Antifungal activity of chitosan/chitooligosaccharides alone and in combination with chemical fungicides against fungal pathogens

Antisopp aktivitet av kitosan/chitooligosakkarider alene og i kombinasjon med kjemiske fungicider mot sopp-patogener

Philosophiae Doctor (PhD) Thesis

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Md. Hafizur Rahman
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Summary

Botrytis cinerea Pers. ex Fr. is an economically important pathogen causing gray mold in over 200 plants species worldwide, including strawberry (*Fragaria* × *ananassa*). In Norway, gray mold is economically the most important disease on strawberry and may cause 30-60% yield loss. In Bangladesh, the main constraint of chickpea production is attack by *B. cinerea*, which may cause up to complete yield loss. Thus farmers are highly concerned to control this disease but they are mainly dependent on commercially available chemical fungicides. In developing countries like Bangladesh, farmers use excessive amounts of chemical fungicides to control *B. cinerea* without having safety knowledge about chemical fungicides and this creates health, environmental and disease resistance problems. This PhD project studied chitosan and chitooligosaccharides (CHOS) as alternatives and means to reduce the use of chemical fungicides without reduced efficacy. Chitosan or CHOS alone and mixtures of chitosan or CHOS combined with reduced amounts of chemical fungicides were tested *in vitro* (in microtiter plates) and *in vivo* (strawberry flower, chickpea and bean leaf assays) against *B. cinerea.*

Chitosan is a biopolymer obtained by partial deacetylation of chitin. The antifungal activity of chitosan depends on factors (Paper I) such as the fraction of acetylation (F_A) (chitosan with low F_A was more inhibitory to *B. cinerea* than high F_A), the pH and cations present in the media (the antifungal activity of chitosan with low F_A was higher at pH 6 than 3-5 and increasing Ca^{2+} and Mg^{2+} concentrations in the media reduced the ability of chitosan to inhibit *B. cinerea*).

The antifungal activity of chitosan also depended on the average degree of polymerization (DP_n) CHOS with DP_n 23 and 40 were the most effective inhibiting germination of all tested pathogens. *Mucor piriformis* and *B. cinerea* BCBD were sensitive (in terms of germination inhibition) to a wide range of CHOS (DP_n 75, 58, 48, 40, 15; DP_n 40, 23, 15 and 11 respectively), but *B. cinerea* BC 101 was only inhibited by DP_n 23-40 (Paper III). As chitosan or CHOS alone were not as effective as chemical fungicides, the combination of chitosan or CHOS with chemical fungicides were tested to control *B. cinerea in vitro* and *in vivo* (Paper II and VI). Our study showed that both chitosan and CHOS showed synergism (in terms of inhibiting *B. cinerea*) with chemical fungicides and CHOS were more effective than chitosan. *In vitro*, CHOS showed high levels of synergism with all tested fungicides, i.e. CHOS DP_n 23 (5 μg mL^{-1}), Amistar and Signum (10 μg mL^{-1}) alone only showed 2-4% germination inhibition of *B. cinerea*

BC 101, but the combinations of CHOS and Amistar or CHOS and Signum, in the aforementioned concentrations and DP_n, showed >90% germination inhibition. Chitosan (DP_n 206) only showed low levels of synergism with Teldor and Switch. *In vivo* bioassay of strawberry flowers and chickpea leaves showed a similar trend as *in vitro* where CHOS showed more synergism with chemical fungicides than chitosan (DP_n 206) in inhibiting *B. cinerea*. However, there was no difference between chitosan and CHOS in combination with chemical fungicides to reduce bean leaf infection by *B. cinerea*.

Our study suggested that CHOS used in combination with chemical fungicides could considerably reduce the need for chemical fungicides by enhancing their efficacy. Application of this knowledge in Integrated Pest Management programs against plant pathogens could reduce the need for chemical fungicides, thus causing less harm for the environment and human health.

Sammendrag

Botrytis cinerea Pers. ex Fr. er en økonomisk viktig patogen som forårsaker gråskimmel på over 200 plantearter over hele verden, deriblant jordbær (*Fragaria* × *ananassa*). I Norge, er gråskimmel den økonomisk viktigste sykdommen på jordbær og kan føre til 30-60% avlingstap. I Bangladesh, er det alvorligste problemet i kikkerter produksjonen, angrep av *B. cinerea*, som kan føre opp til totalt tap av avlingen. Bøndene er derfor svært opptatt av å kontrollere denne sykdommen og de er avhengig av å benytte kommersielt tilgjengelige kjemiske fungicider. I utviklingslandene som Bangladesh, bruker bøndene store mengder kjemiske fungicider for kontroll av *B. cinerea* uten å ha nødvendig sikkerhetsopplæring og dette skaper helse, miljø og sykdomsresistens problemer. I dette PhD prosjektet ble det undersøkt om kitosan og kitooligosakkarider (CHOS) kan være et alternativ for å redusere bruken av kjemiske fungicider uten å redusere sykdomskontrollen. Chitosan eller CHOS alene eller en blanding av kitosan eller CHOS kombinert med redusert mengde kjemiske fungicider ble testet *in vitro* (i mikrotiter plater) og i*n vivo* (på jordbær blomster, kikkerter blad eller bønne blad) mot *B. cinerea.*

Kitosan er en biopolymer fremstilt ved partsiell deasetylering av kitin. Antisopp aktivitet av kitosan er avhengig av faktorer (Paper I) slik som fraksjonen av acetylering (F_A) (kitosan med lav F_A var mer hemmende overfor *B. cinerea* enn kitosan med høy F_A), pH og kationer tilstede i media (den antifungal aktivitet av kitosan med lav F_A var høyere ved pH 6 enn ved 3-5 og økende Ca^{2+} og Mg^{2+} konsentrasjoner i media reduserte evne kitosan hadde til å inhibere *B. cinerea*).

Antisopp aktivitet av kitosan er også avhengig av gjennomsnittlig grad av polymerisering (DP_n) av kitosan. CHOS med DP_n 23 og 40 var de mest effektive hemmere av spiring av alle testede patogener. *Mucor piriformis* og *B. cinerea* BCBD var følsomme (i form av spiring inhibering) til et bredt spekter av CHOS (DP_n 75, 58, 48, 40, 15; DP_n 40, 23, 15 og 11 henholdsvis), mens *B. cinerea* BC 101 bare ble hemmet av DP_n 23-40 (Paper III). Fordi kitosan eller CHOS alene ikke var så effektive som kjemiske fungicider, ble blandinger av kitosan eller CHOS med kjemiske fungicider testet mot *B. cinerea in vitro* og *in vivo*. (Papir II og VI). Vår studier viste at både kitosan og CHOS hadde evnen til å vise synergisme (i form av å hemme *B. cinerea*) med kjemiske soppmidler og CHOS var mer effektiv enn kitosan. *In vitro* viste CHOS høye nivåer av synergisme med alle testede fungicider, dvs. mens CHOS DP_n 23 (5 ug ml-1), Amistar og

Signum (10 µg ml-1) alene bare viste 2-4% spiring hemming av *B. cinerea* BC 101, viste kombinasjoner av CHOS og Amistar eller CHOS og Signum, i de nevnte konsentrasjoner og DPN, > 90% spire hemming. Kitosan (DP_n 206) viste lave nivåer av synergisme med Teldor og Switch. *In vivo* biotester med jordbær blomster og kikertblader viste en lignende trend som *in vitro* hvor CHOS var mer effektivt og vise større synergisme med kjemiske soppmidler enn kitosan (DP_n 206) i hemning av *B. cinerea*. Imidlertid var det ingen forskjell mellom kitosan og CHOS i kombinasjon med kjemiske fungicider for å redusere infeksjon av bønneblader med *B. cinerea*.

Vår studier viser at CHOS brukt i kombinasjon med kjemiske soppmidler kan redusere behovet for kjemiske soppmidler ved å styrke deres effekt. Anvendelsen av denne kunnskapen i Integrert plantevern (IPM) mot plantesykdommer kan redusere behovet for kjemiske soppmidler og dermed forårsaker mindre skade for miljøet og menneskers helse.

List of Papers

I. **Factors affecting the antifungal activity of chitosan against *Botrytis cinerea***

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II. **Effect of combination of chitosan and Teldor (fenhexamid) against *Botrytis cinerea***

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III. **Effect of chitooligosaccharides with different degrees of polymerization on *Botrytis cinerea* and *Mucor piriformis***

Md. Hafizur Rahman, Linda Gordon Hjeljord and Arne Tronsmo

IV. **Inhibition of fungal pathogens by chitooligosaccharides and chemical fungicides alone and in combination**

Md. Hafizur Rahman, Latifur Rahman Shovan, Linda Gordon Hjeljord, Morten Sørlie, Berit Bjugan Aam, Vincent Eijsink and Arne Tronsmo

1. Introduction

In the present study chitosan and chitooligosaccharides (CHOS) were used alone and in combination with chemical fungicides (Teldor, Switch, Amistar and Signum) against plant pathogens such a *Botrytis cinerea, Mucor piriformis, Microdochium majus* and *Alternaria brassicicola.* The study focused on *B. cinerea,* thus the following section will focus on that pathogen.

1.1 Gray mold

1.1.1. Causal agent

Gray mold is caused by the necrotrophic pathogen *Botrytis cinerea* Pers. Fr. (anamorph of *Botryotinia fuckeliana*), which is among the most common and widely distributed plant pathogens.[1]

1.1.2 Morphology

B. cinerea is an ascomycete fungus of the Botryotinia genus, Sclerotiniaceae family and Heliotiales order.[1] *B. cinerea* has light brown, septate and erect conidiophores that produce bunches of 1-2 celled globose or oval conidia.[2] The conidia measure between 4-25×4-18 µm (from infected chickpea) and 4-16×4-10 µm (on potato dextrose agar, PDA).[2]

1.1.3 Disease cycle

When *B. cinerea* conidia reach the host a weak adhesion to the host surface occurs.[3] Secondly, germlings and conidia secrete a film, a fungal sheath that creates a strong binding to the host surface.[3]

Figure 1 shows the disease cycle of *B.* cinerea. The pathogen can overwinter as mycelia in the host[4] or survive for several years as sclerotia in soils[5] or on plants debris.[4] In favourable conditions mycelia and sclerotia produce asexual spores on the conidiophores, but the pathogen can also produce sexual spores (ascospores), which are released from apothesia.[5]

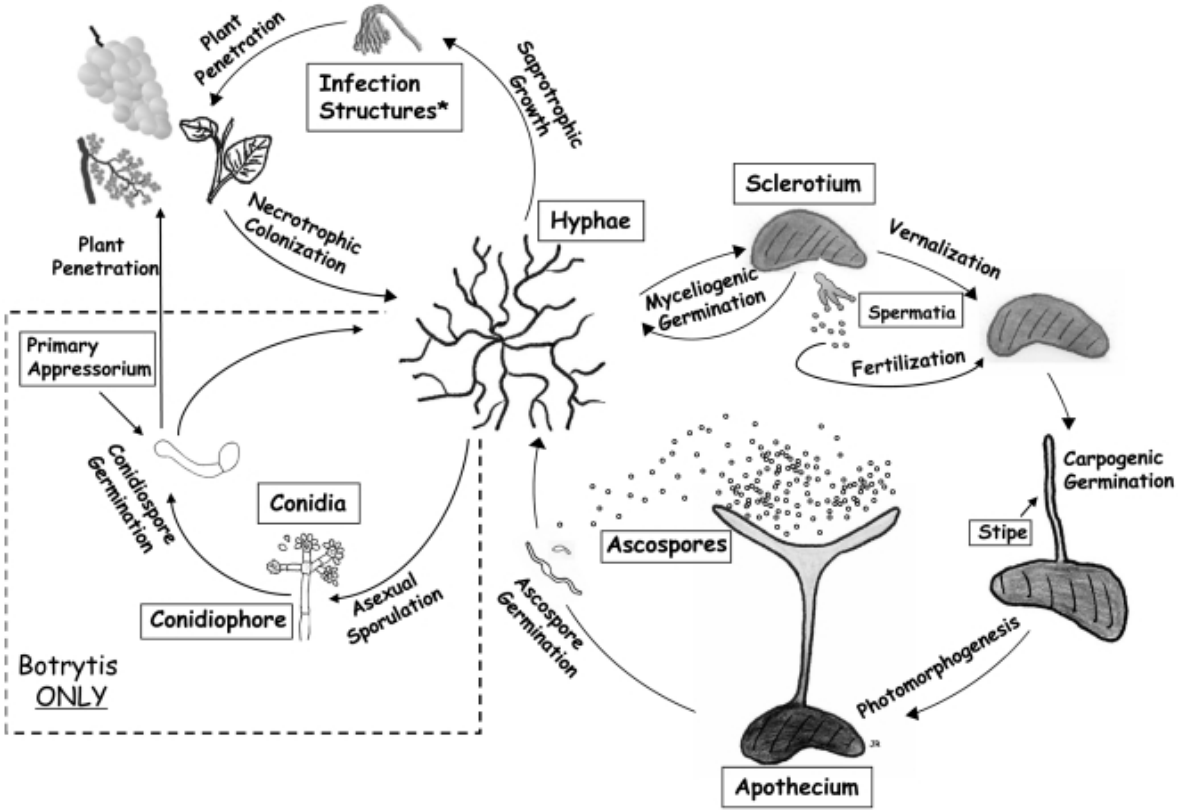

Figure 1. Life cycle of *B. cinerea.*[5]

Germination of conidia depends mainly on free surface water and atmospheric relative humidity (RH), where the optimum RH for germination and growth of *B. cinerea* is above 98% (Peterson et al. as cited by Petäistö and Heiskanen).[6,7] The fungi can germinate in various environments, but optimal is a temperature around 19°C and pH around 5.[8] However, *B. cinerea* is able to grow down to 0°C.[9] Nutrients such as sucrose, glucose and ammonium sulphate may trigger germination of *B. cinerea* conidia.[8]

B. cinerea enters the host by direct penetration or through open stomata or wounds.[10] *B. cinerea* first secrets toxins, enzymes (such as cutinases, pectinases and proteases)[11] and compounds such as oxalic acid to degrade the host cell walls.[1] Research suggest that the stamens are the main infection sites for *B. cinerea* on strawberry.[12] Conidia landing on the stigma may germinate using the stigmatic fluid. Then the germ tubes grow intercellular and reach the carpel within seven days.[13] *B. cinerea* may infect and kill the strawberry flowers, this is termed aggressive blossom blight by Jarvis and Borecka (as cited by Strømeng),[14,15] remains dormant and causes fruit rot

when the fruit starts to ripen (Jarvis and Borecka as cited by Xu et al.).[14,16] Strawberry fruits contain phenolic compounds which are inhibitory to *B. cinerea*.[17] Green strawberry fruits contain at least two antifungal compounds that are not present in white or red fruits, thus antifungal activity decreases with maturity of the fruit.. The *B. cinerea* is able to cause infection in a wide range of temperatures. Generally 15^{o}C is the optimal temperature, but 20^{o}C was optimal for strawberry flower infection (100% infection) at 24 hours wetness.[18]

Sporulation of *B. cinerea* depends on RH, temperature, radiation and wind speed. *B. cinerea* at RH 94% and wind speed 0.6 m sec^{-1} showed high sporulation[19] and near ultraviolet (UV) light induces sporulation in culture.[1] Temperature increase together with sudden raise or decline in humidity make conidiophores eject conidia (Jarvis as cited by Williamson et al.).[1,20] The optimum temperature range for sporulation on strawberry leaves is 17-18^{o}C.[21] *B. cinerea* sporulates abundantly and up to 10^4 spores m^{-3} of air has been recorded.[20] Sporulation normally occurs from newly exposed receptacles and ripe fruit and are dispersed by rain splash or air currents.[20]

1.1.4 Distribution and economic importance

Botrytis cinerea is found in humid, temperate and subtropical areas.[22] The pathogen affects over 200 plant species, mainly dicotyledonous, including legumes, crucifers and horticultural crops, by causing both pre- and post-harvest diseases[1] on nearly all plant parts (flower, buds, leaves, shoots, stems, seedlings and fruits).[23] This results in large economic losses worldwide, especially in grapes, berries and vegetables grown in protected environments.[24] *B. cinerea* affects strawberries worldwide[25] and it is also an economically important disease in chickpea.[2] This study researched the control of *B. cinerea* infection of strawberry, chickpea and bean with main emphasis on strawberry. The following section is thus a brief introduction to strawberry and the main control measures of *B. cinerea*.

1.2 Strawberry affected by gray mold

Strawberry (*Fragaria* × *ananassa*) is a herbaceous perennial. During the flowering new flowers open nearly every day and leaves, with a high number of stomata (300-400 mm^{-2}), are produced

throughout the growing season.[26] In the winter season, most of the above ground plant wilt and die, but young leaves may overwinter.[26]

Strawberry is susceptible to various diseases the entire growing season and the most damaging disease on strawberry in Norway is gray mold, which causes 30-60% yield reductions.[27] Symptoms of *B. cinerea* are diverse, but the most typical on leaves and soft fruits are soft rots, characterized by gray-brown spots, followed by the appearance of gray conidia masses.[1] The main strawberry cultivars in Norway are Corona, Senga Sengana and Bounty.[28] This present study used the variety Corona.

1.3 Control of gray mold

Control measures against *B. cinerea* are mainly taken at three stages in the life cycle of the pathogen (Fig 2): 1) to damage sclerotia, 2) to prevent conidia dispersal following sporulation and 3) to prevent infection of strawberry flowers. As the main infection of strawberry seems to be on the stamens[14], this study focused on inhibiting conidia germination and growth *in vitro* and reducing flower infection by *B. cinerea in vivo*. As the main approach to control gray mold is by chemical fungicides, the following section will discuss chemical control of *B. cinerea*. Chitosan and CHOS alone or in combination with reduced amounts of chemical fungicides were used to inhibit plant pathogens.

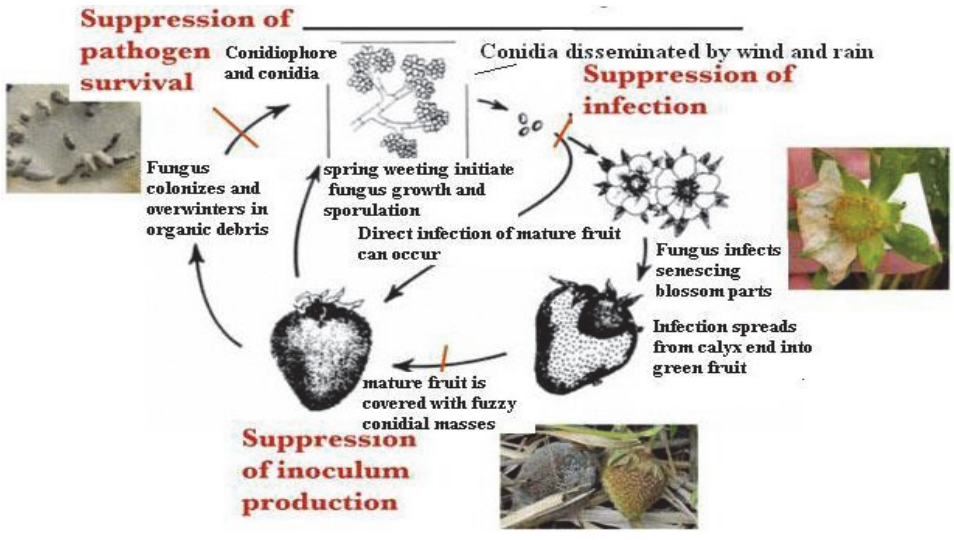

Figure 2. Control measures to break the disease cycle of *B. cinerea* on strawberry.[29]

1.3.1 Chemical control

1.3.1.1 Botryticides

In the 1990s effective compounds to control gray mold were commercialized: phenylpyrrol (fludioxonil), anilinopyrimidines (cyprodinil, pyrimethanil and mepanipyrim) and hydroxyanilide (fenhexamid),[24] but also these botryticides face the risk of resistance development in *B. cinerea* as pathogens often develop resistance to fungicides with a specific mode of action.[30] However, as these botryticides' modes of action are distinct from each other, they can be used in strategies to prevent resistance development.[24]

In Norway, the most commonly used fungicides to control *B. cinerea* in strawberries, based on the size of area treated, are Teldor (applied on 1230 ha), Switch 62.5 WG (1220 ha), Topas 100 EC (1170 ha), Signum (940 ha) and Amistar (229 ha).[31] The average amount of fungicides per application per ha and the total amount used are: Switch 62.5 WG (7.2 g ha^{-1}, total: 872 kg), Topas (465.1 mL ha^{-1}, 593 L), Teldor (19 g ha^{-1}, 2333 kg), Signum (13.3 g ha^{-1}, 1247 kg) and Amistar (total: 226 L).[31] In our study we used Switch (cyprodinil+fludioxonil), Amistar (azoxystrobin), Teldor (fenhexamid) and Signum (boscalid+pyraclostrobin) to control *B. cinerea*, thus the following discussion will focus on those fungicides.

1.3.1.2 Anilinopyrimidines

Anilinopyrimidines were registered in Switzerland in 1995 against gray mold in grapes.[32] This group of botryticides consist of the compounds pyrimethanil, cyprodinil (active ingredient of Switch) and mepanipyrim[33], which have methyl-, cyclopropyl- and propynyl-groups at the pyrimidine ring respectively, but are otherwise closely chemically related.[24]

The anilinopyrimidines inhibit the *B. cinerea* germ tube and mycelium growth[24,34] and can prevent secretion of hydrolytic enzymes, such as protease, cellulase, cutinase and lipase, involved in the pathogenesis.[35] Biochemical studies of *B. cinerea* indicate that the anilinopyrimidines inhibit the biosynthesis of methionin by blocking the cystathionine-β-lyase.[24,36] Cyprodinil (4-cyclopropyl-6-methyl-*N*-phenylpyrimidin-2-amine) is commercialized in mixture with fludioxonil (as in the fungicide Switch) against *B. cinerea* on strawberry.[37] This

non-systemic and protective botryticide inhibits *B. cinerea* spore germination, germ tube and mycelium growth.[45]

Certain isolates of *B. cinerea* have developed resistance towards anilinopyrimidines[34,38,39]and cross-resistance exists between cyprodinil, pyrimethanil and mepanipyrim as they control *B. cinerea* in the same way.[34,40] Cyprodinil has medium risk of resistance development in pathogens and resistance has been found in *B. cinerea*.[33] To avoid resistance development towards Switch consecutive applications should not be used and the fungicide should be used in alternation with a fungicide with different modes of action.[37] In addition, recommendations for Switch are maximum two applications per year of not more than 1 kg ha^{-1} per application and 2 kg ha^{-1} $year^{-1}$.[37]

1.3.1.3 Phenylpyrroles

Certain *Pseudomonas* species produce the antibiotic pyrrolnitrin, which is used to make phenylpyrroles.[24] The phenylpyrrole fludioxonil (4-(2,1-difluoro-1,3-benzodioxol-4-yl)-1H-pyrrole-3-carbonitrile) has a similar chemical structure to pyrrolnitrin, but to increase the light stability of the compound and make it suitable as a foliar botryticide it has substitutions in the phenyl ring.[24] Fludioxonil (active ingredient of Switch), target the mitogen-activated protein (MAP)/Histidine-Kinase in the osmoregulatory signal transduction (os-2, HOG1)[33] and it induces morphological changes of germ tubes like abnormal branching, swelling and cell bursting.[41] Phenylpyrroles have low to medium risk of resistance development in pathogens, but resistance management is still required.[33]

1.3.1.4 Strobilurins

Strobilurins, which originate from natural products, are widely used for pest management in agricultural production. Strobilurins were registered in 1996 and the strobilurin azoxystrobin is now the world most sold fungicide.[42] The strobilurins azoxystrobin (active ingredient of Amistar) ((methyl) (2E)-2-{2-[6-(2-cyanophenoxy) pyrimidine-4-yloxy]phenyl}-3-methoxyacrylate)) and pyrachlostrobin (active ingredient of Signum) (methyl N-(2-(1-(4-chlorophenyl)-1H-pyrazol-3-yloxymethyl) phenyl)-(N-ethoxy) carbamate), in the chemical

groups methoxy-acrylates and methoxy-carbamates respectively, inhibit the respiration of *B. cinerea*.[33] Cross resistance exist between all the strobilurins[33], thus FRAC recommends no consecutive applications, to use the fungicides in alternation with a fungicide with different modes of action and not let strobilurins exceed 30-50% of the total fungicide sprays to the crop per season.[43] Signum applications on strawberry should be no more than 1.8 kg ha^{-1} and 3.6 kg ha^{-1} $year^{-1}$ [44] and recommendations for Amistar is maximum 1-4 applications $year^{-1}$ and 1-6 L ha^{-1}.[45]

1.3.1.5 Succinate dehydrogenase inhibitors

Boscalid (2-chloro-*N*-(4′-chlorobiphenyl-2-yl) nicotinamide) is a succinate dehydrogenase inhibitor (SDHI) in the pyridine-carboxamide group. Boscalid (active ingredient of Signum), inhibits the *B. cinerea* respiration.[33] The risk of resistance development to the botryticide is medium to high, *B. cinerea* resistant to succinate dehydrogenase inhibitors has been found so resistance management is required.[46]

1.3.1.6 Sterol biosynthesis inhibitors

Sterol biosynthesis inhibitors (SBI) are the main group of fungicides used in agriculture, due to their broad spectrum antifungal activity and pathogens' low resistance to these products (Hewitt as cited by Zocco et al.).[47,48] Fenhexamid (*N*-(2,3-dichloro-4-hydroxyphenyl)-1-methylcyclohexanecarboxamide), the only hydroxyanilide fungicide, is the most recent and effective fungicide against *B. cinerea.*[24] Fenhexamid is not effective in inhibiting germination of *B. cinerea* at low concentration, but it effectively inhibits mycelial growth and germ tube elongation.[24] Fenhexamid inhibits the sterol biosynthesis in the cell membrane of the fungus and thereby disrupts cell membrane.[49] The risk of *B. cinerea* resistance to fenhexamid is low to medium[33], still resistance management with no more than 1.5 kg ha^{-1}, (four applications $year^{-1}$)[50] and no more than two sequential applications is required.[51] As fenhexamid does not show cross-resistance towards other fungicides it is an important component in anti-resistance management.[24]

Chemical fungicides are harmful to the environment and human health, so there is growing international awareness about the dangers of their intensive use on crops. Intensive use of chemical fungicides with a specific mode of action increases the chance of resistance

development in *B. cinerea*, thus there is a need to reduce the use and improve the efficacy of chemical fungicides. To address these challenges, this study investigated the possibilities to use chitosan and CHOS to improve the efficacy of chemical fungicides, thus the following section will discuss about the chitin, chitosan, CHOS and modes of action.

1.4 Chitin, chitosan and CHOS

Chitin is a linear biopolymer consisting of β 1,4 linked N-acetyl-D-glucosamine (GlcNAc or A) residues. Chitin has mainly two structures, α and β, where α chitin is the most common. α chitin consist of two antiparallel chains with CO and NH groups in their chains.[52] There is a strong binding between the CO group in one chain and the NH group of another chain by hydrogen bonds.[52] β chitin has a more open structure with parallel chains.[52] The strong intra- and inter-hydrogen bonds make chitin insoluble in water, aqueous acidic solutions and most of the organic solvents.[53]

The annual production of chitin in nature is approximately 1×10^{11} tons, thus it is the world's second most abundant organic compound next after cellulose.[54] Chitin is an important structural material of fungi, algae, protozoa, annelid, molusca, crustaceans and insects.[55] Shells of shrimp and crab contain 15-40% α chitin[56], thus chitin is mainly obtained from shellfish.[57] In 2000, the global production of chitin derivatives was 2000 tons of chitosan, 500 tons of oligosaccharides and 4000 tons of glucosamine.[56] Chitin derivatives are produced in large quantities because they have useful biological and antimicrobial properties and they are an alternative to chemical fungicides[58] as they are biodegradable, non toxic and have antimicrobial activity.[59] Despite the many useful properties, the main constraint to use chitin is its lack of solubility in water.

1.4.1 Solubility and production of chitosan

Chitosan can be obtained after partial deacetylation of chitin and is a heteropolymer consisting of GlcNAc or A unit and D-glucosamine (GlcN or D unit). Chitosan has three reactive, functional groups: the amino group on the C-2 position and both primary and secondary hydroxyl groups at the C-3 and C-6 positions respectively[61] (Fig 3). All are soluble in dilute organic acids at pH below 6.[59]

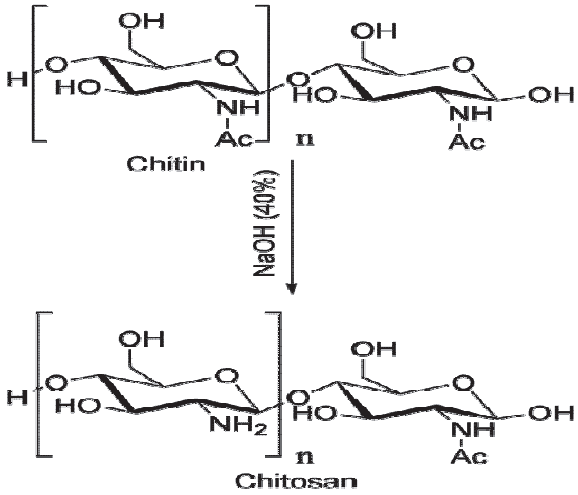

Figure 3. Production of chitosan from chitin.[60]

The presence of amino groups (with pKa 6.3) in chitosan makes chitosan a strong base. This amino group is protonated and positively charged at low pH and become soluble in water. However, when pH is above 6 the amino group becomes deprotonated, loses the positive charge and chitosan becomes insoluble.[59] The organic acids such as lactic acids, acidic acids and formic acids are thus used as solvent of chitosan.[62,63] The most commonly used solvent are acidic acids, but high concentrations are need to dissolve chitosan and concentrated acidic acids solution makes chitosan depolymerized.[62]

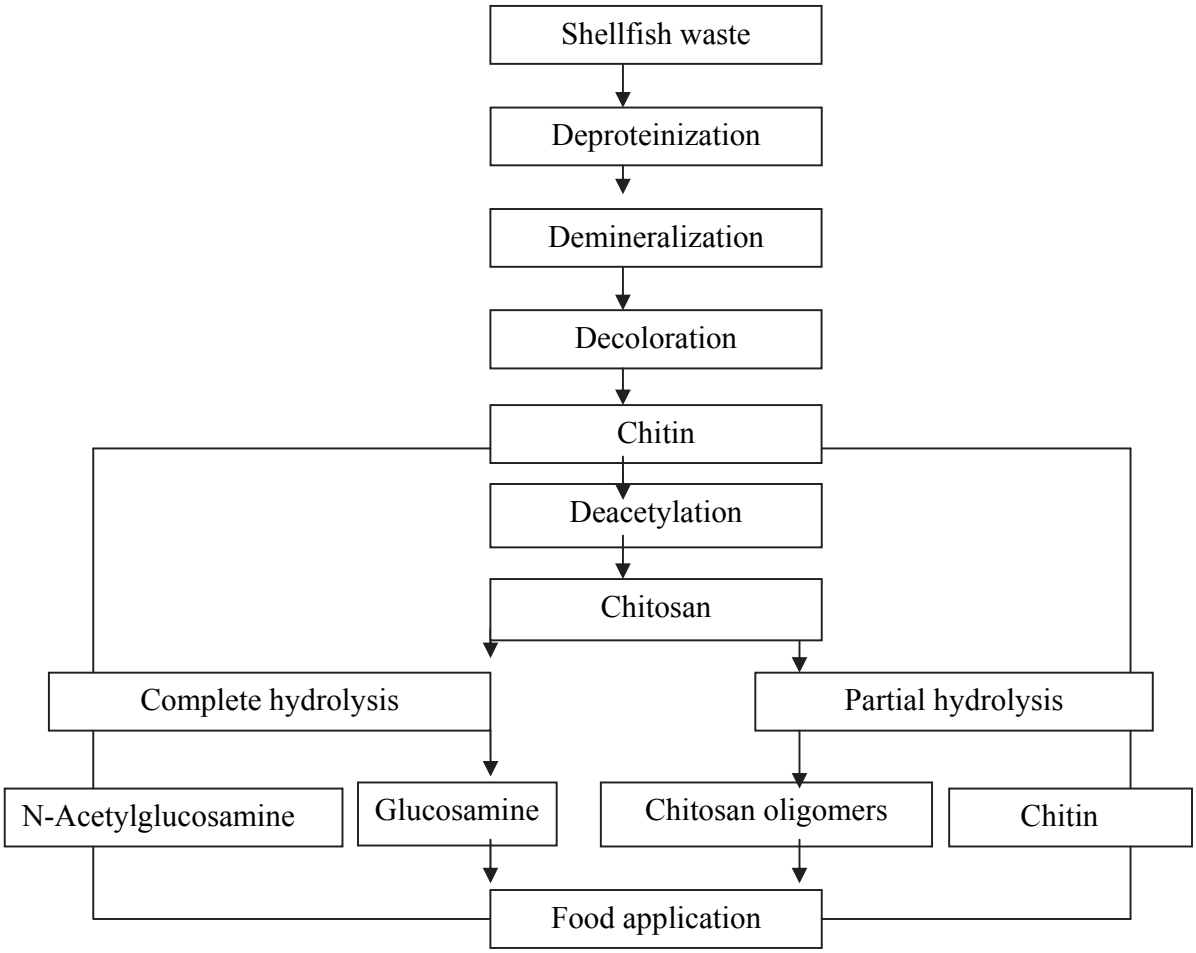

Figure 4. Production of chitin, chitosan and their oligomers from shellfish.[65]

The solubility of chitosan depends on its degree of deacetylation, degree of polymerization.[59,64] Low molecular weight chitosan (called chitooligosaccharides or CHOS) are more soluble than chitosan. There is no standard definition of chitosan or CHOS, but chitosan with molecular weight of 10 kDa or less is termed CHOS.[64]

In our study chitosan obtained by enzymatic hydrolysis of chitosan (DP_n 206) is termed chitooligosaccharides (CHOS). Recently, CHOS have received more interest than chitosan CHOS is soluble in water in addition to be an antitumor[66], antifungal[67] and antimicrobial[68] agent that may promote defence responses in plants.[69] The following section will thus discuss production of CHOS.

1.4.2 Production of CHOS

CHOS can be produced by hydrolyzing glycosidic bonds by different methods such as acid hydrolysis[70], enzymatic hydrolysis[71] and oxidative degradation (with $NaNO_2$).[72] Acid hydrolysis of chitosan to produce CHOS is used for large scale production, but the yield of CHOS from this method is low and mostly D-glucosamine units are produced (Uchida and Ohtakara as cited by Kim and Rajapasha).[64,73] However, acid hydrolysis may cause environmental pollution thus enzymatic hydrolysis is preferred due to lower environmental pollution.[64] Microbial enzymes are the most effective enzymes to produce CHOS, stilllarge scale production of CHOS by microbial enzymes is costly.[64] It is also possible to use low cost enzymes such as cellulose, alphaamylase and proteinase to produce CHOS.[71] In our study we used the glycosyl hydrolase (GH) chitosanase (ScCsn46A) and chitinase (ChiA) to produce CHOS, thus the following section will discuss glycosyl hydrolase used for hydrolysis of chitosan.

The glycosidic bonds of chitosan can be hydrolyzed by GH such as chitosanase and chitinase. Chitosanases are found in organisms such as bacteria[74], fungi[75] plants[76] and viruses.[77] Chitosanase can be classified in six groups based on their amino acid sequence: GH 5, 7 8, 46, 75 and 80.The GH families 5, 7 and 8 were previously called the cellulase family. The GH families 5 and 7 use retaining mechanism and family 8 use invert mechanism to hydrolyze glycocidic bonds of chitosan. However, the GH families 46, 75 and 80 only contain chitosanase. The GH family 46 contains 18 chitosanases where the majority (16) is from bacteria and two are from *Chlorella* viruses.[78]

Chitosan have four different types of glycosidic bonds in their structures: A-A, A-D, D-A and D-D. Chitinase and chitosanase hydrolyze glycosidic bonds differently. On the basis on substrate specificity towards chitosan, chitosanases can be divided in the following three subclasses (Fukamizo et al. as cited by Aam et al.)[79,80]: subclass I comprise of the GH family 46 and 75, which may hydrolyze the A-D and D-A linkages, subclass II only hydrolyze D-D linkages and subclass III can hydrolyze D-A and D-D linkages (Fukamizo et al. cited by Aam et al.).[80,81]

Chitosanase (ScCsn46A) belongs to the GH family 46 and can be obtained from *Streptomyces coelicolor* A3 (2). Heggset and coworkers[82] showed that ScCsn46A initially hydrolysed the D-D linkages of chitosan (F_A 0.32) i.e. with a D unit in the subsites -1 and +1, and produced

deacetylated dimmers, trimers and tetramers. The hydrolysis was initially rapid and then slowed down. ScCsn46A has low subsite specificity towards D or A unit where subsite -1 accepts both D and A units. However, if chitosan is hydrolyzed by ScCsn46A for long time all four linkages (D-D, D-A, A-D and A-A) may be hydrolyzed. ScCsn46A operates according to non-processive endomode of action during hydrolysis of chitosan.[82] Chitinases are mainly found in the GH families' 18 and 19.[80] Chitinase in family 18 hydrolyze A-A and A-D linkages while chitinase in family 19 hydrolyze A-A and D-A.[78] Chitinase (ChiA in the GH family 18) is produced by *Serratia marcescens* and has a deep tunnel like an active site groove. ChiA uses both exo and endo processive modes of action to hydrolyze chitosan. To hydrolyze chitosan ChiA requires A unit in subsite -1, but has no requirements for A or D in subsite +1.[83]

1.4.3 Chitosan mode of action

The precise mechanisms involved in the chitosan antimicrobial activity are not determined[84, 85] but previous research suggest the following three modes of action:

1. The amino groups of chitosan become protonated (NH_3^+) at low pH and interact with the negative cell surface of microorganisms.[86] This electro static interaction results in pore formation on the cell surface[87,88] and increased permeability of the cell membrane[87,89], which causes osmotic imbalance[90] and finally plasmolysis of the microbial cell and cell death.[91] However, the electro static interaction between chitosan and microbes depends on the cell surface negativity; the more negative charges on the cell wall surface, the more interaction the cell wall will have with chitosan.[92] As an example, gram negative bacteria contain lipopolysaccharides, with the anionic functional groups phosphate and carboxyl, in the outer membrane.[93] Gram positive bacteria contain peptidoglycan and teichoic acid in their cell wall.[94] The cell surface negativity is higher in gram negative than gram positive bacteria and this is the reason why chitosan and gram negative bacteria have more interaction than chitosan and gram positive bacteria.[92] Also the surface electronegativity of the fungal cells may influence the electrostatic interaction between cells and chitosan. A previous study showed that the pathogen's susceptibility to chitosan depended on its cell surface electronegativity.[95] The negative charge density on the *Aspergillus niger* cell surface increased with increasing pH, due to deprotonation on the metal binding sites, and caused increased biosorption. At low pH, the positive charge

density on the metal binding sites of the *A. niger* cell surface increased due to high concentration of protons in the solution and resulted in lower biosorption.[96]

2. Chitosan oligomers penetrate the cells of microorganisms where they attack the negatively charged phosphate groups of nucleic acids in the DNA, and prevent cell growth by inhibiting the mRNA synthesis and ultimately the protein synthesis.[97,98] A recent study proved that chitosan can be internalized in fungal cells in an energy dependent manner, but the compound itself is not involved in the endocytosis.[99]
3. A recent study showed that chitosan sensitive and resistant fungi differ in the composition of fatty acids in their cell membrane. While the plasma-membrane of chitosan resistant fungi form a barrier to chitosan, the plasma-membrane of chitosan sensitive fungi contain more polyunsaturated fatty acids, which enhance the permeability of chitosan.[85]

2. Objectives of the study

The objectives of this study are:

a. To find the different factors (such as pH, calcium, magnesium, degree of polymerization, fraction of acetylation) that may influence the antifungal activity of chitosan against *Botrytis cinerea* (Paper I).

b. To assess the synergistic effect of chitosan and Teldor (fenhexamid) inhibiting *Botrytis cinerea* (Paper II).

c. To find the most effective chitooligosaccharides against *Botrytis cinerea* and *Mucor piriformis* (Paper III).

d. To study the synergistic effects of chitooligosaccharides and chemical fungicides on inhibition of plant pathogens (emphasis was given on *Botrytis cinerea*) (Paper IV).

3. Main results

The antifungal activity of chitosan was affected by factors such as pH, calcium and magnesium in the media, degree of polymerization (DP) and fraction of acetylation (F_A). The antifungal activity of chitosan (F_A 0.11, viscosity 19 mPa.s) against *B. cinerea* (isolate BC 101) was higher at pH 6 than 3-5 (Paper 1). Chitosans with low F_A were more effective than chitosans with high F_A. Increasing concentration of calcium reduced the ability of chitosan to inhibit *B. cinerea* BC 101 germination and growth (further germ tube elongation). Chitosan showed complete germination inhibition of *B. cinerea* BC 101 in media without or with a low calcium concentration, but only 15-20% germination inhibition in a high calcium concentration. Magnesium reduced the antifungal activity of chitosan in the same way as calcium (Paper I).

The antifungal activity of chitosan also depended on its DP and chitooligosaccharides (CHOS, produced by enzymatic hydrolysis of chitosan DP_n 206) were more effective against plant pathogens than chitosan (DP_n 206). The CHOS with average degree of polymerization (DP_n) 15-40 were more effective against *B. cinerea* than other CHOS and chitosan used (Paper III and IV). The plant pathogens differed in their sensitivity towards DP_n of CHOS. *Mucor piriformis* and *B. cinerea* (isolate BCBD) were sensitive (in terms of germination inhibition) to wide ranges of DP_n of CHOS (DP_n 15, 23, 40, 48, 58 and 75 and DP_n 11, 15, 23 and 40 respectively), while *B. cinerea* BC 101 was only inhibited by CHOS DP_n 23 and 40 (Paper III).

Both chitosan and CHOS showed synergistic effect with chemical fungicides against *B. cinerea* BC 101 (Paper II and IV). However, the combination of CHOS and chemical fungicides showed higher synergism than the combination of chitosan and chemical fungicides. The CHOS DP_n 23, Amistar and Signum (1% of their recommended doses) only showed 2-4% germination inhibition of *B. cinerea* BC 101 when applied alone, but in combination the germination inhibition was >90% and the levels of synergism were high. In the strawberry flower and chickpea leaf assays, CHOS were also more effective against *B. cinerea* BC 101 and showed higher level of synergism with fungicides than chitosan (DP_n 206). Chitosan showed low levels of synergism when combined with chemical fungicides (1% of their recommended doses) whereas high level of synergism was found when CHOS was combined with the same concentration of chemical fungicides. In the bean leaf assay, there were no significant difference between chitosan (DP_n 206) and CHOS regarding synergism with fungicides.

4. General discussion

Botrytis cinerea is a harmful pathogen causing major losses of crops like berries, fruits and vegetables worldwide.[1,24,100] The pathogen is also the main production constraint in several chickpea growing areas in South Asia, Australia and the Americas and has caused up to complete crop losses.[101] To control *B. cinerea*, farmers mainly depend on chemical fungicides, which are often applied in excessive quantities[102] creating health and environmental problems, like loss of biodiversity. There is thus a need to find natural fungicides to control *B. cinerea*, and chitosan, with antifungal properties, may be such an alternative. Chitosan shows antimicrobial activity is not toxicity, is easily biodegradable[59] and triggers plant defence responses.[69] However, many factors influence the antimicrobial activities of chitosan and the compound is not soluble in water, thus the use of and studies on chitosan are limited. Thus, to find how chitosan could be used as an effective antifungal agent against *B. cinerea*, the four papers in this thesis focus on factors affecting the antifungal activity of chitosan, production of effective CHOS and combination of chitosan or CHOS with chemical fungicides to reduce the need for the latter by enhancing their efficacy.

The antifungal activity of chitosan depends on factors such calcium, magnesium and the pH of the culture media, F_A and molecular weight (or DP). *In vitro,* the presence of calcium and magnesium in the culture media reduced the antifungal activity of chitosan (Paper I). This corresponds to results of Tsai and Su (1999), who suggested that the cations form a complex with chitosan and reduces the antifungal activity. While Tsai and Su (1999) showed that calcium (Ca^{2+}) was more effective than magnesium (Mg^{2+}) in reducing the antimicrobial activity of chitosan, our study did not show significant difference between the two compounds in this regard.

Chitosan needs acidic media to show antifungal activity against *B. cinerea* BC 101 and its (chitosan: F_A 0.11, viscosity 19 mPa.s) antifungal activity was higher at pH 6 than 3-5 (Paper I). Previous study showed that antifungal activity increase with decrease of pH in the media[103] but in our study we found most inhibition by chitosan (with F_A 0.11) at pH 6, which is in accordance with a previous study showing that chitosan had most inhibition of *Phytopthora megasperma* when pH of the media was slightly lower than the pKa (6.2) of chitosan.[104] When pH of the media is slightly lower than its pKa the amino groups of chitosan becomes protonated, interacts

with the negative cell surface resulting in death of microorganism due to leakage of intracellular electrolytes.[87,88] Our *in vitro* and *in vivo* studies showed that chitosan with low F_A was more effective than chitosan with high F_A to inhibit *B. cinerea.* A previous study showed that the lower the F_A, the higher number of amino groups in the chitosan. This causes more protonation in acidic environments resulting in more electrostatic interaction between chitosan and microorganism.[105]

The molecular weight of chitosan also affects its antifungal activity (Paper III), but previous studies show diverging results. Some studies showed that high was more effective than low molecular weight chitosan[106,107], while others showed the opposite.[64,108] In our study we used CHOS with different average degree of polymerization (DP_n) or degree of polymerization (DP) 3-10 obtained from enzymatically hydrolysed chitosan (DP_n 206). DP/DP_n was used as an indicator of molecular weight of chitosan and CHOS. Our study showed that CHOS DP_n 23 and 40 were the most effective in inhibiting *B. cinerea* and *M. piriformis.* However, there was no difference between chitosan (DP_n 206) and CHOS DP 3-10 in terms of germination inhibition at a low concentration (80 µg mL^{-1}), but at a high concentration (1300 µg mL^{-1}) CHOS DP 3-10 was more inhibitory than high DP chitosan (DP_n 206). This corresponds to a previous study stating that chitosan with low DP (DP 3-9) inhibited the growth of *B. cinerea* more than chitosan with high DP (molecular weight 300-500 kDa).[108]

The antifungal activity of chitosan also depends on the tested pathogens' cell wall composition[109], but studies on the correlation between the antifungal activity of chitosan and the cell wall composition of the fungi, show contradictory results. While one study found fungi with cell wall containing chitosan (*Mucor* spp.) to be less sensitive to chitosan (1000 µg mL^{-1}) [109], other studies documented that chitosan (same concentration) inhibited the growth of *Rhizopus stolinifer*[110] and *M. racemosus.*[111] In our study (Paper III), two pathogens with different cell wall compositions (*B. cinerea*: chitin, *M. piriformis*: chitin and chitosan) were tested for their sensitivity to CHOS. The pathogen with chitin and chitosan in the cell wall was less sensitive to high molecular weight chitosan (DP_n 206) and low molecular weight CHOS (DP_n 9 or DP 3-10), but sensitive to a wide range of CHOS in between these DP (DP_n 15, 23, 40, 48, 58 and 75, all chitosan and CHOS mentioned had the same concentration). *B. cinerea* BCBD was sensitive to

CHOS with a narrower range of DP (DP_n 11, 15, 23 and 40) and *B. cinerea* BC 101 was only sensitive to DP_n 23 and 40.

CHOS (DP_n 23 and DP_n 40) were also more effective *in vivo* than chitosan in inhibiting *B. cinerea* than high molecular weight chitosan (DP_n 206). The inhibition of *B. cinerea* in the flower assay by CHOS could be due to direct and indirect effect of chitosan. *In vitro* (microtiter plate assay), CHOS were more effective than chitosan (DP_n 206) in inhibiting germination and growth of *B. cinerea*. In addition to the direct antifungal effects of chitosan observed *in vitro*, chitosan may protect flowers indirectly by eliciting plant defence mechanisms against pathogens.[112] Previous studies showed that chitosan acts as an elicitor of plant defence responses.[113,114] The elicitor property of chitosan depends on its DP.[115] A previous study showed that chitosan stimulates the activity of chitinase in strawberry fruits[110] and this stimulation depends on the DP of chitosan; low was more effective than high DP chitosan in inducing chitinase and glucanase.[115] Thus, in our study CHOS DP_n 23 may have been more effective than high DP chitosan (DP_n 206) in inducing defence responses in strawberry. Our *in vitro* and *in vivo* studies showed that the most effective DP of CHOS against *B. cinerea* on strawberry is somewhere in the range between DP_n 15-40.

Despite CHOS and chitosan's effective direct and indirect antifungal activity against *B. cinerea* the compounds are not as effective as chemical fungicides.[116] However, chemical fungicides have adverse effects on mammals and the environment, thus there is a need to reduce their use and improve their efficacy. Paper II and IV investigated synergism between chitosan or CHOS and chemical fungicides in inhibiting *B. cinerea*. The synergism between chitosan and fungicide *in vitro* and *in vivo* depended on the DP of chitosan and type of fungicide tested. *In vitro,* CHOS showed higher synergism with all tested fungicides than chitosan (DP_n 206). Chitosan DP_n 206 only showed low synergism with Teldor and Switch. The reason for synergism between chitosan or CHOS and fungicides are not known, but could be due to the compounds' different modes of action. The mode of action of chitosan is not known[99,117-119], but previous studies have shown that the electrostatic interaction between positively charged chitosan and a negatively charged microorganism resulted in increased permeability of the cell membrane.[87,88] This permeability, created by chitosan and CHOS, may enable the fungicides to reach the conidia and start their operations earlier than if they were applied alone. Thus, Teldor may reach the conidia earlier and

destroy the cell membrane[33]; Amistar and Signum may inhibit respiration[33] and Switch may destroy the amino acids and hamper the protein synthesis.[33] However, the higher synergism between CHOS and fungicides than between chitosan (DP_n 206) and fungicides may be due to difference in the DP. CHOS may increase cell membrane permeability more than chitosan (DP_n 206) which may facilitate the chemical fungicides to enter the cell. *In vivo* (strawberry flower and chickpea leaf assay) showed that the combinations of CHOS and chemical fungicides were also more effective in reducing infection by *B. cinerea* than the combinations of chitosan and chemical fungicides. Also a previous study on synergism between chitosan and antibiotics found that the DP of chitosan influenced the synergism.[120] Our study showed that if CHOS is used in combinations with fungicides, the fungicide concentration could be reduced considerably and still give the same effect as if only the fungicide (at its recommended dose) was applied alone. Thus the combined use of chitosan or CHOS and chemical fungicides in an integrated pest management (IPM) system may effectively control fungal pathogens by low amounts of chemical fungicides, which would favour the environment, farmers and consumers.

5. Conclusions and recommendations

In this thesis high DP chitosan (DP_n 206) and CHOS (low DP) were used alone and in combination with fungicides to control *B. cinerea*. Many factors are involved in the antifungal activities of chitosan and this present study found that pH, DP of chitosan, calcium and magnesium affected the antifungal activity of chitosan. As previous studies about the effect of DP show contradictory results, we used CHOS with different DP from a single source of chitosan and found that a range of DP (DP_n 15-40) of CHOS were effective against fungal pathogens. The sensitivity of tested pathogens to CHOS may vary with the cell wall composition of the microorganism. Chitosan/CHOS were effective against *B. cinerea* when applied separately and also in combination with fungicides, where they showed synergism. CHOS showed higher synergism (in terms of inhibition of fungal pathogens) and were also more effective to control plant pathogens alone or in combination with fungicides than chitosan. However, further studies on the mechanisms involved in the antifungal activity of chitosan or CHOS when applied alone or in combination with chemical fungicides are needed in order to understand how the combination of chitosan or CHOS and fungicides can be better used in integrated pest management (IPM) systems to control *B. cinerea*.

6. References

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Paper I

Factors affecting the antifungal activity of chitosan against *Botrytis cinerea*

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Factors affecting the antifungal activity of chitosan against *Botrytis cinerea*

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ABSTRACT

Botrytis cinerea is a plant pathogenic fungus and the causative agent of gray mold in a wide variety of economically important crops throughout the world. Chitosan, a derivative of chitin, inhibits the growth of many microorganisms and is a possible alternative to chemical fungicides for controlling *B. cinerea.* In this study, the effects of the fraction of acetylation (F_A), molecular weight (average degree of polymerization (DP_n) and viscosity were indicators of molecular weight), pH and addition of divalent cations in the media on the ability of chitosan to inhibit *B. cinerea* were investigated *in vitro* and *in vivo*. *In vitro*, chitosan with low F_A (0.11 and 0.18) showed greater antifungal activity than chitosan with high F_A (0.39 and 0.40). Chitosan DP_n 22 (obtained by enzymatic hydrolysis of chitosan with DP_n 145 and viscosity 19 mPa.s) significantly (P <0.01) inhibited germination of *B. cinerea* when compared with chitosan (unhydrolyzed, DP_n 145 and viscosity 19 mPa.s). Chitosan (F_A 0.11; DP_n 145 and viscosity 19 mPa.s) showed higher germination inhibition against *B. cinerea* at pH 6 than pH 3-5. Calcium and magnesium reduced the antifungal activity of chitosan when added to a synthetic medium or to potato dextrose broth. *In vivo* tests on strawberry flowers also showed that chitosan with low F_A was more effective than chitosan with high F_A in reducing flower infection by *B. cinerea.* In both *in vitro* and *in vivo* low molecular weight chitosan (viscosity 19 mPa.s and DP_n 145) significantly (P <0.01) inhibited *B. cinerea* when compared with high molecular weight chitosan (viscosity 650 mPa.s) at high concentrations (3000-10000 μg mL^{-1}). In addition, chitosan's ability to reduce flower infection also depended on its time of application; its performance was best when applied 24 hours before inoculation compared to same time or six hours after inoculation with *B. cinerea*.

Key words: Germination inhibition, fungal growth, flower infection, gray mold, chitosan polymers, ionic strength

1. Introduction

The fungal pathogen *Botrytis cinerea* Pers.: Fr. (anamorph of *Botryotinia fuckeliana*) is the causative agent of gray mold. The pathogen is able to grow in diverse climatic conditions and can damage different parts of plants such as flower, buds, leaves, shoots, stems, seedlings and fruits (Williamson et al., 2007). The severe damage by *B. cinerea* in plants causes considerable economic losses (Rosslenbroich and Stuebler, 2000). Gray mold is the economically most important disease of strawberry and causes 30-60% yield reductions in Norway (Land, 2011).

Farmers are highly dependent on chemical fungicides to control *B. cinerea*. In Norway, out of 1730 hectares of cultivated strawberry, 91% were sprayed with chemical fungicides (Strømeng, 2008). The high dependency on chemical fungicides creates environmental pollution and loss of biodiversity, thus alternatives to chemical fungicides are needed to control *B. cinerea*. Alternative management methods of *B. cinerea* include biological control, cultural control (Card, 2005) and plant extracts (Parvu et al., 2010). Biological control methods are environmentally friendly, long term solutions that are highly specific to target pests. However, the main limitations of biological control methods are slow, inconsistent and unreliable disease control compared to chemical fungicides (Bale et al., 2008). Another alternative to chemical fungicides is chitosan, which consists of β (1→4) linked N-acetyl-D-glucosamine (GlcNAc; A) and D-glucosamine (GlcN; D). Chitin is a linear polysaccharide consisting of GlcNAcs and it is one of the most abundant natural biopolymers. It is found mainly in the cell walls of fungi and as a structural polymer in crustaceans, arthropods, insects and parasitic nematodes (Gooday, 1990). Chitosan can be found in fungal cell walls (Ruiz-Herrera, 1992), but can also be produced by partial deacetylation of chitin.

Chitosan is non-allergenic (Kurita, 1998), not toxic towards mammalian cells (Jung et al., 1999; Singla and Chawla, 2001), biodegradable (Badawy and Rabea, 2011) and it may protect plants by direct antimicrobial activity or by eliciting plant defence mechanisms against pathogens (Hadwiger, 1979; Hadwiger and Beckman, 1980). A previous study showed that damage of gray mold in sweet cherry was reduced by pre harvest spraying of or post harvest dipping in chitosan (Romanazzi et al., 2003). Although chitosan is effective as an antimicrobial agent, the compound is only soluble in aqueous acid solutions, not in water, thus the application of chitosan is still limited (Badawy and Rabea, 2011). To avoid this problem, recently chitosan derivatives that are

soluble in water have been used as antifungal agents (Badawy, 2010). However, the antifungal activity of chitosan is influenced by many factors such as its concentration (Palma-Guerrero et al., 2008), molecular weight (Kendra and Hadwiger, 1984) fraction of acetylation (F_A) (Stössel and Leuba, 1984), pH and ionic strength of the media (Jung et al., 1999; Wang, 1992). Studies on the effect of molecular weight of chitosan on plant pathogens show diverging results. While some studies found that polymeric chitosan (high molecular weight) had higher antifungal activity than low molecular weight chitosan (Eikenes et al., 2005; Meng et al., 2010), other studies reported the opposite result (Kim and Rajapakse, 2005; Xu et al., 2007).

The effect of fraction of acetylation (F_A) of chitosan on antimicrobial activity also showed contradictory results. Some studies showed that the antimicrobial activity of chitosan increases with decreasing F_A of chitosan against *Escherichia coli* (Chung and Chen, 2008; Liu et al., 2001; Takahashi et al., 2008) and *Staphylococcus aureus*. However, other researchers showed that the antimicrobial activity of chitosan do not increase with decreasing F_A against *E. coli* (Park et al., 2004). The antimicrobial activity of chitosan depends on pH of the media (Stössel and Leuba, 1984; Tsai and Su, 1999). Tsai and Su showed that the antimicrobial activity of chitosan increases with decreasing pH against *E. coli* (Tsai and Su, 1999), but Stössel and Leuba found that the highest antimicrobial activity was at pH 6 against *Phytopthora megasperma* (Stössel and Leuba, 1984).

Tsai and Su (1999) reported that the addition of metal ions in the growth media considerably reduced the activity of chitosan. The study also showed that divalent cations reduced the activity of chitosan against in *E. coli* in the following order of effectiveness: $Ba^{2+}>Ca^{2+}>Mg^{2+}$ (Tsai and Su, 1999), but other research indicated that calcium has very low or no affinity to chitosan (Delben and Muzzarelh, 1989). The effect of different factors have been studied by many researchers in different pathogens, but there is a need to conduct research of several factors affecting the antimicrobial activity in a single pathogen because the antimicrobial activity of chitosan also depends on the tested pathogen (Chien and Chou, 2006; Hernández-Lauzardo et al., 2008), even on isolates of the same specie (Hirano and Nagao, 1989). As the antifungal activity of chitosan is affected by various factors, this present study investigated the effects of pH; the calcium, magnesium and glucose contents in the media; the fraction of acetylation; and the molecular weight on the antifungal activity of chitosan against *B. cinerea in vitro* and *in vivo*. To

our knowledge, this is the first study of how multiple factors affect the antifungal activity of chitosan against *B. cinerea*.

2. Materials and methods

2.1. Chitosan

Four types of chitosans differing in fraction of acetylation (F_A) and viscocity (η) were obtained from Pronova Biopolymer (Oslo, Norway): chitosan 114 (F_A = 0.11, η 19 mPa·s), chitosan 211B (F_A 0.40, η 72 mPa·s), chitosan 311 (F_A 0.39, η 360 mPa·s) and chitosan 313 (F_A 0.18, η 650 mPa·s). All of them had a dissociation constant (pKa) of 6.5 and they were formulated as dry powder 80% dry weight.

As chitosan 114 in this study had the highest antimicrobial activity of the four chitosans, it was hydrolyzed with chitosanase ScCsn46A to produce chitosan with various average degrees of polymerization (DP_n) having the same F_A and tested for their antifungal activity.

2.2. Production and purification of chitosanase ScCsn46A

As described by Heggset and coworkers (Heggset et al., 2010); chitosanase ScCsn46A, originally from *Streptomyces coelicolor* q9rj88, was purified from the culture supernatant of transformed *E. coli* BL21Star (DE3) according to the published protocol, with the exception that the $(His)_6$-tag was not removed from the enzyme following purification. The enzyme was dialyzed against 20 mM Tris-HCl at pH 8 and stored at 4^{o}C.

2.3. Hydrolysis of chitosan 114

Twenty mg chitosan 114 (due to shortage of chitosan and enzyme we did not hydrolyze more than 20 mg chitosan) was added to 1 ml distilled water and 1 ml hydrolysis buffer (final concentration: 0.04 M sodium acetate + 0.1 M NaCl, pH 5.5) in a 15 ml vial and incubated at 37^{o}C until dissolved. The pH was readjusted to 5.5 with 0.1 M NaOH. Chitosanase ScCsn46A (0.5 μg mg^{-1} chitosan) was added to the chitosan solution and the mixture was incubated for 5 to 20 minutes at 37^{o}C. The enzymatic reaction was stopped by reducing the pH to 2.5 with 0.5 M HCl, followed by immersing the vial in boiling water for 10 minutes to inactivate the enzyme. Hydrolyzed products were dialyzed against deionized water for 48 hours (water was changed

every 12 hours) using a cellulose membrane (cut-off 500 Da, spectra/por® Float-A-Lyzer®, Spectrum® Laboratories, Texas, USA) to remove the salts from the sample. The dialyzed samples were lyophilized and stored at 4°C.

2.4. Measuring the average degree of polymerization (DP_n) of hydrolyzed chitosan 114

Ten mg of lyophilized chitosan 114 was dissolved in 0.5 ml D_2O (deuterium oxide). The solution was adjusted to pH 4.2 with DCl (deuterium chloride) and lyophilized. This process was repeated once. The lyophilized sample was resuspended in 700 µL D_2O, vortexed and transferred to NMR tubes (Sigma-Aldrich®, St. Louis, USA). The ^{1}H-NMR spectra in D_2O at 300 MHz and 85°C were recorded using a Varian Gemini 300 instrument (Varian, USA) with 256 scans.

The DP_n was calculated using the formula (Dα+Dβ+D+Aα+Aβ+A)/(Dα+Dβ+Aα+Aβ)……(1)

where Dα and Dβ are the integral of the reducing end signals of the α and β anomers of the deacetylated (D) and acetylated (A) units respectively; D is the integral of the signals from the internal and non-reducing end deacetylated units; and A is the integral of the signals from the internal and non-reducing end acetylated units (Sørbotten et al., 2005).

2.5. Botrytis cinerea

Botrytis cinerea (isolate BC 101) was originally isolated from an infected field grown strawberry fruit in Grimstad, Norway. Stock cultures were preserved in 20% glycerol at -80°C. For the bioassays, conidia were collected from cultures grown on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) under regular laboratory light for 2 weeks at 23±1°C. The conidia were gently scraped from the surface of the PDA plates, suspended in water and filtered through sterile cotton to remove fragments of mycelia. Conidia concentrations were determined using a hemocytometer counter at 400× magnification (Leica, DM RBE, Germany) and adjusted to required concentrations.

2.6. In vitro bioassay (the effects of medium components and pH on germination and growth of Botrytis cinerea)

2.6.1. The effect of pH on the antifungal activity of chitosan

The antifungal activity (inhibition of conidial germination) of chitosan (114, 211B, 311 and 313 at the concentration 1000 μg mL^{-1}) against *B. cinerea* was tested in 96 wells microtiter plates (Nunc, Denmark) in synthetic medium (SM) following the method of Nelson et al. (Nelson et al., 1988) with different pH (3-6). The final concentrations of components in the wells were 12.5 mM NH_4NO_3, 1.4 mM $CaCl_2 \cdot 2H_2O$, 0.8 mM $MgSO_4 \cdot 7H_2O$, 0.01 mM $MnSO_4 \cdot 4H_2O$, 0.02 mM $ZnSO_4 \cdot 7H_2O$, 5 mM KH_2PO_4, 0.06 mM $FeC_6H_5O_7 \cdot 5H_2O$ and 55.5 mM glucose. The pH of SM with or without chitosan was adjusted to values between 3-6 by HCl (0.1 M) and NaOH (0.5 M). The treatments were: a) control (only SM, no chitosan): there were four control treatments for pH (3-6); and b) SM with chitosan (114, 211B, 311 or 313) having different pH (3-6). One hundred μL of each treatment were added to the wells of the microtiter plates, with two replicate wells per treatment. This was followed by 100 μL of the conidial suspension (final concentration in the wells was 2×10^4 conidia mL^{-1}). The plates were incubated at 23±1°C for six hours. The germination percentage was visually estimated at 400× magnification using an inverted microscope (Fluovert FU, Ernst Leitz Wetzlar GmbH, Wetzlar, Germany). The first 100 conidia observed in each well were counted as germinated when the germ tube length was as long as the diameter of the conidium. The experiments were repeated twice (in total three experiments were conducted).

The germination inhibition percentage was calculated by the following formula:

$$\text{Germination inhibition (\%)} = \{(a-b)/a\} \times 100 \quad (2)$$

where a = number of germinated conidia in the control sample

b = number of germinated conidia in the presence of chitosan.

2.6.2. The effect of medium components on the antifungal activity of chitosan

One magnesium concentration (1.4 mM $MgCl_2 \cdot 6H_2O$), four glucose concentrations (28, 56, 85 and 110 mM) and three calcium concentrations (1.4, 0.34 and 0.14 mM $CaCl_2 \cdot 2H_2O$) were tested

in SM separately to investigate whether these substances affected the antifungal activity of chitosan 114 (24 and 72 hours after inoculation, HAI) against *B. cinerea.* Concentrations given are the final concentration in the wells after adding chitosan and conidia. A calcium concentration of 0 mM indicates SM without calcium. Another experiment with calcium and chitosan in potato dextrose broth (PDB, Difco^{TM}, USA) was performed to test whether the antifungal activity of chitosan 114 would be altered in PDB with added calcium (antifungal activity was observed 24 HAI). Six replicate wells were used for each treatment and the experiment was repeated twice. The pH of the conidia suspension in the microtiter wells was between 5.2 and 5.3 at the start of the experiment and remained about the same 24 HAI. The germination was counted as described above.

Growth was also measured using absorbance readings (A_{595}) in a microtiter plate reader (ASYS Hitech Biocrom, GmbH Austria) immediately after and 72 HAI. The absorbance values were used to calculate the growth inhibition percentage using the following formula:

Growth inhibition (%) = $\{(p-q)/p\} \times 100$……..…… (3)

where p = absorbance value of *B. cinerea* grown in the control sample

q = absorbance value of *B. cinerea* grown in the presence of chitosan.

2.7. The effect of chitosan molecular weight on the inhibition of germination of Botrytis cinerea

The effect of molecular weight (in terms of DP_n) of chitosan 114 (F_A 0.11; DP_n 95, 75, 48 and 22) on the inhibition of germination of *B. cinerea* was studied in modified synthetic medium (MSM) in microtiter plates. SM was diluted to reduce the interference of the ions in SM with chitosan. Final concentrations of MSM in the microtiter plate wells were 2.5 mM NH_4NO_3, 0.28 mM $CaCl_2 \cdot 2H_2O$, 0.16 mM $MgSO_4 \cdot 7H_2O$, 0.002 mM $MnSO_4 \cdot 4H_2O$, 0.002 mM $ZnSO_4 \cdot 7H_2O$, 1 mM KH_2PO_4, 0.06 mM $FeC_6H_5O_7 \cdot 5H_2O$ and 55.5 mM glucose. Only one chitosan concentration (80 μg mL^{-1}) was used. That concentration showed complete germination inhibition at 12 HAI, thus higher concentrations were not needed. Lower concentrations (20 and 40 μg mL^{-1}) were not effective against *B. cinerea*. Germination was counted 12 and 24 HAI. Four replicated wells were used for each treatment and the experiments were repeated twice.

The pH of the conidia suspension in the microtiter wells was between 5.2 and 5.3 at the start of the experiment and remained about the same 24 HAI. The germination was counted as described above.

2.8. In vivo bioassay (the effect of chitosan on strawberry flower infection by Botrytis cinerea)

The ability of chitosans 114, 211B, 311 and 313 (final concentration 80, 5000 and 10000 µg mL^{-1}) to reduce infection by *B. cinerea* on newly opened strawberry (*Fragaria* × *ananassa* cv. Corona) flowers were tested under controlled conditions. Strawberry plants were grown in a greenhouse with controlled temperature (18°C day; 12°C night), light (16 hours, light intensity: 150 µmols m^{-2} sec^{-1}) and relative humidity (65%). Eighteen flowers per treatment (six three-flower replicates) were cut off with a 1½-2 cm stem and placed in empty pipette tip racks set in plastic containers filled with 1-2 cm water. The conidia suspension (with a final concentration of 1×10^6 conidia mL^{-1}) was mixed with each test solution. Aliquots of the mixture consisting of 10 µL drops were placed at the base of three petals on each flower using an automatic pipette (Finnpipette 4027, Thermo Labsystems, Finland). The treatments were randomized in big containers covered with aluminium foil and incubated at 23±1°C. The relative humidity around the flowers was 90-95%, as measured using a thermo-hygrometer (Lambrecht, Germany) (Hjeljord et al., 2011). The experiments were repeated twice. Visual necrotic regions on the abaxial surface under the inoculation points of the flowers were recorded as an infection daily for eight days. The area under the disease progress curve (AUDPC) was calculated on the basis of the cumulative daily infection by the following formula:

$$\text{AUDPC} = \sum_i [(D_i - D_{i-1}) \times \{S_{i-1} + 0.5\,(S_i - S_{i-1})\} \quad (4)$$

where D_i = Days of the i^{th} assessment and S_i = Proportion of the i^{th} infected inoculation point,

The protection index was calculated using the AUDPC values in the following formula (Bardin et al., 2008):

$$100 \times (AUDPC_{\text{control}} - AUDPC_{\text{treatment}})/AUDPC_{\text{control}} \quad (5)$$

where $AUDPC_{\text{control}}$ equals the flowers inoculated with *B. cinerea* alone and $AUDPC_{\text{treatment}}$ equals the flowers treated with chitosan premixed with *B. cinerea* conidia.

2.9. Effect of application timing of chitosan on strawberry flower infection

Chitosans 114, 211B, 311 and 313 (final concentration 5000 µg mL^{-1}) were not premixed with *B. cinerea*, but were instead sprayed (using a hand held atomizer; approximate volume of the test solution applied to each flower: 200 µL) on strawberry flowers at different times. Chitosan was applied 24 hours before, simultaneously or six HAI against *B. cinerea* to find whether the antifungal activity of chitosan 114 and 313 or 211B and 311 differed from each other at different application times. The flower inoculations and the resulting infections were recorded as described for the strawberry flower assay above. The experiments were repeated once (total two experiments were conducted).

2.10. Data analysis

The percent inhibition of *B. cinerea* germination, percent inhibition of growth and percent protection index by chitosan were transformed using an arcsine transformation and tested by one-way ANOVA (analysis of variance); the non-transformed data are presented. For the strawberry flower assay, the cumulative daily infection data over eight days were used to construct disease progress curves. The areas under the curves for different treatments were analyzed by one way ANOVA. When appropriate, means were separated by Tukey's HSD (Honestly Significant Difference) test. All statistical analysis was done using Microsoft Office Excel 2007 or Minitab 16 (MINITAB, USA).

3. Results

3.1. Effect of pH on antifungal activity (germination inhibition) of different chitosan

At all pH levels, *B. cinerea* conidia germination was 100% in the control (SM without chitosan). Chitosan 114 and 313 (F_A 0.11 and 0.18 respectively) showed greater germination inhibition at pH levels 4-6 compared to chitosans 211B and 311 (F_A 0.40 and 0.39 respectively). At all pH levels, there was no significant difference (in terms of germination inhibition) between chitosans 114 and 313 and between chitosan 211B and 311 at 1000 µg mL^{-1}. Chitosan 114 showed significantly ($P < 0.01$) greater germination inhibition at pH 6 than pH 3-5 (Fig. 1).

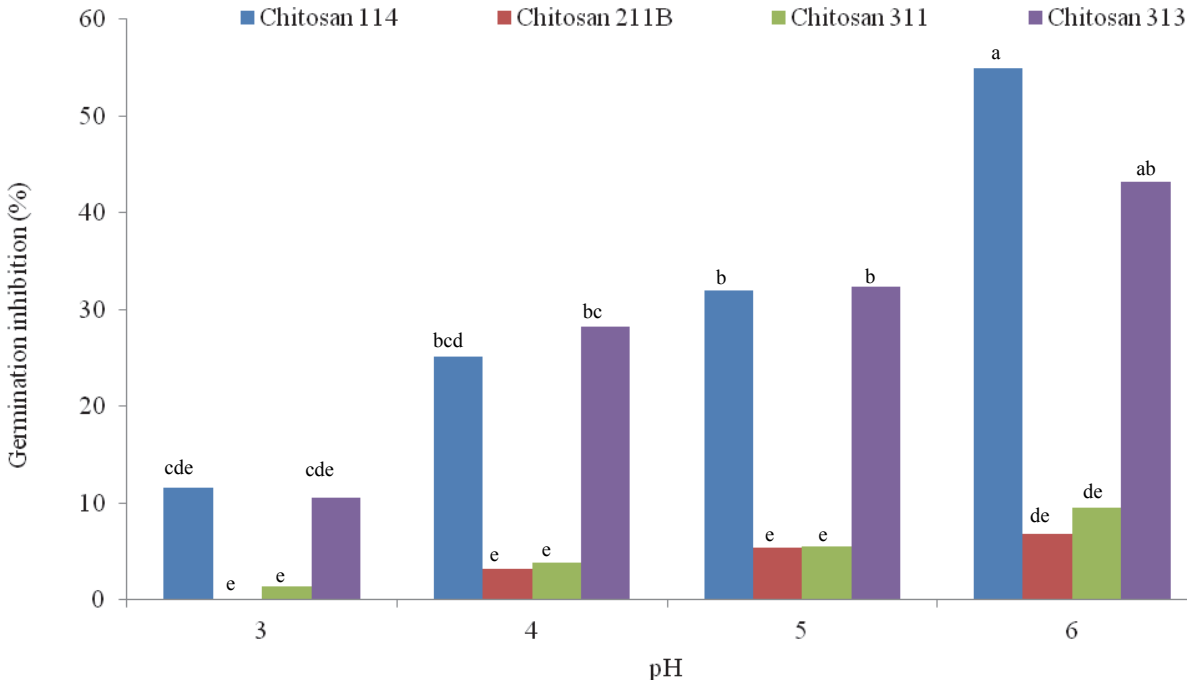

Fig. 1. Inhibition of conidia germination of *B. cinerea* treated with chitosan (1000 µg mL^{-1}) at pH 3 to 6 in SM (6 HAI). Chitosans 114 (F_A 0.11, η 19 mPa·s), 211B (F_A 0.40, η 72 mPa·s), 311 (F_A 0.39, η 360 mPa·s) and 313 (F_A 0.18, η 650 mPa·s). Means in bars without a common letter are significantly different according to Tukey's method at $P< 0.01$.

3.2. The effect of glucose, calcium and magnesium on the antifungal activity of chitosan 114

The four glucose concentrations tested (final concentrations of 28, 56, 85 and 110 mM) did not significantly affect the ability of chitosan 114 (400 µg mL^{-1}) (chitosan 114 showed 15-20% germination inhibition in different glucose concentration) to inhibit *B. cinerea* germination and growth (further germ tube elongation) in SM (data not shown). The calcium concentrations tested did not have any effect on the germination and growth of *B. cinerea* in the absence of chitosan (Fig. 2). When calcium concentrations increased in the SM with chitosan, the antifungal activity of chitosan 114 decreased. In higher concentrations of chitosan 114 (5000 and 3000 µg mL^{-1}) with low calcium concentrations (0 or 0.14 mM), *B. cinerea* germination was completely inhibited 24 HAI. At high calcium concentration (1.4 mM), the germination was only inhibited by 20% and 15% when chitosan concentrations were 5000 and 3000 µg mL^{-1} respectively (Fig. 2). Similar trends were observed in the inhibition of growth (further germ tube elongation data

not shown). Magnesium also showed similar trend as calcium to reduce the antifungal activity of chitosan 114 against *B. cinerea* (data not shown).

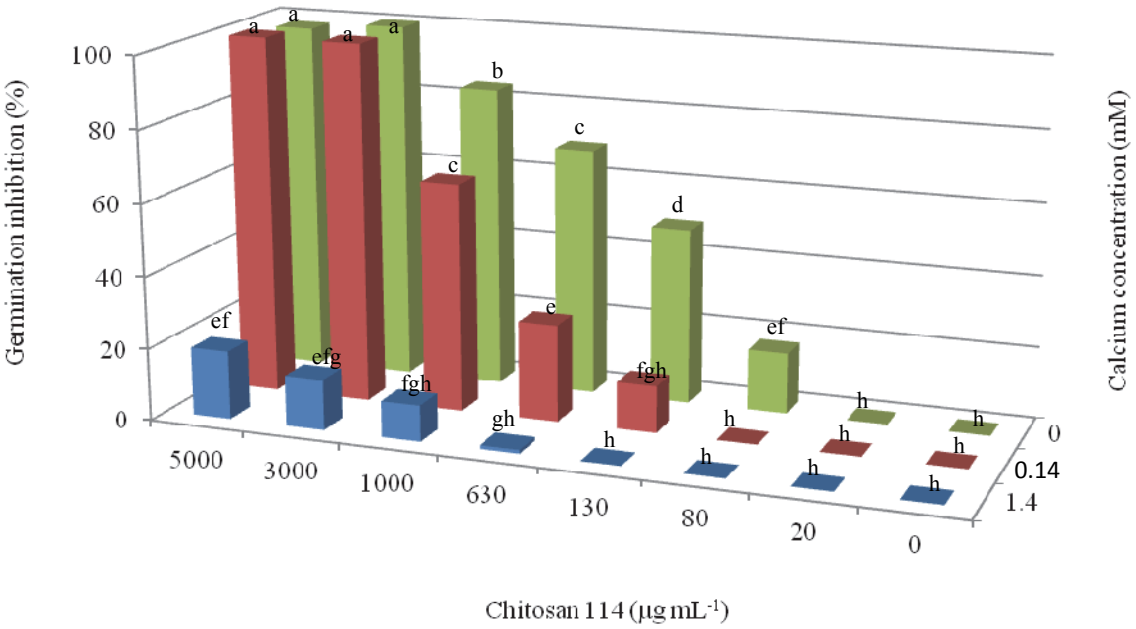

Fig. 2. Effect of calcium concentration on germination inhibition of *B. cinerea* by chitosan 114 (F_A 0.11; η 19 mPa.s) in SM 24 HAI. Means in bars without a common letter are significantly different according to Tukey's method at $P<0.01$.

The effects of calcium in reducing the antifungal activity of chitosan 114 in different media are shown in Table 1. In the absence of calcium, chitosan 114 (80 µg mL^{-1}) showed about complete (98%) germination inhibition of *B. cinerea* in PDB, but only 26% germination inhibition in SM. The presence of calcium reduced the ability of chitosan 114 to inhibit germination of *B. cinerea* in both media. In media with a high calcium concentration (1.4 mM), chitosan 114 (80 µg mL^{-1}) did not inhibit germination in either PDB or SM (Table 1).

Table 1

Effect of calcium concentration on germination inhibition of *B. cinerea* by chitosan 114 (80 μg mL^{-1}) in SM and PDB (24 HAI).

Calcium (mM) [a]	Germination inhibition[b] (% ± SD)	
	PDB	SM
1.4	1 ± 1 d	0 ± 0 b
0.34	36 ± 10 c	0 ± 0 b
0.14	76 ± 4 b	3 ± 2 b
0	98 ± 2 a	26 ± 2 a

[a] Means in column without a common letter are significantly different according to Tukey's method at $P<0.01$.

[b] All data are mean of three experiments ± standard deviation.

3.3. The effects of F_A, molecular weight and time of chitosan application on the antifungal activity of chitosan

The *in vitro* assay showed that chitosan with low F_A was more effective than high F_A of chitosan. Chitosan 114 and 313 (with close F_A, but large difference in chain length) showed similar germination inhibition against *B. cinerea*. Similarly, chitosan 211B and 311 (with close F_A, but large difference in chain length) showed the same germination inhibition at 1000 μg mL^{-1} (Fig. 1). However, at a high concentration (3000 μg mL^{-1}) chitosan 114 was more effective than chitosan 313 (data not shown). *In vivo* assay (strawberry flower assay) also showed that low F_A chitosan was more effective than high F_A chitosan (Table 2). Chitosan 114 (low molecular weight compared to chitosan 313) showed significantly (P <0.01) greater inhibition of flower infection than chitosans 313 at a high concentration (10000 μg mL^{-1}).

Table 2

Disease severity on strawberry flowers inoculated with a mixture of *B. cinerea* conidia and chitosan. AUDPC was used to calculate the protection index.

Chitosan [a]	F_A	Viscosity (mPa.s)	Concentration (μg mL^{-1})	AUDPC [b] ± SD	Protection index (% ± SD)
114	0.11	19	10000	1.7 ± 0.3 g	63 ± 5 a
			5000	3.5 ± 0.7 cdef	24 ± 12 bcd
			80	4.4 ± 0.2 abc	5 ± 4 ef
211B	0.40	72	10000	3.3 ± 0.4 ef	30 ± 6 bc
			5000	3.7 ± 0.3 bcdef	21 ± 3 cde
			80	4.5 ± 0.2 ab	3 ± 3 e
311	0.39	360	10000	3.4 ± 0.3 ef	26 ± 6 bcd
			5000	3.9 ± 0.1 abcde	16 ± 2 cdef
			80	4.4 ± 0.1abcd	6 ± 3 ef
313	0.18	650	10000	2.8 ± 0.3 f	40 ± 4 b
			5000	3.5 ± 0.2 cdef	26 ± 3 bcd
			80	4.1 ± 0.3 abcde	12 ± 3 def
Control	-	-	-	4.7 ± 0.2 a	-

[a] Means in column without a common letter are significantly different according to Tukey's method at $P<0.01$.

[b] The AUDPC values were calculated from cumulative disease incidences recorded up to eight DAI with incubation at 23±1°C. All data are mean of three experiments ± standard deviation.

To find the effect of molecular weight of chitosan with the same F_A (as chitosan 114 and 313 did not have exactly the same F_A) con its antifungal activity against *B. cinerea*, chitosan samples with different DP_n (with the same F_A 0.11) were tested in microtiter plates (Table 3). Low

molecular weight chitosan DP_n 22 and 48 (80 µg mL^{-1}) showed the greatest germination inhibition, followed by DP_n 75 at 12 HAI. High molecular weight chitosan 114 (DP_n 145) at the same concentration did not have any inhibitory effect at 12 or 24 HAI. The most effective molecular weight of chitosan against *B. cinerea* was DP_n 22.

Table 3

Germination inhibition of *B. cinerea* by CHOS and unhydrolyzed chitosan 114 (80 µg mL^{-1}) in MSM.

Hydrolysis time (min)[a]	DP_n	Germination inhibition[b] (% ± SD)	
		12 hours	24 hours
0	145	0 ± 0 c	0 ± 0 c
5	75	59 ± 5 b	26 ± 3 b
10	48	95 ± 4 a	38 ± 5 b
20	22	100 ± 1 a	76 ± 9 a

[a]Means in column without a common letter are significantly different according to Tukey's method at $P<0.01$.

[b]All data are mean of three experiments ± standard deviation.

The timing of chitosan application affected the inhibition of *B. cinerea*. Chitosan 114 (F_A 0.11) inhibited flower infection significantly (P <0.01) (AUDPC 1.1) followed by chitosan 313 (F_A 0.18) (AUDPC 2.3) when applied 24 hours prior to inoculation with *B. cinerea* conidia. Chitosan 211B and 311 (F_A 0.40 and 0.39) did not have any effect on reducing flower infection (AUDPC 3.2-3.5 respectively) when applied 24 hours before inoculation. None of the chitosan was effective to control *B. cinerea* when applied the same time or six HAI (data not shown).

4. Discussion

The natural biopolymer chitosan shows antifungal activity and is an alternative to synthetic fungicides (Trotel-Aziz et al., 2006). However, many factors such as pH and the presence of calcium and magnesium in the media, molecular weight, F_A and application time of chitosan affect its antifungal activity against *B. cinerea*. In our study, the presence of calcium and magnesium in media without chitosan had no effect on the germination and growth (further germ

tube elongation) of *B. cinerea*. When calcium or magnesium were present in the media, the antifungal activity of chitosan decreased. This result is in accordance with Tsai and Su (Tsai and Su, 1999). However, Tsai and Su (1999) showed that calcium is more effective in reducing the antimicrobial activity of chitosan than magnesium against *E. coli*. However, in our study similar trends of effectiveness of calcium and magnesium in reducing the antifungal activity of chitosan were found against *B. cinerea*. The reduction of antifungal activity may be due to a complex formation between chitosan and metal ions, ultimately leaving the chitosan with low or no charge (Tsai and Su, 1999). While some studies suggest that calcium reduces the antifungal activity of chitosan by the formation of cross-linking bonds (involving the -OH and -NH_2 groups of the glucosamine residues) (Rodríguez et al., 2008; Tamura et al., 2004), other studies indicate that calcium has a very low or no affinity for chitosan (Delben and Muzzarelh, 1989). Another explanation for the effects of calcium and magnesium may be that they compete with chitosan for binding sites on the cell surface, and calcium, magnesium do not produce the leakage-inducing effect of chitosan.

In the present study, the antifungal activity of chitosan (F_A 0.11) against *B. cinerea* was higher at pH 6 than at pH 3-5. This is in accordance with a previous study (Stössel and Leuba, 1984) which reported the highest antifungal activity of chitosan (pKa 6.2) against *Phytopthora megasperma* to be at pH 6. When the pH of the media is slightly lower than the pKa value of chitosan, the chitosan amino groups are protonated (Stössel and Leuba, 1984). Our chitosan samples had a pKa of 6.5. One reason for the antifungal activity of chitosan in acidic conditions is that protonated chitosan interact with negatively charged cell surface, causing death of the microorganism by leakage of intracellular electrolytes (Helander et al., 2001; Reddy et al., 1998).

Both our *in vitro* and *in vivo* assays demonstrated that the F_A of chitosan is an important factor influencing the antifungal activity of chitosan. Chitosan with low F_A (0.11 and 0.18) were more effective in inhibiting *B. cinerea* than chitosan with high F_A (39-40). This result is in accordance with Stössel and Leuba (1984), who found that the F_A of chitosan impacted its antifungal activity and that a low F_A (0.09) was more inhibitory than a high F_A (0.34). The strong correlation between the F_A of chitosan (the lower the F_A the higher antimicrobial activity of chitosan) and its antifungal activity is because decreasing F_A increases the number of potentially protonated

amino groups of chitosan, which enables electrostatic interactions with the negative cell surface of microorganisms in acidic environments (Jung et al., 2010).

The molecular weight of chitosan also influences its antimicrobial activity (Kendra and Hadwiger, 1984), but previous research showed contradictory results. While some studies show that the antifungal activity of chitosan increases with decreasing molecular weight of chitosan (Eikenes et al., 2005; Meng et al., 2010), other studies show that high is more effective than low molecular weight chitosan (Kim and Rajapakse, 2005; Xu et al., 2007). Our *in vitro* study showed that low molecular weight chitosan (chitosan 114, F_A 0.11 and viscosity 19 mPa.s) and high molecular weight chitosan (chitosan 313, F_A 0.18 and viscosity 650 mPa.s) with about the same F_A, but with different molecular weight, did not differ in their antifungal activity (at the concentration 1000 μg mL^{-1}, Fig. 1). However, at the concentration 3000 μg mL^{-1}, low molecular weight chitosan (chitosan 114, F_A 0.11, viscosity 19 mPa.s) showed significantly (P<0.01) higher antifungal activity than high molecular weight chitosan (chitosan 313, F_A 0.18, viscosity 650 mPa.s) (data not shown). Our *in vivo* study on strawberry flowers also showed that low molecular weight chitosan (chitosan 114) was more effective against *B. cinerea* than high molecular weight chitosan (chitosan 313) at a high concentration (10 000 μg mL^{-1}), but below that concentration there were no differences regarding antifungal activity between those two chitosans. As the tested chitosans (chitosan 114 and 313) did not have exactly the same F_A, we hydrolyzed chitosan 114 to produce chitosans with different molecular weight with the same F_A and found that low molecular weight chitosan (DPn 22) was more effective than high molecular weight chitosan (DPn 145).

To further investigate the effect of molecular weight on the antifungal activity of chitosan against *B. cinerea* on strawberry flowers *in vivo*, chitosans were applied at different times (24 hours before, at the same time or six hours after inoculation with *B. cinerea*). Low molecular weight chitosan (chitosan 114; η 19 mPa·s) was more effective than high molecular weight chitosan (chitosan 313; η 650 mPa·s) when applied on strawberry flowers 24 hours before inoculation of *B. cinerea* conidia. This study also showed that application of chitosan before inoculation was more effective than applying at the same time or six HAI of *B. cinerea*. This result is in accordance with a previous study which showed that chitosan has fungicidal effect against *B. cinerea* in cucumber plants (Ben-Shalom et al., 2003). When spraying chitosan 24 hours before

inoculation the disease development was reduced by 87%. Our study suggested that both direct (inhibition of germination and further germ tube elongation) and indirect (elicitation effect) antifungal activity of chitosan are needed to control *B. cinerea.* When applying chitosan 24 hours before inoculation the plant has more time to produce defence-related proteins before the *B. cinerea* infection starts to develop (Aziz et al., 2006). An indirect way chitosan may prevent *B. cinerea* infection in the strawberry flower assay may be to form a film, a physical barrier, against *B. cinerea* on the flowers (Romanazzi et al., 2002). The effectiveness of chitosan film against *B. cinerea* on table grapes was not related to the viscosity of chitosan or the thickness of the film (Romanazzi et al., 2009), the direct antifungal activity of chitosan and its elicitor effect may be more important in reducing *B. cinerea* infection than film formation.

In the present study, chitosan was applied to strawberry flowers by two different methods. Chitosan was more effective against *B. cinerea* when co-inoculated with conidia in drops than when sprayed alone on flowers. This result could be due to greater contact between conidia and chitosan when these were mixed prior to application.

This study confirms that chitosan has potential as an alternative to chemical pesticides and thus may be successfully included in integrated pest management (IPM) programs to control *B. cinerea*. As various factors impact the effectiveness of chitosan as an antifungal agent, further research is needed to find the optimal environment for pathogen inhibition.

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Paper II

Effect of combination of chitosan and Teldor (fenhexamid) against of *Botrytis cinerea*

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Effect of combination of chitosan and Teldor (fenhexamid) against *Botrytis cinerea*

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ABSTRACT

Botrytis cinerea is a plant pathogenic fungus causing gray mold on a wide variety of economically important crops throughout the world. Chitosan, a derivative of chitin, inhibits growth of many plant pathogens and is a possible, new alternative to chemical fungicides to control *B. cinerea.* This paper examines synergism between chitosan (fraction of acetylation, (F_A) 0.11; average degree of polymerization (DP_n) 145) and Teldor (Bayer Crop Science, active ingredient: fenhexamid) on antifungal activity against *B. cinerea*. Various concentrations of chitosan and Teldor were mixed together or used separately with *B. cinerea* conidia and incubated in microtiter plates with modified synthetic medium (MSM) (*in vitro* assay), or applied to strawberry flowers (*in vivo* assay). *In vitro,* the combination of Teldor (80 µg mL^{-1}) and chitosan (80 µg mL^{-1}) showed complete germination inhibition with a high level of synergism, but alone the compounds showed none and 3% germination inhibition respectively at 24 hours after inoculation (HAI). Synergism also occurred in the *in vivo* assay, where the combination of low concentrations of Teldor (e.g. 4.5 µg mL^{-1}) and chitosan (80 µg mL^{-1}) reduced flower infection more effectively than either agent alone. The combination of chitosan and Teldor also reduced the sporulation of *B. cinerea.* These findings suggest that chitosan may be used to reduce the input of chemical fungicides in integrated pest management systems (IPM), without loss of pathogen control. To our knowledge this is the first time that synergism between chitosan and Teldor in inhibiting *B. cinerea* has been reported.

Keywords: Interaction effect, disease control, Integrated Pest Management (IPM)

1. Introduction

The fungal pathogen *Botrytis cinerea* Pers.: Fr. (anamorph of *Botryotinia fuckeliana*), may cause gray mold in over 200 plant species, mainly dicotyledonous plants, resulting in severe pre- and postharvest losses in agricultural crops like berries, fruits and vegetables (Williamson et al., 2007). Farmers are highly dependent on chemical fungicides to control *B. cinerea.* Teldor [*N*-(2, 3-dichloro-4-hydroxyphenyl)-1-1methylcyclohexanecarboxamide] (fenhexamid) is a hydroxyanilide chemical fungicide highly effective against *B. fuckeliana*. Teldor only inhibit germination of *B. fuckeliana* at high concentrations, but the fungicide is effective to inhibit further germ tube elongation (Debieu et al., 2001). Chemical fungicides may be harmful to mammals and the environment. Thus, the intensive use of chemical fungicide in crops has made scientists search for alternatives to control *B. cinerea* and biological control and cultural control (pruning, leaf clipping and leaf plucking) (Card, 2005) have been proposed, but none have shown consistent control (Jacometti et al., 2010). Chitosan is another alternative to control *B. cinerea* (Reglinski et al., 2010; Romanazzi et al., 2009).

Chitosan, a heteropolymer obtained by partial deacetylation of chitin, is non toxic, biodegradable (Pillai et al., 2009) and may protect plants through its antimicrobial activity or by eliciting plant defence mechanisms against pathogens (Hadwiger, 1979; Hadwiger and Beckman, 1980; Yang et al., 2010). However, as chitosan is not as effective as chemical fungicides against *B. cinerea* (Bautista-Baños et al., 2006), there is a need to find out an effective way of using chitosan. Previous studies have shown synergism between chitosan and antibiotics (Tin et al., 2010; Tin et al., 2009), thus this paper studied possible synergism between chitosan and Teldor (fenhexamid) in terms of inhibiting germination and strawberry flower infection by *B. cinerea*.

2. Materials and methods

2.1. Chitosan and Teldor

Chitosan 114 (fraction of acetylation (F_A) 0.11; viscosity, η 19 mPa·s; average degree of polymerization (DP_n) 145) with pKa 6.5, was obtained from Pronova Biopolymer Oslo, Norway. Chitosan was dissolved in sterile, distilled water to make a 10000 μg mL^{-1} stock solution (w/v).

Teldor® WG 50 (500 g kg^{-1} fenhexamid; Bayer Crop Science Pty. Ltd., Victoria, Australia) belongs to the chemical group hydroxyanilides. Teldor (10,000 μg mL^{-1}) was suspended in sterile distilled water to make stock solutions.

2.2. Measuring the DP_n and F_A of chitosan

Ten mg chitosan were dissolved in 0.5 ml deuterium oxide (D_2O) and the pH was adjusted to 4.2 with sodium deuteroxide (NaOD) and deuterium chloride (DCl) prior to lyophilization. This process was repeated once. Finally the lyophilized chitosan sample was dissolved in 700 μL D_2O and ^{1}H-NMR analysis was performed on a 300 MHz Varian Gemini 300 instrument (Varian, USA) at 85°C. The ^{1}H-NMR spectra are presented in Figure 1. The DP_n and F_A were calculated by the formulas (Sørbotten et al., 2005):

$(D\alpha+D\beta+D+A\alpha+A\beta+A)/(D\alpha+D\beta+A\alpha+A\beta)$ ……(1)

F_A by the formula $A/(D+A)$ ……(2)

where $D\alpha$, $D\beta$, $A\alpha$ and $A\beta$ are the integral of the reducing end signals of the α and β anomers of the deacetylated (D) and acetylated (A) units respectively, D is the integral of the signals from the internal and non reducing end deacetylated units and A is the integral of the signals from the internal and non reducing end acetylated units.

2.3. Botrytis cinerea

Botrytis cinerea isolate BC 101 was originally collected from an infected field grown strawberry fruit in Grimstad, Norway. Stock cultures were preserved in 20% glycerol at -80°C. For the *in vitro* and *in vivo* bioassays, conidia were collected from cultures pre-grown on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) under regular laboratory light for 2 weeks at

23±1°C. The conidia were gently scrapted from surface of PDA plate and suspended in sterile water and filtered through sterile cotton to remove fragments of mycelia. Conidia concentrations were determined by hemocytometer counter at 400 × magnifications (Leica, DM RBE, Germany) and adjusted to desired concentrations.

2.4. Effect of chitosan and Teldor on Botrytis cinerea germination and growth in vitro

The effects of chitosan alone and in combination with Teldor on germination and growth of *B. cinerea* were investigated in modified synthetic media (MSM) (Rahman et al., 2012). The final concentrations in the microtiter plate wells (after adding chitosan, Teldor and conidia) were thus 2.5 mM NH_4NO_3; 0.28 mM $CaCl_2 \cdot 2H_2O$; 0.16 mM $MgSO_4 \cdot 7H_2O$; 0.002 mM $MnSO_4 \cdot 4H_2O$; 0.002 mM $ZnSO_4 \cdot 7H_2O$; 1 mM KH_2PO_4; 0.06 mM $FeC_6H_5O_7 \cdot 5H_2O$ and 55.5 mM glucose. Treatments were a) control (only MSM), b) chitosan (final concentrations in microtiter wells: 1000 and 80 µg mL^{-1} MSM), or c) chitosan (same final concentrations as in treatment d) in combination with Teldor (final concentrations 160, 80, 40, 20, 10, 4.5, 3, 1.5 and 0.5 µg mL^{-1} MSM). One hundred microliter of each solution were added to microtiter wells in a flat-bottom 96-well microtiter plate (Nunc^{TM}, Roskilde, Denmark). There were four replicate wells of each treatment. One hundred microliter conidial suspensions were added to the contents of the wells for the final concentration 2×10^4 conidia mL^{-1}. The microtiter plates were incubated at 23±1°C for 12 and 24 hours. The germination percentage was visually estimated using an invert microscope (Fluovert FU, Ernst Leitz Wetzlar GmbH, Wetzlar, Germany) at 400× magnification. The first 100 conidia observed in each well were counted as germinated when the germ tube length was at least as long as the diameter of the conidium.

The germination inhibition percentage was calculated by the following formula:

$$\text{Germination inhibition (\%)} = \{(a-b)/a\} \times 100 \quad (3)$$

Where, a = number of germinated conidia in the control sample (MSM)

b = number of germinated conidia in the presence of chitosan or Teldor alone or in combination

The pH of the conidia suspension in the microtiter wells was between 5.2 and 5.3 at the start of the experiment, and remained about the same 24 hours after inoculation (HAI).

The interactions between Teldor and chitosan were determined by Abbott's equation (Levy et al., 1986). Synergistic effect was calculated by the ratio between the observed efficacy E_{obs} (% inhibition) and the expected efficacy (E_{exp}) calculated by Abbott's formula:

$E_{exp} = a+b - (ab/100)$..... (4)

Here a = % germination inhibition by Teldor alone, b = % germination inhibition by chitosan alone. The E_{obs}/E_{exp} ratio 1 indicates additivity, ratios >1 indicate synergy and ratios <1 indicate antagonistic interaction. The experiments were repeated twice.

2.5. Effect of chitosan and Teldor in inhibiting Botrytis cinerea on strawberry flowers in vivo

The ability of chitosan and Teldor alone or in combination to reduce *B. cinerea* flower infection was tested on newly opened strawberry flowers (*Fragaria* × *ananassa*, cv. Corona) under controlled conditions. The strawberry plants were grown in greenhouse with controlled temperature (18^{o}C at day; 12^{o}C at night), light (16 hours, light intensity: 150 µmols m^{-2} sec^{-1}) and relative humidity (65%). Eighteen flowers per treatment (six replications of three flowers) were cut off with a 1½-2 cm stem and placed in empty pipette tip racks set in plastic containers filled with 1-2 cm water. Conidia suspension (final concentration: 1×10^{6} conidia mL^{-1}) was mixed with chitosan (final concentration: 1000 and 80 µg mL^{-1}) or Teldor (final concentration: 60, 4.5, 3, 1.5 and 0.5 µg mL^{-1}) alone or in combination. Conidia suspension (only conidia and water, final concentration: 1×10^{6} conidia mL^{-1}) was used as positive control. Ten microliter drops of each mixture were placed at the base of three petals on each flower using an automatic pipette (Finnpipette 4027, Thermo Labsystems, Finland). The treatments were randomized in big containers and covered with aluminum foil, incubated at 23±1°C and the relative humidity around the flowers was 90-95%. The experiment was repeated twice. The flowers were considered 100% infected when all three points of inoculation displayed necrotic signs.

The visual necrotic regions on the abaxial surface of the flowers under the inoculation points were recorded as an infection daily for eight days. The area under the disease progress curve (AUDPC) was calculated on the basis of the daily cumulative infection by the following formula:

$$AUDPC = \sum_i [(D_i - D_{i-1}) \times \{S_{i-1} + 0.5 (S_i - S_{i-1})\} \quad (5)$$

Where, D_i = Days of the i^{th} assessment and S_i = Proportion of the i^{th} infected inoculation point

The protection index was calculated by using the AUDPC values in the following formula (Bardin et al., 2008):

$$100 \times (AUDPC_{control} - AUDPC_{treatment})/AUDPC_{control} \quad (6)$$

Where $AUDPC_{control}$ were the flowers inoculated with only *B. cinerea* and $AUDPC_{treatment}$ the flowers treated with chitosan or Teldor alone or in combination. The interaction between chitosan and Teldor in the flower assay was determined by Abbott's formula (Levy et al., 1986) as previously described.

3. Results

3.1. Characterization of the chitosan

The ^{1}H-NMR spectrum of chitosan (F_A 0.11, DP_n 145) with α-values 0.01 shows that reducing ends were deacetylated (signals at 5.43 ppm for Dα and 4.92 ppm for Dβ). There were no signals for acetylated reducing ends (Aα at 5.19 ppm and Aβ at 4.74) (Fig. 1).

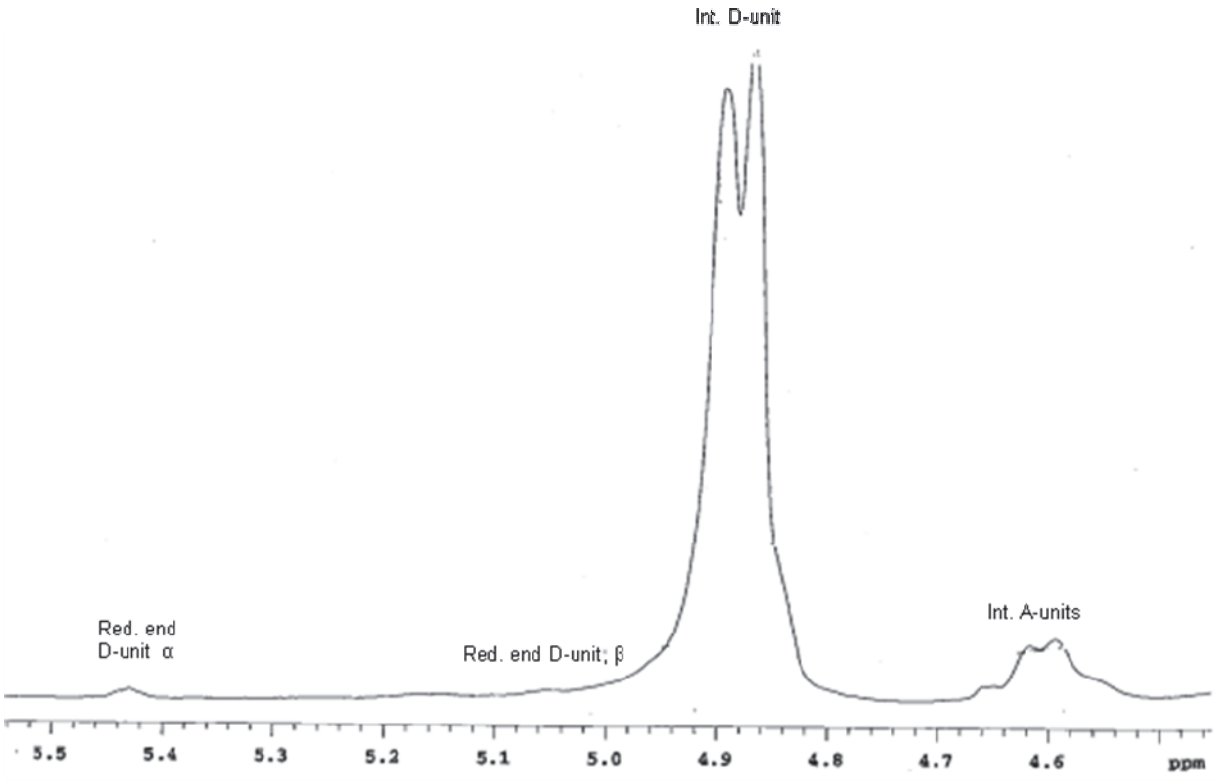

Fig. 1. ^{1}H-NMR spectra of the chitosan. The α-values denote the degree of scission ($\alpha = 1/DP_n$). Dα and Dβ are the integral of the reducing end signals of the α and β anomers of the deacetylated units respectively.

3.2. Effect of chitosan and Teldor on B. cinerea germination in vitro

Chitosan applied alone was not effective in inhibiting *B. cinerea* conidia germination (24 hours after inoculation, HAI) as 1000 μg mL^{-1} chitosan was needed for 50% germination inhibition (data not shown) and 80 μg mL^{-1} chitosan only showed 3% inhibition (Table 1). Teldor is not prescribed for germination inhibition of *B. cinerea*, thus none of the Teldor concentrations tested, not even the recommendation dose (1500 μg mL^{-1}), had inhibitory effects on germination (data not shown). However, all combinations of chitosan (80 μg mL^{-1}) and Teldor (0.5-60 μg mL^{-1}) showed high levels of synergism and the combination of 80 μg mL^{-1} chitosan and 80 μg mL^{-1} Teldor almost completely inhibited germination (24 HAI, Table 1). When the chitosan concentrations were increased (≥80 μg mL^{-1}) and Teldor concentrations remained constant (80 μg mL^{-1}), cytoplasmic disorder occurred (data not shown).

Table 1

Germination inhibition (expressed as percent of control in MSM) of *Botrytis cinerea* by chitosan and/or Teldor.

Teldor ($\mu g\ mL^{-1}$)	Chitosan ($\mu g\ mL^{-1}$)	12 hours after inoculation		24 hours after inoculation	
		Germination inhibition[a] (% ± SD)	E_{obs}/E_{exp}	Germination inhibition (% ± SD)	E_{obs}/E_{exp} [b]
0	80	15 ± 4	-	3 ± 1	-
160	0	0	-	0	-
160	80	98 ± 2	7	97 ± 2	32
80	80	97 ± 2	6	97 ± 4	32
40	80	93 ± 4	6	81 ± 5	27
20	80	81 ± 7	5	68 ± 3	23
10	80	68 ± 7	5	60 ± 8	20
4.5	80	57 ± 9	4	36 ± 7	12
3	80	50 ± 8	3	34 ± 2	11
1.5	80	49 ± 14	3	18 ± 5	6
0.5	80	45 ± 10	3	8 ± 3	3

[a]All data are mean of three experiments ± standard deviation.

[b]The E_{obs}/E_{exp} ratio 1 indicates additivity, ratios >1 indicate synergy.

3.3. Effect of chitosan and Teldor in inhibiting B. cinerea on strawberry flowers

When applied alone, 80 $\mu g\ mL^{-1}$ chitosan only prevented *B. cinerea* infections on strawberry flowers by 8% (Table 2). The recommendation dose of Teldor (1500 $\mu g\ mL^{-1}$) prevented *B. cinerea* infections on strawberry flowers by 62%, and the same level of protection was achieved by the combination of a much lower concentration of Teldor (60 $\mu g\ mL^{-1}$, 0.04 times the recommended dose) and 1000 $\mu g\ mL^{-1}$ chitosan (data not shown). Only combinations of chitosan (80 $\mu g\ mL^{-1}$) and low concentrations of Teldor (0.5 and 1.5 $\mu g\ mL^{-1}$) showed synergism, but the level of protection against flower infection was low (protection index about 20%) (Table 2).

Although the level of protection was low, these combinations affected the sporulation of *B. cinerea* at the eighth DAI (data not shown).

Table 2

Disease severity on strawberry flowers inoculated with a mixture of *Botrytis cinerea* conidia and chitosan and/or Teldor.

Teldor (µg mL^{-1})	Chitosan (µg mL^{-1})	AUDPC (± SD)[a]	Protection index [a] (% ± SD)	E_{obs}/E_{exp} [b]
0	80	4.5 ± 0.2	8 ± 7	-
60	0	2.6 ± 0.3	47 ± 7	-
60	80	2.3 ± 0.3	53 ± 8	1
4.5	0	4.4 ± 0.2	10 ± 8	-
4.5	80	3.9 ± 0.2	20 ± 3	1
3.0	0	4.4 ± 0.2	10 ± 2	-
3.0	80	4.0 ± 0.2	18 ± 1	1
1.5	0	4.6 ± 0.2	5 ± 4	-
1.5	80	3.8 ± 0.3	23 ± 10	2
0.5	0	4.6 ± 0.2	6 ± 5	-
0.5	80	3.9 ± 0.3	20 ± 7	2
control [c]	-	4.9 ± 0.4	-	-

[a] The AUDPC values were calculated from cumulative disease incidences recorded up to eight DAI at 23±1°C. The AUDPC was used to calculate the protection index. All data are mean of three experiments ± standard deviation.

[b] E_{obs}/E_{exp} 1 indicate additivity and ratios >1 indicate synergy.

[c] Control indicates conidia with sterile water.

4. Discussion

The data reported here indicate that chitosan is effective in combination with the chemical fungicide Teldor (fenhexamid) to inhibit *B. cinerea in vitro* and *in vivo*. Teldor and chitosan

applied alone had nearly no effect on the germination of *B. cinerea in vitro*, but the combination of Teldor (80 µg mL^{-1}) and chitosan (80 µg mL^{-1}) showed a high level of synergism with complete germination inhibition. The reasons for synergism between chitosan and Teldor are not clearly understood, as chitosan's mode of action is not yet established and the compound may target several vital functions of the pathogen (e.g. *B. cinerea*) (Palma-Guerrero et al., 2010; Palma-Guerrero et al., 2009; Palma-Guerrero et al., 2008). Synergism may be due to the two compounds' different modes of action. Teldor affects the *B. cinerea* cell membrane through inhibition of the sterol biosynthesis (Debieu et al., 2001), but applied alone, Teldor may not be able to reach the conidia cell membrane prior to germination. The positively charged chitosan causes electrostatic interactions with the negatively charged cell surface (Hadwiger and Beckman, 1980), resulting in loss of cell wall integrity and loosening of the cell wall ultimately increasing the permeability of the cell wall (Helander et al., 2001; Reddy et al., 1998). This permeability may enable Teldor to reach and affect the conidia cell membrane and stop the germination at an earlier stage than if Teldor was applied alone.

The combination of Teldor (60 µg mL^{-1}) and chitosan (80 µg mL^{-1}), which showed high synergism and complete germination inhibition of *B. cinerea* in *vitro* (data not shown), only showed 53% protection against flower infection *in vivo*. However, a protective effect (62 % protection index against flower infection caused by *B. cinerea*) achieved by the recommendation dose of Teldor (1500 µg mL^{-1}), was also achieved by a combination of a much lower concentration of Teldor (60 µg mL^{-1}) and 1000 µg mL^{-1} chitosan. This combination also decreased the sporulation of *B. cinerea*, neither agent did when applied alone, and could thus reduce the severity of the subsequent infection cycle.

Our results suggest that a protective effect against *B. cinerea* compared to that from chemical fungicides may be attained with a combination of a strongly reduced dose of Teldor and a low concentration of chitosan. Chitosan, possessing multiple modes of action against plant pathogens, in combination with Teldor could also reduce the risk of resistance of *B. cinerea.* Thus the combined use of these compounds in an integrated pest management system may reduce the excessive use of chemical fungicides and ultimately benefit the environment, farmers and consumers.

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Paper III

Effect of chitooligosaccharides with different degrees of polymerization on *Botrytis cinerea* and *Mucor piriformis*

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Abstract

BACKGROUND: Chitosan, obtained from chitin by partial *N*-deacetylation, shows little or no toxicity towards mammalian cells, is biodegradable, non-allergenic and protects plants by its antimicrobial activity. Chitosan (fraction of acetylation, F_A 0.15; average degree of polymerization (DP_n) 206) was enzymatically hydrolyzed to produce chitooligosaccharides (CHOS) with DP_n 75, 58, 48, 40, 23, 15, 11, and 9. Then the original chitosan and the individual CHOS fractions were tested for antifungal activity against *Botrytis cinerea* and *Mucor piriformis*.

RESULTS: *In vitro* testing showed that CHOS of DP_n 23 and 40 had the highest germination inhibition against all tested pathogens. The original chitosan (DP_n 206) and a collection of short CHOS (degree of polymerization 3-10) were significantly ($P<0.01$) less effective than those of DP_n 23 and 40. *M. piriformis* M119J showed the most abnormal swelling in presence of CHOS DP_n 40, but all abnormally swelled conidia showed further elongation. *In vivo* testing showed that CHOS DP_n 23 was the most effective in reducing flower infection by two isolates of *B. cinerea*.

CONCLUSION: Chitosan and CHOS inhibit fungal germination and growth and the effect depends highly on the level of polymerization of the oligomers.

Keywords: Chitosan, chitooligosaccharides (CHOS), antifungal, plant protection

1 INTRODUCTION

The pathogenic fungus *Botrytis cinerea* Pers.: Fr. (anamorph of *Botryotinia fuckeliana*) can cause gray mold in over 200 plant species, mainly dicotyledonous plants, resulting in severe pre- and postharvest losses in agricultural crops like berries, fruits and vegetables.[1] *Botrytis cinerea* is also the main production constraint in several chickpea growing areas like South Asia, Australia and the Americas.[2] The pathogenic fungus, *Mucor piriformis* Fischer, also causes postharvest rots on strawberries as well as on several other fruit crops.[3] Chemical fungicides are the primary means to control fungal diseases[4], and in Norway most of the fungicide applications on strawberry aim at preventing gray mold caused by *B. cinerea*.[5] Pathogens may develop resistance towards chemical fungicides, creating a need for even more chemicals.[6] However, as existing chemical fungicides are harmful for mammals and the environment, other alternatives are needed. Alternatives to control *B. cinerea* are biological and cultural control[7] or fungicides that causes no harm to the environment and are not toxic to mammals such as chitosan.[8]

Chitosan is a linear polymer made by partial deacetylation of chitin, and consists of β (1→4) linked N-acetyl-D-glucosamine (GlcNAc; A) and D-glucosamine (GlcN; D). Chitin is a linear polysaccharide consisting of GlcNAcs and it is one of the most abundant biopolymers in nature. It is found mainly in the cell walls of fungi and as a structural polymer in crustaceans, arthropods, insects, and parasitic nematodes.[9] Chitosan, in turn, can be hydrolyzed into shorter chains like chitooligosaccharides (CHOS). Both chitosan and CHOS, which show little or no toxicity towards mammalian cells, are biodegradable[10] and may protect plants by their direct antimicrobial activity or by eliciting plant defence mechanisms against pathogens.[11,12] The antifungal activity of chitosan is influenced by many factors such as its concentration[13], degree of polymerization (DP) or molecular weight,[14] fraction of acetylation (F_A)[15], pH, and ionic strength of the media.[16,17] Previous studies on the effect of molecular weight on the antimicrobial activity of chitosan have given diverging results. While some studies found that polymeric chitosan (high molecular weight) had higher antifungal activity than low molecular weight chitosan[18,19], other studies reported the opposite result.[20,21] However, most studies involved chitosans with only one or very few different molecular weights that were not obtained from the same source or by the same method, thus these data may not give comparable results. The aim of this study is therefore to use well-defined chitosans with respect to molecular weight and

determine which shows most inhibitory effect against the two common plant pathogens *B. cinerea* and *M. piriformis*. Two types of experiments were carried out: an *in vitro* microtiter plate assay on conidial germination and hyphal growth of *B. cinerea* and *M. piriformis*, and an *in vivo* assay on strawberry flower infection by *B. cinerea*.

2 MATERIALS AND METHODS

2.1 Enzymatic production and analysis of chitooligosaccharides (CHOS)

Chitosan (KitoNor; fraction of acetylation (F_A) 0.15; viscosity average molecular weight (MWv) 33.4 kDa and DP_n 206) was obtained from Norwegian Chitosan (Gardermoen, Norway). CHOS with varying level of polymerization were produced from chitosan (DP_n 206) by enzymatic hydrolysis for different lengths of time, using chitosanase ScCsn46A.[22]

Chitosanase was produced as described by Heggset and coworkers[22]; briefly, chitosanase ScCsn46A, originally from *Streptomyces coelicolor* q9rj88, was purified from the culture supernatant of the transformant *Escherichia coli* BL21Star (DE3), following the published protocol, with the exception that the $(His)_6$-tag was not removed from the enzyme after purification. The enzyme was dialyzed against Tris-HCl (20 mM) at pH 8 and stored at 4°C.

2.1.1 Hydrolysis of chitosan to produce CHOS

Chitosan (DP_n 206, 10 mg mL^{-1}) in buffer (0.04M NaAc, 0.1M NaCl, pH 5.5) and HCl (0.5M) was incubated at 37°C and shaken at 225 rpm until the chitosan was dissolved. The pH was further adjusted to 5.5 with NaOH (0.5M). Chitosanase ScCsn46A[22] was added (0.5 µg mg^{-1} chitosan) to the chitosan solution and the mixture was incubated for 10-50 minutes at 37°C and 225 rpm. The enzymatic reaction was stopped by decreasing the pH to 2.5 with HCl (0.5M), followed by immersing the tube in boiling water for at least 10 minutes to inactivate the enzymes permanently. The resulting CHOS samples were dialyzed against dH_2O for 48 hours (water was changed every 12 hours) using a cellulose membrane (Float-A-Lyzer® MWCO 500 Da, from Spectrum Labs, Texas, USA) to remove salts from the sample. Dialyzed samples were sterile filtrated through Filtropur S 0.2 µm sterile filters (Sarstedt, Germany), lyophilized and stored at 4°C.[23]

2.1.2 Measuring the DP_n of CHOS

Lyophilized CHOS (10 mg) were dissolved in deuterium oxide (D_2O, 0.5 ml) and the pH was adjusted to 4.2 with sodium deuteroxide (NaOD) and deuterium chloride (DCl) prior to lyophilization. This process was repeated once. Finally the lyophilized CHOS sample was dissolved in D_2O (700 µL) and ^{1}H-NMR analysis was performed on a 300 MHz Varian Gemini instrument (Varian, USA) at 85^{o}C (^{1}H-NMR spectra is shown in Fig. 1). The DP_n was calculated with the formula (Dα+Dβ+D+Aα+Aβ+A)/(Dα+Dβ+ Aα+Aβ), where Dα, Dβ, Aα and Aβ are the integral of the reducing end signals of the α and β anomers of the deacetylated (D) and acetylated (A) units respectively, D is the integral of the signals from the internal and non reducing end deacetylated units and A is the integral of the signals from the internal and non reducing end acetylated units.[24]

2.1.3 Separation of CHOS DP 3-10 by size exclusion chromatography (SEC)

CHOS DP_n 5, made from enzymatic hydrolysis of chitosan (DP_n 206) with ScCsn46A, was separated on three Superdex^{TM} 30 columns (XK columns from GE Healthcare) with an overall dimension of 2.6×180 cm. The flow rate of the mobile phase (0.15 M NH_4Ac, pH 4.5) was maintained at 0.8 ml min^{-1}.[24] A refractive index detector (Gilson model 133, UK) was used to monitor the relative amounts of the CHOS fractions. CHOS (100 mg) sample was applied in each run. CHOS DP 3-10 was collected from several separate runs and pooled followed by dialysis to remove salts from the buffer. The sample was then sterile filtrated through Filtropur S 0.2 µm sterile filters, and lyophilized.

2.1.4 Matrix assisted laser desorption/ ionization time of flight mass spectrometry

MS spectra were acquired using an Ultraflex^{TM} TOF/TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) with gridless ion optics under control of Flexcontrol 4.1. For sample preparation, 1 µL of isolated CHOS and 2 µL of matrix solution (15 mg mL^{-1} 2,5-dihydroxybenzoic acid) were mixed and 1 µL of the mixed solution was spotted on a target plate.[25] The spotted samples were dried at room-temperature. The MS experiments were conducted using an accelerating potential of 20 kV in the reflector mode.

2.2 Fungal pathogens

The fungal pathogens used in this experiment were: *Botrytis cinerea* isolate BC 101, isolated from infected strawberry fruit in Grimstad, Norway; *B. cinerea* isolate BCBD, from a chickpea leaf in Gazipur, Bangladesh and *M. piriformis* isolate M199J, from an infected strawberry fruit at Hobøl, Norway. Conidia were collected from cultures grown on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) under regular laboratory light for two weeks at 23±1°C. The conidia were suspended in sterile water and were filtered through sterile cotton to remove fragments of mycelia. Conidia concentrations were determined by hemocytometer counter at 400 × magnification (Leica, DM RBE, Germany) and adjusted to the required concentrations.

2.3 *In vitro* microtiter plate assay; effects of chitosan and CHOS against *Botrytis cinerea* and *Mucor piriformis*

The effect of chitosan and CHOS on germination inhibition of *B. cinerea* (isolates BC 101 and BCBD) and *M. piriformis* M199J was studied in modified synthetic medium (MSM) in microtiter plates.[26] The final concentrations of MSM in the microtiter plate wells were 2.5 mM NH_4NO_3; 0.28 mM $CaCl_2 \cdot 2H_2O$; 0.16 mM $MgSO_4 \cdot 7H_2O$; 0.002 mM $MnSO_4 \cdot 4H_2O$; 0.002 mM $ZnSO_4 \cdot 7H_2O$; 1 mM KH_2PO_4; 0.06 mM $FeC_6H_5O_7 \cdot 5H_2O$ and 55.5 mM glucose. The following solutions (100 µL of each) were added separately to microtiter wells in a flat-bottom 96-well microtiter plate (Nunc^{TM}, Roskilde, Denmark): control treatment (only MSM), or chitosan DP_n 206 or CHOS (DP_n 9, 11, 15, 23, 40, 58 and 75) (all 80 µg mL^{-1}) or DP 3-10 (80, 600 and 1300 µg mL^{-1}). There were three replicate wells of each treatment. Conidial suspensions (100 µL) were added to the contents of the wells, giving a final concentration of 2×10^4 conidia mL^{-1}. The microtiter plates were incubated at 23±1°C for 24 hours. The germination percentage 12 and 24 hours after inoculation (HAI) was visually estimated at 400× magnification using an invert microscope (Fluovert FU, Ernst Leitz Wetzlar GmbH, Wetzlar, Germany). The conidia were counted as germinated if the germ tube length was at least as long as the diameter of the conidium. The germination of *M. piriformis* M199J conidia was measured differently since all germinated conidia showed abnormal swelling with amoeba-like structure and one or more protruded portions. When the length of the protruded part was at least as long as the diameter of the swelled conidia 12 HAI, the conidia was considered germinated. The germination inhibition percentage was calculated by the following formula:

Germination inhibition (%) = {(a-b)/a} × 100

Where, a = germinated conidia in the control (MSM)

b = germinated conidia in the presence of chitosan or CHOS in MSM

The pH of the conidia suspension in the microtiter wells was between 5.2 and 5.3 at the start of the experiment, and remained about the same 24 HAI.

Germination and further germ tube elongation were also documented by photographs (10-15, from each treatment with a Canon camera, Japan) taken through the invert microscope at 400× magnification (Fluovert FU, Ernst Leitz Wetzlar GmbH, Wetzlar, Germany) 12 and 44 HAI.

2.4 *In vivo* bioassay: effects of chitosan and CHOS against *Botrytis cinerea* on strawberry flowers

Chitosan (DP_n 206) and CHOS (DP_n 9, 23, 40 and 48) were tested for their ability to reduce infection by *B. cinerea* BC 101 and *B. cinerea* BCBD of newly opened strawberry flowers under controlled conditions. Strawberry plants (*Fragaria* × *ananassa* cv. Corona) were grown in greenhouse under controlled temperature (18^{o}C at day; 12^{o}C at night), light (16 hours, light intensity: 150 µmols m^{-2} sec^{-1}) and relative humidity (65%). Eighteen flowers per treatment (six replications of three flowers) were cut off with a 1½-2 cm stem and placed in empty pipette tip racks set in plastic containers filled with 1-2 cm water. Conidia suspension (final concentration: 1×10^{6} conidia mL^{-1}) was mixed with different test pathogens, and each mixture (10 µL) was placed at the base of three petals on each flower. The plastic containers with flowers were covered with aluminium foil and incubated at 23±1°C and relative humidity 90-95%. The experiment was repeated twice. The visual necrotic regions on the abaxial surface of the flowers under the inoculation points were recorded as an infection daily for eight days, and the area under the disease progress curve (AUDPC) was calculated on the basis of the daily cumulative infection by the following formula:

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

Where, D_i = Days of the i^{th} assessment and S_i = Proportion of the i^{th} infected inoculation point

The protection index was calculated by inserting the AUDPC values in the following formula[27]:

$100 \times (AUDPC_{control}\text{-}AUDPC_{treatment})/AUDPC_{control}$

Where $AUDPC_{control}$ were the flowers inoculated with *B. cinerea* BC 101 or BCBD alone and $AUDPC_{treatment}$ the flowers inoculated with chitosan or CHOS premixed with *B. cinerea* BC 101 or BCBD.

2.5 Data analysis

In the microtiter plate assay, the percentage of germination inhibition of pathogens by chitosan and CHOS were transformed by arcsine transformation and tested by one way ANOVA (only non transformed data are presented). In the strawberry flower assay, the AUDPC was calculated based on the cumulative daily infections from one to eight days, and tested by one way ANOVA. When appropriate, means were separated by Tukey's Honestly Significant Difference method. All statistical analysis was done by Microsoft Office Excel 2007 and Minitab 16 (MINITAB, USA).

3 RESULTS

3.1 Production and characterization of CHOS

CHOS fractions were produced by enzymatic hydrolysis of chitosan with F_A 0.15, viscosity average molecular weight (MWv) 33.4 kDa, and average degree of polymerization (DP_n) 206. DP_n was used as an indicator of molecular weight for all CHOS samples since this is straightforwardly determined by ^{1}H NMR analysis.[24] Using this approach, CHOS fractions with DP_n of 75, 58, 48, 40, 23, 15, 11, and 9 were produced. The ^{1}H-NMR spectra of the CHOS fractions showed that all reducing ends were completely deacetylated (signals at 5.43 ppm for -Dα and 4.92 ppm for -Dβ) (Fig. 1). There were no signals for new acetylated reducing ends (signal for -A α at 5.19 ppm and for DA β at 4.74). A CHOS fraction of DP_n of 5 was also produced and further purified by SEC. CHOS of DP 3-10 were collected and pooled together to serve as the fraction with lowest molecular weights.

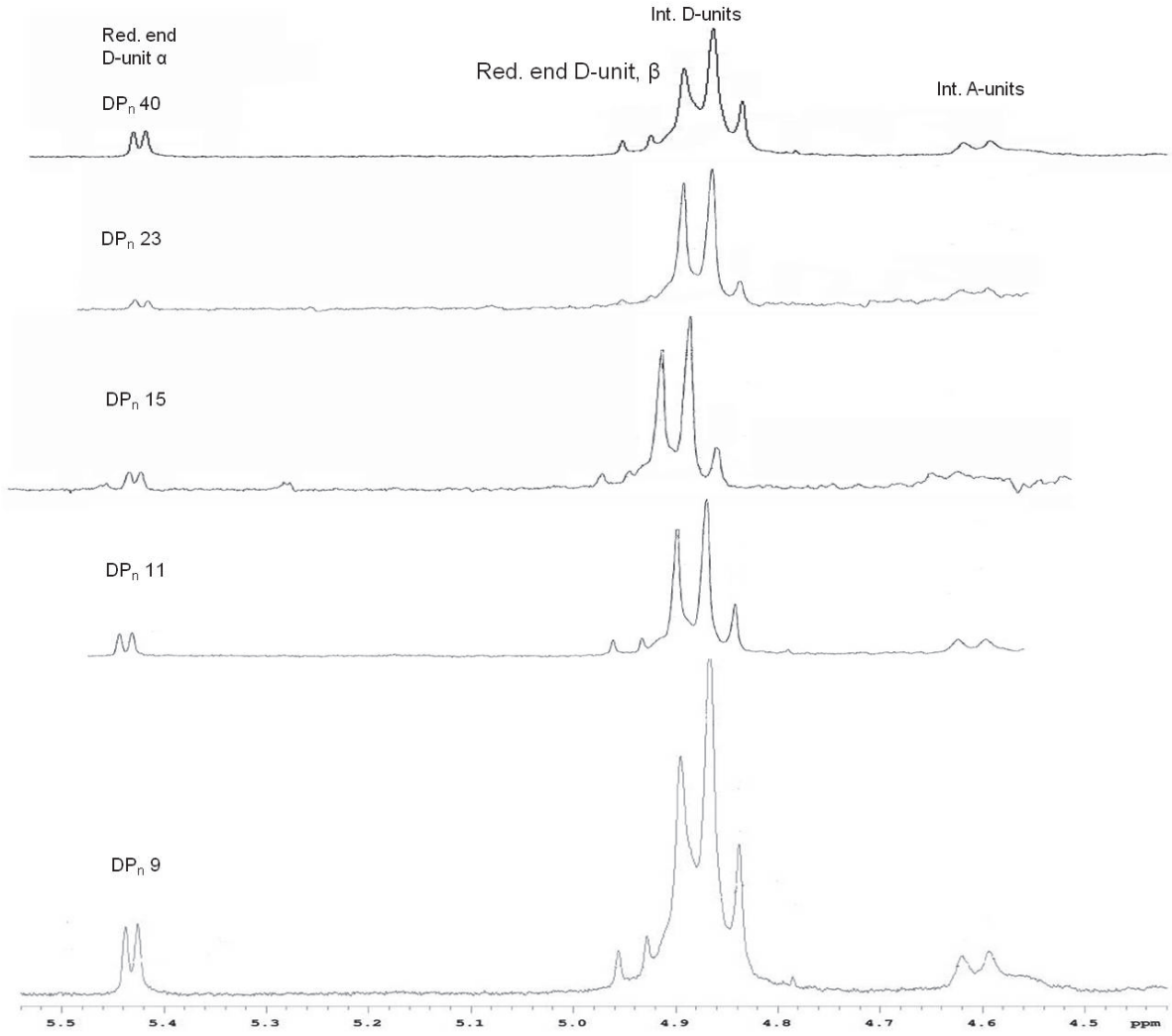

Figure 1. ^{1}H-NMR spectra of CHOS after hydrolysis of KitoNor chitosan (F_A 0.15) with ScCsn46A for DP_n of 40, 23, 15, 11, and 9, respectively (same spectra for DP_n of 75, 58, and 48 was omitted for clarity).

3.2 Effects of chitosan and CHOS on fungal germination and germ tube elongation *in vitro*

The antifungal activities of chitosan and CHOS with different DP_n (80 µg mL^{-1}) against fungal pathogens are shown in Table 1. All three isolates experienced a higher degree of inhibition 12 HAI vs. 24 HAI. *M. piriformis* was sensitive (in terms of germination inhibition) to a wide range of CHOS (DP_n 75, 58, 48, 40, 23, and 15 at 12HAI) while *B. cinerea* BC 101 was only sensitive to CHOS DP_n 23 and 40 (24 HAI). The results clearly show a size dependency for the anti fungal

effect of CHOS. The antifungal activity was also tested for the original chitosan (F_A 0.15, DP_n 206) and CHOS DP3-10 as controls for high and low molecular weight fractions.

Table 1. Characterization and effect of chitosan (DP_n 206; F_A 0.15) and CHOS (both 80 μg mL^{-1}) on germination inhibition (GI)[a] (expressed as percent of control in MSM) of *Botrytis cinerea* (BC 101 and BCBD) and *Mucor piriformis* M199J. All data are means of three experiments.

Chitosan/ CHOS	DP_n [c]	*B. cinerea* BC 101		*B. cinerea* BCBD		*M. piriformis* M199J	
		GI % 12HAI	GI % 24HAI	GI % 12HAI	GI % 24HAI	GI % 12HAI	GI% 24HAI
Chitosan	206	13 b[b]	0 b[b]	5 c[b]	0 c[b]	44 c[b]	0[b] e
CHOS	75 ± 7	12 b	3 b	7 c	0 c	99 a	30 d
	58 ± 3	10 b	2 b	7 c	1 c	99 a	51 c
	48 ± 3	13 b	4 b	6 c	5 c	99 a	50 c
	40 ± 1	90 a	77 a	98 a	99 a	99 a	95 a
	23 ± 3	82 a	80 a	98 a	99 a	99 a	89 a
	15 ± 1	15 b	4 b	100 a	99 a	99 a	66 b
	11 ± 1	7 b	4 b	94 b	45 b	70 b	7 e
	9 ± 1	1 b	1 b	0 c	0 c	21 d	0 e

[a] GI was measured 12 and 24 hours after inoculation (HAI).

[b] Means in columns without common letters are significantly different according to Tukey's method at $P< 0.01$.

[c] DP_n data are mean of two experiments ± standard deviation.

At 80 μg mL^{-1}, there was no significant difference between DP 3-10 and chitosan in terms of germination inhibition (24 HAI), but at a high concentration (1300 μg mL^{-1}) DP 3-10 showed significantly higher germination inhibition of all tested pathogens than chitosan (24 HAI, Table 2).

Table 2. Effect of different concentration of chitosan DP_n 206 and CHOS DP 3-10 on germination inhibition (GI) (expressed as percent of control in MSM) of *Botrytis cinerea* (BC 101 and BCBD) and *Mucor piriformis* M199J. All data are means of two experiments.

Chitosan /CHOS	Concentration ($\mu g\ mL^{-1}$)	*B. cinerea* BC 101		*B. cinerea* BCBD		*M. piriformis* M199J	
		GI % 12 HAI[a]	GI % 24 HAI	GI % 12 HAI	GI % 24 HAI	GI % 12 HAI	GI % 24 HAI
DP_n 206	1300	54 b[b]	36 b[b]	42 b[b]	30 b[b]	100 a[b]	3 c[b]
	600	31 cd	26 b	30 b	18 bc	100 a	2 c
	80	19 d	4 c	4 c	3 c	50 b	1 c
DP 3-10	1300	85 a	88 a	77 a	67 a	100 a	98 a
	600	41 bc	43 b	75 a	26 b	100 a	83 b
	80	3 e	3 c	1 c	0 c	32 c	1 c

[a] Hours after inoculation (HAI).

[b] Means in columns without common letters are significantly different according to Tukey's method at $P<0.01$.

The effects of DP_n on the ability of chitosan and CHOS to inhibit fungal germination and further germ tube elongation were also visually observed (Figs. 2 and 3). Chitosan (DP_n 206) and CHOS (DP_n 40) caused abnormal swelling of *M. piriformis* (Fig. 2), but not of the two isolates of *B. cinerea* conidia. However, CHOS DP_n 40 caused granular inclusions in the cytoplasm of all tested pathogens. In the presence of CHOS DP_n 40, germ tubes from both *B. cinerea* isolates ceased to grow after germination and did not grow further up to 44 HAI (Fig. 3). The *M. piriformis* M199J conidia (treated with CHOS DP_n 40) that were abnormally swelled 12 HAI (Fig. 2), continued to grow 44 HAI, but many non-germinated and non-swelled conidia were also present. Chitosan (DPn 206) and CHOS DP_n 9 did not impact the germ tube elongation of the tested pathogens (Fig. 3).

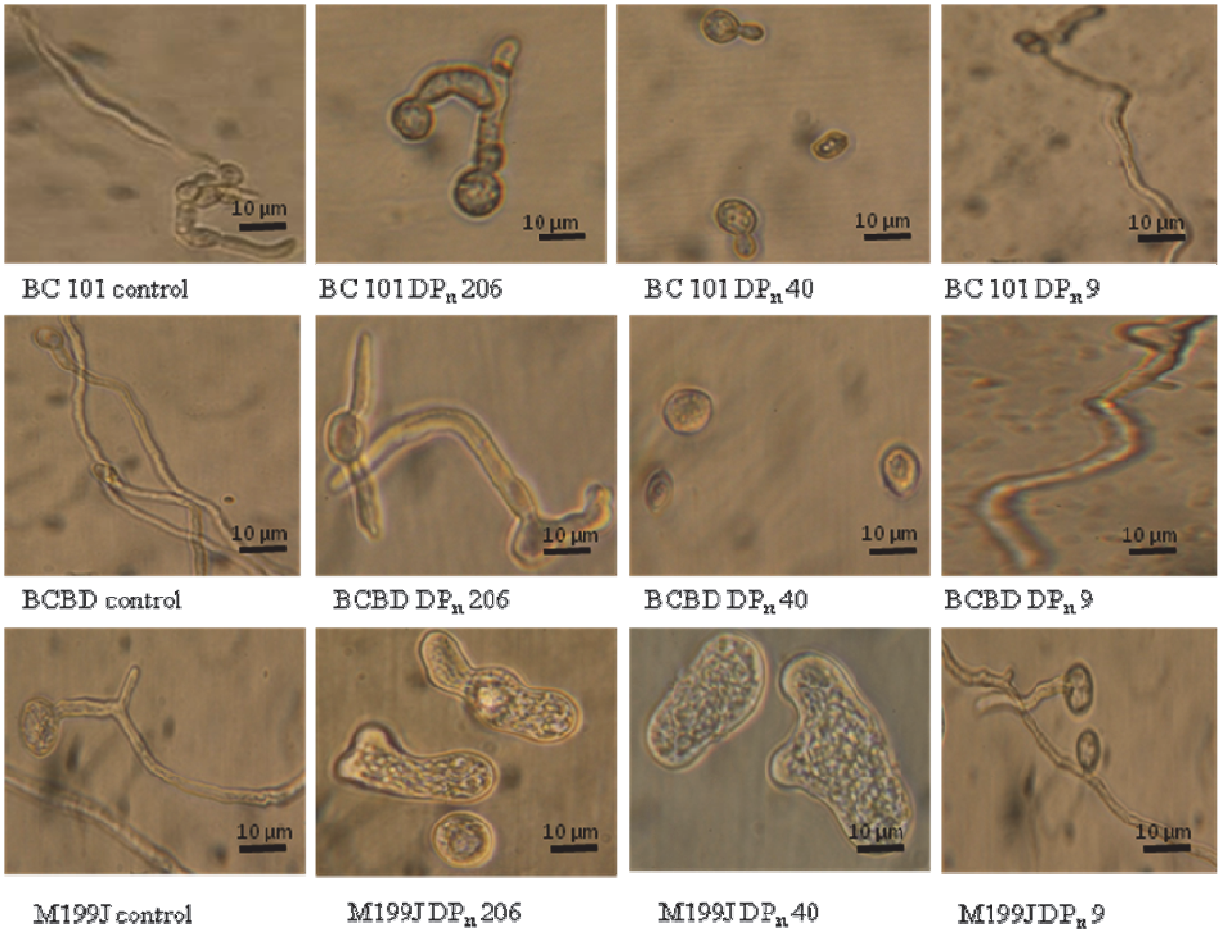

Figure 2. Effect of chitosan DP_n 206 and CHOS DP_n 9 and 40 (all 80 µg mL^{-1}) on conidia germination and further germ tube elongation of *Botrytis cinerea* (BC 101 and BCBD), and *Mucor piriformis* M199J (12 hours after inoculation (HAI), 23±1°C). BC 101: *B. cinerea* BC 101; BCBD: *B. cinerea* BCBD, M199J: *M. piriformis* M199J. Control is conidia in MSM.

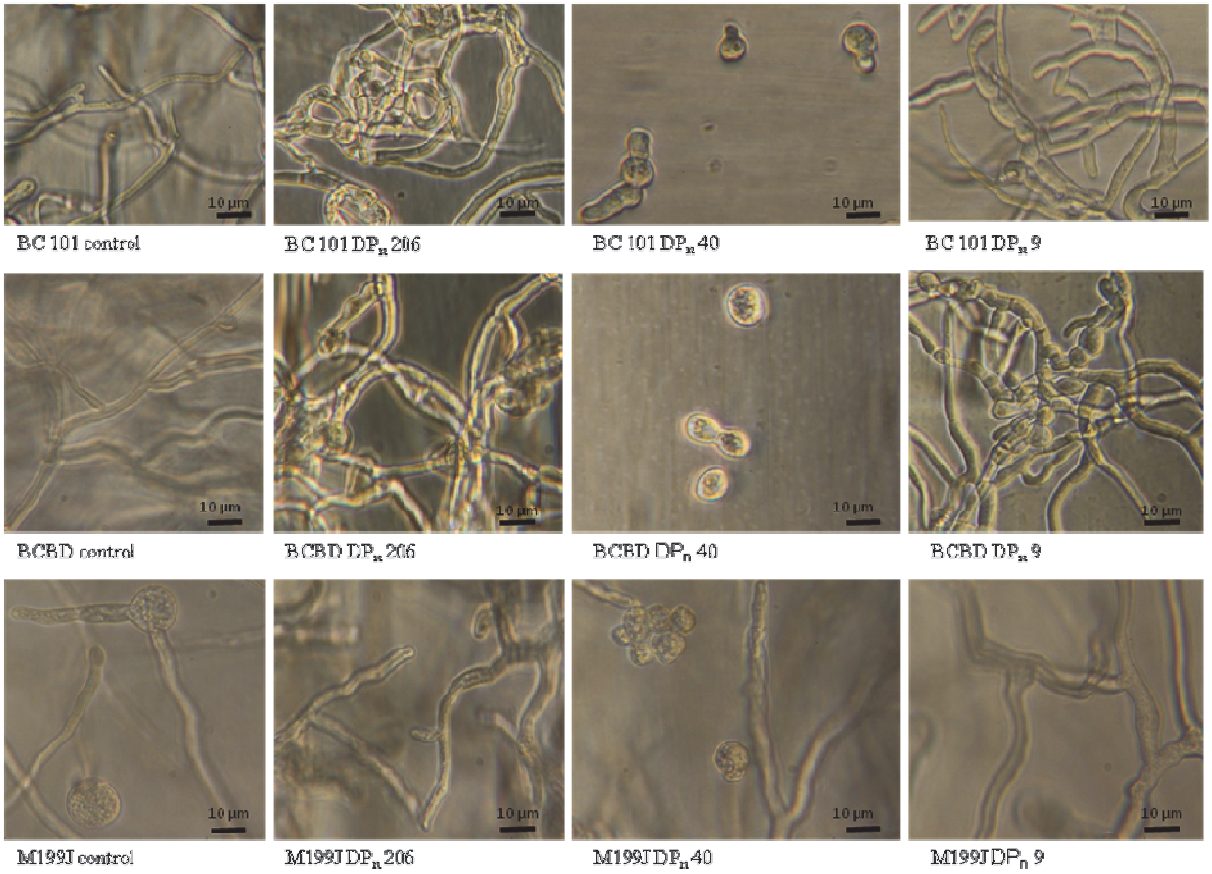

Figure 3. Effect of chitosan (DP_n 206) and CHOS (DP_n 9 and 40) (80 µg mL^{-1}) on germ tube elongation of *Botrytis cinerea* (BC 101 and BCBD), and *Mucor piriformis* M199J (44 hours after inoculation (HAI) at 23±1°C). BC 101: *B. cinerea* BC 101; BCBD: *B. cinerea* BCBD, M199J: *M. piriformis* M199J. Control is conidia in MSM.

3.3 Strawberry flower bioassay

CHOS DP_n 23 followed by DP_n 40 were the most effective chitooligosaccharides in reducing flower infection by *B. cinerea* (BC 101 and BCBD) (Table 3 and Fig. 5) *in vivo.*

Table 3. Disease severity on strawberry flowers inoculated with a mixture of *Botrytis cinerea* (BC 101 or BCBD) conidia and chitosan (DP_n 206) or CHOS with different DP_n (9, 23, 40 and 48) (all 500 μg mL^{-1}). Control indicates conidia in sterile water. All data are means of three experiments.

Chitosan/CHOS	*B. cinerea* BC 101		*B. cinerea* BCBD	
	AUDPC[a]	Protection index (%)	AUDPC[a]	Protection index (%)
Control	4.4 a[b]	-	4.4 a[b]	-
DP_n 206	4.1 a	6 d[b]	3.6 b	18 d[b]
DP_n 48	3.3 b	25 c	2.8 c	36 c
DP_n 40	1.8 c	59 b	1.5 d	66 b
DP_n 23	0.7 d	85 a	0.7 e	84 a
DP_n 9	4.0 a	8 c	3.5 b	21 d

[a] The AUDPC was used to calculate the protection index. The AUDPC values were calculated from cumulative disease incidences recorded up to eight days after inoculation (DAI) with incubation at 23±1°C.

[b] Means in columns without common letters are significantly different according to Tukey's method at $P< 0.01$.

Both *B. cinerea* isolates caused 100% infection on strawberry flowers six DAI in the control and when treated by chitosan DP_n 206 and CHOS DP_n 9 (500 μg mL^{-1}) (Fig. 5). CHOS DP_n 23 and DP_n 40 reduced the *B. cinerea* BC 101 infection to 60% and 30% respectively, and the *B. cinerea* BCBD infection to 43% and 20% respectively at six DAI (data not shown).

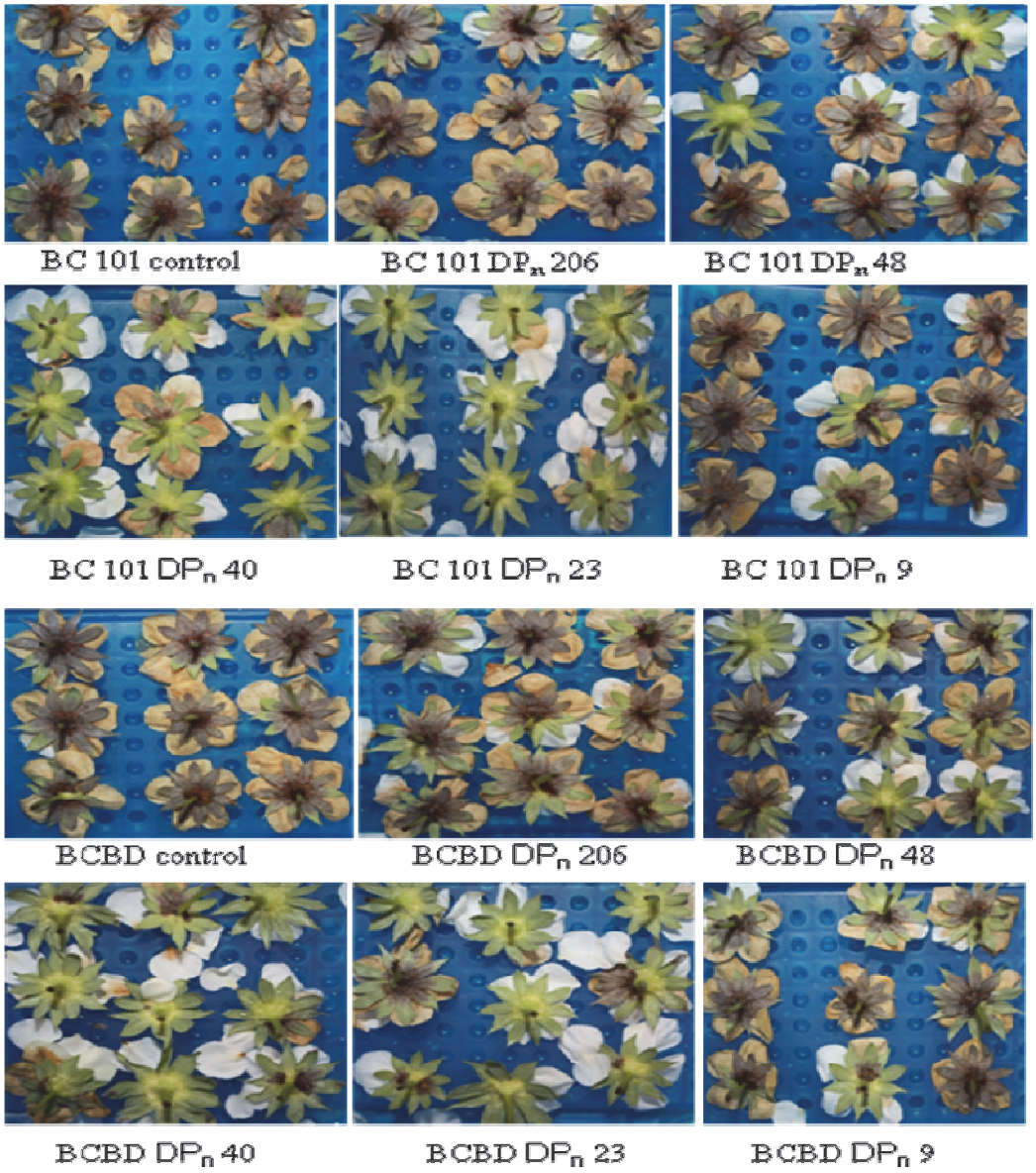

Figure 5. Effect of chitosan (DP_n 206) and CHOS DP_n 9, 23, 40 and 48 (500 µg mL^{-1}) on *Botrytis cinerea* (BC 101 and BCBD) infection of strawberry flowers six days after inoculation (DAI). Control was conidia in sterile water. The flowers were considered 100% infected when all three inoculation points displayed necrotic signs. All treatments included 18 flowers, but only nine flowers are shown here.

4 DISCUSSION

Molecular weight, and consequently the degree of polymerization, of chitosan is an important factor affecting its antifungal activity.[14] In this study, well-defined chitosan and CHOS fractions of different DP/DP_n obtained from the same chitosan were used to test for antifungal activity against *Botrytis cinerea* and *Mucor piriformis in vitro* and *in vivo*. The antifungal activity of chitosan and CHOS varied with the DP_n in both bioassays, and CHOS 23 and 40 were the most effective of the tested pathogens inhibiting germination and further germ tube elongation *in vitro*. Antifungal activity of chitosan has been previously reported to depend on DP, where 6 kDa (DP_n around 40) was the most effective on a range from 5 to 27 kDa in inhibiting *Candida krusei*.[28] In our study, chitosan with high (DP_n 206, MWv 34.4 kDa) and low (CHOS DP 3-10) DP were only effective in inhibiting germination and further germ tube elongation of *B. cinerea* and *M. piriformis* at a high concentration and CHOS DP 3-10 was more effective than chitosan (DP_n 206). This corresponds to a previous study stating that CHOS with low DP (DP 3-9) were more effective inhibiting *B. cinerea* than chitosan with high DP (molecular weight 300-500 kDa).[21]

The effectiveness of chitosan and CHOS also depends on the cell wall composition of the tested pathogen.[29] The cell walls of ascomycetous fungi (*B. cinerea*) contain chitin, whereas *Mucor* spp. (*M. piriformis*) contain both chitin and chitosan.[30] Previous research showed contradictory results regarding the correlation between chitosan's antifungal activity and the fungal cell wall composition. Allan and Hadwiger documented that fungi with cell walls containing chitosan (*Mucor* spp.) were not sensitive to chitosan (1000 µg mL^{-1}).[29] However, at the same chitosan concentration, El-Ghaouth showed germination and growth inhibition of other fungi (*Rhizopus stolinifer* and *Mucor racemosus*) with chitosan in their cell wall.[31] Our results showed that the sensitivity of fungal pathogens to chitosan varied with the DP. *Mucor piriformis* M199J was sensitive (in terms of germination inhibition) to CHOS with a wide range of DP (DP_n 15, 23, 40, 48, 58 and 75), *B. cinerea* BCBD to CHOS with a narrower range of DP (DP_n 11, 15, 23 and 40) and *B. cinerea* BC 101 was only sensitive to DP_n 23 and 40.

Microscopic observations confirmed that the DP of CHOS was important for its ability to inhibit germination and growth (further germ tube elongation) of the tested pathogens, since DP_n 40 showed more cytoplasmic disorder and abnormal swelling of conidia than the other DP_n. *B. cinerea* conidia developed granular substances in the cytoplasm in the presence of CHOS DP_n 40

at low concentration (80 μg mL^{-1}) while higher concentrations of CHOS DP 3-10 (1300 μg mL^{-1}) and chitosan DP_n 206 (≥2500 μg mL^{-1}) were required for the same effect. Similar morphological changes have previously been found in *B. cinerea* treated with chitosan.[32] Still, *B. cinerea* conidia, even at a high chitosan concentration (5000 μg mL^{-1}), did not show abnormal swelling in our study, which has also been observed in a previous study.[31] In contrast, all tested DP of chitosan and CHOS in our study, except the lowest DP (CHOS DP_n 9 and DP 3-10), caused abnormal swelling of *M. piriformis* conidia. The swelling varied with the concentration and DP of chitosan and CHOS where the most abnormal swelling (amoeba like structure) occurred in *M. piriformis* treated with CHOS DP_n 40. Also other studies have confirmed that the DP of chitosan influenced the extent of abnormal swelling of fungal conidia. Chitosan with high DP (molecular weight 30.7 kDa, DP ≈ 150) caused more abnormal swelling of *Rhizopus stolonifer* conidia than chitosan with low DP (molecular weight 17.4 kDa , DP ≈ 90).[33] Low DP (exact molecular weight not mentioned) at a high concentration (15000 μg mL^{-1}) changed the shape of *Rhizoctonia solani* conidia.[34]

No lyses of the cell walls of abnormally swollen *M. piriformis* conidia were observed and all conidia continued to grow (further elongation of germ tube) after germination. Also El-Ghaouth et al.,[31] reported excessive branching and abnormal swelling of *R. stolinifer* treated with chitosan (1500 μg mL^{-1}, molecular weight not mentioned), but no alternation nor lyses of the cells. Other reasons for further growth of the abnormally swelled *M. piriformis* conidia in our study could be that the cells with abnormal swelling belonged to a more resistant subpopulation or survived as the chitosan concentration was reduced through binding to other cells.[35] The reason why conidia of *M. piriformis*, but not *B. cinerea*, showed abnormal swelling could be due to the inherent different cell wall compositions. It could be that the application of chitosan affected new cell wall synthesis of *M. piriformis* and resulted in abnormal swelling of conidia.

In the strawberry flower assay, CHOS DP_n 23 was the most effective in reducing flower infection by *B. cinerea* in line with what was observed *in vitro*. In addition to the direct antifungal effects of chitosan seen in the *in vitro* bioassay, chitosan may protect flowers indirectly by eliciting plant defence mechanisms against pathogens.[12] Previous studies showed that chitosan acts as an elicitor of multiple defence responses of higher plants, and[36,37] that the eliciting effect depends

on the DP of chitosan.[38] Thus, in our study CHOS DP_n 23 may have been more effective in inducing defence responses in strawberry than chitosan (DP_n 206).

The objective of our study was to find the most effective DP of chitosan and CHOS against *B. cinerea* and *M. piriformis*. CHOS was more effective in inhibiting plant pathogens than chitosan (DP_n 206) and the most effective DP of CHOS was within the range of DP_n 15-40.

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Paper IV

Inhibition of fungal pathogens by chitooligosaccharides and fungicides, alone and in combination

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Inhibition of fungal pathogens by chitooligosaccharides and fungicides, alone and in combination

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Abstract

BACKGROUND: Chitosan is a linear heteropolymer consisting of β 1,4-linked N-acetyl-D-glucosamine (GlcNAc or A) and D-glucosamine (GlcN or D). It possesses antifungal properties and is non toxic to mammals. Chitosan (average degree of polymerization (DP_n) 206 and fraction of acetylation (F_A) 0.15) and enzymatically produced chitooligosaccharides (CHOS) of different DP_n were tested alone and in combination with fungicides *in vitro* against several fungal pathogens (emphasis was on *Botrytis cinerea*) and *in vivo* against *B. cinerea*.

RESULTS: CHOS with DP_n in the range of 15-40 were effective against *B. cinerea* and CHOS with DP_n around 30 was the most effective. Both *in vitro* and *in vivo* assays showed that CHOS were more effective and had higher synergistic effect (i.e. more than additive) in combination with chemical fungicides than the combination of chitosan (DP_n 206) and chemical fungicides.

CONCLUSIONS: Our study indicates that CHOS may enhance the activity of chemical fungicides. This may reduce the amounts of chemical fungicides necessary to control plant diseases.

Key words: chitooligosaccharides, chitosan, chitosanase, chitinase, synergism, chemical fungicides

1 INTRODUCTION

Botrytis cinerea Pers.: Fr. (anamorph of *Botryotinia fuckeliana*) causes gray mold in over 200 plant species worldwide, which results in great damage to agricultural crops.[1] In Bangladesh, gray mold has caused near complete yield losses of chickpea[2] and in Norway the pathogen causes 30-60% yield reduction in strawberry production.[3] The pathogenic fungus *Mucor piriformis* Fischer also causes postharvest rots on strawberries as well as on several other fruit crops.[4] *Microdochium majus* (Wollenw.) Glynn and S.G. Edwards (telemorph *Monographella*) (causes seedling blight in many plants)[5] and *Alternaria brassicicola* (Schw.) Wiltshire (causes black spot on crucifers) are also economically important plant pathogens.[6] The control of plant pathogens relies heavily on chemical fungicides. Excessive use of chemical fungicides has caused environmental pollution and development of fungicide resistance in plant pathogens[7], thus there is a need to reduce the use of chemical fungicides by increasing their efficacy.

Chitin, a linear biopolymer consisting of β 1,4-linked *N*-acetyl-D-glucosamine (GlcNAc or A) residues, is insoluble in water, aqueous acidic solutions and most organic solvents due to strong intra- and inter-hydrogen bonds.[8] The fraction of acetylation (F_A) of chitin is usually 0.90[9], meaning there are very few D-glucosamine (GlcN or D) units present. Chitosan, which is obtained by partial deacetylation of chitin, has typical F_A less than 0.35[9] and is thus a heteropolymer consisting of N-acetyl-D-glucosamine and D-glucosamine residues. Chitosan is soluble in aqueous acid solutions.[8-10] Both chitin and chitosan can be hydrolyzed into chito-oligosaccharides (CHOS). CHOS have beneficial biological effects and may be used as fungicides, bactericides, bone-strengtheners in osteoporosis, vectors for gene delivery, hemostatic agents in wound-dressings,[11] antimicrobial agents,[12] and as inducers of plant defence responses against pathogens.[13] Hydrolysis of chitosan into CHOS can either be done chemically or by the glycosyl hydrolases (GH) chitinase or chitosanase. Chitinases are found in the GH18 and 19 families while chitosanases are found in the GH 5, 7, 8, 46, 75 and 80 families.[14] The GH46, GH75 and GH80 families only contain chitosanase. Chitinase can hydrolyze A-A, but not D-D bonds, while chitosanase in the GH46 and GH75 families can hydrolyze both A-D and D-D bonds. [14]

Recently, we showed that CHOS (DP_n of 40 and 23) obtained from enzymatic hydrolysis of chitosan (fraction of acetylation, F_A 0.15; average degree of polymerization (DP_n) 206) by a family 46 chitosanase significantly inhibited germination of isolates of *B. cinerea* and *M.*

piriformis.[15] The present study thus investigates the antifungal effect of these CHOS in combination with commercially available fungicides against *B. cinerea and M. majus.*

2 MATERIALS AND METHODS

2.1 Fungal cultures

B. cinerea (isolate BC 101), *A. brassicicola* (isolate A 328), and *M. piriformis* (isolate M119J) were obtained from the culture collection at the Norwegian University of Life Sciences (UMB) and *M. majus* was collected from Bioforsk (Ås, Norway). For the *in vitro* and *in vivo* bioassays, conidia were collected from cultures grown on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) under regular laboratory light for 2 weeks at 23±1°C. Concentrations of conidia in aqueous suspensions were determined by haemocytometer count at 400× magnification and adjusted to the required concentration with sterile water.

2.2 Commercially available fungicides

Four fungicides were tested: (1) Teldor® WG 50 (Bayer Crop Science Pty Ltd., Germany): active ingredient: 500 g kg^{-1} fenhexamid, chemical group: hydroxyanilide; (2) Switch® 62.5 WG (Syngenta Crop Protection Pty. Ltd., Switzerland): active ingredients: 375 g kg^{-1} cyprodinil and 250 g kg^{-1} fludioxonil, chemical groups: anilinopyramidine and phenylpyrrole respectively; (3) Amistar® (Syngenta Crop Protection Pty. Ltd.): active ingredient: 500 g kg^{-1} azoxystrobin, chemical group: strobilurin; and (4) Signum® WG (BASF, Germany): active ingredients: 26.7% w/w boscalid and 6.7% w/w pyraclostrobin, chemical groups: pyridinecarboximide and methoxy-carbamate respectively.

2.3 Enzymatic production of CHOS

Chitosan (KitoNor, F_A 0.15, DP_n 206) was obtained from Norwegian Chitosan, Gardermoen, Norway. This chitosan was used for all experiments in this research. CHOS were produced by enzymatic hydrolysis of chitosan by chitosanase or chitinase. Chitosanase ScCsn46A was produced as described by Heggset and coworkers[16]; briefly**,** chitosanase, originally from *Streptomyces coelicolor* q9rj88, was purified from the culture supernatant of the transformant *Escherichia coli* BL21Star (DE3), following the published protocol, with the exception that the $(His)_6$-tag was not removed from the enzyme after purification. The enzyme was dialyzed against Tris-HCl (20 mM) at pH 8 and stored at 4°C. Chitinase ChiA from *Serratia marcesecens* was produced according to Brurberg and coworkers.[17]

2.3.1 Production of CHOS

Chitosan (10 mg mL^{-1}) in buffer (0.04M NaAc, 0.1M NaCl, pH 5.5) was adjusted to pH 5.5 with HCl (0.5M) and incubated at 37°C and 225 rpm until the chitosan was dissolved (approximately 20 minutes). The pH was adjusted back to 5.5 with NaOH (0.5M). ScCsn46A[16] or ChiA[17] (0.5 µg mg^{-1} chitosan) were added to the chitosan solution and the mixture was incubated for various lengths of time at 37°C and 225 rpm. The enzymatic reaction was stopped by decreasing the pH to 2.5 with HCl (0.5M), followed by immersing the tube in boiling water for at least 10 minutes to inactivate the enzymes permanently. CHOS samples were dialyzed against distilled water for 48 hours (water was changed every 12 hours) using a cellulose membrane (Float-A-Lyzer® MWCO 500 Da from Spectrum Labs, USA) to remove buffer salts from the sample. Dialyzed samples were sterile filtered through Filtropur S 0.2 µm sterile filters (Sarstedt, Germany), lyophilized and stored at 4°C.[11] ChiA was used to produce CHOS with a GlcNAc (A unit) on the reducing ends and ScCsn46A was used to produce CHOS with GlcN (D unit) on the reducing ends.

2.3.2 ^{1}H-NMR analysis CHOS

Lyophilized CHOS (10 mg) were dissolved in deuterium oxide (D_2O) (0.5 ml) and the pH was adjusted to 4.2 with sodium deuteroxide (NaOD) and deuterium chloride (DCl) prior to lyophilization. This process was repeated once. Finally the lyophilized CHOS sample was dissolved in D_2O (700 µL) and ^{1}H-NMR analysis was performed on a 300 MHz Varian Gemini instrument (Varian, USA) at 85°C. The DP_n was calculated by the equation (Dα+Dβ+D+Aα+Aβ+A)/(Dα+Dβ+Aα+Aβ), where Dα, Dβ, Aα and Aβ are the integral of the reducing end signals of the α and β anomers of the deacetylated (D) and acetylated (A) units respectively, D is the integral of the signals from the internal and non reducing end of the deacetylated units (GlcN) and A is the integral of the signals from the internal and non reducing end of the acetylated units (GlcNAc).[18]

2.4 *In vitro* bioassay: germination inhibition of conidia

2.4.1 Effect of CHOS on germination of Botrytis cinerea, A. brassicicola and M. piriformis

CHOS with different DP_n (96, 62, 49, 40, 28, 15 and 9.5) were produced by enzymatic hydrolysis (ScCsn46A) of chitosan to test their antifungal activity against *B. cinerea* in MSM.[19] The final concentrations in the microtiter plate wells were 2.5 mM NH_4NO_3; 0.28 mM $CaCl_2 \cdot 2H_2O$; 0.16 mM $MgSO_4 \cdot 7H_2O$; 0.002 mM $MnSO_4 \cdot 4H_2O$; 0.002 mM

$ZnSO_4 \cdot 7H_2O$; 1 mM KH_2PO_4; 0.06 mM $FeC_6H_5O_7 \cdot 5H_2O$ and 55.5 mM glucose. One hundred microliters of CHOS or chitosan were added to wells in a flat-bottom 96-well microtiter plate (Nunc^{TM}, Roskilde, Denmark). There were three replicate wells of each treatment. Conidial suspensions (100 mL) were added to the wells to a final concentration of 2×10^4 conidia mL^{-1} MSM. The microtiter plates were incubated at 23±1°C for 24 hours. The germination percentage was visually estimated at 400× magnification using an invert microscope (Fluovert FU, Ernst Leitz Wetzlar GmbH, Wetzlar, Germany). The conidia were counted as germinated when the germ tube length was at least as long as the diameter of the conidium.

The germination inhibition percentage was calculated by the following equation:

Germination inhibition (%) = {(a-b)/a} × 100

Where, a = number of germinated conidia in the control (only MSM)

b = germinated conidia in the treatment (in MSM)

The pH of the conidia suspension in the microtiter wells was between 5.2 and 5.3 at the start of the experiment, and remained about the same 24 hours after inoculation (HAI).

The dose response curve of germination inhibition was estimated at different chitosan or CHOS concentration levels. Afterwards, the IC_{50} (the concentration required for 50% inhibition of germination) level was calculated using four degree polynomial regression (R^2 was close to 1). The minimum inhibitory concentration (MIC: the minimum concentration of the antimicrobial agent needed to completely inhibit germination) and IC_{50} were measured at 24 HAI.

CHOS DPn 37 was used to test antifungal activity against *B. cinerea, M. piriformis* and *A. brassicicola* in MSM. All germinated *M. piriformis* M199J conidia showed abnormal swelling with amoeba-like structure and one or more protruded portion. These conidia were thus counted as germinated when the length of the protruded part was at least as long as the diameter of the swelled conidia 12 HAI. Conidia of *M. majus* and *A. brassicicola* were counted as germinated when the length of the germ tube was half of the conidia length.

Another experiment was conducted to test whether the presence of GlcN or GlcNAc at reducing ends of CHOS (having same DP_n) would impact its antifungal activity against *B. cinerea* in MSM. CHOS DP_n 33.5 (having GlcN at the reducing ends) and CHOS DPn 34.6 (having GlcNAc at the reducing ends) were used in this experiment.

2.4.2 Synergism between fungicides and chitosan or CHOS in inhibiting Botrytis cinerea and Microdochium majus in vitro

Treatments were a) control treatment (only MSM), b) chitosan or CHOS in MSM, c) chitosan or CHOS combined with chemical fungicides (Teldor, Switch, Amistar or Signum) in MSM, and d) the individual chemical fungicides in MSM.

The interaction between chemical fungicides and chitosan or CHOS was determined by Abbott's equation.[20] The interaction effect was calculated by the ratio between the observed efficacy E_{obs} (% inhibition) and the expected efficacy (E_{exp}), which was calculated by Abbott's formula: $E_{exp} = a+b - (ab/100)$. Here a = % germination inhibition by chemical fungicides (Teldor, Switch, Amistar or Signum) alone, b = % germination inhibition by chitosan or CHOS alone. The E_{obs}/E_{exp} ratio 1 indicates additivity, ratios >1 indicate synergy and ratios <1 indicate antagonistic interaction.

2.5 *In vivo* bioassay on the effect of fungicides and chitosan or CHOS on infection of strawberry flowers and chickpea leaves by *Botrytis cinerea*

Synergism between chemical fungicides and chitosan or CHOS in inhibiting flower infection by *B. cinerea* was tested on newly opened strawberry (*Fragaria* × *ananassa*) flowers (cv. Corona) under controlled conditions. The strawberry plants were grown in greenhouse with controlled temperature (18°C at day; 12°C at night), light (16 hours, light intensity: 150 µmols $m^{-2}sec^{-1}$) and relative humidity (65%). Eighteen flowers per treatment (six replications of three flowers) were cut off with a 1½-2 cm stem and placed in empty pipette tip racks sets in plastic containers filled with 1-2 cm water. Conidia suspensions of the pathogen (final concentration: 2×10^6 conidia mL^{-1}) were mixed with test ingredients (chemical fungicides, CHOS or chitosan separately or mixtures of chemical fungicides and CHOS or chitosan) or sterile water (control). Ten microliters of each mixture were placed at the base of three petals on each flower with a pipette. The treatments were randomized in containers and were then covered with aluminium foil and incubated at 23±1°C. The relative humidity around the flowers was 90-95%, measured by a thermo-hygrometer (Lambrecht, Germany). The visual necrotic regions on the abaxial surface of the flowers under the inoculation point were recorded daily for eight days, and the area under the disease progress curve (AUDPC) was calculated on the basis of the cumulative daily infection by the following equation:

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

Where, D_i = Days of the i^{th} assessment, S_i = Proportion of the i^{th} infected inoculation point

The protection index was calculated by using the AUDPC values in the following equation[21]:

$100 \times (AUDPC_{control}-AUDPC_{treatment})/AUDPC_{control}$

Where $AUDPC_{control}$ represents flowers inoculated with only *B. cinerea* conidia and $AUDPC_{treatment}$ represents flowers inoculated with conidia premixed with chemical fungicides and/or CHOS.

Similar tests were performed on detached chickpea (*Cicer arientinum* L.) and bean (*Vicia faba* L.) leaves. Chickpea and bean were grown in green house with temperature 22±3^{o}C under twelve hours light. Nine complex chickpea leaves were used for each treatment (three replications with three complex leaves in each). Each complex leaf had six inoculation points on their leaflets. Three bean leaves with nine inoculation points on each were used for each treatment. There were three replications. The chickpea and bean leaves were inoculated (10 µL of 2×10^6 mL^{-1}) with *B. cinerea* conidia (control) or chitosan or CHOS (DP_n 30) alone or in combination with Switch or Signum. The development of the disease was counted up to eight days after inoculation (DAI).

Sporulation of *B. cinerea* from chickpea leaves was recorded on the eighth DAI. All leaves from each treatment were soaked in sterile water (10 mL) for approximately 20 minutes and vortexed several times. Finally the conidia concentration in the water was determined by counting samples in a hemacytometer.

3 RESULTS

3.1 Enzymatic production of CHOS

Chitosan (DP_n of 206 and F_A 0.15) was incubated with the family 46 chitosanase, ScCsn46A, for different time intervals to yield CHOS fractions of DP_n between 96 and 9. Using this approach, the majority of the CHOS produced will have a deacetylated unit at the reducing end, since ScCsn46A prefers to hydrolyse the glycosidic bond between two deacetylated units.[13] Since the most reliable studies have shown that, in addition to DP and F_A, the sequence of A and D units (pattern of acetylation, P_A) are central in several of the observed biological effects[11], a CHOS fraction of DP_n 35 was produced by enzymatic hydrolysis by

ChiA from *Serratia marcescens* of the same chitosan. ChiA is a GH 18 chitinase and yields exclusively an acetylated unit at the reducing end.

A common method to produce CHOS from chitin or chitosan is to treat the polymers with concentrated HCl in an acid catalyzed hydrolysis.[22] Due to intrinsic properties of the polymers, hydrolysis after an acetylated unit is favoured 115 times higher compared to that of a deacetylated unit, resulting in CHOS with a higher degree of a mixture of both acetylated and deacetylated units at the reducing end compared to when enzymes are used for hydrolysis.

3.2 Effect of the reducing end sugars (deacetylated vs. acetylated) on the ability of CHOS to inhibit germination of *Botrytis cinerea*

Our previous work showed that CHOS with DP_n 23 and 40 had the highest inhibitory effect on *B. cinerea*.[15] To test the effect of reducing end sugars (GlcN vs GlcNAc) of chitosan or CHOS on their antifungal activity against germination of *B. cinerea*, chitosan (DP_n 206) with 85% GlcN on reducing ends, CHOS DP_n 33.5 (from chitosan DP_n 206 hydrolysed by ScCsn46A) with approximately 95% GlcN on reducing ends and CHOS DP_n 34.6 (from chitosan hydrolysed by ChiA) with 100 % GlcNAc on reducing ends were tested (Fig. 1). The CHOS with 95% GlcN on the reducing ends were more inhibitory than CHOS with 100 % GlcNAc on the reducing ends. As an example; 80 μg mL^{-1} CHOS (DP_n 33.5) with GlcN on 95% of the reducing ends prevented further hyphal growth completely, whereas 310 μg mL^{-1} of the CHOS (DP_n 34.6) with 100 % GlcNAc on the reducing ends was required to obtain the same effect (data not shown). Because of this, CHOS obtained from ScCsn46A degradation of chitosan was used for the rest of this work.

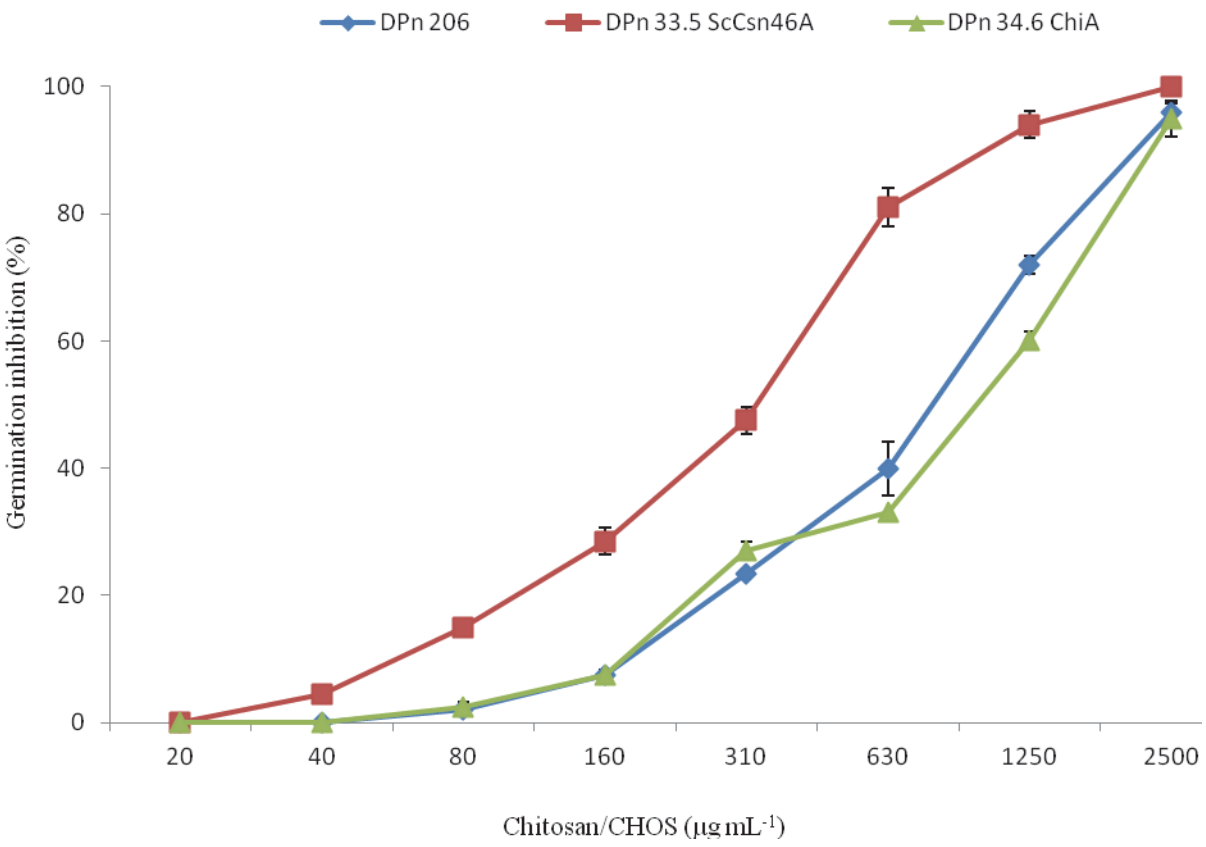

Figure 1. Effect of the reducing end sugars (GlcN vs GlcNAc) on the ability of CHOS to inhibit germination of *B. cinerea* (24 hours after inoculation, HAI) Data points are mean of three replicate wells ± standard deviation.

3.3 The minimum inhibitory concentration of CHOS on *Botrytis cinerea,*

Alternaria brassicicola and *Mucor piriformis*

CHOS with different DP_n were tested against *B. cinerea* germination. The most active fractions of CHOS had DP_n values around 30, but also other CHOS samples with DP_n values in the range 15-40 showed good antifungal activities (Table 1). All tested CHOS (except DP_n 9) were more inhibitory than unhydrolysed chitosan (Table 1 and Fig. 2).

Table 1. Effect of chitosan, or chitooligosaccharides (CHOS) obtained by hydrolysis of chitosan (unhydrolyzed, DP_n 206), on germination of *Botrytis cinerea*.

DP_n of chitosan/CHOS	MIC (µg mL^{-1}) [a]	IC_{50} [b] (µg mL^{-1} ± SD) [c]
206	5000	2467 ± 14
96	2500	1267 ± 16
62	2500	625 ± 6
49	2500	470 ± 20
40	1200	254 ± 7
28	310	98 ± 7
15	310	109 ± 9
9.5	>2500	>2500

[a] MIC: the minimum concentration of the antimicrobial agent needed to completely inhibit germination

[b] IC_{50}: the concentration required for 50% inhibition of germination

[c] All data are mean of three experiments ± standard deviation.

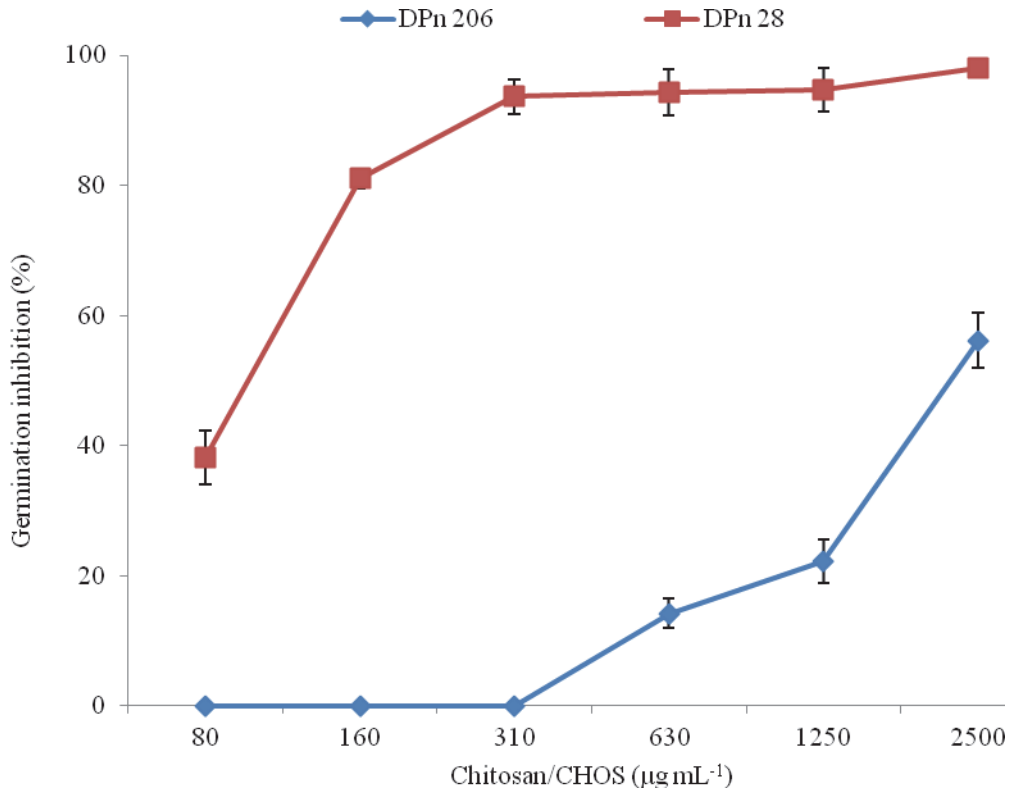

Figure 2. Dose-response relationships of chitosan and CHOS (DP_n 28) on germination inhibition of *Botrytis cinerea.* Data points are mean of three experiments ± standard deviation.

Moreover, the effects of CHOS DP_n 37 on three genera of plant pathogenic fungi were also tested. All tested genera showed different dose-response relationships (Fig. 3). While *B. cinerea* and *M. piriformis* showed decreasing germination over a broad concentration range of CHOS (DP_n 37) (20–2500 µg mL^{-1}), *A. brassicicola* was completely inhibited by 80 µg mL^{-1} CHOS DP_n 37. CHOS DP_n 37 showed 50% germination inhibition of *A. brassicicola* at 40 µg mL^{-1}, whereas CHOS concentrations of 630 and 160 µg mL^{-1} were needed for 50% inhibition of *B. cinerea* and *M. piriformis* respectively.

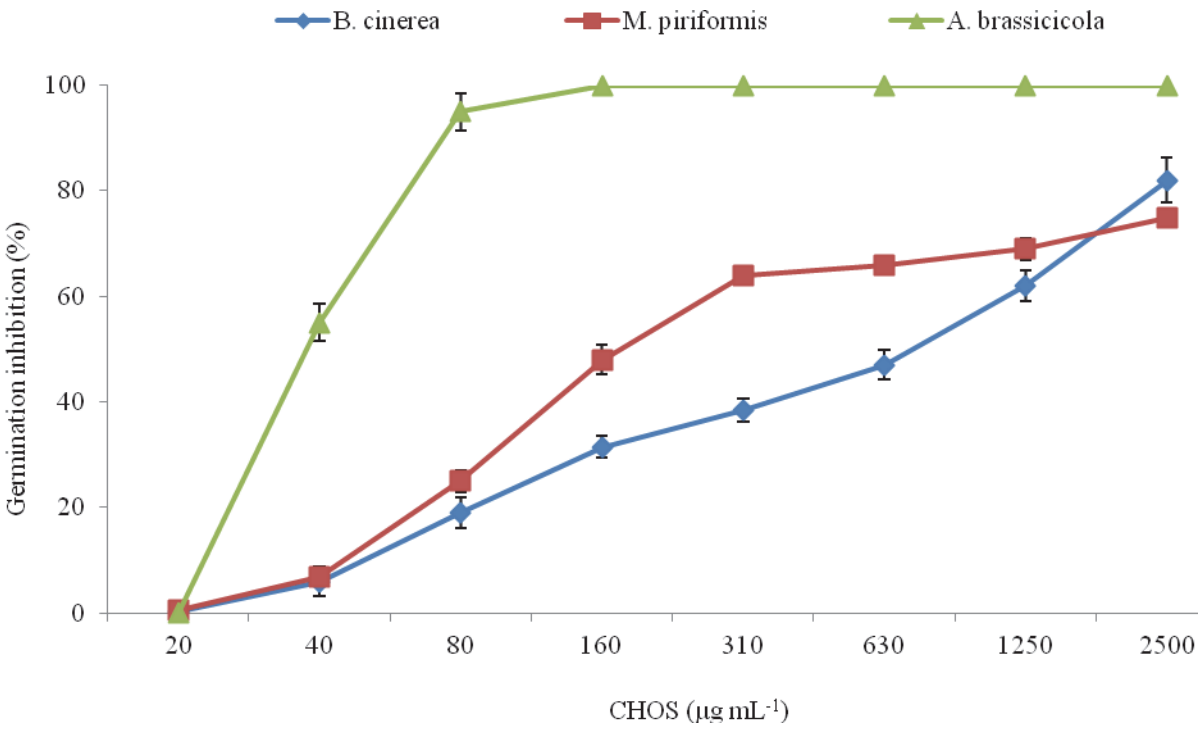

Figure 3. Dose-response relationships of CHOS DP_n 37 on germination of *Botrytis cinerea*, *Alternaria brassicicola* and *Mucor piriformis*. Data points are mean of three replicates wells ± standard deviation.

3.4 Interactions between chemical fungicides and chitosan or CHOS in inhibiting *Botrytis cinerea* and *Microdochium majus in vitro*

The combinations of chitosan and chemical fungicides showed either synergism or additivity in inhibiting germination of *M. majus* (Table 2). Signum was very effective against *M. majus* when applied in combination with chitosan. Chitosan (80 µg mL^{-1}) and Signum (1 µg mL^{-1}) applied alone showed 3% and 26% germination inhibition of *M. majus* respectively, but the combination of these low concentrations showed synergism and more than 50% germination

inhibition (Table 2). Also chitosan (640 µg mL^{-1}) showed only 3 % germination inhibition when applied alone, but combined with Signum (1 µg mL^{-1}) complete germination inhibition occurred (data not shown).

Table 2. Effect of chitosan (unhydrolyzed, DP_n 206), and chemical fungicides on germination inhibition (expressed as percent of control in MSM) of *Microdochium majus* recorded 24 hours after inoculation (HAI).

Treatment (µg mL^{-1})	Germination inhibition (% ± SD)[a]	E_{obs}/E_{exp}[b]
Chitosan 80	3 ± 1	-
Teldor 60	4 ± 1	-
Teldor 15	2 ± 1	-
Chitosan 80 + Teldor 60	13 ± 1	2
Chitosan 80 + Teldor 15	9 ± 1	2
Switch 5	93 ± 5	-
Switch 1	71 ± 1	-
Chitosan 80 + Switch 5	100 ± 0	1
Chitosan 80 + Switch 1	93 ± 1	1
Amistar 1	11 ± 2	-
Amistar 0.5	7 ± 1	-
Chitosan 80 + Amistar 1	20 ± 1	1
Chitosan 80 + Amistar 0.5	15 ± 1	2
Signum 1	26 ± 2	-
Signum 0.5	26 ± 1	-
Chitosan 80 + Signum 1	57 ± 1	2
Chitosan 80 + Signum 0.5	39 ± 2	1

[a]All data are mean of three replicate wells ± standard deviation.

[b]The E_{obs}/E_{exp} ratio 1 indicates additivity, ratios >1 indicate synergy.

When applied separately, 60 µg mL^{-1} Teldor and 80 µg mL^{-1} chitosan showed 1% and about 10% germination inhibition of *B. cinerea* respectively (24 HAI), but their combination showed synergism, with germination inhibition above 60%. At 25 µg mL^{-1} Switch showed 75% germination inhibition of *B. cinerea*, but when chitosan (80 µg mL^{-1}) was added, the

germination inhibition increased to 92%. Amistar or Signum and chitosan did not show synergism in germination inhibition (Table 3).

Table 3. Inhibition of *Botrytis cinerea* germination (expressed as percent of control in MSM) recorded 24 hours after inoculation (HAI) by chitosan (unhydrolyzed, DP_n 206) and chemical fungicides alone and in combination.

Treatment (μg mL^{-1})	Germination inhibition (% ± SD)[a]	E_{obs}/E_{exp}[b]
Chitosan 80	10 ± 3	-
Teldor 60	1 ± 1	-
Teldor 15	1 ± 1	-
Chitosan 80 + Teldor 60	64 ± 8	6
Chitosan 80 + Teldor 15	36 ± 5	3
Switch 25	75 ± 6	-
Switch 5	35 ± 4	-
Chitosan 80 + Switch 25	92 ± 8	2
Chitosan 80 + Switch 5	56 ± 8	2
Amistar 100	20 ± 4	-
Amistar 10	4 ± 3	-
Chitosan 80 + Amistar 100	32 ± 2	1
Chitosan 80 + Amistar 10	15 ± 4	1
Signum 10	18 ± 2	-
Signum 2	4 ± 3	-
Chitosan 80 + Signum 10	25 ± 4	1
Chitosan 80 + Signum 2	18 ± 6	1

[a]All data are mean of three experiments ± standard deviation

[b]The E_{obs}/E_{exp} ratio 1 indicates additivity, ratios >1 indicate synergy.

Almost all combinations of CHOS (DP_n 23) and chemical fungicides showed synergism in inhibiting germination of *B. cinerea*. Amistar (10 μg mL^{-1}) and Signum (10 μg mL^{-1}) applied alone showed only 2% germination inhibition, but with the addition of CHOS (DP_n 23, 5 μg mL^{-1}), germination inhibition increased to more than 90% (Table 4).

Table 4. Effect of chitooligosaccharides (CHOS) (DP_n 23) and chemical fungicides on germination inhibition of *Botrytis cinerea* (expressed as percent of control in MSM 24 hours after inoculation).

Treatment (μg mL^{-1})		Germination inhibition (% ± SD)[a]	E_{obs}/E_{exp}[b]
CHOS	5	4 ± 3	-
Teldor	150	4 ± 4	-
Teldor	15	1 ± 1	-
CHOS	5 + Teldor 150	21 ± 5	3
CHOS	5 + Teldor 15	12 ± 6	2
Switch	25	82 ± 5	-
Switch	5	18 ± 12	-
CHOS	5 + Switch 25	94 ± 6	1
CHOS	5 + Switch 5	96 ± 3	5
Amistar	100	5 ± 1	-
Amistar	10	2 ± 1	-
CHOS	5 + Amistar 100	96 ± 3	11
CHOS	5 + Amistar 10	96 ± 4	16
Signum	10	2 ± 1	-
Signum	2	2 ± 1	-
CHOS	5 + Signum 10	93 ± 7	16
CHOS	5 + Signum 2	89 ± 8	15

[a]All data are mean of three experiments ± standard deviation

[b]The E_{obs}/E_{exp} ratio 1 indicates additivity, ratios >1 indicate synergy.

3.5 Combination of chemical fungicides and chitosan or CHOS in inhibition of *Botrytis cinerea in vivo*

In the strawberry flower assay, Amistar (10 μg mL^{-1}, 1% of the recommended dose) showed little inhibition of *B. cinerea* when applied alone, but when combined with chitosan (400 μg mL^{-1}) the level of protection was similar to the protection level achieved by the recommended dose (Table 5). However, none of the tested combinations of chemical fungicides and chitosan showed synergism.

Table 5. Disease severity on strawberry flowers inoculated with a mixture of *Botrytis cinerea* conidia and chitosan (unhydrolyzed, DP_n 206) and/or chemical fungicides.

Treatment (μg mL^{-1})	AUDPC (± SD)[a]	Protection index (% ± SD)[a,b]
Control[c]	5.0 ± 0.2	-
Chitosan 400	3.8 ± 0.2	24 ± 3
Teldor 1500[d]	1.5 ± 0.3	70 ± 6
Teldor 15	3.8 ± 0.5	23 ± 7
Chitosan 400 + Teldor 15	2.4 ± 0.2	53 ± 11
Switch 500[d]	1.0 ± 0.2	80 ± 5
Switch 5	3.2 ± 1.0	36 ± 10
Chitosan 400 + Switch 5	2.1 ± 0.3	58 ± 4
Amistar 1000[d]	2.0 ± 0.3	60 ± 5
Amistar 10	3.5 ± 0.1	31 ± 2
Chitosan 400 + Amistar 10	2.0 ± 0.3	60 ± 4
Signum 1000[d]	1.3 ± 0.3	74 ± 5
Signum 10	3.7 ± 0.2	26 ± 7
Chitosan 400 + Signum 10	2.5 ± 0.1	50 ± 4

[a]All data are mean of two experiments ± standard deviation.

[b]The AUDPC was used to calculate the protection index.

[c]Conidia in sterile water.

[d]Recommended dose.

When applied alone, CHOS (DP_n 23, 10 μg mL^{-1}), Teldor, Switch, Signum or Amistar (1% of the recommended doses) showed very low levels of protection against flower infection by *B. cinerea*. However, when CHOS (DP_n 23, 10 μg mL^{-1}) was combined with the low doses of fungicides, the levels of protection were high (Table 6), and similar to the protection levels achieved by the recommended doses of the tested chemical fungicides (data not shown).

Table 6. Effect of chitooligosaccharides (CHOS) (DP_n 23) and/or chemical fungicides on disease severity (on detached strawberry flowers) caused by *Botrytis cinerea*.

Treatment (μg mL^{-1})		AUDPC (± SD)[a]	Protection index (% ± SD) [a,b]	E_{obs}/E_{exp} [c]
Control[d]		4.7 ± 0.2	-	-
CHOS	10	4.4 ± 0.2	5 ± 3	-
Teldor	150	2.8 ± 0.5	39 ± 11	-
Teldor	15	4.4 ± 0.1	5 ± 1	-
CHOS 10 + Teldor	150	0.6 ± 0.2	87 ± 5	2
CHOS 10 + Teldor	15	0.9 ± 0.4	80 ± 8	8
Switch	25	4.3 ± 0.1	9 ± 3	-
Switch	5	4.5 ± 0.2	3 ± 1	-
CHOS 10 + Switch	25	0.6 ± 0.4	87 ± 4	6
CHOS 10 + Switch	5	0.4 ± 0.4	92 ± 8	12
Amistar	100	4.5 ± 0.2	3 ± 1	-
Amistar	10	4.6 ± 0.2	1 ± 1	-
CHOS 10 + Amistar	100	0.9 ± 0.3	79 ± 8	10
CHOS 10 + Amistar	10	0.9 ± 0.4	80 ± 10	13
Signum	10	4.4 ± 0.1	4 ± 2	-
Signum	2	4.6 ± 0.1	2 ± 1	-
CHOS 10 + Signum	10	0.7 ± 0.3	85 ± 7	10
CHOS 10 + Signum	2	0.6 ± 0.4	86 ± 8	12

[a]All data are mean of two experiments ± standard deviation.

[b]The AUDPC was used to calculate the protection index.

[c]E_{obs}/E_{exp} 1 indicates additivity, $E_{obs}/E_{exp}>1$ indicate synergy.

[d]Conidia in sterile water.

B. cinerea showed 100% infection of strawberry flowers within 3-4 DAI in the control treatment and when CHOS (DP_n 23, 10 μg mL^{-1}), Teldor (15 μg mL^{-1}), Switch (5 μg mL^{-1}), Amistar (10 μg mL^{-1}) or Signum (10 μg mL^{-1}) were applied alone. However, when the inoculated flowers were treated with combinations of CHOS DP_n 23 and fungicides (at the mentioned concentrations) no infection occurred before the sixth DAI (Fig. 4).

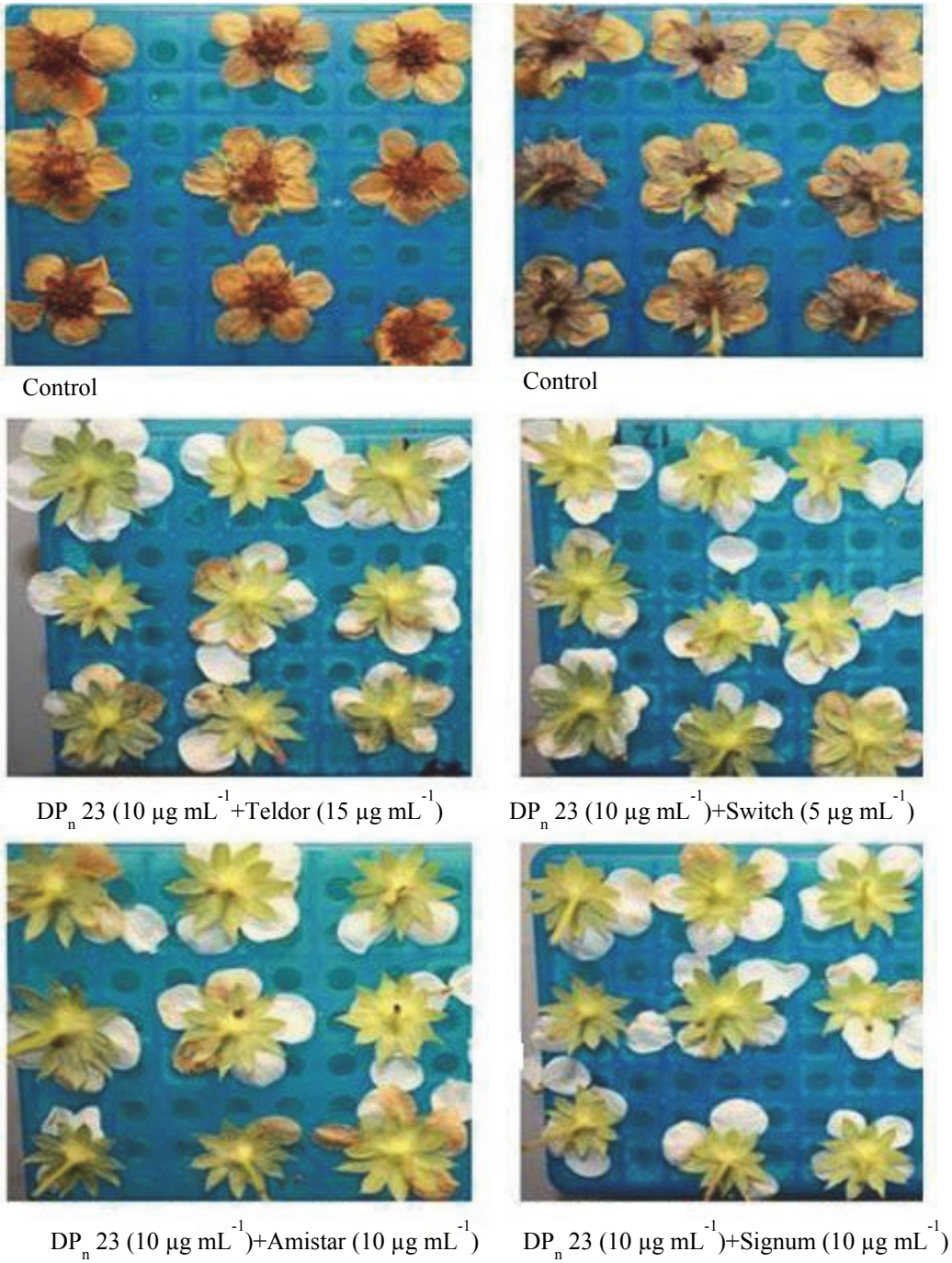

Figure 4. Effect of combinations of chemical fungicides (Teldor, Switch, Amistar and Signum 15, 5, 10 and 10 µg mL^{-1} respectively) and CHOS (DP$_n$ 23, 10 µg mL^{-1}) in inhibiting disease caused by *Botrytis cinerea* on detached strawberry flowers six days after inoculation (DAI). The flowers were considered 100% infected when all three inoculation points displayed necrotic signs. All treatments included 18 flowers, but only nine flowers are shown here. Control flowers were inoculated with conidia in sterile water.

In a chickpea leaf bioassay, chitosan, CHOS DP_n 30 and Switch were used alone and in combination against *B. cinerea*. The combinations of chitosan (320 µg mL^{-1}) or CHOS DP_n 30 (320 µg mL^{-1}) and Switch (1% of the recommended dose) showed synergism and better effect in reducing the *B. cinerea* infection on chickpea leaves than each compound applied alone. The combination of CHOS and Switch was as protective as the recommended dose of Switch against (500 µg mL^{-1}) *B. cinerea*. The combination of Switch (10 µg mL^{-1}) and CHOS (DP_n 30, 320 µg mL^{-1}) showed higher reduction of leaf infection by *B. cinerea* than the combination of Switch (10 µg mL^{-1}) and chitosan (320 µg mL^{-1}) (Table 7).

Table 7. Effect of combinations of chitosan (unhydrolyzed, DP_n 206) or chitooligosaccharides (CHOS) (**(**DP_n 30) and Switch on *Botrytis cinerea* infection of detached chickpea leaves.

Treatment (µg mL^{-1})	AUDPC (± SD)[a]	Protection index (% ± SD)[a,b]	E_{obs}/E_{exp}[c]
Control[d]	6.5 ± 0	-	-
Chitosan 2500	4.4 ± 0.4	33 ± 7	-
Chitosan 320	6.1 ± 0.2	5 ± 2	-
CHOS 2500	2.8 ± 0.4	58 ± 7	-
CHOS 320	5.5 ± 0.1	15 ± 2	-
Switch 500[e]	0.1 ± 0.1	98 ± 1	-
Switch 10	1.3 ± 0.5	80 ± 7	-
Switch 5	3.5 ± 0.4	46 ± 6	-
Chitosan 320 + Switch 10	1.4 ± 0.1	79 ± 2	1
Chitosan 320 + Switch 5	1.3 ± 0.2	80 ± 4	2
CHOS 320 + Switch 10	0.3 ± 0.1	96 ± 1	1
CHOS 320 + Switch 5	0.7 ± 0.3	90 ± 5	2

[a]All data are mean of three replicates (each replication contains three complex leaves) ± standard deviation.

[b]The AUDPC was used to calculate the protection index.

[c]E_{obs}/E_{exp} 1 indicates additivity, $E_{obs}/E_{exp}>1$ indicate synergy.

[d]Conidia in sterile water.

[e]Recommended dose.

Also Signum, chitosan, and CHOS DP_n 30 were tested against infection of *B. cinerea* on chickpea leaves. The combinations of chitosan (320 µg mL^{-1}) or CHOS DP_n 30 (320 µg mL^{-1}) with Signum (5 or 10 µg mL^{-1}) showed better effect in reducing disease severity than each component alone. The combinations of CHOS DP_n 30 (320 µg mL^{-1}) and Signum (5 µg mL^{-1}) showed greater inhibition of *B. cinerea* than the combination of chitosan and Signum at the same concentrations (Table 8). None of the combinations showed synergism.

Table 8. Effect of the combination of chitosan (DP_n 206) or chitooligosaccharides (CHOS) DP_n 30 and Signum on *Botrytis cinerea* infection of detached chickpea leaves up to eight days after inoculation (DAI) at 23±1°C.

Treatment (µg mL^{-1})	AUDPC (± SD)[a]	Protection index (% ± SD)[a,b]
Control[c]	6.5 ± 0.0	-
Chitosan 320	3.7 ± 1.6	43 ± 24
CHOS 320	2.2 ± 1.0	67 ± 16
Signum 1000[d]	0.0	100 ±0.0
Signum 10	3.1 ± 0.7	52 ± 10
Signum 5	5.0 ± 0.4	23 ± 5
Chitosan 320 + Signum 10	1.0 ± 0.8	84 ± 12
Chitosan 320 + Signum 5	2.2 ± 0.6	66 ± 10
CHOS 320 + Signum 10	0.1 ± 0.1	98 ± 2
CHOS 320 + Signum 5	0.6 ± 0.3	91 ± 4

[a]All data are mean of three replicates (each replication contains three complex leaves) ± standard deviation.

[b]The AUDPC was used to calculate the protection index.

[c]Conidia in sterile water.

[d]Recommended dose.

Another experiment was conducted to assess the effects of combinations of chitosan or CHOS DP_n 30 with the chemical fungicide Switch on *B. cinerea* infections on bean leaves. In this assay the combinations of 2.5 µg mL^{-1} Switch and 160 µg mL^{-1} CHOS DP_n 30 or chitosan completely controlled the infection, while each compound alone (at the mentioned concentrations) was less effective (data not shown). There was no difference between the

combinations of 'Switch and CHOS' and 'Switch and chitosan' in reducing bean leaf infection by *B. cinerea*. None of the combinations showed synergism (data not shown).

Sporulation of plant pathogenic fungus on infected plant parts is an important source of secondary inoculum. Thus an experiment was performed to assess the effects of the combination of chitosan or CHOS (DP_n 30) with Signum on the sporulation of *B. cinerea* on infected chickpea leaves. The combinations of chitosan or CHOS (DP_n 30) and Signum reduced sporulation of *B. cinerea* more than each component alone, and the combination of CHOS (DP_n 30) and Signum (10 μg mL^{-1}) was the most effective of all tested combinations in reducing the sporulation of *B. cinerea* (Table 9).

Table 9. Effect of combinations of chitosan (DP_n 206), or chitooligosaccharides (CHOS) DP_n 30 and Signum on sporulation of *Botrytis cinerea* on infected chickpea leaves.

Treatment (μg mL-1)	Conidia mL^{-1}
Control [a]	2×10^5
Signum 10	4×10^4
Chitosan 320	8×10^4
CHOS 320	4×10^4
Chitosan 320 + Signum 10	8×10^3
CHOS 320 + Signum 10	3×10^2

[a]Control indicates conidia in sterile water.

4 DISCUSSION

Our study showed that CHOS with deacetylated (GlcN or D unit) reducing ends were more inhibitory against *B. cinerea* than CHOS with similar DP_n and acetylated (GlcNAc or A unit) reducing ends. Many studies have reported that deacetylation of chitosan is an important factor affecting antifungal activity of chitosan.[23,24] The GlcN in chitosan have an amino group which becomes protonated in acidic environments and interaction with the negatively charged cell surface results in pore formation on the cell surface and ultimately cell death due to leakage of intracellular electrolytes.[25,26] To our knowledge there are no previous reports showing that the presence of GlcN at the reducing ends of CHOS impacts its antifungal activity.

Previous studies showed that CHOS are more inhibitory than polymeric chitosan due to better solubility in water.[27,28] However, our study showed that not only solubility but also the degree of polymerization (DP) of chitosan is an important factor impacting its antifungal activity. Our *in vitro* assay showed that CHOS obtained after hydrolysis (by chitosanase: ScCsn46A) of chitosan (DP_n 206) was more inhibitory than the chitosan (DP_n 206) against *B. cinerea*, and CHOS ranging from DP_n 15-40 were the most effective. Antifungal activity of chitosan has been previously reported to depend on DP, where 6 kDa (DP_n around 40) was the most effective on a range from 5 to 27 kDa in inhibiting *Candida krusei*.[29]

CHOS alone or in combination with fungicides were more effective against *B. cinerea* than chitosan alone or in combination with chemical fungicides. CHOS showed high levels of synergism and high germination inhibition of *B. cinerea* with all tested chemical fungicides. The mechanisms for synergism in inhibiting *B. cinerea* germination are not known, but it could be due to the compounds' different modes of action. Teldor inhibits sterol biosynthesis, Switch inhibits protein synthesis (cyprodinil) and signal transduction (fludioxonil), while Amistar and Signum inhibit respiration.[30] The mode of action of chitosan is not clearly understood[31-34], but previous studies suggest that positively charged chitosan may cause electrostatic interactions with the negatively charged cell surface of microorganisms[23,35] resulting in loss of integrity and loosening of cell wall[36], which ultimately increases the cell wall permeability.[25,37] Increased cell wall permeability may have enabled Teldor (fenhexamid) to reach the conidial membrane earlier and thereby stop the germination at an earlier stage than if Teldor was applied alone. The increased cell membrane permeability by chitosan or CHOS may also have enabled Amistar and Signum to inhibit respiration or Switch to inhibit protein synthesis[30] earlier than if the fungicides were applied alone. However, the reason for higher synergism between CHOS and fungicides than between chitosan and fungicides is not known, but it could be that CHOS are more effective to increase cell wall permeability than chitosan, enabling the fungicide to reach its site of activity earlier than if the fungicide was applied alone.

Also *in vivo*, combinations of CHOS and chemical fungicides had higher levels of synergism in inhibiting *B. cinerea* on strawberry flowers and chickpea leaves than combinations of chitosan and fungicides. Reasons may be as described above. Our study showed that the synergistic effects between CHOS and fungicides in inhibiting *B. cinerea* could reduce the need for chemical fungicides up to 99% of their recommendation doses while maintaining their efficacy. Thus, combinations of CHOS and chemical fungicides could be used in

integrated pest management (IPM) programs against *B. cinerea*, where even small amount of CHOS would considerably reduce the need for chemical fungicides and this will ultimately benefit humans and the environment.

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