et al., 2002). When the weather is favorable, hosts are susceptive and inoculums levels are high, this disease develops as an epidemic causing maximum yield loss of pods.

Figur 1. *Botrytis* grey mould (*Botrytis ciinera*) of chickpea: (A) infected plant without pods; (B) grayish colony on twig and (C) lesions on leaf and flower and strawberry: (D) infected fruit. Source: chickpea (Photos: ICRISAT: http://cropgenebank.sgrp.cgiar.org/index.php?option=com_content&view=article&id=457&Itemid=639)

Strawberry (Linda G. Hjeljord)

1.3 *Microdochium majus*

1.3.1 Taxonomy

Kingdom: Fungi, Phylum: Ascomycota, Subphylum: Pezizomycotina, Class:Sordariomycetes, Subclass: Xylariomycetidae, Order: Xylariales, Family: Incertae sedis, Genus: Microdochium

1.3.2 Economic important

Microdochium nivale is an important fungal pathogen which causes seedling blight of many cereal crops (Humphreys et al., 1995) and causes head blight when infecting the ears of wheat (scab). It has been reported that *M. nivale* can reduce up to 74% of seedling emergence of untreated wheat seed as a seed-borne infection of the stem base and airs (Pettitt et al., 1993). Foot rot and ear infection of mature wheat plants can also be caused by this pathogen; recently foliar lesions have been identified as possible sources of conidial inoculum for ear infection (Jenkinson and Parry, 1994). When *M. nivale* attacks in winter wheat, it can reduce the yield 15 to 28% without affecting the grain quality (Humphreys et al., 1995). *Microdochium nivale* can be classified into two sub-groups on the basis of PCR-based molecular methodology (Lees et al., 1995) such as *M. nivale* var *nivale* and *M. nivale* var. *majus*. The two varieties may have host preference. It was found from the National disease survey that *M. nivale* var. *majus* are more prevalent on the stem base and ear of wheat (Parry et al., 1995) whereas var. nivale isolates are more frequently found on rye (Miedaner et al., 1993) and turf grasses (Mahuku et al., 1998).

1.4 *Mucor piriformis*

1.4.1 Taxonomy

Kingdom: Fungi, Phylum: Zygomycota, Class: Zygomycetes, Order: Mucorale, Family: Mucoraceae, Genus: *Mucor*

1.4.2 Morphology

Zygospore: - colonies on MEA at 20^0C 45 - 70 mm high, whitish to olivaceous-buff, odour aromatic, sporangiophore can be short with sympodially branched or tall with lateral branches. Sporangia blackish depend on darkness, 300 - 350 μm diameter; columellae either large and obovoid, or small and cylindrical-ellipsoidal, pyriform or subglubose, 190 x 150 μm, sometimes with brownish content; sporangiospores grayish, ellipsoidal, 7-9.5 x 4-7 μm, intermixed with some subglobose to globose, more strongly pigmented ones, originating from short sporangiophores. Chlamydospore absent. Zygospores formed on cherry decoction agar at 15^0C near the agar surface, black colour, 210 - 240 μm diameters, covered with irregular warts. Growth and sporulation occur in the range 5-20^0C; the optimum is in the range 10-15^0C (Domsch et al., 1980).

1.4.3 Economic importance

There are more than 360 species of genus *Mucor.* Mainly *Mucor* spp causes post harvest disease. Among all species *M. piriformis* Fischer causes fruit rot of strawberry, gooseberry, pears, apple, peaches and nectarian while *M. heimalis* Whelmer causes rot in raspberry and guava; *M. strictus* Hagem causes disease in pear; *M. racemosus* Fres causes rot of various fruits and vegetables; *M. circinelloides* Tieghem causes rot of tamato (Smith et al., 1979). Ripe soft fruit can be infected by *M. piriformis* before harvest and remain dormant in certain period, but after harvest this fungus can cause serious damage in storage condition. Severe infection can be caused if the fruits are damaged mechanically (Smith et al., 1979). Mucor rot can occur every year, but serious disease outbreak may occur when environment factors are favorable and improper handling of fruits during harvesting and packaging (Michailides and Spotts, 1986).

1.5 *Alternaria brassicicola*

1.5.1 Taxonomy

Kingdom: Fungi, Phylum: Ascomycota, Class: Dothideomycetes, Order: Pleosporales, Family: pleosporaceae, Genus: *Alternaria*.

1.5.2 Morphology

On PDA, spores were produced in rarely branched chains of up to 15 and had no beak. They were dark olivaceous brown, nearly cylindrical (24·88 ± 7·38 X 10·93 ± 1·71μm; range 13–44 X 8–21) with 1–6 transverse septa and up to 2 longitudinal septa. Colonies on PDA were velvety and dark olivaceous brown. They grew at an average rate of 7·8–8·8 mm per day and sporulated copiously (Pattanamahakul and Strange, 1999b).

1.5.3 Economic importance

The genus *Alternaria* is pathogenic and saprophytic and able to damage fruits in the field and causes spoilage during storage and transportation. *Alternaria* species can grow at low temperature; even they can damage fruits during storage in the refrigerator (Tournas and Stack, 2001). *Alternaria brassicicola* is the most serious fungal pathogen of important cruciferous vegetable crops which causes severe damage in many countries in the world (Pattanamahakul and Strange, 1999a). Principally *A. brassicicola* are thought to be causal agent, but *A. brassicae and A. raphani* may also cause disease. *A. brassicicola* is important pathogens in crops such as broccoli, brussels sprouts, cabbage and cauliflower in Australia (Sivapalan and Browning, 1992). This fungus reduces the marketable value of cauliflower by causing black sport in the curd. Though *A. brassicicola* is a seed-born pathogen, the disease can transmit through infected crops residue, wind-blow or rain splash (Humphreys et al., 1995). It was reported 26 out of 44 seed sample of *Brassica oleracea* were infected with the fungus *A. brassicicola* (Sivapalan and Browning, 1992*).*

1.6 Disease management

1.6.1 Cultural control

There are many cultural steps that can help to reduce the inoculums level in the field and produce less suitable conditions for fungal infections. It is recommended to use pathogen free seeds and propagation material and before sowing seed should be sterilized (Jones, 1987). All

disease plants and weeds and plant debris should be carefully removed from the crop and destroyed. High relative humidity (RH), free moisture on plant surface and cool weather are the most important environment factors which promote infection by the pathogen (Elad et al., 1996). It is very important to provide adequate plant spacing in order to avoid excessive foliage density that can lead to increase levels of relative humidity (Palti, 1981). There are a number of other cultural techniques for suppressing the pathogen which are specific to certain crops such as, pruning, training, crop rotation and removal of alternative host plant etc. However, in years when the weather is conductive for disease development, good cultural practices are not sufficient for disease control.

1.6.2 Chemical control

Chemical control is the main way of controlling grey mould as well as other fungal diseases throughout the world. The synthetic botryticides can be classified according to their biochemical modes of action. Five categories are recognized, namely those affecting fungal respiration, microtubule assembly, osmoregulation, fungicides whose toxicity is reversed by amino acids, and sterol biosynthesis inhibitors (Leroux, 2007). In fungi, the final step of the catabolic process takes place in mitochondria which provide energy for the survival of the fungal species. Several groups of fungicides disturb the energy supply in *B. cinerea* and inhibit conidia germination. In 1996 fungicides were introduced in the market that can inhibit mitochondrial respiration by binding to cytochrome b, a part of the cytochrome bc 1 comples (Leroux, 2007). Most of them are synthetic analogues of natural strobilurins produce by Bacidiomycete wood-rotting fungi. Strobilurin fungicides are members of QoIs because of their binding at the Qo site of cytochrome b (Bartlett et al., 2002). Some of them such as azoxystrobin control *B. cinerea* on various crops, including vegetables, ornamentals and strawberries. The systemic fungicide boscalid is a novel broad spectrum carboximide which can reduce grey mould disease of grapevine very effectively by inhibiting the respiration of *B. cinerea*. it was reported that cyprodinil whose activity is reversed by methionine belongs to novel class of chemicals, as its mode of action is lack of cross-resistance potential with current market products and it is highly effective against *Botrytis* and *Alternaria* by inhibiting both their penetration and their mycelia growth on the surface of and inside leaves (Heye et al., 1994). Another active ingredient of Switch is fludioxynil which induces morphological alteration of germ tubes: i.e. swelling, branching and cell bursting (Leroux, 1996). Fenhexamid is sterol biosynthesis inhibitors which do not prevent conidial germination of *B. cinerea* but at low concentrations they inhibit the elongation of germ tubes and mycelial

growth (Leroux, 2007). Additionally, the germ tubes produced on fungicide-supplimented media are distorted, bulge and their cytosol has a granular appearance (Leroux et al., 1999).

During the 1980s reports of *B. cinerea* strains resistance against dicarboximides started to appear (Leroux, 2007, O'Brien and Glass, 1986). Later, resistance towards other groups of fungicides has also been reported (Leroux, 2007). Fungicide resistance, fungicides residue problems and environmental concerns, have encouraged the attempt to reduce the use of fungicide application by use of different approaches. It was reported in Denmark and Norway that application of half rate of fungicide of the recommended dose written on the labels can do sufficient control of grey mould disease (Nestby, 1992). It is necessary to repeat fungicide applications throughout the growing season for effective disease control and consequently strawberries are among the crops most likely to be contaminated with pesticide residues (Sutton, 1994).

1.6.3 Biological control

In broad sense biological control means, controlling the disease or reducing the effect of pathogens by relying on biological mechanisms or organisms other than man (Campbell, 1989). In plant pathology the expression `biological control` usually refers to the control of one organism by another. The concept of biological control differs between entomologist and plant pathologists. The entomologists in the beginning introduced a specific parasitic insect from another country to control an insect pest, whereas the plant pathologist put emphasis on management of naturally antagonistic soil microorganisms through such practices as crop rotation or organic amendments. Biological control of plant pathogen can be achieved in different ways: 1) through cultural practices that create an environment favorable to antagonists, host plant resistance or both; 2) through plant breeding to improve resistance to the pathogen or suitability of the host plant to activities of antagonists: or 3) through the mass introduction of antagonists, nonpathogenic strains, or other beneficial organisms or agents (Cook and Baker, 1996).

1.6.3.1 The need for biological control agents

In modern agriculture, the farmer could successfully use chemical fungicide in the crop field to control disease epidemic. But the continuous and indiscriminate use of chemical fungicide to manage the crop disease results in accumulation of harmful chemical residues in the soil, water and grains. Moreover, the farmers faced new problems with pathogens resistant to

available chemical fungicides. Furthermore, there is a need for efficient measures to combat soil borne diseases and inoculums buildup.

Alternative way to control plant diseases using biological control agent have gained interest in the scientific society. Recently, in many research works biological control agent has been used in broad scale trails to demonstrate control of different diseases, to attempt at understanding the mechanisms by which biological agents reduce the impact of pathogens (Tronsmo, 1995).

1.6.3.2 *Trichoderma* as a Biological control agent

Trichoderma is a ubiquitous fungus, as it can be found in soil all over the world. It has a high degree of ecological adaptability. It can survive under different environment condition. It is a fast growing saprophyte and generally grows on various substrates. It is characterized by its fast growth, ability to use different substrates and resistance to toxic chemicals (Klein and Eveleigh, 1998). *Trichoderma* spp. can reduce plant pathogenic fungi through several ways; they can induce resistance or plant defense mechanism or by antagonistic interaction which is classified as antibiosis, mycoparasitism as well as competition (Tronsmo, 1986). *Trichoderma* and *Gliocladium* are being used against diseases in many different crops, e.g., cotton, grapes, sweet corn, lettuce, onion, peas, plum, apples, and carrots, caused by pathogens such as *Pythium, Phytophthora, Rhizoctonia, Sclerotinia, Botrytis* and *Fusarium* (Nielsen et al., 2001). *Trichoderma* and *Gliocladium* have also been used successfully to competitively control colonization by *Botrytis* on strawberry (Tronsmo and Dennis, 1977) and apple (Tronsmo and Raa, 1977). Recently *Trichoderma* have been marketed in formulated biological control products for controling many plant pathogenic fungi.

1.6.3.3 Mechanism of action

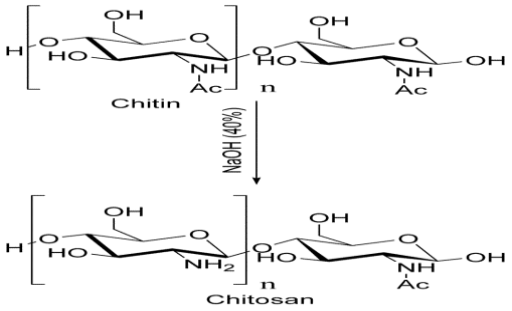
The mechanism of action of biocontrol microorganism is competition, antibiosis, mycroparasitsm and host induced resistance. The microorganism can show one or more modes of action against plant pathogenic fungi. Certain groups of microorganism such as *Trichoderma* sp, *Gliocladium* sp produce low molecular weight volatile and non-volatile compounds as secondary metabolites. These antibiotics have fungicidal or fungistatic activities towards some plant pathogenic fungi (Tronsmo and Dennis, 1978). There are four distinguished stages which are collectively called mycoparasitism by which one fungus shows parasitism to another fungus (Chet, 1990). These are : "(a)chemotropic growth, in which a chemical stimulus from the target fungus attracts the antagonist; (b) specific recognition,

probably mediated by lectins on the cell surfaces of both pathogen and antagonist; (c) attachment and coiling of the *Trichoderma* hyphae around its host; and (d) secretion of lytic enzyme that degrades the host wall" (Hjeljord and Tronsmo, 1998). Fungi can compete with one another for limited resources for the growth and survival which may lead to disease control. They can compete for oxygen (Hjeljord and Tronsmo, 2003), carbon, nitrogen, infection sites, iron, vitamins, sugar etc (Paulitz, 1990). The aggressive growth and ability in substrate utilization makes *Trichoderma* and *Gliocladium* to successful biocontrol agents (Hjeljord and Tronsmo, 1998). Recently host induced resistance is considered as an effective disease control mechanism. Here some antagonistic fungi act as elicitor of the host owns biochemical defense mechanisms and as a result the host can restrict the plant pathogenic fungi at the infection site (Harman, 2000).

Though biological control agents such as *Trichoderma* can be used to reduce the use of synthetic pesticide, it has been found in several studies that biological control agent cannot replace the use of chemical treatments (Elad, 2000, Elad et al., 1993, Thrane et al., 1997). Plant disease control provided through the biological control agent is highly inconsistent compared to chemical control (Tronsmo, 1995), so there is a need for more effective control method, one possibility is the use of chitosan alone or together with fungicide.

1.6.4 Chitin

Chitin is a linear polysaccharide consisting of β- (1-4)-linked 2 acetamido-2-deoxy-β-D-glucopyranose which is also called N-acetyl-D-glucosamine. After cellulose chitin is the second most abound organic compound in the world which can be found in the exoskeleton of crustaceans and insects, in the eggs of nematodes and in algae, protozoa, mollusks and fungi (Tronsmo, 1995). Primarily chitin is extracted from shellfish (Khor and Lim, 2003) and the annual production of chitin is estimated to 1x 10^{11} tons. Among crustaceans, the shells of shrimp and crab contain 15-40% α chitin, 20-40% protein and 20-50% calcium carbonate (Kurita, 2006). Chitin is the main source of commercially produced chitosan.



Figur 2. Production of chitosan from chitin (Rabea et al., 2003)

1.6.4.1 Chitosan

Chitosan (2-amino-2-deoxy-β-D-glucopyranase or GlcN) is the deacetylated form of chitin. There is no clear definition of degree of deacetylation that distinguishes chitin from chitosan, but the difference is that chitosan is soluble in acetic acid (Shahidi et al., 1999) or in water (Qin et al., 2006), whereas chitin is not. For production of 1 kg of 70% deacetylated chitosan from shrimp shells, 6.3 kg of HCl and 1.8 kg of NaOH are required in addition to nitrogen, process water and cooling water (Kumar, 2000). In 2000 worldwide production of chitosan from chitin was 2000 tones (Kurita, 2006). Production of chitosan is abundant because of its unique biological properties such as biocompatibility, biodegradability, haemostatic activity and it has also a wide variety of industrial applications like food preservation/nutrition, chelating agent in water purification, medicinal use and cosmetics (Kumar, 2000).

1.6.4.2 Chitosan as antimicrobial agents

Continuous use of synthetic chemicals to reduce the disease severity has taken its toll environmentally and on human health. There is growing international awareness about the adverse effect of intensive use of synthetic fungicides on crops. Scientists worldwide are trying to explore new alternatives of synthetic fungicide that control pathogenic disease and avoid negative side effects on human health. Chitosan has achieved a greatest interest as an alternative to synthetic fungicides for its antifungal activity. The important antifungal properties of chitosan have been found to be concentration, degree of deacetylation, molecular weight and pH of the growth media (Liu et al., 2007, Stössel and Leuba, 1984, Wang, 1992). The antifungal activity of chitosan is influenced by the particle size of chitosan. The antifungal activity of chitosan decreased with increasing particle diameter because smaller particles of chitosan can penetrate in to the cell wall of pathogens more easily compare to large particle (Stössel and Leuba, 1984, Cooke, 1980). Chitosan may protect plant by its fungicidal effect or by elicitation of defence mechanisms in plant tissues (Terry and Joyce, 2004). Fungal spores are more sensitive to chitosan than hyplae (Palma Guerrero et al., 2008). An *in vitro* study was conducted on controlling grey mould disease caused by *B. cinerea* and blue mould caused by *Penicillium expansum* and it was found that chitosan strongly inhibited spore germination, germ tube elongation and mycelia growth of *B. cinerea* and *P. expansum* (Liu et al., 2007). Chitosan can also significantly reduce the mycelia growth and spore viability of *Alternaria* (Reddy et al., 1998).

1.6.4.3 Mechanism of action

The precise methanisms by which chitosan exhibits its antimicrobial acitivity has not yet been established, but several hypotheses have been proposed over the years by different scientists. Initially two hypotheses were assumed for the antifungal activity of chitosan; one suggests that the positively charged amino groups interact with the negatively charged cell wall in the target organism (Sudarshan et al., 1992), while the other suggests that chitosan oligomers penetrate into the microbial cell and prevent DNA transcription by degrading negatively charged phosphate groups of nucleic acids (Hadwiger and Loschke, 1981). Later it is demonstrated by some other scientists that the mode of action of chitosan is probably more complex than initially assumed, involving a number of events that may ultimately lead to a killing process. After initial contact between polycationic chitosan macromolecule and the negatively charged cell wall polymer, the teichoic acids play a major role for disruption of the cell wall (Raafat et al., 2008). It was found through electron microscopy photographs that 50 KDa chitosan able to destroy the cell membrane and penetrate the inner cell of *A. niger* (Li et al., 2008). In the same study it was found that chitosan inhibit DNA to RNA transcription.

2 Aims of the study

The main aim was to investigate alternative control measures to reduce the application of fungicide against *Botrytis cinerea* which causes grey mould of strawberry and chickpea, as well as many other plants. The alternative control measures tested were biological control and use of chitosan. The specific objectives were:

1. Morphological and molecular identification of fungal isolates.
2. To determine the optimal growth of fungal isolates in different temperatures and pH.
3. Assessment of antagonistic activity *in vitro* of two *Trichoderma* isolates against *B. cinerea*101 and *B. cinerea*-BD as well as two other plant pathogenic fungi on a nutrient rich and a nutrient poor media.
4. To determine the sensitivity of *Trichoderma* isolates to different fungicides in synthetic media
5. To determine the antifungal activity of fungicides and unhydrolyzed chitosan against the test fungal pathogens in synthetic media
6. To examine the possible synergistic effects of unhydrolyzed chitosan and fungicides against the test fungi in synthetic media and on detached strawberry flowers.
7. To compare antifungal activity of unhydrolyzed and hydrolyzed chitosan against *B. cinerea* 101.

3 Materials and Methods

The experiments were conducted at department of chemistry, biotechnology and food science (IKBM), Norwegian University of Life Sciences (UMB) from November 2009 to November 2010.

3.1 Morphological and molecular characterization of the fungal isolates

3.1.1 Collection of fungal isolates

All together 7 fungi isolates were used in the experiments. I had isolated *Botrytis cinerea*-BD from chickpea leaf at Gazipur, Bangladesh and *Trichoderma*-BD was found as microparasite on *Botrytis cinerea*-BD. *Botrytis cinerea*101 which I obtained from the culture collection at IKBM (UMB) was isolated from strawberry leaf at Grimstad, Norway; *Mucor* 199J which was also obtained from the culture collection at IKBM (UMB), was isolated from strawberry fruits at Hobøl, Norway; *Alternaria* 328 and *Trichoderma*-P1 were obtained from the culture collection of IKBM (UMB). *Microdochium majus* was obtained from the culture collection of Bioforsk, Ås. All fungi were cultured on Potato Dextrose Agar (PDA) (Difco Laboratories, Detroit, MI).

3.1.2 Morphological Identification

Morphological identification of *Trichoderma* spp was done with the help of an interactive key for strain identification (Samuels, G.J., Chaverri, P., Farr, D.F., & McCray, E.B. http://nt.ars-grin.gov/taxadescriptions/keys/TrichodermaIndex.cfm) based on morphological characteristics, differences in growth rates on potato dextrose agar (PDA) and microscopic measurements of mycelia parts. Size and shape of conidia, absence or presence of sterile hairs, all of these morphological characters were measured from slide mounts prepared by the tape touch method (Harris, 2000) in a drop of lactofuchsin. Newly inoculated PDA plates with *Trichoderma* spp were incubated at 25, 30 and 35^0C in dark and radial growth was measured at 24, 48 and 72 h.

3.1.3 Molecular Identification

3.1.3.1 Extraction of DNA from Mycelia

Fungal mycelium was taken from 4 - 10 days old cultures on PDA except *Microdochium majus* which was collected from 6 days old culture grown on cellophane on PDA. A small scalpel was used to collect mycelium from the PDA plates. The mycelium was transferred to a 2 ml Fast Prep (MP-Bio) tube containing a small amount of glass beads. 500 µl cetrimonium bromide (CTAB) extraction buffer (50 ml 10% (wt/wt) CTAB in 0.7 M NaCl, 50 ml 240 mM KH_2PO_4/ K_2HPO_4, pH 8.0 with 1 M NaOH) was added to the sample tube. After shaking, 500 µl mixture of phenol: chloroform: isoamylalcohol 25: 24: 1 was added to the sample tube that was placed on ice. The sample was macerated using "fast Prep ®$^{24"}$ machine for 30 seconds at a speed of 5.5 m/s. The sample was centrifuged in a centrifuge (Model-3500, Kubota corporation, Japan) for 5 minutes at 14,000 rpm at 4^0C. The supernatant was transferred to a new 1.5 ml eppendorf tube and an equal volume of chloroform: isoamylalcohol 24: 1 was added to remove the phenol. The suspension was mixed by inverting the tube. The sample was centrifuged for 5 minutes at 14,000 rpm at 4^0C. The aqueous phase from top was transferred to a new 1.5 ml eppendorf tube and DNA was precipitated with 1 volume of isopropanol. The sample was left two hours at room temperature and then stored at -20^0C over night. The sample was centrifuged for 5 minutes at 14,000 rpm at 4^0C and the supernatant was removed. The pellet was washed carefully with 0.5 ml ice-cold 70% ethanol (EtOH). The sample was again centrifuged for 5 minutes at 14,000 rpm at 4^0C and the ethanol was removed. In order to remove all ethanol the sample was dried in vacuum centrifuge for 20 minutes. The pellet was dissolved in sterile MilliQ water and stored at -20^0C.

3.1.3.2 PCR (Polymerase Chain Reaction)

The DNA extracted from mycelium was amplified with PCR by using ITS1F (forward) and ITS4 (reverse) primer.

Ingredients in Mastermix X 10 reaction:

10X reaction buffer	50 µl
dNTP mixture (2.5 mM of each dNTP)	40 µl
Primer 1 – forward primer (25 µM)	20 µl
Primer 2 – reverse primer (25 µM)	20 µl
1% bovine serum albumin	5 µl
milliQ water	310 µl
Total	445 µl

The PCR reaction contained 44.5 µl Mastermix, 5 µl DNA sample and 0.5 µl HotMaster *Tag* DNA polymerase (5, Prime). One positive and one negative control samples were processed together with the samples containing DNA template. DNA from bacteria (*Rhodococcus* sp) was used for positive control. For negative control, 5 µl MilliQ-water was added instead of the DNA template.

The following PCR program was used for amplification of DNA isolated from different fungal mycelia:

94 ^{0}C	5 minutes	
94 ^{0}C	1 minute	
50 ^{0}C	1 minute	repeated 35 times
72 ^{0}C	1 minute	
72 ^{0}C	10 minutes	
4 ^{0}C	∞	

3.1.3.3 Agarose gel electrophoresis

Gel electrophoresis was conducted to determine whether the PCR was successful on a 1% agarose minigel containing ethidium bromide (EtBr). 0.5 g agarose was mixed with 50 ml Tris-acetate-EDTA (TAE) buffer in an Erlenmeyer flask. The mixture was melted in microwave oven and it was checked frequently to avoid over boiling. The mixture was cooled around 60^0 under running tap water. 1 drop (3 µl) EtBr was added to the mixture. The mixture was then poured into a gel tray with an inserted "fixed-height" comb with 8 or 15 teeth and was left to solidify for half an hour. When the gel was solidified the comb was removed carefully and the gel tray was placed in an electrophoresis tank with wells closest to black (negative) electrode. 1X TAE –buffer was poured into the tank until it covered the gel completely.

A strip of parafilm was put on the bench. 3 µl loading buffer was placed on strip. 4 µl of each sample was mixed properly with loading buffer and it was applied to wells in gel. In the same way 4 µl 1 kb DNA ladder (New England BioLabs) with 3 µl loading buffer was also applied in one well. The electrophoresis tank was then closed with a lid and it was turned on with 80 volt for about 1 hour. After that the gel was photographed under UV light in a Gel Doc (Bio-Rad)

3.1.3.4 Cleaning up PCR products

The PCR products were cleaned up with an 'E.Z.N.A Cycle-Pure Kit' from Omega Bio-Tek (Doraville, Georgia, USA). 200 µl (about 4 times sample volume) of Buffer CP (Carrier protein reaction buffer) was added to the PCR product and vortexed thoroughly. The sample was put in a labeled DNA column that was placed in a collection tube and centrifuged for 1 minute at 14000 rpm at room temperature. After discarding the liquid the column was placed again in the same collection tube. 700 µl Wash Buffer was added and centrifuged for 1 minute at 14000 rpm and the liquid was discarded. This step was repeated with 500 µl Wash Buffer. Then the column was centrifuged for 1 minute at 14000 rpm to dry it out. The column was placed in a new labeled eppendorf tube with the lid cut off. 40 µl Elution Buffer was added directly onto column matrix and centrifuged for 2 minutes at 14000 rpm to elute DNA. The eluate was transferred into a new labeled eppendorf tube and stored at 4^0C for sequencing PCR.

3.1.3.5 Sequencing PCR

'BigDye®Terminator v3.1 Cycle Sequencing kits' from AB applied Biosystems was used for sequencing PCR. Two separate reaction mixtures, one with the forward primer and one with the reverse primer, were placed in separate PCR tubes for each sample.

The reaction mixture consists of the following reagents:

DNA template (cleaned PCR product)	5 µl
Forward or reverse primer (3.2 µM)	1 µl
BigDye® ready reaction mixture	2 µl
BigDye® Terminator Sequencing bufferX5	3 µl
MilliQ	9 µl
Total	20 µl

The sequencing PCR program was:

96^0C	30 sec	
50^0C	15 sec	repeated 25 times
60^0C	4 min	
4^0C	∞	

For sequencing PCR, the same primers, ITS1F and ITS4 were used. After sequencing PCR, the amplified DNA was kept in freezer for cleaning up

3.1.3.6 Cleaning up and precipitating sequencing PCR samples

The amplified DNA samples were transferred from PCR tubes to 1.5 ml eppendorf tubes. 2 µl EDTA 125 mM (pH 8.0), 2 µl sodium acetate (NaAc) 3M (pH 5.2) and 52 µl 96% EtOH was added to the PCR samples and incubated at room temperature for 15 minutes. The samples were centrifuged at 14000 rpm for 30 minutes putting the eppendorf tubes lid hinge

outside to make sure that the pellet will be on the same side as the hinge. The supernatant was discarded and the pellet was washed very carefully with 70 µl 70% EtOH and centrifuged at 14000 rpm for 15 minutes. The EtOH was removed carefully and the eppendorf tubes were put in a laminar flow cabinet at room temperature for drying out. After 1 hour when the tubes were completely dry, they were put at -20^{0}C until they were sent to the sequencing lab (IKBM, UMB).

After the sequencing process, the sequences were manually edited using the software BioEdit (Tom Hall). Sequences were edited by aligning the two sequences from a sample (one created by the forward primer and one by the reverse). BLAST (Basic Local Alignment Search Tool) analyses were performed to detect sequence similarity in the ITS region for species identification from the National Center for Biotechnology Information available online (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.1.4 Optimal growth conditions of the fungi

Two isolates of *Trichoderma* spp and two isolates of *Botrytis* spp were used in this experiment to find their optimal growth conditions. A 5 mm mycelium disc was cut with a cork borer from the growing edge of a 3 days old culture grown on PDA at room temperature and inoculated in the centre of new PDA plate. The plates were incubated at different temperatures (5, 11, 15, 20, 25, 30 and 37^{0}C). Three replicated plates were used for each temperature and fungus. The radial growth was measured daily in two directions minus inoculum disk to find average growth rate.

To find out the optimum pH for fungal growth, conidiagermination was counted in Synthetic Media (SM) adjusted to pH 2, 3, 4, 5, 6, 7, 8 and 9. In this case, a suspension containing 4X10^4 conidia ml^{-1} was made from 10 days old culture of *Botrytis* spp. 100 µl conidiasuspension (final concentration 2x10^4 conidia ml^{-1}) and 100 µl 2xSM (final concentration in microtiter plate: 2.5 mM NH_4NO_3; 028 mM $CaCl_2{\cdot}H_2O$; 0.16 mM $MgSO_4{\cdot}$7H2O; 0.002 mM $MnSO_4{\cdot}4H_2O$; 0.002 mM $ZnSO_4{\cdot}7H_2O$; 1 mM KH_2PO_4; o.06 mM $FeC_6H_5O_7{\cdot}5H_2O$ and 55.5 mM glucose) was added in the wells of microtiter plate (preparation of 2xSM is presented in Appendix H). Three replicated wells were used for each pH and fungus.

3.2 Assessment of antagonistic activity and sensitivity to fungicides using in vitro tests

3.2.1 Dual cultures

The antagonistic activity of the two *Trichoderma* isolates was assessed against two *Botrytis cinerea* isolates, *Microdochium majus* and *Alternaria brassicicola.* For all in vitro tests, 5 mm mycelium discs were used to inoculate 9 cm petridishes with PDA and SNA at 25^0C. SNA is a low sugar medium which consists of 1 g of KH_2PO_4, 1 g of KNO_3, 0.5 g of Mg $SO_4 \cdot 7H_2O$, 0.5 g KCl, 0.2 g of sucrose and 20 g of agar per 1 liter distilled water (Nirenberg, 1976). In the first test, the ability of *Trichoderma* sp. to produce non-volatile inhibitors was tested using a dual culture technique (Dennis and Webster, 1971c). In this technique one mycelial disc of the *Trichoderma* isolate and one disc of test fungal pathogen were placed simultaneously 6 cm apart on PDA and SNA plates. Three replicated PDA and SNA plates were used for each *Trichoderma* isolate and test fungi. The plates that received only one mycelia disc of the test pathogens served as control.

Inhibition percentages of the test pathogens were calculated based on the growth of the pathogen on PDA and SNA plates following the formula:

% inhibition= X-Y/X × 100

Where X= Mycelial growth of pathogen in absence of *Trichoderma* isolates

Y= Mycelial growth of pathogen in presence of *Trichoderma* isolates

3.2.2 Production of water-soluble inhibitory metabolites

The second test was conducted to measure the ability of *Trichoderma* isolates to produce water-soluble inhibitors against test pathogen using cellophane technique (Dennis and Webster, 1971a). The PDA and SNA plates were covered by the cellophane (Q 80 mm, 1000 Kp1). The *Trichoderma* discs were placed in the centre of PDA and SNA plates covered by cellophane membrane and incubated at 25^0C. The plates were observed at regular intervals so that the hyphae of *Trichoderma* isolate did not reach the uncovered agar. After 48 hours of inoculation cellophane and adhering mycelium were removed. Test fungal discs were inoculated on the center of the PDA and SNA medium where *Trichoderma* mycelia had been previously growing. For control plates, test fungi were cultured on the PDA and SNA medium

which had been covered with cellophane membranes, but without *Trichoderma* spp. Mycelial growth of the test fungi was recorded every 24.

3.2.3 Production of inhibitory volatile metabolites

The third test was conducted to see the production of inhibitory volatile metabolites produced by *Trichoderma* spp. (Dennis and Webster, 1971b). In this case PDA and SNA plates were inoculated with 5mm discs of *Trichoderma* isolates and test fungi were inoculated in the same way on separate PDA and SNA plates. The lids of the plates were taken off and plates of *Trichoderma* isolates were taped together with the plates of test fungi by adhesive tape. For control, the test fungi were cultured in the same way but without *Trichoderma* isolates. Mycelial growth of the test fungi was recorded every 24 hours. This experiment was repeated twice.

3.2.4 Sensitivity to fungicides

To find out the sensitivity of two *Trichoderma* isolates against fungicides, conidia germination was counted in Synthetic Media (SM). A suspension containing 4 x 10^4 conidia ml^{-1} (final concentration 2 x 10^4 conidia ml^{-1}) was made from 7 days old culture of *Trichoderma* spp in MilliQ water. Four different fungicides at the recommended dose (final concentration) and 1/10 and 1/100 times thereof were mixed with 2 x SM. 100 µl conidia suspension and 100 µl SM with fungicide were added to the wells of microtiter plates. Three replicated wells were used for each concentration of fungicides. Conidia germination was counted 24 hours after inoculation.

3.3 Inhibition of fungi by unhydrolyzed chitosan (Kitoflokk, former name Kitonor) and fungicides

3.3.1 Fungal isolates and fungicides

Five fungi: (1) *Botrytis cinerea*101, (2) *Botrytis cinerea*-BD, (3) *Alternaria brassicicola*, (4) *Microdochium maju*s, (5) *Mucor piriformis*; four fungicides: (1) **Teldor**® WG 50: Active ingredient: 500 g/kg fenhexamid; Chemical Group: Hydroxyanilide (Bayer CropScience Pty Ltd,), (2) **Switch**® 62.5 WG: Active ingredient: 375 g/kg cyprodinil, 250 g/kg fludioxonil; Chemical Group: anilinopyramidine, phenylpyrrole (Syngenta Crop Protection Pty Limited), (3) **Amistar**: Active ingredient: 500 g/kg azoxystrobin; Chemical Group: strobilurin

(Syngentra Crop Protection Pty Limited) and (4) **Signum**® WG: Active ingredient: 26.7% w/w boscalid and 6.7% w/w pyraclostrobin (BASF, the chemical company); and **chitosan** (unhydrolyzed and hydrolyzed) (Kitoflokk, Norwegian chitosan company, Gardermoen, Norway) were used in this experiment.

3.3.2 Conidia production

To ensure a supply of fresh conidia during the experimental period, all fungi were re-inoculated on new PDA plates every 7 days. All fungi except *Microdochium majus* readily produce conidia after 7 to 14 days on PDA at room temperature under regular laboratory lighting. As *M. majus* shows poor sporulation on PDA plate, cellophane covered PDA was used to culture this fungus (Cooke, 1980). Cellophane discs 8 cm Ø were autoclaved in distilled water to remove plasticisers and to sterilize the cellophane, and each disc was placed flat on the agar surface in separate 9 cm petridishes. Inverted plugs of mycelium, 5 mm diameter, taken from the edge of a growing colony were placed in the centre of the cellophane covered PDA plates and incubated at room temperature for 7 days under black light blue lamp (SYLVANIA, F 18w/BLB-T8, PROLYS AS). Conidial suspension was made in sterile water by gently scraping off the fungal colony to avoid scraping into media and mixing it with sterile water. If the conidial suspension contained mycelia fragments, it was shaken and filtered through sterile cotton before use. Conidia concentrations were determined by hemacytometer at x 400 magnification (Leica, DM RBE). The conidia suspensions were diluted with sterile water to a final concentration of $4x10^4$ conidia/ml.

3.3.3 Stock solution of chitosan, fungicide

One percent (w/v) stock solutions of chitosan and the fungicides were made by diluting them in sterile water. The chitosan and fungicides were mixed with 2x Synthetic Media (SM) to make treatment concentrations. In most of the cases it was difficult to count germinated conidia as the conidia did not visualize properly when fungicides were used from the 1% stock solution. To overcome that problem 0.1% and 0.01% diluted solutions were made from top suspension of 1% the stock solution without shaking.

3.3.4 In vitro fungal conidia germination and germ tube growth assay against fungicide, chitosan and their combination

Conidia germination and germ tube growth of different plant pathogenic fungi in vitro were investigated by mixing conidial suspensions with 2 x SM and incubating in 96-well, flat bottom microtiter plates (Nunc^{TM}, Rockilde, Denmark) with fungicides, chitosan and their combination. Six different concentrations of chitosan (final concentration: 0.25%, 0.128%?, 0.064%, 0.032%, 0.016%, 0.008%, 0.004% and 0.001%) were used in this experiment. Recommended dose of fungicide (final concentration) and 1/10 and 1/100 times thereof were used. To see the synergistic effect, LD_{50} (50% inhibition of conidia germination after 24 hours) and MIC (the lowest concentration that gives 100% inhibition of conidia germination after 24 hours) were determined for all the chemicals and chitosan against all of the test fungi. The LD_{50} concentration and 1/10 of that concentration of the chemicals and chitosan dissolved in 2 x SM . One hundred µl of chitosan or chemical or their combination in 2xSM were added to specific wells followed by adding 100 µl $4x10^4$ conidia ml^{-1} (final concentration $2x10^4$) in microtiter plates. The average pH was measured by adding 5 µl from each well to an automatic pH-meter (Sentron®, The Netherlands) just after inoculation of conidial suspension. Absorbance reading (A_{595}) of all treatments was measured in a microtiter plate reader (ASYS Hitech Biocrom, GmbH Austria) at 0 h and 72 h after inoculation for growth measurement at Bioforsk Ås, Norway. The fungal growth was stopped by floating the microtiter plates on a water bath at 60^0C for 20 minutes (Md. Hafizur Rahman, personal communication). Growth was stopped after 24 h and 72 h for individual treatments, but for combinations it was stopped only after 24 h and 72 h. Conidia germination was counted after 24 h, and fungal growth was observed after 72 h of inoculation by using an invert microscope (Fluovert FU, Ernst Leitz Wetzlar GmbH, Wetzlar, Germany) at 400X magnification. A minimum of 100 randomly selected conidia in each of two replicate wells per treatment were evaluated and counted as germinated when the germ tube exceeded the diameter of the conidium (Hjeljord et al., 2001). Conidia of *Mucor piriformis* showed abnormal swelling and looked like amoeboid structures with one or more protruded portions after 12 hours of incubation in >0.008% chitosan. When length of any protruded part became double of diameter of the swollen conidia after 12 hours of incubation, the conidia was considered as germinated. Three replicated wells were used for each treatment. Synergistic effect was calculated by the ratio between observed efficacy (inhibition %) and the expected

efficacy (calculated by Abbott's formula) of the combination of fungicides and chitosan (Cohen and Levy, 1990). Abbott's formula:

$$E_{exp} = a+b-(ab/100)$$

Here, a = % germination inhibition by fungicide

b = % germination inhibition by chitosan

3.3.5 Detached flower assay

The test was performed as described in Hjeljord et al., (2001). Newly opened strawberry flowers (cv. Corona) from the greenhouse were picked with 1.5 – 2 cm stems and brought to the laboratory. 18 flowers for each treatment were placed in empty pipette tip racks set in plastic containers filled with 1-2 cm water. The plastic containers were then placed in large trays that were filled with 3-4 cm of water to maintain high humidity during incubation. Inoculation of single flowers was carried out by placing 10 µl drops of 2 x 10^6 *Botrytis cinerea* 101 conidia ml^{-1} on three points close to the receptacle base using Finnpipette 4540 (Finnpipette-Labsystems, Helsinki, Finland). Conidia suspensions were tested with chitosan, fungicides or the combination thereof. Control flowers were inoculated with water instead of conidia suspension. The trays containing racks of inoculated flowers were covered with aluminium foil and incubated at room temperature. The inoculated flowers were inspected every 24 hours for necrosis on the abaxial surface of the sepals. The inspection was done very carefully using a sterile forceps, so that petal did not fall down. Data were recorded daily up to 8 days after inoculation to calculate the area under disease progress curve (AUDPC). AUDPC was calculated by following formula:

$$AUDPC = \sum [(D_i - D_{i-1}) * \{S_{i-1} + 0.5(S_i - S_{i-1})\}]$$

D_i = Days of the ith assessment

S = Proportion of the infected inoculation points

3.4 Production of chitosan oligomers

3.4.1 Production and purification of chitosanase ScCsn46A

Two litres LB medium {10 g Bacto-Tryptone (BD), 5 g Bacto-Yeast extract (Merck), 10 g NaCl in 1 L MilliQ water} were prepared in a conical flask and the pH was 7. Four sterile conical flasks (2L) were filled with 300 ml LB medium and 5 ml LB medium was filled into 5 test tubes. The conical flask and test tubes containing LB medium were autoclaved at 121°C for 20 min. The *Escherichia Coli* BL21Star (DE3) transformant containing a 749bp fragment (a single gene) from Streptomyces coelicolor Q9RJ88 coding for chitosanase ScCsn46A (Heggset et al., 2010). It was kindly provided by Elinor Heggset, NTNU, Norway. The transformant was inoculated into 5 test tubes containing 5 ml LB medium with 50 ng/ml sterile filtered Kanamycin (Sigma). The test tubes were shaken at 225 rpm at 37^0 C over night. 4.5 ml of the overnight culture was added to the 2 L conical flasks containing 300 ml LB medium with 50 ng/ml Kanamycin and shaken at 225 rpm and 37^0C to it reached an OD of 0.77 - 0.78. Then 120 µl 1 M iPTG (Isopropyl-β-D-thigalactopyranoside) (Sigma) was added to each of the conical flask (final concentration 0.4 mM iPTG) and the incubation was continued for another 4 hours at 225 rpm and 37^0C.

The sample was centrifuged at 4 ^{0}C and 8000 rpm for 10 min (BECKMAN COULTER $_{TM}$ Avanti ^{TM} J-25 with Rotor JA14) and then supernatant was removed. 30 ml spheroplastbuffer (0.5 mM EDTA; 100 mM Tris/HCl pH 8; 20 mM sucrose; 0.05 mM Phenylmethanesulfonyl fluoride (PMSF)) was mixed with the pellet and incubated in ice for 5 minutes. Again it was centrifuged at 4 ^{0}C; 8000 rpm for 10 min, and the supernatant was removed. The pellet was incubated at room temperature for 10 min. Then the pellet was resuspended with 25 ml ice cold sterile MQ water and incubated in ice for 45 second. Then 1.25 ml 20 mM $MgCl_2$ was added and the tube was centrifuged for 10 minutes at 4 ^{0}C and 8000 rpm. The supernatant which contain $(His)_6$-tagged was collected and then filtered on a membrane filter 0.20 µm. PMSF (2 µl for each ml filtered sample) was added and the enzyme was stored at 4^0C. Unlike in (Heggset et al., 2010), the $(His)_6$-tagged was not removed from enzyme.

The enzyme was purified by using a column (Glass econo column, BIORAD) containing 2ml Ni-NTA super Flow column material. The column can bind 5 mg/ml tagged protein per ml packing materials. Two type of buffer, Buffer A (50 ml 1 M Tris HCL, 10 ml 1 M Imidazol and 440 ml Milli Q water) was used as a washing buffer to remove non- attached element in side column and Buffer B (50 ml 1 M Tris HCL, 50 ml 1 M Imidazol and 400 ml Milli Q

water) was used as a elution buffer to elute protein which was attached to the column during purification of the protein. Flow rate was 2 ml/min.

Then 10 µl Nu PAGE buffer (2 µl Nu PAGE sample buffer (Invitrogen), 3 µl Nu PAGE Reducing agent (Invitrogen), 5 µl water) and 10 µl sample was added to an eppendorf tube and incubated in boiling water for 4 min. Bench mark ladder 5 µl, and 20 µl samples was run on a sodium dodecyl sulphate polyacrylamide gel at 200 volt for 50 min. The gel was washed with water, then stained with 1% Comassic Brilliant Blue 0.25 g, 50% Methanol 125 ml, 10% acetic acid 25 ml and dH_20 100ml for 30 min and then destained in 10% (v/v) methanol 50 ml, 10% acetic acid 50 ml and dH_2O 400 ml for 2 hours. During staining and destaining the gel was incubated on a shaker (30 times/minute) in a laminar flow cabinet. Thereafter the gel was photograph under UV light in a Gel Doc (Bio-Rad). Only one band of 30 KDa was detected from the chitosanase samples on the gel. Protein concentration was measured by using the Qunat-IT™ protein assay kit and Qubit™ fluorometer (Invitrogen). The chitosanase enzyme was stored at 4^0C (Heggset et al., 2010).

3.4.2 Hydrolysis of Chitosan

Ten mg chitosan (Kitoflokk) was dissolved by adding 0.5 ml dH_2O and 0.5 ml hydrolysis buffer (0.08M NaAcetate + 0.2M NaCl, pH 5.5) in a 15 ml tube (Greiner). The tube was incubated in incubator (Multitron eco, INFORS) at 220 rpm and 37^0C until the chitosan was dissolved. The pH was adjusted to 5.5 with 0.5 M HCl and 10 µl BSA (Bovine Serum Albumin, Promega) was added to the dissolved chitosan. One µg chitosanase ScCsn46A was added to the 10 mg chitosan solution and incubated for 10, 20, 30, 40 and 50 min. at 220 rpm and 37^0C. The enzyme reaction was stopped by adding 0.5 M HCl until pH 2.5 and then by incubating the tube in boiling water for 10 minutes. Chitosan without enzyme was used as a control. Salt was removed from hydrolyzed products by dialyzing the sample with deionised water in cellulose membrane (cut-off 500 Da, spectra/por® Float-A-Lyzer®)) for 48 hours. Dialysis water was changed every 12 hours. The hydrolyzed samples were freeze dried and stored at 4^0C before proton NMR measurements.

3.4.3 Nuclear Magnetic Resonance (NMR) spectroscopy

Ten mg of freeze dried chitosan (hydrolyzed) was placed in an eppendorf tube. Then 0.5 ml D_2O was added to the chitosan to dissolve it. The pH of the chitosan was adjusted to 4.2 by using NaOD (Sodium deuteroxide) or DCl (Deuterium chloride) and the samples were freeze

dried in Maxi dry (Savant, Speed Vac). These steps were repeated twice. Finally 0.7ml D_2O were added to the freeze dried sample. The NMR spectra were recorded on a Varian Gemini 300 instrument in D_2O at 300 MHz (256 scan). Average degree of polymerization (DPn) was measured by using equation (Dα+Dβ+D+A)/ Dα+Dβ where Dα is the area of the reducing end of α anomer of A-unit, Dβ is the area of reducing end of β anomer of D-unit, D is the area of internal D-unit and A is the area of internal A-unit. The degree of scission (α) was estimated as 1/DPn (Sørbotten et al., 2005).

3.4 4.Comparison of hydrolyzed and unhydrolyzed chitosan

Comparison of the antifungal activity of hydrolyzed and unhydrolyzed chitosan was done by mixing six different concentrations of hydrolyzed and unhydrolyzed chitosan (0,25%, 0,128%, 0.064%, 0.032%, 0.016%, 0.008%, 0.004% and 0.001%) with SM in a mitrotiter plate following the method described above

3.5 Data Analysis

Minitab 16 and Microsoft office Excel were used for the statistical analysis in this thesis. Whenever necessary data were transformed using Arcsine transformation before testing one way ANOVA (analysis of variance) and non transformed data are presented as well. The significance of differences between treatment means was tested by Tukey's Protected Least Significance Difference (comparing among all treatments). The significance level was set to P = 0.01 (1% level) for all experiments analyzed.

4 Results

4.1 Morphological and molecular characterization of the fungal isolates

Morphological and cultural characters (size and shape of conidia and phialides, presence or absence of sterile hair, coconut odor and radial growth in PDA) of the two *Trichoderma* isolates used in this study are shown in Table 1. According to the interactive key (Samuels, G.J., Chaverri, P., Farr, D.F., & McCray, E.B. http://nt.ars-grin.gov/taxadescriptions/keys/TrichodermaIndex.cfm) *Trichoderma* isolate *Trichoderma*-BD was identified as *Trichoderma asperellum* and *Trichoderma*-P1 was identified as *Trichoderma atroviride*.

Table 1. Morphological characters used for identification of *Trichoderma* isolates

Trichoderma isolates	Conidia			Phialide (µm)			Sterile hair	Coconut odour	Radius growth on PDA (mm)		
	Shape	Length (µm)	Width (µm)	Length	Width at middle	Width at base			Temperature (^{0}C)		
									25	30	35
T. asperellum	Subglubose to ovoidal	3.5-4.5	3-4	10-11.5	3-4	2-2.5	Absent	Absent	53	67	26
T. atroviride	Glubose to subglubose	3-3.5	2.5-3	8-10	2.5-3	1-1.5	Absent	Present	65	45	< 2

Molecular Identification was performed on DNA extracted from *B. cinerea* 101, *B. cinerea*-BD, *Trichoderma*-BD, *Trichoderma*-P1, *Alternaria* 328 *Mucor* 199J, and *Microdochium majus.* The DNA extract was amplified with ITS1F (forward) and ITS4 (reverse) primers. When DNA was tested by gel electrophoresis, only DNA from *Trichoderma*-BD showed band in Gel A, DNA from *B. cinerea* 101 and *B. cinerea*-BD in Gel B, DNA from *Trichoderma*-P1 and *Alternaria* 328 in Gel C and DNA from *Mucor* 199J showed band in Gel D (Figure 3). Except for *Microdochium majus,* that didn't show any band. The band for all fungal DNA had a size of about 600 bp. In Gel D, Probably negative control was contaminated because it showed band. For some isolates repetition of extraction procedure was done when there was no band the first time.

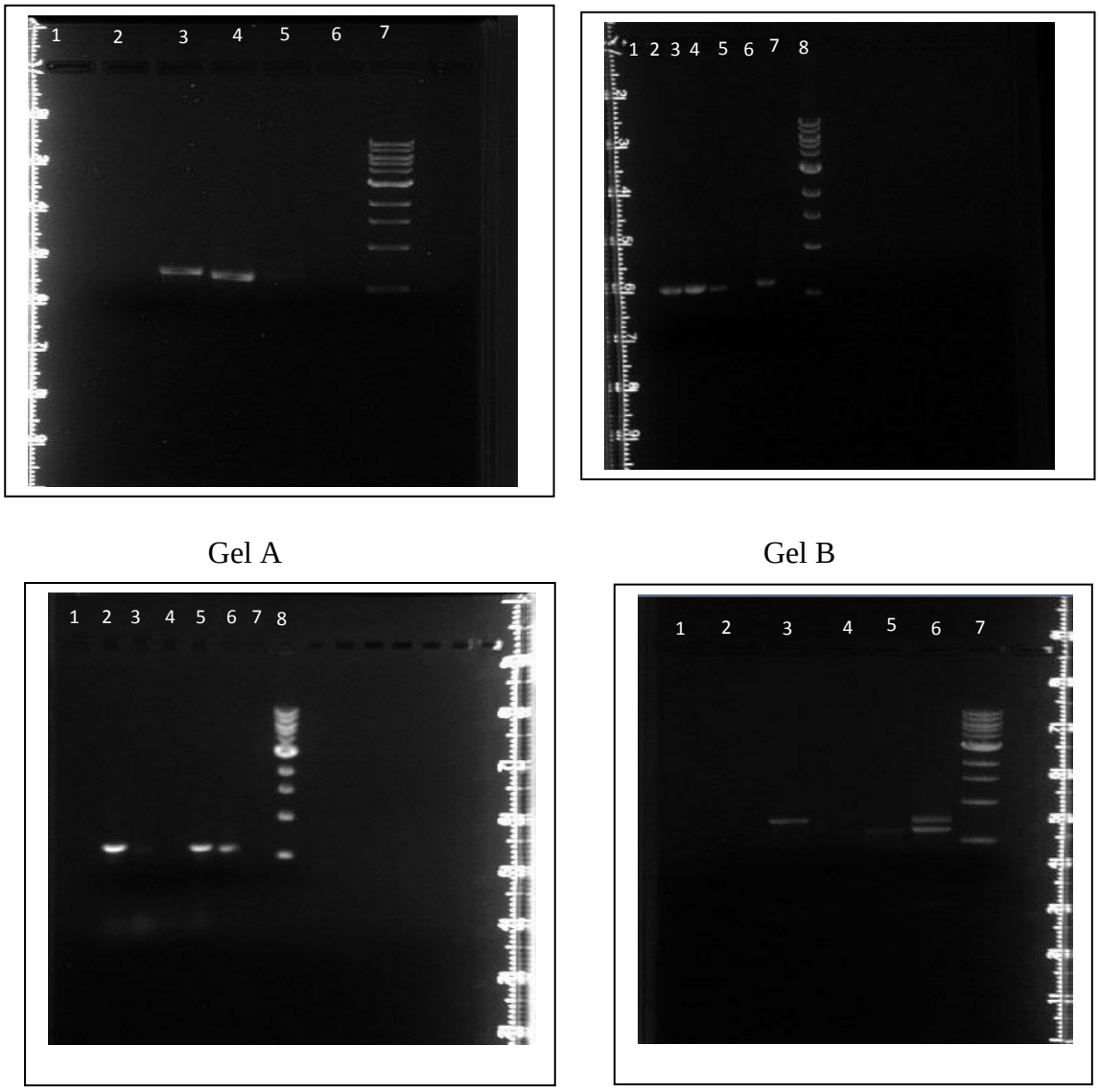


Figure 3. Gel electrophoresis after amplification of fungal DNA with ITS1F and ITS4 primers and an annealing temperature of 48^0C. Gel A: Lane 2 - *B. cinerea*-BD, lane 3 - *Trichoderma*-BD, lane 4 - *Rhodococcus* sp (positive control), lane 5 - *Rhodococcus* sp., lane 6 - negative control and lane 7 - ladder. Gel B: Lane 2 - *B. cinerea* 101, lanes 3 & 4 - *B. cinerea* - BD, lane 5 - *Trichoderma*-P1, lane 6 - *Rhodococcus* sp. (positive control), lane 7 - negative control and lane 8 - ladder. Gel C: Lane 2 - *Trichoderma*-P1, lane 3 – *Mucor* 199J, lane 4 - *Microdochium majus*, lane 5 - *Alternaria* 328, lane 6 - *Rhodococcus* sp (positive control), lane 7 - negative control and lane 8 - ladder. Gel D: Lane 2 – *Mucor* 199J, lane 3 – *Mucor* 199J, lane 4 - *Microdochium majus*, lane 5 - *Rhodococcus* sp (positive control), lane 6 - negative control and lane 7 - ladder. Lane 1 was not used in any gel.

When extracted DNA was sequenced, DNA of *B. cinerea* 101, *B. cinerea*-BD, *Trichoderma*-BD, and *Alternaria* 328 resulted in spectra with little noise. The spectra from *Trichoderma*-P1 and *Mucor* 199J contained some noise. The length of the final edited sequences after alignment is shown in Appendix (B-G), along with the 20 first BLAST results from NCBI (National Center for Biotechnology Information) for each sample. Among the first 20 nucleotide sequencing results the species which came in first and contained the highest score with good expected value (E-Value) was taken into consideration. *B. cinerea* 101 was identified as *Botryotinia fuckeliana* (*B. cinerea*) by nucleotide sequence. When sequenced 8 out of 20 first identification results were *B. fuckeliana*, 6 were uncultured soil fungus or uncultured fungus, 3 *Sclerotinia sclerotiorum* 1 fungal endophyde, 1 *B. fabae* and 1 uncultured ascomycete. The species with the highest score and first hit was *B. fuckeliana* (Appendix B).

B. cinerea-BD was identified as *Botryotinia fuckeliana* (*Botrytis cinerea*). Among the first 20 identification results, 7 were *B. fuckeliana*, 6 were uncultured fungus or uncultured soil fungus, 6 were *S. sclerotiorum* or *sclerotiorum* sp and 1 was fungal endophyte, but the first hit and highest score showed *B. fuckeliana* (Appendix C).

Trichoderma-BD was identified as *Trichoderma asperellum*. Among the 20 BLAST search results 17 were *T. asperellum*, 2 were uncultured hypocreales and 1 were fungal sp. The first hit and highest score was *T. asperellum* (Appendix D).

Trichoderma-P1 was identified as *Trichoderma atroviride*. Among 20 sequence results 9 were *T. atroviride*, 3 were *T. viride*, 3 were uncultured fungus, 2 were *Hypocrea atroviridis*. 1 was *Trichoderma* sp, 1 was uncultured *Trichoderma* and 1 *Rhizopus stolonifer*. The first hit and highest score was *T. atroviride* (Appendix E).

Alternaria 328 was identified as *Alternaria brassicicola*. Among 20 sequence results 8 were *A. brassicicola*, 4 were *Pleospora papaveracea*, 2 were *Brachycladium papaveris*, 1 was *A. mimicula*, 1 was *A. japonica*, 1 was *Embellisia conoidea*. 1 was *Ulocladium botrytis*, 1 was *Crivellia papaveracea* and 1 was *Dendryphiella triticicola*. The first hit and highest score was *A. brassicicola* (Appendix F).

Mucor 199J was previously identified by the Centraalbureau voor Schimmelcultures (CBS) as *Mucor piriformis.* Among the 20 sequencing results 2 were *M. piriformis*. 11 were *Pilaira anomala* or *Pilaira* sp. 2 were uncultured compost fungus. 4 were *Mucor mucedo* and 1 was *M. flavus*. The first hit and highest score was in case of *M. piriformis* (Appendix G).

4.1.1 Effect of temperature on the growth and morphology of the test fungi

Figure 4 and 6 shows that the temperature markedly affects the growth rate and the morphology of the fungi, such as sporulation, and sclerotia formation. Although some fungi can grow at relatively high (37^0C) and low (5^0C) temperatures, their growth is very slow at such temperatures. *B. cinerea*-BD and *B. cinerea* 101 and *T. atroviride* grew at 5^0C, but they did not grow at 37^0C, whereas *T. asperellum* grew at 37^0C, but did not grow at 5^0C. The optimum temperature for the growth of *B. cinerea*-BD and *B. cinerea* 101 was about 25^0C, and 25 – 30^0C for *T. asperellum and T. atroviride* (Figure 5). At 20 and 25 ^{0}C *T. asperellum* formed concentric rings of sporulation and at 37^0C colony color was yellowish (Figure 6). At 30^0C, *B. cinerea*-BD and *B. cinerea* 101 showed distorted mycelium (Figure 6). After 3 weeks at the tested temperatures, all cultures were incubated at room temperature. *T. asperellum*, which had been incubated at 5^0C, started to grow at room temperature. Except for *T. asperellum*, the fungi that had not grown at 37^0C did not grow when they were subsequently incubated at room temperature. *B. cinerea*-BD and *B. cinerea*101 produced sclerotia at 5 to 20^0C. The *B. cinerea* strains grew better than the *Trichoderma* strains at 5 and 11^0C, whereas *Trichoderma* grew better than *Botrytis* at 30^0C.

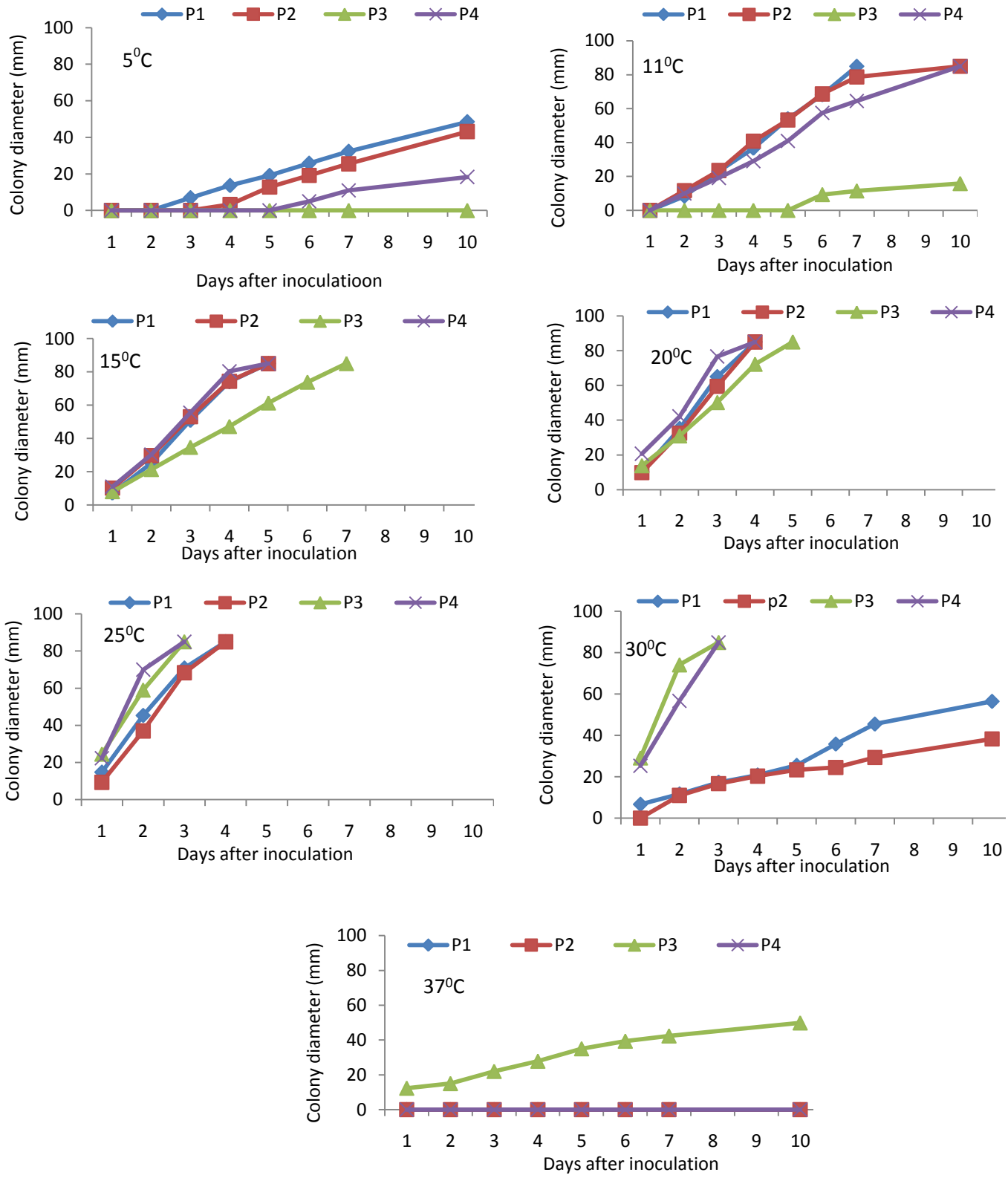


Figure 4. Effect of temperature on the growth rate of fungi. Here P1= *B. cinerea*-BD, P2= *B. cinerea* 101, P3= *T. asperellum* and P4= *T. atroviride*

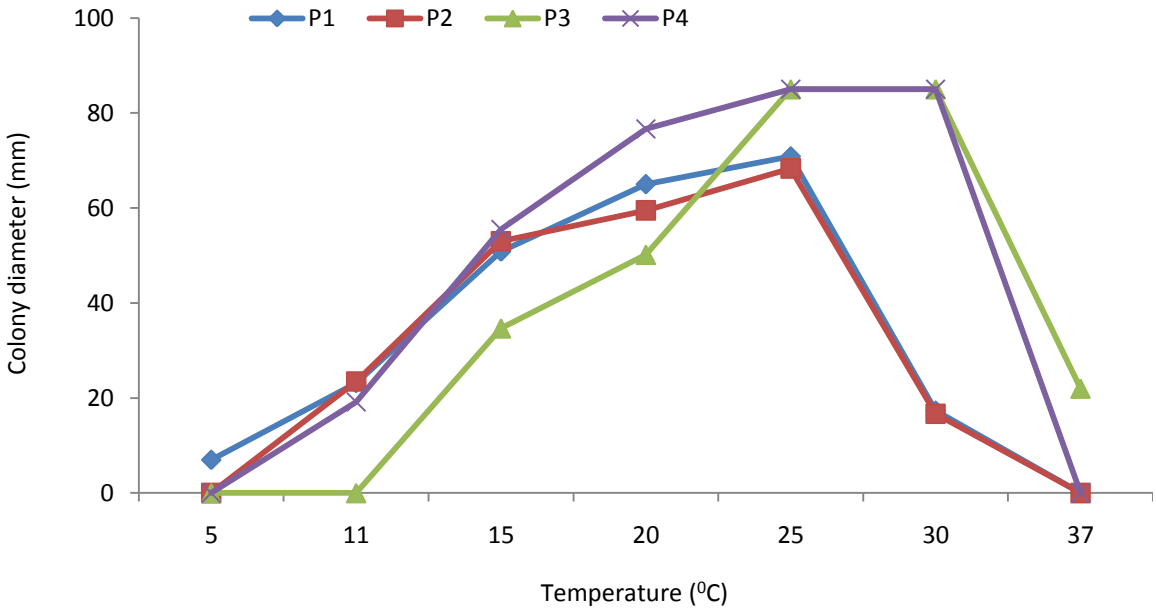


Figure 5. Colony growth of fungi at different temperatures 3 days after inoculation. Here P1= *B. cinerea*-BD, P2= *B. cinerea* 101, P3= *T. asperellum* and P4= *T. atroviride*

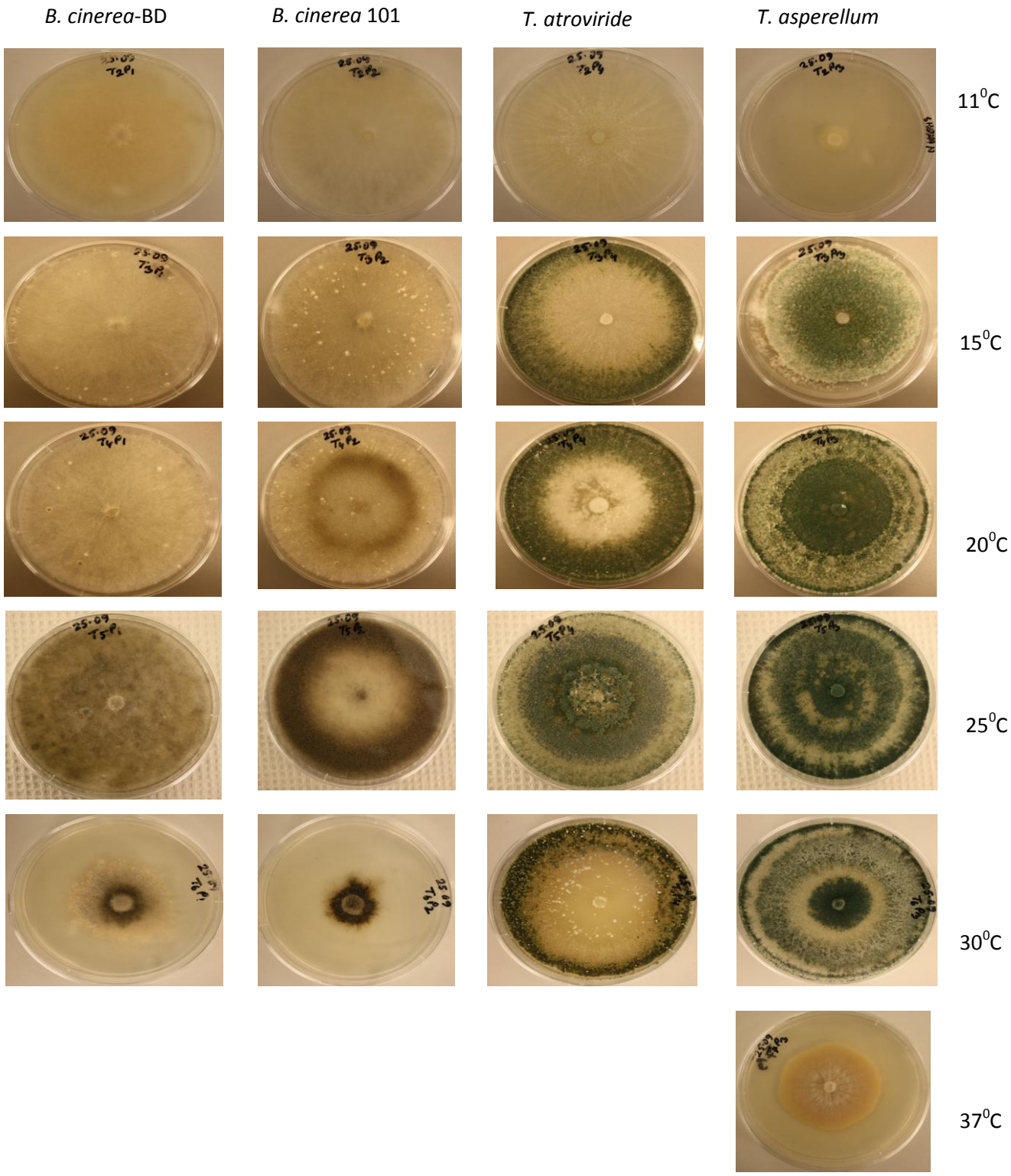


Figure 6. Colony morphology of the test pathogens on PDA at different temperatures 10 days after inoculation

Figure 7 shows that the conidia of *B. cinerea*-BD and *B. cinerea* 101 could germinate well in a pH range of 3 to 8.5 after 12 hours incubation. *B. cinerea*-BD germinated more quickly at a wider pH range than *B. cinerea* 101. At pH 2 conidia of both strains germinated poorly. At pH 9, *B. cinerea* 101 did not grow, while *B. cinerea*-BD showed almost 88% germination 12 hours after inoculation. After 6 hours only 9% of *B. cinerea*-BD conidia and 2% of *B. cinerea* 101 conidia had germinated at pH 2, whereas at pH 9 they did not germinate. It seemed 6 hours after inoculation, the optimum pH of *B. cinerea* 101 was 5.

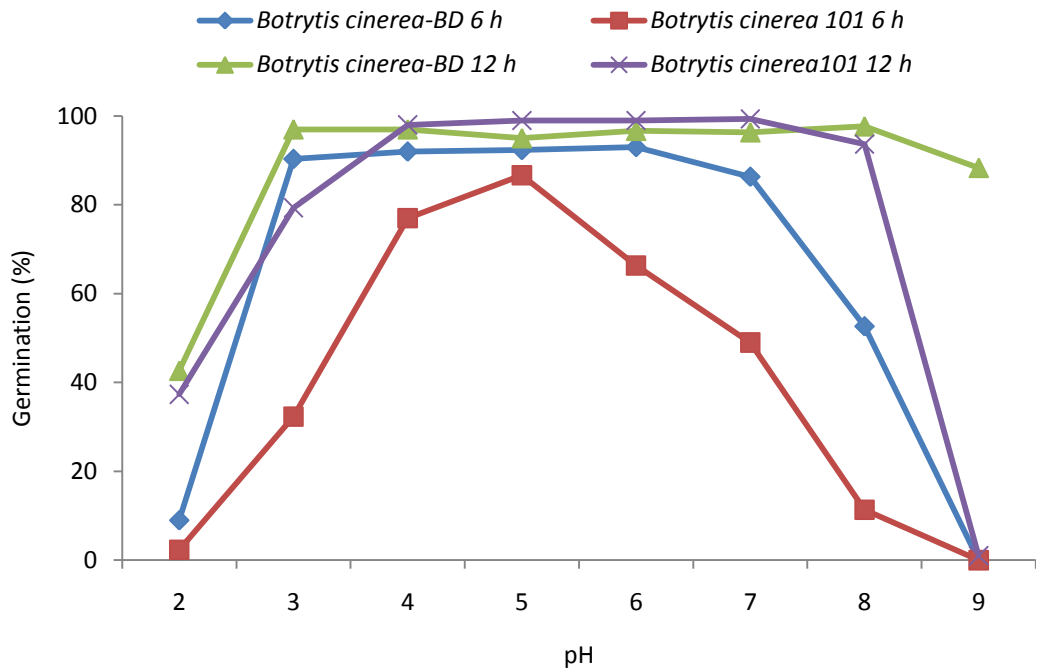


Figure 7. Effect of pH on germination rate of *Botrytis cinerea*-BD and *Botrytis cinerea* 101

4.2 Assessment of antagonistic activity and sensitivity to fungicides using *in vitro* tests

4.2.1 Dual cultures

In dual culture technique, *T. atroviride* showed most inhibition of *M. majus* whereas *T. asperellum* showed most inhibition of *B. cinerea* 101 in PDA media (Table 2 & Figure 8). *T. atroviride* is more effective in PDA than in SNA in reducing the radial growth of the test pathogens. *T. asperellum* was almost equally effective both in PDA and SNA against test pathogens except in case of *A. brassicicola* where it showed less inhibition in SNA. No inhibition zone was clearly noticed between *Trichoderma* spp and the test pathogens and the *Trichoderma* isolates grew over the pathogens and then covered the whole plate. Although the *Trichoderma* isolates covered the whole area, up to a certain period sporulation of *Botytis* isolate was visible over the plate, and that was most pronounced in the SNA media. This experiment was repeated with similar results.

Table 2. Percent inhibition of radial growth of plant pathogenic fungi by *Trichoderma atroviride* and *Trichoderma asperellum* in dual culture technique at 25^0C measured after 3 days.

Biocontrol agent	Pathogens	% Inhibition of growth	
		PDA	SNA
T. atroviride	*B. cinerea* 101	30 b c	10 a
	B. cinerea-BD	26 c	14 a
	M. majus	41 a	14 a
	A. brassicicola	35 a b	13 a
T. asperellum	*B. cinerea* 101	33 a	24 a
	B. cinerea-BD	22 b	27 a
	M. majus	32 a	30 a
	A. brassicicola	19 b	9 b

Means with different lettering within the same biocontrol agent and same media vary significantly (P=0.01)

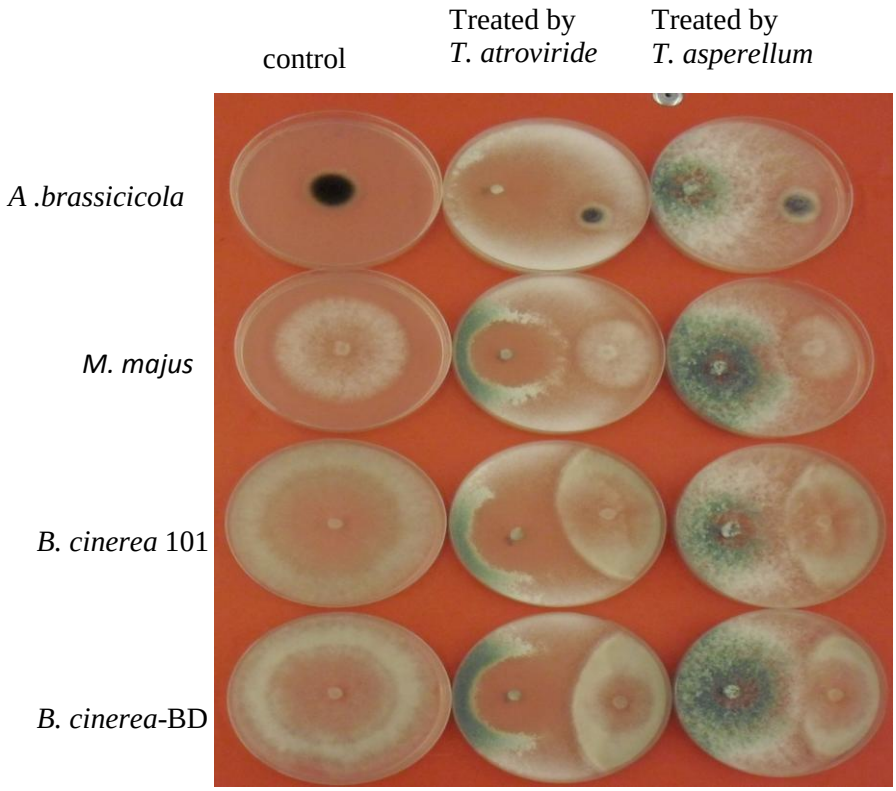


Figure 8. Inhibition of radial growth of test pathogens by *T. atroviride* and *T. asperellum* after 4 days at 25^0C

It was observed from the above figure, that sporulation of *Trichoderma* isolates and test fungi were influenced by the competition as well.

4.2.2 Production of water-soluble inhibitory metabolites measured with the cellophane technique

Trichoderma isolates produced water-soluble metabolites that had fungicidal or fungistatic effects on all of the tested pathogens. Metabolites released from *T. atroviride* that had grown for 2 days on cellophane completely inhibited radial growth of all test pathogens in PDA media (Table 3 & Figure 9), whereas in SNA it was less effective. *T. asperellum* also produced water-soluble inhibitors that had great influence on the radial growth of test pathogens both in PDA and SNA. *T. asperellum* completely inhibited the growth of *M. majus* in PDA. Inhibition percent of test pathogens by *T. asperellum* was very noticeable in SNA as well. Inhibition of fungal growth in this experiment was not due to unfavorable pH, as the pH in the media was 5.2 – 5.5 throughout the experiment. After 8 days, inoculum discs of the test fungi, which were completely inhibited, were transferred to a new PDA plate, but the fungi did not resume growth. This experiment was repeated with similar results.

Table 3. Effect of water soluble inhibitor produced by *Trichoderma atroviride* and *Trichoderma asperellum* after 3 days at 25^0C on the percent inhibition of radial growth of plant pathogenic fungi

Biocontrol agent	Pathogens	% Inhibition of growth	
		PDA	SNA
T. atroviride	*B. cinerea* 101	100 a	4 c
	B. cinerea-BD	100 a	5 c
	M. majus	100 a	14 b
	A. brassicicola	100 a	21 a
T. asperellum	*B. cinerea* 101	88 b	65 b
	B. cinerea-BD	75 d	60 c
	M. majus	100 a	94 a
	A. brassicicola	81 c	44 d

Means with different lettering within the same biocontrol agent and same media vary significantly (P=0.01)

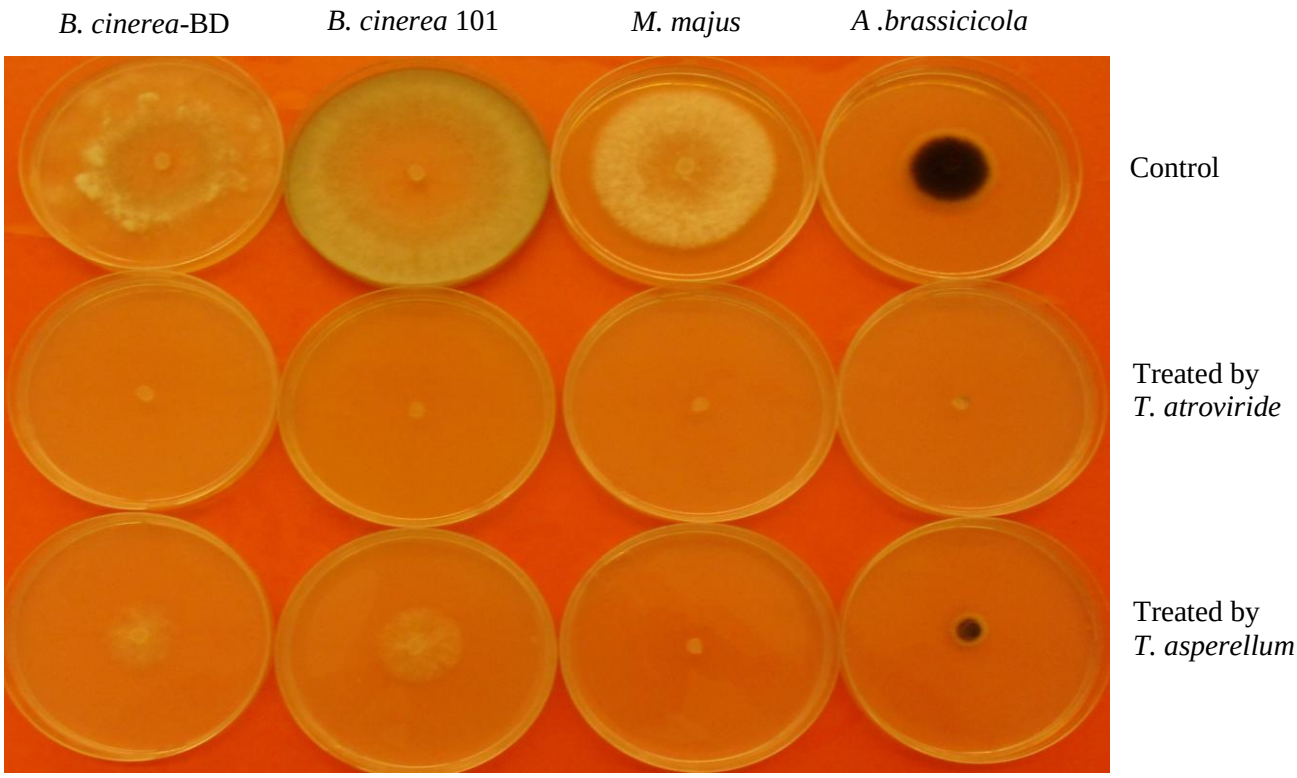


Figure 9. Inhibition of radial growth of fungi by water soluble inhibitor produced by *Trichoderma* isolates after two days growth on cellophane and PDA at 25^0C.

No radial growth of test pathogens was noticed when treated by *T. atroviride*. Only *M. majus* was completely inhibited by *T. asperellum*. The picture was taken 4 days after inoculation.

4.2.3 Production of inhibitory volatile metabolites

The *Trichoderma* strains produced inhibitory volatile metabolites which caused some reduction in growth of plant pathogenic fungi (Table 4 & Figure 10). In most of the cases, inhibition of the test fungi was greater in PDA media than in SNA media. Volatile inhibitors from *T. atroviride* P1 showed most inhibition against *M. majus*, whereas volatile inhibitors from *T. asperellum* showed most inhibition aginst *B. cinerea* 101 in PDA. Both *Trichoderma* strains exhibited poor production of volatile inhibitors in SNA. The volatile inhibitor caused stunted mycelia and frequent branching in all tested fungi. Sporulation of test fungi was slowed down. In this experiment, it was also found that *B. cinerea* 101 and *B. cinerea*-BD slightly inhibited the mycelium growth of each other (data not shown). This experiment was repeated with similar results.

Table 4. Effect of inhibitory volatile metabolite produced by *Trichoderma atroviride* P1 and *Trichoderma asperellum* after 3 days of inoculation at 25^0C on colony growth of plant pathogenic fungi

Biocontrol agent	Pathogens	% Inhibition	
		PDA	SNA
T. atroviride (P1)	*B. cinerea* 101	29 b	23 a
	B. cinerea-BD	19 d	13 b
	M. majus	46 a	10 b
	A. brassicicola	23 c	11 b
T. asperellum (Tri-BD)	*B. cinerea* 101	37 a	28 a
	B. cinerea-BD	11 c	11 b
	M. majus	31 b	11 b
	A. brassicicola	15 c	7 b

Means with different lettering within the same biocontrol agent and same media vary significantly (P=0.01)

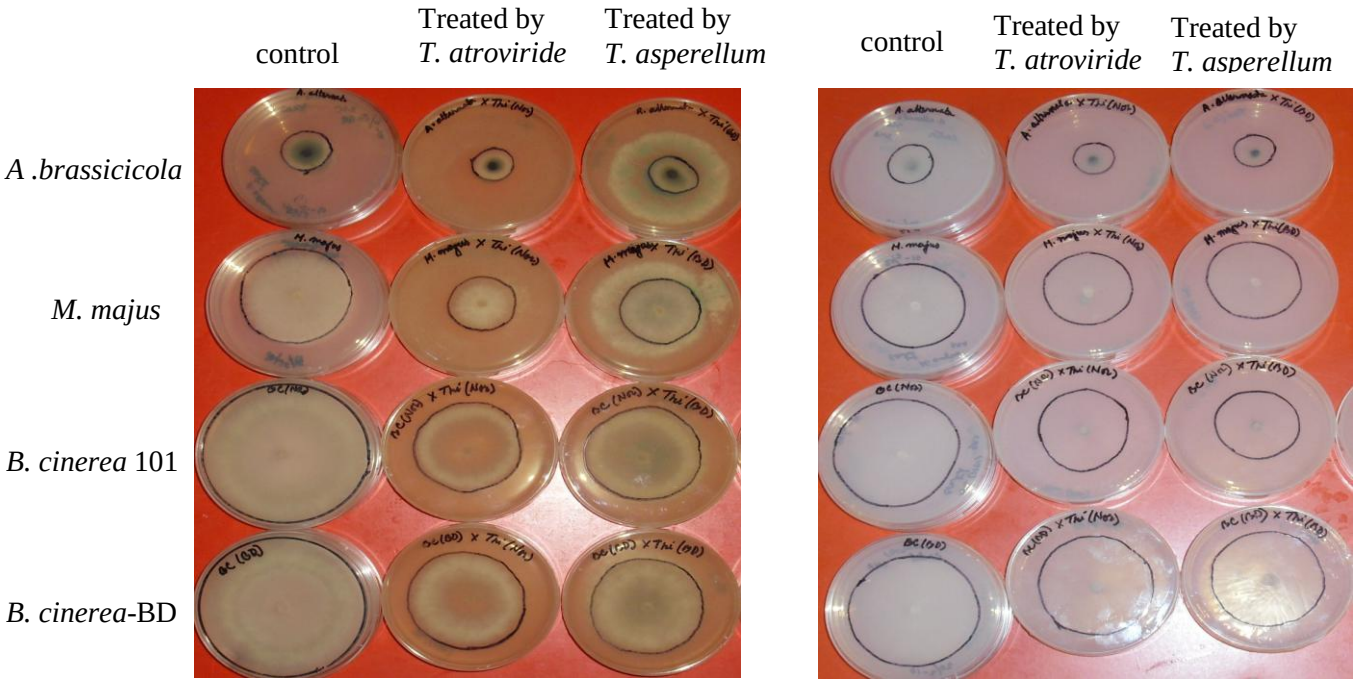


Figure 10. Inhibition of radial growth of test fungi by the volatile metabolite produce by *Trichoderma* isolates in PDA and SNA media at 25^0C. The pictures were taken 4 days after inoculation.

4.2.4 Sensitivity to fungicides

Four different fungicides were used to test the sensitivity of the *Trichoderma* isolates to fungicides (Figure 11). Teldor did not reduce the germination of the *Trichoderma* isolates; in fact it accelerated conidia germination of both the *Trichoderma* isolates. They were moderately affected by Switch. *T. atroviride* was highly sensitive to Amistar and Signum, whereas *T. asperellum* was not sensitive to the low concentrations of those fungicides.

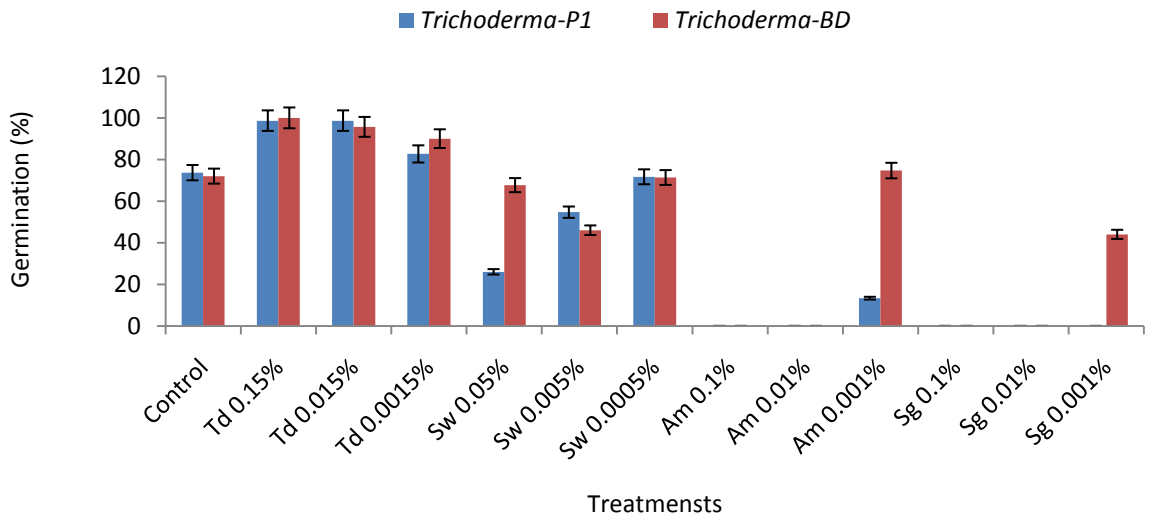


Figure 11. Effect of fungicides on conidia germination of *Trichoderma atroviride* and *Trichoderma asperellum.* Vertical bars indicate ± S.D. (Here, Td=Teldor, Sw= Switch, Am= Amistar, Sg= Signum).

4.3 Inhibition of fungi by chitosan and fungicides

4.3.1 Effect of fungicides on fungi

Three concentrations of four fungicides (recommended dose and 1/10 and 1/100 of the recommended dose) were tested against conidia germination of four plant pathogens (Figure 12 & Appendix I). Among these four fungicides, Teldor was effective against *M. piriformis* and *M. majus* in the recommended dose (0.15% Teldor) and less effective against *B. cinerea* 101 and *A. brassicicola.* None of the tested concentrations of Teldor inhibited the germination of *B. cinerea* 101 conidia, but the elongation of the germ tubes was inhibited.

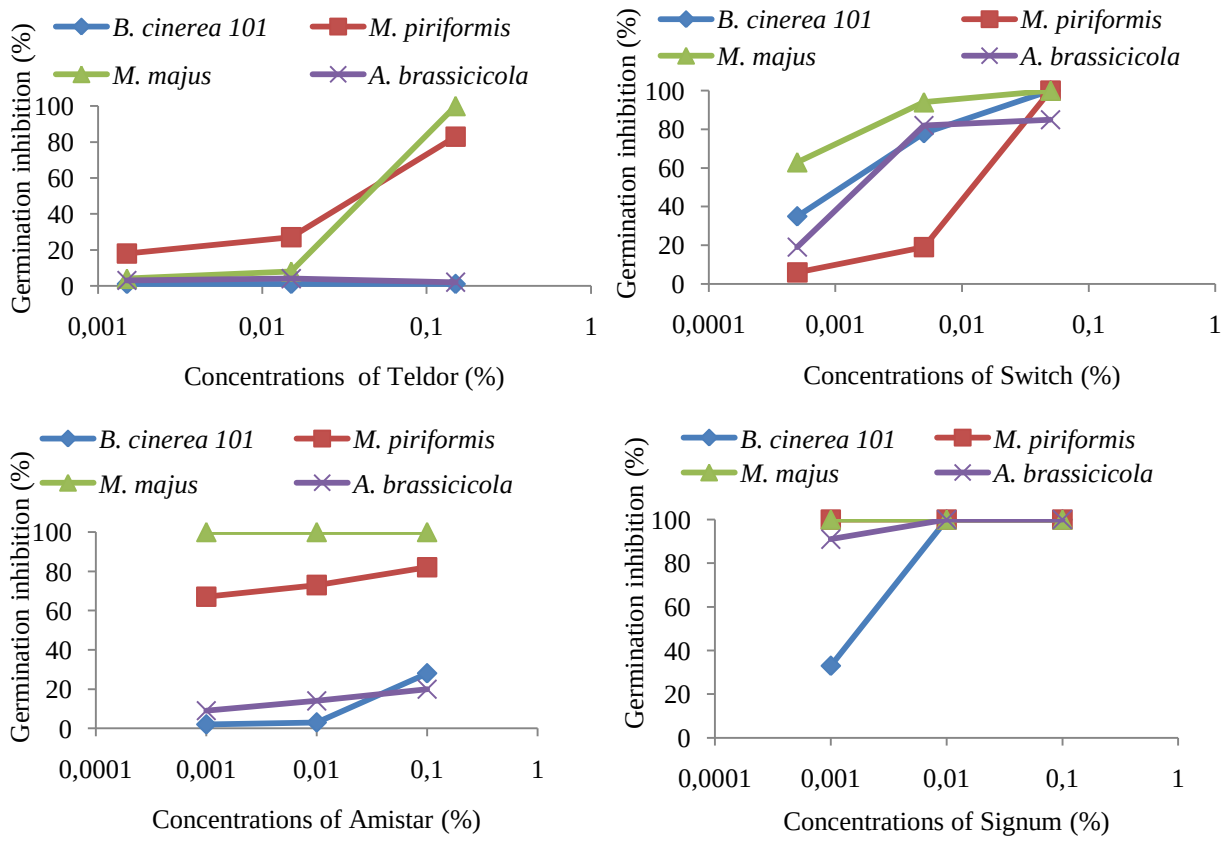


Figure 12. Effect of fungicides on conidia germination of plant pathogens recorded 24 hours after inoculation

Almost all tested concentrations of Switch were very effective against all of the test fungi. Switch showed dose response with all tested concentration against *B. cinerea* 101 and 0.05% Switch completely inhibited conidia germination of *B. cinerea* 101. *M. majus* was very sensitive against Amistar. 0.001% Amistar completely inhibited conidia germination of *M. majus.* Amistar was less effective against *B. cinerea* 101 and *A. brassicicola*. Among the tested fungicides, Signum was the most effective fungicide against all of the test pathogens.

0.001% Signum completely inhibited *M. piriformis* and *M. majus* and 0.01% Signum completely inhibited conidia germination of *B. cinerea* 101 and *A. brassicicola.*

4.3.2 Effect of unhydrolyzed chitosan on fungi

Effect of chitosan on inhibition of conidia germination of four test fungi is shown in Figure 13 and Appendix J. Different fungal isolates showed different sensitivity to chitosan. Chitosan was very effective to inhibit the conidia germination of *A. brassicicola* as 0.008% chitosan showed 100% inhibition of conidia germination of *A. brassicicola*. Chitosan followed the dose response effect against *B. cinerea* 101 with all tested concentrations and 0.25% chitosan completely inhibited conidia germination of *B. cinerea.* 0.064 % chitosan inhibited 42% conidia germination of *B. cinerea* which is near lethal dose 50 (LD_{50}) against *B. cinerea.* All tested concentrations of chitosan were less effective against *M. piriformis* and *M. majus.*

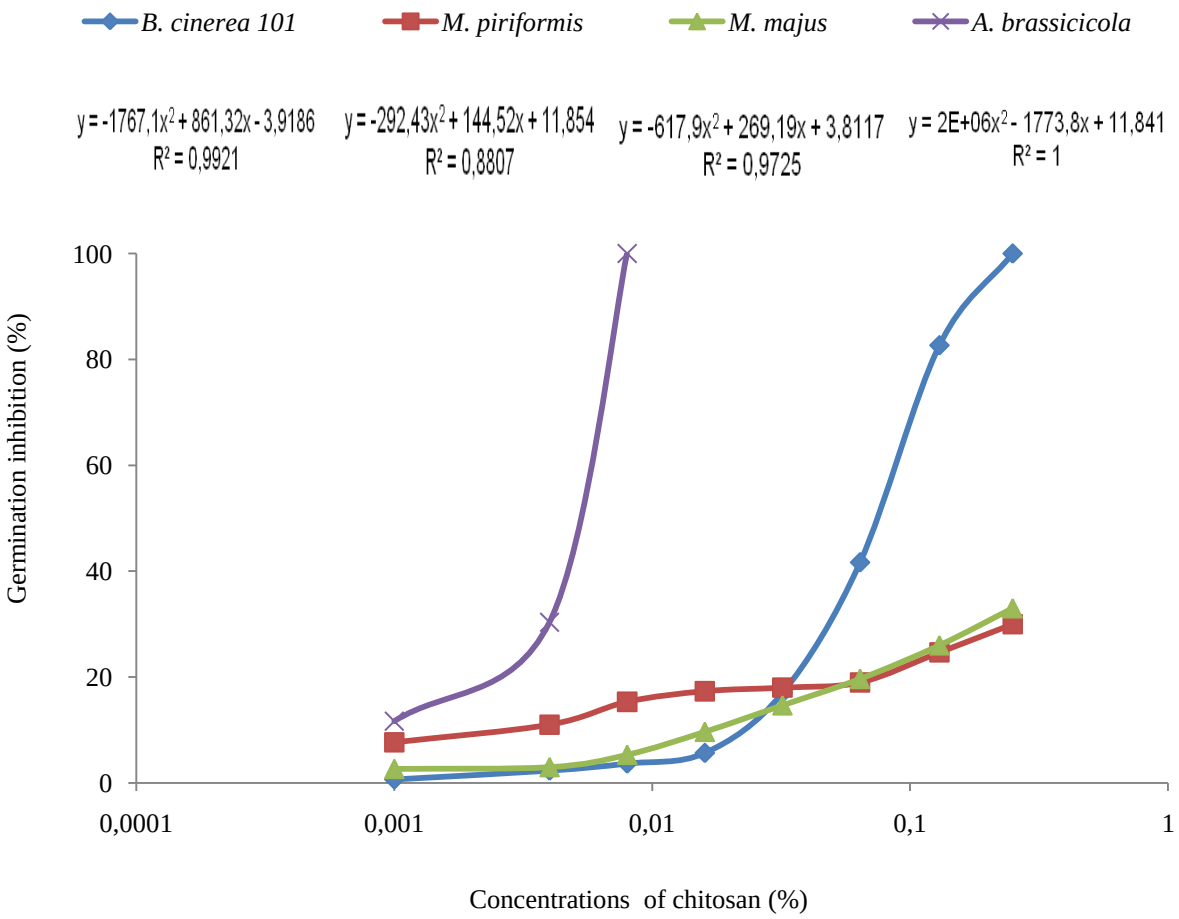


Figure 13. Effect of chitosan on conidia germination of plant pathogens recorded 24 hours after inoculation.

4.3.3 Synergistic effects of chitosan and fungicides

Effects of combining chitosan and fungicides on conidia germination of *B. cinerea* 101 and *B. cinerea*-BD are shown in Table 5. In most of the cases the combination was more effective against both *B. cinerea* 101 and *B. cinerea*-BD than the components alone. Fungicide combined with chitosan showed synergism in most of the cases (Effect >1), additivity in few cases (Effect = 1) and antagonism (Effect <1.0) in one case against *B. cinerea* 101, and 3 cases against *B. cinerea*-BD. Combination of all concentrations of Teldor with chitosan showed good synergism. The combination treatment of 0.006% Teldor + 0.008% chitosan showed 74% germination inhibition (highest synergism) against *B. cinerea* 101, whereas individually 0.006% Teldor showed 2% inhibition and 0.008% chitosan showed 11% germination inhibition. *B. cinerea*-BD followed the same trend as *B. cinerea* 101 in most of the cases. Conidia germination inhibition of *B. cinerea*-BD was greatly affected by the combination of 0.006% Teldor + 0.008% chitosan as well and the synergism was highest (4.4).

Table 5. Effect of chitosan and fungicides on inhibition of conidia germination of *Botrytis cinerea* 101 and *B. cinerea* -BD recorded 24 hours after inoculation. Effect above 1.0 is regarded as a synergistic effect

Treatments	% inhibition	Effect*	% inhibition	Effect*
	B. cinerea 101		*B. cinerea*-BD	
Chitosan 0.064%	32 e		17 c d	
Chitosan 0.008%	11f		5 e	
Teldor 0.006%	2 g		1 f	
Teldor 0.0015%	0.3 g		1 f	
Teldor 0.006% + chitosan 0.064%	78 a	2.4	42 a	2.3
Teldor 0.006% + chitosan 0.008%	74 b	5.8	26 b	4.4
Teldor 0.0015% + chitosan 0.064%	45 c	1.4	18 c	1
Teldor 0.0015% + chitosan 0.008%	42 d	3,7	15 d	2.8
Chitosan 0.064%	32 e		17 f	
Chitosan 0.008%	11 f		5 g	
Switch 0.0025%	82 b		43 d	
Switch 0.0005%	67 d		19 f	
Switch 0.0025% + chitosan 0.064%	100 a	1.2	100 a	1.9
Switch 0.0025% + chitosan 0.008%	100 a	1.2	90 b	2
Switch 0.0005% + chitosan 0.064%	78 c	1	82 c	2.5
Switch 0.0005%% + chitosan 0.008%	65 d	1	25 e	1.1
Chitosan 0.064%	32 a b		17 b	
Chitosan 0.008%	11 c d		5 d	
Amistar 0.01%	21 b c		14 c	
Amistar 0.001%	0.00 d		1 e	
Amistar 0.01% + chitosan 0.064%	42 a	0.9	21 a	0.7
Amistar 0.01% + chitosan 0.008%	33 a b	1.1	12 c	0.7 #
Amistar 0.001% + chitosan 0.064%	40 a	1.3	18 b	1
Amistar 0.001% + chitosan 0.008%	10 c d	0.9 #	12 c	2
Chitosan 0.064%	32 c		17 d	
Chitosan 0.008%	11 e		5 e	
Signum 0.001%	19 d		26 c	
Signum 0.0002%	0.33 f		2 e	
Signum 0.001% + chitosan 0.064%	68 a	1.5	47 a	1.3
Signum 0.001% + chitosan 0.008%	21 d	0.8	24 c	0.8 #
Signum 0.0002% + chitosan 0.064%	41 b	1.3	39 b	2.1
Signum 0.0002% + chitosan 0.008%	12 d	1.1	3 e	0.5 #

* Ratio between the observed efficacy and the expected efficacy of the combination. Ratio close to 1 indicates additivity, whereas ratio greater than 1 indicates synergy. (Cohen and Levy, 1990). Values within a column with common letters do not differ significantly (P=0.01)

means antagonistic

Effect of chitosan and fungicides on hyphal growth inhibition of *B. cinerea* 101 and *B. cinerea* -BD was measured 72 hours after inoculation (Figure14 & 15). In most of the cases the combination was more effective on growth inhibition of *B. cinerea* 101 & *B. cinerea* -BD than the component alone and it was also complementary to the result of germination inhibition (Table 5). If the germination inhibition was high, growth inhibition was high as well. In a few cases the growth inhibition result did not follow the result of germination inhibition, for example the individual tested concentrations of Teldor did not inhibit conidia germination, but prevented germ tube elongation. 0.008% chitosan slightly stimulated the growth of *B. cinerea* 101, but inbihited conidia germination slightly.

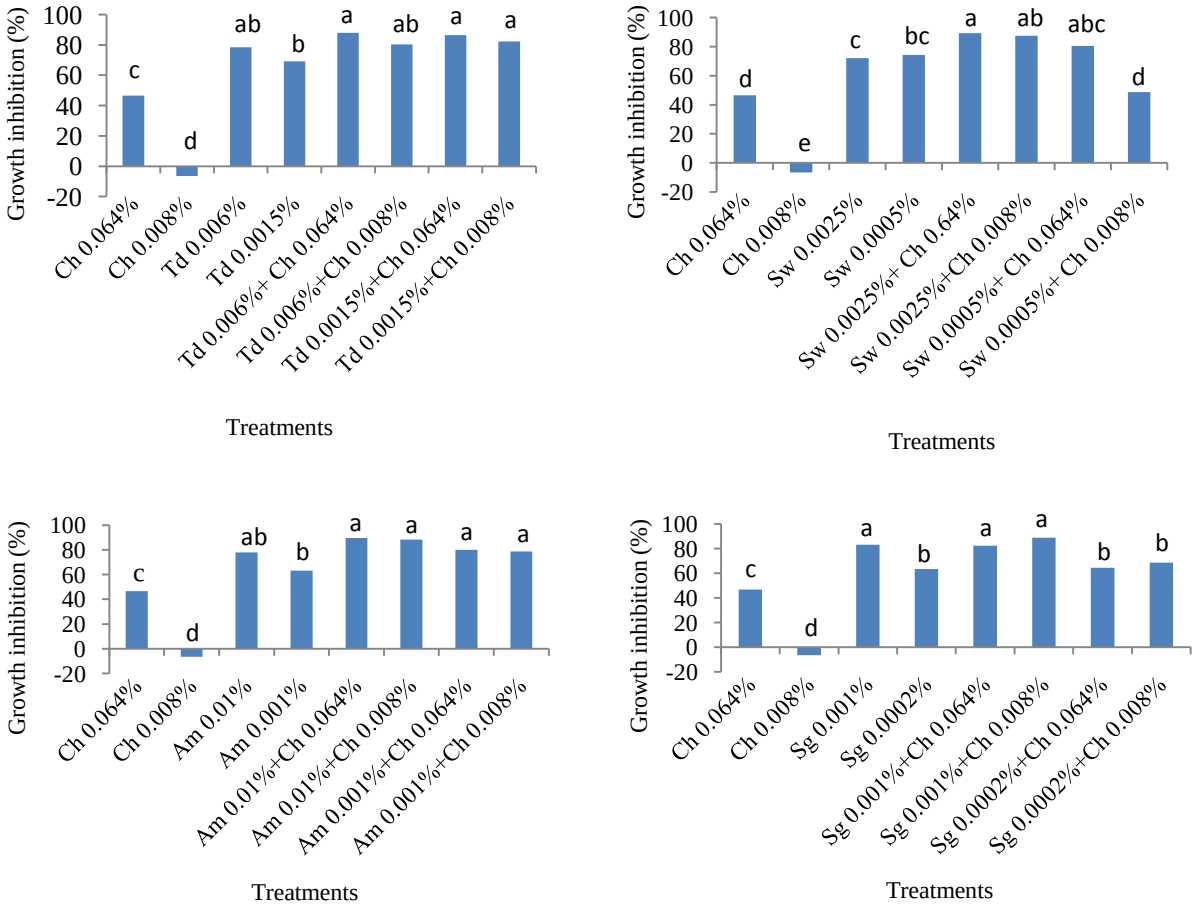


Figure 14. Effect of chitosan and fungicides on growth inhibition of *Botrytis cinerea* 101. Statistical analysis was done separately on each fungicide, its combinations with chitosan and chitosan alone. Bars having the same letter within each fungicide group are not statistically different (P=0.01). Here, Ch= chitosan, Td=Teldor, Sw= Switch, Am= Amistar, Sg= Signum

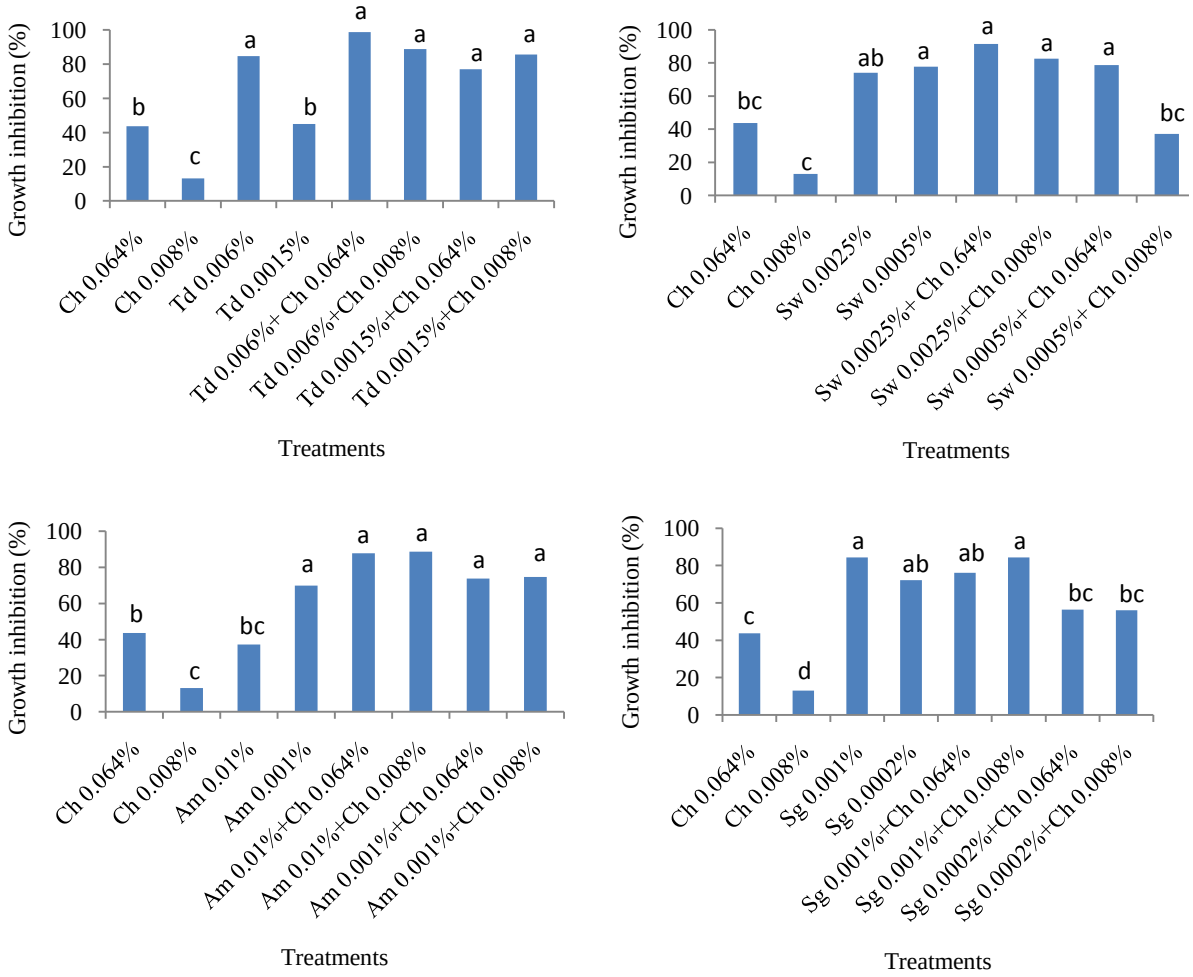


Figure 15. Effect of chitosan and fungicides on growth inhibition of *Botrytis cinerea*-BD. Statistical analysis was done separately on each fungicide, its combinations with chitosan and chitosan alone. Bars having the same letter within each fungicide group are not statistically different (P=0.01). Here, Ch= chitosan, Td=Teldor, Sw= Switch, Am= Amistar, Sg= Signum

Effect of combination of chitosan and fungicides on conidia germination of *M. majus* is shown in Table 6. Some combination treatments showed synergism and some combinations showed additivity. Combination treatments of chitosan with Signum showed more effectiveness than other chitosan and fungicide treatment combinations. The combination of 0.001 % Amistar + 0.064 % chitosan showed synergism which was significantly different from all other individual and combinations of Amistar and chitosan. The combination of 0.0001% Signum + 0.064% chitosan showed high synergism (100% germination inhibition), whereas 0.0001% Signum alone showed 26% and 0.064% chitosan showed 3% germination inhibition. Though complete inhibition of conidia germination was achieved by some combinations of Switch and chitosan, they show little synergism. This is probably because Switch was so effective alone.

Table 6. Effect of chitosan and fungicides on conidia germination of *Microdochium majus* recorded 24 hours after inoculation

Treatment	% inhibition of *M. majus*	Effect*
chitosan 0.064%	3 c	
chitosan 0.008%	3 c	
Teldor 0.006%	4 c	
Teldor 0.0015%	2 c	
Teldor 0.006%+ chitosan 0.064%	9 b	1.4
Teldor 0.0015%+chitosan 0.064%	4 c	0.7
Teldor 0.006%+chitosan 0.008%	13 a	1.8
Teldor 0.0015%+chitosan 0.008%	9 b	1.7
chitosan 0.064%	3 d	
chitosan 0.008%	3 d	
Switch 0.0005%	93 b	
Switch 0.0001%	71 c	
Switch 0.0005%+ chitosan 0.64%	100 a	1
Switch 0.0001+ chitosan 0.064%	100 a	1.4
Switch 0.0005%+ chitosan0.008%	100 a	1
Switch 0.0001%+ chitosan 0.008	93 b	1.4
chitosan 0.064%	3 f	
chitosan 0.008%	3 f	
Amistar 0.0001%	11 d	
Amistar 0.00005%	7 e	
Amistar 0.0001%+chitosan 0.064%	54 a	4
Amistar 0.00005%+chitosan 0.064%	22 b	2.3
Amistar 0.0001%+chitosan 0.008%	20 b	1.4
Amistar 0.00005%+chitosan 0.008%	15 c	1.5
chitosan 0.064%	3 e	
chitosan 0.008%	3 e	
Signum 0.0001%	26 d	
Signum 0.00005%	26 d	
Signum 0.0001%+chitosan 0.064%	100 a	3.6
Signum 0.00005%+chitosan 0.064%	98 a	3.5
Signum 0.0001%+chitosan 0.008%	57 b	2
Signum 0.00005%+ chitosan 0.008%	39 c	1.4

* Ratio between the observed efficacy and the expected efficacy of the combination. Ratio close to 1 indicates additivity, whereas ratio greater than 1 indicates synergy. (Cohen and Levy, 1990). Values within a column with a common letters do not differ significantly (P=0.01)

4.3.4 Effect of chitosan and fungicide on *B. cinerea* 101 infection in detached strawberry flower

When detached strawberry flowers were treated with chitosan and fungicides, the infection *of B. cinerea* 101 was recorded daily up to 8 days after inoculation to calculate the area under disease progress curve (AUDPC). The AUDPC value helped to measure the disease severity. The lower the AUDPC value, the lower the disease severity. It was found that all combinations of chitosan and fungicides had better effects compared to their individual effect (Figure 16).

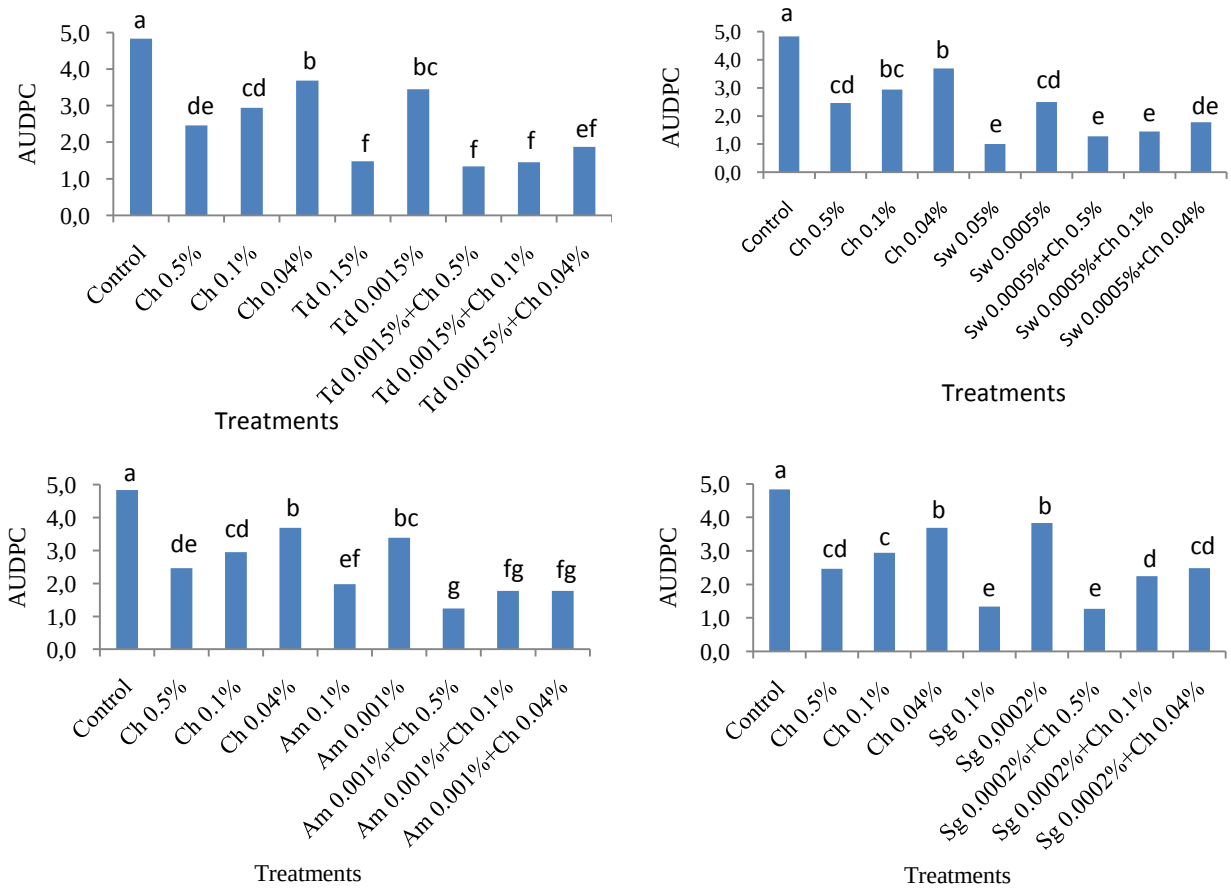


Figure 16. Effect of chitosan, fungicides and their combinations on the disease severity caused by *Botrytis cinerea* 101 in the detached strawberry flowers assay (Here, Ch= chitosan, Td=Teldor, Sw= Switch, Am= Amistar, Sg= Signum). Statistical analysis was done separately on each chitosan, fungicide, its combinations with chitosan and control alone. Bars having the same letter within each fungicide group are not statistically different (P=0.01)

The control flowers that were treated with only conidial suspension of *B. cinerea* 101 showed visible sign of infection 3 days after inoculation and they were completely (100%) infected 4 days after inoculation. 0.1% chitosan combined with 0.0015% Teldor reduced the disease severity as much as the recommended dose (0.15%) of Teldor and there was no significant difference among the recommended dose and the combination treatments. The combination of

0.5% chitosan with 0.001% Amistar showed better disease reduction than the recommended dose of Amistar (0.1%).

Detached strawberry flower assay was repeated to see the effect of chitosan, fungicide and combination of chitosan and funcides on *B. cinerea* 101 infection (Figure 17). In this case only 0.04% chitosan combined with different fungicides concentrations was used, as these combinations showed good result in previous experiment (Figure 16). Almost all combinations of 0.04% chitosan and fungicdes showed better effect in reducing the disease severity than the components alone. Combination of chitosan with Teldor showed better disease reduction than combination of chitosan with other fungicides. 0.0005% Switch+0.04% chitosan and 0.0002% Signum+0.04% chitosan showed antagonism.

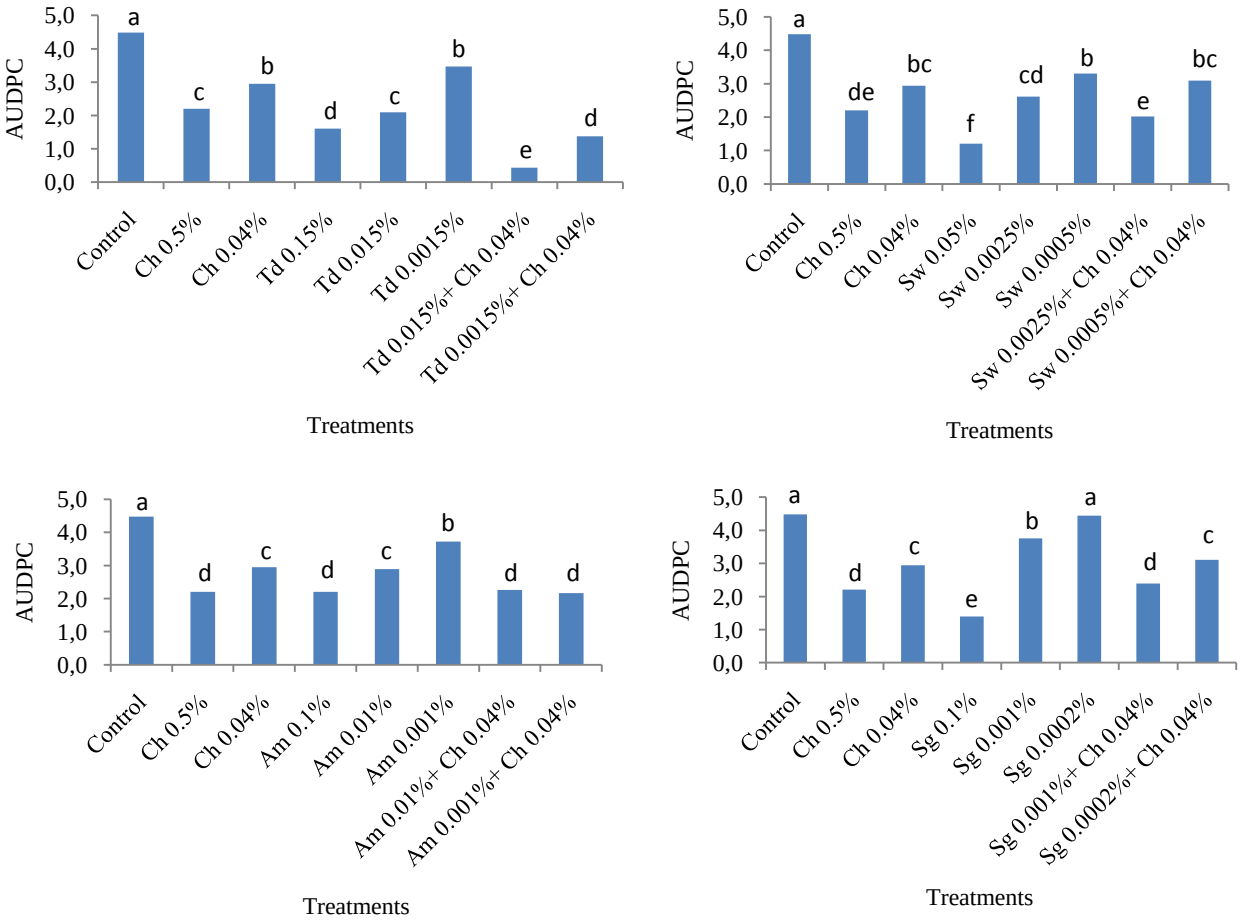
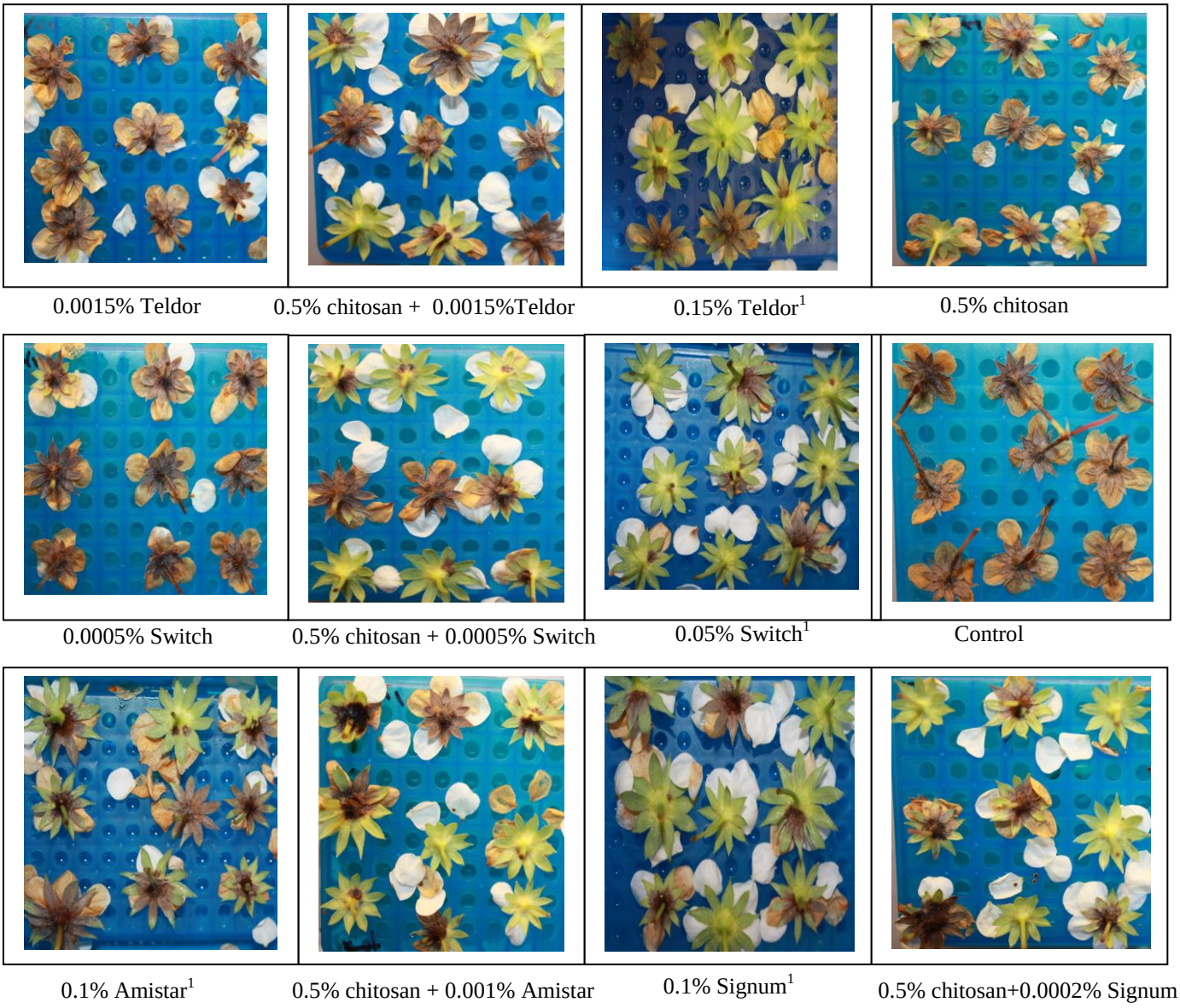


Figure 17. Effect of chitosan, fungicides and their combinations on the disease severity caused by *Botrytis cinerea* 101 in the detached strawberry flowers assay (Here, Ch= chitosan, Td=Teldor, Sw= Switch, Am= Amistar, Sg= Signum). Statistical analysis was done separately on each chitosan, fungicide, its combinations with chitosan and control alone. Bars having the same letter within each fungicide group are not statistically different



[1] recommended dose

Figure 18. Effect of chitosan, fungicides and their combinations on disease severity caused by *Botrytis cinerea* 101 in detached flowers 8 days after inoculation. Disease severity is indicated by the number of lesions on the flower sepals. The photographs illustrate some of the treatments shown in Figure 16.

In all cases combination treatments showed better reduction of the disease severity than the individual treatments (Figure 18). 8 days after inoculation, sporulation of *B. cinerea* 101 was seen on inoculated control flowers (data not shown). Inoculated flowers treated with 1/100 of recommended doses of Teldor, Switch and Amistar and 1/500 of Signum looked like inoculated control flowers. However, when these fungicide concentrations were combined with 0.5% chitosan there was no more infection than with the recommended fungicide dose.

4.4 Hydrolysis of chitosan

When chitosan was hydrolyzed by chitosanase ScCsn46A, as the hydrolysis time increased the average degree of polymerization (DPn) was reduced and degree of scission (α) was increased (Table 7).

Table 7. Characterization of chitosan following hydrolysis by 0.1µl/ml chitosanase ScCsn46A. α is degree of scission

Hydrolyzed time in min.	DPn	α value
10	56	0.02
20	49	0.02
30	41	0.02
40	34	0.024
50	25	0.04

4.4.1 Comparison of hydrolyzed and unhydrolyzed chitosan

Antifungal activity of hydrolyzed (DPn 41) and unhydrolyzed chitosan (DPn 206) depends on their concentrations (Figure 19 & Appendix K). Less than 0.016% hydrolyzed or non-hydrolyzed chitosan had almost no effect on conidia germination of *B. cinerea*101. Percent inhibition of conidia germination increased significantly as chitosan concentrations exceeded 0.016%, and almost 100% germination inhibition was observed with 0.25% concentration of both chitosan samples. Hydrolyzed and non-hydrolyzed chitosan both showed a clear dose response.

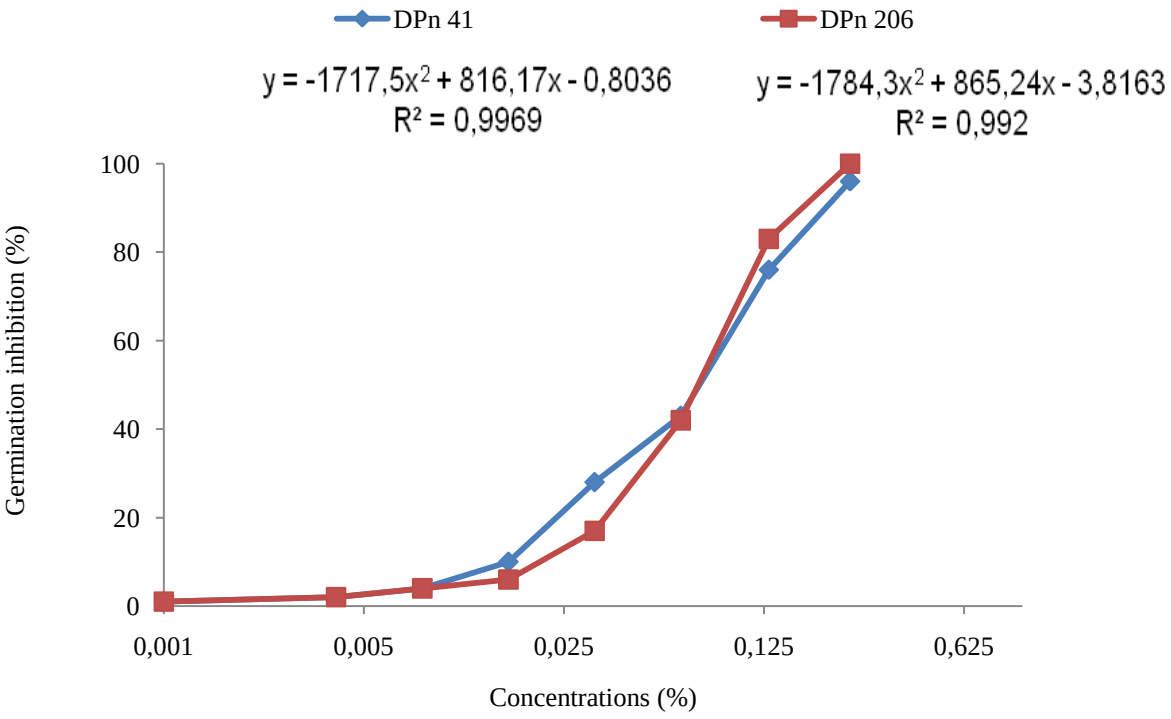


Figure 19. Effect of hydrolyzed chitosan (DPn 41) and unhydrolyzed chitosan (DPn 206) on conidia germination inhibition of *Botrytis cinerea* 101 recorded 24 hours after inoculation

5 Discussion

5.1 Characterization of the fungal isolates

Five of the fungal isolates used in this study are considered to be important pathogens of nursery plants, vegetables, ornamental, field and orchard crops and stored and transported agricultural products. Considering their importance and significant damage to agricultural products, their management is necessary. For this purpose the first and foremost step required is recognition of a pathogen. Morphological and molecular identification are two tools that help us to distinguish pathogens.

Morphological characteristics are greatly influenced by environmental and cultural conditions, so there is always some doubt about their effectiveness to identify the fungal species. Though morphological features such as sclerotial size and form and conidial size are useful for recognizing some *Botrytis* species, many species are morphologically similar and heterogeneous growing conditions also significantly influence variation (Beever et al., 2004). It is reported that identification of *Botrytis* species based on traditional criteria can be fraught as there is no key available to all recognized species (Nielsen et al., 2001). As the morphological characters with limited variation can mislead the species identification (Kullnig et al., 2001), the present study emphasizes on DNA based molecular identification. In this study an online interactive key (Samuels, G.J., Chaverri, P., Farr, D.F., & McCray, E.B. http://nt.ars-grin.gov/taxadescriptions/keys/TrichodermaIndex.cfm) was used for morphological identification of *Trichoderma* isolates, because it is widely used and coupled with molecular database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) it helped to identify the *Trichoderma* isolates in an ideal way.

5.1.1 Molecular Identification

In the present study amplification and sequencing of the ITS region of some fungal isolates in culture led to almost successful identification to the species level. *Botrytis cinerea* 101 was identified as *Botryotinia fuckeliana* (*Botrytis cinerea*) by nucleotide sequence. This strain was previously identified on the basis of carbon source utilization (Biolog system) as *B. cinerea* (L. Hjeljord, personal communication). *Botrytis cinerea*-BD, which was obtained from Gazipur, Bangladesh under the name *B. cinerea,* was identified as *Botryotinia fuckeliana* (*Botrytis cinerea*) by molecular identification. *Trichoderma*-BD was identified as *Trichoderma asperellum* by morphological characteristics. The molecular identification confirmed it as *T. asperellum* using BLAST analysis. When *Trichoderma*-P1 was identified

by morphological and molecular basis, the morphological identification was complemented by molecular identification and it was identified as *Trichoderma atroviride*. It was first reported as *T. harzianum* Rafai strain P1 (Tronsmo, 1989). Now it has been demonstrated that this strain belongs to *T. atroviride* (Kullnig et al., 2001). It was also identified as *T. atroviride* by morphological and molecular basis identification (Anees et al., 2010). *Alternaria*-328 was identified as *Alternaria brassicicola. Muco*r 199J was identified as *Mucor piriformis* which confirmed previous identification as *M. piriformis* by the Centraalbureau voor Schimmelcultures (CBS). Extraction of DNA from mycelium of *Microdochium majus* grown on cellophane was not successful, though it is reported that extraction of DNA from a filamentous fungus grown on cellophane is a rapid and economic method (Cassago et al., 2002, Lecellier and Silar, 1994). It might be that the amount of mycelium used to extract DNA was insufficient in the case of *M. majus*. This strain was obtained from Bioforsk under the name of *M. majus*.

Molecular identification using the Gene Bank database may not always identify the correct species, as some other species with the same sequence may exist in the database. The Gene Bank database may contain sequences from incorrectly identified fungal species, or many inexperienced researchers may put some sequences in the database considering the E-value of 0.00 without scrutinizing whether the sequence is identical or only highly similar (Druzhinina and Kubicek, 2005). That might be the reasons that different species under the same genera, different fungal species or uncultured fungal with the same E-value and same score appeared after the BLAST analysis in the present study.

5.1.2 Optimal growth conditions of the fungi

In the present study it was found that temperature and pH had effect on growth condition of fungi. *B. cinerea*-BD and *B. cinerea* 101 could grow within a temperature range of 5 to 30^0C with some morphological changes and varying growth rates. It was reported that the optimum temperature for growth of *B. cinerea* is (21-) 22-25 (-30) ^{0}C (Domsch et al., 1980) and conidia of *B. cinerea* can germinate in the pH range 2.0 – 9.8 on sugar beet decoction, with a broad optimum between pH 3 and 7 (Domsch et al., 1980). The result of the present study is in agreement with above statement. *T. asperellum* did not grow at 5^0C up to 21 days of incubation, but when it was put at room temperature it started to grow again. This indicates that it was dormant at 5^0C temperature. The optimum temperature for growth is in the wide range 15 – 35 (Domsch et al., 1980), but 30^0C represent a good average for most isolates which supports the result of the present study. As *T. atroviride* grew even at 5^0C, it can be

used as biological control agent to reduce the disease severity of *B. cinerea* in temperate region, as *B. cinerea* may be more susceptible to the inhibitors produce by *T. atroviride* at low temperature (Tronsmo and Dennis, 1978). It is reported that *T. atroviride* could successfully prevent cold storage fungal disease(Tronsmo, 1989). *T. asperellum* can be effectively used in tropical region as it could grow at 37^0C in this study.

5.2 Assessment of antagonistic activity and sensitivity to fungicides using in vitro tests

5.2.1 Dual cultures

Both of the *Trichoderma* isolates seemed to be moderately effective in controlling the test pathogens in dual culture technique. No clear inhibition zone was noticed between the radial growth of test fungi and *Trichoderma* isolates. Therefore temperature effects come into consideration. It might be possible that *Trichoderma* isolates did not produce enough inhibitory substance at 25^0C or diffusion of inhibitory substance might be affected by temperature or the aggressiveness of the test fungi was higher at 25^0C. An experiment was carried out and it was found that most of the *Trichoderma* species produced bigger inhibition zone at the lowest temperature (Tronsmo and Dennis, 1978). Though in present study no inhibition zone was noticed, *Trichoderma* isolates suppressed the radial growth of test pathogen through the over growth. It may be *Trichoderma* isolates parasitize the test pathogens by competition or coiling around pathogen hyphae, penetration and subsequently dissolution of the host cytoplasm (Shalini and Kotasthane, 2007). In some cases, without penetration *Trichoderma* causes the collapse and death of the hyphae of pathogen by coiling (Card, 2005). It is also reported that competitiveness of *Trichoderma* isolates depends on rapid growth and germination (Hjeljord and Tronsmo, 1998). In our study it was found that although *Trichoderma* isolates covered the whole area, up to a certain period sporulation of *Botytis* isolate was visible over the plate. It might be that the sporulation of *Botytis cinerea* started before the onset of antagonistic activity of the *Trichoderma* isolates (Strømeng, 2008).

5.2.2 Production of water-soluble inhibitory metabolites

It was reported that *Trichoderma* isolates produce water soluble metabolites that have fungicidal or fungistatic effect against plant pathogens (Tronsmo and Dennis, 1978). In this study it was found that water soluble metabolites produced by *T. atroviride* completely inhibited all test fungi and *T. asperellum* completely inhibited *M. majus* in PDA. By transferring inoculum discs of the test fungi to new PDA media, it was confirmed that

*Trichoderm*a isolates had fungicidal effect. *T. atroviride* showed less effectiveness in SNA (low nutrient media), where as *T. asperellum* was effective in both PDA and SNA. It could be that *T. atroviride* produced more water soluble metabolites in PDA than in SNA. *Trichoderma* isolates that are able to produce water soluble metabolites *in vitro* are also able to reduce disease *in vivo* (Anees et al., 2010). As *T. asperellum* produced water soluble metabolites in SNA as well, it could be a potential biocontrol agent in low nutrient areas where plant may be more susceptible to disease.

5.2.3 Production of inhibitory volatile metabolites

The volatile inhibitors produced by *Trichoderma* isolates were not very effective in reducing the radial growth of the test pathogens. But there is sufficient evidence that antibiotics may play an active role in biological control in nature (Fravel, 1988). In the present study in all cases radial growth inhibition of test pathogen by *Trichoderma* isolates was higher in PDA than SNA. When two plates were separated, very strong smell was emitted from inside the plates. Coconut odour came out from those plates where test fungi were inoculated with *T. atroviride*. Recently biochemical analysis has been carried out on antibiotics and it was found that many *Trichoderma* isolates produce volatile polyketide, 6-n-pentyl-2H-pyran-2-one (6PAP), which has a coconut-like aroma, and this volatile compound was partly responsible for antagonism on the high nutrient media (Card, 2005). The inhibition level of the test fungi might be slightly influenced by CO_2 in all cases as well. It also reported that CO_2 from *Trichoderma* species slightly reduced mycelial growth of test fungi (Tronsmo and Dennis, 1978). In this study it was also found that sporulation of test fungi was slowed down by inhibitory volatile metabolites. The effect on sporulation could be produced by the CO_2, but not by any other volatile components (Glen and Hutchinson, 1969).

Trichoderma isolates showed more antagonistic activity in PDA than in SNA. In most of the cases *T. atroviride* showed more antagonistic activity than *T. asperellum*. It was found that *T. atroviride* had higher competitive activity in high nutrient media than low nutrient media (Schubert et al., 2008). Findings of the present investigation are in agreement with the findings of the above investigators.

5.2.4 Sensitivity to fungicides

Telodor stimulates conidia germination of both *Trichoderma* isolates. They are moderately sensitive to Switch. Both *Trichoderma* isolates could be used in integrated strategies for the management of diseases. Use of *Trichoderma* isolate with fungicide to protect plant pathogen may provide consistent result and can reduce the risk of fungal pathogen to become resistant against fungicides. Fungicides can stress, weaken the pathogen and render pathogen propagules more susceptible to subsequent attack by antagonistic fungi (Tronsmo, 1995).

5.3 Inhibitory effect of unhydrolyzed chitosan and fungicides against fungi

5.3.1 Fungicides against fungi

According to the definition of conidia germination used in this experiment (when germtube exceeds the diameter of the conidium (Hjeljord et al., 2001) Teldor showed no inhibition of conidia germination of *B. cinerea* 24 hours after inoculation, but 72 hours after inoculation it showed that the germtube did not elongate further in all concentrations used in this experiment. It is reported that fenhexamid did not stop conidia germination of *B. fuckeliana,* but the germtubes did not elongate and seemed to be swollen, distorted and strongly branched (Guido et al., 2007). When Teldor comes in contact with the cell wall of the germ tube, sterone accumulation occurs which affects cell membrane fluidity and subsequently increases the permeability and fragility of the cell membrane (Debieu et al., 2001). This mode of action might prevent further growth of the fungus. Amistar was less effective against *B. cinerea* as well, whereas it was effective against *M. piriformis* and *M. majus.* Amistar is a systemic fungicide which can inhibit mitochondrial respiration of fungi and reduce spore production (Young, 2008). Cyprodinil, active ingredient of Switch, is highly effective against *Botrytis* and *Alternaria* by inbiting their penetration and their mycelia growth on the surface of and inside leaves (Heye et al., 1994). Another active ingredient of Switch is fludioxynil which induces morphological alteration of germ tube: i.e. swelling, branching and cell bursting (Leroux, 1996). In this study Switch showed clear dose response against *B. cinerea* and it was also effective against other test fungi. Under control condition, Switch and Teldor can significantly reduce incidence of sporulation on leaf surfaces by 44 to 51% (Strømeng, 2008). She also reported that Teldor can reduce the number of conidia produce by sclerotia by 81 to 96%. %. Signum was very effective against *B. cinerea* and *Rhizopus stolonifer in vitro,* field experiment and postharvest trial (Sallato et al., 2007). In this present study it was also found

that among all tested fungicides, Signum was the most effective fungicide against all of the test pathogens. Although all the fungicides which have been used in these experiments were name as botryticide, they were also effective against other plant pathogens. Their effectiveness varied from fungus to fungus. As only three concentrations of fungicide were used in the present study, it was difficult to find out the dose response with the different fungicides against different fungi and to calculate the LD_{50}. Why recommended dose of some fungicides did not work against some fungi is a question. It might be fungicide has specificity with specific fungus or the specific fungus has become resistant because of continuous use of that fungicide. It is also questionable why concentration of the recommended dose is so high when 1/100 times concentration of recommended dose can completely inhibit the conidia germination and growth of fungi. It could be because in vitro the fungicide can come in more direct contact with the conidia than in the field.

5.3.2 Chitosan against Fungi

When chitosan was applied to investigate its effectiveness against four test pathogens, it showed a dose response effect against *B. cinerea* 101, and 0.25% chitosan completely inhibited conidia germination of *B. cinerea* 101.Chitosan concentrations greater than 0.004% were effective against *A. brassicicola.* But less than 0.005% concentration of chitosan was not effective against *Penicillium expansum* (Yu et al., 2007). Chitosan showed less effectiveness against *M. piriformis* and *M. majus*. The antifungal activity of chitosan depends on its concentration (Palma Guerrero et al., 2008). Molecular weight of chitosan can be an important factor for its antifungal activity. "Aspergillus niger is a fungus whose cell wall mainly consists of chitin and chitosan and therefore it holds plenty of chitinase, by which chitosan excites much of its activity in fungus. Only the chitosan with proper molecular weight and concentration can highly excite the expression of chitinase in Aspergillus niger, make the cell distortion and finally killing the cell."(Li et al., 2008). This same hypothesis might be applicable in case of *M. piriformis* whose cell wall is composed of chitin as well (Allan and Hadwiger, 1979). In this study, when *M. piriformis* was treated with non-hydrolyzed chitosan (high molecular weight), it was not effective, only abnormal swelling of conidia was observed and after 72 hours hyphal network was seen in all concentrations of chitosan.

5.3.3 Synergistic effects of chitosan and fungicides

Possible synergistic interactions of chitosan and fungicides on inhibition of conidia germination of test fungi were studied in order to reduce the concentration of individual dose of chitosan and fungicides. In case of *B. cinerea* 101 and *B. cinerea*-BD combinations of chitosan with Teldor showed better synergism than the other treatment combinations of chitosan and other fungicides, whereas in case of *M. majus* a combination of 0.001% Amistar + 0.064% chitosan showed highest synergism. It was also reported that combination of chitosan with ethanol had synergistic effect to reduce the infection of *B. cinerea* in table grapes (Romanazzia et al., 2007). Level and importance of synergism depend on factors such as test methods, ratio and type of components in the mixture, target fungus or disease, and climatic conditions (Gisia et al., 1985). There is no specific explanation for different levels of synergism in different mixtures. Efficiency of synergism can depend on mode of action of the components. One component may facilitate another component's activity on cell wall or cell membrane of fungi (Waard and Nistelrooy, 1982). In only a few cases did the combination show slight antagonism.

5.3.4 Detached flower assay

The effect of combination of chitosan and fungicide on infection of detached strawberry flower showed good results. In most of the cases when the flowers under control and individual treatments started to show visible signs of infection, the flowers under combination treatments remained symptom-free. Although 8 days after inoculation the flowers treated with low concentration of chitosan and fungicide combination showed 100% infection, the disease severity was less than in the flowers treated with either chitosan or fungicide alone. So it was clear that combination treatment slowed down the infection rate as well as disease severity. In most of the cases there was no significant difference between the effect of recommended doses of fungicides and the combination effect of low amounts of fungicide and chitosan in term of disease severity.

In case of flower assay higher concentration of chitosan was used with fungicides than the concentration used in microtiter plate to see the synergistic effect since the two growth environments differ. In the microtiter plate liquid media (SM) was used where fungal conidia always remained in contact with the chitosan and fungicides even after germination, whereas on plant surface after germination the germtube penetrated the plant tissue and reduced the effect of chitosan and fungicide (Elad and Evensen, 1995). That might be the reason why *B.*

cinerea is more sensitive against combinations of chitosan and fungicides in microtiter plate than in detached strawberry flowers. In all cases, sporulation of *B. cinerea* was not seen on strawberry flowers 8 days after inoculation in the combination treatments. This means combination of chitosan and fungicides might reduce disease severity by reducing the conidia dispersal.

5.4 Comparison of hydrolyzed and unhydrolyzed chitosan

In this study hydrolyzed chitosan with DPn 41 was compared with unhydrolyzed chitosan with DPn 206 against *B. cinerea* to determine whether the degree of polymerization has any effect on inhibition of conidia germination. The two different DPn chitosans showed almost the same effectiveness regarding conidia germination inhibition and only two high (0.25% and 0.13%) concentrations showed effectiveness. Previous reports showed that chitosan with DPn 40 inhibited 100% conidial germination of *B. cinerea* at low concentration (0.008%) 24 hours after inoculation (Md. Hafizur Rahman, personal communication). Our hypothesis was DPn 41 would show similar inhibition as DPn 40. However DPn 41 showed the similar result as unhydrolyzed chitosan and less effectiveness compared to chitosan with DPn 40. The different antifungal activity of chitosan with DPn 40 and DPn 41 may be due to the distribution of chitosan chains (Gerasimenko et al., 2004). They used chitosan with MW 4 and 5 KDa and found different antimicrobial effects. In this study low enzyme concentration (0.1 µg/ mg chitosan) was used to hydrolyze chitosan to get DPn 41. Chitosan with DPn 40 used by Md. Hafizur Rahman was obtained using high enzyme concentration (0.5 µg/ mg chitosan). The concentration of enzyme used for hydrolysis of chitosan may be responsible for different chain lengths of similar DPn.

Conclusion and future study

Based on the present study it may be concluded that growth and morphology of the *Trichoderma* species and *Botrytis* strains were affected by temperature. *T. atroviride* grew at 5^0C, so it could be an effective biocontrol agent against *B. cinerea* in temperate region, as *B. cinerea* appeared to be susceptible to the inhibitors produce by *T. atroviride*. *T. asperellum* could be effective in tropical regions as it could grow at 37^0C. At 25^0C, which was the optimum temperature of the tested *Botrytis* strains and *Trichoderma* species, both of the *Trichoderma* isolates were found to be effective in reducing the radial growth of *B. cinerea* as well as other plant pathogenic fungi *in vitro*. Water soluble metabolites produced by the *Trichoderma* isolates completely inhibited the mycelial growth of the test fungi in high nutrient media. In future, a superior genetically manipulated *Trichoderma* strain that produces higher levels of water soluble metabolite might successfully be implemented under field conditions. As Teldor stimulated conidia germination in both *Trichoderma* isolates and they were moderately affected by Switch, both *Trichoderma* isolates may be used with Teldor or Switch in the integrated control of *B. cinerea* in future study.

Teldor, Switch, Signum could successfully control *B. cinerea* in synthetic media, although Amistar could not. All of the fungicides were effective against other test pathogens as well. Among the tested fungicides, Signum was the most effective against all of the test pathogens. Teldor was very effective in inhibiting germ tube elongation at low concentration. Unhydrolyzed chitosan effectively inhibited conidia germination of *B. cinerea* and *A. brassicicola*. Hydrolysis did not improve the antifungal activity of chitosan against *B. cinerea*. Combination of chitosan with each of the tested fungicides showed synergistic effect in reducing condia germination of *B. cinerea* 101, *B. cinerea*-BD and *M. majus* in synthetic media. Combination of chitosan and Teldor showed better synergism in inhibiting conidial germination of *B. cinerea* 101 and *B. cinerea*-BD in synthetic media and reducing disease severity of *B. cinerea* 101 on strawberry flower than combination of chitosan with other fungicides. As hydrolysis did not improve the antifungal activity of chitosan in this study, further research is required to make out the precise mode of action of chitosan against fungi. The results showing synergistic effect of chitosan and fungicide were very promising in this study. In future, a broad scale research to study the synergistic effects of fungicide and chitosan in integrated control is recommended. Integrated control using chitosan with *Trichoderma* spp. may provide alternatives to control plant diseases in the future. Moreover, further investigations on fungal resistance mechanisms to chemical and chitosan are warranted.

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Appendix index

Appendix A- Primers

Primers	Sequence	Producer	Catalogue no.
Primer ITS1F	5’ CTTGGTCATTTAGAGGAAGTAA 3’	Iinvitrogen	10336022/X4445E12
Primer ITS4	5’ TCCTCCGCTTATTGATATGC 3’	Iinvitrogen	10336-022/H4393D08

Appendix B - Nucleotide sequences and BLAST results for *Botrytis cinerea* 101

Query Length 523

Sequences producing significant alignments		Score (bits)	E Value	Max ident
HQ171053.1	*Botryotinia fuckeliana*	940	0.0	99%
HQ171052.1	*Botryotinia fuckeliana*	940	0.0	99%
GU054208.1	Uncultured fungus	940	0.0	99%
GQ999375.1	Uncultured fungus	940	0.0	99%
HM537028.1	Fungal endophyte	940	0.0	99%
HM037653.1	Uncultured soil fungus	940	0.0	99%
HM037648.1	Uncultured soil fungus	940	0.0	99%
EU821471.1	*Botrytis fabae*	940	0.0	99%
FJ820814.1	Uncultured fungus	940	0.0	99%
AM901713.1	Uncultured ascomycete	940	0.0	99%
EU128649.1	*Botryotinia fuckeliana*	940	0.0	99%
EU128648.1	*Botryotinia fuckeliana*	940	0.0	99%
EF207415.1	*Botryotinia fuckeliana*	940	0.0	99%
EF207414.1	*Botryotinia fuckeliana*	940	0.0	99%
EF207413.1	*Botryotinia fuckeliana*	940	0.0	99%
AM260931.1	Uncultured fungus	940	0.0	99%
DQ491491.1	*Botryotinia fuckeliana*	940	0.0	99%
AF455526.1	*Sclerotinia sclerotiorum*	940	0.0	99%
AF455523.1	*Sclerotinia sclerotiorum*	940	0.0	99%
AF455456.1	*Sclerotinia sclerotiorum*	940	0.0	99%

>Contig-0

CAAGGTTTCNGTAGGTGAACCTGCGGAAGGATCATTACAGAGTTCATGCCCGAAA
GGGTAGACCTCCCACCCTTGTGTATTATTACTTTGTTGCTTTGGCGAGCTGCCTTC
GGGCCTTGTATGCTCGCCAGAGAATACCAAAACTCTTTTTATTAATGTCGTCTGAG
TACTATATAATAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGA
AGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATC
GAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGGGGGGCATGCCTGTTCGA
GCGTCATTTCAACCCTCAAGCTTAGCTTGGTATTGAGTCTATGTCAGTAATGGCAG
GCTCTAAAATCAGTGGCGGCGCCGCTGGGTCCTGAACGTAGTAATATCTCTCGTT
ACAGGTTCTCGGTGTGCTTCTGCCAAAACCCAAATTTTTCTATGGTTGACCTCGGA
TCAGGTAGGGATACCCGCTGAACT

Appendix C – Nucleotide sequences and BLAST results for *Botritis cinerea*-BD
Query Length 544

Sequences producing significant alignments		Score bits)	E Value	Max ident
HQ171053.1	*Botryotinia fuckeliana*	973	0.0	99%
HQ171052.1	*Botryotinia fuckeliana*	973	0.0	99%
GU054208.1	Uncultured fungus	973	0.0	99
GQ999375.1	Uncultured fungus	973	0.0	99%
HM537028.1	Fungal endophyte	973	0.0	99%
HM037653.1	Uncultured soil fungus	973	0.0	99%
HM037648.1	Uncultured soil fungus	973	0.0	99%
FJ820814.1	Uncultured fungus	973	0.0	99%
EU128648.1	*Botryotinia fuckeliana*	973	0.0	99%
EF207415.1	*Botryotinia fuckeliana*	973	0.0	99%
EF207414.1	*Botryotinia fuckeliana*	973	0.0	99%
EF207413.1	*Botryotinia fuckeliana*	973	0.0	99%
AM260931.1	Uncultured fungus	973	0.0	99%
DQ491491.1	*Botryotinia fuckeliana*	973	0.0	100%
AF455526.1	*Sclerotinia sclerotiorum*	973	0.0	99%
AF455523.1	*Sclerotinia sclerotiorum*	973	0.0	99%
AF455456.1	*Sclerotinia sclerotiorum*	973	0.0	99%
AF455439.1	*Sclerotinia sclerotiorum*	973	0.0	99%
AF455413.1	*Sclerotinia sclerotiorum*	973	0.0	99%
AJ279480.1	*Sclerotinia sp*	973	0.0	99%

>Contig-0

GTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACAGAGTTCATGCCC
GAAAGGGTAGACCTCCCACCCTTGTGTATTATTACTTTGTTGCTTTGGCGAGCTGC
CTTCGGGCCTTGTATGCTCGCCAGAGAATACCAAAACTCTTTTTATTAATGTCGTC
TGAGTACTATATAATAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCG
ATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAAT
CATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGGGGGGCATGCCTGTT
CGAGCGTCATTTCAACCCTCAAGCTTAGCTTGGTATTGAGTCTATGTCAGTAATGG
CAGGCTCTAAAATCAGTGGCGGCGCCGCTGGGTCCTGAACGTAGTAATATCTCTC
GTTACAGGTTCTCGGTGTGCTTCTGCCAAAACCCAAATTTTTCTATGGTTGACCTC
GGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCATTAAAG

Appendix D - Nucleotide sequences and BLAST results for *Trichoderma*-BD
Query Length 590

Sequences producing significant alignments		Score (bits)	E Value	Max ident
FJ004799.1	*Trichoderma asperellum*	1052	0.0	99%
FJ612738.1	Fungal sp.	1052	0.0	99
EU280132.1	*Trichoderma asperellum*	1052	0.0	99%
EU280109.1	*Trichoderma asperellum*	1052	0.0	99%
HM545083.1	*Trichoderma asperellum*	1047	0.0	99%
HM545082.1	*Trichoderma asperellum*	1047	0.0	99%
HM545081.1	*Trichoderma asperellum*	1047	0.0	99%
HM545080.1	*Trichoderma asperellum*	1047	0.0	99%
HM545079.1	*Trichoderma asperellum*	1047	0.0	99%
GU318216.1	*Trichoderma asperellum*	1047	0.0	99%
FJ797513.1	*Trichoderma asperellum*	1047	0.0	99%
EF087981.1	Uncultured Hypocreales	1047	0.0	99%
DQ767600.1	Uncultured Hypocreaceae	1047	0.0	99%
AJ230669.1	*Trichoderma asperellum*	1045	0.0	99%
EU272525.1	*Trichoderma asperellum*	1041	0.0	99%
EU280110.1	*Trichoderma asperellum*	1041	0.0	99%
EU077227.1	*Trichoderma asperellum*	1041	0.0	99%
AF278789.1	*Trichoderma asperellum*	1040	0.0	99%
AF278788.1	*Trichoderma asperellum*	1040	0.0	99%
AJ230680.1	*Trichoderma asperellum*	1040	0.0	99%

>Contig-0

CCAGCGGAGGGATCATTACCGAGTTTACAATTCCCAAACCCAATGTGAACGTTAC
CAAACTGTTGCCTCGGCGGGGTCACGCCCCGGGTGCGTCGCAGCCCCGGAACCAG
GCGCCCGCCGGAGGAACCAACCAAACTCTTTCTGTAGTCCCCTCGCGGACGTATT
TCTTACAGCTCTGAGCAAAAATTCAAAATGAATCAAAACTTTCAACAACGGATCT
CTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTG
CAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCT
GGCGGGCATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCCCTCCGGGGGATCG
GCGTTGGGGATCGGGACCCCTCACACGGGTGCCGGCCCCGAAATACAGTGGCGG
TCTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGCACAACTCGCACCGGGAGCGCGG
CGCGTCCACGTCCGTAAAACACCCAACTTTCTGAAATGTTGACCTCGGATCAGGT
AGGAATACCCGCTGAACTTAAGCATATCAATTAAGCGGAGG

Appendix E - Nucleotide sequences and BLAST results for *Trichoderma* P1

Query Length 430

Sequences producing significant alignments		score (bits)	E Value	Max ident
FN646616.1	*Trichoderma atroviride*	176	1e-40	79%
GQ999243.1	Uncultured fungus	176	1e-40	79%
GQ999242.1	Uncultured fungus	176	1e-40	79%
FN812781.2	Uncultured fungus	176	1e-40	79%
GU060132.1	Uncultured Trichoderma	176	1e-40	79%
HM236005.1	*Trichoderma atroviride*	176	1e-40	79%
AB570248.1	*Trichoderma atroviride*	176	1e-40	79%
HM176575.1	*Trichoderma atroviride*	176	1e-40	79%
AB563715.1	*Hypocrea atroviridis*	176	1e-40	79%
AB563713.1	*Hypocrea atroviridis*	176	1e-40	79%
HM051191.1	*Trichoderma* sp	176	1e-40	79%
HM047764.1	*Trichoderma atroviride*	176	1e-40	79%
HM037987.1	*Rhizopus stolonifer*	176	1e-40	79%
HM037976.1	*Trichoderma viride*	176	1e-40	79%
HM037937.1	*Trichoderma viride*	176	1e-40	79%
HM037930.1	*Trichoderma atroviride*	176	1e-40	79%
HM037928.1	*Trichoderma viride*	176	1e-40	79%
GU947796.1	*Trichoderma atroviride*	176	1e-40	79%
AM981214.1	*Trichoderma atroviride*	176	1e-40	79%
GU595060.1	*Trichoderma atroviride*	176	1e-40	79%

>Contig-0

CTNGTGGACGNGNNACGCTCCCGGTGNGAGTTGTGCAAACTACTTCGCAAGAGA
NGGTGNNGCGAAACCGNCACTGNATTTCGGGGGCAGGGGATCCCNNNTTANGGG
NTCCCGAGGNCCCCAACGNCGACCCCNCGGAGGGGTTCGAGGGTNNANATGANN
NTGANACAGGTTTGCCCCACAGANNTCTCGGGGNTNNGGTGNGTTGAAAAAANC
TACGATAATNNAAATAATAATGGAAATTGCANAAATCATTGGAATCTTGCNAATC
TTTTTAANCCGNANTNNGCNCCACAAGNTTTTGGGGGGGAANTCCTNTTNAANTT
TTTTTTCAACCCTGNAAACCNNNAAGGGGGGGGGGGGGNGNNCANATAGAANA
NNTGANTAGGGCCTNCCGGCGNCCACCNNATTTNGGGGNCTGCNACNNAC

Appendix F - Nucleotide sequences and BLAST results for *Alternaria* 328

Query Length 598

Sequences producing significant alignments		score (bits)	E Value	Max ident
AY154707.1	*Alternaria brassicicola*	1058	0.0	99%
AF392986.1	*Alternaria brassicicola*	1011	0.0	100%
FJ266477.1	*Alternaria mimicula*	985	0.0	100%
FJ348226.1	*Embellisia conoidea*	985	0.0	100%
U05198.1	*Alternaria brassicicola*	976	0.0	100%
FJ465174.1	*Alternaria brassicicola*	975	0.0	100%
AF229462.1	*Alternaria brassicicola*	975	0.0	100%
AF392985.1	*Alternaria brassicicola*	971	0.0	100%
AY154703.1	*Alternaria japonica*	967	0.0	95%
DQ885386.1	*Pleospora papaveracea*	958	0.0	95%
GU983654.1	*Alternaria brassicicola*	946	0.0	99%
GQ496082.1	*Alternaria brassicicola*	946	0.0	100%
AF455497.1	*Pleospora papaveracea*	946	0.0	95%
AF455453.1	*Pleospora papaveracea*	946	0.0	95%
AF102888.1	*Pleospora papaveracea*	942	0.0	95%
AY625070.1	*Ulocladium botrytis*	921	0.0	95%
GQ995478.1	*Brachycladium papaveris*	913	0.0	95%
FJ357311.1	*Crivellia papaveracea*	913	0.0	95%
FJ357310.1	*Brachycladium papaveris*	913	0.0	95%
AJ876894.1	*Dendryphiella triticicola*	913	0.0	95%

>Contig-0

CTTGGTCATTTTTAGGGAAGTAAAAGTCGTAACAAGGTCTCCGTAGGTGAACCTG
CGGAGGGATCATTACACAATATGAAAGCGGGCTGGACTCACCTCAGCAGCATCT
GCTGTTGGGGCCAGCCTTGCTGAATTATTCACCCGTGTCTTTTGCGTACTTCTTGT
TTCCTTGGTGGGCTCGCCCACCACAAGGACAAACCATAAACCTTTTGTAATTGCA
ATCAGCGTCAGTAACAACATAATAATTACAACTTTCAACAACGGATCTCTTGGTT
CTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAAT
TCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGG
CATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGTCTT
GTCTCCAGTTTGCTGGAGACTCGCCTTAAAGTCATTGGCAGCCGGCCTACTGGTTT
CGGAGCGCAGCACAAGTCGCGCTCTCTTCCAGCCAAGGTCAGCATCCATAAAGCC
TTTTTTCAACTTTTGACCTCGGATCAGGTAGGGATACCCGCTGAACT

Appendix G - Nucleotide sequences and BLAST results for *Mucor* 199J

Query Length 489

Sequences producing significant alignments		score (bits)	E Value	Max ident
EU484276.1	*Mucor piriformis*	812	0.0	96%
FM173096.1	Uncultured compost fungus	796	0.0	96%
AM711393.1	Uncultured compost fungus	796	0.0	96%
AJ278359.1	*Mucor piriformis*	760	0.0	94%
EU484207.1	*Mucor mucedo*	482	7e-133	88%
EU484199.1	*Mucor mucedo*	479	8e-132	88%
AF412289.1	*Mucor mucedo*	479	8e-132	88%
EU484287.1	*Mucor mucedo*	471	1e-129	88%
EF555502.1	*Pilaira* sp.	385	1e-103	84%
FJ160942.1	*Pilaira anomala*	383	5e-103	84%
FJ160941.1	*Pilaira anomala*	383	5e-103	84%
AM778602.1	*Pirella circinans*	383	5e-103	80%
EF555499.1	*Pilaira anomala*	383	5e-103	84%
EF555498.1	*Pilaira anomala*	383	5e-103	84%
EF555497.1	*Pilaira anomala*	383	5e-103	84%
EF555496.1	*Pilaira anomala*	383	5e-103	84%
EF555494.1	*Pilaira anomala*	383	5e-103	84%
EF555486.1	*Pilaira anomala*	383	5e-103	84%
EF555485.1	*Pilaira anomala*	383	5e-103	84%
EU484282.1	*Mucor flavus*	379	6e-102	82%

>Contig-0

AAAAGTTTCTAGGCAGACAGAAGAACAATCTTAGCCAAATATTATTAAGGAGAA
CTGCCCGTATGAAGGGGCGGTTAATCTCCCAAGACCATCGTTAAAAAAAAAAAA
TTTTTAAGATGTGGGGTGTTTTTGATACTGAAACAGGCGTGGGCGGTGGAATACC
AACCACCNCAAGATGCGTTCAAAGACTCGATGATTCNCTGAATATGCAATTCACA
CTAGTTATCGCACTTTGCTACGTTCTTCATCGATGCGAGAACCAAAAGATCCGTTG
TTAAAAGTTGTTTTAATGGTTTGTTAAGCCATATTAAAATATTTTAAAATGAATTC
TTTTGGATAATAATAATTTTTTAAGGACACCAAGCCTAAGCTTGATCNCGACTCG
GTTAACATCTTTTCTGGCCTGGCCNTTATGACCAGAGAAGCATTCCTCAAGCGCC
GCGCAATAATACAGTTCACGGTAAAAAATGATATGAGTGAAAAGAGAAG

Appendix H - preparation of 2 X Synthetic Media (2 SM)

The SM media was made from the following stock solutions;

Solutions and dilutions	
10 x A (without iron):	10 g NH_4NO_3 2 g $KCl.2H_2O$ 2 g $MgSO_4.7H_2O$ 0.02 g $MnSO_4.4\ H_2O$ 0.02 g $ZnSO_4$ 1000 ml H_2O
10 x B (0.1M):	6.8 g KH_2PO_4 1000 ml H_2O
1% Ferric Citrate	1 g $FeC_6H_5O_7.5H_2O$ 100 ml H_2O
20% Glucose:	20 g glucose 100 ml H_2O

20% glucose, 10 x A and 10 X B solutions were autoclaved separately at 121^0C for 15 minutes. 1% ferric citrate solution was sterilized by filtration through a 0.22 µm sterite syringe filter.

We used 2 X SM to make conidial suspention, with and without chitosan and/or fungicides Final concentration 1X SM in the microtiter plate.

Solutions and dilutions	
10 ml 2 X SM	0.4 ml A 0.4 ml B 1 ml 20% glucose 0.04 ml 1% Ferric Citrate 8.16 ml water

Appendix I - Effect of fungicides on inhibition of conidia germination recorded 24 hours after inoculation

Fungicide	Concentrations (%)	Inhibition of conidia germination (%)			
		B. cinerea (BC101)	*M. piriformis*	*M. majus*	*A. brassicicola*
Teldor	0.15 [1]	1 e*	83 b	100 a	2 h
	0.015	1 e	27 e	8 d	4 g h
	0.0015	1 e	18 f	4 e	3 h
Switch	0.05 [1]	100 a	100 a	100 a	85 c
	0.005	78 b	19 f	94 b	82 c
	0.0005	35 c	6 g	63 c	19 d e
Amistar	0.1 [1]	28 d	82 b	100 a	20 d
	0.01	3 e	73 c	100 a	14 e f
	0.001	2 e	67 d	100 a	9 f g
Signum	0.1 [1]	100 a	100 a	100 a	100 a
	0.01	100 a	100 a	100 a	100 a
	0.001	33 c	100 a	100 a	91 b

[1] recommended dose

* Values within a column with common letters do not differ significantly (P=0.05)

Appendix J - Effect of chitosan (Kitoflokk) on inhibition of conidia germination recorded 12 hours and 24 hours after inoculation.

Concentration of chitosan (%)	% germination inhibition							
	B. cinerea (BC101)		*M. piriformis*		*M. majus*		*A. brassicicola*	
	12 h	24 h	12 h	24 h	12 h	24 h	12 h	24 h
0.25	100 a*	100 a	100 a	30 a	75 a	33 a	100 a	100 a
0.13	100 a	83 b	100 a	25 b	45 b	26 b	100 a	100 a
0.064	77 b	42 c	100 a	19 c	23 c	20 c	100 a	100 a
0.032	33 c	17 d	100 a	18 c	17 d	15 d	100 a	100 a
0.016	14 d	6 e	100 a	17 c	16 de	10 e	100 a	100 a
0.008	7 e	4 e f	100 a	15 c	13 d e	5 e f	100 a	100 a
0.004	3 f	2 e f	100 a	11 d	12 e	3 f	48 b	30 b
0.001	2 f	1 f	19 b	8 d	3 f	3 f	17 c	12 c

* Values withing a column with common letters do not differ significanty (P=0.05)

Appendix K - Effect of hydrolyzed chitosan (DPn 41) and non-hydrolyzed chitosan (DPn 206) on inhibition of conidia germination of *Botrytis cinerea* (BC101) recorded 24 hours after inoculation

Chitosan concentration (%)	% germination inhibition	
	DPn 41	DPn 206
0.25	96 a*	100 a
0.13	76 b	83 b
0.063	43 c	42 c
0.031	28 d	17 d
0.016	10 e	6 e
0.008	4 f	4 f
0.004	2 f	2 f
0.001	1 f	1 f

*Values within a column with common letters do not differ significantly (P=0.05)