DEVELOPING CHITOSAN BASED GREEN FUNGICIDES TO CONTROL PRE- AND POSTHARVEST ANTHRACNOSE OF DRAGON FRUIT

By

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A thesis submitted in fulfilment of the requirement for the degree of Doctor of Philosophy

School of Biosciences, Faculty of Science

THE UNIVERSITY OF NOTTINGHAM MALAYSIA CAMPUS

AUGUST, 2013

DEDICATION

I dedicate this humble effort to my Parents and loving Husband whose support has been a great source of motivation and inspiration

ABSTRACT

DEVELOPING CHITOSAN BASED GREEN FUNGICIDES TO CONTROL PRE AND POST HARVEST ANTHRACNOSE OF DRAGON FRUIT

A study was conducted to develop an ecofriendly and sustainable fungicide based on submicron chitosan dispersions using low molecular weight chitosan and locally prepared chitosan. The physicochemical properties of both submicron dispersions were determined and their antimicrobial effect on *Colletotrichum gloeosporioides* was also investigated. The antifungal effect was further demonstrated by the production of fungal cell wall degrading enzymes and by the production of defence related enzymes in the plants along with the effect of submicron chitosan dispersions on the vegetative growth of plants. The effect of submicron chitosan dispersions on biochemical and physiological responses of dragon fruit during storage at 10 ± 2 °C and 80 ± 5% relative humidity for 28 days was also investigated.

In vitro antifungal activity of submicron chitosan dispersions showed that low molecular weight chitosan has better antifungal properties than the locally prepared chitosan and therefore was selected for further studies. However, 600 nm droplets of 1.0% chitosan showed promising results in terms of suppressing mycelial growth (90.2%), conidial germination (93.1%) and reducing dry weight of mycelium (37.9%). Similarly, *in vivo* studies showed that the 600 nm droplets of 1.0% chitosan helped to reduce the disease incidence by up to 33.0 %.

Beside the direct antifungal effect, this study suggests that the submicron chitosan dispersions enhanced host resistance through the production of

defence related compounds, such as total phenols, peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) and also the activity of pathogenesis related proteins, such as *β*, 1-3 glucanase and chitinase. Treatments with submicron chitosan dispersions as 600 nm droplets of 1.0% chitosan showed more vigorous growth of plants as compared to the control.

The submicron chitosan dispersions with 600 nm droplets of 1.0% chitosan helped to reduce the incidence of disease on fruit by up to 94% as compared to the control. The biochemical and physiological studies of dragon fruit after 28 days of storage showed that weight loss of the fruit treated with 600 nm droplets of 1.0% chitosan compared with the control reduced two fold but this was not significantly different from the conventional chitosan. The same trend was observed in all the parameters tested, including antioxidant activity. Control of disease using submicron chitosan dispersions has the potential to add market value to fresh produce.

The findings from all the experiments showed that 600 nm droplets of 1.0% chitosan help to reduce anthracnose in the field and also during postharvest storage. Thus, 600 nm droplets of 1.0% chitosan could be used commercially in fields of dragon fruit as a green fungicide. Being non-toxic and biodegradable, chitosan has the potential to become an alternative to synthetic fungicides for protecting fruit crops and thus assisting sustainable agriculture.

ACKNOWLEDGEMENTS

Up and above, everything, all gratitude to ***Almighty Allah***, the compassionate, the merciful, who enabled me to elucidate a drop from the existing ocean of knowledge, and the ***Holy Prophet Muhammad (peace be upon him)*** the most perfect and exalted among and even of ever born on the surface of earth, who is, far ever a torch of guidance and knowledge for the humanity as a whole.

I am highly obliged to my worthy supervisor ***Dr. Asgar Ali***, Professor and Director of Centre of Excellence for Postharvest Biotechnology (CEPB), School of Biosciences, The University of Nottingham Malaysia Campus, for his pleasing temperament, personal interest, inspiring guidance, enlightened supervision and constructive criticism throughout the course of this research endeavor. I could not have imagined having a better advisor and mentor for my Ph D studies.

I wish to express my deep sense of gratitude to my Co-supervisor ***Dr. Sivakumar Manickam***, Professor in Department of Chemical and Environmental Engineering, The University of Nottingham Malaysia Campus, for his keen interest, kind cooperation, scholastic guidance and precious suggestions throughout the course of my research.

I also wish to express my deep sense of gratitude to my Co-supervisor ***Dr. Yasmeen Siddiqui***, Senior Lecturer, University Putra Malaysia, for her insight full comments and precious suggestions during my research.

In addition I would like to thank ***Dr. Ajit Singh***, Associate Professor, School of Biosciences, The University of Nottingham, Malaysia Campus who

introduced me to the statistical programme and taught me from the basics of statistics.

I like to articulate my humble gratitude to my internal assessor ***Dr. Peter G. Alderson***, Associate Professor, School of Biosciences, The University of Nottingham Sutton Bonington Campus, United Kingdom.

Furthermore, I would also like to thank the Chief Lab Technician, ***Mr. Wan Ghani*** and other lab technicians, ***Ms. Radha***, ***Ms. Siti Norazlin***, ***Ms. Shankari***, for providing me the required equipments and materials, to complete this task and ***Ms. Filzah***, for providing guidance during SEM studies. A special thanks goes to my exquisite fellows ***Dr. Ong Mei Kying, Ms. Nurul Aalya Alwi, Ms. Maysoun, Mr. Wei Keat*** and Faculty Office Staff ***Ms. Sabariah*** and ***Mr. Marcus*** for their assistance and help during my PhD programme.

The most profound thanks go to the ***Ministry of Agriculture, Government of Malaysia (MOA)*** represented by The University of Nottingham Malaysia Campus, for providing me the Research Assistantship (RA) and financial support under the project grant (05-02-12-SF1003).

I would like to express my weighty esteem and appreciation to my sweetest daughter ***Doa Fatima*** and dearest son ***Mohammad Afnan Mehdi*** for their serenity and patience during the entire phase of my studies.

I do not have words at command to express my gratitude and profound admiration to my dearest parents, venerated brother ***Mr. Aafaq Zahid*** and valued in laws for their spiritual and intellectual inspiration and moral support which made it possible for me to achieve the present knowledge in my life and wished to see me glittering high in the skies of success.

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DECLARATION

I hereby declare that the thesis is based on my original work except for the quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at the University of Nottingham Malaysia Campus or other institutions.

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NOOSHEEN ZAHID

Date:

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FIELD PLOT DESIGN

LIST OF ABBREVIATIONS

µm	Micrometer
ANOVA	Analysis of variance
AUDPC	Area under disease progress curve
°C	Celsius
Ca	Calcium
$CaCl_2$	Calcium chloride
CaO	Calcium oxide
CEC	Cation exchange capacity
cm	Centimeters
CO_2	Carbon dioxide
Cp	Centipoise
CRD	Completely randomized design
cv	Cultivar
DD	Degree of deacetylation
DI	Disease incidence
DMRT	Duncan multiple range test
DNA	Dioxyribonucleic acid
DS	Disease severity
EDS	Emulsion dispersion size
Fig	Figure
FRAP	Ferric reducing antioxidant power
FTIR	Fourier transform infrared spectroscopy
g	Gram
g	Gravitational force
GC	Gas chromatograph
h	Hour
H_2O	Water
H_2O_2	Hydrogen peroxide
HPMC	Hydroxypropyl methylcellulose
HR	Hypersensitive response
K	Potassium

K_2O	Potassium oxide
KCl	Potassium chloride
kDa	Kilo Dalton
kg	Kilogram
l	Litre
Mg	Magnesium
mg	Milligram
MgO	Magnesium oxide
$MgSo_4.7H_2O$	Magnesium sulphate heptahydrate
ml	Milliliter
mm	Millimeter
mM	Millimolar
Mol	Mole
$Na_2\ CO_3$	Sodium carbonate
N	Newton
N	Normality
n	Total number of sampled plants
$Na_2HPO_4.7H_2O$	Sodium monohydrogen phosphate heptahydrate
NaOH	Sodium hydroxide
NH_2	Amine group
nm	Nanometer
OD	Optical density
OH	Hydroxyl group
ONGP	*O*-nitrophenyl-β-$_D$-galactoside
P	Probability
P_2O_5	Phosphorus pentoxide
PAL	Phenylalanine ammonia lyase
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PIRG	Percent inhibition in radial mycelial growth
OH	Hydroxyl group
ONGP	*O*-nitrophenyl-β-$_D$-galactoside
POD	Peroxidase

ppm	parts per million
PPO	Polyphenol oxidase
PR	Pathogenesis related
PVP	Polyvinylpyrrolidone
PVPP	Polyvinylpolypyrrolidone
r^2	Coefficient of determination
RM	Ringgit Malaysia
S.E	Standard error
SAS	Statistical analysis system
sp	Specie
SSC	Soluble solids concentration
TA	Titratable acidity
TBZ	Thiabendazole
TCD	Thermal conductivity detector
TFA	Trifluoroacetic acid
TGA	Thioglycolic acid
TPTZ	2,4,6-tripyridyl-s-triazine
Tris HCl	Tris hydrochloride
U	enzyme unit $\left(U/_{ml} = \frac{\Delta\ OD/_{min} \times dilution\ factor}{ml\ of\ enzymes\ used\ in\ assay} \times 100\right)$
UK	United Kingdom
UNIVARIATE	One variable
USA	United States of America
UV-Vis	Ultraviolet – visible
v	Volume
var	Variety
w	Weight
Z	Highest rating scale

CHAPTER 1

INTRODUCTION

Dragon fruit is a newly introduced fruit crop, also known as pitaya. It is widely distributed in the tropics from coastal areas to high mountains and in tropical rain forests. Dragon fruit belongs to the genera *Hylocereus* and *Selenicereus* in the Cactaceae family from the subfamily Cactoidea of the clan Cactea (Raveh *et al*., 1993). Buah naga (Malaysia), night blooming cereus and strawberry pear (English) are some other names for this climbing cactus (Crane and Balerdi, 2005). Three species of dragon fruit are grown in the world:

1. Red skin, white flesh dragon fruit (*Hylocereus undatus*)
2. Red skin, red flesh dragon fruit (*Hylocereus polyrhizus*)
3. Yellow skin, white flesh dragon fruit (*Selenicereus megalenthus*)

Only two species of the genus *Hylocereus* are grown in Malaysia. The suitable tropical climate, rainfall, light intensity and soil type are contributing to the successful cultivation of this exotic fruit in the country (Luders and McMahon, 2006). It is widely cultivated in South East Asian countries including Malaysia, Thailand and Vietnam (Nguyen, 2006).

Red fleshed dragon fruit has attracted global attention due to its striking purple red colour, economic value as a source of food and antioxidative activity associated with its high betacyanin content (Le Bellec *et al*., 2006). It is an attractive fruit crop with a short shelf life of 6 days. Pre- and post-

harvest diseases may significantly lower the quality and value of this commodity (Masyahit *et al*., 2009).

Golden Hope Company at Sungai Wangi Estate, Perak, was the pioneer for introducing dragon fruit in Malaysia at the end of the 1990's. Some factors limit the growth and yield of dragon fruit, including abiotic factors, such as chilling and mechanical injuries, higher summer temperature, sunburn, and water loss. In addition, the infestation of pests and diseases has also influenced its cultivation (Cheah and Zulkarnain, 2008).

Recently, the fungus *Colletotrichum gloeosporioides* has posed serious problems to Malaysian producers because of favorable environmental conditions for this pathogen (Masyahit *et al*., 2009). The red fleshed species of dragon fruit seems to be more frequently affected by anthracnose than the white fleshed species in Malaysia. Infected plants and fruits have small, water-soaked, sunken and round spots that become blackish, emit spore masses and cause rot (Masyahit *et al*., 2009). This disease poses a serious threat as the spores infect the fruit in the field and the infection remains latent until the fruit is in storage. This ubiquitous fungus causes more than 50% losses in fresh vegetables and fruit (Paull *et al*., 1997).

Spraying conventional fungicides onto the plants is the only available control method and is widely used. The most commonly used fungicides to control anthracnose of dragon fuit are carbendazim, difenoconazole, propineb and benomyl (Hoa, 2008). The increasing public awareness on the effect of toxicological properties of fungicides on human health (Klein and Lurie, 1991), the buildup of chemical tolerance of pathogens towards fungicides (Adaskaveg *et al*., 2002) as well as environmental impacts such

as the poisoning of soils necessitate the development of non-toxic biofungicides (Northover and Zhou, 2002). Several fungicides have been removed from the market by the Environmental Protection Agency (EPA), Florida because of ground water contamination and its detrimental effects on human health and wild life (Crnko *et al*., 1992). For this reason several chemical fungicides were removed from the market in Germany in 2005; whereas in developing countries, including Malaysia, by 2020 under the Montreal Protocol these fungicides are scheduled to be phased out (UNDP, 2003). Therefore, it has become necessary to develop a more ecological-based system for producing high quality dragon fruit with reduced fungicide inputs. To maintain the marketable quality of fruit, alternative approaches are necessary, hence the evaluation of chitosan.

Chitosan is a polysaccharide obtained from the exoskeleton of crustaceans, such as shrimp and crab shells (No and Meyers, 1997). The highly positive charge on chitosan makes it unique and in great demand in a wide array of industries, such as cosmetology (body creams, hair additives, lotions) (Zhao *et al*., 2004), food (coating, preservative, antimicrobial) (Shahidi *et al*., 2001; Benjakul *et al*., 2000; Roller and Covill, 1999), biotechnology (chelator, emulsifier, flocculent) (Agulló *et al*., 2003) medicine and pharmacology (artificial organs, membranes, drugs) (Liu *et al*., 2001; Nishimura, 1997).

In the agricultural industry, it is used as a soil modifier to improve seed and flower quality and increases crop yields (Ren *et al*., 2001; Makino and Hirata, 1997). Another important attribute of this natural compound is associated with its antimicrobial properties against a large number of

pathogens (Bhaskara-Reddy *et al*., 1997). Several researchers have reported that chitosan could be used as an antifungal agent against a number of pathogens isolated from vegetables and fruit (Ali *et al*., 2010; Chien *et al*., 2007a; Romanazzi *et al*., 2007). Studies carried out on the preharvest application of chitosan have shown chitosan induced systemic resistance against *Pythium*, *Phytophthora* spp. and *Fusarium oxysporium* (Stone *et al*., 2003; Romanazzi *et al*., 2002; Benhamou *et al*., 1994). Ait Barka *et al*. (2004) reported the maximum *in vitro* antifungal activity (64 ± 1.01%) of chitosan at 5.0% against *Botrytis cinerea* in *Vitis vinifera*.

However, chitosan showed some negative effects on plantlet growth, shoot length and also caused death of plantlets when used at concentrations higher than 1.75% (Ait Barka *et al*., 2004). Benhamou *et al*. (1994) also reported the death of tomato plants when sprayed with 1.0% chitosan. In a recent study by Ali *et al*. (2010), it was reported that 1.5% chitosan controlled postharvest anthracnose of papaya caused by *Colletotrichum gloeosporioides* by up to 93%. However, at less than 1.5% there were limited antifungal properties, while at concentrations more than 1.5% the quality of papaya fruit deteriorated (Ali *et al*., 2011). Thus, the use of chitosan at low concentration and viscosity could be a new approach to overcome its limited antifungal properties.

Knowing the potential of chitosan and the low viscosity of its solutions, a few studies have been reported on chitosan edible films (Ziani *et al.,* 2009), where different techniques have been used to improve the stability and quality of the coatings. Nanotechnology is a new and emerging technique, whereby the viscosity of the solution could be reduced to a desired extent.

Furthermore, innovative nanotechnology has been used successfully for packaging of food (Traver, 2006). Submicron dispersion is the class of emulsions where the droplet size is in the range of 200-1000 nm (Winhab *et al*., 2005). These are widely used in various industries, such as in cosmetics, pharmaceuticals, health care and agrochemicals (Sonneville-Aubrun *et al*., 2004; Schulz and Daniels, 2000).

The small droplet size and high kinetic stability make submicron dispersions suitable for efficient delivery of active ingredients (due to their large surface area) and for penetration through the “rough” texture of the skin (Amselem *et al*., 1998). The formulation of such a sub micro carrier could result in a slow release of chitosan so that sudden accumulation of chitosan on the fruit surface and in the plant tissues never occurs.

So far, no study has been reported on the use of submicron chitosan dispersions for the control of pre- and postharvest anthracnose of dragon fruit. Therefore, this study has been aimed at developing a novel biofungicide using submicron chitosan dispersions to control pre- and postharvest anthracnose.

1.1 Hypothesis

The application of submicron chitosan dispersions will help control *C. gloeosporioides* and supress disease of dragon fruit plants and fruit due to its small size and low viscosity.

1.2 Research objectives

The main objective is to develop submicron chitosan dispersions and to study their efficacy on pre- and postharvest anthracnose and the quality of dragon fruit.

1. Formulation of submicron chitosan dispersions and measurement of their physical and chemical properties.
2. To determine the antifungal activity of submicron chitosan dispersions against anthracnose disease of dragon fruit plants.
3. To study the mechanism of action of submicron chitosan dispersions for the control of *C. gloeosporioides*.
4. To evaluate the efficacy of submicron dispersions against anthracnose disease of dragon fruit.
5. To evaluate the effect of submicron chitosan dispersions on physiological responses, antioxidant activity and gaseous exchange analysis of dragon fruit during cold storage.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Dragon fruit

This epiphytic tropical cactus is known as pitaya or pitahaya (Spanish), thang loy (Vietnamese), strawberry pear, night blooming cactus and dragon fruit. Ancient Chinese called this fruit “jaina” which translates literally to “the sweetest and best tasting”. In Malaysia, it is commonly known as ‘buah naga’ or ‘buah mata naga’.

The true origin of dragon fruit is Mexico, Central and South America. It is the most widely distributed cactus, and is now found in six continents. Southeast Asian countries, such as Vietnam, Thailand, Malaysia, Taiwan and the Southeast coast of China, are known for its cultivation. Spaniards and Filipinos introduced this fruit in the Philippines through trading and exchange of goods. High economic value of this fruit indicates its competitive benefit for the local fruit industry (Teddy, 2008).

Plants of dragon fruit are epiphytic, fast growing, terrestrial, perennial and vine-like cacti. The stem has many green, fleshy and triangular branches. Each stem segment has three wavy spineless wings or may have 1-3 spines. Pitaya stems form aerial adventitious roots to adhere to the surface on which they grow. Each stem can reach a length of about 6.1 m. (Crane and Balerdi, 2005).

Some species of dragon fruit are self-incompatible, however, flowers of some cultivars are hermaphrodite. The plant bears very large bell-shaped flowers (23 cm wide and 36 cm long) which are extremely showy (white or

pink), fragrant and only receptive at night. The stamens and lobed stigmas are cream coloured. They are usually cross-pollinated by bats, bees and other nocturnal insects or animals. For the fruit to mature from anthesis requires 30 to 35 days. The plant has fleshy berry fruit, which are oblong with red or yellow skin (almost 0.31 cm thick) and scales. Fruit pulp contains numerous small black seeds (Masyahit *et al*., 2009; Chien *et al*., 2007 b).

The dragon fruit grows very well in tropical climates. Hence, it is suitable to be grown in Malaysia as a commercial crop. The plant requires rainfall of 500 to 1500 mm annually and temperature between 25 and 30°C. It also needs well-drained loose soils (Cheah and Zulkarnain, 2008).

The preferred method of propagation is through stem cuttings, however, such plants possess variable characteristics of stem and fruit. Usually whole stem segments of 12-38 cm are used. Cuttings grow very fast and produce fruit 6 to 9 months after planting. Fruit production by 3-4 years old plants may reach 100 kg per year and the estimated life of a dragon fruit plant is 20 years (Crane and Balerdi, 2005).

Dragon fruit is a popular commercial fruit, which can be eaten fresh and used for culinary and confectionary purposes. It can also be fermented as wine and for the extraction of functional enzymes. The fruit is mostly consumed fresh, however, the frozen pulp may be used to make yogurt, candies, ice cream, marmalade, jelly, juice and pastries. Unopened flower buds can be used as a vegetable. The mild laxative activity of dragon fruit is due to its seeds, which contain oil (Cheah and Zulkarnain, 2008; Crane and Balerdi, 2005). Its products have several useful properties, including as

colouring agent, thickening properties, high antioxidant capacity and dietary fibre (Le Bellec *et al*., 2006).

Here the focus is on the red dragon fruit which is scientifically known as *Hylocereus polyrhizus.* Red dragon fruit have attracted significant attention worldwide. This is because of the unique red-purple colour of the flesh, economic value as a food source and high betacyanin content. Other benefits, such as cancer chemopreventives, anti-inflammatory agents and anti-diabetics, can also be found in dragon fruit. It reduces cardiovascular mortality rates (Cos *et al*., 2004). It contains less sugar than most popular tropical fruits, and thus is more suitable for diabetics and high blood pressure patients (Lau *et al*., 2008).

In Malaysia, the total value of red dragon fruit production for 2006 was RM 12 million. The cultivation area has been increased about 20 fold in 4 years from 2002 to 2006, indicating that the demand for the fruit has increased at a remarkable rate (Cheah and Zulkarnain, 2008).

In a seminar on Pitaya Production, Market and Export - Issues and Challenges (2008), it was stated that, although the demand is increasing and red dragon fruit cultivation is lucrative, very few studies have been done on fungal and bacterial infections on the fruit after harvest. These infections can drastically reduce the shelf life of the fruit, making it difficult to commercialize it globally (Masyahit *et al*., 2009; Cheah and Zulkarnain, 2008).

2.2 Anthracnose

Scientific reports on pathogenic diseases infecting dragon fruit plants are still lacking, especially on anthracnose. Masyahit *et al.* (2009) noted that

there had been only three previous studies on the occurrence of fungal diseases in dragon fruit and only in white-fleshed species in Japan and USA and yellow species in Brazil (Takahashi *et al*., 2008; Taba *et al*., 2006).

Anthracnose caused by *Colletotrichum gloeosporioides* is a devastating disease not only on dragon fruit but also on other tropical fruit. Damage to plants results in decreased total yield and reduced quality and value of the fruit. This disease is more dominant during rainy seasons. The fungal pathogen not only attacks postharvest dragon fruit but also the live stems, branches, flower buds and fruit (Hoa, 2008).

Masyahit *et al*. (2009) reported that, in Malaysia, anthracnose disease on dragon fruit plants is caused by a facultative fungus, *Colletotrichum gloeosporioides*, which belongs to the order *Melanconiales*. This disease is regularly seen in the field on ripe or overripe fruit and reduces the economic value of crops in tropical, subtropical and temperate regions. The most significant economic losses occur when the fruiting stage is attacked. Anthracnose appears in developing and mature plant tissues. It can affect the developing fruit in the field and also damage the mature fruit during storage. The ability to cause latent infections has made it one of the most important postharvest pathogens (Agrios, 2004; Freeman *et al*., 1998).

The symptoms that can be observed on the plants are round, water soaked and sunken spots. Lesion centres are covered with pinkish-orange conidial masses. Symptoms are sharply defined with irregular to circular spots, and reddish-brown in colour. These lesions are referred to as "chocolate spots." As the plant matures, these spots enlarge rapidly to form the characteristic circular sunken lesions (Masyahit, *et al*., 2009).

Chau and Alvarez (1983) examined the infection process of *Colletotrichum gloeosporioides* using light microscopy and transmission and scanning electron microscopy. The conidia are dumbbell shaped or slightly curved, one-celled, oblong or ovoid, 5-7 µm in width and 10-15 µm in length.

Production of sunken lesions is a consistent feature of anthracnose, which causes the death and maceration of infected tissues (Baiely *et al*., 1992). *Colletotrichum* species secrete a range of enzymes, which destroy and sometimes kill plant cells. Two enzymes are encountered most frequently; one which is responsible for degradation of carbohydrates and dissolve cell walls, and the second hydrolyzes the cuticle. Cell wall degrading enzymes, such as polygalacturonases, pectin lyases and proteases, play an important role in starting infection and softening of tissues (Bailey and Jeger, 1992).

Environmental conditions favouring the pathogen are high temperature (28 ^{o}C being optimal) and high humidity. Presence of abundant moisture helps in the release of spores. Spores can only ooze out in the presence of high moisture and their dispersal usually takes place with rain. Disease severity is related to weather conditions. Extreme temperatures, low humidity, dry weather and sunlight rapidly inactivate fungi (Agrios, 2004).

2.3 Techniques used to control pre harvest anthracnose of fruit plants

In recent years there has been strong market competition in the agriculture sector due to developments in technology. Extra attention has been given to provide high quality fresh products without using synthetic

chemicals. However, despite all these efforts and techniques being used, modern agriculture is still heavily dependent on synthetic fungicides (Schirra *et al*., 2011). In order to minimize the use of fungicides and to find some safer alternatives, research is being conducted on improved cultural practices, biological control and plant defense promoters. To reduce the losses due to anthracnose, techniques such as crop rotation, chemical or biological control have been adopted (Jirak-Peterson and Esker, 2011; Pinto *et al*., 2010; Cowan, 1999).

2.3.1 Crop rotation and other cultural practices

In recent years, crop rotation and other cultural technical practices have been used to control anthracnose throughout the world. Crop rotation is the first step in a successful cropping system. It is also beneficial for improvement of soil conditions and reduction of weed and insect populations, resulting in high yields of crops. Implementation of crop rotations is helpful to farmers who use conservation tillage. Bergstrom and Nicholson (1999) concluded that corn crop residues are the important source of inoculum for anthracnose disease dispersal. Lipps (1983) demonstrated that anthracnose is negatively correlated with increasing distance from a residue source, indicating that local sources of inoculum were most important for disease development. The use of rotation or tillage can reduce the level of soil inoculum. Previous study has shown that corn and soy bean rotation instead of continuous corn reduced the incidence of corn anthracnose (Lipps, 1983). However, there are a few drawbacks to these conventional cultural practices, for example tillage resulted in higher incidence of anthracnose stalk rots

compared to no tillage (Lipps and Deep, 1991; Byrnes and Carroll, 1986; Doupnik *et al*., 1975). The major disadvantage of crop rotation is that it needs a lot of expertise, equipment and differing management practices. Nutrient requirements vary from crop to crop which results in alteration of nutrient management programmes and weed control practices.

2.3.2 Chemical control

Application of antifungal agents is the most common and effective method to control fungal diseases (Sommer, 1985) and synthetic fungicides are the most viable option. Commonly used synthetic fungicides are thiabendazole and benomyl (Khan *et al.*, 2001). However, due to the consistent use of these synthetic chemicals some resistant strains of *Colletotrichum* have emerged (Mari *et al*., 2003) and environmental and health risks are very high (Janisiewicz and Korsten, 2002; Mari and Guizzardi, 1998; Ragsdale and Sisler, 1994; Wilson and Wisniewski, 1989; Eckert and Ogawa, 1985).

The demand for non-chemically treated products has increased due to consumer awareness of these chemicals. Thus, an alternative approach for safe and efficient control of anthracnose by using plant and animal derived products is needed.

2.3.3 Biological control

Many research studies have shown the potential of biological control agents for the prevention of plant diseases, but there are only a few commercialized systems for bio control of plant diseases, especially for foliar parts (Elad 2000; Elad *et al*., 1998). Nevertheless, progress has been

substantial and *Trichoderma* spp. have been used for the control of *B. cinerea* for grapes (Dubos, 1984). Spadaro and Gullino (2004) reviewed the main agents that have been assayed as antagonistic microorganisms, including *Pseudomonas syringae* Van Hall, which are active against the genera *Botrytis*, *Penicillium*, *Mucor* and *Geotrichum*. The yeast *Candida oleophila* Montrocher was found to be effective against *Botrytis* and *Penicillium spp*., while other yeasts such as *Aureobasidium pullulans*, *Candida saitoana*, *Candida sake* and *Metschnikowia pulcherrima* are under development. It is important that evaluation of these microorganisms is carried out in a product formulation, since the formulation may improve or diminish antagonistic efficacy depending on the concentration and the duration of exposure to the treatment. *Bacillus brevis* protected Chinese cabbage from anthracnose by reducing the wetness of leaves (Edwards and Seddon, 1992).

Another approach to control plant diseases is the use of compost extracts. These extracts are helpful in controlling *Sphaerotheca fuliginea* on cucumbers and *B. cinerea* in strawberries and beans (Weltzien, 1992; Tränkner, 1991). Activity of dried extracts of compost could be enhanced with the addition of proteins against *B. cinerea* (Urban and Tränkner, 1993). The compost extracts help by inducing resistance and by direct inhibition of the pathogen (Weltzien, 1992). Watery extracts of compost originated from animal and plant resources (cattle, chicken manure and grape marc) suppressed the activity of grey mould of pepper and tomato by up to 56-100% depending on the fermentation of the compost (Elad and Shtienberg, 1994).

Several plant extracts have also been used for the biocontrol of anthracnose (Pinto *et al*., 2010). Sweetflag (*Acorus calamus* L.) and Betel pepper (*Piper betel* L.) were used against four species of *Colletotrichum* isolated from chilli plants. The mixture of these plant extracts helped to reduce anthracnose on the plants but they caused some phytotoxic effects on chilli leaves (Charigkapakorn, 2000).

The degree of control obtained by these biological control agents alone is often not satisfactory, so the use of additives or chemical fungicides at low concentrations can enhance biocontrol activity. Bioactivity of yeast antagonists could be enhanced by several methods. For example, bicarbonate salts have been shown to possess broad-spectrum antimicrobial properties for controlling pathogens. Postharvest decay caused by *P. expansum* and *A. alternata* in pear fruit can be reduced significantly by the addition of 2% (w/v) sodium carbonate to suspensions of the antagonistic yeasts *Cryptococcus laurrentii* or *Trichosporon pullulans* (Yao *et al*., 2004).

Reasons for the paucity of biocontrol agents include the low cost, availability and effectiveness of fungicides, the surface conditions of plant parts not favouring survival and activity of introduced microorganisms (Fokkema and Schippers, 1986), the inconsistent effectiveness of biocontrol agents, the relatively narrow spectrum of diseases controlled and the difficulty of formulating and distributing biocontrol agents as compared with chemicals. In addition, registration is required by regulatory agencies, e.g. Environmental Protection Agency (EPA) and European Agencies, before any biocontrol agent can be used commercially.

The doses of these compounds to be applied need to be determined, particularly in relation to any potential mammalian toxicity. Furthermore, the efficacy of these compounds can decrease under commercial conditions when used as a stand-alone treatment (Droby *et al*., 2002). Thus, due to these drawbacks, interest has increased in developing alternative control methods, which are biodegradable and environmentally sound.

2.4 Techniques used to control postharvest anthracnose

For reduction of postharvest losses of fresh produce efforts are also being made to use some physical methods, such as ultraviolet irradiation, radiofrequency treatment, heat treatments (heat therapy), biological control and storage techniques (Narayanasamy, 2006; Barkai-Golan, 2001).

2.4.1 Physical control

Chilling injury and rot development of fruit can be reduced by using postharvest heat treatments, such as hot water treatment, short hot water rinsing and brushing and hot air treatment (Kumah *et al*., 2011; Barkai-Golan and Phillips, 1991). These treatments enhance the shelf life of fresh produce and also provide quarantine security. Heat treatment as a postharvest technique was used in the first decade of the 20th century after the First World War in the citrus industry in the United States. Subsequently, postharvest heat treatments have been used commercially on a limited scale to control fungal diseases and pest infestations of horticultural crops. In general, heat treatments are applied as pre-storage treatments prior to short or long cold storage by using hot water, hot air or vapour heat (Yimyong *et*

al., 2011; Fallik, 2010; Fan *et al*., 2008; Kremer-Köhne, 1999). However, there are limitations, for example different commodities require different temperatures and exposure times (Kumah *et al*., 2011). There are some situations in which the application of inappropriate hot water treatments leads to tissue damage, abnormal softening, lack of starch breakdown, flesh darkening and development of internal cavities (Maqbool, 2012).

2.4.2 Chemical control

Use of synthetic fungicides is the most common method for controlling anthracnose (Young, 2008; Leroux, 2004; Rosslenbroich and Stuebler, 2000). The emergence of resistant strains of fungi (Mari *et al*., 2003) and very high environmental and health risks are the major reasons for the removal of such fungicides from the market (Janisiewicz and Korsten, 2002; Mari and Guizzardi, 1998; Ragsdale and Sisler, 1994; Wilson and Wisniewski, 1989; Eckert and Ogawa, 1985).

2.4.3 Natural compounds

Natural compounds, particularly of plant and animal origin, with antimicrobial properties have gained considerable attention during the past decade (Ncube *et al*., 2008; Valgas *et al*., 2007; Tripathy and Dubey, 2004). Acetic acid, jasmonates, flavour compounds (e.g. hexane, acetaldehyde, and benzaldehyde), fusapyrone and deoxyfusapyrone, glucosinolates, chitosan, essential oils and propolis are examples of natural compounds that have been exploited to manage postharvest diseases of fresh fruits and vegetables (Tripathy and Dubey, 2004). *In vitro* trials of some plant volatiles,

e.g. benzaldehyde, benzyl alcohol, acetaldehyde, ethanol, 2-nonanone and nerolidol, have proved them to be anti-fungal agents against the fruit and vegetable pathogens, *R. stolonifer*, *C. musae, P. digitatum* and *Erwinia carotovora* (Utama *et al*., 2002). Similarly, essential oils obtained from the leaves of *Citrus medica, Melaleuca leucadendron and Ocimum canum* are able to control deterioration of stored food caused by *Aspergillus versicolor* and *A. flavus* (Dubey and Kishore, 1988). Most of the studies showed that several essential oils can inhibit fungi in *in vitro* conditions (Hidalgo *et al*., 2002; Bellerbeck *et al*., 2001; Singh and Tripathi, 1999; Bishop and Reagan, 1998). Lima et al. (1998) reported that the growth of *B. cinerea* and *P. expansum* could be inhibited by propolis obtained from poplar and conifer trees.

These compounds need to be assessed for mammalian toxicity. Their efficacy is limited and, under commercial conditions, they may not be effective when used alone (Droby *et al*., 2001). Thus, there is urgency to develop alternative control methods that are biodegradable and environmentally safe.

One fascinating area of study is the use of natural compounds such as edible films and coatings. Edible films and coatings have been used since the 1800's (Guilbert *et al*., 1996) and extensive research on them has paved the way for effective treatments.

2.5 Chitosan

Chitosan is derived from *N*-deacetylated chitin, which is obtained from the outer shell of crustaceans (Sandford, 1989). It is a natural polymer composed

of *β*-(1,4)-2 acetylamino-2-deoxy-$_D$-glucose and *β*-(1,4)-2-amino-2-deoxy-$_D$-glucose units. The positive charge of chitosan is responsibe for its distinctive physiological and biological properties. From its biological activity and mode of action, it is postulated as an antimicrobial compound that can be applied against fungi, bacteria and viruses, and as an inducer of the defense mechanisms of plants (Guerra-Sánchez, 2009; Fisk *et al*., 2008; Terry and Joyce, 2004).

Chitin and chitosan are natural polysaccharides and the presence or absence of nitrogen distinguishes them from cellulose. Chitosan is in high demand as a natural compound used in various industuries, such as agriculture (films, fungicide and elicitor) (Ren *et al*., 2001; Makino and Hirata, 1997; Hoagland and Parris, 1996; Lafontaine and Benhamou, 1996), food (coating, antimicrobial, preservative and antioxidant,) (Shahidi *et al.*, 2001; Benjakul *et al*., 2000, Roller and Covill, 1999; Sapers, 1992, Pennisi, 1992), pharmacology and medicine (fibre, membranes, fabrics, drugs and artificial organs) (Liu *et al*., 2001; Kulpinsky *et al*., 1997; Nishimura, 1997; Muzarelli, 1989), biotechnology (emulsifier, chelator and flocculent) (Hirano 1989; Sandford, 1989), and cosmetology (hair additives, lotions, facial and body creams) (Lang and Clausen, 1989).

2.5.1 Antifungal properties of chitosan

The antifungal properties of chitosan are correlated with its concentration (Ali *et al*., 2010), suggesting a link between its polycationic nature and its antifungal properties. It is also possible that chitosan plays an important role

in the synthesis of certain fungal enzymes. Changes in fungal morphology, such as abnormal shapes of conidia, excessive mycelial branching, abnormal shapes of hyphae and reduction in the size of hyphae, are due to the effect of chitosan (Bautista-Baños *et al*., 2006; Bautista-Baños *et al*., 2003). After treatment with chitosan, *Rhizopus stolonifer* and *Sclerotinia sclerotiorum* showed abnormal shapes of conidia and swelling and reduction in size of hyphae (Hernández-Lauzardo *et al*., 2008; Cheah *et al*., 1997).

Based on *in vitro* and *in vivo* studies, the antifungal properties of chitosan have been highly corelated with fungal inhibition (Ali and Mahmud, 2008; Bautista-Baños *et al*., 2003; Ben-Shalom *et al*., 2003; Cheah *et al*., 1997; El Ghaouth *et al*., 1992; Kendra and Hadwiger, 1984).

Mycelial growth is inhibited when the culture medium contains chitosan, for example *Sclerotinia sclerotiorum* was inhibited by 4% when chitosan concentration was increased (Cheah *et al*., 1997). The radial expansion of *Alternaria alternata*, *Botrytis cinerea*, *Colletotrichum gloeosporioides* and *Rhizopus stolonifer* declines as the concentration of chitosan increases (Ali and Mahmud, 2008; El Ghaouth *et al*., 1992). Other reports showed similar results, i.e. growth of *F. oxysporum*, *C. gloeosporioides*, *R. stolonifer* and *Penicillium digitatum* was inhibited by 3.0% chitosan. Generally, fungal spores treated with chitosan germinate less well compared with untreated spores, as shown for *C. gloeosporioides* (Bautista-Baños *et al*., 2003).

Various investigations have confirmed the antifungal potential of chitosan. Dipping of tomato seedlings and seeds significantly reduced the diseases caused by *P. aphanidermatum* and *F. oxysporum* f. sp. *radicis-lycopersici* (Lafontaine and Benhamou, 1996; Benhamou *et al*., 1994; El Ghaouth *et al*.,

1994a). An increase in chitosan concentration (0.5 to 2.0 mg ml^{-1}) helped reduce root lesions on tomato seedlings caused by *F. oxysporum* f. sp. *lycopersici* (Benhamou *et al*., 1994).

Benhamou *et al*. (1994) reported that no root lesions in tomato were observed when a combination of chitosan-amended soil and chitosan-treated tomato seeds were used. In contrast, for cucumber plants only the growing medium was amended with 400 mg ml^{-1} chitosan and no symptoms of root infection were observed (El Ghaouth *et al*., 1994a). Lower incidence of disease caused by *F. graminearum* and better germination of wheat was recorded for chitosan-treated (2 to 8 mg ml^{-1}) seeds in comparision with untreated controls (Bhaskara Reddy *et al*., 1999).

The preventive antifungal effect of chitosan has been documented on cucumber and groundnut plants infected by *B. cinerea* and *Puccinia arachidis*, respectively. For both studies, chitosan (0.1% and 1000 ppm, respectively) was sprayed 24h before inoculation (Ben-Shalom *et al*., 2003; Sathiyabama and Balasubramanian, 1998).

Daikon radish (*Raphanus sativus* L.) showed an increase in root and shoot growth (Tsugita *et al*., 1993). Various crops, such as soybean sprouts (Lee *et al*., 2005), cabbage (*Brassica oleracea* L. var. 'Capitata') and sweet basil (Kim, 2005), showed vigorous growth after treatment with chitosan. Frequent application of chitosan and the concentration applied markedly increased the growth in various crops, including chilli, Chinese cabbage, celery, bitter cucumber and rice (Boonlertnirun *et al*., 2005; Chandrkrachang *et al*., 2003).

Increase in the growth of orchids (*Paphiopedilum* and *Dendrobium* Sensational 'Purple') was observed with the use of chitosan at 2.5-40.0 mg l^{-1} (Chandrkrachang, 2002 and 2005). Limpanavech *et al*. (2003) noted that the development and growth of cut flower *Dendrobium* Sonia Jo 'Eiskul' was affected by the concentration and degree of deactylation of chitosan. The reduced severity of leaf spot and increase in inflorescences in three and half year old *D. misteen* were reported by Win *et al*. (2005), however there was no effect on size of floret and new shoot growth.

In bell pepper, production of cell wall macerating enzymes such as polygalacturonases is markedly reduced by chitosan (El Ghaouth *et al*., 1997). Chitosan effectively impaired the assembly of fungal virulence factors, such as organic acids (fumaric and oxalic acids), cell wall degrading enzymes (pectate lyase, cellulose and polygalacturonase), and host specific toxins (alternariol and alternariol monomethylether) and induced rishitin production (Bhaskara Reddy *et al*., 2000).

2.5.2 Chitosan as an elicitor of response mechanisms

Chitosan is considered as a potential candidate for inducing phenolics (Bautista-Baños *et al*., 2006) and defense-related enzymes in plants (Benhamou, 1996). It is regarded as a potent elicitor of plant defense reactions because it induces activity of chitinases, phytoalexins, protease inhibitors and structural compounds such as cellulose and lignin (Benhamou, 1996).

Enzymatic responses of plants are highly correlated with induced defense reactions. Previous studies have demonstrated that chitosan is responsible

for eliciting exogenous host defense responses, e.g. phenolic compounds, *β*-1,3-glucanases, chitinases, synthesis of phytoalexins and lignin induction (Zhang and Quantick, 1998; Bhaskara Reddy *et al*., 1997; Fajardo *et al*., 1998; Arlorio *et al*., 1992; Tejchgraber *et al*., 1991). Gagnon and Ibrahim (1997) reported an increase in the amount of 20-hydroxygenistein monoprenyls, genistein and isoflavonoids after chitosan treatment in roots and exudates of white lupin, respectively.

Application of chitosan as root and seed dressings and foliar spray helps in inducing resistance by eliciting defense reactions when applied in susceptible tomato plants against *F. oxysporum* (Benhamou *et al*., 1998). This induced resistance may be due to the accumulation of fungitoxic compounds at penetration sites of the pathogen.

Phenolics encompass a wide range of substances that have an aromatic ring with at least one hydroxyl group. They represent one of the most abundant groups of compounds found in nature and are of particular interest in postharvest due to their defense mechanism and their role in colour and flavour in many crops. They have been identified as antimicrobial and antifungal agents. In general, phenolics have been implicated as detrimental allelopathic agents and feeding deterrents for fungi. Due to these functions, plant phenolic compounds have been advocated to play a number of roles in plant defense mechanisms against several pathogens. The amount of phenolics varies widely in postharvest products (Hammerschmidt, 2005; Kays, 1997).

De Ascensao and Dubery (2000) investigated the tolerance in banana against *Fusarium* wilt caused by race four of *Fusarium oxysporum* f. sp.

cubense. They observed that tissue cultured cv. Goldfinger roots treated with chitosan showed a prominent increase in total soluble phenolic acids, ester-bound phenolic acids and cell wall-bound phenolic acids, but a relatively small increase in free phenolic acids and a moderate increase in glycoside-bound phenolic acids, as compared to the corresponding controls. This could be explained by the fact that esterification with phenols, such as hydroxycinnamic acids, modifies the cell wall polysaccharides and they may become cross-linked and provide a platform for later lignification to resist the action of lytic enzymes produced by fungal pathogens (Lewis and Yamamoto, 1990). Success or failure of plant resistance may, therefore, depend on the relative rate and extent of the host's lignification response.

A plant cell wall is composed of lignin and cellulose. Chitosan induces plants to increase their lignin and cellulose synthesis, and the greater lignification results in a stronger cell wall that is less penetrable by a fungal pathogen (Vander *et al*., 1998; Prospieszny and Zielinska, 1997). Chitosan increases lignification in wheat plants and chitosan oligomers elicit lignification in pea tissue (Barber *et al*., 1989). The invasion by a pathogen in the plants was restricted by the induction of structural barriers at fungal penetration sites. In some plants, cellular suberisation and lignification occurred during the course of infection. Chitosan treatments after inoculation with *B. cinerea* caused moderate lignification of cell walls after 48 and 72 h in wheat leaves (Barber *et al*., 1989; Pearce and Ride, 1982). The evidence of formation of abnormal structures, such as agglomerates of hyphae, was confirmed by transmission electron microscopy of *Fusarium oxysporium* f. sp. *radicis-lycopersici*. Chitosan treated tomato leaves and roots showed some

changes in cell structure, including (i) formation of papillae (wall appositions) into the endodermis tissues and the cortex, (ii) xylem vessels sealed with bubble-like structures which were milky in appearance and (iii) thickening of pit membranes (Lafontaine and Benhamou, 1996).

Tomato roots treated with chitosan plus *Bacillus pumilus* showed an increase in host defense (Benhamou *et al*., 1998). The host defense response of inoculated (*P. aphanidermatum*) cucumber plants was similar to those observed on tomato plants, such as formation of papillae and fibrillar material to protect the cell wall and intercellular space, respectively (El Ghaouth *et al*., 1994a).

Some studies have reported the involvement of self-defense enzymes in controlling disease and preventing infection caused by pathogens (Vander *et al*., 1998; Bohland *et al*., 1997). Chitin and chitosan have been reported to enhance resistance systems, such as lignin formation, phenylalanine ammonia lyase and lipoxygenase activities, in wheat leaves (Vander *et al*., 1998; Bohland *et al*., 1997). Some oxidative enzymes, such as polyphenol oxidase (PPO) and peroxidase (POD), are also involved in defense mechanisms of plants against pathogens. These enzymes catalyse the formation of oxidative phenols and lignin that contribute to the formation of defense barriers for reinforcing the cell structure (Zhu and Ma, 2007).

The natural resistance of plants towards diseases is based on preformed defenses and on induced mechanisms. The induced mechanisms are associated with local changes of pathogen infection site, such as the hypersensitive response (HR), which is one of the most efficient forms of plant defense (Kortekamp and Zyprian, 2003). Besides the accumulation of

antimicrobial compounds, such as phenolic compounds and phytoalexins (Ortega *et al.,* 2005), the HR also leads to an increase in the activity of peroxidase (POD) (Kortekamp and Zyprian, 2003) and polyphenol oxidase (PPO) enzymes involved in defense responses (Agrios, 2004).

PPO belongs to the group of enzymes called oxidoreductases. It is present in almost all plants, but most abundant or active in fruit, being distributed in almost all fruit parts (Vamos-Vigyazo, 1981). PPO catalyses two reactions; the oxidation of diphenol to *o*-benzoquinones and hydroxylation of the monophenol oxidase of hydroxyl group adjacent to the *o*-position. Both reactions utilise molecular oxygen as a co-substrate (Rupasinghe, 2008).

POD, like PPO, is also an oxidoreductase (Vamos-Vigyazo, 1981). It is widely distributed in nature and catalyses the decomposition of hydrogen peroxide (H_2O_2) in the presence of a hydrogen donor.

It has been observed that the wounding or softening of fruit results in an increase of POD and PPO activity (Cantos *et al.,* 2002). These enzymes along with total phenolics may result in quality losses by inducing changes in the flavour, colour, texture and nutrient value of the fruit. Oxidation of phenolic compounds by PPO and POD causes fruit to undergo enzymatic browning (Rupasinghe, 2008).

Reduction of PPO levels in plants or fruit could contribute to reduced oxidation of phenolic compounds, which could result in reduced resistance to pathogens. Examination of the sensitivity of plants to pathogens revealed a dramatic increase in their susceptibility when there were lower amounts of PPO in the plants (Mayer, 2006). While the exact mechanisms have yet to be

elucidated, PPO and POD are known to play a part in increasing the defenses of a plant against pathogens.

El Ghaouth *et al.* (1997) found that, in bell pepper, chitosan reduced the secretion of polygalacturonases by *B. cinerea* that results in decreased maceration of host cell walls. Bhaskara Reddy *et al.* (2000) reported increased acitivities of phenolic compounds, POD and PPO in strawberry fruit treated with chitosan. However, chitosan increased the total phenolic compounds and protein in wounded tomato but the PPO activity decreased (Badawy and Rabea, 2009). Ben-Shalom *et al.* (2002) also found that the increased resistance of cucumber against *B. cinerea* was due to elicited activity of POD. In a recent study, Liu *et al*. (2007) observed that chitosan not only inhibited the *in vitro* growth of *P. expansum* and *B. cinerea* but also induced defense reactions in tomato fruit. The findings of previous studies suggest that activity of chitosan is not only associated with fungitoxic properties, but is also involved in elicitation of defense enzymes in fruit and plants.

Evidence strongly suggests that esterification of phenols to cell wall materials is a common theme in the expression of plant resistance, and the crosslinking of such phenylpropanoid esters leads to the formation of lignin-like polymers (Fry, 1987). In plants, the key enzyme for phenylpropanoid metabolism is phenylalanine ammonia lyase (PAL). PAL activity in plant tissue may rapidly change under the influence of various factors, such as pathogen attack and treatment with elicitors (Dixon and Harrison, 1990). However, POD is a phenol oxidizing enzyme. The activities of PAL and POD may be rapidly enhanced under the influence of elicitors or pathogen attack.

As a biodegragable agent, chitosan is a potential antimicrobial candidate and is involved in eliciting activities (Benhamou, 1996). The fungicidal activities of chitosan have been shown to be equivalent to synthetic fungicides such as thiabendazole (TBZ) and ipriodione (El Ghaouth *et al*., 1992).

Extensive literature documents the biochemical changes of defense mechanisms against fungal pathogens, as they cause physiological changes induced in plants by treatment with chitosan. One of the most studied biochemical changes is an increased production of antifungal hydrolases, such as *β*-1,3-glucanase, chitinase and chitosanase, in leaves and roots of bell pepper. Hydrolases are the major defense mechanisms in a plant's arsenal against fungal attack, as the enzymes are able to attack the cell walls of the invading fungal germ tubes. Marked increases in the anti-fungal hydrolases (0.13 U mg^{-1} protein) after treatment with chitosan have been noted in strawberries and raspberries (Zhang and Quantick, 1998; El Ghaouth *et al*., 1997).

Tejchgraber *et al*. (1991) reported that exposure time of soyabean seeds to chitosan glutamate was crucial for increased germination and chitinase activity. Some other studies indicated that the *de-novo* synthesis of phenolic compounds was triggered by chitosan, and these phenolic compounds serve as the first defensive line for inhibiting fungal growth, while *β*-1,3-glucanase serves as the second barrier for inhibiting the penetration of fungal hyphae into plant cells (Lanfontaine and Benhamou, 1996; Benhamou *et al*., 1994). In contrast, chitosan was unable to produce significant amounts of defense enzymes in cucumber to reduce grey mould (Ben-Shalom *et al*., 2003).

2.5.3 Mode of action of chitosan

Based on the positive charge of the glucosamine monomer on the C2 below pH 6, chitosan showed more antimicrobial activity as compared to chitin (Chen *et al*., 1998). The actual antimicrobial mechanism of chitin and chitosan is unknown, but diverse theories have been reported.

The leakage of intracellular and proteinaceous constituents occurred as a result of the interaction between polycatoinic chitosan and anionic microbial cell membranes (Chen *et al*., 1998; Fang *et al*., 1994; Seo *et al*., 1992; Hadwiger *et al*., 1986). The outer surface of a pathogen is in direct contact with chitosan. At concentrations lower than 0.2 mg ml^{-1}, the positively charged chitosan may not interact fully with the negatively charged microbial surface to form accretion while, at higher concentrations, chitosan molecules form a positively charged network on the bacterial surface and keep them in suspension. Chitosan also inhibits microbial growth and binds trace metals, which proves chitosan to be a chelating agent (Cuero *et al*., 1991).

It also inhibits the production of various fungal enzymes and helps in triggering numerous defense processes in the host tissue (El Ghaouth *et al*., 1992). Application of chitosan on plant tissues results in two major effects. The first is prevention of the pathogen invading inside the plant tissue by the formation of a physical barrier. This phenomenon is similar to the abscission zones observed on potato leaves, which prevent necrotrophic pathogens from further spread (El Hadrami *et al*., 2009). The second is cell wall fortification, which occurs as a response of H_2O_2 accumulation and serves as an alert signal for healthy plant parts.

Chitosan is a chelator for metals and minerals and used for purification of fresh and salt water. This ability to chelate is also of value as it prevents the access of pathogens to minerals and nutrients. Chitosan can also bind mycotoxins and can reduce the damage of tissues due to toxins (Bornet and Teissedre, 2005).

Owing to the ability of chitosan to bind to biological membranes and their positive charges, it is helpful in wound healing following pathogen attack or mechanical injury (Hirano *et al*., 1999). Chitosan is reported to trigger the formation of defense-related enzymes including peroxidase and phenylalanine ammonia-lyase and PR-proteins. These enzymes are involved in lignin assembly and formation of tyloses that accelerate wound healing.

2.5.4 Chitosan as an edible coating

Another advantage of chitosan is its barrier properties, which make it a good coating agent to prolong the shelf life of fresh fruits and vegetables. The chitosan coatings on fruits and vegetables are able to form a semi-permeable layer that can adjust the gaseous exchange, reduce transpiration loss and hold back fruit ripening. Reductions in respiration rate and water loss, when chitosan is applied as a coating, eventually lead to the retention of freshness as well as a slower decrease in weight loss, as evidenced by studies with strawberry, peach, longan and papaya (Ali *et al*., 2011; Jiang and Li, 2001; Du *et al.,* 1997; El Ghaouth et al., 1992).

Decreased respiration of fruit results in the reduction of internal CO_2 in pears due to the efficacy of chitosan coatings (Du *et al.,* 1997), with a consequent decrease in ethylene production. Similar effects were reported in

chitosan coated tomatoes and peaches (Li and Yu, 2000). The physical quality of crop commodities that have been treated with chitosan coating improved significantly, such as external appearance and retention of colour (Ali *et al*., 2011). Chitosan coated fruit, such as strawberries, litchi and raspberries, were able to retard anthocyanin degradation (Zhang and Quantick, 1997 & 1998). Chitosan treated papayas were more firm at the end of storage in comparision with the control fruit (Bautista-Baños *et al.,* 2003).

Chitosan enhances the quality of vegetables and fruits, but may also decrease the quality depending on the variety, cultivar and type of crop. Bell pepper and cucumbers treated with chitosan were darker green than control fruit, while a slight colour enhancement was observed in papayas and pears (Jiang and Li, 2001; Du *et al.,* 1997; Woods *et al*., 1996: El Ghaouth *et al.,* 1992).

2.6 Submicron chitosan dispersions

Dispersion of a liquid in another immiscible liquid by mechanical shear forms an emulsion (Bibette *et al*., 1990). These forms of emulsion are commonly known as dispersions (Kobayashi *et al*., 2001) and contain uniform and extremely small droplets from 1-1000 nm (Wells and Goldberg, 1992). Submicron dispersions are kinetically stable systems that can be transparent (emulsion dispersion size (EDS < 200 nm)) or ‘‘milky” (EDS ~500 nm) (Tadros *et al*., 2004; Izquierdo *et al*., 2002).

Properties of dispersions, such as low viscosity, high kinetic stability against creaming or sedimentation and a large interfacial area (Solans *et al*.,

2002; Buszello and Muller, 2000), make them increasingly used in many applications (Solans *et al*., 2005).

2.6.1 Formation of submicron dispersions

Formation of submicron dispersions requires energy inputs by means of mechanical devices, such as high pressure homogenizers, ultrasound generators and high-shear stirring, or by chemical potential of the components. It has been reported that the smallest sizes of dispersions are obtained by the machines which supply energy for a short time (Walstra, 1996).

The most efficient method for producing small sized droplets is ultrasound emulsification but it is only suitable for small batches (Walstra, 1996). In preparing submicron dispersions, sonication time and amplitude are two important components. Time of sonication depends on the type of liquid, as more hydrophobic monomers require a longer period of sonication (Landfester *et al*., 2004).

The properties of submicron dispersions depend on liquids and on the emulsification path or sonication time. Optimization studies are required for achieving the best emulsifiers and required droplet sizes. The aim behind this optimization is to increase the efficacy of submicron dispersions with respect to conventional emulsions (i.e. macro dispersions). Therefore, in general, the smallest droplet size and minimum polydisperity is obtained by proper optimization of the variables. Stability is another problem for these smaller sized particles that can be managed by optimization.

For different combinations of Span-Tween surfactants, an optimum surfactant composition presenting a water solubility maximum is chosen, and droplet size variation is studied with respect to water concentration (Uśon, 2004).

The small droplet size of dispersions confers stability against sedimentation (or creaming) because the Brownian motion and consequently the rate of diffusion are higher than the rate of sedimentation (or creaming) induced by gravity force. Ostwald ripening or molecular diffusion, which arises from dispersion polydispersity and different solubility levels between smaller and larger droplets, is the main mechanism for the destabilization of submicron dispersions (Tadros *et al*., 2004).

The theory of Lifshitz and Slyozov (1961) and Wagner (1961) assumes that the droplets of the dispersed phase are spherical, the distance between them is higher than the droplet diameter and the kinetics is controlled by molecular diffusion of the dispersed phase in the continuous phase. According to this theory, the Ostwald ripening rate in O/W dispersions is directly proportional to the solubility of the oil in the aqueous phase.

2.6.2 Applications of submicron dispersions

Higher kinetic stability, low viscosity and optical transparency of submicron dispersions make them very attractive systems for many industrial applications. They are used in the pharmaceutical field as drug delivery systems (Taha *et al*., 2004; Wu *et al*., 2001), in cosmetics as personal care formulations (Sonneville-Aubrun *et al*., 2004), in agrochemicals for pesticide delivery (Lee and Tadros, 1982) and in the chemical industry as

polymerization reaction media (Liu *et al*., 2004). The use of submicron dispersions as colloidal drug carriers is well documented (Sadurni *et al*., 2005; Taha *et al*., 2004; Nicolaos *et al*., 2003). The bioavailability of drugs was reported to be strongly enhanced by solubilization in small droplets (below 0.2 μm) (Nicolaos *et al*., 2003). The formation of submicron dispersions is generally attributed to phase instabilities during emulsification, where the presence of lamellar liquid crystallites and/or bicontinuous microemulsions are thought to play critical roles (Forgiarini *et al*., 2001a; Forgiarini *et al*., 2001b; Forgiarini *et al*., 2000).

The attraction of submicron dispersions for applications in various industrial fields (Izquierdo *et al*., 2002), for example as reaction media for polymerization, health care and agrochemicals, is due to their small droplet size, high kinetic stability and optical transparency compared to conventional emulsions, giving advantages for their use in many technological applications. Submicron dispersion applications deal with the preparation of polymeric small particles using a monomer as the dispersant (Antonietti and Landfester 2002; Asua 2002; El-Aasser and Miller 1997).

Surfactants containing a polymerisable group (surfmers) are used in dispersion polymerisation to protect, stabilise and functionalize the polymer particles. Guyot *et al*. (2003) reported the dispersion polymerization of methylmethacrylate or styrene in the presence of acrylic surfmers which gave the latexes a good stability. However, a non-polymerisable surfactant was required to obtain stable dispersions.

An important application of dispersion polymerisation is the production of low viscosity and high solid content latexes. Do Amaral and Asua (2004) and

Asua (2002) prepared submicron dispersions using acrylic monomers and reported that particle size is mainly controlled by droplet size of the dispersion, which is dependent on the type and concentration of surfactant.

The small droplet size and the high kinetic stability make dispersions suitable for efficient delivery of active ingredients (due to their large surface area) and for penetration through the “rough” texture of the skin (Amselem and Friedman, 1998).

With respect to agrochemical applications, Wang *et al*. (2007) proposed dispersions for solubilizing water-insoluble pesticides. Submicron dispersions are formed before the application by dilution of a concentrate containing oil, surfactant, the active and 50% water. It is claimed that the dispersions obtained showed good stability but the diameter of droplets increased about five times in 14h, from 40 to 200 nm. Therefore, application should be carried out a few hours after preparation to obtain the advantages of small droplet size.

Chitosan dissolves in a mixture of water and acetic acid, but it cannot be dissolved in oil. Therefore, to prepare submicron chitosan dispersions, particles have to be bound by an emulsifier. The emulsifier aggregates around the chitosan micelles and entraps them, conferring further stability. Hydrophilic emulsifiers which contain a hydroxyl group are usually suitable for polysaccharides (Navarro-Tarazaga *et al*., 2008).

Thus, on the basis of this literature review, the present study has been designed to evaluate the effect of chitosan and submicron chitosan dispersions on pre- and post-harvest anthracnose of dragon fruit, production of inducible compounds, plant growth and the yield of dragon fruit.

CHAPTER 3

PRELIMINARY STUDY FOR SCREENING OF CHITOSAN, FORMULATION OF SUBMICRON CHITOSAN DISPERSIONS AND DETERMINATION OF THEIR PHYSICOCHEMICAL PROPERTIES

3.1 Introduction

Antimicrobiol activity of chitosan has been detected towards many bacteria, fungi and yeasts. Several studies show that the biological activity of chitosan considerably depends on degree of deacetylation (DD) and physicochemical properties such as molecular weight (MW) (Ueno *et al*., 1997; Hirano and Nagao, 1989; Uchida, *et al*., 1989; Kendra and Hadwiger, 1984). These features have a great impact on the solubility of chitosan and its contact with the cell walls of target microorganisms. These factors independently affect the antimicrobial properties of chitosan, though it has been advocated that the effect of molecular weight is greater than the degree of deacetylation (Sekiguchi *et al*., 1994).

Antimicrobial properties of chitosan can be enhanced by decreasing the deacetylation (Andres *et al*., 2007; Tsai *et al*., 2004). In studies using several fungi, including *Aspergillus parasiticus*, *Candida albicans*, *A. fumigatus and Fusarium oxysporum*, *and* Gram-negative bacteria (*Escherichia coli*, *Salmonella tiphymurium*, *Pseudomonas aeruginosa*, *Shigella dysenteriae*, *Vibrio cholerae*, *V. parahaemolyticus*) and Gram-positive bacteria (*Staphylococcus saprophyticus* and *S. aureus*), it was found that antimicrobial activity increased with the lower degree of deacetylation (Hongpattarakere and Riyaphan, 2008; Tsai *et al*., 2002).

The application of this polysaccharide is limited due to its low solubility in aqueous dilute acids and the high viscosity of its solutions (Tikhonov *et al*.,

2006). Chitosan at concentrations higher than 1.5% showed limited antimicrobial effect due to high viscosity (Davis, 2011), therefore it is essential to increase the efficacy of chitosan by reducing its viscosity and surface tension.

The main objectives of the present study were:

1. To screen different molecular weight chitosans and to evaluate their efficacy against *C. gloeosporioides*.
2. To formulate submicron chitosan dispersions and to study their physicochemical properties.

3.2 Materials and methods for screening of different types of conventional chitosan against *C. gloeosporioides*

3.2.1 Materials

Locally prepared crab shell chitosan (MW 350 kDa; 95% deacetylated) was purchased from Chitin-Chitosan Research Centre of Universiti Kebangsaan Malaysia. Low molecular weight chitosan from crab shell (MW 50 kDa; 75–85% deacetylated), medium molecular weight chitosan from shrimp shell (MW 400 kDa; 75–85% deacetylated) and high molecular weight chitosan from shrimp shell (MW 760 kDa; ≥ 85% deacetylated) were purchased from Sigma-Aldrich, USA. Brij 56 and Span 20 used as emulsifiers were purchased from Merck KGaA, Darmstadt, Germany and Sigma-Aldrich, USA, respectively.

3.2.2 Isolation of *C. gloeosporioides* and preparation of inoculum

Isolation of *C. gloeosporioides* was carried out from infected dragon fruit plants and fruit. Small slices of the fruit skin and infected plant with disease lesions were placed in Petri dishes containing potato dextrose agar (PDA, Merck KGaA, Darmstadt, Germany) and incubated at room temperature (28 ± 2°C). To obtain pure cultures, the colonies were re-isolated after mycelial growth was observed. The isolates were identified on the basis of their morphological and cultural characteristics (Barnett and Hunter, 1972). The identified cultures of *C. gloeosporioides* were preserved for further use on PDA slants.

3.2.3 Pathogenicity test

Pathogenicity test was carried out according to the method developed by Melanie *et al*. (2004) and Ali (2006), on healthy stems and fruit, respectively. The isolated pathogen (Section 3.2.2) was cultured in potato dextrose broth (PDB) and incubated for 7 days on a rotary shaker at 150 rpm. Mycelium was removed from the harvested conidia by filtering through four layers of cheesecloth. Sterile purified water was used to adjust the conidial concentration to 1×10^{6} conidia ml^{-1}, determined using a hemacytometer. Healthy plant stems were surface disinfected with 1% sodium hypochlorite (NaOCl) for 2 min. A sterile sharp tooth pick was used to made 2 mm deep artificial wounds and each wound was inoculated with 20 µl of conidial suspension using a sterile syringe needle. These stems were laid on moist filter paper in trays wrapped with transparent plastic and incubated at room temperature for 14 days. Symptom development was observed regularly.

For the pathogenicity test on fruit, healthy fruit were surface sterilised using 0.5% sodium hypochlorite solution followed by washing with purified water. Using a sharp sterile cork borer, 5-6 mm diameter plugs were cut from the fruit surface and replaced with mycelial plugs from a 10 days old culture of *C. gloeosporioides.* The fruit were placed separately in plastic bags and incubated for 10 days at 28 ± 2 °C. They were observed daily for lesion development.

3.2.4 Preparation of conventional chitosan solution

Chitosan powder (1.0 g) was dissolved in 100 ml purified water containing 0.5 ml (v/v) glacial acetic acid using an overhead stirrer (Model: IKA® RW 14 basic, Fisher Scientific Sdn Bhd., Malaysia). The pH of the solution was adjusted to 5.6 by adding 1N NaOH, using a digital pH meter (Model: Knick 646). Four types of chitosan were used for the screening experiment, i.e. locally prepared chitosan, low molecular weight chitosan, medium molecular weight chitosan and high molecular weight chitosan.

The following treatments were used:

T_1: Control

T_2: 0.5% chitosan

T_3: 1.0% chitosan

T_4: 1.5% chitosan

T_5: 2.0% chitosan

3.2.5 *In vitro* antifungal assay of conventional chitosan against *C. gloeosporioides* isolated from dragon fruit plants

3.2.5.1 Inhibition of radial mycelial growth

The *in vitro* antifungal activity of conventional chitosan was determined by placing a 5 mm diameter disc from the pure culture of *C. gloeosporioides* in the centre of PDA dishes containing chitosan solution at 0.5, 1.0, 1.5 and 2.0%. Petri dishes containing only PDA were used as controls. All the Petri dishes were incubated at room temperature (28°C) until the radial mycelial growth of the control dishes reached the edge of the dish. The percentage

inhibition in radial mycelial growth (PIRG) was calculated using the formula described by Al-Hetar *et al*. (2010).

$$PIRG = \frac{R_1 - R_2}{R_1} \times 100$$

Where R_1 = mycelial growth in control plates

R_2 = mycelial growth in treated plates

3.2.5.2 Conidial germination inhibition test

The conidial germination inhibition test was carried out using the cavity slide technique. Briefly, an aliquot of 40 µl of each of the chitosan concentrations was pipetted onto cavity slides. Ten µl of a freshly harvested conidial suspension of *C. gloeosporioides* (adjusted to 10^5 conidia ml^{-1}) was then pipetted onto each cavity slide and the slide was covered with a cover slip and kept in the dark for 7 h at 25 ± 2°C. Cavity slides containing purified water served as the control. After 7 h of incubation, the conidia were killed by adding 10 µl of 2.0% sodium azide and observed for germination under a light microscope at 40× magnification. The number of germinated conidia was counted in 10 microscopic fields in 20 replicated dishes and presented as percent inhibition. A conidium was considered germinated if its germ tube was longer than the conidium itself. The percentage inhibition in germination was calculated by the method of Cronin *et al*. (1996).

3.2.5.3 Dry weight of mycelium

Potato dextrose broth (PDB) and chitosan solutions were separately autoclaved at 121°C for 20 min and 100 ml of chitosan was added to the PDB (100 ml) in 250 ml Erlenmeyer flasks. Three mycelial discs (5 mm) from

a 10 days old culture were added to each flask and incubated at room temperature for 14 days. Pre-weighed Whatman no. 1 filter paper was used to filter mycelium and dried in an oven at 100°C for 24 h, and then weighed. The dry weight of mycelium was calculated by the following formula:

$$\text{Dry weight of mycelium} = \frac{\text{Biomass of control sample} - \text{Biomass of treated sample}}{\text{Biomass of control sample}}$$

3.2.5.4 Viability of spores

Viability of spores was measured using growth media containing chitosan as described above (Section 3.2.5.3). A spore suspension of *C. gloeosporioides* (adjusted to 200 spores ml^{-1}) was prepared using 10 days old culture plates incorporated with different concentrations of conventional chitosan along with submicron chitosan dispersions of different droplet sizes. 1 ml of suspension was added to PDA dishes and incubated at room temperature for 4 days. After incubation, the spore population density in each treatment was measured as a percent of sporulation using the formula described by Al-Hetar *et al*. (2010).

$$\% \text{ Sporulation} = \frac{\text{Number of spores in treated sample}}{\text{Number of spores in control sample}} \times 100$$

3.3 Methods for the preparation of submicron chitosan dispersions

Submicron chitosan dispersions were prepared using a new nano emulsifying alcohol free system. Chitosan was dissolved separately in 0.5% (v/v) glacial acetic acid (Sigma-Aldrich, USA) and was used as an oil phase. The emulsifiers, Brij 56 (polyethylene glycol hexadecyl ether) and Span 20 (sorbitan monolaurate), were mixed with each other in a ratio of 1:1. The

chitosan solution and emulsifier mixture (4:1) were mixed together and subjected to ultrasonication in an ultrasound water bath (Model: Bandelin Sonorex, Germany) at 25 °C. Subjecting to ultrasound for different time periods, submicron chitosan dispersions with droplet sizes of 200, 400, 600, 800 and 1000 nm were obtained. The droplet size was measured using a dynamic light scattering (DLS) technique [Zetasizer NanoZS, Malvern, UK; utilising an argon laser (λ=633 nm) at a scattering angle of 173°] (Jafari *et al*., 2007).

Before using Brij 56 and Span 20, a few other emulsifiers at different temperatures and sonication times were also tested for the preparation of submicron dispersions (Appendix A 3.1).

3.3.1 Physicochemical analysis of submicron chitosan dispersions

3.3.1.1 Zeta potential and stability determination

The zeta potential values of submicron chitosan dispersions were measured using a Zetasizer (Freitas and Müller, 1998). The stability of submicron chitosan dispersions was determined by measuring the size of the droplets in nm at 25°C and was expressed in days.

3.3.1.2 Measurement of viscosity

Viscosity of submicron chitosan dispersions was measured using a Brookfield Viscometer (Brookfield LVDV-I Prime, Brookfield Engineering Laboratory Inc., USA) equipped with different spindles. The operating principle of the viscometer is to drive the spindle through the calibrated spring

immersed in the testing liquid. By using the spindle No. 62 and 250 ml of the liquid in a glass beaker of 400 ml, the viscosity was measured and the data was expressed in centipoises (cp) in a range that a torque required to rotate the spindle is 10% or more by reacting at 100 rpm at 25°C for 1 minute.

3.3.1.3 Determination of pH

The pH of submicron chitosan dispersions was determined using a pH meter (Model: Cyber Scan pH 510, Eutech Instruments, Singapore).

3.3.1.4 Release of chitosan from submicron dispersions

The release of chitosan from submicron dispersions was measured using the method described by Nitschke *et al*. (2011) with slight modifications. Briefly, samples (3 ml) were dissolved in 100 ml of 1M NaOH solution. The mixture was boiled for two hours at 100°C in a water bath with continuous agitation. The mixture was filtered and the residues collected were dissolved in 20 ml of 10% acetic acid. The mixture was again boiled for one hour in a water bath. This mixture served as the standard solution for chitosan determination. A 2 µl sample of the standard solution was dyed with 1 µl of 1.0% lugol's solution and the absorbance was determined at 450 nm using a UV-Vis spectrophotometer (Varioskan Flash Multimode Reader, Thermo Fisher Scientific, USA) for 3 min. A standard curve was prepared using 0.5 to 5 ml of chitosan solution (Appendix B 3.2).

3.4 Statistical analyses

A completely randomised design (CRD) with factorial combination was used for all the experiments and the data were subjected to analysis of variance (ANOVA) using statistical analysis system SAS® version 9.1. Duncan's multiple range test (DMRT) was used for comparing treatment means and statistical significance was assessed at $P < 0.05$ (Appendix C3.1-3.9). Four replicates of twenty units for each treatment were used in all the parameters tested and all the experiments were repeated twice. The differences between repeated measures were tested using the UNIVARIATE procedure in SAS. Variations among different sets of experiments were negligible and the treatment effect was similar, so the data was pooled before analysis.

3.5 Results and discussion

3.5.1 Isolation and identification of *C. gloeosporioides* from dragon fruit plants

The fungus was isolated from anthracnose lesions present on dragon fruit plants and fruit. The isolates grown on PDA medium at 28 ± 2°C for 10 days were identified on the basis of previous studies (Masyahit *et al*., 2009 & 2008) (Fig. 3.1a, b). The fungus had whitish-orange colonies (Fig. 3.1c), with acervuli having conidiogenous cells bearing capsule shaped conidia (Fig. 3.1 d). This pathogenic fungus was identified as *C. gloeosporioides* (Penz.) Penz. & Sacc. (Sutton, 1992). Its morphology was the same as that described previously by Masyahit *et al*. (2009). This demonstrated that *C. gloeosporioides* is the causal agent of anthracnose in dragon fruit plants and is the most common fungus causing latent infections in dragon fruit during postharvest storage.

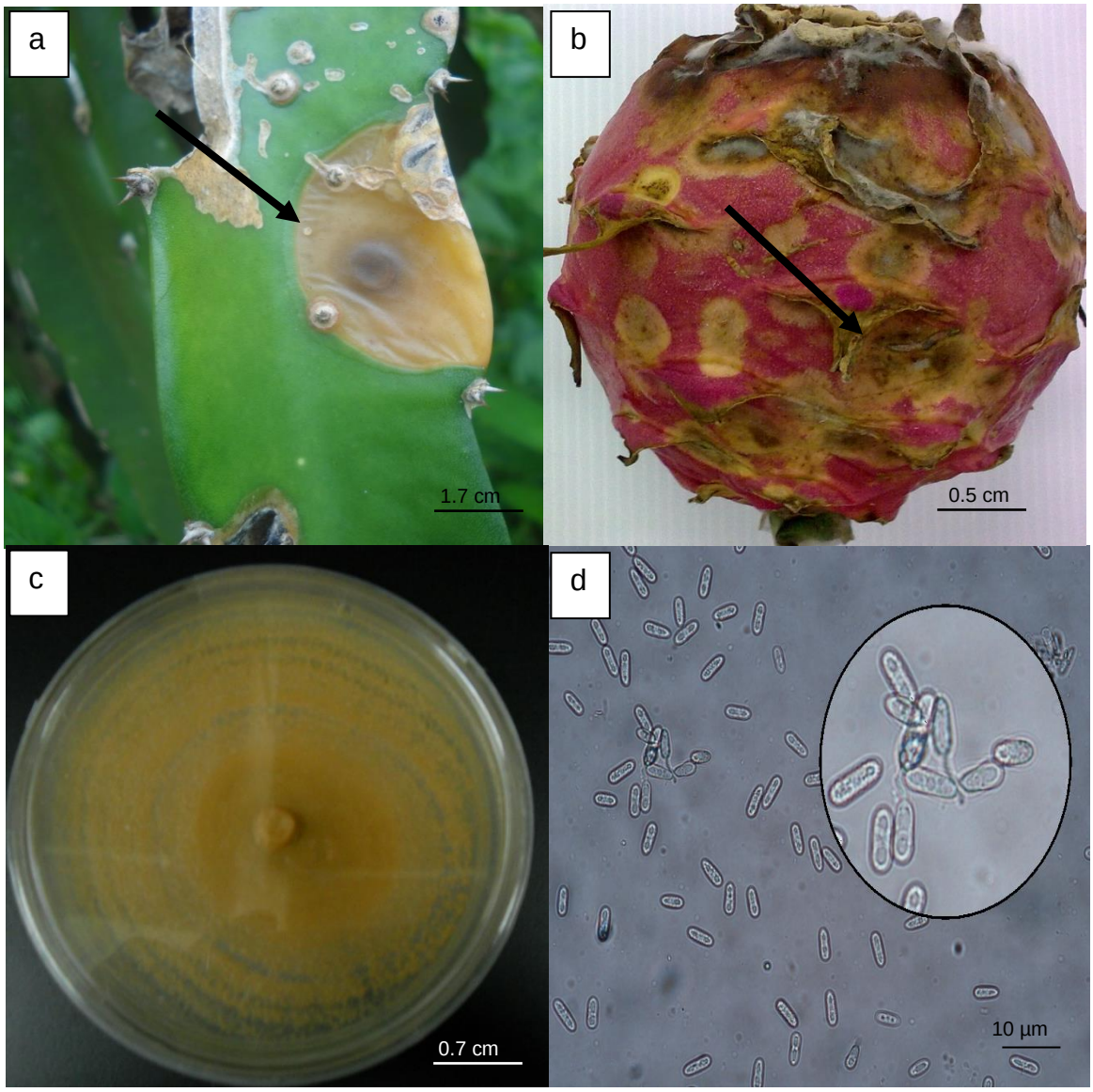


Figure 3.1: Symptoms of anthracnose on dragon fruit: a) Arrow showing anthracnose lesion on shoot, b) Arrow showing lesion on fruit, c) Pure culture of *C. gloeosporioides* and d) Conidia.

3.5.2 Pathogenicity test

The anthracnose symptoms produced on inoculated stems were similar to those found in natural field conditions. Typical symptoms appeared on stems 3 days after inoculation (Fig. 3.2 a) and the inoculated stem showed severe rotting after 10 days (Fig. 3.2 b).

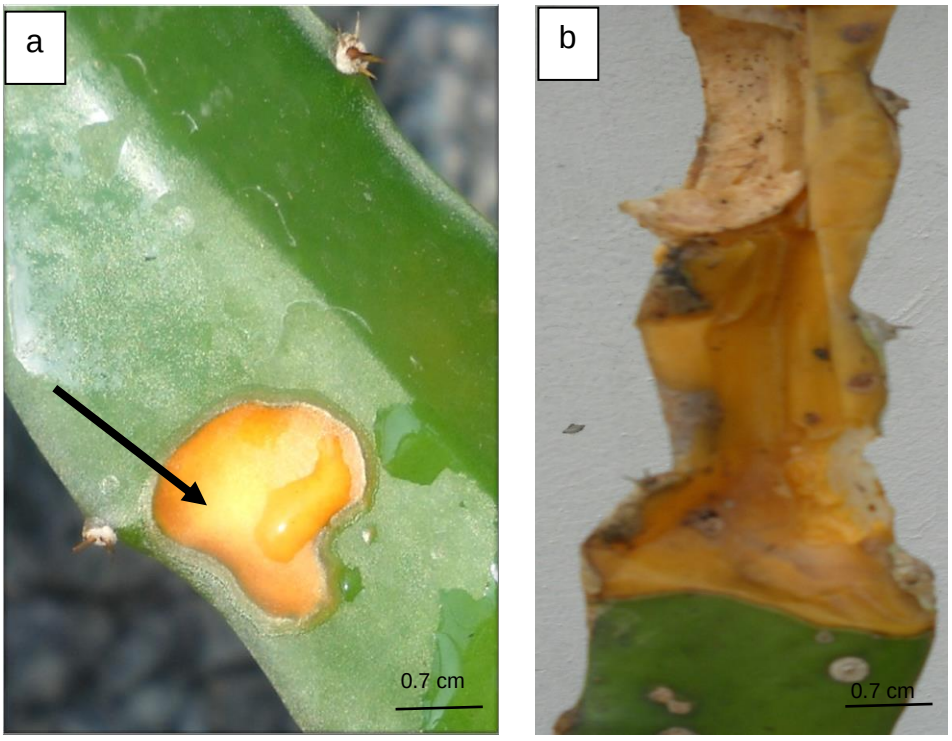


Figure 3.2: Pathogenicity test showing lesion on shoot a) 3 days and b) 10 days after inoculation.

Similar results were reported by Masyahit *et al*. (2009) where they observed necrotic lesions with yellowish brown centres which later coalesced to an extensive rot. *C. gloeosporioides* was also found to be the causal organism of anthracnose in white fleshed dragon fruit plants in Florida, USA (Palmateer *et al*., 2007) and in Taiwan (Taba *et al*., 2006). In Brazil, *C. gloeosporioides* was found to be the causal organism of anthracnose in yellow genotypes of dragon fruit plants (Takahashi *et al*., 2008).

In the present study, the fungus developed some water soaked lesions on the fruit by the second day after inoculation, which increased in size by day 5 (Fig. 3.3a, b).

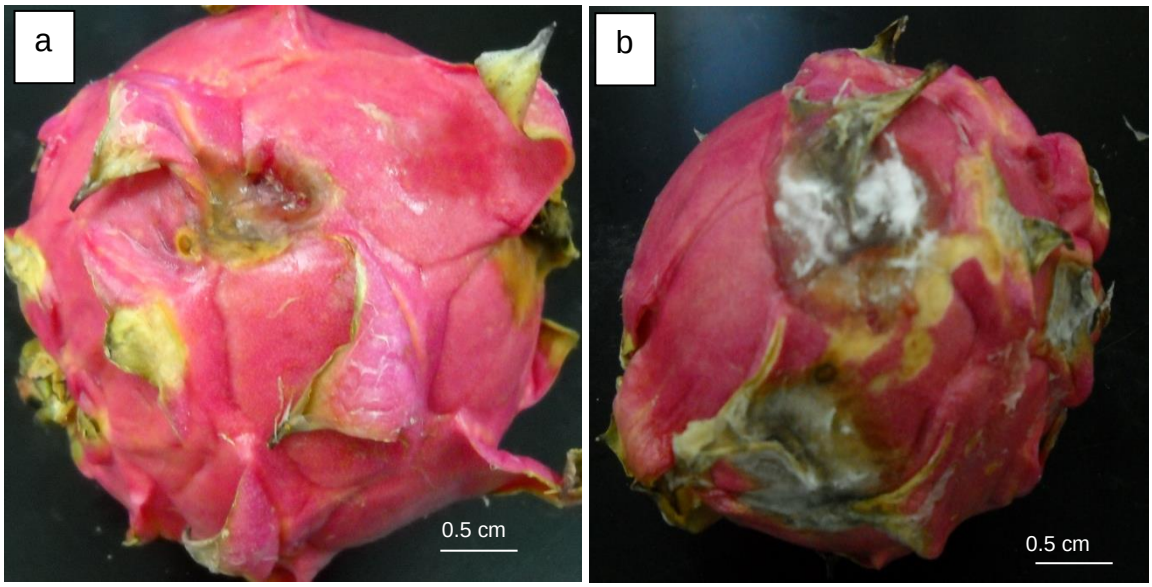


Figure 3.3: Pathogenicity test showing lesions on fruit a) 2 days and b) 5 days after inoculation.

This evidence that *C. gloeosporioides* is the causal organism of anthracnose in dragon fruit is in agreement with previous reports (Awang *et al*., 2011; Awang *et al*., 2010; Masyahit *et al*., 2009). Awang *et al*. (2010) reported that latent infections occur in the field on immature fruit but that the pathogen remains quiescent until the postharvest stage. The symptoms occur on the fruit surface as brown to black sunken spots with white to orange masses of conidia (Sijam *et al*., 2008).

3.5.3 Effect of chitosan on radial mycelial growth of *C. gloeosporioides*

Inhibition of radial mycelial growth of *C. gloeosporioides* was observed after 10 days of incubation at 28 ± 2°C using different types of chitosan (locally prepared, low molecular weight, medium molecular weight and high

molecular weight). The growth was decreased significantly ($P < 0.05$) by the different types of chitosan at all concentrations tested as compared to the control (Fig. 3.4). The maximum percentage inhibition in growth was observed with 2.0% low molecular weight chitosan followed by 2.0% locally prepared chitosan, respectively. Medium and high molecular weight chitosans also inhibited the radial mycelial growth of *C. gloeosporioides* but with lower efficacy.

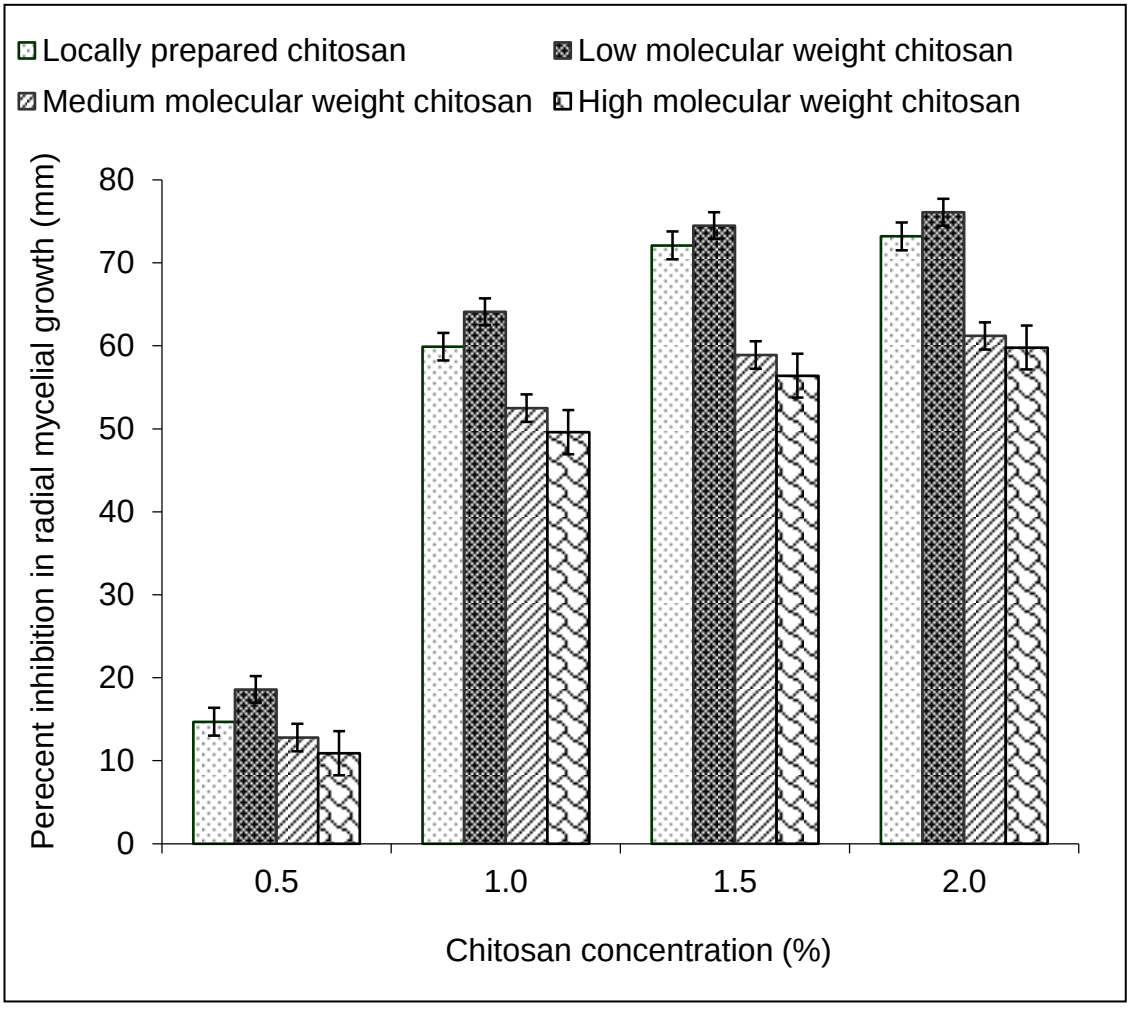


Figure 3.4: Effect of concentration and type of chitosan on radial mycelial growth after 10 days of incubation. The vertical bars represent the standard error of means for four replicates.

Similar results were obtained when chitosan was used to inhibit the radial mycelial growth of *Rhizopus stolonifer* (El Ghaouth *et al*., 1992) and various

fungal species (Hirano and Nagao, 1989). It is believed that the antifungal activity of chitosan varies with the degree of deacetylation, molecular weight, concentration and pH of the solvents (Junang *et al*., 2009; Guo *et al*., 2006; Sekiguchi *et al*., 1994). In a recent study by Hernández-Lauzardo *et al.* (2008), the inhibition of mycelial growth of *R. stolonifer* was more in low molecular weight chitosan as compared with high molecular weight chitosan, while the high molecular weight chitosan affected sporulation more than low and medium molecular weight chitosan. In an earlier study it was shown that a lower level of deacetylation would enhance the antifungal property of chitosan (Stössel and Leuba, 1984). In addition, the intensity of action of chitosan on fungal cell walls depends on the concentration and pH of the chitosan solution (Stössel and Leuba, 1984).

3.5.4 Effect of chitosan on conidial germination of *C. gloeosporioides*

The germination of *C. gloeosporioides* conidia was significantly ($P < 0.05$) inhibited by all types of chitosan tested at all concentrations as compared to the control (Fig. 3.5). The maximum percentage inhibition in conidial germination was observed with 2.0% low molecular weight chitosan followed by 2.0% locally prepared chitosan. Medium and high molecular weight chitosans also inhibited the conidial germination of *C. gloeosporioides* but with lower efficacies.

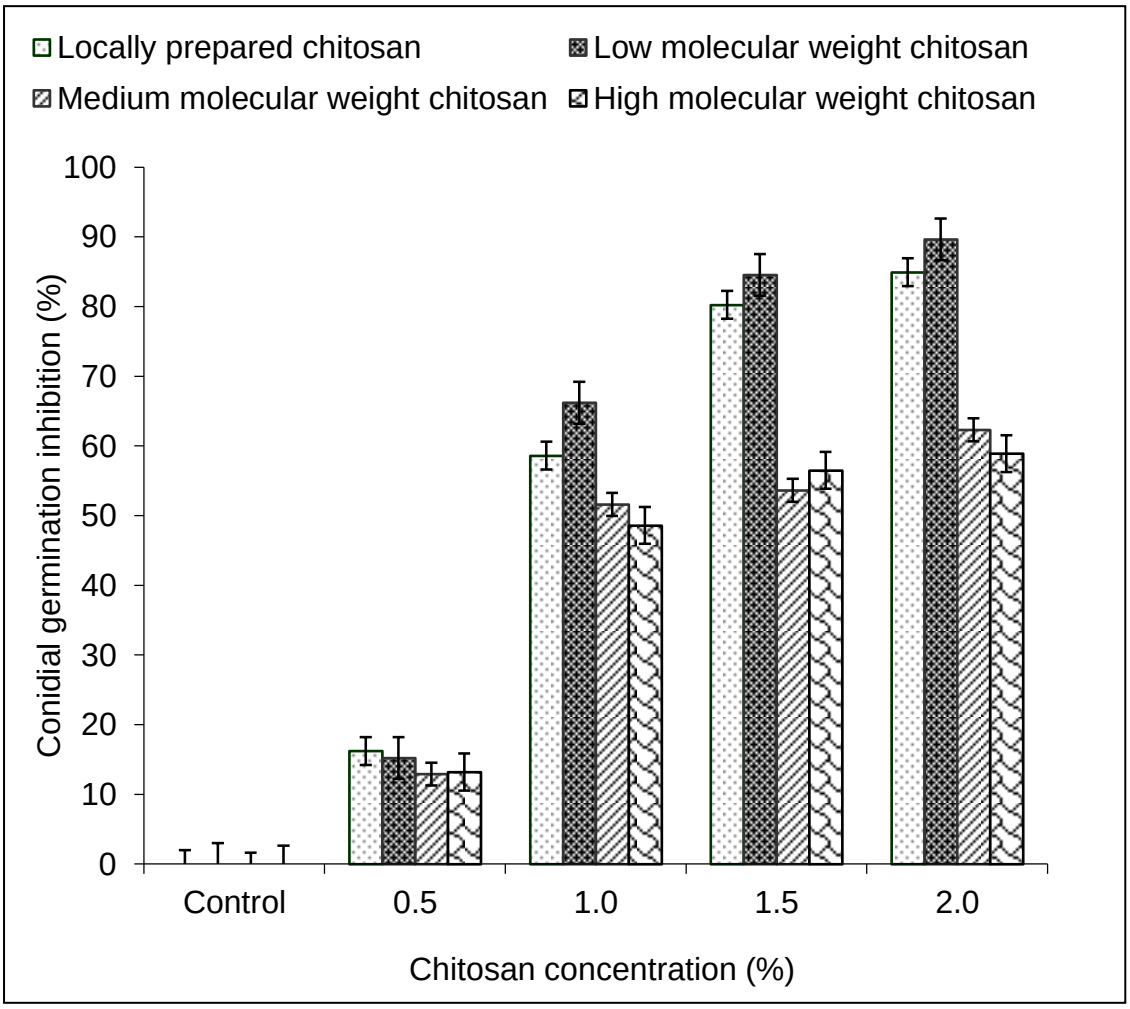


Figure 3.5: Effect of concentration and type of chitosan on conidial germination inhibition (%) of *C. gloeosporioides*. The vertical bars represent the standard error of means for four replicates.

Chitosan concentrations are directly proportional to the conidial germination inhibition (Al-Hetar *et al*., 2010). Studies conducted on cultures of *R. solani* and *S. rolfsii* revealed that the percentage germination of fungal spores decreased with increased concentration of chitosan in the medium and also with the degree of deacetylation. Zhang *et al.* (2003) reported 80% inhibition of conidial germination for *Phomopsis asparagi* and 95% for *Fusarium oxysporum, Rhizoctonia solani* and *Cucumerinum owen* with the decrease in molecular weight and degree of deacetylation of chitosan.The

degree of deacetylation is a basic factor in solubility and charge development of chitosan, where the dominating reactive sites are NH_2 and OH groups. Hence, as the degree of deacetylation is reduced, more free amino groups present in chitosan lead to higher antimicrobial activity (Andres *et al*., 2007). The antifungal mechanism of chitosan involves cell wall morphogenesis with chitosan molecules interfering directly with fungal growth (El Ghaouth *et al*., 1992).

3.5.5 Effect of different types of chitosan on dry weight of mycelium and spore viability of *C. gloeosporioides*

The dry weight of mycelium of *C. gloeosporioides* was significantly ($P < 0.05$) reduced by all types and concentrations of chitosan as compared to the control (Fig. 3.6). The minimum dry weight of mycelium was observed in Petri dishes treated with 2.0% low molecular weight chitosan followed by 2.0% locally prepared chitosan (0.94 g and 0.98 g), respectively. In contrast, medium and high molecular weight chitosans resulted in lower dry weight of mycelium but with lower efficacy.

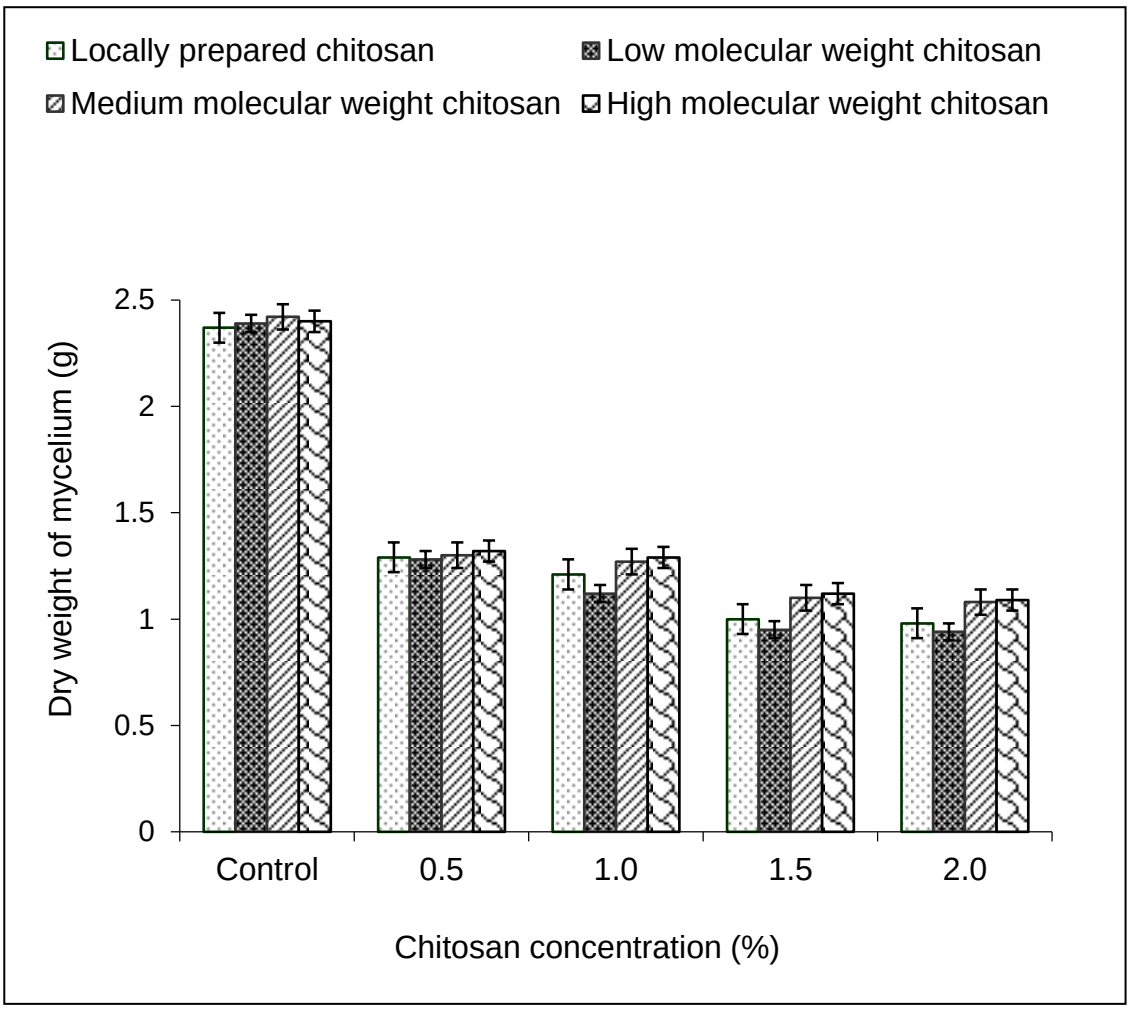


Figure 3.6: Effect of concentration and type of chitosan on dry weight (g) of mycelium of *C. gloeosporioides*. The vertical bars represent the standard error of means for four replicates.

The spore viability of *C. gloeosporioides* was significantly ($P < 0.05$) reduced by all types and concentrations of chitosan as compared to the control (Fig. 3.7). The highest loss in viability was observed in Petri dishes treated with 1.5% and 2.0% low molecular weight chitosan and locally prepared chitosan. Medium and high molecular weight chitosans also showed inhibition in spore viability but with lower efficacies.

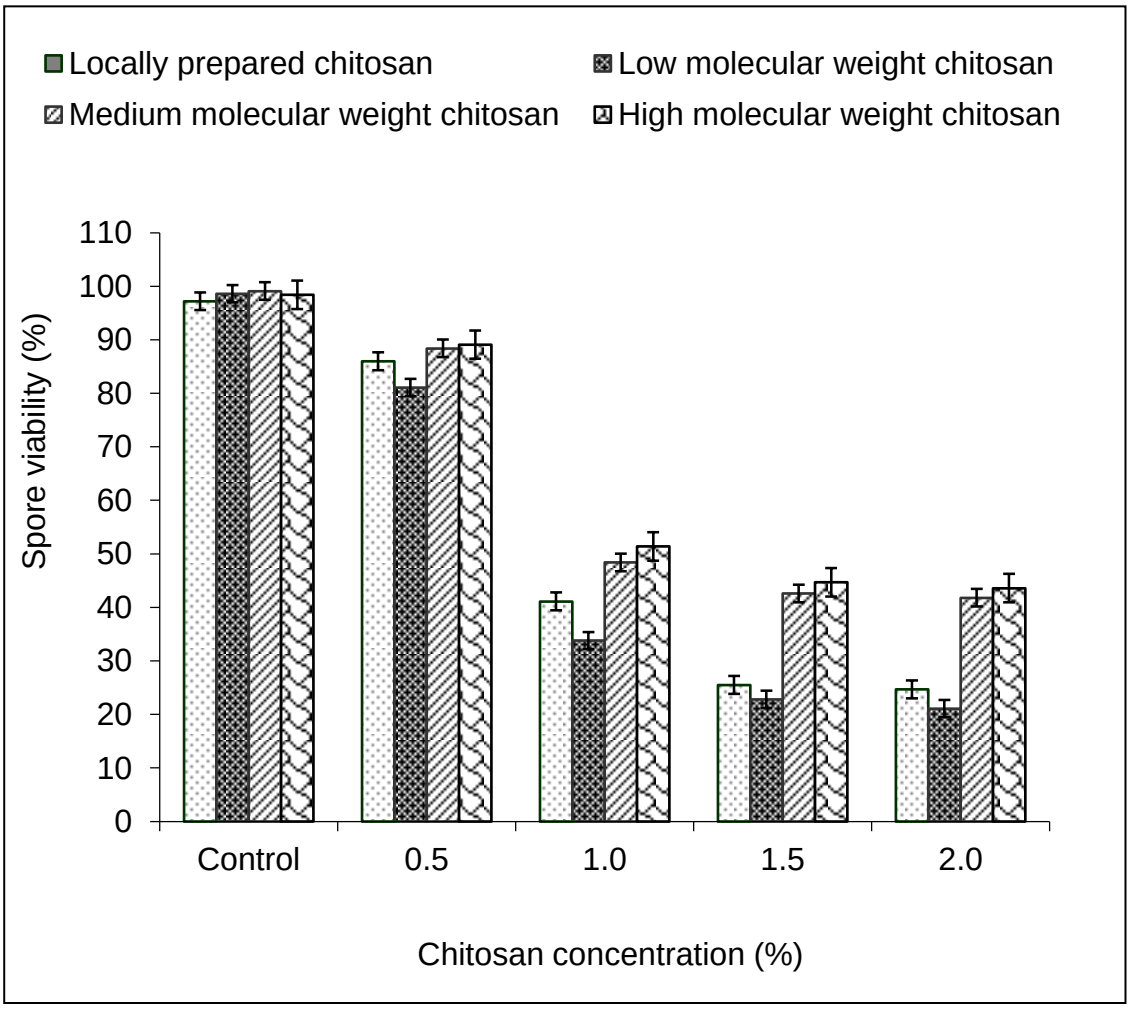


Figure 3.7: Effect of concentration and type of chitosan on spore viability (%) of *C. gloeosporioides*. The vertical bars represent the standard error of means for four replicates.

Chitosan affected the dry weight of the mycelium and sporulation, demonstrating its effects on various developmental stages of *C. gloeosporioides*. As discussed earlier, molecular weight and degree of deacetylation strongly affect antimicrobial activity of chitosan, with varied effect based on the growth stage of the microorganism (Andrew, 2001). Generally, viability of spores has been reduced very effectively by chitosan (Sashai and Manocha, 1993; El Ghaouth *et al*., 1992). However, chitosan has the potential to induce regulatory changes in fungi, but it is presumed to

be fungistatic rather than fungicidal (Raafat *et al*., 2008; El Ghaouth *et al*., 1992).

After an initial screening experiment, two types of chitosan (low molecular weight and locally prepared) were selected to prepare submicron dispersions and for further *in vitro* evaluation against *C. gloeosporioides* isolated from dragon fruit plants.

3.6 Formation of submicron chitosan dispersions

To obtain smaller droplets, a longer period of sonication was applied. Similarly, with an increase in chitosan concentration, a longer time was required for sonication (Table 3.1). In contrast, to achieve larger droplets at low chitosan concentration required less time of sonication. Locally prepared chitosan required a little more time for sonication as compared to low molecular weight chitosan for all of the concentrations and droplet sizes.

The results regarding formation of submicron chitosan dispersions could be explained by the increased sonication time also increasing the energy input into the system, which resulted in a decrease of the droplet size (Jafari *et al*., 2007). It has previously been reported that the time of sonication required to prepare submicron dispersions varies with the type and concentration of the material (Lobo and Svereika, 2003; Karbstein and Schubert, 1995).

Table 3.1: Time of sonication (min) to obtain different droplet sizes at various concentrations of chitosan.

Type of chitosan	Chitosan concentration (%)	Droplet size (nm)				
		200	400	600	800	1000
		Time of sonication (min)				
Low molecular weight	0.5	20.4 g	16.9 ij	13.6 k	11.0 m	8.4 n
	1.0	25.9 de	23.2 f	18.9 h	16.4 j	12.2 l
	1.5	30.1 bc	25.6 de	22.1 fg	18.1 h	15.5 k
	2.0	35.2 a	30.4 bc	24.5 e	20.2 g	18.5 h
Locally prepared	0.5	21.3 g	17.2 i	14.7 jk	12.4 l	13.3 k
	1.0	27.8 de	25.7 de	22.0 fg	17.8 i	16.0 j
	1.5	32.5 b	28.7 c	25.2 de	21.1 g	18.4 h
	2.0	36.4 a	32.1 b	28.5 c	23.0 f	21.4 g

Means with different letters are significantly different using DMRT test ($P < 0.05$).

3.6.1 Physicochemical properties of submicron chitosan dispersions

3.6.1.1 Zeta potential

With an increase in the concentration of chitosan tested, the value of zeta potential also increased significantly ($P < 0.05$) (Table 3.2; Appendix C 3.6). The zeta potential was highly dependent on the energy input in terms of time of sonication, however, the values for low molecular weight chitosan droplets were higher than those for locally prepared chitosan. The zeta potential value decreased with an increase in droplet size for both of the chitosans tested. The values also increased with the increase in sonication time. The zeta potential value of 200 nm droplets of 0.5% chitosan was lower than the same droplet size of 2.0% chitosan. The larger droplet sizes had low zeta potential values due to low energy input. 1000 nm droplets of 2.0% chitosan had higher zeta potential as compared to 200 nm droplets of 0.5% chitosan. The locally prepared chitosan showed slightly lower values of zeta potential. The difference in zeta potential values for low molecular weight chitosan and locally prepared chitosan were probably due to the effect of molecular weight (Rusu-Balaita *et al*., 2003; Rinaudo *et al*., 1999).

Zeta potential is a parameter characterizing electrochemical equilibrium on interfaces (McNaught and Wilkinson, 1997), which depends on the properties of the liquid and surfactant. Electrostatic repulsion between droplets increases with an increase in the value of zeta potential (Lagaly, 1984). In the present study, the increase in zeta potential with the decrease in droplet size could be ascribed to increased energy input from sonication (Riddick, 1968).

Table 3.2: Zeta potential (mV) of different droplet sizes and various concentrations of chitosan.

Type of chitosan	Chitosan concentration (%)	Droplet size (nm)				
		200	400	600	800	1000
		Zeta potential (mV)				
Low molecular weight	0.5	20.1 e	17.0 i	15.2 l	15.9 k	14.8 m
	1.0	21.3 d	19.1 ef	17.5 h	15.3 kl	16.6 j
	1.5	23.2 bc	22.3 c	22.7 c	21.1 d	18.2 gh
	2.0	25.5 a	24.4 b	24.1 b	21.2 d	22.1 c
Locally prepared	0.5	16.7 j	14.3 m	13.9 n	12.2 o	12.6 no
	1.0	17.1 i	15.3 l	14.9 m	15.8 k	14.1 m
	1.5	19.3 f	18.8 g	16.2 j	16.5 j	16.2 j
	2.0	21.2 d	20.7 d	18.8 g	17.4 i	18.5 gh

Means with different letters are significantly different using DMRT test ($P < 0.05$).

3.6.1.2 Stability

The stability of dispersions decreased with increase in droplet size for both of the chitosans tested (Table 3.3) and also depended on the concentration of the chitosan. Stability of the low molecular weight submicron chitosan dispersions was higher than that of the locally prepared chitosan, i.e. 6.0 and 5.0 days for 200 nm droplets of 0.5% low molecular weight and locally prepared chitosan, respectively. However, stability decreased with increase in concentration.

Stability of the submicron dispersions was directly proportional to the zeta potential value (Fig. 3.8). The equation shows that each unit increase in zeta potential resulted in 2.3546 units increase in stability of low molecular weight chitosan (Fig. 3.8 a). For locally prepared chitosan, every unit increase in zeta potential resulted in 1.586s unit increase in stability (Fig. 3.8 b).This is in agreement with the previous findings of Riddick (1968), where a reduction in physical stability was observed with a reduction in zeta potential. It reflects that electrical potential of submicron dispersions is influenced by dispersion medium and its composition. Dispersions with high zeta potential retain their stablity in suspension, as the similar charges on the surface of dispersions prevent accumulation of the particles (Rajendran *et al*., 2010).

The difference in stability of different types of submicron chitosan dispersions mainly depends on the degree of deacetylation (Manufacturer provided the details of deactylation; written on packaging). Chitosan with less deacetylation possesses more positive charge on its surface, such that the similar charges resist the formation of aggregates (Jansson, 2010).

Table 3.3: Stability (days) of droplet sizes at various concentrations of chitosan.

Type of chitosan	Chitosan concentration (%)	Droplet size (nm)				
		200	400	600	800	1000
		Stability (days)				
Low molecular weight	0.5	6.0 b	5.0 c	5.0 c	5.0 c	4.0 d
	1.0	6.0 b	6.0 b	5.0 c	5.0 c	4.0 d
	1.5	7.0 a	6.0 b	6.0 b	6.0 b	5.0 c
	2.0	7.0 a	7.0 a	7.0 a	6.0 b	6.0 b
Locally prepared	0.5	5.0 c	5.0 c	4.0 d	4.0 d	4.0 d
	1.0	5.0 c	5.0 c	4.0 d	4.0 d	4.0 d
	1.5	6.0 b	5.0 c	5.0 c	5.0 c	5.0 c
	2.0	6.0 b	6.0 b	5.0 c	5.0 c	5.0 c

Means with different letters are significantly different using DMRT test ($P < 0.05$).

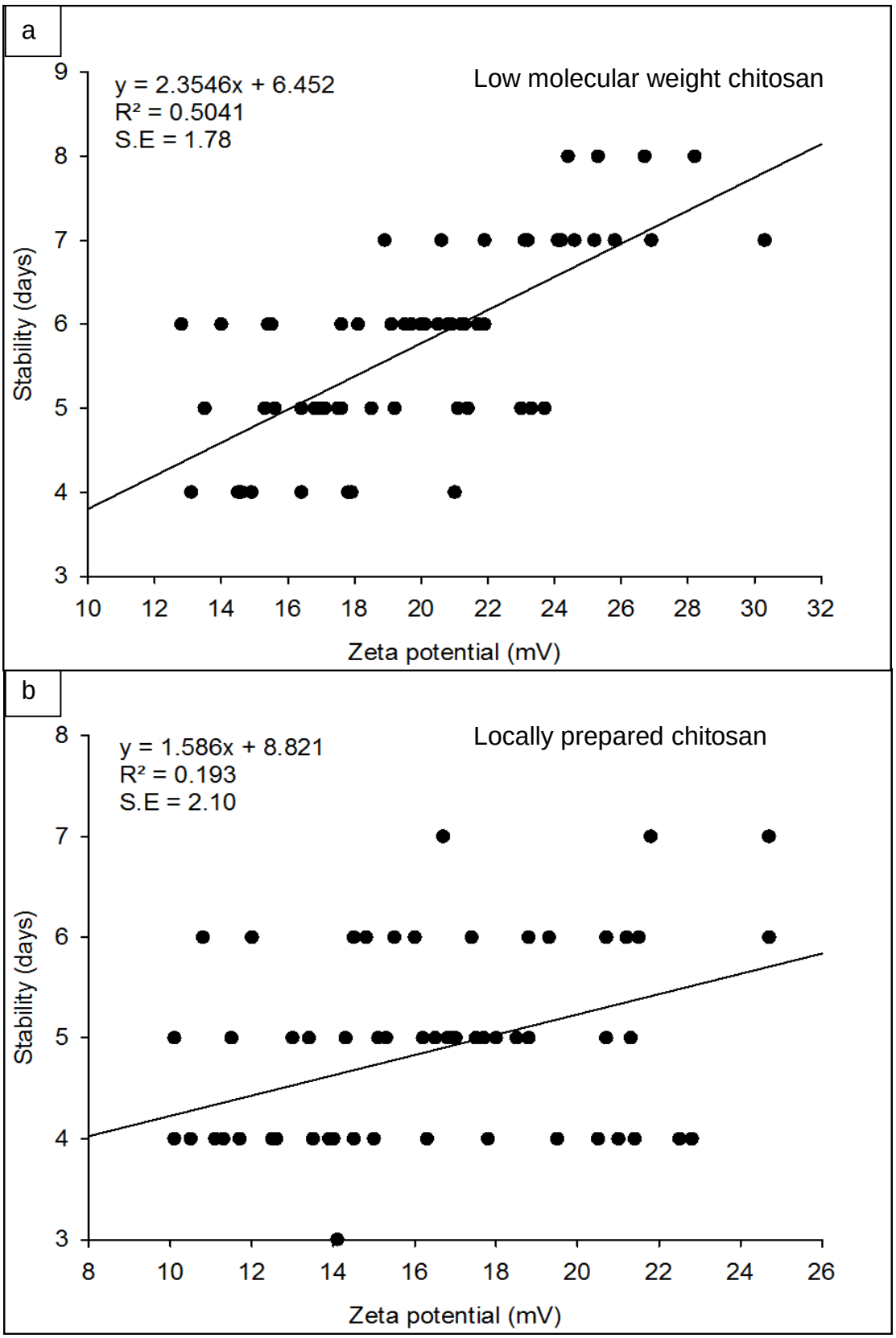


Figure 3.8: Relationship between zeta potential and stability of submicron chitosan dispersions of a) low molecular weight chitosan and b) locally prepared chitosan at $P < 0.05$.

3.6.1.3 Viscosity and pH

The viscosity of the dispersions also increased with increase in the concentration of chitosan and in droplet size for both of the chitosans tested (Table 3.4). The solutions prepared with locally prepared chitosan had higher viscosity than the low molecular weight chitosan solutions. The increase in viscocity with the increase in droplet size and chitosan concentration could be ascribed to the greater time of sonication and therefore the greater energy input required to obtain smaller submicron dispersion droplets. Increased sonication time leads to higher temperature of the submicron dispersion, thus reducing the viscosity of the solution. The temperature was monitored using a thermometer and constantly maintained at 25 °C by adding water in the sonicator as it is believed that temperature is the most important factor which reduces the viscosity and interfacial tension of dispersions (McClements, 2005). Schuhmann (1995) also reported that higher temperatures caused the destabilisation of droplets and decreased the viscosity.

There was no effect on the pH of submicron chitosan dispersions after formulation of dispersions and the pH at 5.6.

Table 3.4: Viscosity (cp) of different droplet sizes and various concentrations of chitosan.

Type of chitosan	Chitosan concentration (%)	Droplet size (nm)				
		200	400	600	800	1000
		Viscosity (cp)				
Low molecular weight	0.5	12.5 w	19.6 w	24.4 v	36.3 u	42.2 tu
	1.0	17.6 w	22.8 v	48.4 t	69.1 s	75.1 r
	1.5	204.4 o	323.4 n	428.6 m	546.2 k	728.3 g
	2.0	541.5 k	582.7 j	756.3 g	1092.5 e	1462.4 c
Locally prepared	0.5	38.2 u	53.1 t	64.2 s	92.9 qr	119.1 q
	1.0	49.1 t	72.8 r	89.4 r	118.4 q	156.5 p
	1.5	324.6 n	510.9 kl	614.5 i	782.7 g	1096.6 e
	2.0	645.5 h	894.7 f	1292.2 d	1564.1 b	1783.7 a

Means with different letters are significantly different using DMRT test ($P < 0.05$).

3.6.1.4 Release of chitosan from submicron dispersions

The release of chitosan from submicron dispersions decreased with increase in the concentration of chitosan and in the droplet size for both of the types of chitosan tested (Table 3.5). The solutions prepared with locally prepared chitosan showed lower release of chitosan as compared to the low molecular weight chitosan solutions. The results of this study showed that the release of chitosan is related to the chitosan concentration, droplet size and molecular weight of chitosan (Hejazi and Amiji, 2003). The release of chitosan from submicron dispersion at lower concentrations was faster. This could be attributed to the network of emulsifier and chitosan at lower concentration containing a large volume of free moving liquid molecules, which results in quick release of active ingredient from the emulsifiers (Desai and Park, 2005). The molecular weight of chitosan also affects the release of chitosan from the matrix of emulsifier. Chitosan of higher molecular weight has higher chain relaxation ability which results in an increase in the polymer chain entanglement of chitosan and emulsifiers (Agnihotri *et al*., 2004). Therefore, variation in the molecular weight of chitosan results in different release profiles.

Table 3.5: Release of chitosan (mg ml^{-1}) from submicron chitosan dispersions at various droplet sizes and chitosan concentrations.

Type of chitosan	Chitosan concentration (%)	Droplet size (nm)				
		200	400	600	800	1000
		Chitosan release (mg ml^{-1})				
Low molecular weight	0.5	2.11 ab	2.07 ab	1.54 d	1.28 ef	1.08 hij
	1.0	1.12 gh	1.10 ghi	1.22 ef	1.037 g-k	0.962 jkl
	1.5	0.657 jkl	0.717 lm	0.807 klm	0.78 lm	0.64 lm
	2.0	0.55 m	0.47 m	0.49 m	0.577 m	0.557 m
Locally prepared	0.5	2.32 a	2.18 a	2.31 a	1.84 bc	1.82 bc
	1.0	1.26 ef	1.29 ab	1.28 ef	1.36 e	1.18 fg
	1.5	1.14 gh	1.16 g	1.07 ghi	1.035 g-k	1.04 g-k
	2.0	1.04 g-k	1.064 ghi	0.987 jkl	0.687 klm	0.695 lm

Means with different letters are significantly different using DMRT test ($P < 0.05$).

This study showed that the antimicrobial properties of chitosan depend on the degree of deacetylation and molecular weight of the chitosan. Chitosan with low molecular weight showed better antifungal activity than chitosan with medium or high molecular weight. Similar to the antifungal effects, the properties of submicron chitosan dispersions were also highly dependent on the molecular weight of chitosan. Further studies are required to investigate the antifungal effects of submicron chitosan dispersions and also to elucidate the mechanism behind their antimicrobial activities.

CHAPTER 4

PREHARVEST ANTIFUNGAL EFFECTS OF SUBMICRON CHITOSAN DISPERSIONS ON DRAGON FRUIT PLANTS

4.1 Introduction

Colletotrichum is one of the most important pathogens on crops, causing the serious disease anthracnose on numerous vegetables, legumes, cereals and fruits (Bailey and Jeger, 1992). Dragon fruit is an important fruit crop affected by anthracnose with yield losses of 20-80% (Kays, 1997).

This fungus enters plants through natural openings, by mechanical force or wounds on the surface of plants (Chau, 1981; Van den Ende and Linskens, 1974). It is hemibiotrophic (Wijesundera *et al*., 1989) and takes nutrients from dead and living cells. It produces several types of cell wall degrading enzymes which play a major role in the infection process and development of symptoms (Wijesundera *et al*., 1984). These enzymes help the fungal hyphae penetrate the mechanical barrier of the plant cell wall (Köller *et al*., 1982) resulting in death and maceration of infected tissues (Fernando *et al*., 2001).

Cultural practices, such as crop rotation, enhancement of soil quality, water management, growing of resistant varieties, field sanitation, habitat management and chemical fungicides, are commonly used to control the disease (Waller, 1992). Cultural or chemical control strategies have not been very effective against this disease (Backman, 1997).

The use of natural compounds, such as chitosan, for the control of anthracnose is a novel approach. Chitosan is well known for its versatile antifungal, biodegradable, nontoxic and biocompatible properties (Kurita,

1998). However, some drawbacks, such as its high viscosity, have been reported (Tikhonov *et al*., 2006). Chitosan in the form of submicron dispersions, which are low in viscosity and penetrate tissues more rapidly, is a novel approach to control anthracnose of dragon fruit plants. To our knowledge no studies have been conducted on the antifungal activities of submicron chitosan dispersions.

Therefore, the main objectives of the present study were:

1. To test the *in vitro* efficacy of submicron chitosan dispersions and emulsifiers against *C. gloeosporioides*.
2. To evaluate the efficacy of submicron chitosan dispersions against the cell wall degrading enzymes produced by *C. gloeosporioides*.
3. To determine the *in vivo* antifungal activity of submicron chitosan dispersions against anthracnose.

4.2 Materials and methods

Four concentrations (0.5, 1.0, 1.5 and 2.0%) of chitosan were used to prepare submicron dispersions with droplet sizes of 200, 400, 600, 800 and 1000 nm, as described in Section 3.3. Two types of chitosan (low molecular weight chitosan and locally prepared chitosan) were used for the *in vitro* antifungal assay of submicron chitosan dispersions and emulsifiers (Brij 56 and Span 20), alone and in combination.

4.2.1 Inhibition in radial mycelial growth

The *in vitro* antifungal activity of submicron chitosan dispersions was determined by the food poison technique described in Section 3.2.5.1.

4.2.2 Conidial germination inhibition test of *C. gloeosporioides*

The inhibition of conidial germination by submicron chitosan dispersions was determined by the cavity slide technique described in Section 3.2.5.2.

4.2.3 Dry weight of mycelium and viability of spores

Dry mycelial weight and viability of spores were determined as described in Sections 3.2.5.3 and 3.2.5.4, respectively.

The conidial morphology was also observed using a camera attached to a light microscope (Nikon Eclipse 80i) at 40× magnification.

4.3 Methods for *in vitro* extraction of hydrolytic enzymes by *C. gloeosporioides*

4.3.1 Extraction of cellulolytic enzymes

Fifty ml of Czapek's broth (Sigma Aldrich) containing 0.5% carboxy methyl cellulose (w/v) (acting as a sole carbon source) with submicron dispersions of chitosan at 0.5, 1.0, 1.5 and 2.0% was added to 250 ml Erlenmeyer flasks. The flasks were inoculated with 9 mm mycelial disks from ten days old cultures of *C. gloeosporioides* and incubated for 10 days at room temperature. The contents of the flasks were filtered after 10 days using Whatman filter paper number 1. The 150 ml of filtrate was mixed with 350 ml of 0.1 M potassium phosphate buffer pH 6.8 followed by centrifugation at 5000 x *g* for 20 minutes at 4°C. The supernatant was lyophilized and served as the source for enzyme assays (Fischer *et al*., 1995).

4.3.1.1 *β*-galactosidase

β-galactosidase activity was determined by hydrolysis of the chromogenic substance, *O*-nitrophenyl-*β*-$_D$-galactoside (ONGP) as a substrate (Byrde and Fielding, 1968). An extract of 100 µl was mixed with 0.9 ml of Z-buffer and 2 ml of breaking buffer. The mixture was incubated in a water bath at 28 °C for 5 minutes. ONGP (0.2 ml) was added and the mixture incubated again at 28°C in a water bath. The reaction was stopped after 30 minutes by adding 0.5 ml of Na_2CO_3. The hydrolysis was monitored spectrophotometrically at 420 nm using a UV-Vis spectrophotometer (Varioskan Flash Multimode

Reader, Thermo Fisher Scientific, USA). One *β*-galactosidase activity unit (U) was defined as the quantity of enzyme required for hydrolysis of 1 µmol of substrate (ONGP) per minute (U mg^{-1} protein) under given experimental conditions (Gueguen *et al*., 1997). Specific activity was measured using the formula:

$$\beta\text{-galactosidase activity} = \frac{OD_{420} \times 1.7}{0.0045 \times \text{protein} \times \text{extract volume} \times \text{time}}$$

Where,

OD_{420} is optical density of product at 420 nm

Factor 1.7 corrects the reaction volume

Factor 0.0045 was optical density of *O*-nitrophenol at 420 nm

Z-buffer contained 60 mM $Na_2HPO_4.7H_2O$, 40mM $NaH_2PO_4.H_2O$, 10mM KCl and 1mM $MgSO_4.7H_2O$ adjusted to pH 7. After preparation, the buffer was stored at 4°C. Before use, 2.7 ml of 2-mercaptoethanol was added in 1 litre of the Z-buffer.

Breaking buffer contained 50 ml of 100 mM Tris HCl (pH 8), 1 ml of 1mM dithiothreitol and 50 ml of 20% glycerol. The protein content of the filtrate was measured according to Bradford (1976) with bovine serum albumin (BSA) as standard (Appendix B 4.1).

4.3.1.2 *β*-1, 4-glucanase

β-1,4-glucanase activity was measured spectrophometrically by determining the amount of reducing sugars released from 1 g of filter paper (Whatman no. 1) as substrate, using dinitrosalicylic acid as a reagent (Gopinath *et al*., 2006). Crude extract (0.5 ml) was added to 1 ml of Na-

citrate buffer pH 4.8 and incubated at 50°C for 60 minutes. After incubation, 3.0 ml of 1.0% 3,5-dinitrosalicylic reagent was added to the reaction mixture and boiled at 100°C for 5 minutes, followed by immediate incubation on ice. The amount of reducing sugars produced was determined by a UV-Vis spectrophotometer (Varioskan Flash Multimode Reader, Thermo Fisher Scientific, USA) at 540 nm. The enzyme activity was expressed as mg glucose released per min. Boiled enzyme extract served as a control (Jayaraj and Radakrishnan, 2003). A standard curve was prepared using a glucose stock solution (Appendix B 4.2). Specific activity was measured using the following formula after correcting the values with the glucose standard curve

$$\beta\text{-1, 4-glucanase activity} = \frac{0.185}{\text{enzyme concentration to release 2.0 mg glucose}}$$

Where,

Factor 0.185 is the optical density of 1.0% 3, 5-dinitrosalicylic acid at 540 nm

4.3.2 Extraction of pectinolytic enzymes

Pectinolytic enzymes were extracted using the method described in Section 4.3.1 but with 1.0% citrus pectin (w/v) as a carbon source (Jayaraj and Radhakrishnan, 2003). The supernatant obtained was precipitated by adding 75% chilled acetone and centrifuged for 10 minutes at 10,000 rpm and 4°C. The pellets obtained after were washed twice with 95% chilled ethanol and used for further determination of enzyme activity (Ayers *et al*., 1966).

4.3.2.1 Polygalacturonase activity

The pellets (Section 4.3.2) were re dissolved in 1ml of 50 mM potassium phosphate buffer at pH 5.5, incubated in a water bath for five h at 30 °C and absorbance was measured at 556 nm (ε_M = 54176 mol^{-1} l^{-1} cm) with a UV-Vis spectrophotometer (Varioskan Flash Multimode Reader, Thermo Fisher Scientific, USA) (Cooper and Wood, 1975). One unit of polygalacturonase was defined as the amount of enzyme that liberates 1 µmol of reducing groups per ml at 30°C using *D*-galacturonic acid as a standard (Appendix B 4.3).

4.3.2.2 Pectin lyase activity

The pellets (Section 4.3.2) were dissolved in a mixture of 1 ml of 50 mM Tris-HCl and 1ml of 1mM $CaCl_2$ pH 8.5. One hundred µl of the dissolved sample was mixed with reaction mixture, which contained 200 µl of 1.0% polygalacturonic acid dissolved in 1.0% pectin from citrus and 50µl of 50mM Tris-HCl, and incubated at 30°C for five hours. The absorbance was measured at 550nm with a UV-Vis spectrophotometer (Varioskan Flash Multimode Reader, Thermo Fisher Scientific, USA) (Gainvors *et al*., 1994). One unit of pectin lyase was defined as the amount of enzyme producing one µmol of unsaturated product per ml at 30°C. A standard curve of bovin serum albumin was used to determine protein content (Appendix B 4.1). Specific acitivity was defined as U mg^{-1} protein.

4.4 Preliminary field trial to screen potential treatments

4.4.1 Plant material and experimental site preparation

The size of the experimental area was 87 x 45 metres. A system of poles was already installed in the field with the poles 2 metres high and 3 metres apart.

Cuttings of 0.5 m in length, from mature, healthy red dragon fruit plants (2 years old), were obtained from a commercial orchard located at Puchong, Selangor, Malaysia. These cuttings were rooted and grown in a shade house at The University of Nottingham, Malaysia Campus. After 2 months, they were planted in the field plot at Farm no B2, Taman Pertanian University, UPM, Serdang, Malaysia. The soil of the field site was Serdang series (Table 4.1).

The first trial was carried out from April 2011 to March 2012 and the second from August 2011 to July 2012. For these trials, the plants were raised separately. Planting holes were prepared by digging the soil to a depth of 14 cm and four plants were planted at each pole.

4.4.1.1 Fertilizer applications

The rate and schedule of fertilizer application was done according to the recommendations made by Yusoff (2006). Organic fertilizer Amino-Q® (1.38% N: 3.02% P_2O_5: 0.98% K_2O: 0.87% MgO: 5.74% CaO) was applied at 350 g $plant^{-1}$ at intervals of one month.

Table 4.1: Physicochemical characteristics of Serdang Series soil of Universti Putra Malaysia.

Parameter	Value
pH (H_2O)	3.93
Total N %	0.11
Total organic carbon (OC %)	0.94
Available P (g kg^{-1})	2.50
K (C mol (+) kg^{-1})	0.06
Ca (C mol (+) kg^{-1})	0.17
Mg (C mol (+) kg^{-1})	0.10
CEC (C mol (+) kg^{-1})	8.32
Bulk density (pB) (g cm^{-1})	1.75
Clay (µm)	28.80
Silt (µm)	5.39
Sand (µm)	11.58
Texture	Sandy Clay Loam (SCL)

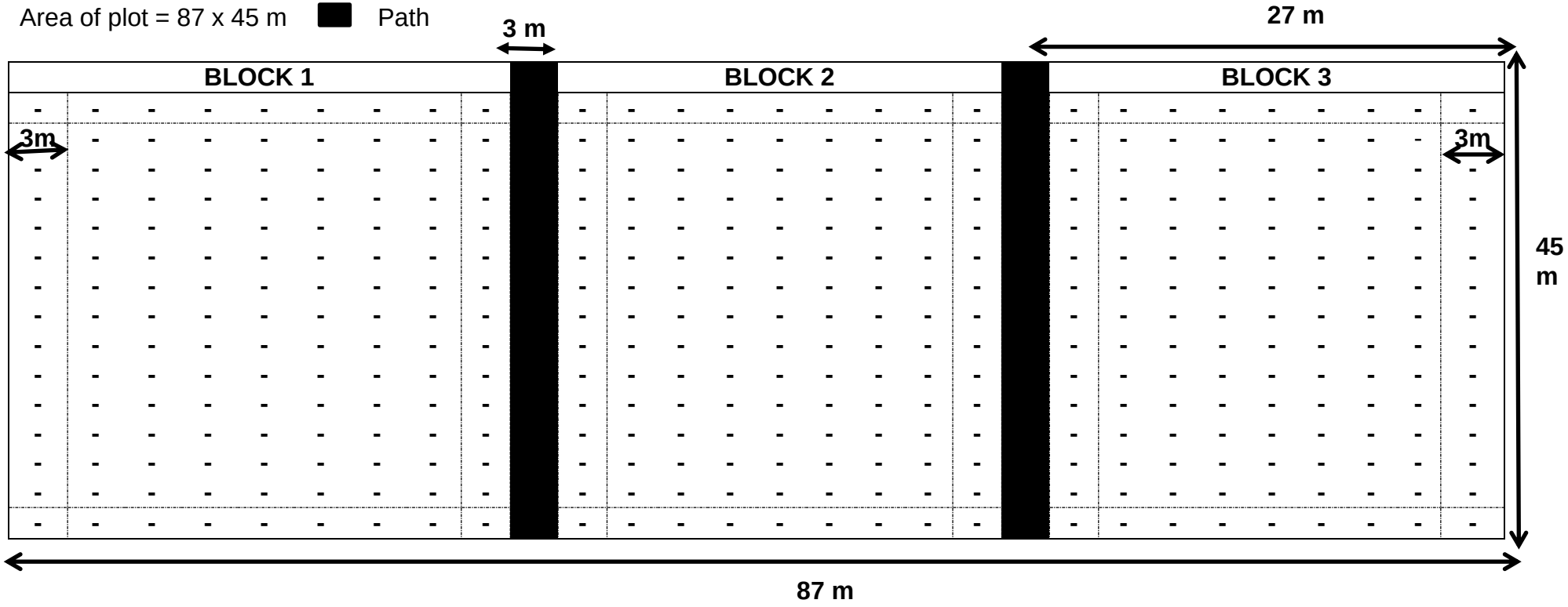
Source: Siddiqui (2005), PhD Thesis, Universiti Putra Malaysia, Serdang.

4.4.1.2 Water management

Watering was carried out daily in the evening through a sprinkler irrigation system for the first two months and subsequently at three days' intervals to ensure adequate moisture in the soil. On rainy days, irrigation was not carried out.

4.1 FIELD LAYOUT FOR PRELIMINARY SCREENING

Total number of treatments = 17, No of plants in each treatment = 15
No of replications = 3, Total number of plants = 255
Plant to plant distance = 3 m
No of rows = 15
No of columns = 27
Area of plot = 87 x 45 m ■ Path



4.4.2 Disease incidence

Plants used for assessment of disease incidence were inoculated artificially using a toothpick method (Melanie *et al*., 2004). A sterile sharp toothpick was used to make wounds approximately 2 mm deep and a sterile syringe needle was used to inoculate 20 µl of spore suspension (1 x 10^6 spores ml^{-1}) in each wound site. Five areas on five stems of each plant were inoculated. Starting one week after inoculation, the plants were treated at monthly intervals for 3 months with conventional chitosan solution and submicron chitosan dispersions applied as spray until run off (at each pole, 150 ml for the first month, 250 ml for the second and 400ml for the third). Development of foliar symptoms as water soaked necrotic lesions was assessed at intervals of 30 days for three months. Disease incidence was expressed as the percentage of stems showing necrotic symptoms out of the total number of inoculated stems in each treatment (Cooke, 2006).

$$\text{DI \%} = \frac{\text{Number of infected plant units}}{\text{Total number of plant units assessed}} \times 100$$

4.4.3 Area under disease progress curve

The effectiveness of the chitosan treatments was assessed as reduction in the disease incidence and was expressed by plotting a disease progress curve. The area under the disease progress curve (AUDPC) was calculated using the formula described by Campbell and Madden (1990).

$$\text{AUDPC} = \sum_{i=1}^{n-1} [(t_{i+1} - t_i)(y_i + y_{i+1})/2]$$

Where, t = time interval between assessments (days)

y = disease incidence; n = number of assessments

4.4.4 Disease severity

Disease severity (DS) was recorded using a scale of 1-5 at intervals of thirty days, where 0 = no disease, 1 = 1-20%, 2 = 21-40%, 3 = 41-60%, 4 = 61-80% and 5 = 81-100% disease symptoms (Bowen, 2007). It was expressed as a percentage (%) calculated using the formula proposed by Kranz (1988).

$$DS\ (\%) = \frac{\sum (\text{number of symptomatic plants} \times \text{severity scale})}{N \times Z} \times 100$$

$$= \frac{\sum\ (n \times 0) + (n \times 1) + (n \times 2) + (n \times 3) + (n \times 4) + (n \times 5)}{N \times Z} \times 100$$

Where, N = Total number of sampled plants

Z = Highest rating scale

The *in vitro* experiments were arranged in a completely randomized design (CRD) with four replicates, each consisting of twenty units. The experimental layout for DI and DS was a randomized complete block design (RCBD) with three replicates and each replicate consisted of 17 plots (treatments) with five plants per plot. Extra plants were planted on all the four sides of each experimental block in order to minimize the border effect. Data for DI and DS were subjected to arcsine and square root transformation, respectively and then back transformed for presentation. Data collected were subjected to UNIVARIATE procedure in SAS for testing the normality of residuals. Analysis of variance (ANOVA) was carried out and tested for significant differences among treatments by Duncan's multiple range test (DMRT) at $P < 0.05$, using statistical analysis system SAS® version 9.1 (Appendix C 4.1 to 4.16).

4.5 Results and discussion

4.5.1 Effect of different types of submicron chitosan dispersions on *in vitro* growth of *C. gloeosporioides*

4.5.1.1 Percent inhibition in radial mycelial growth of *C. gloeosporioides*

Neither of the emulsifiers tested (Brij 56 and Span 20), either alone or in combination, showed inhibition in radial mycelial growth of *C. gloeosporioides* (Fig. 4.1).

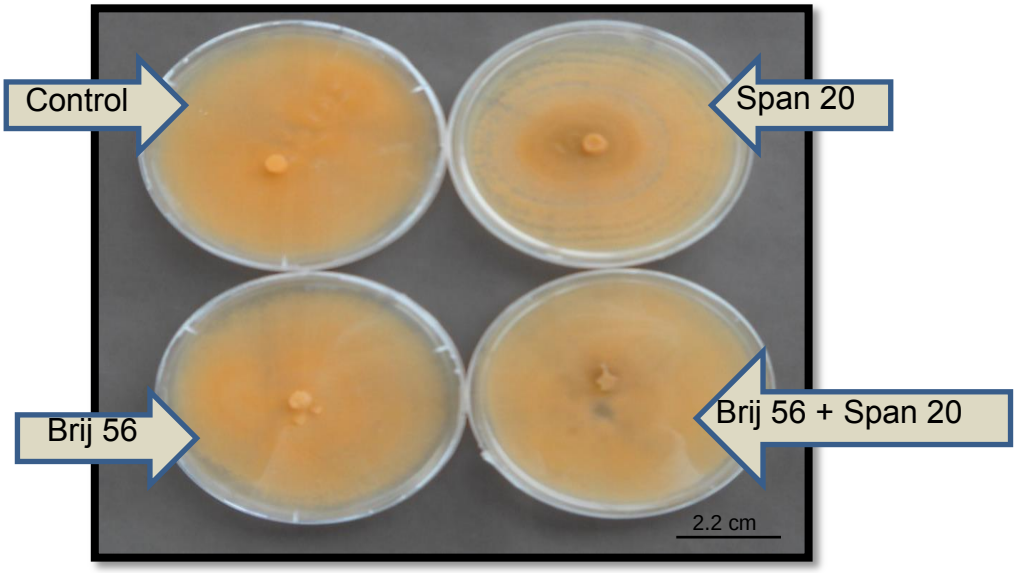


Figure 4.1: Comparison between control and emulsifiers on mycelial growth of *C. gloeosporioides*.

Radial mycelial growth of *C. gloeosporioides* was significantly ($P < 0.05$) inhibited by all the concentrations and droplet sizes for submicron chitosan dispersions of both chitosans (Figs 4.2 to 4.4). Droplets of 600 nm showed best results at all concentrations of both chitosans. Significant ($P < 0.05$, Appendix C 4.1) inhibition was noted for 600 nm droplets of 1.0% low

molecular weight chitosan (Figs 4.2a, 4.3) and for 600 nm droplets of 1.5% chitosan (Figs 4.2b, 4.4; Appendix C 4.2).

The 600 nm submicron chitosan dispersions had the greatest effects against *C. gloeosporioides* in *in vitro* experiments and, as the concentration of chitosan increased, the growth of *C. gloeosporioides* was decreased. Comparable results were obtained when chitosan was used on strawberry to control growth of *Rhizopus stolonifer* and *Botrytis cinerea* (El Ghaouth *et al*., 1992), and when chitosan was increased from 0.75 to 6.0 mg ml^{-1} to decrease the mycelial growth of *Alternaria alternata* and *C. gloeosporioides* (Muñoz *et al*., 2009; Ali and Mahmud, 2008; Wade and Lamondia, 1994). Chitosan has also been reported to affect *Sclerotinia sclerotiorum* on carrot (Cheah *et al*., 1997), and at 3% to inhibit completely *F. oxysporium*, *R. solani*, *Pencillium digitatum* and *C. gloeosporioides* isolated from fruit and vegetables (Bautista-Baños *et al*., 2004 & 2003). Liu et al. (2007) showed that low molecular weight chitosan at 0.01-1.0% markedly inhibited radial mycelial growth of *B. cinerea* and *Penicillium expansum*.

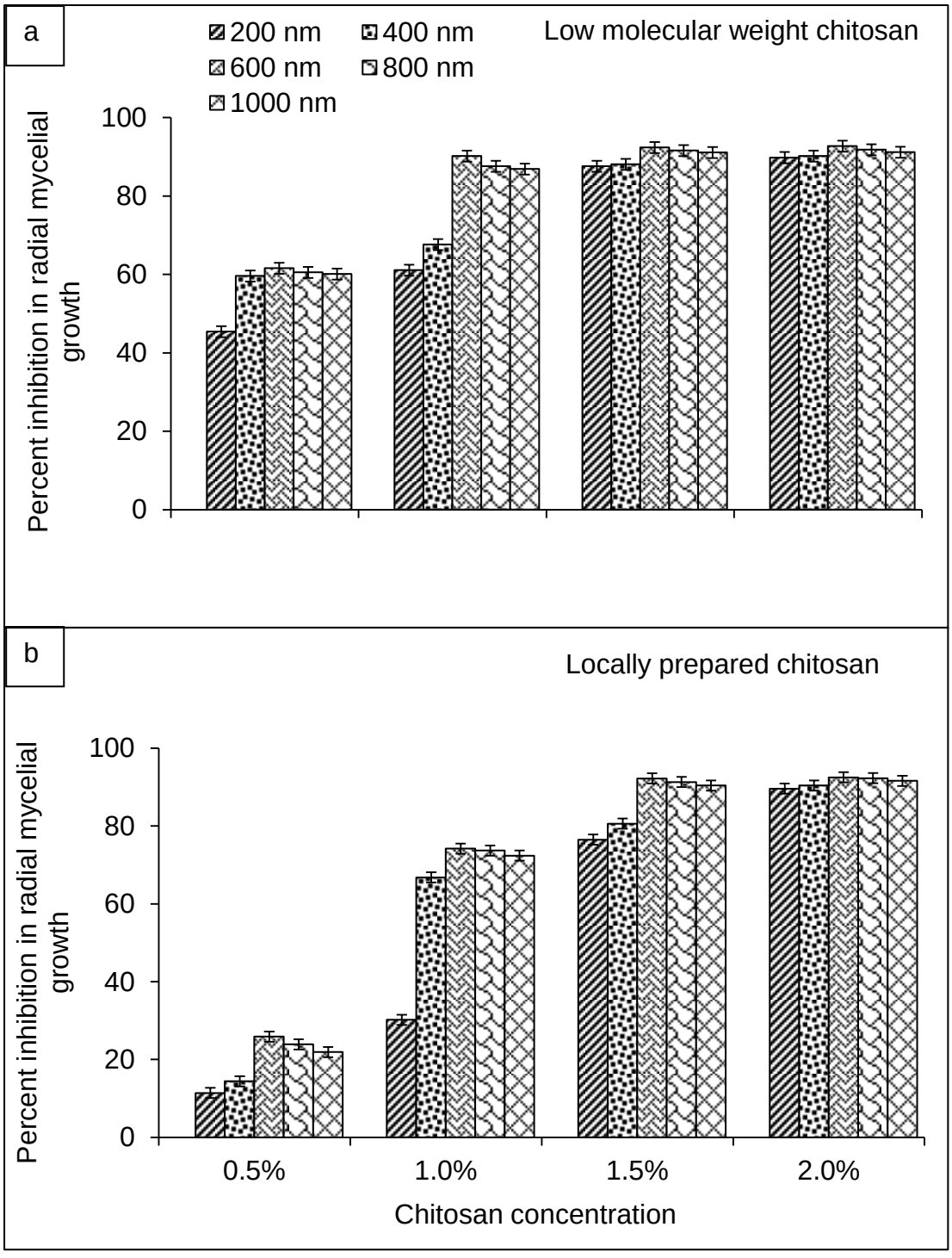


Figure 4.2: Effect of submicron dispersions of a) low molecular weight chitosan and b) locally prepared chitosan on percent inhibition in radial mycelial growth after 10 days of incubation. The vertical bars represent the standard error of means for four replicates.

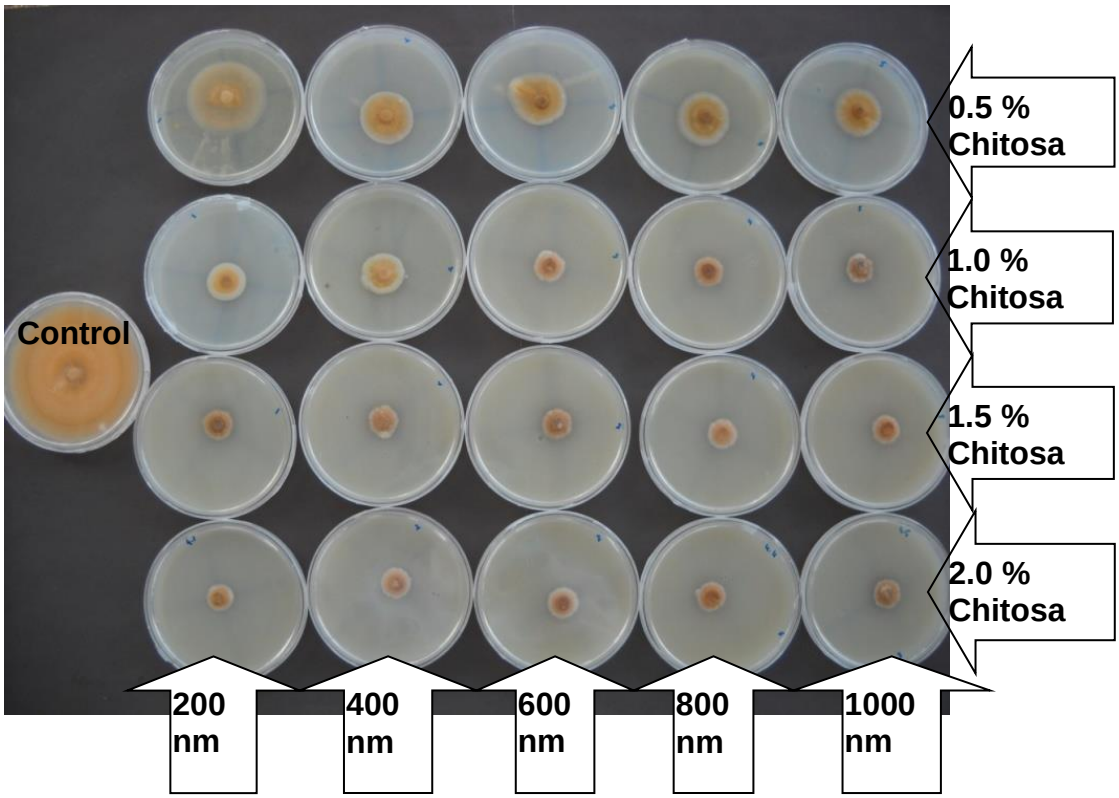


Figure 4.3: Effect of concentration and droplet size of low molecular weight submicron chitosan dispersions on mycelial growth of *C. gloeosporioides*.

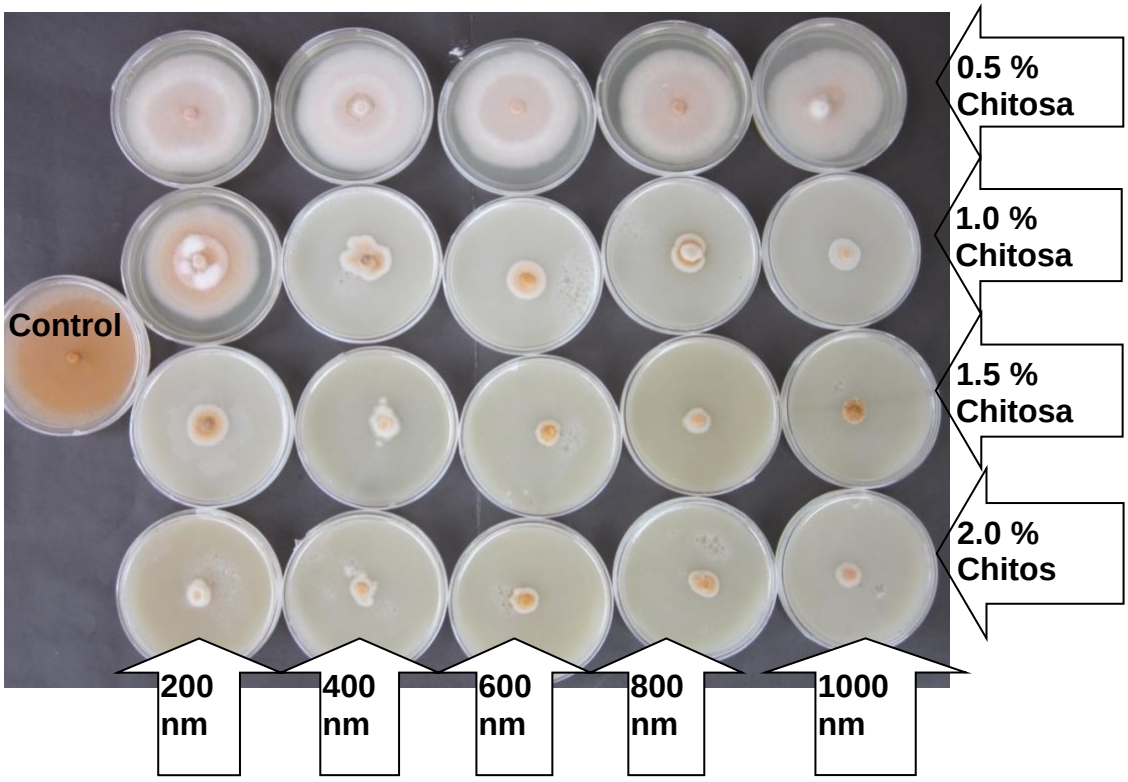


Figure 4.4: Effect of concentration and droplet size of locally prepared submicron chitosan dispersions on mycelial growth of *C. gloeosporioides*.

Antimicrobial activity of chitosan has been detected for many yeasts, filamentous fungi and bacteria (Ueno *et al*., 1997; Hirano and Nagao, 1989; Uchida, *et al*., 1989; Kendra and Hadwiger, 1984). These studies show that physicochemical properties of chitosan, such as molecular fraction of glucosamine units and molecular weight, influence significantly its biological activity. These factors affect the interaction of chitosan with the cell wall of targeted microorganisms and its solubility. However, in some applications, this polysaccharide is restricted by its high molecular weight causing a highly viscous solution and low solubility even at low concentrations. Numerous studies have supported the antimicrobial activity of chitosan (Badawy *et al*, 2004; Muzzarelli *et al*., 2001; Muzzarelli *et al*., 1990; Rhoades and Roller, 2000) and some debateable evidence has been found for a parallel connection between antimicrobial activity and chitosan molecular weight.

In the present study, 600, 800 and 1000 nm droplets gave the best inhibition of radial mycelial growth which showed that the amount of chitosan in these droplet sizes was enough to combat the fungus and inhibit its growth. In contrast, 200 and 400 droplets did not perform well in reducing mycelial growth, possibly due to the high stability of these droplets which delayed the release of chitosan (Section 3.6.1.2). Low molecular weight submicron chitosan dispersions resulted in reduction of radial mycelial growth at 1.0% chitosan concentration while locally prepared chitosan gave the similar results at 1.5%. These results are in line with earlier studies which showed that increase in the chitosan molecular weight results in decreased antimicrobial activity (Gerasimenko *et al*., 2004; Zheng and Zhu *et al*., 2003).

4.5.1.2 Percent inhibition in conidial germination of *C. gloeosporioides*

The emulsifiers Brij 56 and Span 20, either alone or in combination, did not inhibit conidial germination of *C. gloeosporioides* (Data not shown). The submicron chitosan dispersions significantly ($P < 0.05$) inhibited the conidial germination as compared to the control (Fig. 4.5). For both types of chitosan, 600 nm droplets were the most effective in inhibiting conidial germination. Low molecular weight chitosan showed significant ($P < 0.05$) inhibition at 1% with 600 nm droplets (Fig. 4.5a, Appendix C 4.3) and locally prepared chitosan at 1.5% chitosan with 600 nm droplets (Fig. 4.5b, Appendix C 4.4).

Rappussi *et al*. (2009) reported that conidial germination and appressorium formation of *Guignardia citricarpa* were stimulated when treated with chitosan as compared to the control. However, the conidial germination and appressorium formation were abnormal with the germ tube and appressorium deformed. Plascencia-Jatomea *et al*. (2003) also showed that chitosan caused spore aggregation and morphological anomalies, such as swelling of germ tube and spore polarization of *Aspergillus niger.*

Reglinski *et al.* (2010) observed that chitosan inhibited *B. cinerea* growth and also induced morphological changes in conidia. Similarly, 75% and 90% reduction in conidial germination and germ tube elongation was observed by El Ghaouth *et al*. (1992) when they used chitosan at 10 and 15 mg ml^{-1} against *B. cinerea* and *R. stolonifer,* respectively.

In this investigation 600, 800 and 1000 nm droplets showed more inhibition of conidial germination with both types of chitosan. This suggests that droplets 600 nm or larger are capable of encapsulating sufficient

chitosan to inhibit conidial germination and appressorium formation. They can also induce changes in conidial morphology and cause hyphal agglomeration.

The light microscopy study provided evidence that the germ tube, in the presence of emulsifiers alone and in combination, grew to the same degree as in the control (Fig. 4.6).

Based on the findings of conidial germination inhibition, the morphology of conidia was observed only with 600 nm droplets of 1.0% low molecular weight chitosan. There was no inhibition of germ tubes in the control (Fig. 4.7a), wheras chitosan dispersions markedly inhibited the growth of conidia (Fig. 4.7b) and induced changes in *C. gloeosporioides* morphology characterised by hyphal agglomeration (Fig. 4.7c) and the presence of large vesicles in the hyphae (Fig. 4.7d).

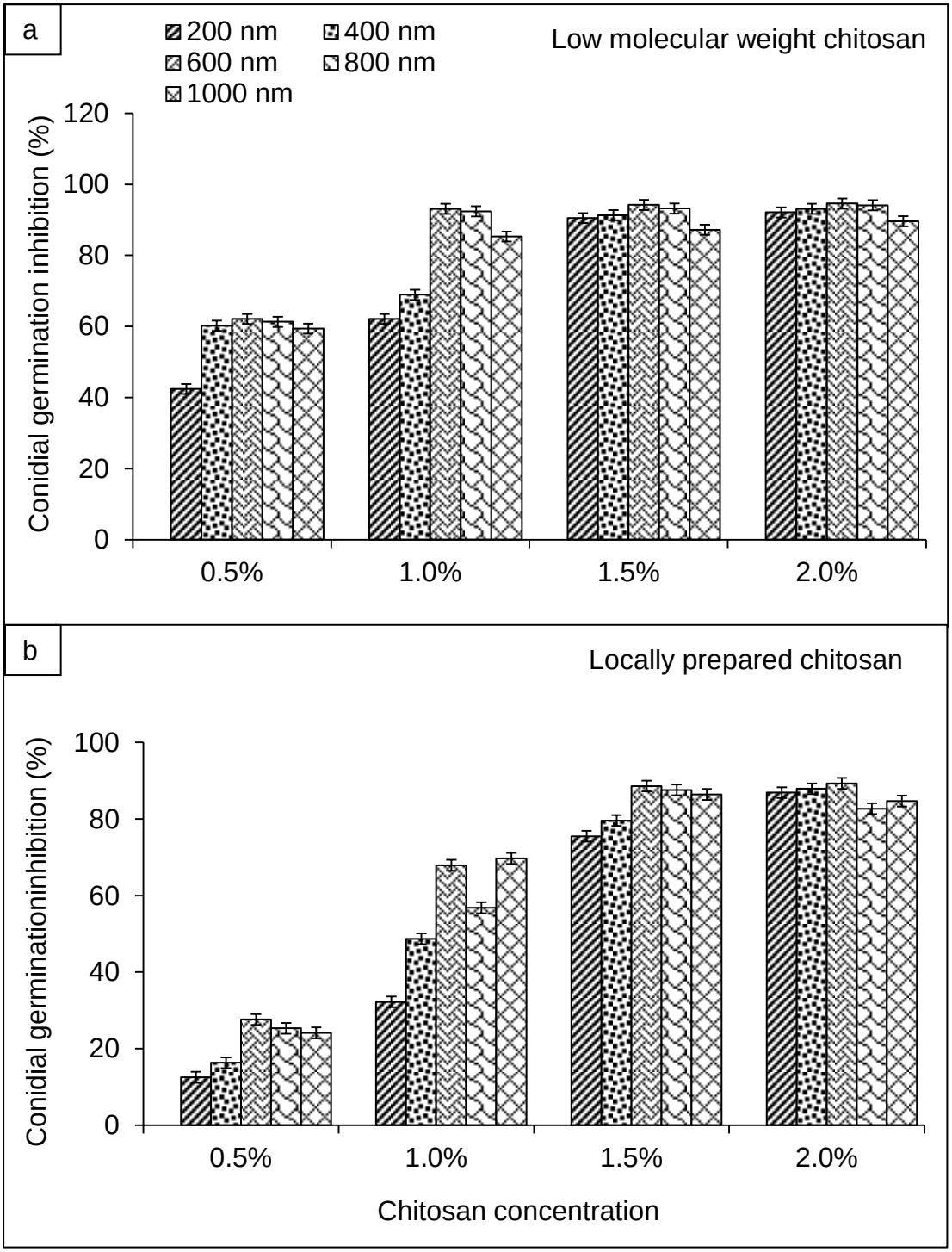


Figure 4.5: Effect of submicron dispersions of a) low molecular weight chitosan and b) locally prepared chitosan on conidial germination inhibition (%) after seven hours of incubation. The vertical bars represent the standard error of means for four replicates.

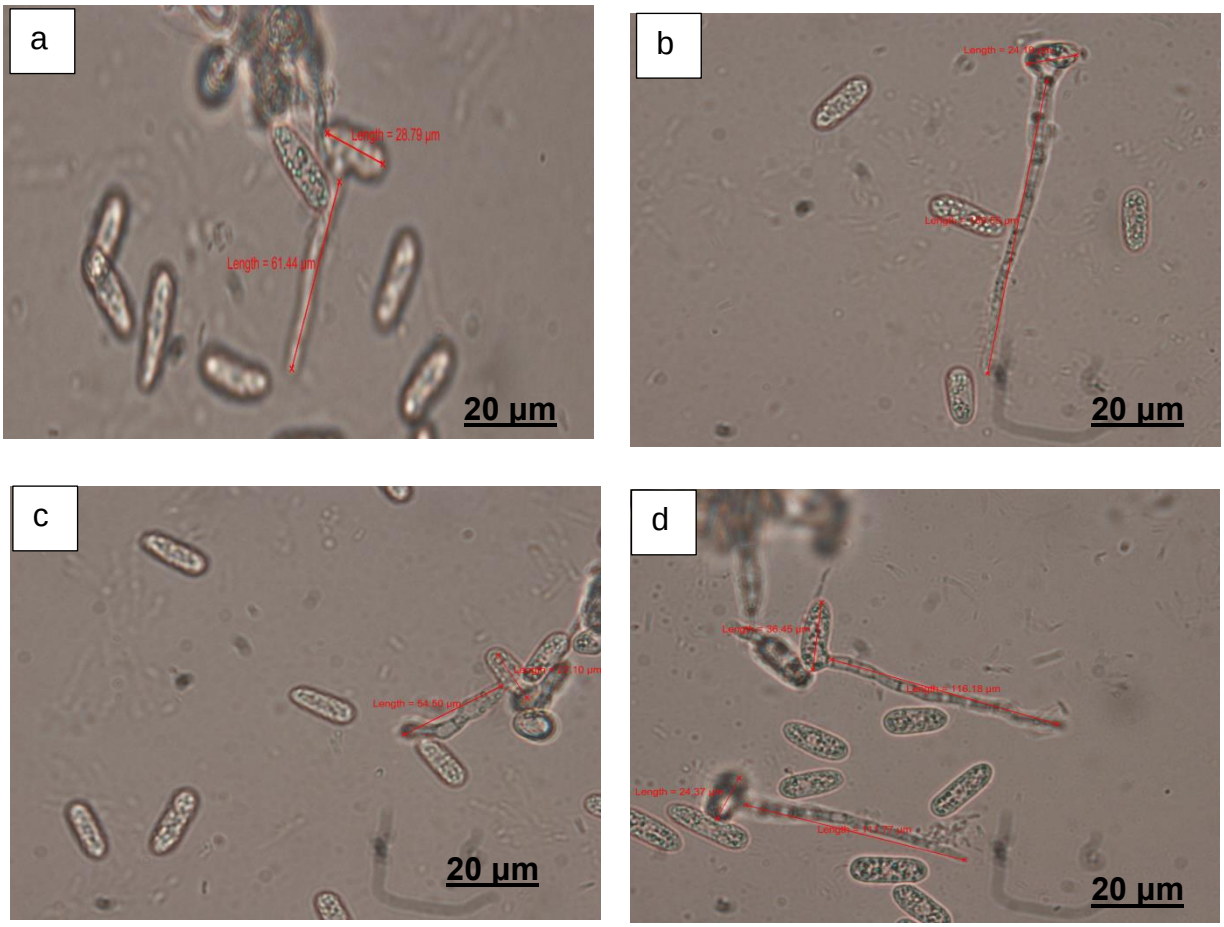


Figure 4.6: Light microscopy study of germ tube of *C. gloeosporioides* treated with emulsifiers: a) control, b) spores treated with Brij 56, c) spores treated with Span 20 and d) spores treated with combination of Brij 56 and Span 20.

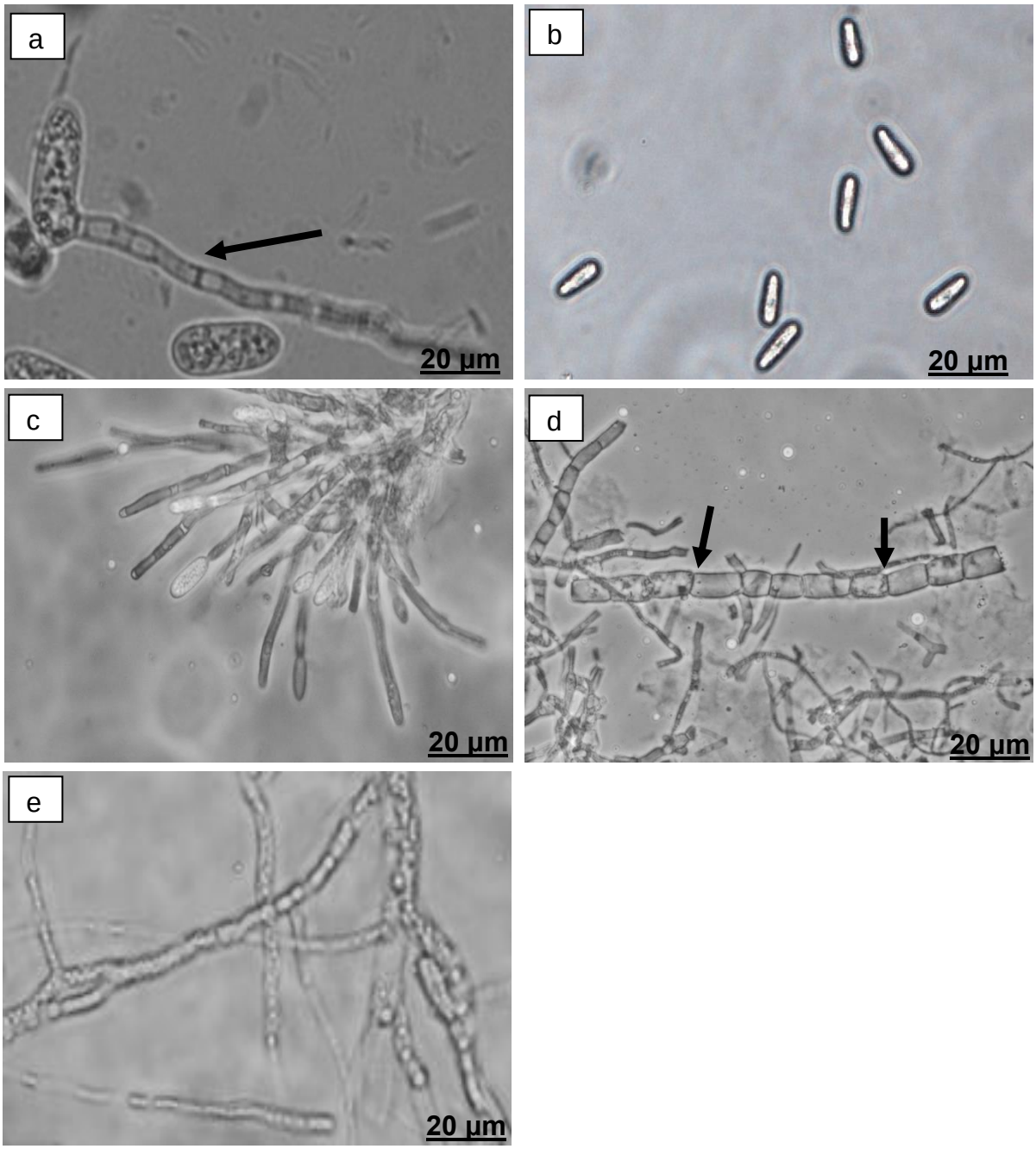


Figure 4.7: Light microscopy of conidia and mycelium of *C. gloeosporioides* treated with submicron chitosan dispersions: a) germ tube growth in control (arrow), b) treated conidia, c) hyphal agglomeration from treated conidia, d) large vesicles in treated hyphae (arrows), e) normal growth of hyphae in control.

In a recent study by Ali *et al*. (2010), it was found that conidial germination of *C. gloeosporioides* was inhibited and morphologically affected by chitosan. El Ghaouth *et al*. (1992) also found that, at concentrations higher than 0.3%, chitosan induced abnormal hyphal growth and reduced the growth of germ tubes and viability of spores of *R. stolonifer* and *B. cinerea* isolated from strawberry fruit. Similar effects were reported for *F. oxysporum* f. sp. *radicis lycopersici* (Benhamou, 1996), *F. oxysporum, F. acuminatum* and *Cylindrocarpon destructans* (Laflamme *et al*., 1999), *F. oxysporum* f.sp. *albedinis* (El Hassni *et al*., 2004) and *Phytophthora capsici (Xu et al., 2007).*

4.5.1.3 Effect of submicron chitosan dispersions on dry weight of mycelium and sporulation of *C. gloeosporioides*

Dry weight of mycelium and sporulation were assessed only with submicron chitosan dispersions. The 600 nm droplets for both chitosans proved to be the most effective in reducing dry weight of mycelium with significant reduction ($P < 0.05$; Appendix C 4.5, 4.6) by 1.0% low molecular weight chitosan and 1.5% locally prepared chitosan (Fig. 4.8a and b).

A similar trend in inhibiting sporulation was observed for both chitosans (Fig 4.9a and b). Sporulation inhibition was significantly ($P < 0.05$; Appendix C 4.7, 4.8) higher with 600 nm droplets of 1.0% low molecular weight chitosan and 1.5% locally prepared chitosan in comparision with the control.

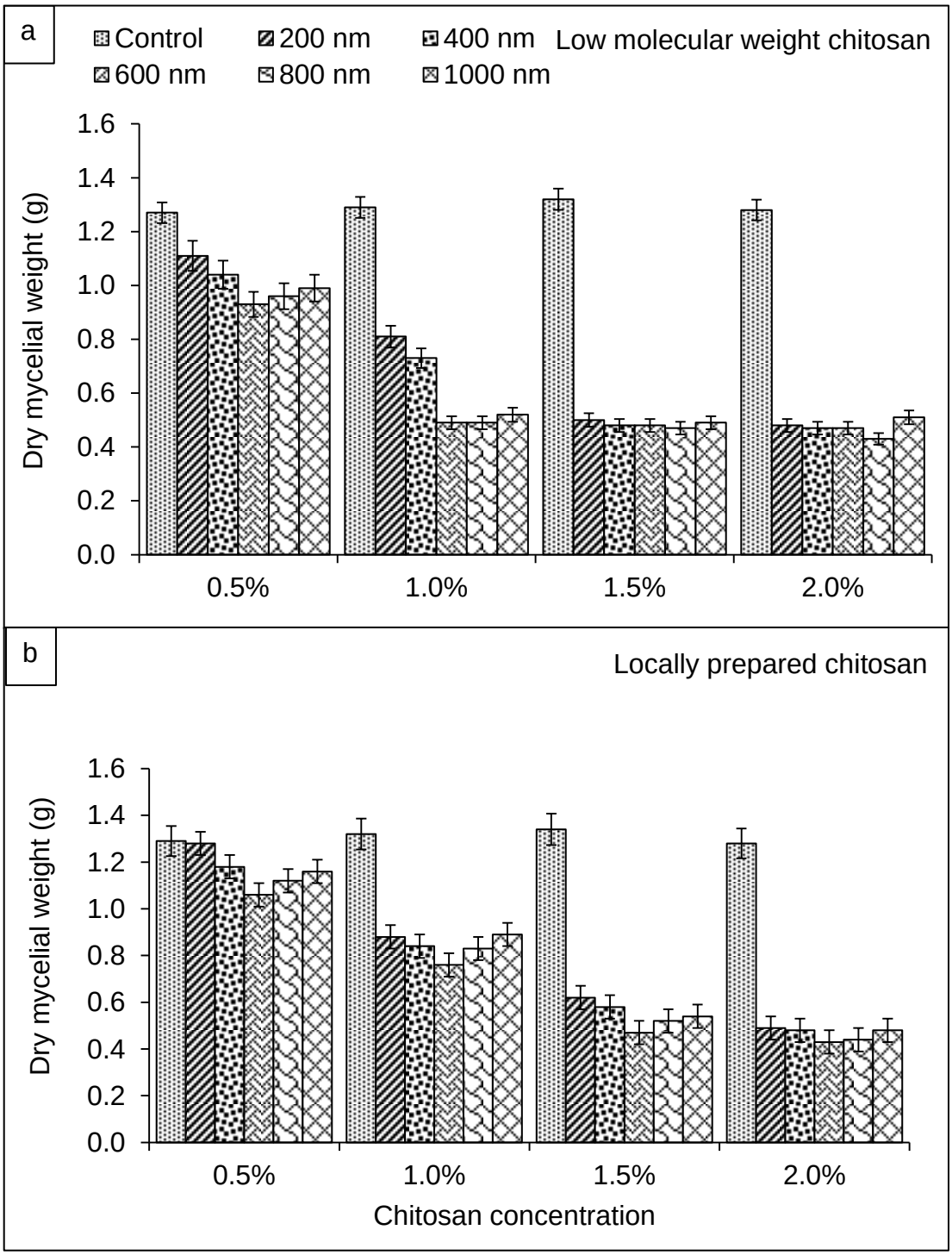


Figure 4.8: Effect of submicron dispersions of a) low molecular weight chitosan and b) locally prepared chitosan on dry weight of mycelium (g). The vertical bars represent the standard error of means for four replicates.

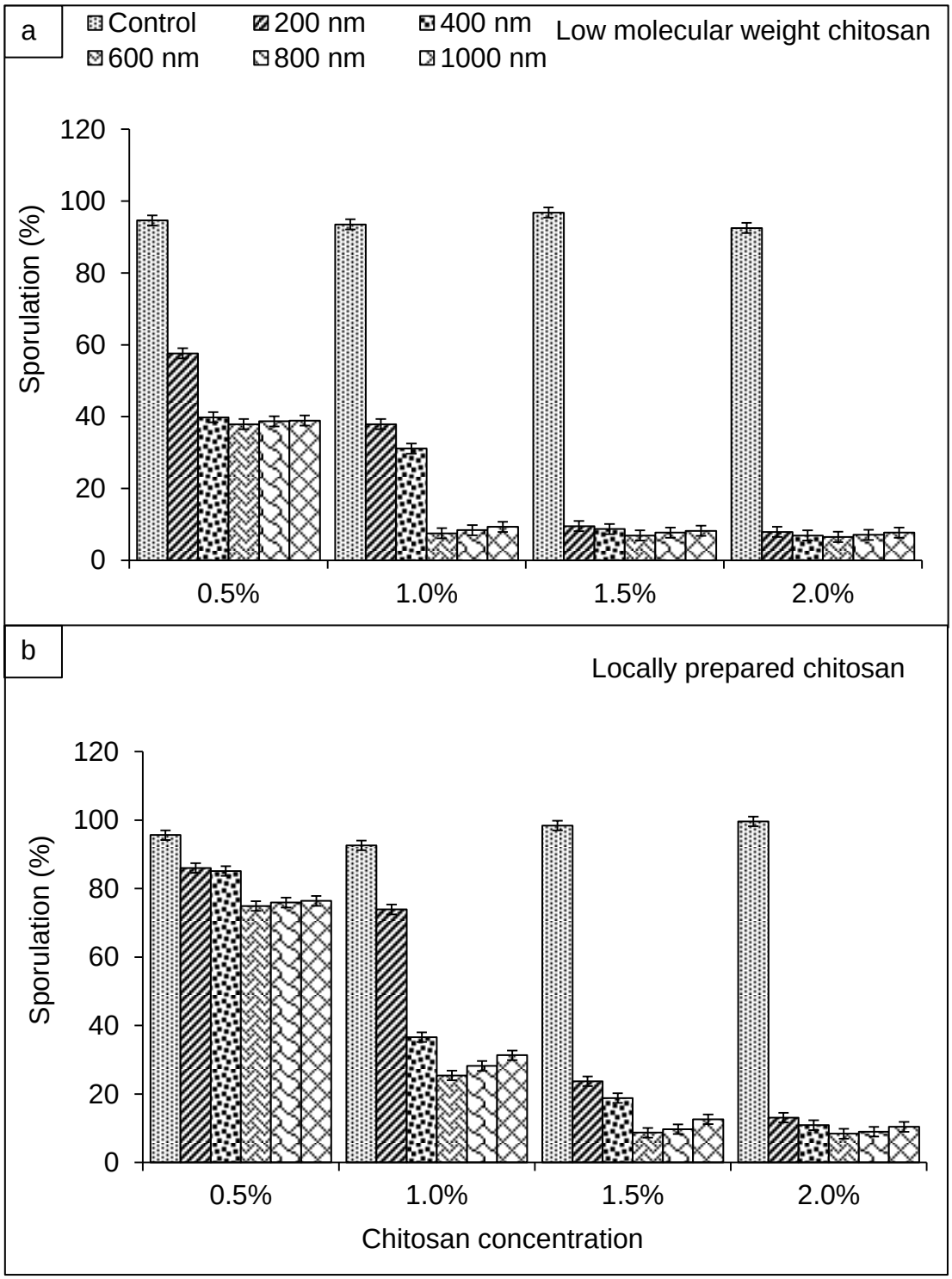


Figure 4.9: Effect of submicron dispersions of a) low molecular weight chitosan and b) locally prepared chitosan on sporulation (%). The vertical bars represent the standard error of means for four replicates.

Mycelial growth, spore germination, dry mycelium weight and sporulation were affected by chitosan indicating its effects on various growth and developmental stages of *C. gloeosporioides*. Previous studies have shown similar effects in *F. oxysporum* isolated from papaya (Bautista-Baños *et al*., 2004), *F. solani* (Kauss *et al*.,1989) and *Phytophthora capsici* (Xu *et al*., 2007). In contrast, some studies showed increase in sporulation of *A. alternata* (Bhaskara Reddy *et al*., 1999) and *C. gloeosporioides* (Bautista-Baños *et al*., 2006) and that high sporulation could be associated with a stress response caused by chitosan. High sensitivity of conidia of *C. gloeosporioides* to chitosan was observed with no germination when they were plated on media amended with chitosan (Bautista-Baños *et al*., 2004). Spore germination of *B. cinerea* (Ben-Shalom *et al*., 2003), *F. oxysporum* f. sp. *radicis lycopersici*, (Tikhonov *et al*., 2006), *C. gloeosporioides*, (Bautista-Baños *et al*., 2004) and *P. capsici* (Xu *et al*., 2007) was completely inhibited in the chitosan amended media. Similar results for broth cultures were obtained by Prapagdee *et al*. (2007) for *F. solani* f. sp. *glycines*.

The mechanism of action of chitosan against microorganisms is still not known. It is a common belief that the antifungal effect of chitosan is due to its polycationic nature, which makes it positively charged with great affinity to the negatively charged residues of macromolecules on the microbial cell surface. Therefore, chitosan adheres rapidly to the surface of microorganisms, causing leakage of the intercellular and proteinaceous constituents and electrolytes as a consequence of the increased membrane permeability, and causes death by the loss of essential fluids (Tsai and Su, 1999; Muzzarelli, 1996; Fang *et al*., 1994).

Another possible mechanism whereby chitosan inhibits microbial growth is its activity as a chelating agent. It may bind to trace metal ions, trace elements or essential nutrients in the intracellular fluid and substances that are essential in the biochemistry of microbes. Deficiency of metal ions can result in the inhibition of the production of toxins and enzymes (Cuero *et al*., 1991). In this study, the enlarged vesicles observed suggested higher membrane permeability, whereas the chelating activity of chitosan was supported by the results of conidial germination and growth in broth culture.

The third possible mechanism for chitosan to inhibit microbial growth is that it binds DNA and inhibits production of protein and mRNA upon invasion into the nuclei (Hadwiger *et al*., 1986). The inhibition of mycelial growth and conidial germination by different concentrations of chitosan has also been explained by histological studies in which chitosan applied was accumulated in the fungal cell wall to hinder its growth (Kendra *et al*., 1989; Kendra and Hadwiger, 1987; Hadwiger *et al*., 1981).

Thus, in the present study, low molecular weight submicron chitosan dispersions showed better antifungal activity than locally produced chitosan for all of the parameters assessed. That may have been due to the difference in molecular weight of the chitosans, since low molecular weight chitosan has lower viscosity which enables it to enter into the cell's nucleus, interfering in mRNA synthesis by interacting with DNA, and affecting protein production and inhibiting various enzymes (Martìnez-Camacho *et al*., 2010; Rabea *et al*., 2003).

Due to higher antifungal activity in comparision with locally prepared chitosan, low molecular weight chitosan, in conventional form and as 200 nm,

600 nm and 1000 nm droplets, was selected for further studies. Selection of 200 nm droplets was based on the putative better penetration and higher antifungal activity. Selection of 600 nm droplets was due to the best performance in all the parameters assessed, whereas 1000 nm droplets were selected for their antifungal effects being similar to the 600 nm droplets.

4.5.2 Effect of submicron chitosan dispersions on activity of cellulose degrading enzymes

The activity of cell wall degrading enzymes was decreased with the increase in chitosan concentration. The activity of *β*-galactosidase was significantly ($P < 0.05$; Appendix C 4.9) lower in all the conventional chitosan and submicron chitosan dispersions as compared to the control after 10 days of incubation (Fig. 4.10). Significantly ($P < 0.05$) lower activity of *β*-galactosidase was recorded with 600 nm droplets of 1.0% chitosan as compared to other treatments.

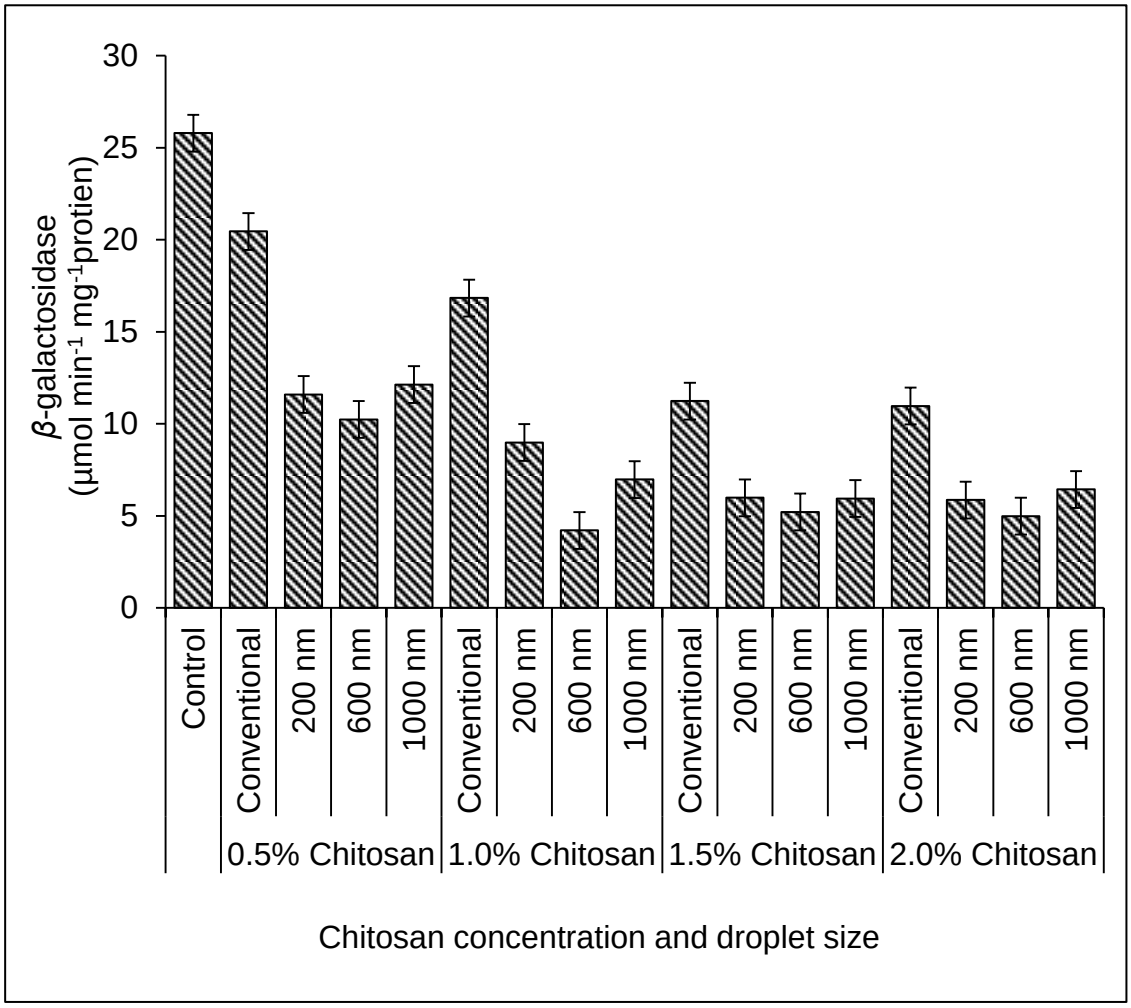


Figure 4.10: Effect of concentration of conventional chitosan and droplet size of submicron chitosan dispersions on *β*-galactosidase activity after 10 days of incubation. The vertical bars represent the standard error of means for four replicates.

The activity of *β*-1,4-glucanase was significantly ($P < 0.05$, Appendix C 4.10) lower in all the conventional chitosan plus submicron chitosan dispersions after ten days of incubation. The highest inhibition in the activity was observed with 600 nm droplets of 1.0% chitosan (Fig. 4.11).

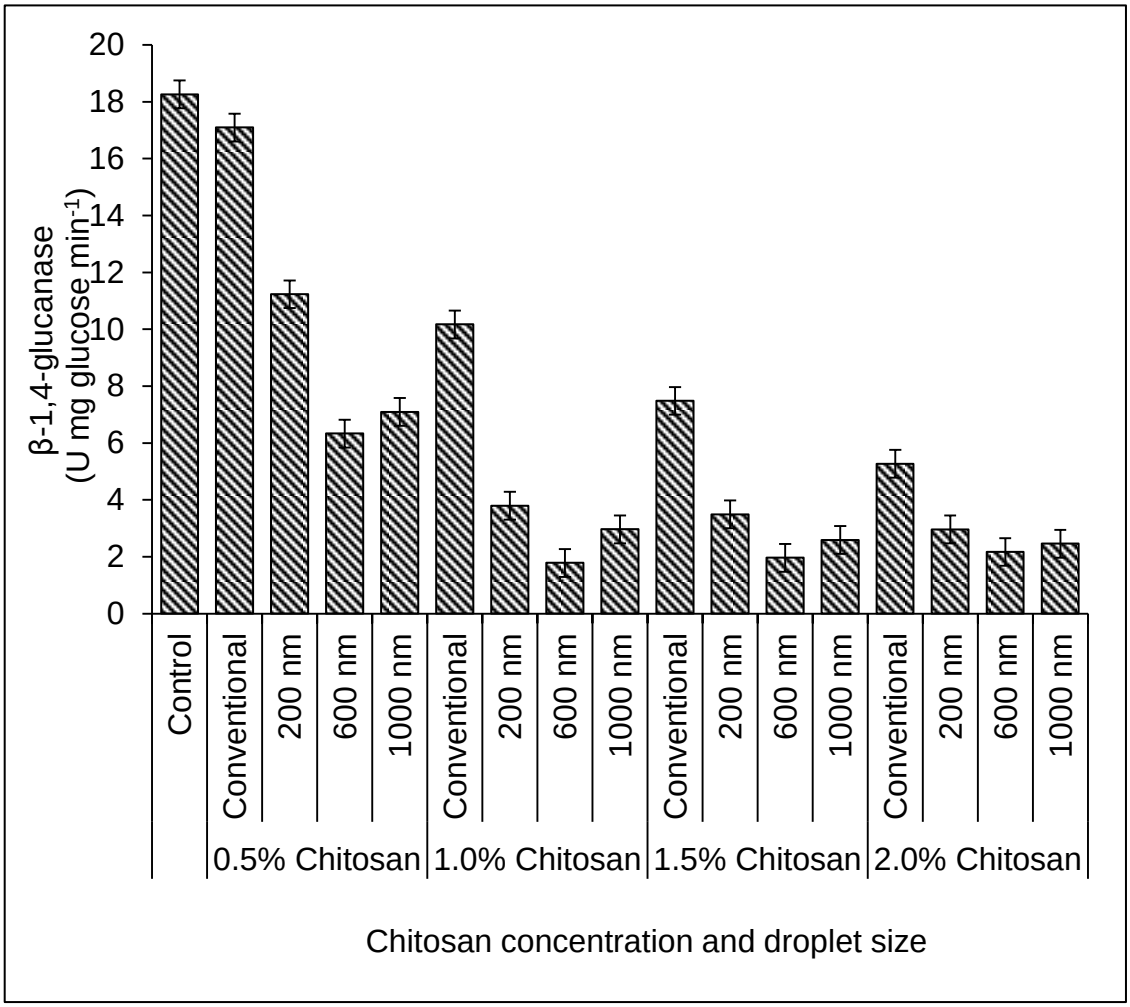


Figure 4.11: Effect of concentration of conventional chitosan and droplet size of submicron chitosan dispersions on *β*-1,4-glucanase activity after 10 days of incubation. The vertical bars represent the standard error of means for four replicates.

The production of cellulose degrading enzymes by various *Colletotrichum* spp. has been reported by Senaratne *et al*. (1991) and Kanakaratne and Adikaram (1990). *β*-galactosidase and *β*-1,4-glucanase are the enzymes responsible for cleavage of cellulose in plant tissues (Fernando *et al*., 2001). The physiological roles of these enzymes are extremely diverse, being involved in catabolism of human tissues and plant cell walls and defense against pathogens in plants (Leclerc *et al*., 1987). These enzymes are very helpful for plant pathogens as they play an important role in the infection

process and development of symptoms, tissue maceration and cellular death (Wijesundera *et al*., 1989). In this study, chitosan treatments reduced the production of cellulolytic enzymes which might be linked with the antifungal activity of chitosan. In addition to the inhibition of mycelial and conidial growth, chitosan has the ability to interfere with the production of host specific toxins, organic acids and cell wall degrading enzymes. Several studies have confirmed direct antifungal action of chitosan (El Ghaouth *et al*., 1992 & 1991; Allan and Hadwiger, 1979). Even though information is available on the antimicrobial effects of chitosan, the mechanism of its involvement in the pathogenesis of disease causing organisms in plants is still unknown.

4.5.3 Effect of submicron chitosan dispersions on activity of pectin degrading enzymes

The activity of polygalacturonase tested was significantly ($P < 0.05$, Appendix 4.11) lower in all the conventional chitosan plus submicron chitosan dispersions as compared to the control after ten days of incubation (Fig. 4.12). The highest activity of polygalacturonase was observed in control samples. In contrast, the lowest activity was recorded with 600 nm droplets of 1.0% chitosan.

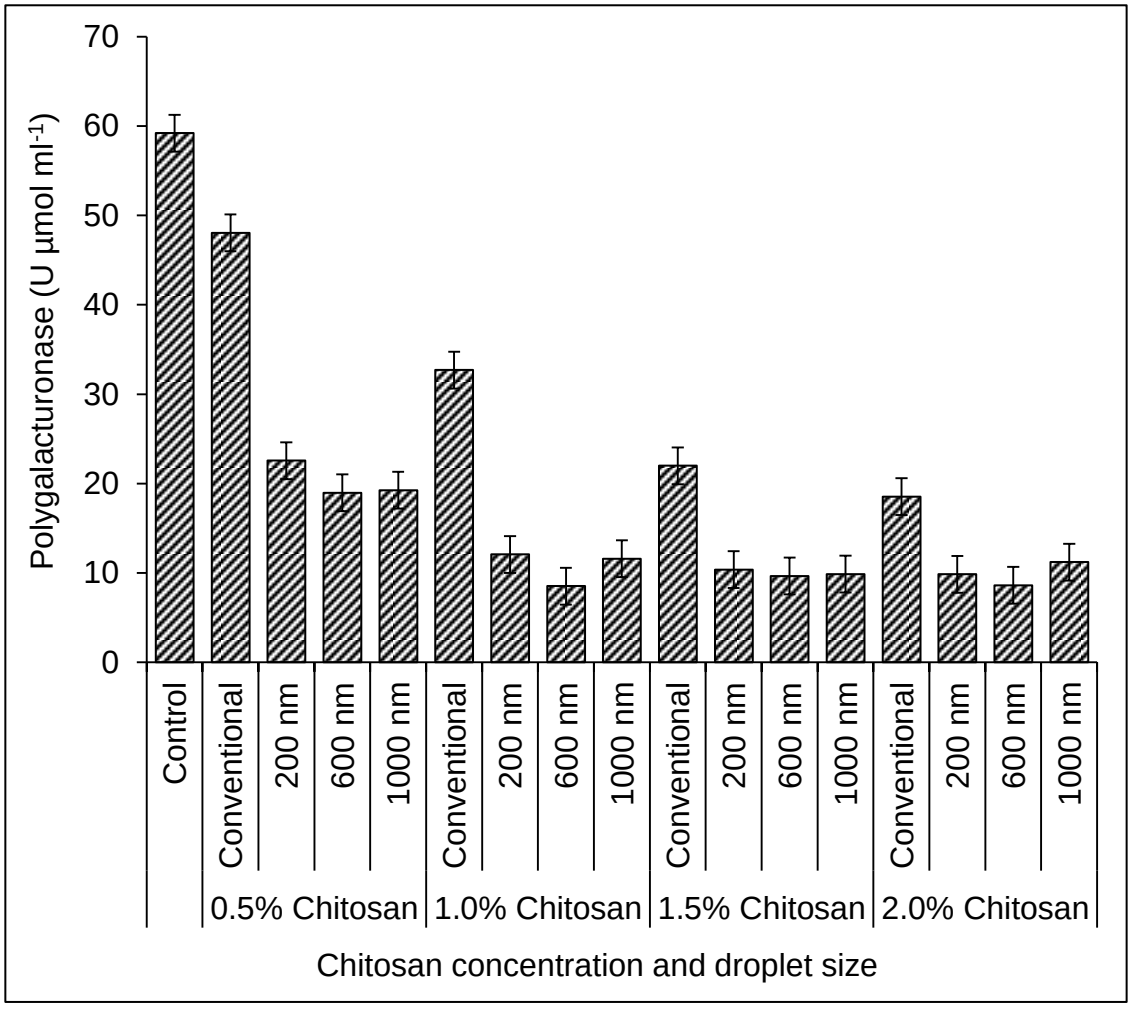


Figure 4.12: Effect of concentration of conventional chitosan and droplet size of submicron chitosan dispersions on polygalacturonase activity after 10 days of incubation. The vertical bars represent the standard error of means for four replicates.

Pectin lyase activity was significantly ($P < 0.05$, Appendix C 4.12) lower with all conventional chitosan and submicron chitosan dispersions as compared to the control (Fig. 4.13). The lowest activity was observed with 600 nm droplets of 1.0% submicron chitosan dispersions, however this did not differ from the enzyme activity with 600 nm and 1000 nm droplets of 1.5% and 2.0% submicron dispersions.

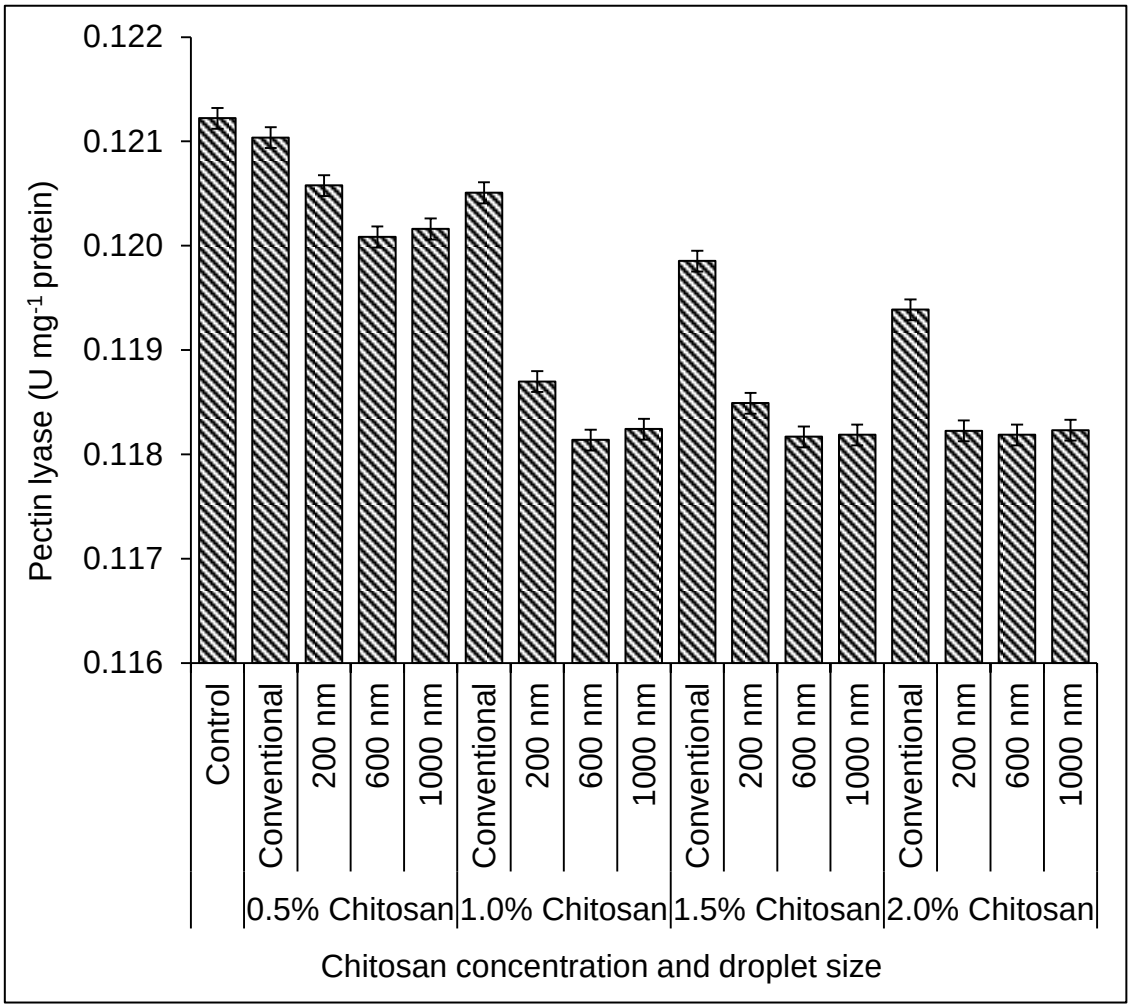


Figure 4.13: Effect of concentration of conventional chitosan and droplet size of submicron chitosan dispersions on pectin lyase activity after 10 days of incubation. The vertical bars represent the standard error of means for four replicates.

Pectin degrading enzymes have been recognized as major factors in necrotrophic pathogenesis (Schafer, 1994; Ikotun, 1984). Plant cell wall architecture is maintained by pectin substances and the degradation of other cell wall polymers is dependent on depolymerisation of pectin (Bauer *et al*., 1977). Degradation of pectin and other enzyme activities caused loss in turgor due to desiccation of cells in tomato (Tucker and Grierson, 1987). In the present study, less protein in the chitosan treated samples of the fungus suggests that there was a reduction in fungal growth, which eventually

reduced the production of macerating enzymes by *C. gloeosporioides*. These results support the previous findings of Reddy *et al*. (2000) which showed that chitosan treatments on *A. alternata* reduced the cell wall degrading activity. Polygalacturonase solubilized the pectin substances in plants and thus enabled the fungal appressoria to penetrate inside the cell wall (Bennett *et al*. 1993). However, the fungus in direct contact with chitosan showed less activity of pectin degrading enzymes, possibly due to solubilisation of polygalacturonase formed in the presence of chitosan also being reduced, as shown with the *Erwinia* system in potato (Reddy *et al*., 1997). Hence, reduction in the fungal virulence occurred as the signals required by the pathogen to produce toxins may not have been generated in the presence of chitosan.

Up till now there has been no study reported on the uptake of chitosan into the cells of microorganisms. However, in the present study, submicron chitosan dispersions at 1.0% with 600 nm droplets showed the best results in the parameters studied, which suggests that this size might permit penetration and release inside the fungus more easily. This submicron dispersion affected the morphology of the spores at various stages, reduced mycelial growth and dry weight and also inhibited growth of the spores. At this droplet size, the production of pathogenic factors by the fungus, such as cell wall degrading enzymes, was also reduced. In contrast, 200 nm droplets might also penetrate but the higher stability of smaller droplets could have deferred the release of chitosan and adversely affected its antifungal properties.

4.5.4 Effect of conventional chitosan and submicron chitosan dispersions on anthracnose of dragon fruit plants

4.5.4.1 Disease incidence

The initial symptoms of disease appeared in control plants and the plants treated with 0.5 and 1.0% conventional chitosan and submicron chitosan dispersions thirty days after inoculation. In plants treated with 1.5 and 2.0% conventional chitosan and submicron chitosan dispersions, the disease symptoms appeared sixty days after inoculation (Table 4.2). The higher concentrations of conventional chitosan and submicron dispersions formed a thick layer on the plant surface, which later either dropped off or was washed away with sprinkler water or rain.

Disease incidence reached up to 46.8% after ninety days in control plants. Significant differences ($P < 0.05$) in disease incidence were recorded between the plants treated with conventional chitosan and submicron chitosan dispersions (Table 4.3; Fig. 4.14, Appendix 4.13). The highest fungicidal effect occurred in plants treated with 600 nm droplets of 1.0% chitosan. Disease incidence recorded for 1.5 and 2.0% submicron chitosan dispersions was higher than for 1.0% dispersions.

Disease development was less in the 600 nm submicron chitosan dispersions at all the four concentrations tested based on the area under the disease progress curve (AUDPC). The AUDPC at the 90^{th} day after inoculation with *C. gloeosporioides* was lowest for 600 nm droplets of 1.0% chitosan and highest for control plants (Table 4.3).

Table 4.2: Effect of conventional chitosan and submicron chitosan dispersions on disease incidence (%) of dragon fruit plants.

Chitosan concentration	Droplet size (nm)	Day 0	Day 30	Day 60	Day 90
Control		0.00	7.033	29.03	46.80
	Conventional	0.00	4.833	24.76	43.03
0.5%	200	0.00	3.19	21.56	36.50
	600	0.00	1.80	21.23	29.56
	1000	0.00	3.15	23.50	36.36
	Conventional	0.00	3.05	19.33	36.26
1.0%	200	0.00	2.10	16.86	18.65
	600	0.00	1.55	8.76	15.46
	1000	0.00	2.73	15.26	17.86
	Conventional	0.00	0.00	15.50	31.73
1.5%	200	0.00	0.00	15.96	20.70
	600	0.00	0.00	14.93	18.53
	1000	0.00	0.00	15.06	21.70
	Conventional	0.00	0.00	15.10	30.86
2.0%	200	0.00	0.00	16.76	23.66
	600	0.00	0.00	15.90	20.73
	1000	0.00	0.00	16.40	22.80

Table 4.3: Effect of conventional chitosan and submicron chitosan dispersions on disease incidence (%) and area under disease progress curve (AUDPC) of dragon fruit plants, 90 days after inoculation with *C. gloeosporioides.*

Chitosan concentration	Droplet size (nm)	Disease incidence (%)	Total AUDPC (units per square)
Control		46.80 a	1783.89 a
	Conventional	43.03 b	1533.24 b
0.5%	200	36.50 c	1289.10 c
	600	29.56 d	1134.30 d
	1000	36.36 c	1326.30 bc
	Conventional	36.26 c	1205.70 cd
1.0%	200	18.65 f	848.55 f
	600	15.46 h	544.50 h
	1000	17.86 g	799.50 fg
	Conventional	31.73 d	940.95 e
1.5%	200	20.70 e	789.30 fg
	600	18.53 ef	725.85 g
	1000	21.70 e	777.30 g
	Conventional	30.86 d	915.90 ef
2.0%	200	23.66 e	857.70 f
	600	20.73 e	787.95 g
	1000	22.80 e	834.00 f

Values with different letters are significantly different according to ANOVA and Duncan's multiple range test (DMRT).

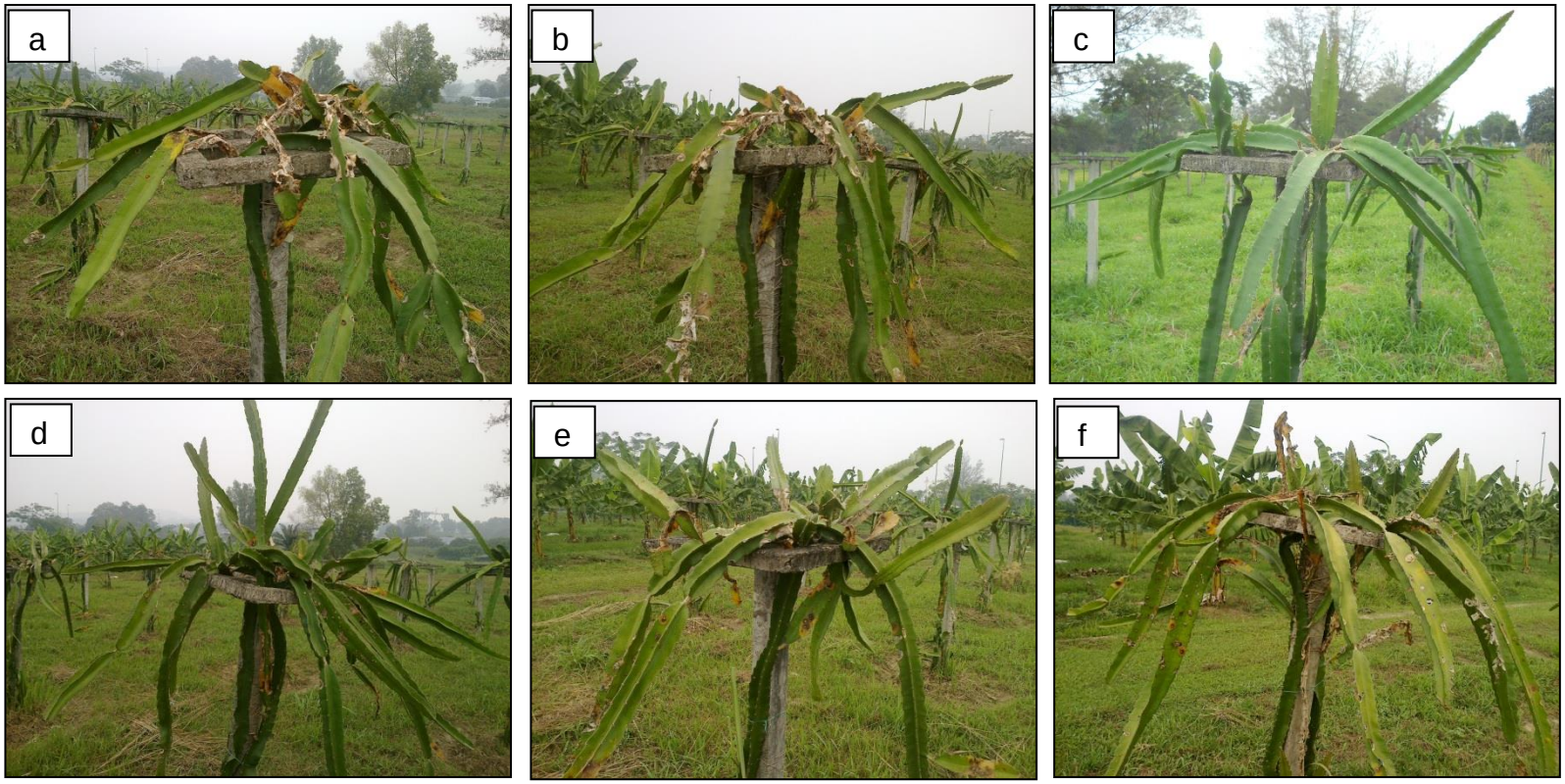


Figure 4.14: Dragon fruit plants inoculated with spore suspension of *C. gloeosporioides* and sprayed with conventional chitosan and submicron chitosan dispersions after 90 days of treatments. (a: Control; b: 600 nm droplets of 0.5% chitosan; c: 600 nm droplets of 1.0% chitosan; d: 600 nm droplets of 1.5% chitosan; e: 600 nm droplets of 2.0% chitosan; f: 2.0% conventional chitosan).

4.5.4.2 Disease severity

Disease severity was significantly ($P < 0.05$) reduced in all treatments compared with the control plants (Table 4.4, Appendix C 4.15). The highest fungicidal effect was found in plants treated with 600 nm droplets of 1.0% chitosan followed by the plants treated with 600 nm droplets of 1.5% chitosan 90 days after inoculation.

The fungicidal activity of chitosan and submicron chitosan dispersions has been clearly shown in *in vitro* as well as *in vivo* experiments. Chitosan has been found to induce a delay in appearance of disease, leading to a reduction in plant wilting (Benhamou *et al*., 1994). The polycationic nature and molecular weight of chitosan are the major factors in its fungicidal properties (Hirano and Nagao, 1989). The effect of chitosan has also been documented in several pathosystems which include numerous plant species and a wide range of pathogens comprising fungi, bacteria, viruses and nematodes.

In a recent study by Reglinski *et al*. (2010), efficacy of chitosan was assessed against *B. cinerea* in a vineyard. Plants treated with chitosan showed more vigour and disease incidence was reduced by up to 84.2%. Chitosan has also been shown to possess antifungal activity against downy mildew (Aziz *et al*., 2006) and *F. oxysporium* f. sp. *lycopersici* of tomato seedlings (Benhamou and Thériault, 1992). Disease suppression has also been observed in cucumber and groundnut against *B. cinerea* and *P. arachidis* (Sathyabama and Balasubramanian, 1998; Ben-Shalom *et al*., 2003).

Table 4.4: Effect of conventional chitosan and submicron chitosan dispersions on disease severity (score) and disease severity (%) of dragon fruit plants 90 days after inoculation with *C. gloeosporioides.*

Chitosan concentration	Droplet size (nm)	Disease severity (score)	Disease severity (%)
Control		3.26 a	65.2 a
	Conventional	2.50 b	50.00 b
0.5%	200	2.23 cd	41.60 c
	600	2.13 de	27.69 ef
	1000	2.26 c	39.17 cd
	Conventional	2.26 c	42.10 c
1.0%	200	1.83 gh	34.16 d
	600	1.60 h	25.60 f
	1000	1.90 f	38.00 cd
	Conventional	1.86 gh	31.72 e
1.5%	200	1.90 f	30.40 e
	600	1.70 g	27.20 ef
	1000	1.93 f	33.40 d
	Conventional	2.13 de	42.60 c
2.0%	200	2.00 d	37.30 cd
	600	1.92 f	30.70 e
	1000	2.10 d	39.20 cd

Values with different letters are significantly different according to ANOVA and Duncan's multiple range test (DMRT).

Earlier studies have shown that various host defense mechanisms induced by chitosan are involved in controlling anthracnose (El Ghaouth *et al*., 1994 b). Induced resistance in plants has been enhanced by increasing the activity of some enzymes, such as chitinase and *β*-1,3-glucanase (Fajardo *et al*., 1998; Zhang and Quantick, 1998), and by increasing levels of phytoalexins (Reglinski *et al*., 2004), phenylalanine ammonia lyase (PAL) activity (Romanazzi *et al*., 2002) and total phenolics (Aziz *et al*., 2006).

In the present study, the conventional form of chitosan and all the submicron chitosan dispersions showed antifungal effects, however, submicron chitosan dispersions of 600 nm droplets at 1.0% concentration showed the most promising results in terms of delaying the onset of anthracnose on dragon fruit plants. Agglutination of conventional chitosan around the penetration sites on the plant tissues might act as a physical barrier to prevent the pathogen invading healthy tissues. This phenomenon is similar to abscission zones on leaves which inhibit the spread of some pathogens (El Hadrami *et al*., 2009). Secondly, hydrogen peroxide accumulates around the isolated zone and elicits the hypersensitive response which acts as an alarm signal for healthy plant tissues. This second phenomenon is due to the binding capacity of chitosan with several metals and initiation of the healing of wounds (Hirano *et al*., 1999).

It can be concluded from the present investigation that submicron chitosan dispersions have better fungicidal effects than using chitosan in a conventional form and, therefore, they could provide an alternative to synthetic fungicides for controlling anthracnose of dragon fruit plants.

CHAPTER 5

MECHANISM OF SUPPRESSION OF *Colletotrichum gloeosporioides*, THE CAUSAL ORGANISM OF ANTHRACNOSE IN DRAGON FRUIT PLANTS

5.1 Introduction

Dragon fruit (*Hylocereus polyrhizus*) is a commercial fruit crop, grown throughout tropical regions. Disease management is one of the most important components in the production system since this crop is prone to many fungal and bacterial diseases, such as stem and fruit rot, stem spots and cactus virus X. Anthracnose, caused by *Colletotrichum gloeosporioides,* has been reported to cause significant losses in the yield and quality of the crop (Masyahit *et al*., 2009) and, so far, chemical pesticides have been widely used to control this disease. The ecologically unnatural and unstable conditions of monoculture, cost of traditional pesticides as well as rising environmental risk due to xenobiotics have promoted studies on alternative strategies in crop protection.

Plants also possess complex structures and inducible reactions which can successfully protect them against pathogens (Trotel-Aziz *et al*., 2006). To detect possible pathogen attack these inducible reactions need the alarm signals, after which the plant generates a wide range of defense mechanisms which can be useful tools of protection against the assaulting pathogens. This plant defense mechanism could involve the production of defense-specific chemicals, such as jasmonates or salicylic acid or follow the stimulation of fatty acids and phenylpropanoid pathways. Plant components having antimicrobial properties, such as pathogenesis-related (PR) proteins and phytoalexins, have also been reported (Kombrink and Somssich, 1995). Over

the past decade, the use of exogenous elicitors to stimulate natural defense responses has become an important tool (Métraux *et al*., 1991). Some distinct oligosaccharides, such as oligomers derived from chitosan and chitin, algal or microbial *β*-1,3 glucans, have been shown to enhance non-host plant resistance against pathogens (Manjunatha *et al*., 2008; Côté *et al*., 1998; Roby *et al*., 1987).

The use of eco-friendly and biodegradable agents, such as chitosan, is gaining importance as an alternative safe means to manage pests and diseases, and to sustain productivity in fruit production systems (Gozzo, 2003). As an antimicrobial agent, chitosan has been tested against pathogens such as *A. alternata*, *B. cinerea*, *C. gloeosporioides* and *R. stolonifer* (Ali and Mahmood, 2008; El Goauth *et al*., 1992). However, variation in the extraction methods, as well as different sources of chitinous material, affect the functional properties and bioactivity of chitosan (Wu *et al*., 2004). Chitosan, a deacetylated derivative of chitin, induces various defense responses in several plants, including phytoalexin production in pea pods (Hadwiger and Beckman, 1980), synthesis of PR proteins in rice seedlings (Agrawal *et al*., 2002) and lignification of wheat leaves (Vander *et al*., 1998; Barber *et al*., 1989). Consequently, there may be inhibition of fungal growth and defense from further infection (Hadwiger and Beckman, 1980).

The main drawback of using this natural compound is its macromolecule with high viscosity, which reduces its penetration into tissues/cells and its antimicrobial properties (Tikhonov *et al*., 2006). Thus, the use of submicron dispersions of chitosan is a novel approach to its application for the control anthracnose of dragon fruit plants. The objective of this study was to

investigate the efficacy of submicron dispersions of chitosan on anthracnose disease of dragon fruit plants and on the production of inducible compounds and their possible mechanism in controlling anthracnose. In addition, the effect of submicron chitosan dispersions on growth responses of plants was determined.

5.2 Materials and methods

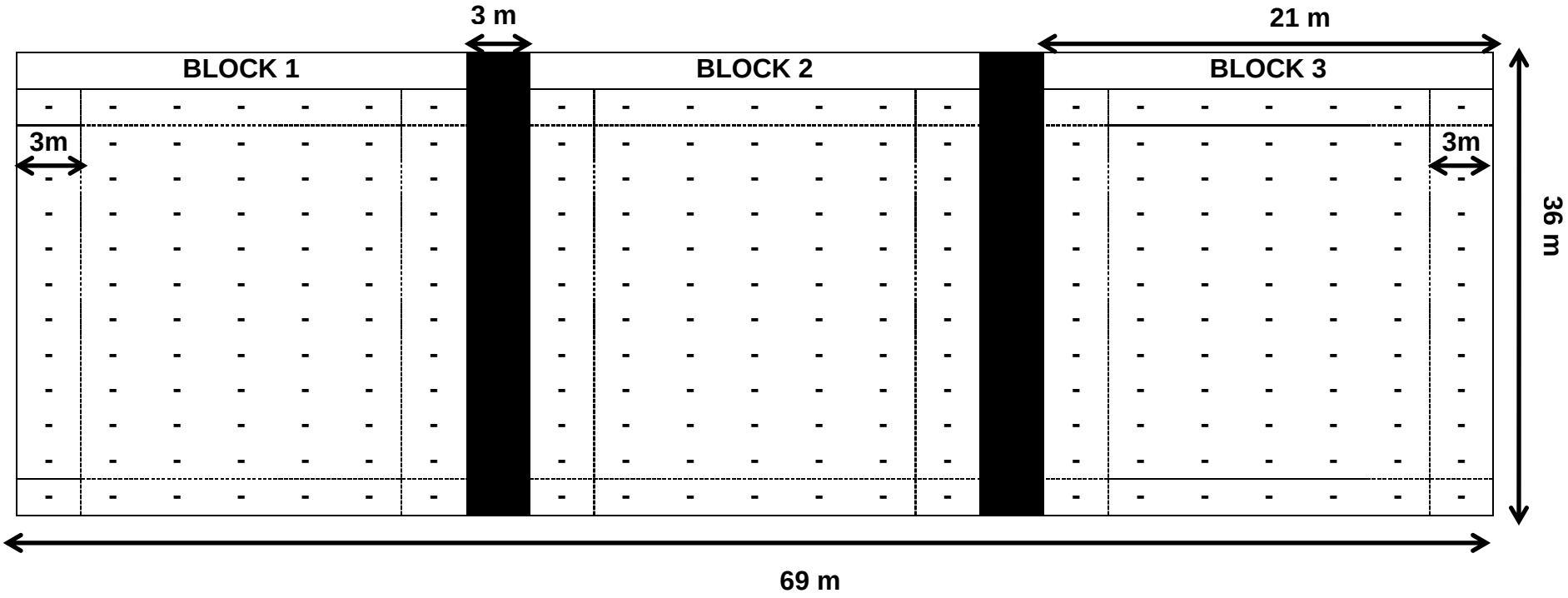
Conventional chitosan and different droplet sizes of submicron dispersions (200, 600 and 1000 nm) were prepared as described in Sections 3.2.4 and 3.3. The plant material and experimental site were prepared and managed as described in Section 4.4.1. The experimental site was Farm B1 at Taman Pertanian, University Putra Malaysia, Serdang, Malaysia.

The size of the experimental site was 69 x 36 metres with a pole system already installed. Two trials were carried out in order to ensure the reproducibility of results. The first trial was from December 2011 to September 2012 and the second from February 2012 to November 2012. Extra plants were planted on all four sides of the field in order to minimize the border effect. Five treatments (untreated control, 1.0% conventional chitosan and 200 nm, 600 nm and 1000 nm submicron chitosan dispersions) were selected after the preliminary field study (Section 4.5.4) and were evaluated based on eight parameters, including disease incidence (DI%), lignification, total phenolic compounds, peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL), *β*-1,3-glucanase and chitinase activities. Plants were inoculated artificially by using the tooth pick method (Section 4.4.2). The plants were sampled for analysis of enzymes on day 0 and then at intervals of 3 days until day 30.

The experiments were arranged in a randomized complete block design (RCBD) with three replicates of ten plants. Data were subjected to analysis of variance (ANOVA) and tested for significant differences among treatments by Duncan's Multiple Range Test (DMRT) at $P < 0.05$ using SAS® software (Appendix C 5.1 – C 5.35).

5.1 FIELD LAYOUT FOR PLANT AND FOR ENZYME ANALYSIS

Total number of treatments = 5, No of plants for each treatment = 10
No of replications = 3, Total number of plants = 150
Plant to plant distance = 3 m
No of rows = 12
No of columns = 21
Area of plot = 69 x 36 m Path



5.2.1 Determination of disease incidence and area under disease severity curve

Disease incidence (DI %) was recorded at 3 day intervals for one month as described in Section 4.2.2. AUDPC was calculated using the formula in Section 4.4.3 to evaluate the efficacy of the treatment.

5.2.2 Determination of lignin compounds

Lignin compounds were extracted using thioglycolic acid (TGA) with slight modification of the method given by Bruce and West (1982). Briefly, 2 g of stem tissues were homogenized in 20 ml of 80% methanol. The mixture was then filtered with Whatman filter paper no. 4. The residues were washed with 80% methanol and then dried at 60 °C for 24 hours. Dried insoluble residue served as the sample and was used for lignin determination. The reaction mixture contained 5 ml of 2N HCl and 0.5 ml of thioglycolic acid (TGA). The reaction mixture was mixed with 50 mg of sample in glass vials. The mixture was boiled in a water bath for 4 hours and then centrifuged at 10,000 x *g* for 30 min. The supernatant was drained and the resulting pellets were washed in 5 ml of distilled water. The pellets were then suspended in 5 ml of 0.5 N NaOH and shaken on an orbital shaker for 2 hours at 25 °C followed by 30 min of centrifugation at 10,000 x *g*. The supernatant was mixed with 1 ml of lignin-thioglycolic acid and 12N HCl followed by precipitation at 4 °C for 4 hours. The mixture was centrifuged for 10 min at 10,000 x *g* and orange-brown pellets were obtained after discarding the supernatant. An aliquot of 10 ml of 0.5 N NaOH was used to dissolve these pellets and was centrifuged again for 10 min. The absorption of thioglycolic

acid was determined in the supernatant at 280 nm using a UV-Vis spectrophotometer (Varioskan Flash Multimode Reader, Thermo Fisher Scientific, USA). The results were expressed as increase in thioglycolic acid g^{-1} fresh weight (Mandal, 2010).

5.2.3 Penetration of conventional chitosan and submicron chitosan dispersions inside the plants and determination of total phenolic content

Bromocresol purple was used to dye the submicron emulsions of chitosan. The dye was prepared by adding 100 mg of bromocresol purple to 5 ml of ethanol (Abou-Shoer, 2010) and 20 ml was added per 100 ml of submicron chitosan dispersions. The solutions were sprayed on the dragon fruit plants and the plants were destructively sampled after three days. Ultra-thin slices of the stem were observed under a research microscope, and the images were captured using a camera (Nikon Eclipse 80i) attached to a light microscope at 20 x magnification.

The total phenolic content in dragon fruit plants was estimated by the method of Ramamoorthy and Bono (2007). Samples of stem (2 g) were homogenised with 10 ml of 80% (v/v) methanol and agitated for 15 min at 70°C. The crude enzyme extract (1 ml) was mixed with 5 ml of purified water and 250 µl of 1N Folin-Ciocalteau reagent and incubated for 5 min at room temperature (25 ± 3°C). Saturated sodium carbonate (1 ml of 7% Na_2CO_3) was added to the mixture, incubated for 1 h at 25°C followed by measurement of absorbance at 725 nm using a UV-Vis spectrophotometer (Varioskan Flash Multimode Reader, Thermo Fisher Scientific, USA). A blank

reagent was prepared and the total phenolic content in the extract was measured using a standard curve (Appendix B 5.1) from freshly prepared gallic acid in 80% ethanol. The results were stated in µg of gallic acid equivalent per gram fresh weight of plant sample.

5.2.4 Determination of enzymatic activities

5.2.4.1 Extraction of peroxidase and polyphenol oxidase

A crude enzyme extract was prepared by the method described by Liu *et al*. (2007) with minor modifications. Plant samples (2 g) from each treatment were homogenised at 4°C in 10 ml sodium phosphate buffer (100 mM, pH 6.4) containing 0.2 g of polyvinylpyrrolidone (PVP) using a mortar and pestle. The homogenized samples were centrifuged for 30 min at 12,000 × *g* and 4°C. The activity of peroxidase and polyphenol oxidase enzymes was determined in the supernatant obtained after centrifugation.

5.2.4.1.1 Assay of peroxidase (PO) activity

The activity of peroxidase was determined by the oxidation of guaiacol to tetraguaiacol in the presence of H_2O_2, which results in the development of a brown colour. Crude extract (0.1 ml) was mixed with 2 ml of O-methoxyphenol (guaiacol) in 100 mM sodium phosphate buffer (pH 6.4) followed by incubation at 30°C for 30 min, then 1 ml of hydrogen peroxide (24 mM) was added. The increase in absorbance was monitored at 470 nm using a UV-Vis spectrophotometer (Varioskan Flash Multimode Reader, Thermo Fisher Scientific, USA) for one minute. The PO activity was expressed as U

g^{-1} fresh weight (Liu *et al.,* 2007) where one unit is defined as the difference in absorbance at 470 nm per gram fresh weight per minute.

5.2.4.1.2 Assay of polyphenol oxidase (PPO) activity

PPO activity was determined by measuring the conversion of catechol to quinone mediated by PPO. Crude enzyme extract (0.1 ml) was added in catechol substrate (3 ml). Catechol substrate was prepared by adding 500 mM of catechol to 100 mM sodium phosphate buffer pH 6.4, and the increase in absorbance was measured at 398 nm using a spectrophotometer (Varioskan Flash Multimode Reader, Thermo Fisher Scientific, USA) for 3 minutes. The PPO activity was expressed as U g^{-1} fresh weight, where one unit is defined as an increase of activity at OD_{398} per gram fresh weight per 30 minutes (Liu *et al.,* 2007).

5.2.4.2 Extraction and assay of phenylalanine ammonia-lyase (PAL) activity

A crude enzyme extract was prepared by the method described by Morelló *et al*. (2005) with minor modifications. Plant samples (1 g pericarp tissue) from each treatment were homogenised for 30 sec at 4°C in 25 ml of 0.05 M potassium phosphate buffer (pH 6.6) containing 0.2 g of Triton X-100 using a mortar and pestle. Polyvinylpolypyrrolidone (PVPP) (25 mg) was added and the suspension was centrifuged for 30 min at 12,000 × *g* and 4°C. The supernatant was kept on ice, filtered through glass wool and used as a source of crude enzyme.

PAL activity in the crude enzyme extracts was assayed by the method of Zucker (1965) as reported by McCallum and Walker (1990). The assay mixture consisted of 0.06 M sodium borate buffer (4.1 ml; pH 8.8) and crude enzyme (0.4 ml) and the reaction was initiated by adding 1 ml of 10 mg ml^{-1} (final concentration 11 mM) L-phenylalanine. The tubes were incubated at 37°C for 1 h. The reaction was stopped by adding 0.5 ml of 35% (w/w) trifluoroacetic acid (TFA) and the tubes were centrifuged for 5 min at 5000 × *g*. The cinnamic acid yield was estimated by measuring the absorbance at 290 nm of the supernatant using a spectrophotometer (Varioskan Flash Multimode Reader, Thermo Fisher Scientific, USA). One unit of PAL activity is defined as the formation of 1 mmol cinnamic acid per hour.

5.2.4.3 Extraction of *β*-1,3-glucanase and chitinase

A crude enzyme extract was prepared using the method given by Zheng *et al*. (2011). Plant samples from each treatment were homogenized with 0.1M acetic acid buffer (pH 5.0) and centrifuged at 10,000 x *g* and 4 °C for 5 min. The supernatant was the enzyme extract.

5.2.4.3.1 Assay of *β*-1,3-glucanase activity

The activity of *β*-1,3-glucanase was assayed using laminarin as a substrate (Siefert *et al*., 1994). The assay mixture consisted of 100 µl of crude enzyme mixed with 50 µl of 0.4% laminarin, followed by incubation for 1 hour at 37 °C. One ml of 1.0% 3,5-dinitrosalicylic acid was added and the reaction mixture was boiled for 5 min to stop the reaction, followed by immediate incubation on ice. The increase in absorbance was measured at

500 nm using a UV-Vis spectrophotometer (Varioskan Flash Multimode Reader, Thermo Fisher Scientific, USA) for 3 min. The *β*,1-3-glucanase activity was measured as the amount of reducing glucose released from laminarin using glucose as a standard and determined from the standard curve described in Section 4.3.1.2. Enzyme activity is expressed as U g^{-1} fresh weight, where one unit is defined as the formation of 1 µmol glucose released from laminarin per hour under these assay conditions.

5.2.4.3.2 Assay of chitinase activity

Chitinase activity was measured using the method described by Tonon *et al*. (1998). An aliquot (600 µl) was mixed with 6 mg of glycol chitosan and 1 ml of 0.3 M sodium acetate buffer (pH 5.2) and shaken at 50 rpm for half an hour. The mixture was then incubated at 37 °C for 2 hours followed by centrifugation at 12,000 x *g* for 20 min. The supernatant (500 µl) was mixed with 2 ml of 0.6 M sodium borate buffer and heated at 100 °C for 7 min. After cooling to room temperature, 1 ml of Erlich's reagent diluted with glacial acetic acid (1:8 v/v) was added and the mixture was incubated at 37 °C for 2 hours. The absorbance was measured at 540 nm using a UV-Vis spectrophotometer (Varioskan Flash Multimode Reader, Thermo Fisher Scientific, USA) for 3 min. A standard curve was prepared using *N*-acetyl-*D*-glucosamine (Appendix B 5.2). Chitinase activity was expressed as U g^{-1} fresh weight, where one unit is defined as 10^{-9} mol *N*-acetyl-*D*-glucosamine produced per hour under these assay conditions.

5.2.4.4 Determination of vegetative growth of dragon fruit plants

The number and length of new shoots per cutting was recorded after the main stem reached the height of the pole.

Stem diameter of the main stem was measured using a digital calliper (CD-6C, Mitutoyo).

5.2.4.5 Chlorophyll content

Chlorophyll was measured using the method described by Ali *et al*. (2012) with slight modifications. Stem samples (1 g) were ground with 10 ml of 80% chilled acetone in a mortar and pestle. The extract was centrifuged for 15 min at 1157 x *g*. The absorbance of the supernatant was determined at 646.6, 663.6 and 750 nm using a UV-Vis spectrophotometer (Varioskan Flash Multimode Reader, Thermo Fisher Scientific, USA) for 3 min. Readings at 646.5 and 663.6 were corrected by subtracting light absorption at 750 nm. Chlorophyll contents were measured using the following equations:

$$\text{Chlorophyll a } \left(^{g}/_{ml}\right) = 12.25 \times A_{663.6} - 2.55 \times A_{646.6}$$

$$\text{Chlorophyll b } \left(^{g}/_{ml}\right) = 20.31 \times A_{646.6} - 4.91 \times A_{663.3}$$

$$\text{Total chlorophyll } \left(^{g}/_{ml}\right) = 17.76 \times A_{646.6} + 7.34 \times A_{663.6}$$

Where:

12.25, 2.55, 20.31, 4.91, 17.76 and 7.34 = constant

$A_{663.6}$ = absorbance at wavelength 663.6

$A_{646.6}$ = absorbance at wavelength 646.6

5.3 Results and discussion

5.3.1 Disease incidence

On the basis of symptomatic lesions, disease development was recorded for 30 days on the plants inoculated with *C. gloeosporioides*. The disease started 9 days after inoculation. Significant ($P < 0.05$) differences were observed in the plants treated with chitosan and submicron chitosan dispersions and the untreated controls (Fig. 5.1, Appendix C 5.1, 5.2). Disease incidence increased with time in all the plants and reached 7.03 % in the control plants after 30 days. In contrast, plants treated with 600 nm submicron chitosan dispersions showed the least symptoms 30 days after inoculation which were significantly ($P < 0.05$) different from all other treatments.

The area under the disease progress curve (AUDPC) was measured 30 days after inoculation with *C. gloeosporioides* (Table 5.1). Disease development was highest in the control plants and the lowest AUDPC was recorded for 600 nm droplets.

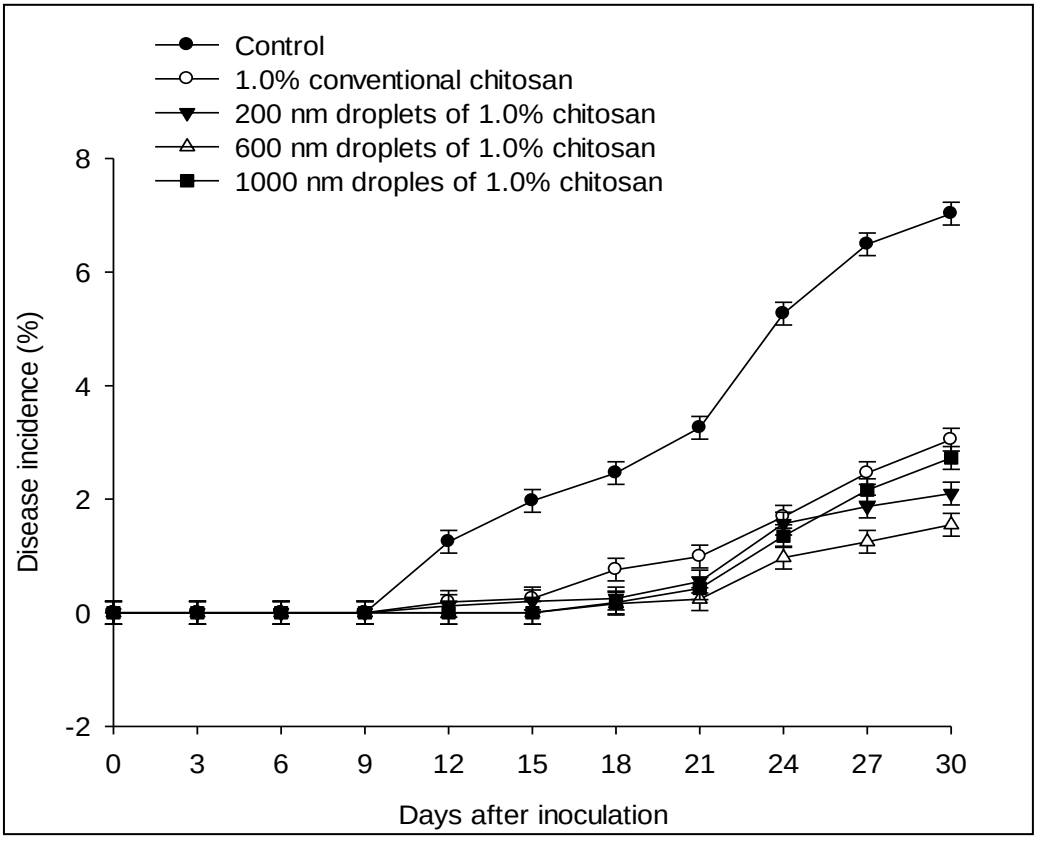


Figure 5.1: Disease incidence in dragon fruit plants treated with conventional chitosan and submicron chitosan dispersions after inoculation with *C. gloeosporioides*. Each value represents a mean of 3 replicates and vertical bars indicate standard errors.

Table 5.1: Effect of conventional chitosan and submicron chitosan dispersions on disease severity (%) and area under disease severity curve (AUDPC) of anthracnose on dragon fruit plants 30 days after treatment.

Treatment	Disease severity (%)	AUDPC (units square^{-1})
T1	11.3 a	105.495 a
T2	8.00 b	3.4534 b
T3	6.40 c	2.8665 c
T4	6.02 d	1.7430 e
T5	8.20 b	2.0418 d

Values with different letters are significantly different at $P < 0.05$.

T1 = Control; T2 = 1.0% conventional chitosan; T3 = 200 nm droplets of 1.0% chitosan; T4 = 600 nm droplets of 1.0% chitosan; T5 = 1000 nm droplets of 1.0% chitosan.

5.3.2 Effect of conventional chitosan and submicron chitosan dispersions on lignin induction

A general increase in lignin content was recorded in all the treatments on the 9th day after inoculation (Fig. 5.2). The highest increase was observed in plants treated with 600 nm droplets 24 days after treatment which was significantly different ($P < 0.0001$; Appendix C 5.4) from the control. A highly significant difference (Appendix C 5.5) was observed between untreated control and treated plants (Fig. 5.2b). Lignin content was highest in the plants inoculated with *C. gloeosporioides* on day 21 after treatment with 600 nm droplets. A negative correlation was observed between lignin content and disease incidence (Fig. 5.3). The data suggests that, with the increase in lignin content of 3.5 g, disease incidence becomes less than 4% regardless of treatment. From the r^2 value $\{(-0.317)^2 \times 100\}$, the contribution of lignin induction to variation in disease in uninoculated plants was 10%. In contrast, the r^2 value $\{(-0.393)^2 \times 100\}$ in the plants inoculated with *C. gloeosporioides* suggested that, with the increase in lignin content, the incidence of disease decreased by 15.4%. The higher r^2 value in the inoculated plants suggests a curative effect of chitosan against anthracnose.

Lignin is a complex polymer of phenylpropanoids and is primarily deposited in cell walls (Whetten and Sederoff, 1995). Lignification occurs in response to infection by microorganisms and mechanical wounding in many plants (Vance *et al*., 1980). Lignin deposition is known to act as a physical barrier to further invasion of a pathogen inside the host plant (Ride, 1975).

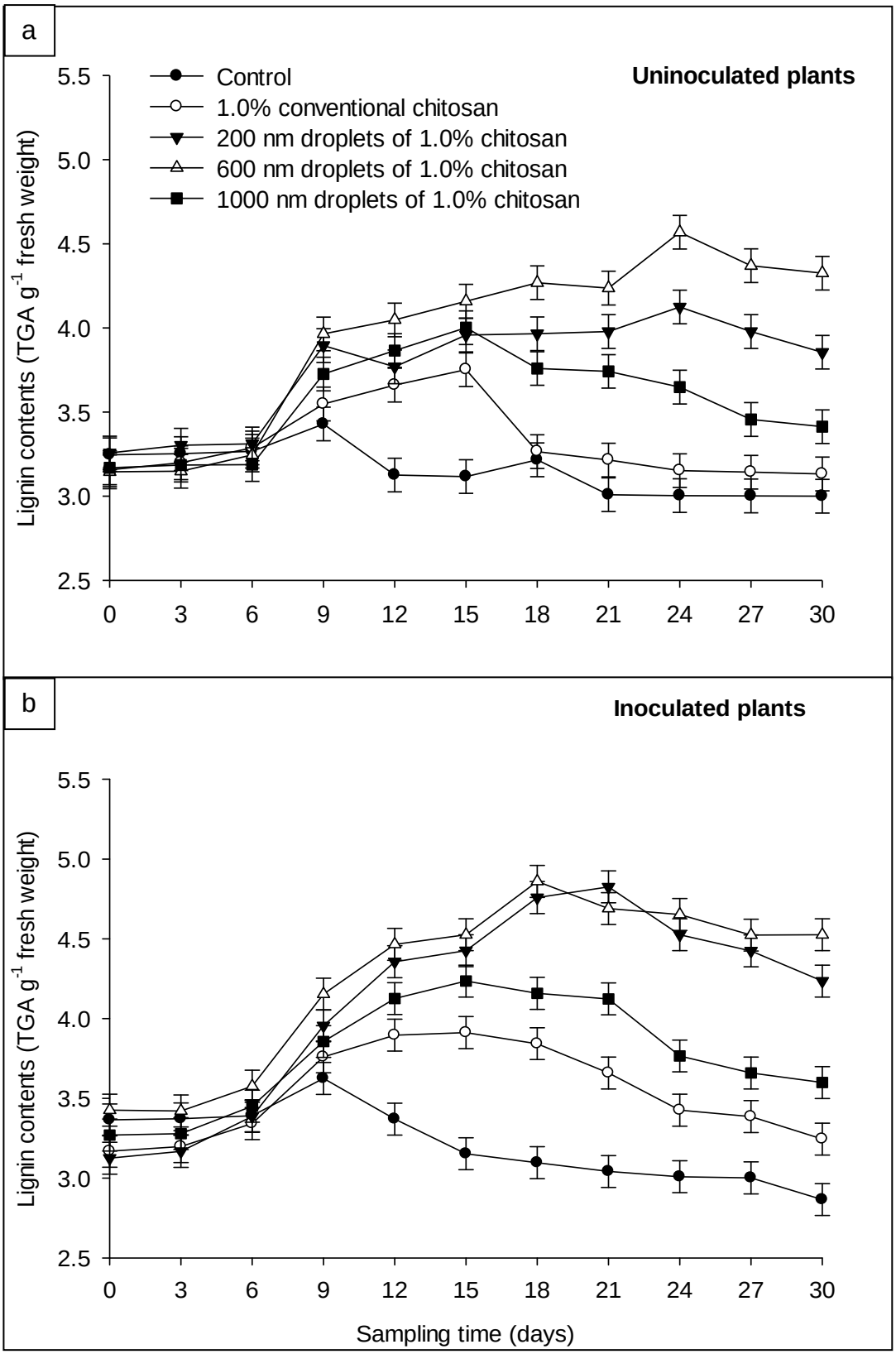


Figure 5.2: Lignin content of dragon fruit plants treated with conventional chitosan and submicron chitosan dispersions after inoculation with *C. gloeosporioides*. Each value represents a mean of three replicates and vertical bars indicate standard errors.

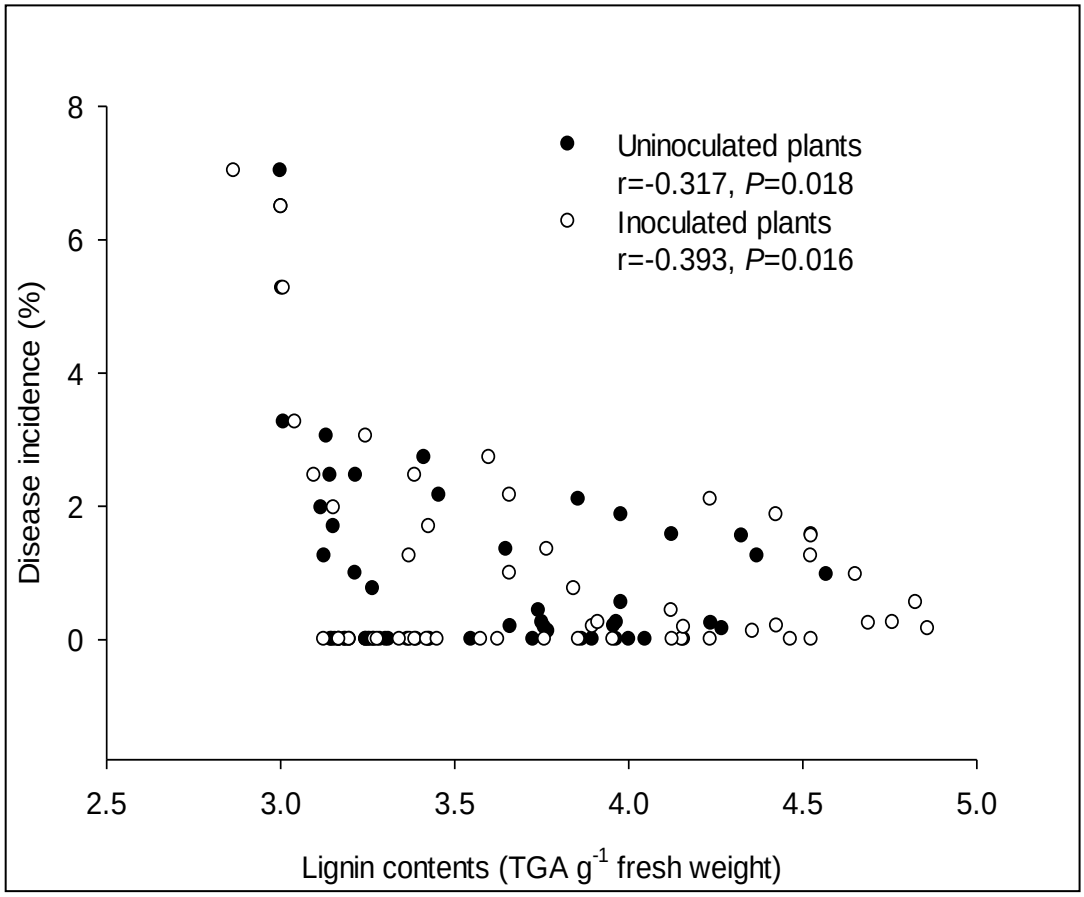


Figure 5.3: Relationship between lignin content and disease incidence (%) in dragon fruit plants.

High lignin content in the treated dragon fruit plants (Fig. 5.1) suggested that they may have acquired some sort of tolerance against *C. gloeosporioides* through cell wall reinforcement by the deposition of lignin (Mandal, 2010).

Chitosan is an active elicitor in defense related lignification in wounded and intact leaves of wheat (Gotthardt and Grambow, 1992). It elicits phenolic compounds which are deposited in the cell wall and form ether-/ester-link of polysaccharides or polymerized into lignin (Lewis and Yamamoto, 1990). These cross linked cell wall polysaccharides serve as a backbone matrix of plants. The localized response of plants to fungal and bacterial attack, such as lignification, involves a multiple matrix of components which includes

phenolics and proteins (McLusky *et al*., 1999). In the present study, the increase in lignin content in response to chitosan treatment is significant in respect to cross linking of phenolic acids in cell wall material. The increase in total phenolics resulted in increased deposition of lignin in plants (Mandal, 2010).

Increase in lignification was significant in wheat plants after chitosan treatments which reduced infection by *Fusarium graminearium* (Reddy *et al*., 1999). Previous studies showed that the lignified cells are more resistant to cellulase and pectinase enzymes secreted by fungi (Ride and Pearce, 1979). Biochemical studies showed that lignin content increased with the increase of key enzymes such as phenyl ammonia lyase (PAL) during the expression of resistance to various fungi (Tiburzy and Reisener, 1990).

5.3.3 Penetration of chitosan inside dragon fruit plants and its effects on total phenolic

Green colour pigments were visible in the stem samples of the control plants (Fig. 5.4a). The stem samples treated with 200 and 600 nm submicron droplets showed large patches of purple blue pigments of dyed chitosan inside the plants (Fig. 5.4c, 5.4d). In contrast, plants treated with 1000 nm droplets and 1.0% conventional chitosan solutions showed very small purple brown patches (Fig. 5.4b, 5.4e). This indicates that smaller sized droplets enter the plants more effectively than larger droplets and conventional chitosan. This lower permeation inside the plants may be due to the higher viscosity of the chitosan solution or to incompatibility with the pore size of the cell wall.

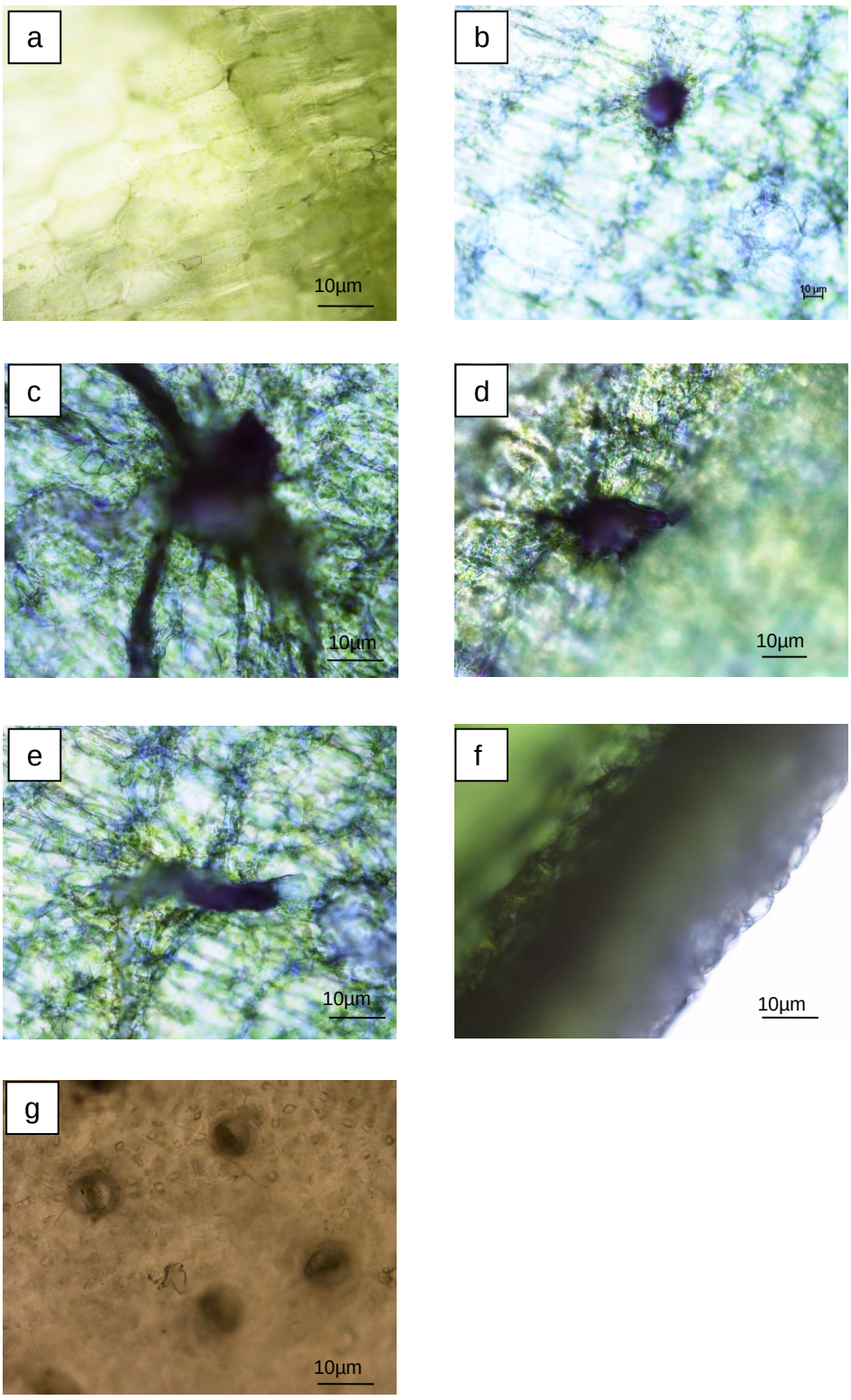


Figure 5.4: Visualization of dye-binded conventional chitosan and submicron chitosan dispersions inside stems of dragon fruit plants. Dye absent in a) control but visible in plants treated with b) 1.0% conventional chitosan, c) 200 nm droplets of 1.0% chitosan, d) 600 nm droplets of 1.0% chitosan and e) 1000 nm droplets of 1.0% chitosan. Stem f) showing accumulation of dye at the edge and g) stomata on the surface.

The penetration of chitosan inside plants is still unclear. There is no previous study supporting the uptake and movement of chitosan inside the plant stem. It may penetrate through the plant epidermis, as the edge of stems showed more purplish coloured cells (Fig 5.4f), or through natural openings (stomata) (Fig 5.4 g). Further studies would be required in order to untangle the mechanism behind binding of the dye with chitosan and also its penetration and movement inside the plants.

The total phenolic content increased with the progress of disease in all the plants. There was a significant interaction between treatments and days for total phenolics in inoculated (Appendix C 5.6) and uninoculated plants (Appendix C 5.7). An increase was observed on day 9 after treatment in all the plants which was significantly ($P < 0.05$) different from the control plants (Fig. 5.5a). A large increase in total phenolics was observed in the inoculated plants. The phenolic content in plants increased gradually from day 9 until day 12, but decreased gradually after day 18 with the progression in disease in the plants treated with 1.0% conventional chitosan and 200 nm submicron chitosan dispersion (Fig. 5.5b). The plants treated with 600 nm droplets showed an increase in total phenolics until day 27 and remained constant until the end. A negative correlation was observed in total phenolic content and disease incidence (Fig. 5.6), which suggested that an increase in 0.2 units of total phenolics resulted in a 2% decrease in disease incidence. The r^2 value showed that the total phenolics resulted in 2.7% decrease in disease in uninoculated plants and 11% decrease in inoculated plants. The differences in the onset of phenolics in uninoculated and inoculated plants suggested that the higher concentration of total phenolics may be allied with

less disease. A negative correlation between phenol levels and the degree of disease resistance has been reported previously (Schlosser, 1980).

There is no information available on the antifungal effects of submicron chitosan dispersions. In the present study, it was thought that the increased amount of total phenols with 600 nm droplets was due to penetration of chitosan inside the plants through natural openings and more effective triggering of the enzymes (Fig. 5.4g). While 200 nm droplets can also penetrate inside the plant, the amount of chitosan in the droplet may not be enough to trigger the plant defense mechanism. Stability of smaller droplet size could be another factor contributing to a slower release of chitosan from the 200 nm droplets and consequent failure to stimulate the plant defense mechanism.

There are several reports which have shown that chitosan has some eliciting properties (Manjunatha *et al*., 2008; Liu *et al.,* 2007; Ali, 2006; Bautista-Baños *et al.,* 2006; Ben-Shalom *et al.,* 2002). Growth of a pathogen can be restricted or slowed down when high levels of phenolic compounds accumulate at the site of pathogen invasion (Reimers and Leach, 1999). El Ghaouth *et al*. (1994b,c) found that there was some elicitation of disease resistance responses by chitosan in tomato and bell pepper. This may be due to the induction of various enzymes, the formation of physical barriers and plugging of intercellular spaces with antifungal phenolic-like compounds. Liu *et al.* (2007) reported the induction of phenolic compounds in chitosan treated tomato plants.

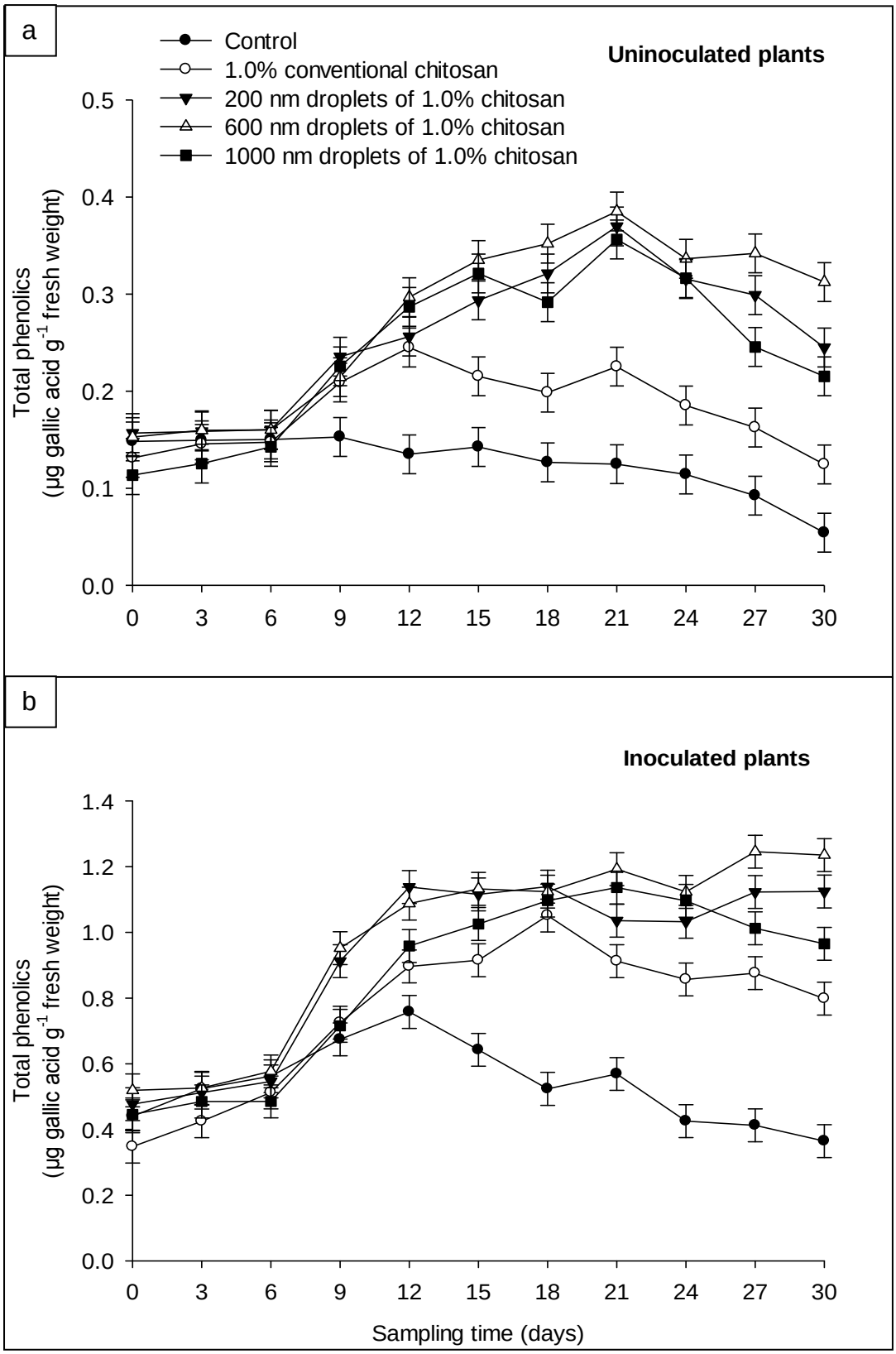


Figure 5.5: Total phenolic content in a) uninoculated dragon fruit plants and b) plants inoculated with *C. gloeosporioides* after treatment with conventional chitosan and submicron chitosan dispersions. Each value represents a mean of three replicates and vertical bars indicate standard errors.

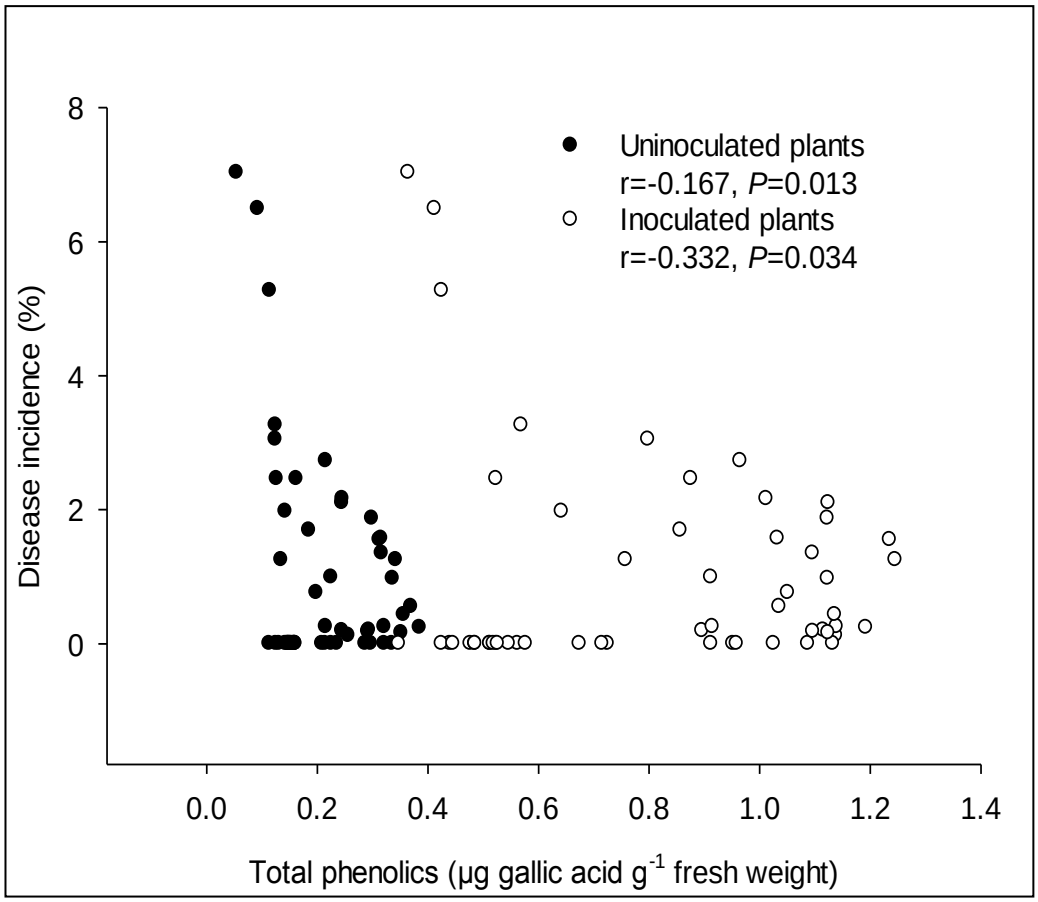


Figure 5.6: Relationship between total phenolics and disease incidence in dragon fruit plants.

The accumulation of phenolic compounds to enhance resistance of plants against pathogens with the use of chitosan has been reported in tomato and Chinese water chestnut (Liu *et al.,* 2007; Pen and Jiang, 2003).

Enzyme activities and accumulation of phenols are linked with biochemical defense mechanisms. The metabolism of phenolic compounds also involves oxidative enzymes, such as PPO and POD, which catalyze the oxidation of phenols to quinones (Campos-Vargas and Saltveit, 2002). It is also possible that an increase in phenolic metabolism may be induced by the action of other enzymes, such as PAL, which contribute to the biosyntyhesis of phenolic compounds (Rösler *et al*., 1997). In addition, the ability of chitosan to induce phenolic compounds is allied with its acetylation and

polymerization and with the physiology of the plant. Further study at the molecular level is required to elucidate the mechanism of submicron chitosan dispersions against fungal pathogens.

5.3.4 Effect of conventional chitosan and submicron chitosan dispersions on enzymatic activities

5.3.4.1 Peroxidase (PO) activity

Significantly more PO activity ($P < 0.05$) was observed in treated as compared with untreated plants (Appendix C 5.8, 5.9). Plants treated with 200 nm and 1000 nm submicron chitosan dispersions showed a maximum increase in PO activity on day 21 (Fig. 5.7a) and on day 24 with 600 nm submicron chitosan dispersions. Plants inoculated with *C. gloeosporioides* showed higher PO activity. There was a constant decrease over time in PO activity in control plants ($P < 0.05$) (Fig. 5.7b). The PO activity was negatively correlated with disease incidence (Fig. 5.8). The data suggests that regardless of treatment, with an increase in 0.1 U g^{-1} of PO activity disease incidence reduced to 3%. From the r^2 value $\{(-0.182)^2 \times 100\}$, the contribution of PO to variation in disease in uninoculated plants was 3%. While the r^2 value $\{(-0.295)^2 \times 100\}$ in the inoculated plants suggested that with the increase in PO activity the disease incidence decreased by 8.7%. The higher r^2 value in the inoculated plants suggests that chitosan induced resistance against anthracnose.

Chitosan being a polycationic polymer may interact with negatively charged phospholipids, upsetting the plasma membrane integrity of plant cells and, consequently, increasing the elicitor activity (Amini, 2009; Eikemo *et al*., 2003; Paz-Lago *et al*., 2000). Production of peroxidases in plant

tissues is dependent on environmental conditions, possibly the timing of the treatment, methods of assay and the choice of substrate (Reuveni, 1995). Induced resistance is a common response of a host plant when inoculated or treated with avirulent or non-pathogenic isolates or chemicals, prior to or associated with inoculation of a pathogen (Xue *et al*., 1998). The involvement of PO has been implicated in defense responses of many crop/pathogen interactions (Anguelova *et al*., 1999; Tonon *et al*., 1998; Hammerschmidt and Yang Cashman, 1995; Geiger *et al*., 1993; Khan *et al*., 2003; Agrawal *et al*., 2002; Kazana *et al*., 1998; Rybka *et al*., 1998). Peroxidases oxidize phenolics to quinones (Campos-Vargas and Saltveit, 2002) and, during this process, hydrogen peroxide is generated which is toxic to many pathogens (Peng and Kuc, 1992) and releases highly reactive free radicals (Mader *et al*., 1980).

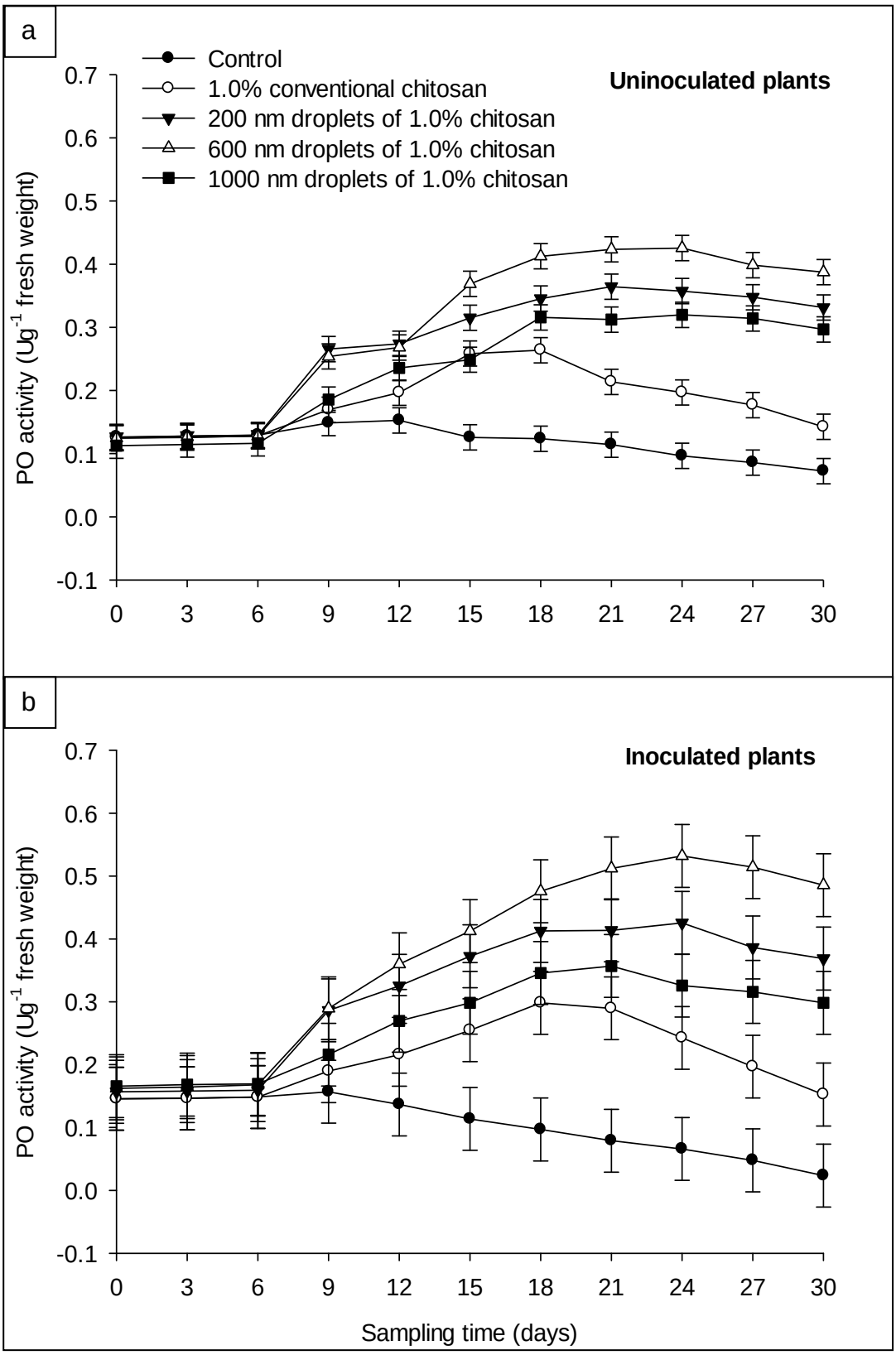


Figure 5.7: Peroxidase (PO) activity in a) uninoculated dragon fruit plants and b) plants inoculated with *C. gloeosporioides* treated with conventional chitosan and submicron chitosan dispersions. Each value represents a mean of three replicates and vertical bars indicate standard errors.

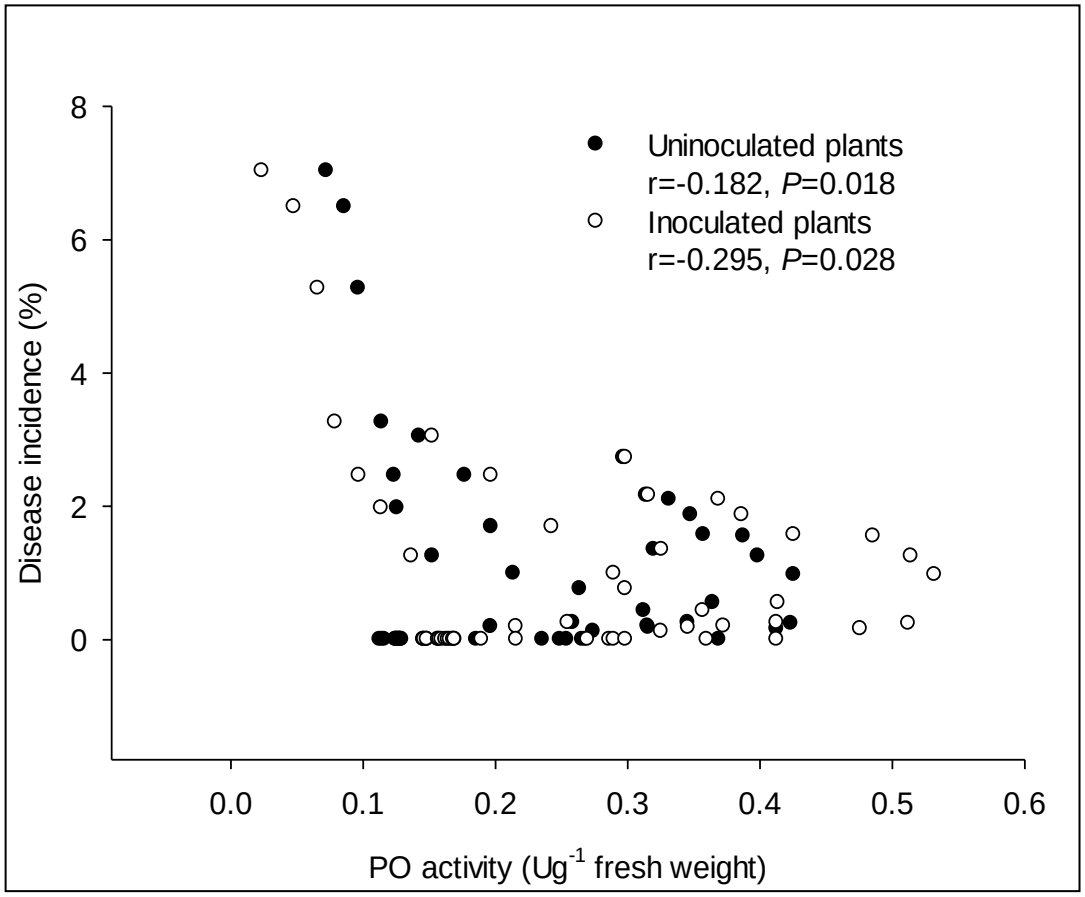


Figure 5.8: Relationship between PO content and disease incidence in dragon fruit plants.

Peroxidases also increase the rate of polymerisation of phenolic compounds into lignin-like substances which form barriers against pathogen invasion by being deposited in cell walls to inhibit their enzymatic digestion and restrict progress of the pathogen (Nandeeshkumar *et al*., 2008). Thus, they constitute a part of the host resistance mechanism (Amini, 2009; Khan *et al*., 2003; Paz-Lago *et al*., 2000). Okey *et al*. (1997) also demonstrated that high activity of PO in cacao clones constituted a part of their resistance mechanism against *Phytophthora palmivora* by enhancing phenol and lignin accumulation. It has also been shown biochemically (Mader *et al.*, 1980) and cytochemically (Hepler *et al*., 1972) that PO isozymes bound to the cell wall

play a role in lignification. Liu *et al*. (2007) found that the levels of PO increased significantly at 25°C and at 2°C in chitosan-treated tomato fruit.

PO has also been reported to be involved in many processes that lead to morphological changes associated with disease resistance (Dalisay and Kuc, 1995). Mayer (2006) observed that the susceptibility of plants to pathogens dramatically increased when there were lower amounts of PO in the plants. PO played a significant role in increasing the defense of a plant against pathogens.

Therefore, the current observation on the increased activity of PO suggests that it is one of the manifestations of resistance mechanisms induced by 600 nm droplets as compared to other treatments.

5.3.4.2 Polyphenol oxidase (PPO) activity

An initial increase in PPO activity occurred in all the treatments including the control (Fig. 5.9). Differences between the treated and untreated plants were significant ($P < 0.05$; Appendix C 5.10, 5.11) with the highest activity occurring in plants treated with 200 nm and 600 nm droplets of 1% chitosan. The plants treated with 1.0% conventional chitosan and 1000 nm submicron chitosan dispersions showed their highest PPO activity on or after day 18 after treatment (Fig. 5.9a). The activity of PPO was higher in all the treated plants that had been inoculated with *C. gloeosporioides* (Fig 5.9b). .

There was a negative correlation between PPO activity and disease incidence (Fig. 5.10). The value of r^2 $\{(-0.25)^2 \times 100\}$ showed that the increase of PPO activity resulted in a 6.25% decrease in disease in uninoculated plants and $\{(-0.395)^2 \times 100\}$ 15.6% decrease in inoculated plants. This indicates that 0.2 units increase in PPO activity lowered the

incidence of disease to 3% in uninoculated and inoculated plants regardless of the treatment.

Chitosan acts as an eliciting compound as it increases the activity of oxidative enzymes (Ben-Shalom *et al*., 2002). Oxidative enzymes, such as PPO and PO, are responsible for the oxidation of phenolic compounds into anti-microbial quinones in plant cells infected by phytopathogens, thus conferring disease resistance during the incompatibility reactions (Chittoor *et al*., 1990). The lower disease incidence in plants treated with 600 nm submicron chitosan dispersions may signify the expression of induced resistance (Ben-Shalom *et al*., 2002). The effectiveness in eliciting compounds involved in reducing disease incidence may indicate that the induced resistance can be effective, particularly in early stages of a disease (Amini, 2009; Eikemo *et al*., 2003; Hoffland *et al*., 1996).

The significance of PPO activity in disease resistance is its property to oxidize phenolic compounds to quinones, which are more toxic to pathogens than the original phenolics (Campos-Vargas and Saltveit, 2002). A high activity of PPO reported in wheat clones against *F. graminearum* was considered to be a mechanism of induced resistance by the induction of phenols and lignin accumulation (Falcón-Rodríguez *et al*., 2009).

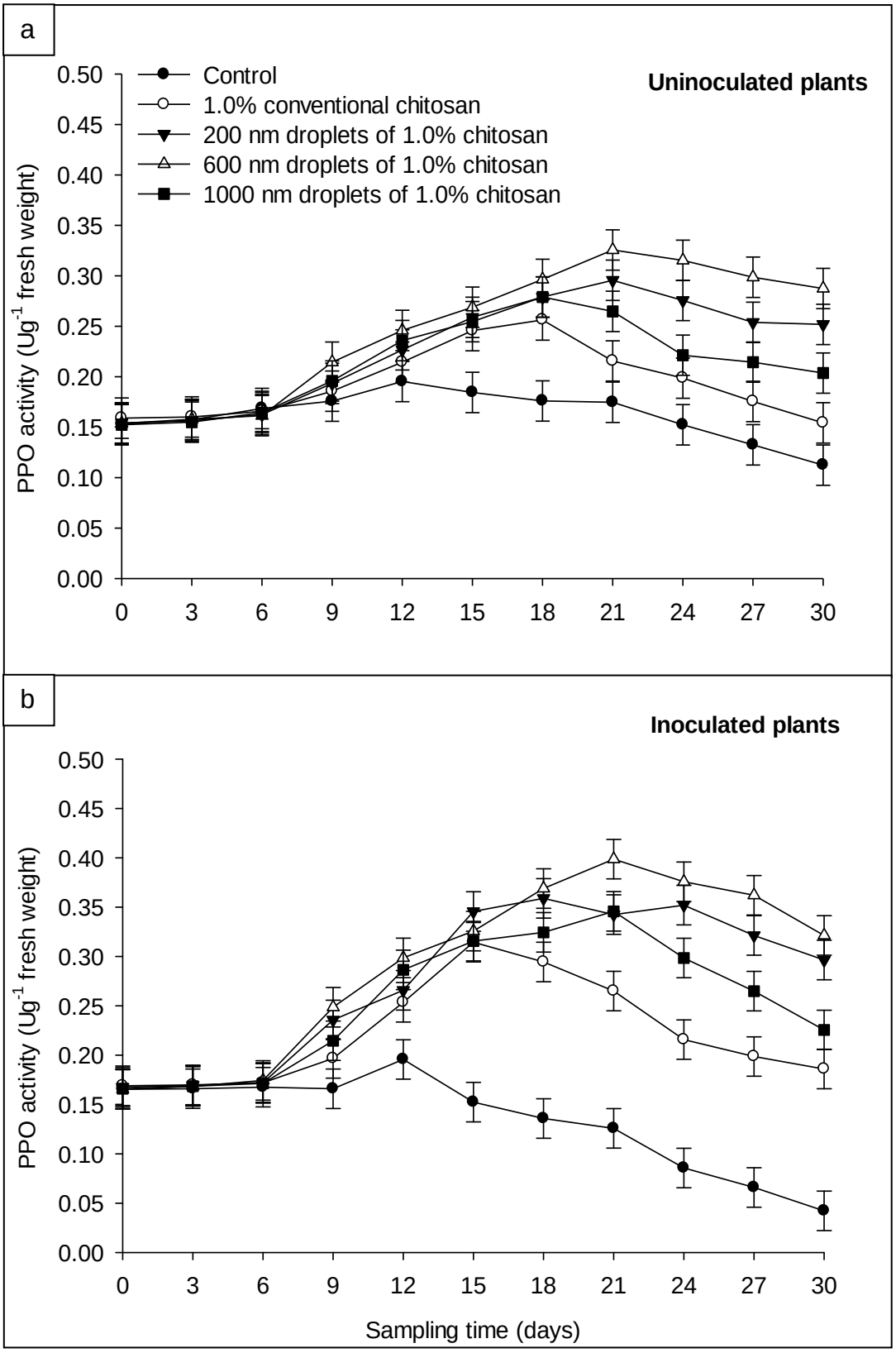


Figure 5.9: Polyphenol oxidase (PPO) activity in a) uninoculated dragon fruit plants and b) plants inoculated with *C. gloeosporioides* treated with conventional chitosan and submicron chitosan dispersions. Each value represents a mean of three replicates and vertical bars indicate standard errors.

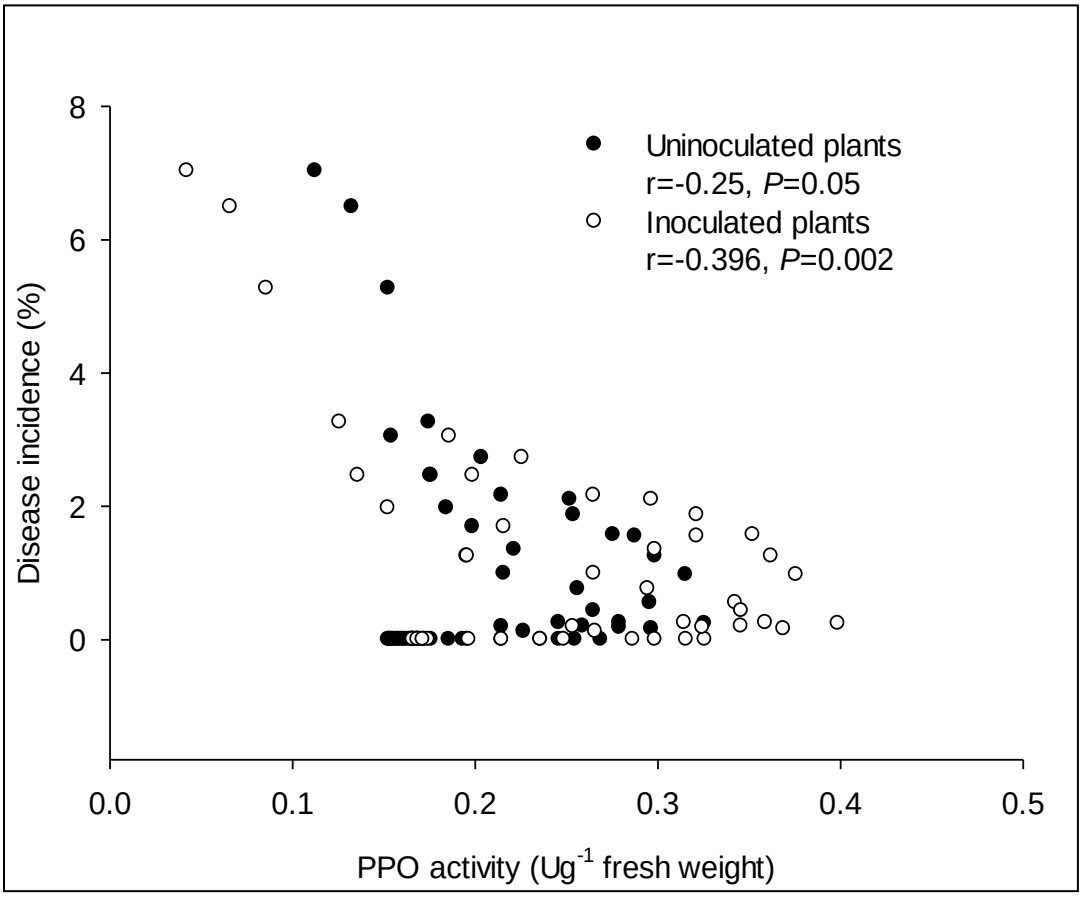


Figure 5.10: Relationship between PPO content and disease incidence in dragon fruit plants.

Nandeeshkumar *et al*. (2008) stated that PPO inhibits the enzymatic digestion of cell walls of plants by increasing the rate of polymerization of phenolic compounds into lignin, and thus reducing the invasion by a fungus and consequent restriction of the progress of a pathogen. Mandal (2010) reported the elicitation of PPO in egg plant roots after treatment with chitosan, and also the possible involvement of PPO in defense management of egg plants. Therefore, it is reasonable to assume that an upturn in PPO activity in the plants sprayed with 600 nm droplets will result in a higher amount of toxic products after oxidation, leading to a greater degree of resistance to infection.

5.3.4.3 Effect of conventional chitosan and submicron chitosan dispersions on phenylalanine ammonia lyase (PAL) activity

PAL activity was significantly ($P < 0.05$) higher in treated plants (Appendix C 5.12, 5.13). Conventional chitosan at 1.0% concentration showed a maximum PAL activity (1.762 U g^{-1} fresh weight) on day 18 after treatment, while all the submicron chitosan dispersions showed maximum PAL activity on day 21 after treatment (Fig. 5.11a). A similar trend in PAL activity was observed in the plants inoculated with *C. gloeosporioides* (Fig. 5.11b).

There was a negative correlation between PAL activity and disease incidence (Fig. 5.12). The r^2 value $\{(-0.657)^2 \times 100\}$ showed that PAL activity resulted in a 43% decrease in disease in uninoculated plants and $\{(-0.811)^2 \times 100\}$ 65.7% decrease in inoculated plants. The differences in r^2 values in uninoculated and inoculated plants suggest that PPO activity increased with the increase in disease and may be associated with the reduction in disease incidence.

PAL is a key enzyme in the metabolism of phenylpropanoids, that catalyses phenylalanine to *trans*-cinnamic acid. It is the first step in phenylpropanoids biosynthesis, which leads to secondary metabolites, such as phytoalexins, lignins and flavonoids (Nandeeshkumar *et al*., 2008). Various biotic factors (e.g. infection by fungi, bacteria and viruses) and abiotic factors (e.g. wounding, UV-B light and high and low temperatures) are responsible for induction of PAL (Strack, 1997; Dixon and Paiva, 1995; Hahlbrbrock and Scheel, 1989), which suggests that PAL plays an essential role in modulating the resistance of plant tissues to such stresses.

Chitosan treatments have been reported to enhance disease resistance by regulating defense enzymes, such as *β*-1,3-glucanase, chitinase, PAL,

POD, PPO and SOD, in table grapes (Meng *et al*., 2008; Romanazzi *et al*., 2002), litchi (Wang *et al*., 2007), mango (Kongkaew *et al*., 2005), tomato (Badawy and Rabea, 2009) and potato (Sun *et al*., 2008).

Meng and Tian (2009) found that spraying chitosan at 10 days prior to harvest significantly enhanced PAL activity in table grapes compared with control fruit at harvest. In addition, preharvest spraying of table grapes with chitosan reduced natural decay of table grapes during storage, perhaps due to direct antifungal activity and induction of disease resistance (Meng *et al*., 2008; Romanazzi *et al*., 2002). Trotel-Aziz *et al*. (2006) found that increase in activity of PAL in chitosan treated grape vine leaves is biphasic. An early short peak was observed 2-4 h after treatment which was followed by a further longer increase after 8h.

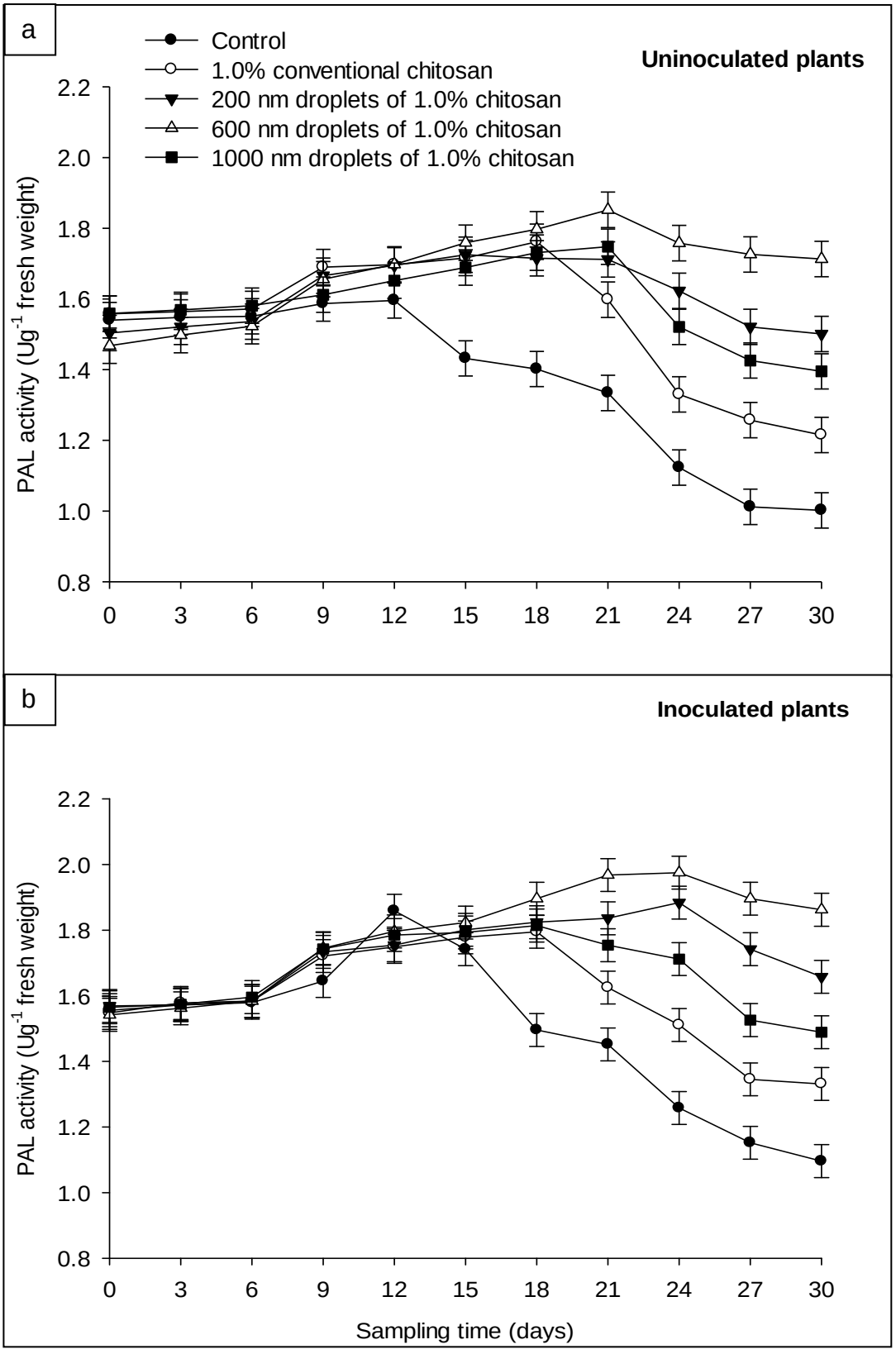


Figure 5.11: Phenylalanine ammonia lyase (PAL) activity in a) uninoculated dragon fruit plants and b) plants inoculated with *C. gloeosporioides* treated with conventional chitosan and submicron chitosan dispersions. Each value represents a mean of three replicates and vertical bars indicate standard errors.

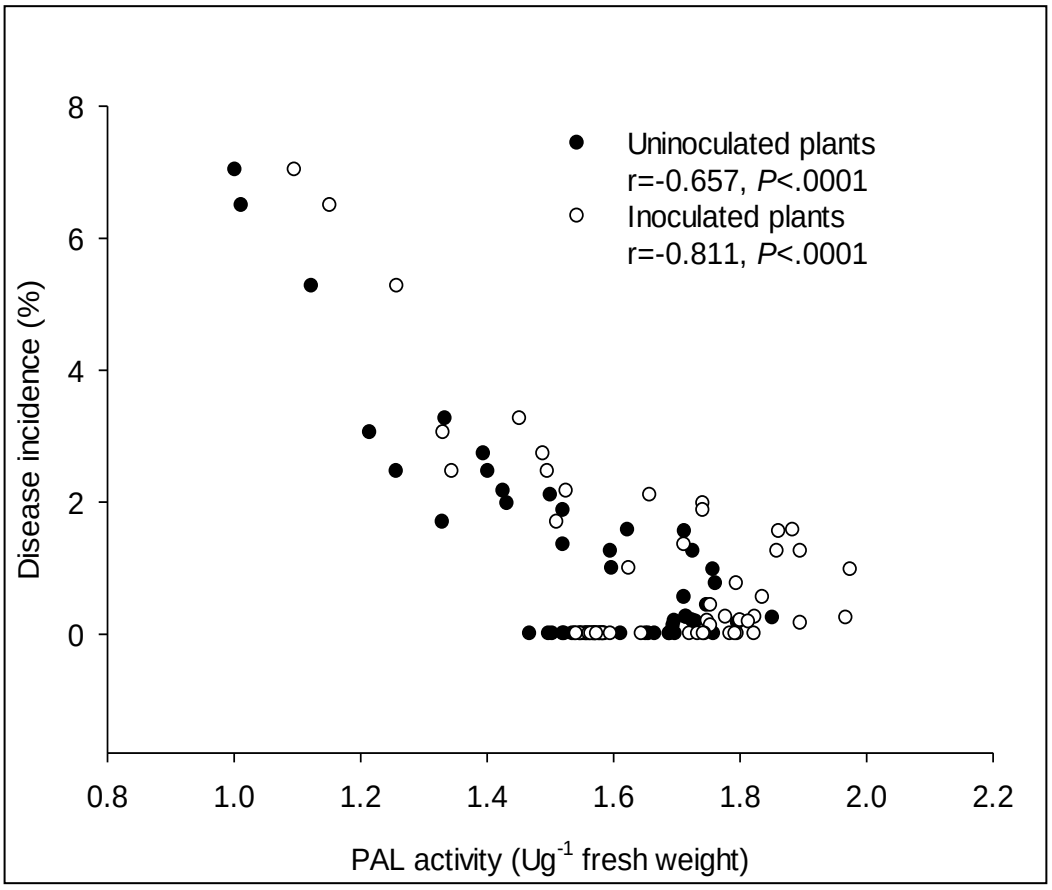


Figure 5.12: Relationship between PAL content and disease incidence in dragon fruit plants.

Synthesis of secondary metabolites (e.g. salicylic acid and phytoalexins) depends on PAL activity (Shadle *et al*., 2003; Lee *et al*., 1995), and it has been proposed that these secondary metabolites are involved in reducing disease incidence through activation of plant defense responses (Jeandet *et al*., 2002; Reymond and Farmer, 1998). The improvement of antifungal activity and enhancement of plant defense responses has been reported for interactions of chitosan with tobacco and wheat (Klarzynski *et al*., 2000; Vander *et al*., 1998; Bohland *et al*., 1997). It has been suggested from these studies that salicylic acid biosynthesis is due to early PAL activation, which in turn is responsible for increased activity of PAL, thereby strengthening the defense responses.

5.3.4.4 Effect of conventional chitosan and submicron chitosan dispersions on *β*-1,3-glucanase and chitinase activity

All the chitosan treated plants showed the highest *β*-1,3-glucanase activity on day 18 after treatment (Fig. 5.13a) for uninoculated plants and on day 21 for the plants inoculated with *C. gloeosporioides* (Fig. 5.13b). The activity of *β*-1,3-glucanase activity in control plants decreased after day 12. There was a significant interaction between treatments and days after treatment (Appendix C 5.14, 5.15). *β*-1,3-glucanase activity in dragon fruit plants was negatively correlated with incidence of disease (Fig. 5.15a). With every 0.5 unit increase in *β*-1,3-glucanase activity, the disease incidence reduced by 2%. The r^2 value $\{(-02.58)^2 \times 100\}$ showed that *β*, 1-3 glucanase activity contributed 6.6% in reduction of disease incidence in uninoculated plants and $\{(-0.329)^2 \times 100\}$ 10% in inoculated plants.

Differences in chitinase activity between all the treatments were significant ($P < 0.05$) (Appendix C 5.16, 5.17). The plants treated with 1.0% conventional chitosan and 200 nm and 1000 nm submicron chitosan dispersions showed the highest increase in chitinase activity on day 15 after treatment and then it gradually declined, whereas the plants treated with 600 nm droplets showed highest activity on day 18 (Fig. 5.14a). An increased chitinase activity in the plants inoculated with *C. gloeosporioides* was recorded on day 18. The maximum increase occurred in the plants treated with 600 nm droplets while the maximum decline in chitinase activity was recorded in the plants sprayed with water (Fig 5.14b). There was a negative relationship between chitinase activity and disease incidence (Fig. 5.15b). The data suggests that regardless of treatment and of inoculation with *C. gloeosporioides,* whenever chitinase acitivity exceeds 0.20 units, the

incidence of disease is limited to below 3%. The values obtained for r^2, {(-0.276) x 100} and $\{(-0.337)^2 \times 100\}$, showed that, with chitinase activity, disease decreased by 7.6% and 11.0% in uninoculated and inoculated plants, respectively.

Some proteins, known as pathogenesis related proteins (PR), are also induced by stress, such as pathogen attack or wounding (Zheng *et al*., 2011). *β*-1,3-glucanases belong to PR-3, -4, -8 and -11 families and chitinases belong to the PR-2 family and are associated with defense responses (Van Loon and Van Strien, 1999). Previous studies have shown that *de-novo* synthesis of phenolic compounds has been triggered by chitosan which serves as the first defensive route to inhibit fungus, while *β*-1,3-glucanase serves as a mechanical barrier to inhibit fungal invasion inside the plant tissue and also protects the tissues against phytotoxic substances produced by the fungus. A significant increase in the intercellular *β*-1,3-glucanase and chitinase was observed in ground nut plants treated with chitosan (Sathiyabama and Balasubramanian, 1998).

The accumulation of PR proteins in plants may lead to the hydrolysis of *β*-1,3-glucan which is the major component of fungal cell walls (Neuhaus, 1999). The stimulation of chitinase and *β*-1,3-glucanase in pea seeds after treatment with *Penicillium fluorescens* resulted in fungal cell wall degradation (Benhamou, 1996).

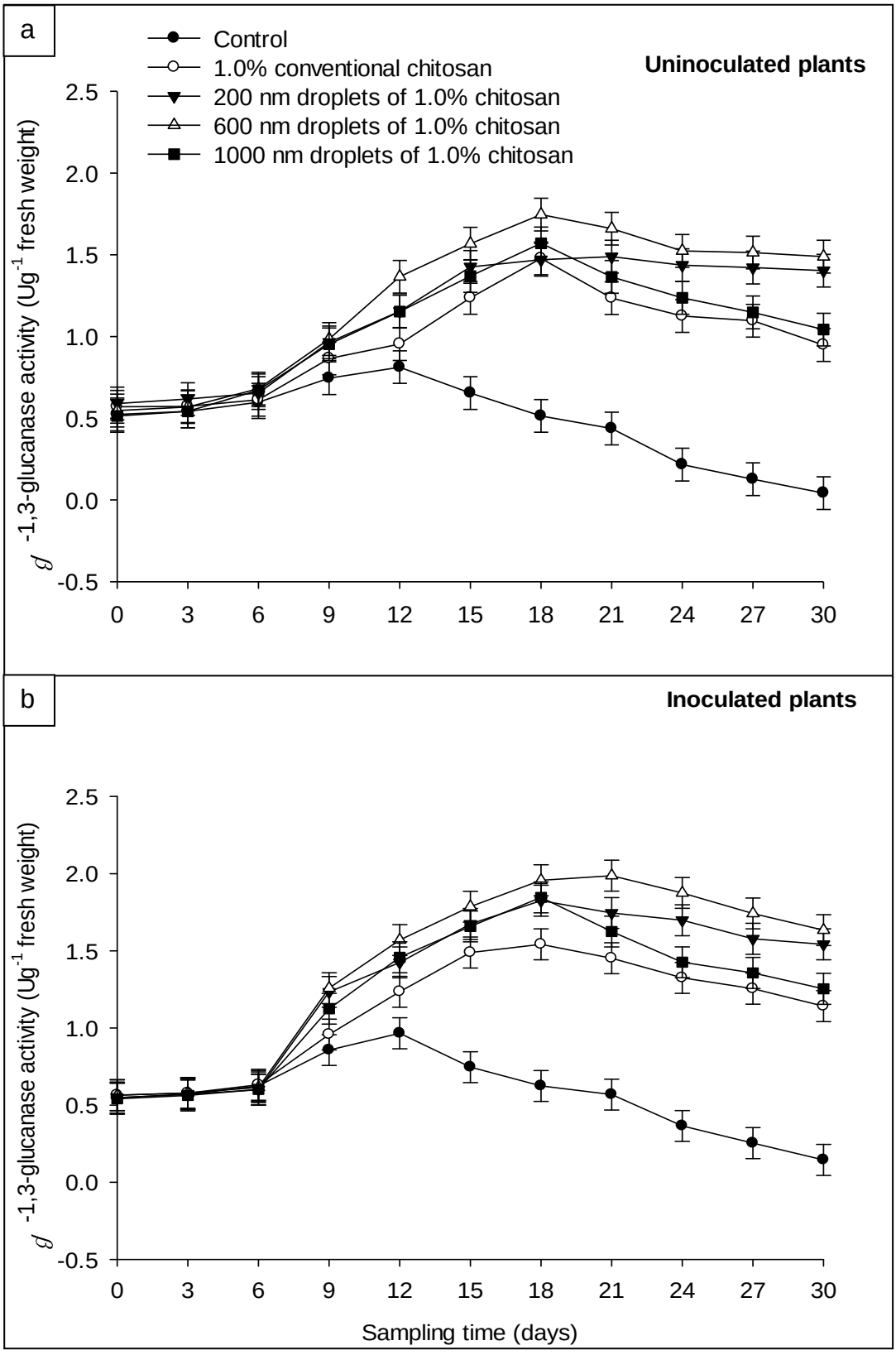


Figure 5.13: *β*-1,3-glucanase activity in a) uninoculated dragon fruit plants and b) plants inoculated with *C. gloeosporioides* treated with conventional chitosan and submicron chitosan dispersions. Each value represents a mean of three replicates and vertical bars indicate standard errors.

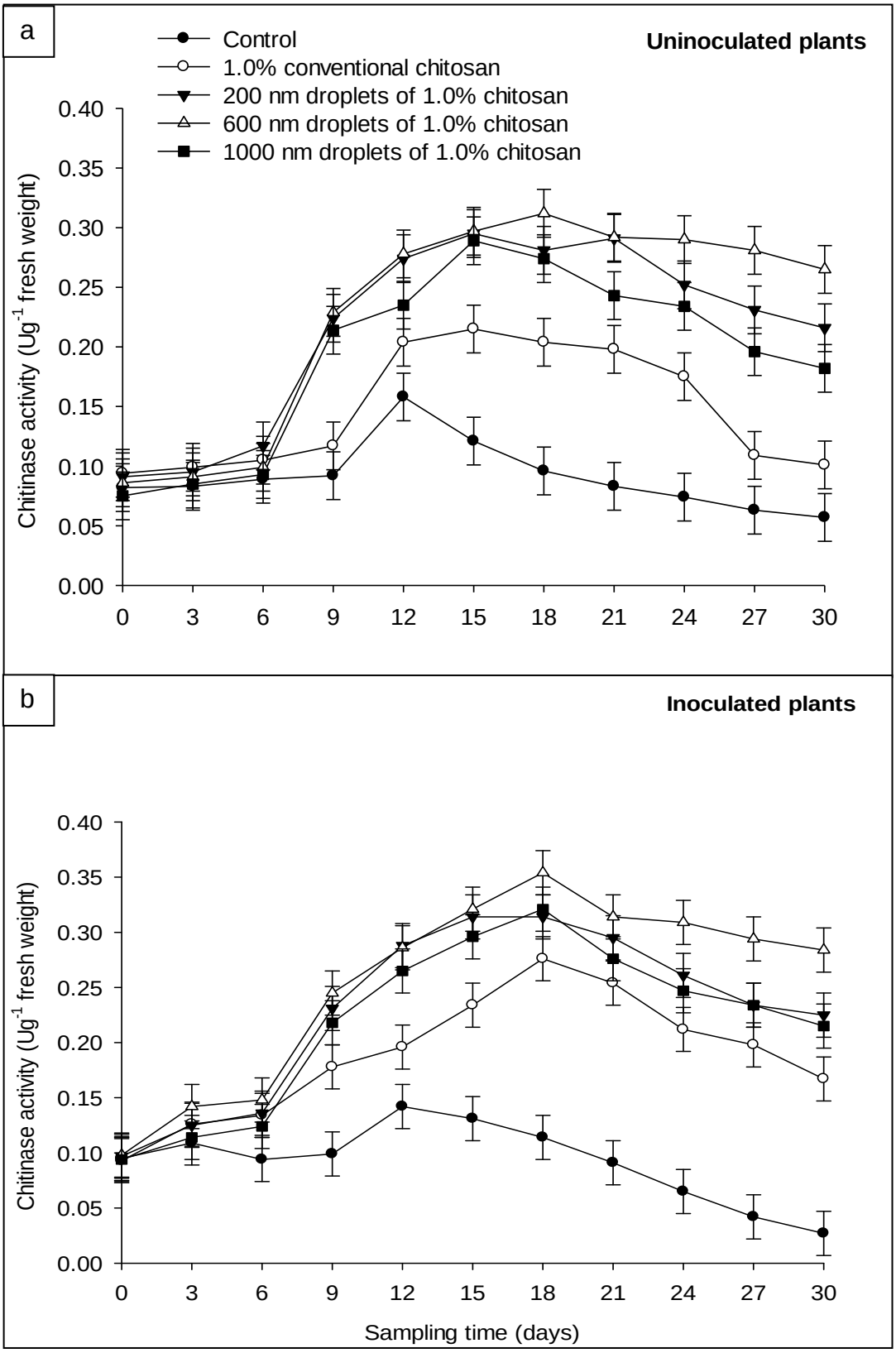


Figure 5.14: Chitinase activity in a) uninoculated dragon fruit plants and b) plants inoculated with *C. gloeosporioides* treated with conventional chitosan and submicron chitosan dispersions. Each value represents a mean of three replicates and vertical bars indicate standard errors.

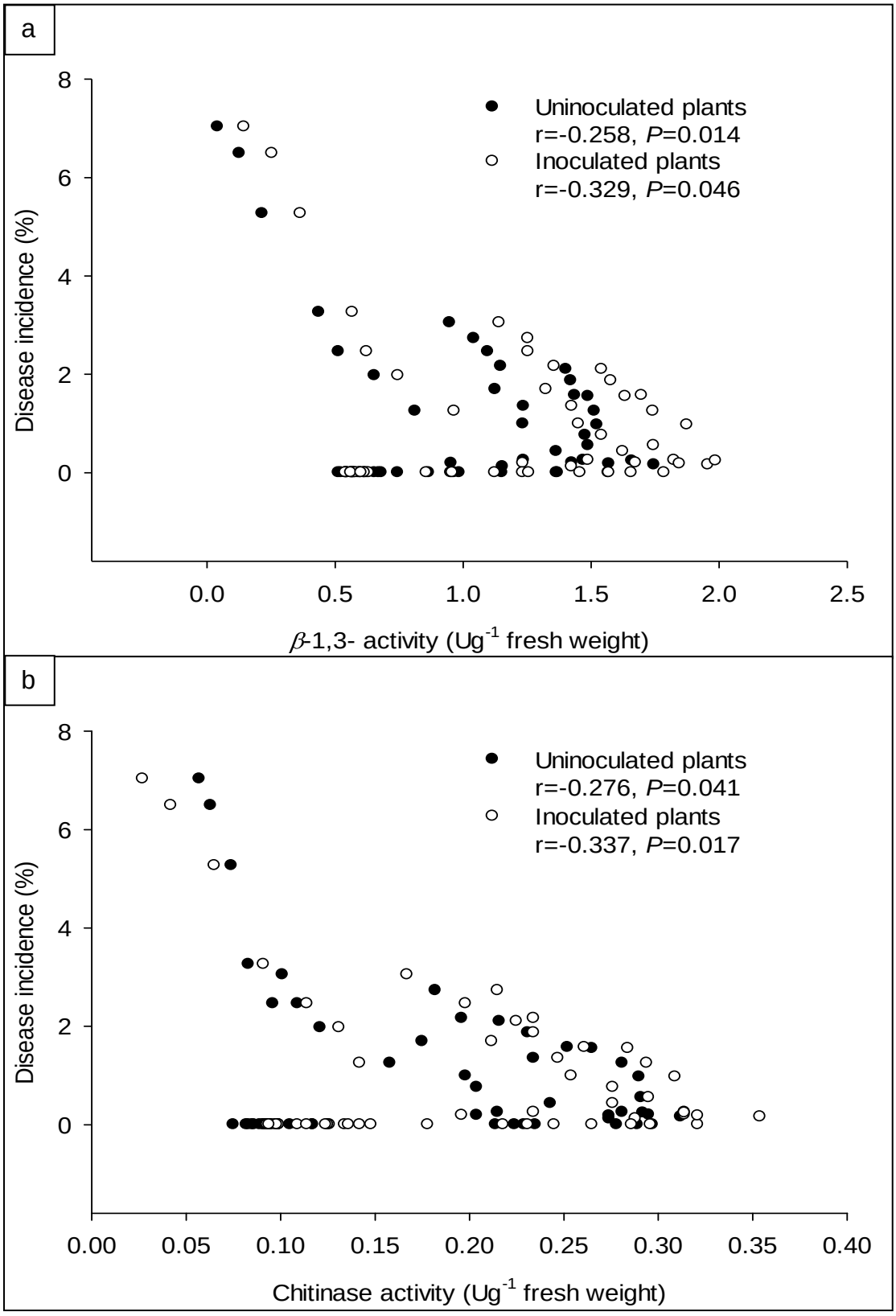


Figure 5.15: Relationships between (a) *β*, 1-3 glucanase activity and disease incidence and (b) chitinase activity and disease incidence in dragon fruit plants.

In tomato plants, production of PR proteins induced by chitosan was responsible for hyphal degradation of *Fusarium oxysporum* f. sp. *radices lycopersici* (Benhamou and Thériault, 1992).

The systemic nature and consistent elicitation of defense enzymes in host plant tissues by chitosan can be important to retard the resumption of quiescent infections which typically become active only when the decline in tissue resistance occurs (Nandeeshkumar *et al*., 2008; Reglinski *et al*., 2004). The actual mechanisms by which chitosan activates plant genes and inhibits fungal growth are not known yet. However, it is known that for both functions the optimal polymerization of glucosamine should involve seven or more units. The altered positive charges along the length of the polymer, due to alternating orientation of the glucosamine units, and its affinity for DNA indicate that chitosan may be partially responsible for structural changes in plant cell nuclei (Kendra *et al*., 1989; Kendra and Hadwiger, 1987 & 1984).

The control of anthracnose in dragon fruit plants using submicron chitosan dispersions and the induction of defense enzymes in plants may offer a new strategy for disease control. This study has shown that 600 nm submicron chitosan dispersions elicit maximum enzyme activity. This might be due to the 600 nm droplets being optimal for penetrating plant tissues and for effective release of the chitosan.

The efficacy of submicron chitosan dispersions suggests that chitosan acted as a natural fungicide which delayed the onset of disease on dragon fruit plants. The actual mechanism of chitosan action is still unknown but the size of submicron dispersions may permit penetration inside the fungus and the release of the active ingredient from the emulsifier matrix (Lim *et al*.,

1997). Release of chitosan from submicron dispersion depends on the viscosity of the solution and the interaction of the active ingredient with the emulsifier (Desai and Park, 2005).

5.3.5 Effect of chitosan on the growth of plants

In terms of number of shoots per cutting, stem diameter and shoot length, the growth of the uninoculated plants was significantly ($P < 0.05$) greater than the plants inoculated with *C. gloeosporioides* (Table 5.2; Appendix C 5.18-5.26).

Treatment with 600 nm droplets with 1.0% chitosan gave the highest number of shoots per cuttings on each sampling day in uninoculated plants, which was significantly ($P < 0.05$) higher than for inoculated plants. The lowest number of shoot cuttings occurred in control plants after day 90. A significant difference in the stem diameter of uninoculated plants and plants inoculated with *C. gloeosporioides* was observed in the treatment with 600 nm droplets of 1.0% chitosan after day 90.

A similar trend was observed for shoot length, with the uninoculated plants treated with 600 nm droplets of 1.0% chitosan having longer shoots than with the 200 nm and 1000 nm droplets treatments. Significantly ($P < 0.05$) longer shoots were observed in uninoculated plants as compared with inoculated plants in all the treatments.

Table 5.2: Effect of conventional chitosan and submicron chitosan dispersions on the number of shoots, stem diameter and shoot length of dragon fruit plants inoculated with *C. gloeosporioides* and of uninoculated plants at thirty day intervals after treatment.

	Sampling time (days)	Treatments				
		T1	T2	T3	T4	T5
		Number of shoots per cutting				
Inoculated	30	7.00 b	7.33 b	7.00 b	7.66 b	7.66 b
Uninoculated		8.00 a	8.00 a	8.66 a	9.30 a	9.30 a
Inoculated	60	9.3 d	9.3 d	10.0 c	11.6 bc	9.0 d
Uninoculated		11.6 bc	11.3 bc	12.0 b	14.0 a	11.3 bc
Inoculated	90	12.3 f	13.0 e	14.6 d	17.0 b	13.6 de
Uninoculated		13.0 e	14.0 d	15.6 c	19.0 a	14.9 c
		Stem diameter (m)				
Inoculated	30	0.051 c	0.08 bc	0.101 d	0.085 b	0.059cd
Uninoculated		0.085 b	0.092 a	0.089 a	0.086ab	0.089 a
Inoculated	60	0.102 e	0.155cd	0.102 e	0.178 a	0.168 b
Uninoculated		0.147 d	0.161 c	0.170 b	0.178 a	0.169 b
Inoculated	90	0.169 e	0.186 c	0.179 d	0.204 b	0.178 d
Uninoculated		0.177 d	0.188 c	0.195 c	0.231 a	0.189 c
		Shoot length (m)				
Inoculated	30	1.71f	1.908 c	1.77 e	1.80 d	1.77 e
Uninoculated		2.11 a	2.12 a	2.10 ab	2.08 b	1.99 c
Inoculated	60	1.84 f	2.04 d	2.07 d	1.98 e	2.07 d
Uninoculated		2.28 b	2.31 b	2.25 bc	2.40 a	2.24 c
Inoculated	90	2.15 f	2.33 e	2.59 c	2.70 ab	2.25 e
Uninoculated		2.41 d	2.39	2.63 b	2.804 a	2.47 cd

Each value is the mean of three replicates. The values with different letters within same day are significantly different at $P < 0.05$ using DMRT.

T1 = Control; T2 = Conventional chitosan; T3 = 200 nm droplets of 1.0% chitosan; T4 = 600 nm droplets of 1.0% chitosan; T5 = 1000 nm droplets of 1.0% chitosan.

The superior vegetative growth in the plants treated with submicron chitosan dispersions as compared to control plants may have occurred because of the lower disease incidence (Section 4.5.4). These positive effects of chitosan on plant growth were probably due to the fact that chitosan provides molecular signals that serve as plant growth promoters and induce disease resistance (Chookhongkha *et al*., 2012). Chitosan has been reported to increase growth of various crops, such as sweet pepper (Ghoname *et al.,* 2010), sweet basil (Kim, 2005), soybean sprouts (Lee *et al.,* 2005), strawberry (Abdel-Mawgoud *et al.,* 2010) and cabbage (Hirano, 1988). For some crops, such as bitter cucumber, chilli, celery and Chinese cabbage, the response varies with the frequency of application and concentration of chitosan (Chandrkrachang *et al.,* 2003 and Boonlertnirun *et al.,* 2005).

The improved vegetative growth of plants treated with submicron dispersions was probably related to the fact that poly *D*-glucosamine chitosan enhances carbon sources by stimulating the growth of beneficial microbes in soil. The highly acidic nature of chitosan may break down mineral elements in the soil that are unavailable to plants and may enhance the uptake of nutrients, such as nitrogen, potassium and phosphorus, which results in higher plant growth (Farouk *et al*., 2011). Results from the Fourier transform infrared spectroscopy (FTIR) showed that phosphorus, sulphur and silicon traces are also present in chitosan, which effectively play important roles in the vegetative growth of plants (Chookhongkha *et al*., 2012). Another reason for the increased plant growth due to chitosan application is the free amino group in the chitosan chain which provides extra nitrogen to plants (Chibu

and Shibayama, 2001). The easier release of chitosan from the emulsifier matrix as 600 nm droplets might be helpful in increased vegetative growth.

5.3.6 Effect of chitosan on chlorophyll content of dragon fruit plants

Application of chitosan submicron dispersions resulted in significant ($P < 0.05$) differences in the chlorophyll contents of uninoculated and inoculated plants (Table 5.3).The chlorophyll content was highest on day 60, but decreased by day 90 (Table 5.3). Application of 600 nm droplets did not affect total chlorophyll and chlorophyll b in plants inoculated with *C. gloeosporioides* and in uninoculated plants.

An increase in chlorophyll in plants increases their tolerance of disease and results in higher photosynthetic rate and significantly more vegetative growth (Borkowski *et al*., 2007). In the present study, the decrease in chlorophyll on day 90 may have been due to the increase in disease on the plants with the passage of time (Section 4.5.4). The increase in chlorophyll in the stems treated with chitosan and chitosan dispersions was probably due to the chelating effect of chitosan, which can increase ion exchange and increase the uptake of nutrients from the soil (Farouk *et al*., 2011).

Table 5.3: Effect of conventional chitosan and submicron chitosan dispersions on chlorophyll a, chlorophyll b and total chlorophyll in dragon fruit plants inoculated with *C. gloeosporioides* and uninoculated plants at 30 day intervals after treatment.

	Sampling time (days)	Treatments				
		T1	T2	T3	T4	T5
		Chlorophyll a (g ml^{-1})				
Inoculated	30	3.76 c	5.12 b	5.67 b	6.09 ab	5.76 c
Uninoculated		3.97 c	5.40 b	5.70 b	6.64 a	5.97 ab
Inoculated	60	4.76 g	5.70 f	7.99 d	9.91 b	7.65 de
Uninoculated		5.21 f	7.28 e	8.30 c	10.81 a	7.72 d
Inoculated	90	4.49 i	5.34 g	7.14 c	7.60 b	6.43 e
Uninoculated		4.91 h	5.62 f	6.95 c	7.92 a	6.75 d
		Chlorophyll b (g ml^{-1})				
Inoculated	30	1.81 d	3.03 c	3.22 b	3.24 b	3.96 a
Uninoculated		1.16 e	1.84 d	3.22 b	3.21 b	3.93 a
Inoculated	60	2.21 f	4.27 d	5.30 b	6.65 a	4.86 c
Uninoculated		2.83 e	4.03 d	5.43 b	6.69 a	4.86 c
Inoculated	90	1.67 h	2.49 f	4.86 b	5.46 a	3.42 e
Uninoculated		2.13 g	2.13 g	4.49 c	5.59 a	3.61 d
		Total Chlorophyll (g ml^{-1})				
Inoculated	30	5.58 d	8.15 bc	8.90 b	9.33 ab	9.72 a
Uninoculated		5.14 d	7.72 c	8.94 b	9.97 a	9.90 a
Inoculated	60	6.71 e	8.76 d	13.39 b	16.5 a	12.5 bc
Uninoculated		8.45 d	11.3 c	13.73 b	17.5 a	12.5 bc
Inoculated	90	6.44 g	7.89 e	12.0 b	13.0 a	9.85 d
Uninoculated		7.05 f	7.75 e	11.3 c	13.5 a	10.3 d

Each value is the mean of three replicates. The values with different letters within same day for each parameter tested are significantly different at $P < 0.05$ using DMRT.

T1 = Control; T2 = Conventional chitosan; T3 = 200 nm droplets of 1.0% chitosan; T4 = 600 nm droplets of 1.0% chitosan; T5 = 1000 nm droplets of 1.0% chitosan.

The increase in photosynthetic pigments with the use of chitosan has been reported by El-Tantawy (2009), Khan *et al*. (2002) and Chibu and Shibayama (2001). The 600nm droplets showed more accumulation of photosynthetic pigments probably because of the better penetration of chitosan droplets inside the plants (see Fig 5.4d), or the amount of chitosan inside this droplet was enough to induce biochemical reactions inside the plants. These results are in line with Chibu and Shibayama (2001) who showed that 0.5% chitosan was beneficial for upland rice and soya bean while a lower concentration (0.1%) was helpful for the growth of lettuce and cherry tomatoes.

In conclusion, 600 nm droplets of 1.0% chitosan gave the best results in all the parameters tested. This formulation and amount of chitosan was sufficient to combat microbial organisms, resulting in reduction in disease and vigorous growth of the plants.

CHAPTER 6

EFFECT OF SUBMICRON CHITOSAN DISPERSIONS ON POSTHARVEST ANTHRACNOSE OF DRAGON FRUIT DURING STORAGE

6.1 Introduction

Pathogens, transpiration and senescence are the main causes of loss in quality of fresh vegetables and fruit during postharvest storage (Valero and Serrano, 2010; Mulas and Schirra, 2007). Among these factors, the postharvest losses due to microbiological diseases are the most important because they pose higher economic losses (Adaskaveg and Förster, 2010; Tripathi and Dubey, 2004; Kader, 2002). It is difficult to estimate the total amount of postharvest losses due to diseases, however, conservative estimates place U.S. losses to fruits and vegetables from spoilage are around 24% of the harvested crop (Goletti, 2003). In less developed countries, postharvest losses are even greater because of the lack of adequate refrigeration and poor sanitation. Worldwide, postharvest losses have been estimated to be 50% of the harvested crop and much of this due to rots caused by microorganisms (Goletti, 2003).

Dragon fruit is a non-climacteric fruit that is highly prone to diseases causing enormous postharvest losses during transportation and storage (Masyahit *et al*., 2009). About 50% of postharvest losses are due to anthracnose caused by *C. gloeosporioides* (Awang *et al*., 2011).

Control methods, such as chemical control, biological control, physical methods (ultraviolet illumination, radiofrequency treatment, heat treatments) and storage technologies, have been devised that are capable of reducing the growth of pathogens and ultimately inhibiting, or at least lessening, losses

(Adaskaveg and Forster, 2010; Smilanick *et al*., 2008; Fallik and Lurie, 2007; Mulas and Schirra, 2007; Narayanasamy, 2006; Fallik, 2004; Barkai-Golan, 2001; Schirra *et al*., 2000). Despite the benefits of these methods, the complete control of decay is rarely accomplished and, most importantly, they are costly to run on a commercial scale (Tripathi and Dubey, 2004). However, chemical control has been found to be effective which can ensure product protection (Schirra *et al*., 2011; Fallik and Lurie, 2007). It is allowed only on a small scale and, in addition, public opinion demands a reduction in the use of chemical products (Adaskaveg and Forster, 2010). The appearance of pathogens resistant to fungicides has contributed to greater attention to developing substitutes for chemicals controlling the microorganisms responsible for postharvest losses (Tripathi and Dubey, 2004; Janisiewicz and Korsten, 2002; Barkai-Golan, 2001). Therefore, there is an immediate need for effective and new means of controlling postharvest diseases that pose less risk to human health and the environment.

During this study a novel technique of using submicron dispersions of chitosan as an edible coating was used to control postharvest anthracnose of dragon fruit. The main objective of this part of the study was to test the *in vitro* and *in vivo* efficacy of conventional chitosan in comparison with the submicron chitosan dispersions to control *C. gloeosporioides* during storage of dragon fruit.

6.2 Materials and methods

6.2.1 *In vitro* antifungal assay of conventional chitosan and submicron chitosan dispersions

Four concentrations (0.5, 1.0, 1.5 and 2.0%) of chitosan were used to prepare different droplet sizes of submicron dispersions (200, 400, 600, 800 and 1000 nm) as described in Sections 3.2.4 and 3.3, respectively.

6.2.1.1 Inhibition of mycelial growth and conidial germination

The antifungal assay of conventional chitosan and submicron chitosan dispersions was done using the food poison technique as described in Sections 3.2.5.1 and 3.2.5.2, respectively.

6.2.1.2 Scanning electron microscopy

Alterations in conidial and mycelial morphology of *C. gloeosporioides* were detected using a scanning electron microscope (FEI Quonta 400 FE-SEM). Samples (1.5 mm^3) of *C. gloeosporioides* from 10 days old control cultures and cultures grown on media containing submicron chitosan dispersions were mounted on aluminium stubs and viewed immediately using a scanning electron microscope.

6.2.2 *In vivo* antifungal assay of conventional chitosan and submicron chitosan dispersions

Healthy, mature, uniform sized fruit without any detectible signs of infection were selected to evaluate the antifungal effects of conventional chitosan and submicron chitosan dispersions. The fruit were rinsed with purified water followed by air-drying at room temperature for two hours. They were then dipped in a spore suspension (1 x 10^5 spores ml^{-1}), followed by complete surface drying at ambient temperature. After drying, they were dipped in conventional chitosan or submicron chitosan dispersions and dried again at room temperature for five hours. Then they were packed in cardboard boxes and stored at 10 ± 2 °C, 80 ± 5% RH for 28 days.

6.2.2.1 Disease incidence

Disease incidence (DI) based on the anthracnose symptoms on the fruit surface was recorded weekly for 28 days during cold storage. The data was expressed as a percentage (Sivakumar *et al*., 2002).

$$\text{Disease incidence (\%)} = \frac{\text{Number of infected fruit}}{\text{Total number of fruit assessed}} \times 100$$

6.2.2.2 Disease severity

Disease severity (DS) was scored at intervals of 7 days using the scale 1 = 0% of fruit surface rotten, 2 = 1-25% rotten, 3 = 26-50% rotten, 4 = 51-75% rotten and 5 = 76-100% rotten (Sivakumar *et al*., 2002).

The experiments were arranged in a completely randomized design with four replicates, each consisting of twenty units. DI and DS data were transformed using arcsine and square root transformation, respectively. Presentation of data was done after back transformation. Normality of residues was checked with UNIVARIATE procedure in SAS. The data was subjected to analysis of variance and tested for significant differences among treatments by the Duncan's multiple range test (DMRT) at $P < 0.05$ (Appendix C 6.1 – C 6.4) using Statistical analysis system SAS® version 9.1.

6.3 Results and discussion

6.3.1 Inhibition of radial mycelial growth

Neither of the emulsifiers tested (Brij 56 and Span 20), either alone or in combination, showed any inhibition of radial mycelial growth after incubation for 10 days. Mycelial growth was significantly ($P < 0.05$) inhibited by all the treatments of chitosan tested as compared to the control (Fig. 6.1). Droplet size significantly ($P < 0.05$) affected radial mycelial growth, with the droplets 600 nm or larger providing the greatest benefits.

Increasing the concentration of chitosan appeared to reduce the growth of the fungus, showing a similar pattern of inhibition to that noted in Section 4.5.1.1. The fungal isolates obtained from the fruit were more resistant to chitosan treatment in comparison with the fungal isolates obtained from plants.

Droplet sizes of 600, 800 and 1000 nm were found to be more effective in controlling mycelial growth of both isolates of *C. gloeosporioides,* perhaps due to the amount of chitosan in these droplet sizes being enough to contest the fungus and inhibit its growth.

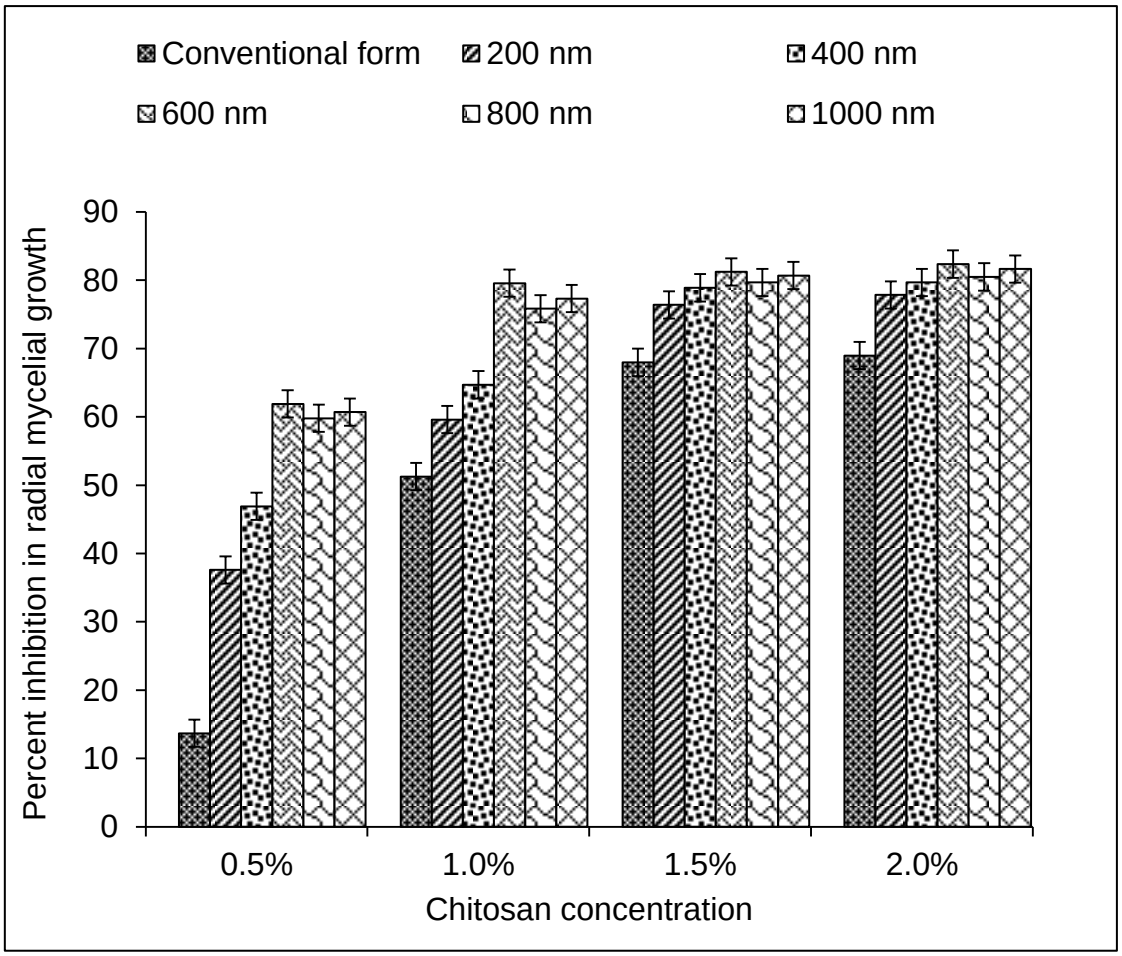


Figure 6.1: Effect of concentration of chitosan and droplet size of submicron chitosan dispersions on percent inhibition of radial mycelial growth of *C. gloeosporioides* after 10 days of incubation. The vertical bars represent standard error of means for four replicates.

6.3.2 Conidial germination inhibition test

The conidial germination of *C. gloeosporioides* from dragon fruit was significantly ($P < 0.05$) inhibited by all the treatments of conventional chitosan and submicron chitosan dispersions as compared to the control (Fig. 6.2). Significant ($P < 0.05$) effects of 600 nm and larger droplets were noted.

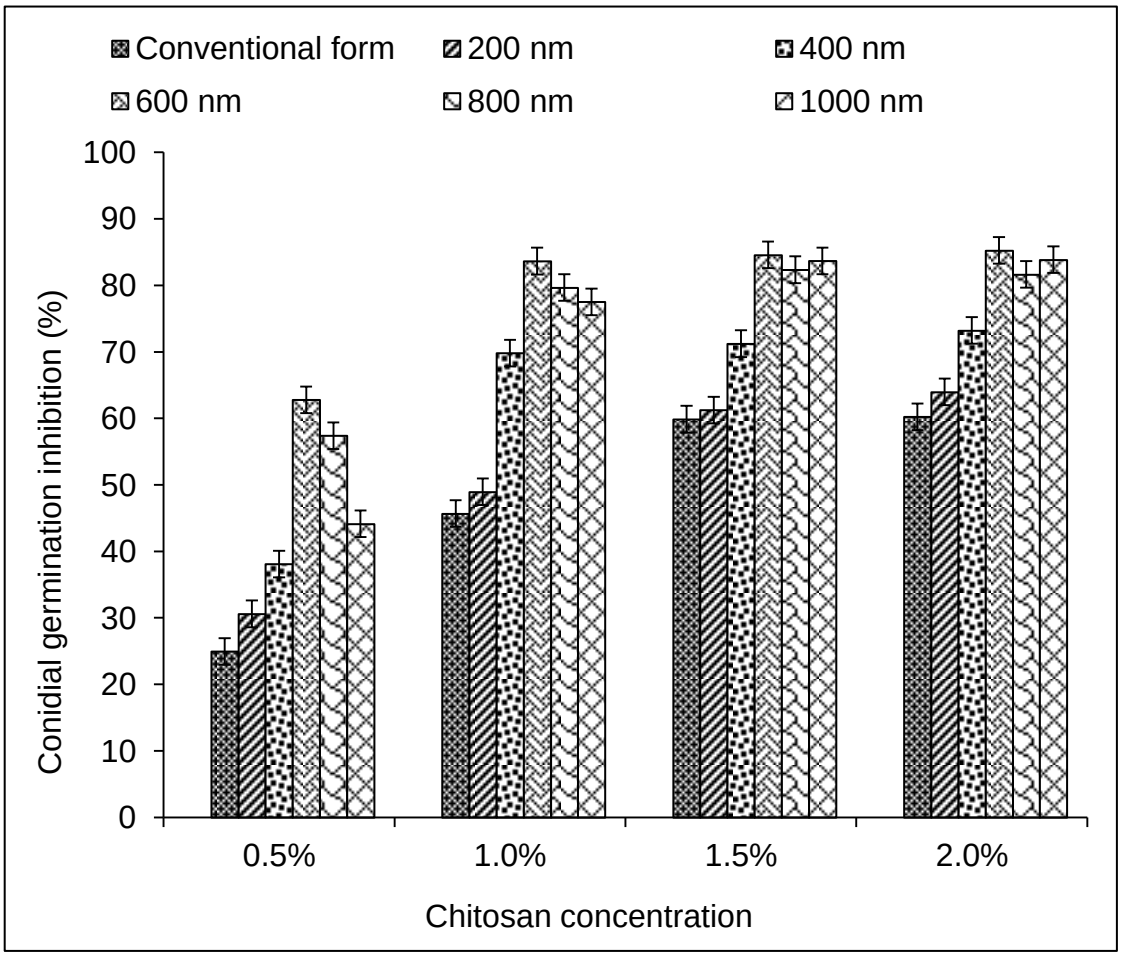


Figure 6.2: Effect of chitosan concentration and droplet size of submicron chitosan dispersions on conidial germination inhibition (%) of *C. gloeosporioides* after 7 hours of incubation. The vertical bars represent standard error of means for four replicates.

These results suggest that chitosan helps to inhibit conidial germination of *C. gloeosporioides*. Reglinski *et al*. (2010) reported a reduction in growth of *B. cinerea* and a change in morphology of conidia after treatment with chitosan. These results support the inhibition of the germination of conidia reported in Figure 4.5a. Conidial inhibition was greater for fungal isolates from plants than for those from fruit. The differences in the *in vitro* growth of fungal isolates may have been due to temperature effects. High temperature in the field has been shown to cause lysis of fungal spores of *Colletotrichum* species on strawberry (Leandro *et al*., 2003).

The effectiveness of 600 nm and larger droplets suggests that these droplet sizes encapsulate enough active ingredient to inhibit conidial germination (Zahid *et al*., 2012). In contrast, the smaller droplets penetrate more easily but, due to their higher stability, the release of active ingredient was delayed (Section 3.6.1.2).

6.3.3 Scanning electron microscopy

From observations under the scanning electron microscope (Fig. 6.3), the growth of mycelium in the control was normal (Fig. 6.3a), whereas there was hyphal agglomeration (Fig. 6.3b) after treatment with 600 nm droplets of 1.0% submicron chitosan dispersions. This treatment also induced changes in the length and shape of conidia (Figs 6.3c & 6.3d). Out of twelve Petri plates of each treatment, nine of the samples showed similar changes in hyphae and conidia.

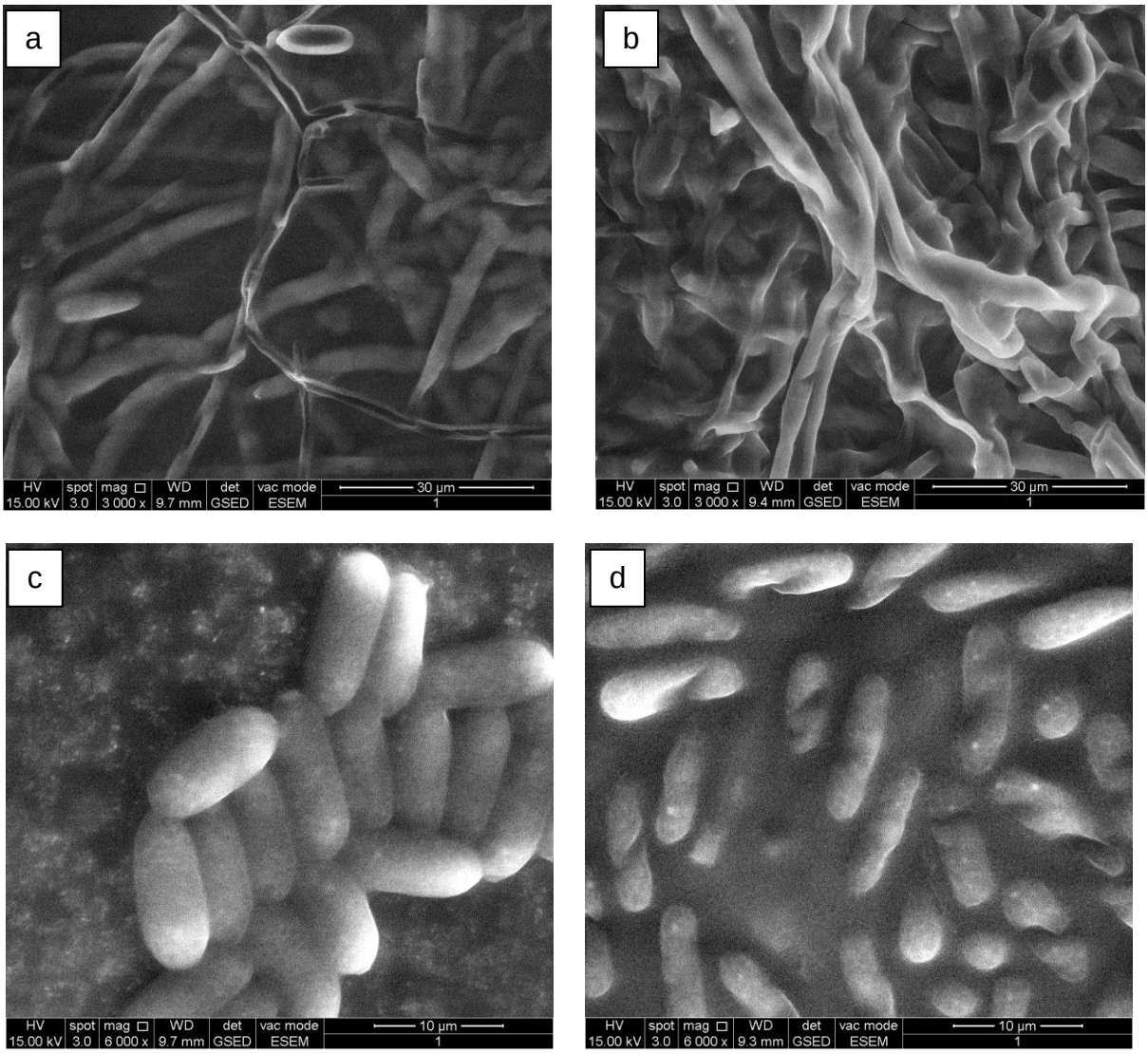


Figure 6.3: Scanning electron microscopy of normal conidia and conidia of *C. gloeosporioides* treated with submicron chitosan dispersions: a) normal hyphae, b) cluster of hyphae, c) normal conidia and d) abnormal conidia extracted from dragon fruit.

The clustering of hyphae and abnormal shapes of conidia in response to submicron dispersions may have been due to cellular changes. Similar marked cellular changes were observed in *F. oxysporum* f. sp. c*ubense* after using media amended with chitosan (Al-Hetar *et al*., 2010). Changes in the morphology of other pathogenic microorganisms have also been reported when grown on chitosan (Benhamou *et al*., 1994; El Ghaouth *et al*., 1997).

Several mechanisms have been proposed for the antifungal action of chitosan. Its activity appears to be an interaction between an ammonium group that provides the positive charge to chitosan and phospholipid head groups of the fungus, which then confers negative charge to the fungus cell wall residues and leads to increase in plasma membrane permeability (Leuba and Sotossel, 1986; Kauss *et al*. 1989).

The inhibition might be due to direct interaction with chitosan, which enters the cell wall of the fungus and interacts with DNA, to alter its configuration, thus blocking the production of mRNA and proteins (Hadwiger and Loschke, 1981). In the present study, conidial germination inhibition and agglomeration of hyphae supported the concept of toxicity of chitosan towards fungi. The results may be corroborated by Al-Hetar *et al*. (2010) who found that the chelating activity of chitosan inhibited the growth of *Fusarium oxysporum* f. sp. c*ubense* in liquid cultures.

6.3.4 *In vivo* antifungal effects of conventional chitosan and submicron chitosan dispersions

During the 28 days of storage, disease incidence (DI) was significantly reduced ($P < 0.05$) by all of the chitosan treatments compared with the control (Table 6.1). In control fruit, DI reached 99.9%. The submicron chitosan dispersions were consistently more effective than conventional chitosan at reducing DI and 600 nm droplets of 1% chitosan was the best treatment.

Differences in disease severity (DS) among all treatments were significant ($P < 0.05$) after 28 days of storage (Table 6.1). DS score for control fruit

reached the maximum value of 5 and all of the chitosan treatments resulted in reduced DS. DS increased with time in storage and for conventional chitosan the highest reduction (68.0%) occurred with the 2.0% treatment. With submicron chitosan dispersions, the highest reduction (75.4%) was with 600 nm droplets of 1.0% chitosan (Table 6.1).

These results suggest that chitosan was effective for disease control and also demonstrate the fungistatic activity of chitosan. They are in agreement with the findings of Ali *et al*. (2010) who reported that, with an increase in chitosan concentration, there is a reduction in anthracnose on Ekotica II papaya. In a previous study on carrots, chitosan showed higher antifungal effects in comparision with synthetic fungicides for the control of *Sclerotinia sclerotiorum* (Cheah and Page, 1997).

Although all the submicron chitosan dispersions showed better results than the conventional chitosan treatments, chitosan at 1.0% with 600 nm droplets showed the best reduction in DI and DS. The effectiveness of the 600 nm droplets is attributed to the appropriate size required for penetrating inside the fungus and to faster release of the active compound delaying the onset of disease (Zahid *et al*., 2013).

The results of the present study showed that the 1.0% submicron chitosan dispersion with 600 nm droplets was the most promising for controlling postharvest anthracnose of dragon fruit caused by *C. gloeosporioides*. However, further studies are required to elucidate the mechanism behind the antimicrobial activity of submicron dispersions.

Table 6.1: Effect of chitosan and submicron chitosan dispersions on disease incidence (%) and disease severity (score) of dragon fruit during 28 days of storage at 10 ± 2 °C and 80 ± 5% RH.

	Droplet size (nm)	Disease incidence (%)	Disease severity (score)
Control		99.92 a	5.00 a
0.5% chitosan	Conv. chitosan	80.50 b	4.53 b
	200	64.30 c	3.40 c
	600	57.33 e	2.90 de
	1000	61.10 d	3.06 d
1.0% chitosan	Conv. chitosan	41.63 f	2.40 e
	200	11.46 hi	1.50 g
	600	6.46 m	1.23 i
	1000	8.80 jk	1.30 hi
1.5% chitosan	Conv. chitosan	32.60 g	1.80 f
	200	12.43 h	1.70 fg
	600	7.68 l	1.40 h
	1000	8.26 k	1.60 g
2.0% chitosan	Conv. chitosan	19.03 h	1.60 g
	200	13.23 h	3.40 c
	600	7.43 l	2.90 de
	1000	9.60 j	3.06 d

Values with different letters are significantly different according to ANOVA and Duncan's multiple range test (DMRT).
*Conv. stands for conventional.

CHAPTER 7

EFFECT OF SUBMICRON CHITOSAN DISPERSIONS ON PHYSICO-CHEMICAL CHARACTERISTICS AND ANTIOXIDANT PROPERTIES OF DRAGON FRUIT DURING STORAGE

7.1 Introduction

Fresh vegetables and fruit are alive after harvesting and also continue most biochemical and physiological processes during storage. The physiological activity is mainly noticed as softening changes that occur during fruit ripening (Valero and Serrano, 2010). Respiration and transpiration are the most important processes causing postharvest losses in the quantity and quality of fresh vegetables and fruit. Thus, in order to delay the ripening process and senescence, the respiratory and metabolic activities of fruit must be restrained after harvesting (Kays, 1991).

There have been many storage techniques developed to minimise the rates of respiration and transpiration, including low temperature, controlled atmosphere and hypobaric storage, but most of them have their own limitation of use (Zhang *et al*., 2010; Artés-Hernández *et al*., 2007).

Dragon fruit being a non-climacteric fruit has no peak of ethylene or carbon dioxide production (Le Bellec *et al*., 2006). At ambient temperature, the fruit stays fresh for a few days after which its physical appearance deteriorates and disease infestation sets in resulting in spoilage within 6 to 7 days. Lau *et al.* (2008) reported that fruits remained fresh and disease-free for 6 days when stored at 10°C with 90% relative humidity, but started deteriorating and rotting after the 15th day. Therefore, there is a need for new

and effective means to control the metabolic activities of fruit resulting in reduction of fruit decay.

Thus, during this study the novel technique of using edible coatings obtained from natural products such as chitosan has been adopted to maintain quality and extend storage life of dragon fruit. As described previously, submicron chitosan dispersions help reduce infection of the fruit (Chapter 6). It is now necessary to determine whether it alters the physiology of disease free fruit. Therefore the main objectives of the present study were:

1. To evaluate the effect of conventional chitosan and submicron chitosan dispersions on quality attributes of dragon fruit, such as weight loss, soluble solids concentration and titratable acidity during storage.
2. To observe the effect of submicron chitosan dispersions on preservation of antioxidants in coated and uncoated dragon fruit during storage.

7.2 Materials and methods

7.2.1 Fruit samples

Mature dragon fruit (purplish pink colour) grown in a commercial orchard located at Puchong, Selangor, State of Malaysia were harvested and transported to the laboratory within two hours.

7.2.2 Coating solutions

Chitosan and submicron chitosan dispersions were prepared as described in Sections 3.2.4 and 3.3, respectively.

Treatments for the fruit consisted of untreated control, 1.0% conventional chitosan, 200 nm, 600 nm and 1000 nm submicron chitosan dispersions of 1.0% chitosan, and the experiment was laid out in a completely randomized design (CRD). These treatments were selected on the basis of the results obtained from *in vivo* antifungal effects of submicron dispersions (Section 6.3.4). The treatments were replicated four times with twenty fruit per replication. Data collected were subjected to analysis of variance (ANOVA) using statistical analysis system SAS® version 9.1. Duncan's multiple range test (DMRT) was carried out for mean separation and statistical significance was assessed at $P < 0.05$. All the experiments were repeated twice and the data were pooled before analysis.

7.2.3 Physicochemical quality changes in fruit coated with conventional chitosan and submicron chitosan dispersions

7.2.3.1 Sample preparation

Mature, healthy and uniform fruit were rinsed with purified water and then dipped in solutions of conventional chitosan or submicron chitosan dispersions for 2-3 minutes in order to apply the solution uniformly over the whole fruit surface. The fruit in the control treatment were only dipped in purified water. After dipping, the fruit were allowed to dry at ambient temperature and then were packed in cardboard boxes and stored at 10 ± 2 °C, 80 ± 5% RH for 28 days. The data were recorded before treatment (Day 0) and at seven day intervals for 28 days.

7.2.3.2 Determination of physical quality

Weight loss was determined by weighing 20 fruit from each treatment using a digital balance (model GF-6100, A&D Co. Ltd., Japan). The same fruit were used for determining weight loss from day 0 at intervals of 7 days until the end of the experiment. The difference between initial and final weight was considered as the total weight loss during the storage interval and was expressed as percentage weight loss using the standard AOAC (1984) method by the equation:

$$\% \text{ Weight loss} = \frac{W_1 - W_2}{W_1} \times 100$$

where W_1 = Initial weight of the fruit (day 0); W_2 = Weight of fruit after 7 days of storage

Fruit firmness was determined on 4 fruit in each treatment with an Instron texture analyser (Instron 2519-104, Norwood, MA) having an 8 mm plunger tip. Firmness was evaluated by subjecting fruit to a puncture test at a constant speed of 20 mm min^{-1}. The maximum amount of force required to penetrate the fruit was recorded on each fruit at three random positions and the average of these results on fruit firmness was expressed in Newtons (*N*).

After performing the firmness assay, the same fruit were used for determination of chemical quality and antioxidant activity.

7.2.3.3 Determination of chemical quality

Soluble solids concentration (SSC) of dragon fruit pulp was determined using a Palette Digital Refractometer (Model: PR-32α, Atago Co, Ltd. Japan). Fruit pulp (10 g) was sampled from a mixture of 4 fruit in each treatment homogenized in a kitchen blender with 40 ml of purified water. The mixture was centrifuged at 5000 × *g* for 5 min and then filtered through cotton wool. A drop of the filtrate was placed on the prism glass of the refractometer to obtain the % SSC reading. The refractometer was calibrated with purified water to give a 0% reading before analysis. The readings were multiplied by the dilution factor to obtain the SSC of the dragon fruit pulp.

Titratable acidity was measured using the remaining filtrate of SSC. A 5 ml aliquot of filtrate was titrated with 0.1 N NaOH until the pH reached 8.1. Titration was not done until the colour changed to pink as the sample was already pink and pH 8.1 of the sample acted as an indicator to determine the total acidity (Yusoff, 2006).

The titratable acidity was measured using the following equation:

$$\text{Titratable acidity} = \frac{\text{Volume of NaOH used}}{\text{Weight of sample}} \times 0.1\text{N}$$

7.2.3.4 Determination of antioxidants

The total phenolic content was estimated using the Folin Ciocalteau (FC) spectrophotometric method, established by Ramamoorthy and Bono (2007). Fruit pulp (50 g) was homogenized in a kitchen blender and squeezed through a muslin cloth to obtain fruit juice. Fruit juice (0.1 ml) was mixed with 1.5 ml of 7% sodium carbonate and 0.5 ml of FC. Volume of the mixture was adjusted to 10 ml by adding purified water. The mixture was incubated at 40 °C for 2 hours. The absorbance was recorded at 750 nm using a UV-Vis Spectrophotometer (Varioskan Flash Multimode Reader, Thermo Fisher Scientific, USA). The results were stated in mg gallic acid equivalent per 100 g of fresh weight of fruit, using values calculated from the standard curve in Section 5.2.3.

The spectrophotometric method developed by Froehlicher *et al*. (2009) was adopted to measure the total flavonoids in dragon fruit. Samples (0.5 mg) of ground fruit pulp were mixed with 1.5 ml of methanolic $AlCl_3.6H_2O$ in sealed tubes and kept in the dark for 15 min. The absorbance was measured at 430 nm using a UV-Vis Spectrophotometer (Varioskan Flash Multimode Reader, Thermo Fisher Scientific, USA). A standard curve was prepared using quercetin stock solution (Appendix B 7.1) and the results were expressed as µg of quercetin equivalent to one g fresh weight of fruit sample.

Lycopene extraction was done by the method developed by Fish *et al.* (2002). Briefly, 0.6 ml of fruit juice (from juice extracted for total phenolics) was mixed with 10 ml of hexane, 5 ml of 0.05% butylated hydroxytoluene in acetone and 5 ml of 95% ethanol. The reaction mixture was agitated at 180 rpm for 15 min on ice. Then, 3 ml of ice cold water was added and it was shaken again for 5 minutes. The absorbance was recorded at 503 nm using a UV-Vis Spectrophotometer (Varioskan Flash Multimode Reader, Thermo Fisher Scientific, USA). The amount of lycopene was calculated using the formula of Javanmardi *et al*. (2006).

$$\text{Lycopene} = \left({}^{x}/_{y}\right) \times A_{503} \times 3.12$$

Where,

x= amount of hexane

y= weight of fruit

The results were expressed in µg g^{-1} fresh weight of fruit sample.

The total antioxidants activity was measured by ferric reducing antioxidant power (FRAP) assay using the juice extracted for total phenolics. The FRAP reagent contained 2.5 ml of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCL, 2.5 ml of 20 mM $FeCl_3$ and 25 ml of 0.03 mM acetate buffer pH 3.6. The reaction mixture consisted of 40 µl of fruit extract mixed with 3 ml of FRAP reagent and was incubated at 37°C for 4 min. Absorbance was recorded at 593 nm and the reducing power was estimated using the standard curve for ferrous sulphate ($FeSO_4$) (Appendix B 7.2). The results were expressed as the concentration of antioxidants having a ferric reducing activity equivalent to 1 mg $FeSO_4$ g^{-1} fresh weight of fruit sample (Ahmed and Baig, 2009).

7.2.4 Gaseous exchange analysis

7.2.4.1 Determination of respiration rate

The rates of respiration and ethylene evolution were measured using the methods described by Ali (2006), with slight modification. Briefly, respiration was measured by placing one dragon fruit in a 1 litre plastic container for 1 h and a gas tight hypodermic syringe was used to withdraw 1 ml of gas sample from the headspace and analysed with a gas chromatograph (GC) (Claru-500, Perkin Elmer, USA) equipped with a stainless steel column (Porapak R 80/100). Helium at a flow rate of 20 ml min^{-1} served as the carrier gas. The oven, injector and thermal conductivity detector (TCD) were set at 60, 100 and 200°C, respectively. Calibration was done using one ml of 1.0% CO_2 gas (Scotty Gases, Beltifonte, PA USA) as standard. The amount of CO_2 production was expressed in ml kg^{-1} h^{-1}.

7.2.4.2 Determination of ethylene evolution

Ethylene evolution was measured by taking a 1 ml gas sample from each jar using a hypodermic syringe and injecting it into a GC fitted with a stainless steel column (Porapak T, 100/120) and a flame ionisation detector (FID). Nitrogen, hydrogen and air flow rates were 20 ml min^{-1}. The nitrogen served as a carrier gas. Oven, injector and FID were set at 150, 200 and 200°C, respectively. One ml ethylene gas (10 µl ml^{-1}) (Scotty Gases, Beltifonte, PA USA) was injected for calibration, and served as an external standard gas. The amount of ethylene was expressed in µl kg^{-1} h^{-1}.

The experiment was conducted using a completely randomized design (CRD) with four replicates in each treatment. Each replicate consisted of twenty fruit. The data were subjected to analysis of variance (ANOVA) using SAS® software, while least DMRT was used to compare differences among treatments at 95% confidence level for each variable. The whole experiment was repeated twice, and data were pooled before analysis.

7.3 Results and discussion

7.3.1 Physical quality of fruit treated with conventional chitosan and submicron chitosan dispersions

7.3.1.1 Weight loss

There were significant differences ($P < 0.05$) in weight loss among the treatments during the 28 days of storage compared to the control (Fig. 7.1). In control fruit, the weight loss reached a maximum of 27.8%. Weight loss increased with time in storage and the smallest weight loss (15.4%) was observed with 1.0% conventional chitosan treatments, but differences compared with 600 nm (18.1%) and 1000 nm (17.2%) droplets were not significant (Fig. 7.1).

Weight loss during ripening is a common and unavoidable phenomenon for all fruits and vegetables. When first picked from a tree, the fruit remains ‘normal’ for a few days or even weeks, during which it undergoes a series of ‘background’ metabolic processes in preparation for ripening. During this time, the fruit continues to respire and transpire, losing some of its substrate and water which results in some weight loss. The basic mechanism of weight loss in fresh fruit and vegetables is due to vapour pressure differentials, and coatings maintain the quality of fruit by maintaining the turgidity through water retention in the fruit (Chien *et al*., 2007b).

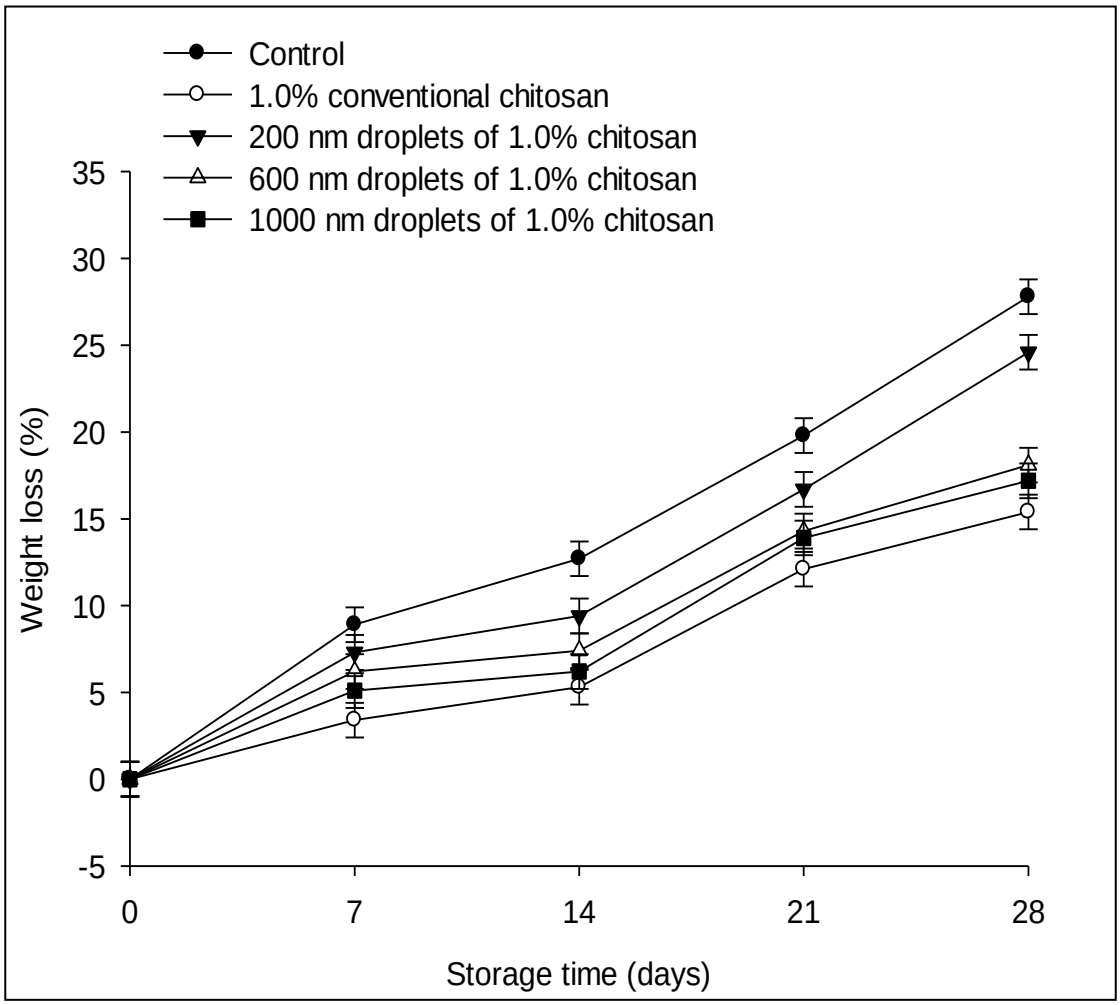


Figure 7.1: Effect of conventional chitosan and submicron chitosan dispersions on weight loss in dragon fruit during 28 days of storage at 10 ± 2 °C; vertical bars indicate standard errors; means of four replicates.

The results of the present study suggest that conventional chitosan has the ability to form a coating on the surface of the fruit which serves as a barrier against diffusion through the stomata (Paull and Chen, 1989). These results are in agreement with the findings of Ali *et al*. (2011) who reported that chitosan coatings reduced weight loss and helped in maintaining the quality of papaya fruit. However, the application of submicron chitosan dispersions as 200 nm droplets may not be helpful in reducing water loss in the fruit. This suggests that the water barrier properties were impaired by the submicron chitosan dispersions, resulting in increased water permeability

and excessive water loss (Lin and Zhao, 2007). The reduction in water barrier properties of 200 nm droplets might be due to the lower viscosity as compared with the other droplets (Table 3.4). This lower viscosity may reduce adhesion properties of the coating material and might not be appropriate to form a smooth layer of coating on the fruit surface. Therefore, an incomplete film may have been formed.

7.3.1.2 Fruit firmness

There were significant differences ($P < 0.05$) in fruit firmness of all the treatments compared to the control during the 28 days of storage (Fig. 7.2). In the control fruit, firmness declined rapidly to 10.4 N. The fruit firmness decreased with time, but the smallest decrease (23.5 N) was observed with 1.0% conventional chitosan but it did not differ significantly ($P > 0.05$) from 600 nm (19.8 N) and 1000 nm (21.8 N) droplets.

The results are in agreement with the earlier findings of Ali *et al*. (2010) and Zhu *et al*. (2008), where fruit retained their firmness with chitosan coatings. Fruit softening mainly occurs due to deterioration in the structural composition of cell walls and in the intracellular materials (Seymour *et al*., 1993). This biochemical process involves several enzymes which lead to depolymerisation of pectin substances, resulting in an increase in the activity of pectinesterase and polygalacturonase.

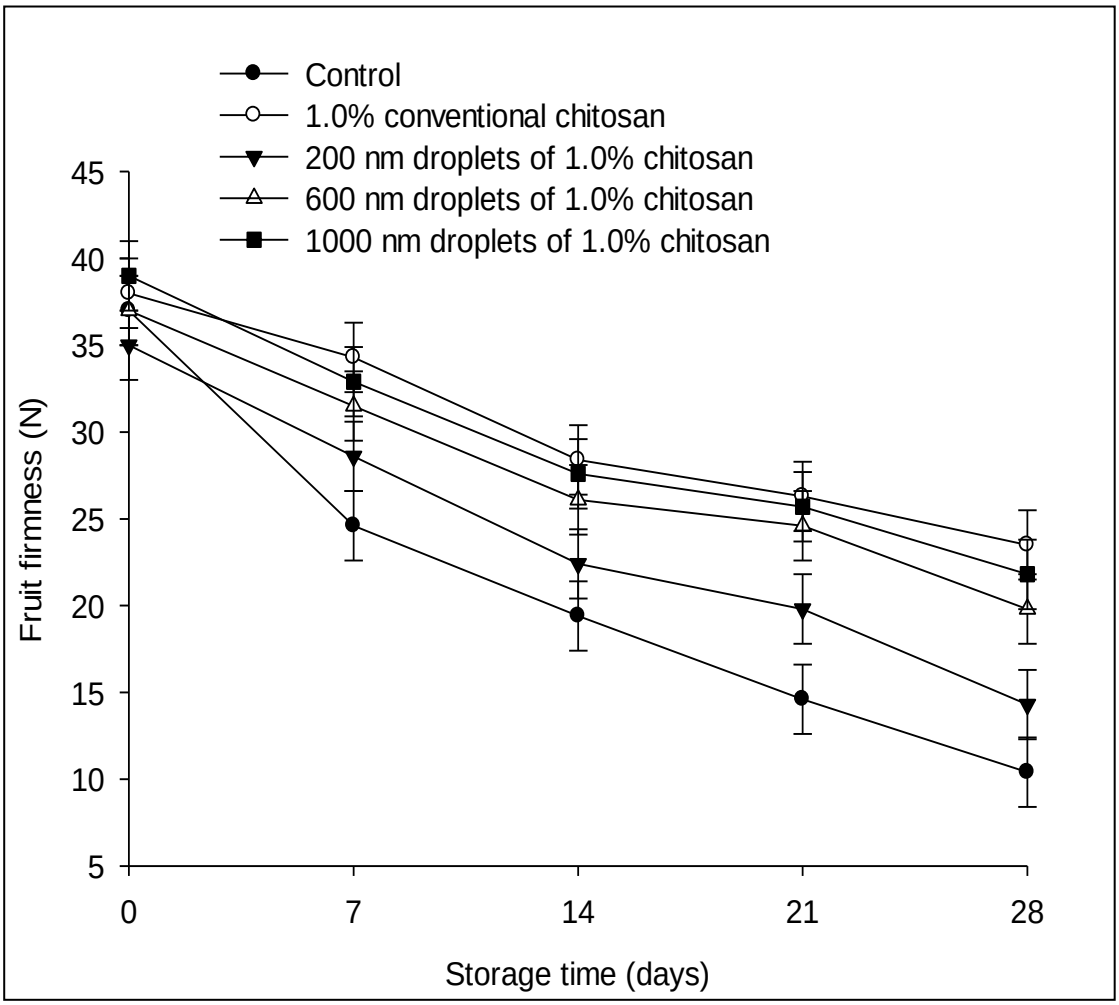


Figure 7.2: Effect of conventional chitosan and submicron chitosan dispersions on fruit firmness of dragon fruit during 28 days of storage at 10 ± 2 °C; vertical bars indicate standard errors; means of 4 replicates.

The increase in softening of fruit with submicron chitosan dispersions may have been due to a decrease in cohesion between the coating and the fruit surface such that the permeability towards O_2 and CO_2 increased. Thus, the internal atmosphere of the fruit would not have changed. Jafarizadeh *et al*. (2011) also reported that hydroxypropyl methylcellulose (HPMC) coatings on banana fruit increased O_2 and CO_2 permeability due to reduced cohesion of the coating material and thus were unable to retain fruit firmness. Fruit firmness can be retained with high levels of CO_2 and low levels of O_2 that limit the activity of the enzymes (Salunkhe *et al*., 1991).

7.3.2 Chemical quality of fruit treated with conventional chitosan and submicron chitosan dispersions

7.3.2.1 Soluble solids concentration

There was a greater increase in SSC content in the untreated control fruit as compared with the treated fruit (Fig. 7.3). In control fruit, SSC reached a maximum of 60.0% after 28 days of storage. The lowest increase in SSC (39.8%) was observed with 1.0% conventional chitosan.

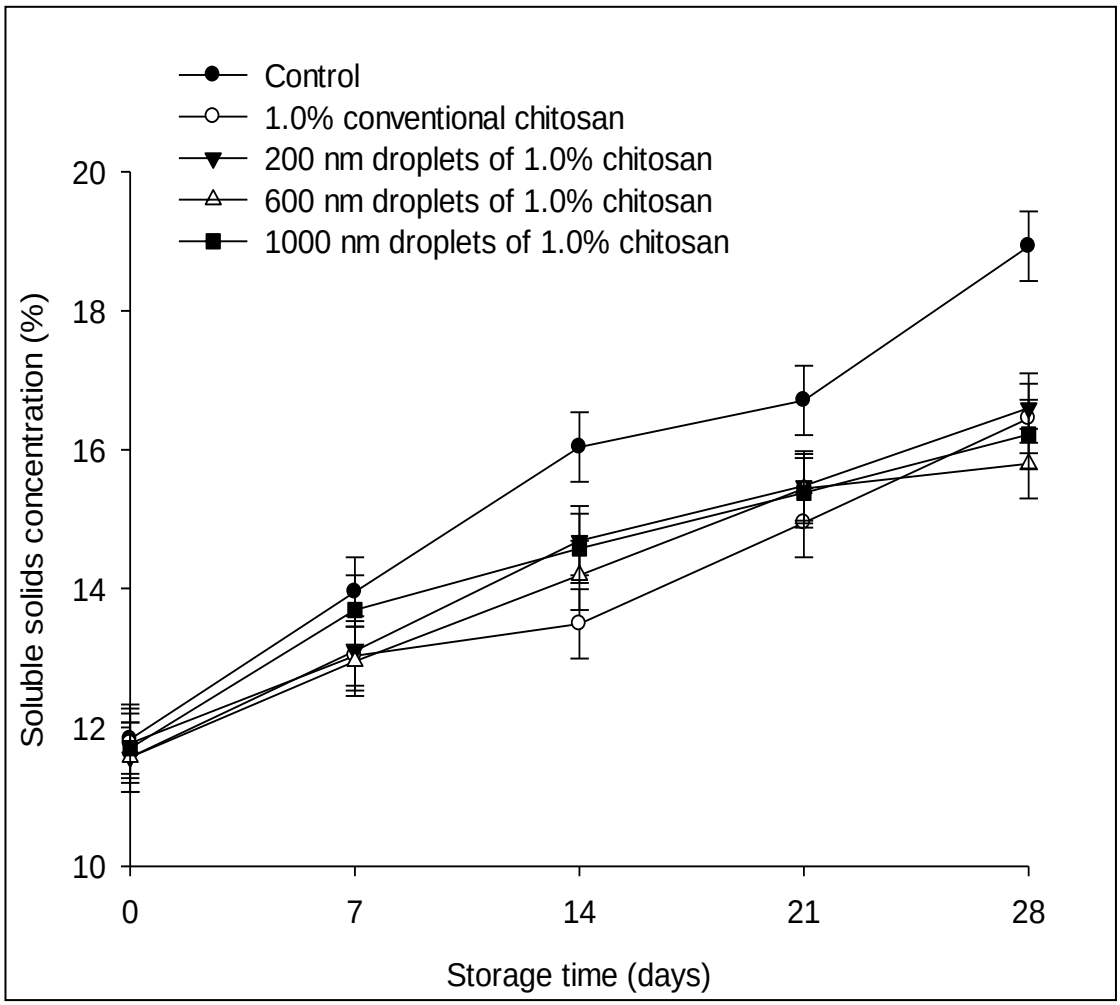


Figure 7.3: Effect of conventional chitosan and submicron chitosan dispersions on soluble solids concentration of dragon fruit during 28 days of storage at 10 ± 2 °C; vertical bars indicate standard errors; means of four replicates.

The increase in SSC values reflected the carbohydrate metabolism in the climacteric fruit, particularly conversion of starch to glucose (Bashir and Abu-Goukh, 2003). A higher SSC value is observed when there is higher respiration, which results in the hydrolysis of sugars and organic acids (Jafarizadeh, *et al*., 2011). It is suggested that chitosan treatments formed a semi-permeable film around the fruit, which suppressed ethylene evolution and maintained their SSC content (Ali *et al*., 2010).

7.3.2.2 Titratable acidity

Major organic acids in ripe dragon fruit are citric and L-lactic acids. There was a major decrease in TA in the control (Fig. 7.4) and a slower decrease in treated fruit. The decrease in TA in control fruit reached the lowest value (0.12) after 28 days of storage. The smallest decrease (0.45) in TA occurred with 1.0% conventional chitosan coating but it did not differ significantly ($P > 0.05$) with 600 nm (0.41) and 1000 nm (0.44) droplets.

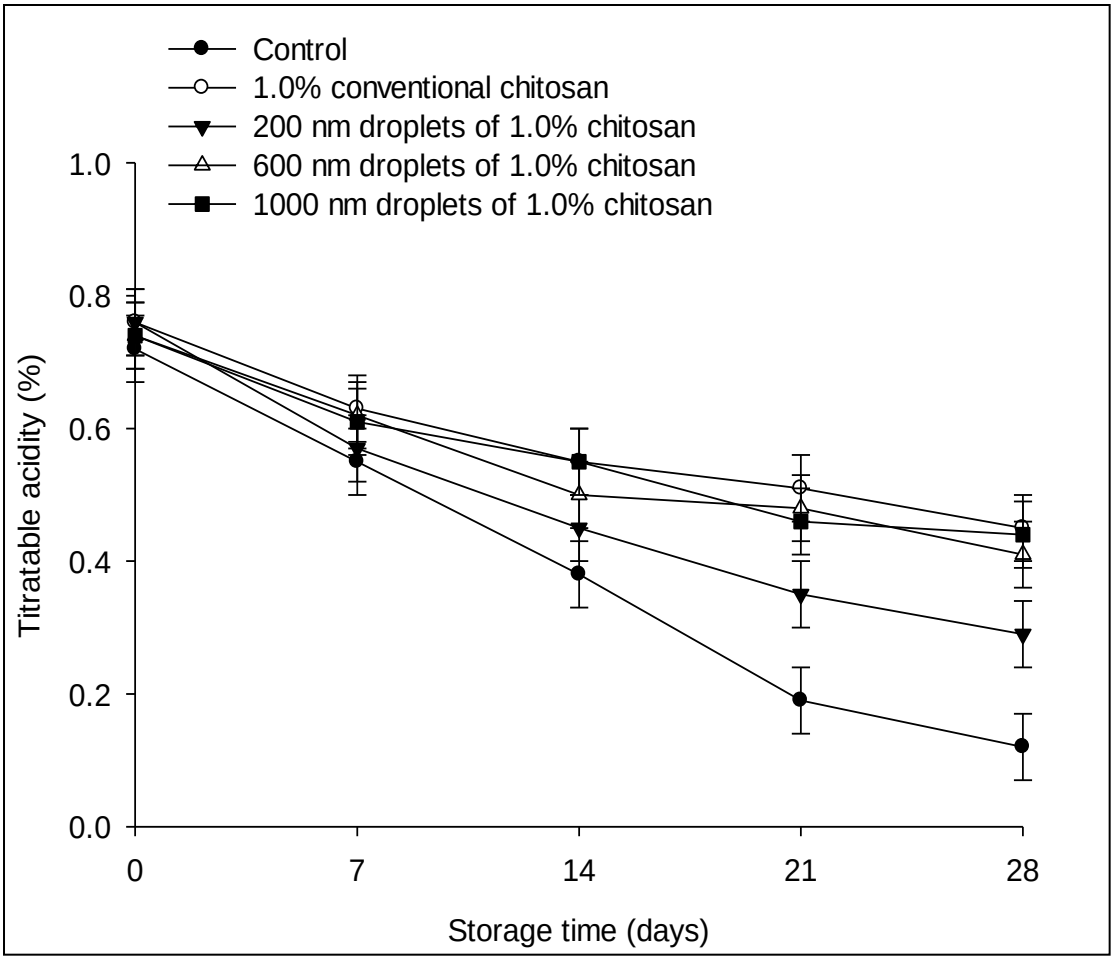


Figure 7.4: Effect of conventional chitosan and submicron chitosan dispersions on titratable acidity of dragon fruit during 28 days of storage at 10 ± 2 °C; vertical bars indicate standard errors; means of four replicates; DMRT at $P < 0.05$.

In general, TA decreases due to the metabolism of organic acids. Han *et al*. (2004) reported a slow decrease in TA in fruit coated with chitosan. A rapid decrease in TA accompanies senescence. The decrease in TA in fruit treated with chitosan as 200 nm droplets may be attributed to the pore size of the coating produced by dispersions, which may be inappropriate for forming a smooth layer of coating on the surface of fruit. Thus, larger pore size and less cohesion of these coatings due to emulsifiers would result in higher respiratory activity which causes rapid metabolic changes in the fruit (Echeverria and Valich, 1989).

7.3.3 Antioxidant capacity of fruit treated with conventional chitosan and submicron chitosan dispersions

7.3.3.1 Total phenolic content

The total phenolic content declined in the fruit after harvest in all treatments (Fig. 7.5). A significantly lower ($P < 0.05$) content of total phenolics was observed in control fruit (73.3%) and the 200 nm droplet treatment. Less of a decrease occurred in fruit treated with 1.0% conventional chitosan (35.0%), which was similar to the submicron chitosan dispersions as 600 and 1000 nm droplets.

The decrease in total phenolic compounds in control fruit could have been due to higher rate of respiration and fungal infection (El Ghaouth *et al*., 1991), which would result in the degradation of some phenolic compounds (Day, 2001). In this study, the decrease in total phenolics with 200 nm droplets might be due to increase in water loss (Fig. 7.1) which results in reduced cellular turgidity (Saladié *et al*., 2007) and may cause the deterioration of cell structure. Many biotic and abiotic stresses are responsible for the decrease in total phenolics (Artés-Hernández *et al*., 2007). These stresses have been associated with senescence and breakdown of cell structure during storage (Macheix *et al.,* 1990).

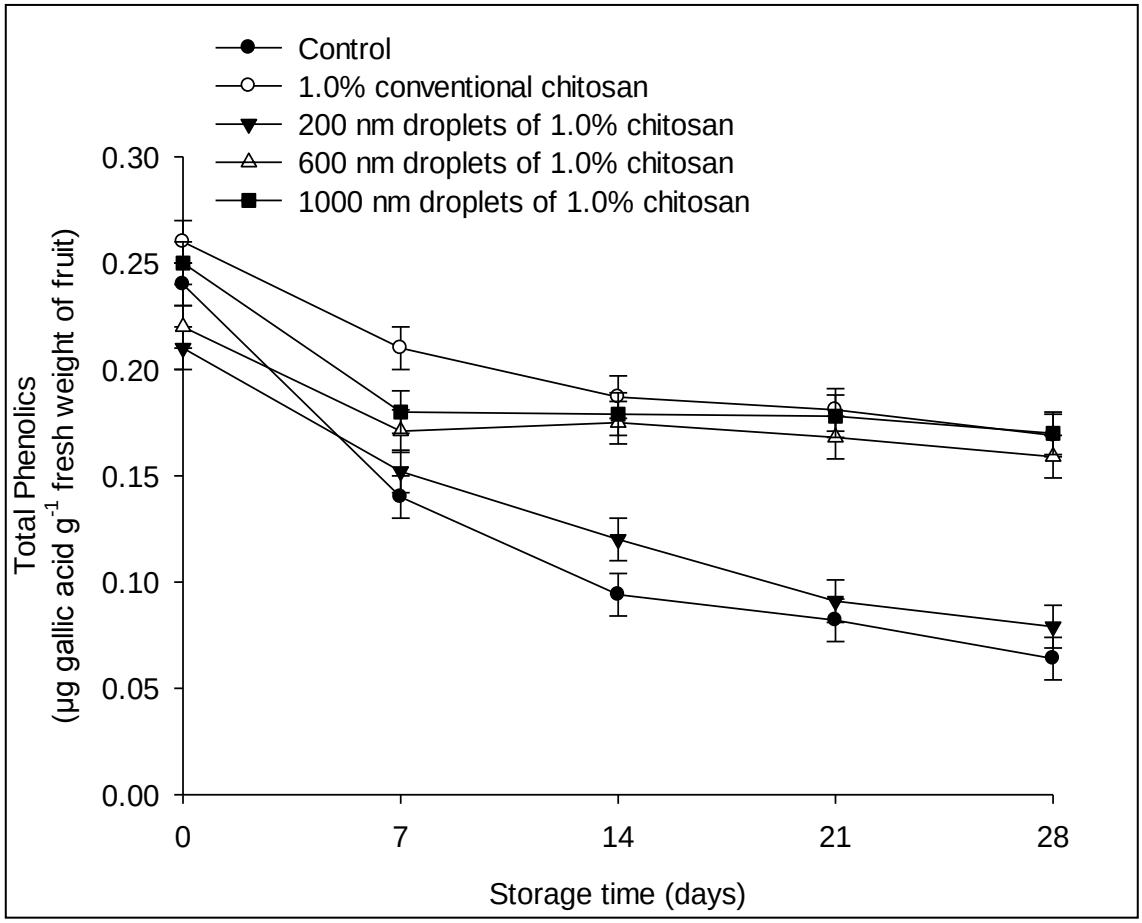


Figure 7.5: Effect of conventional chitosan and submicron chitosan dispersions on total phenolics of dragon fruit during 28 days of storage at 10 ± 2 °C; vertical bars indicate standard errors; means of four replicates.

7.3.3.2 Total flavonoids

Similar to total phenolics, flavonoid content declined in the fruit after harvest in all treatments (Fig. 7.6). A significant ($P < 0.05$) decrease in total flavonoids was observed in control fruit (79.4%). Less of a decrease was observed in fruit treated with 1.0% conventional chitosan (19.9%), which did not differ from 600 and 1000 nm droplets of 1.0% chitosan.

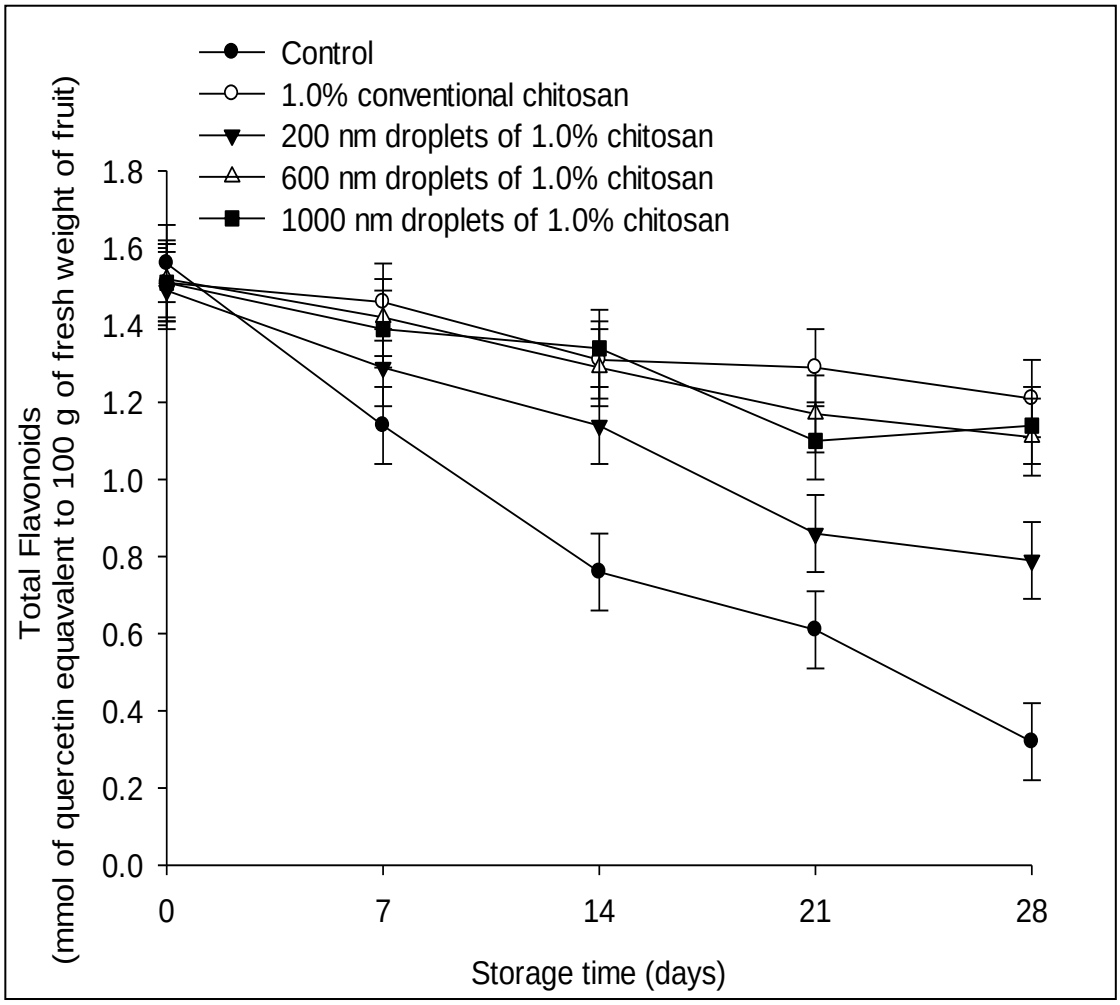


Figure 7.6: Effect of conventional chitosan and submicron chitosan dispersions on total flavonoids of dragon fruit during 28 days of storage at 10 ± 2 °C; vertical bars indicate standard errors; means of four replicates.

The degradation in total flavonoids may have been due to the high respiration at the end of the storage period. It may also relate to tissue degradation (DuPont *et al*., 2000).

7.3.3.3 Lycopene

A significant ($P < 0.05$) decrease in lycopene (78.6%) was observed in control fruit during their storage. In fruit treated with conventional chitosan, the decrease (25.7%) was less and was similar in the treatments with 1.0% submicron chitosan dispersions as 1000 nm and 600 nm droplets (Fig. 7.7).

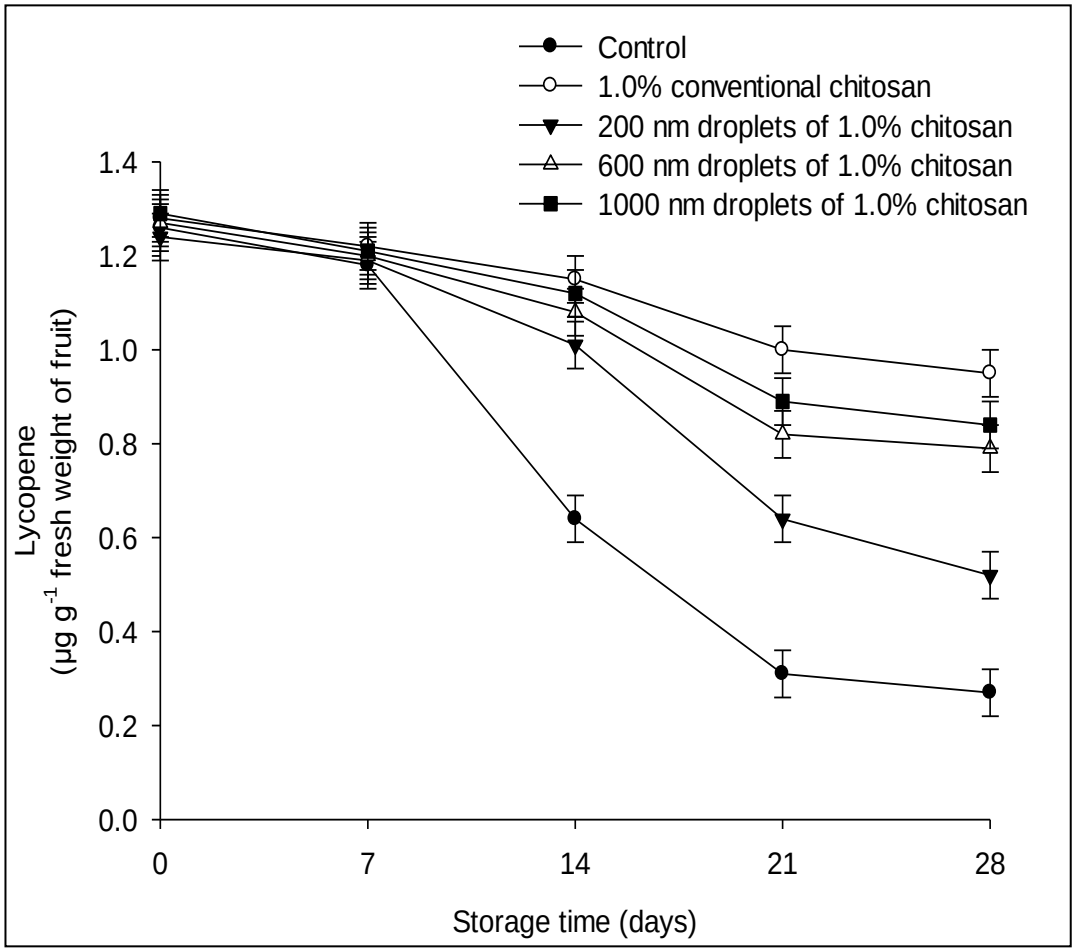


Figure 7.7: Effect of conventional chitosan and submicron chitosan dispersions on lycopene content of dragon fruit during 28 days of storage at 10 ± 2 °C; vertical bars indicate standard errors; means of four replicates.

Gil *et al*. (1999) reported that lycopene synthesis increased with the ripening of tomato. The higher retention of lycopene in tomatoes during storage is due to low oxidation of carotenoids (Özlem and Bayindirli, 1998). It has also been reported that lycopene content depends on the rate of respiration (Javanmardi and Kubota, 2006). In the present study, the degradation of lycopene in control fruit was due to the high respiration rate at the end of storage, while its degradation in the treatment with 200 nm droplets was probably due to the low viscosity of submicron dispersions which reduces its filmogenic properties.

7.3.3.4 Total antioxidant activity

A decrease in total antioxidants was observed in all the treatments but the decrease was faster in control fruit (77.1%), followed by the fruit treated with 200 nm droplets (66.7%). The slower decrease in fruit treated with conventional chitosan (33.3%) was not significantly different ($P > 0.05$) from submicron chitosan dispersions with 600 and 1000 nm droplets (39.7 and 37.4%, respectively) (Fig. 7.8).

In general, a positive correlation occurs between total phenolic content and total antioxidant activity (Artés-Hernández *et al*., 2007). The antioxidants in fresh fruits and vegetables depend on several factors, such as species, cultivar, environmental conditions, geographical origin, cultural practices and analytical methods (Chun *et al*., 2005). In the present study, the decrease in total antioxidants complements the earlier findings of Ghasemnezhad *et al*. (2010). They reported that the decrease in total antioxidant activity of apricot was due to heat production in the fruit and breakdown of cell structure during storage.

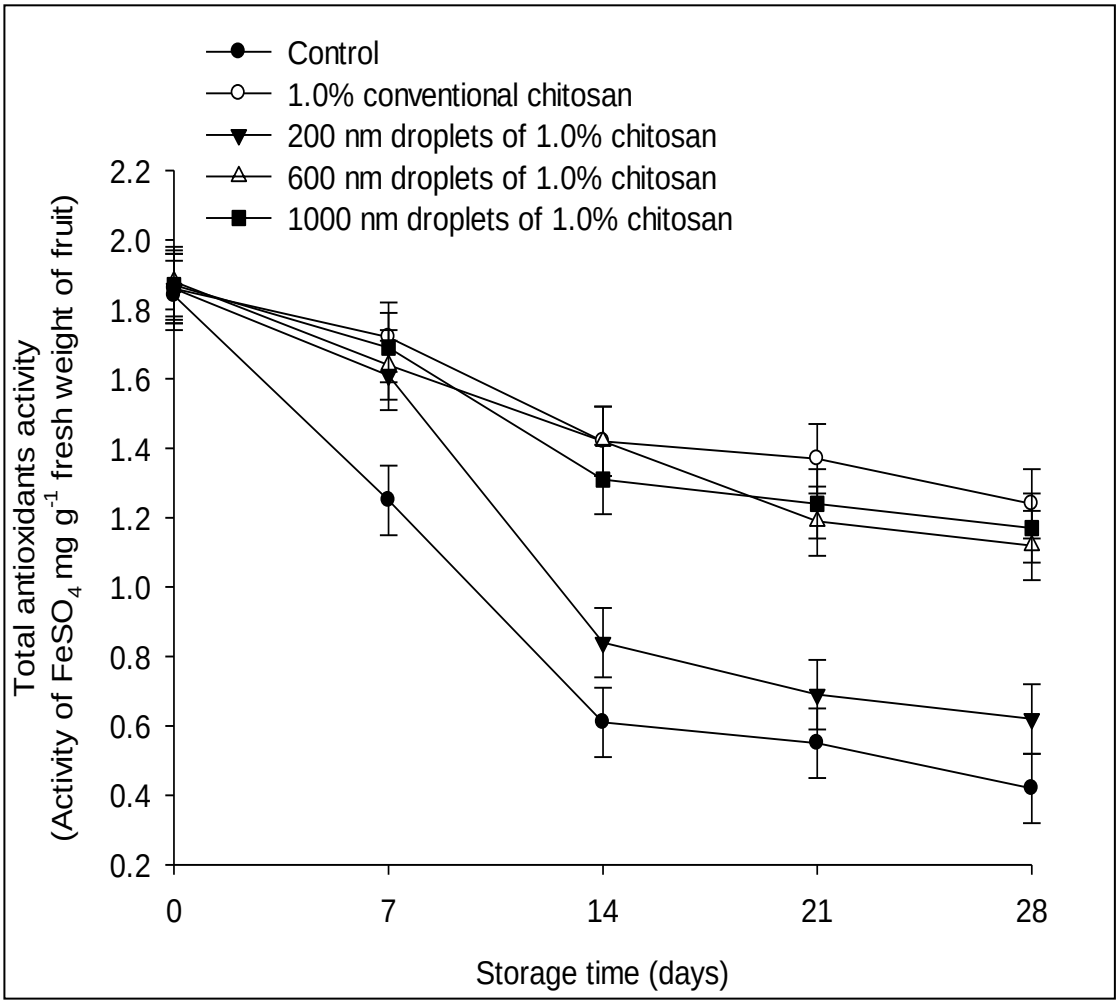


Figure 7.8: Effect of conventional chitosan and submicron chitosan dispersions on total antioxidants of dragon fruit during 28 days of storage at 10 ± 2 °C; vertical bars indicate standard errors; means of 4 replicates.

This study has shown that the good retention of total phenols, total flavonoids and lycopene in fruit treated with 1.0% conventional chitosan and the submicron chitosan dispersions as 1000 nm and 600 nm droplets is important for maintaining the total antioxidants capacity.

7.3.4 Gaseous exchange analysis

7.3.4.1 Respiration rate

No peaks of respiration and ethylene were recorded in the fruit during 28 days of storage. A continuous decrease in carbon dioxide (CO_2) production was observed in all treatments (Fig. 7.9 a). A sudden increase in CO_2 was observed after 14 days in control fruit and the increase in CO_2 concentration was 98.0% on the 28^{th} day. An increase in CO_2 was observed in all other treatments after 21 days.

7.3.4.2 Ethylene evolution

A decrease in ethylene concentration was observed in all treatments (Fig. 7.9 b) until day 21 after which it remained constant or slightly increased. The changes in the ethylene production of the control fruit were more pronounced than in the other treatments. On day 28, ethylene production in the control had increased and was significantly ($P < 0.05$) higher than in the other treatments.

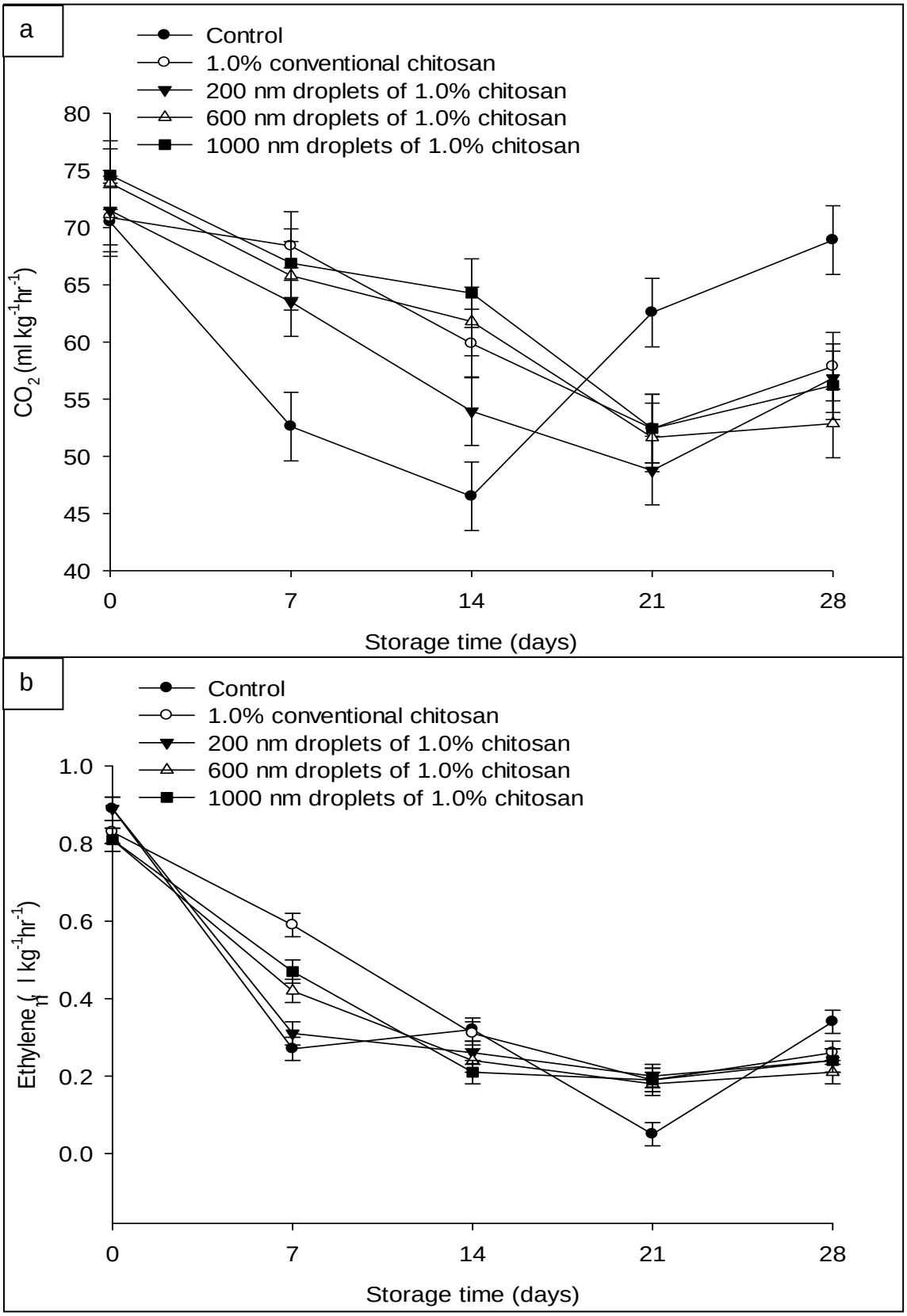


Figure 7.9: Effect of conventional chitosan and submicron chitosan dispersions on (a) carbon dioxide (CO_2) production (b) ethylene evolution in dragon fruit during 28 days of storage at 10 ± 2 °C; vertical bars indicate standard errors; means of four replicates.

This pattern of respiration and ethylene production could be due to the growth of fungus on the skin of the fruit (Pérez-Tello *et al*., 2001). An increase in respiration due to disease has previously been reported in strawberries (El Ghaouth *et al*., 1991) and in longan fruit (Jiang and Li, 2001).

Minor differences in the decrease in quality of the fruit treated with submicron chitosan dispersions when compared with conventional chitosan could be due to the low viscosity of submicron chitosan dispersions that may have reduced the filmogenic properties of the solutions (Navarro-Tarazaga *et al*., 2008).

The results of the present study showed that 1.0% conventional chitosan helped to maintain the quality of fruit and was comparable with the submicron chitosan dispersions as 600 and 1000 nm droplets. In addition, these dispersions did not show any detrimental effects on the quality of the fruit.

CHAPTER 8

CONCLUSION

Four chitosan solutions of different molecular weight were tested against *C. gloeosporioides* during this study and the low molecular weight chitosan showed best antifungal activity. Using low molecular weight chitosan led to application of less time for sonication in preparing submicron chitosan dispersions. Besides the use of less energy, it maintained a higher zeta potential due to its lower degree of acetylation which helped the stability of the dispersions. The low viscosity of low molecular weight chitosan submicron dispersions may allow it to penetrate into the cells more easily.

Submicron chitosan dispersions based on low molecular weight chitosan showed promising effects in controlling *C. gloeosporioides* isolated from dragon fruit plants in comparsion with locally prepared chitosan. This could be attributed to its low degree of acetylation and high cationic nature which interacts with the negative charges of the fungal cell wall and causes leakage in the fungal cells.

The maximum reduction in disease of dragon fruit plants with the use of 600 nm droplets could be endorsed with the curative effect of chitosan, as it was helpful in reducing the disease even when applied one week after inoculation. This reduction in disease could also be ascribed to the multi-mode action of chitosan which has the potential to elicit the production of inducible compounds, such as total phenolics, PO, PPO, PAL and PR proteins, and consequently enhances the resistance of the plant against anthracnose. There are different threshold levels of these enzymes in

inoculated and uninoculated plants which suggests that higher concentrations of these enzymes may be associated with lower disease incidence.

Droplets at 600 nm reduced the onset of disease and this resulted in increased vegetative growth. This increase in vegetative growth might be due to the increased chlorophyll content. As chlorophyll is the major photosynthetic pigment of the plant, the increase in chlorophyll increased the vigour of the plants and consequently their vegetative growth.

Postharvest application of submicron chitosan dispersions showed that 600 nm droplets of 1.0% chitosan caused a reduction in disease incidence and disease severity. It is proposed that the matrix of this droplet size contains enough of the active ingredient which penetrates the fungal cell wall and causes leakage from the fungal cells.

Fruits coated with 600 nm droplets of 1.0% chitosan exhibited less weight loss, maintained fruit firmness, had slower increase in soluble solids and retained antioxidant properties without any negative effects.

The findings of this study suggest that use of 600 nm droplets of 1.0% chitosan is an inexpensive method with potential to control pre- and postharvest anthracnose of dragon fruit. Future studies should be conducted at the genetic level to observe the effect of these submicron dispersions on the production of inducible compounds against anthracnose. These submicron chitosan dispersions should also be tested on a commercial scale in relation to the development of new formulations and their application to a range of crops.

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APPENDICES
APPENDIX-A

A 3.1: Emulsifiers, sonication time and temperature used for the preparation of chitosan loaded nanoemulsions.

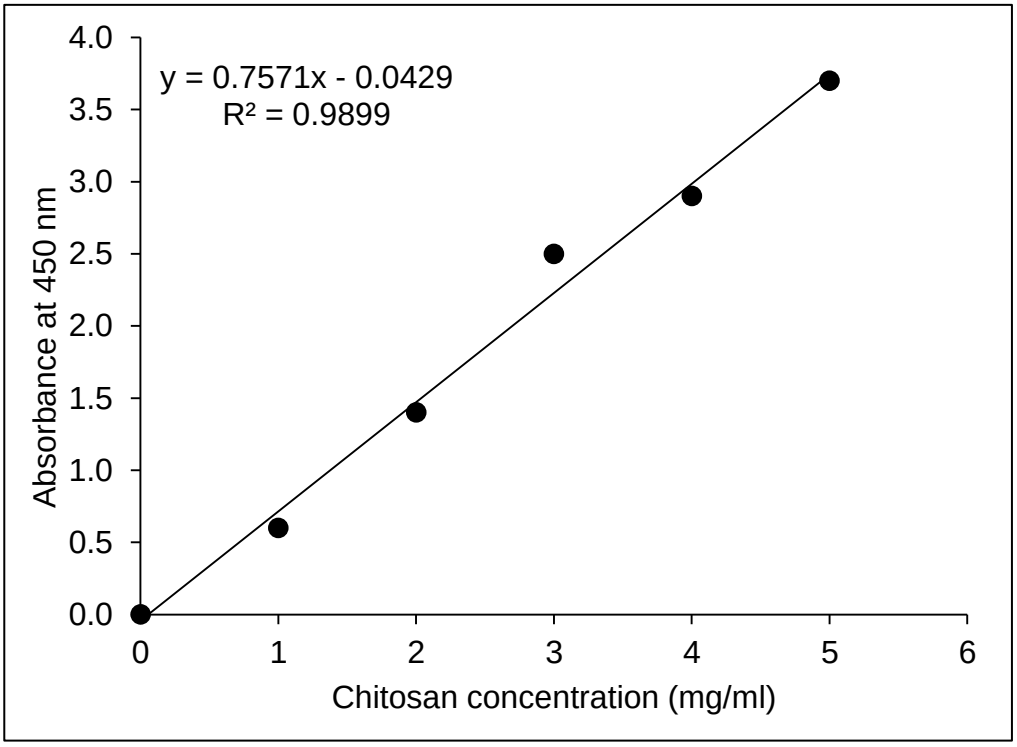
Chitosan conc. (%)	Temp (°C)	Sonication Time (min)	Emulsifier	Droplet size (nm)
0.5	**60**	10	**Tween 80**	9350
0.5	60	20	Tween 80	9143
0.5	60	30	Tween 80	8246
0.5	60	40	Tween 80	Not detected
0.5	**45**	10	Tween 80	9465
0.5	45	20	Tween 80	9123
0.5	45	30	Tween 80	8163
0.5	45	40	Tween 80	8150
0.5	45	50	Tween 80	8134
0.5	45	60	Tween 80	8119
0.5	45	1hr, 30 min	Tween 80	Not detected
0.5	**30**	10	Tween 80	9264
0.5	30	20	Tween 80	9134
0.5	30	30	Tween 80	9132
0.5	30	40	Tween 80	9192
0.5	30	60	Tween 80	9113
0.5	**25**	10	Tween 80	9257
0.5	25	20	Tween 80	9143
0.5	25	30	Tween 80	9127
0.5	25	40	Tween 80	9347

0.5	25	50	Tween 80	Not detected
0.5	25	60	Tween 80	Not detected
0.5	25	10	**Tween 20**	6623
0.5	25	20	Tween 20	6142
0.5	25	30	Tween 20	5921
0.5	25	40	Tween 20	$1.18x10^4$
0.5	25	50	Tween 20	Not detected
0.5	25	60	Tween 20	Not detected
0.5	25	10	**Glycerol**	Not detected
0.5	25	20	Glycerol	Not detected
0.5	25	30	Glycerol	9564
0.5	25	40	Glycerol	9568
0.5	25	50	Glycerol	9694
0.5	25	60	Glycerol	9693
0.5	25	10	**Brij 56 ,Span 20**	1000
0.5	25	15	Brij 56 ,Span 20	800
0.5	25	20	Brij 56 ,Span 20	600
0.5	25	25	Brij 56 ,Span 20	400
0.5	25	30	Brij 56, Span 20	200
1.0	25	15	Brij 56 ,Span 20	1000
1.0	25	20	Brij 56 ,Span 20	800
1.0	25	25	Brij 56 ,Span 20	600
1.0	25	30	Brij 56 ,Span 20	400

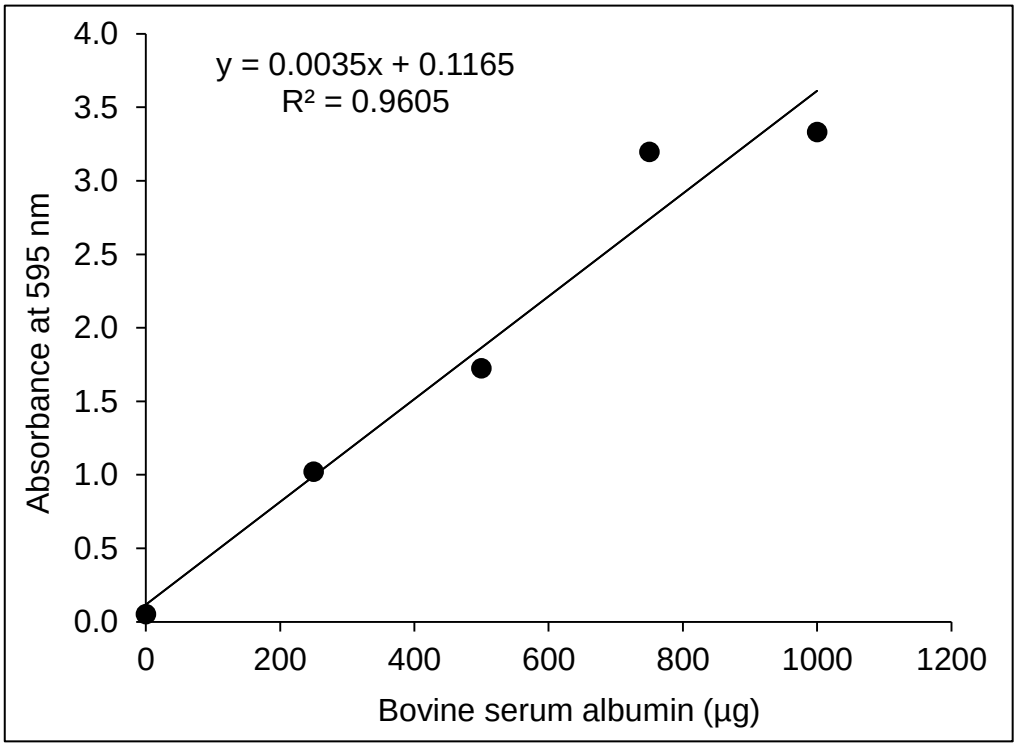
1.0	25	35	Brij 56, Span 20	200
1.5	25	10	Brij 56 ,Span 20	1000
1.5	25	15	Brij 56 ,Span 20	800
1.5	25	20	Brij 56 ,Span 20	600
1.5	25	25	Brij 56 ,Span 20	400
1.5	25	30	Brij 56, Span 20	200
2.0	25	10	Brij 56 ,Span 20	1000
2.0	25	15	Brij 56 ,Span 20	800
2.0	25	20	Brij 56 ,Span 20	600
2.0	25	25	Brij 56 ,Span 20	400
2.0	25	30	Brij 56, Span 20	200

APPENDIX-B

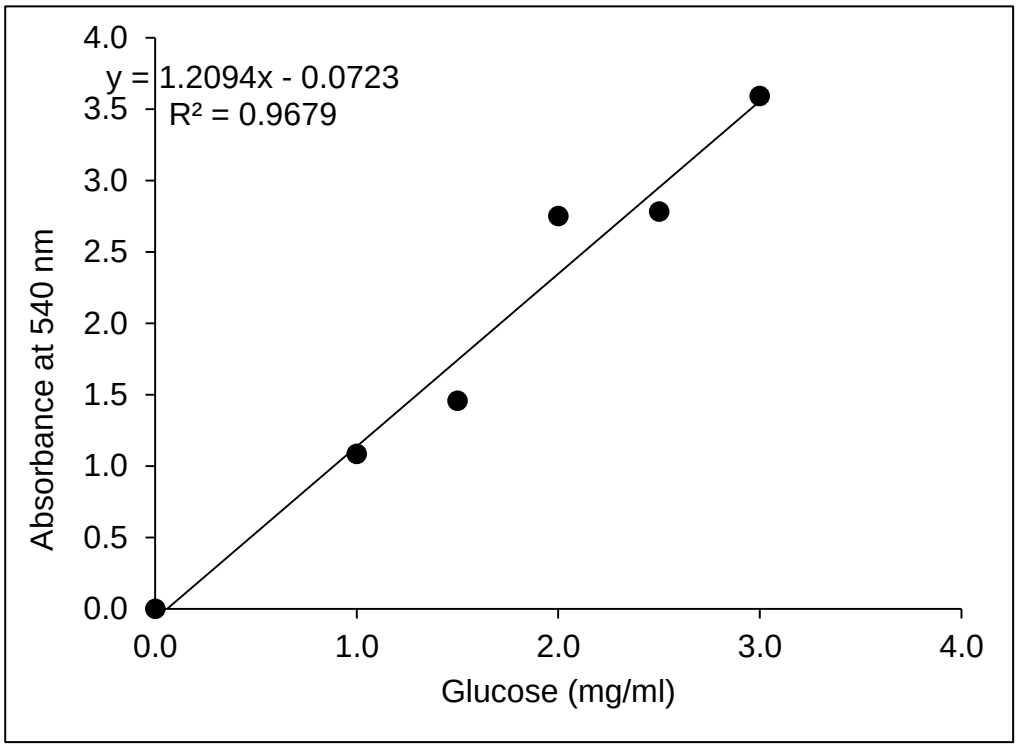
B 3.1: Chitosan standard curve.



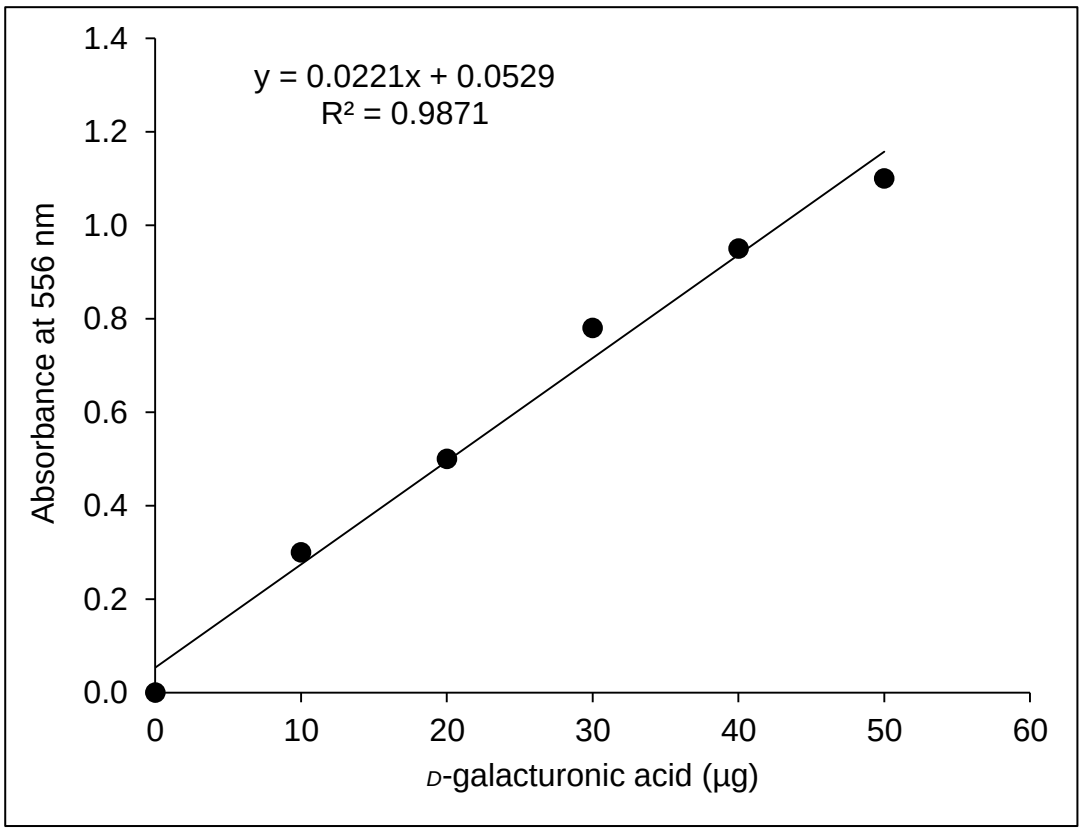
B 4.1 Bovine serum albumin standard curve.



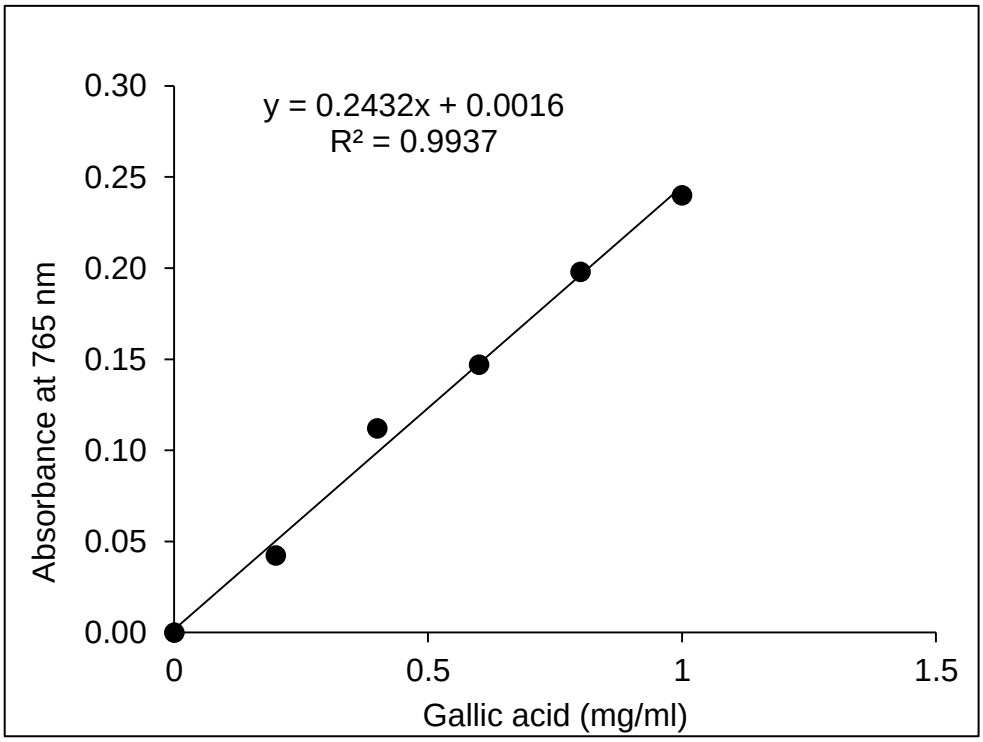
B 4.2 Glucose standard curve.



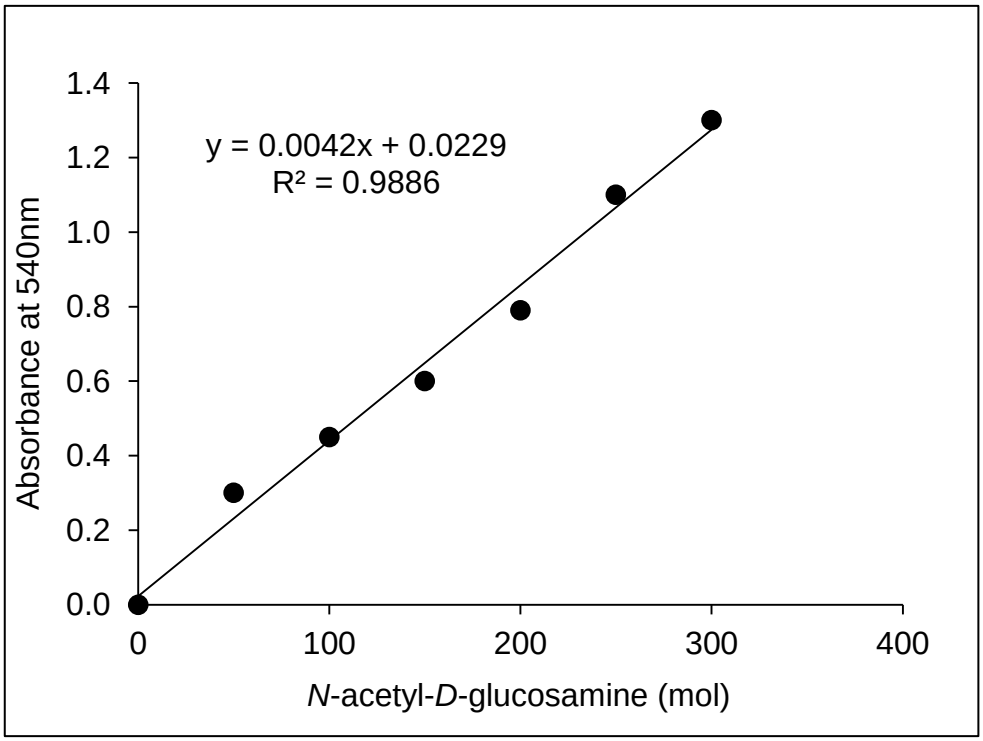
B 4.3 *D*-galacturonic acid standard curve.



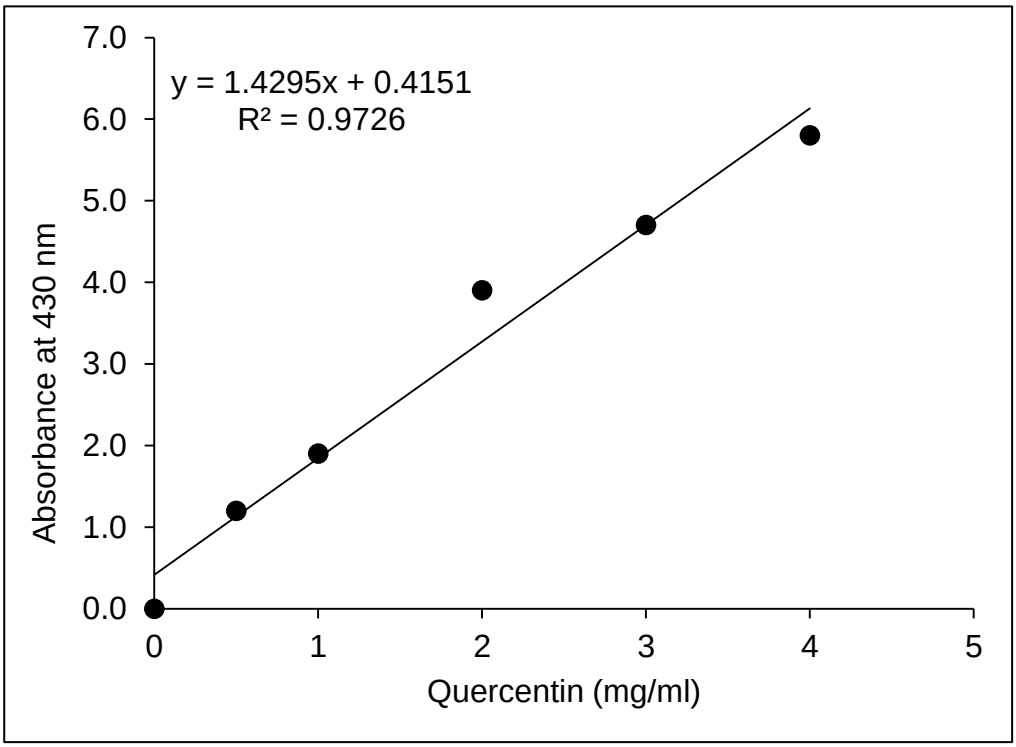
B 5.1 Gallic acid standard curve.



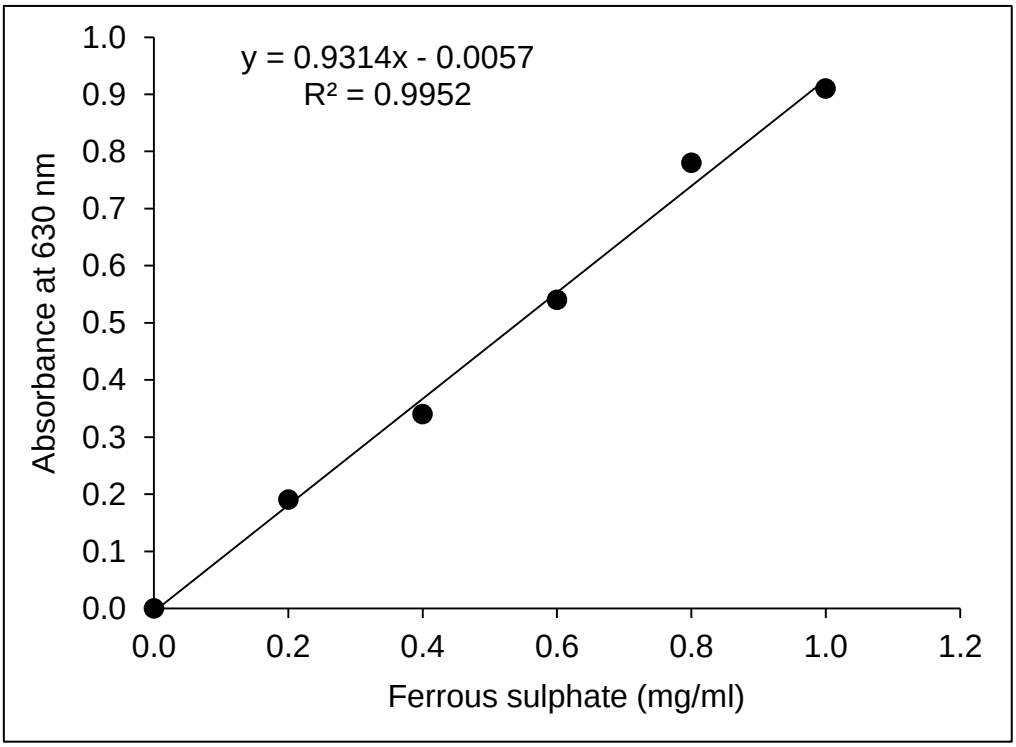
B 5.2 *N*-acetyl-*D*-glucosamine standard curve.



B 7.1 Quercetin standard curve.



B 7.2 Ferrous sulphate standard curve.



APPENDIX-C

C 3.1 Analysis of variance for radial mycelial growth of *C. gloeosporioides* using different types of chitosan

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Chitosan concentration (Conc)	4	61097.96	15274.49	542.59	< .0001
Type of chitosan (Tc)	3	3051.84	1017.28	36.14	< .0001
Trt x Tc	12	1606.37	133.86	4.76	< .0001
Error	60	1689.06	28.15		
Corrected Total	79	67445.24			

C 3.2 Analysis of variance for conidial germination inhibition of *C. gloeosporioides* using different types of chitosan

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Chitosan concentration (Conc)	4	70380.97	17595.24	609.60	< .0001
Type of chitosan (Tc)	3	3559.75	1186.58	41.11	< .0001
Trt x Tc	12	3029.04	252.42	8.75	< .0001
Error	60	1731.81	28.86		
Corrected Total	79	78701.59			

C 3.3 Analysis of variance for dry mycelia weight of *C. gloeosporioides* using different types of chitosan

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Chitosan concentration (Conc)	4	22.66	5.66	65.05	< .0001
Type of chitosan (Tc)	3	0.184	0.06	0.71	0.552
Trt x Tc	12	1.90	0.15	1.90	0.05
Error	60	5.22	0.08		
Corrected Total	79	29.96			

C 3.4 Analysis of variance for spore viability of *C. gloeosporioides* using different types of chitosan

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Chitosan concentration (Conc)	4	63561.94	15890.48	3109.79	< .0001
Type of chitosan (Tc)	3	2288.26	762.75	149.27	< .0001
Conc x Tc	12	707.34	58.94	11.54	< .0001
Error	60	306.59	5.109		
Corrected Total	79	66864.13			

C 3.5 Analysis of variance for time of sonication to obtain different droplet sizes using different types of chitosan

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Type of chitosan (Tc)	1	306.08	306.08	51.79	< .0001
Chitosan concentration (Conc)	3	3059.44	1019.81	172.55	< .0001
Tc x Conc	3	7.64	2.54	0.43	0.731
Droplet size(Ds)	4	3598.14	899.53	152.20	< .0001
Concx Ds	4	65.50	16.37	2.77	0.0303
Tc x Ds	12	261.10	21.75	3.68	0.0001
Tc x Conc x Ds	12	134.57	11.21	1.89	0.04
Error	120	709.24	5.91	32.02	< .0001
Corrected Total	159	8141.11			

C 3.6 Analysis of variance for zeta potential of different droplet sizes using different concentrations and types of chitosan

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Type of chitosan (Tc)	1	529.62	529.62	56.03	< .0001
Chitosan concentration (Conc)	3	752.54	250.84	26.54	< .0001
Tc x Conc	3	35.34	11.78	1.25	0.2961
Droplet size(Ds)	4	290.25	72.56	7.68	< .0001
Concx Ds	4	110.48	27.63	2.92	0.024
Tc x Ds	12	210.68	17.54	1.85	0.047
Tc x Conc x Ds	12	233.21	19.43	2.05	0.025
Error	120	1134.27	9.45		
Corrected Total	159	3296.39			

C 3.7 Analysis of variance for stability of different droplet sizes using different concentrations and types of chitosan

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Type of chitosan (Tc)	1	26.08	26.08	24.57	<.0001
Chitosan concentration (Conc)	3	41.55	13.85	13.05	<.0001
Tc x Conc	3	14.63	4.86	4.58	0.004
Droplet size(Ds)	4	28.07	7.01	6.61	<.0001
Concx Ds	4	20.9	5.23	4.93	0.001
Tc x Ds	12	4.83	0.40	0.38	0.9684
Tc x Conc x Ds	12	71.98	5.99	5.64	<.0001
Error	120	127.37	1.06		
Corrected Total	159	335.41			

C 3.8 Analysis of variance for viscosity of different droplet sizes using different concentrations and types of chitosan

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Type of chitosan (Tc)	1	115659.08	115659.08	2196.16	<.0001
Chitosan concentration (Conc)	3	26999207.09	8999735.7	17088.8	<.0001
Tc x Conc	3	659675.63	219891.88	417.53	<.0001
Droplet size(Ds)	4	4015958.26	1003989.5	1906.39	<.0001
Concx Ds	4	137360.64	34340.16	65.21	<.0001
Tc x Ds	12	3375212.34	281267.69	534.07	<.0001
Tc x Conc x Ds	12	218692.60	18224.38	34.60	<.0001
Error	120	63197.36	63197.36		
Corrected Total	159	36625898.99			

C 3.9 Analysis of variance for release of chitosan with different droplet sizes using different concentrations and types of chitosan

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Type of chitosan (Tc)	1	11.56	11.56	245.86	<.0001
Chitosan concentration (Conc)	3	20.43	6.81	144.78	<.0001
Tc x Conc	3	1.84	0.62	13.08	<.0001
Droplet size(Ds)	4	1.48	0.37	7.88	<.0001
Concx Ds	4	1.28	0.32	6.82	<.0001
Tc x Ds	12	1.05	0.08	1.86	0.045
Tc x Conc x Ds	12	1.48	0.12	2.63	0.003
Error	120	5.64	0.04		
Corrected Total	159	44.79			

C 4.1 Analysis of variance for percent inhibition radial mycelial growth of *C. gloeosporioides* using low molecular weight chitosan

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Chitosan (Conc)	3	12254.32	4084.77	1292.92	<.0001
Droplet size (Ds)	5	86390.51	17278.10	305.66	<.0001
Conc x Ds	15	4029.67	268.64	20.10	<.0001
Error	72	962.18	13.36		
Corrected Total	95	103636.69			

C 4.2 Analysis of variance for percent inhibition radial mycelial growth of *C. gloeosporioides* using locally prepared chitosan

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Chitosan (Conc)	3	54959.96	18319.98	1915.75	<.0001
Droplet size (Ds)	5	61050.33	12210.06	1276.82	<.0001
Conc x Ds	15	13866.15	924.41	96.67	<.0001
Error	72	688.52	9.5628		
Corrected Total	95	13054.97			

C 4.3 Analysis of variance for conidial germination inhibition of *C. gloeosporioides* using low molecular weight chitosan

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Chitosan (Conc)	3	13483.72	4494.57	2877.92	<.0001
Droplet size (Ds)	5	88614.03	17722.80	729.85	<.0001
Conc x Ds	15	4625.35	308.35	50.07	<.0001
Error	72	443.39	6.15		
Corrected Total	95	107166.49			

C 4.4 Analysis of variance for conidial germination inhibition of *C. gloeosporioides* using locally prepared chitosan

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Chitosan (Conc)	3	46373.41	15457.80	1236.55	<.0001
Droplet size (Ds)	5	53198.22	10639.64	851.12	<.0001
Conc x Ds	15	11404.46	760.29	60.82	<.0001
Error	72	900.05	12.50		
Corrected Total	95	111876.15			

C 4.5 Analysis of variance for dry mycelia weight of *C. gloeosporioides* using low molecular weight chitosan

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Chitosan (Conc)	3	3.03	1.01	1433.27	<.0001
Droplet size (Ds)	5	5.59	1.11	1586.74	<.0001
Conc x Ds	15	0.94	0.06	89.18	<.0001
Error	72	0.05	0.0007		
Corrected Total	95	9.62			

C 4.6 Analysis of variance for dry mycelia weight of *C. gloeosporioides* using locally prepared chitosan

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Chitosan (Conc)	3	5.01	1.67	302.40	<.0001
Droplet size (Ds)	5	4.44	0.88	160.88	<.0001
Conc x Ds	15	0.93	0.06	11.32	<.0001
Error	72	0.39	0.005		
Corrected Total	95	10.78			

C 4.7 Analysis of variance for sporulation of *C. gloeosporioides* using low molecular weight chitosan

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Chitosan (Conc)	3	12915.58	4305.19	1142.91	<.0001
Droplet size (Ds)	5	76703.43	15340.68	4072.52	<.0001
Conc x Ds	15	4978.08	331.87	88.10	<.0001
Error	72	271.21	3.76		
Corrected Total	95	94868.32			

C 4.8 Analysis of variance for sporulation of *C. gloeosporioides* using locally prepared chitosan

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Chitosan (Conc)	3	48837.40	16279.13	2746.73	<.0001
Droplet size (Ds)	5	51970.41	10394.08	1753.76	<.0001
Conc x Ds	15	14324.81	954.98	161.13	<.0001
Error	72	426.72	5.92		
Corrected Total	95	115559.35			

C 4.9 Analysis of variance for *β*-galactosidase of *C. gloeosporioides* using low molecular weight chitosan

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Chitosan (Conc)	4	18328.55	6109.51	11.32	<.0001
Droplet size (Ds)	3	43302.92	14434.30	26.73	<.0001
Conc x Ds	9	28924.58	3220.50	5.96	<.0001
Error	51	27537.21	27537.21		
Corrected Total	67	145307.16	145307.16		

C 4.10 Analysis of variance for *β*-1,4-glucanase of *C. gloeosporioides* using low molecular weight chitosan

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Chitosan (Conc)	4	617.51	205.83	215.02	<.0001
Droplet size (Ds)	3	304.46	101.48	106.02	<.0001
Conc x Ds	9	538.01	59.77	62.45	<.0001
Error	51	48.82	0.95		
Corrected Total	67	2464.80			

C 4.11 Analysis of variance for polygalacturonase of *C. gloeosporioides* using low molecular weight chitosan

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Chitosan (Conc)	4	30020.91	7505.22	438.56	<.0001
Droplet size (Ds)	3	74597.88	24865.96	1453.03	<.0001
Conc x Ds	9	5407.86	600.87	35.11	<.0001
Error	51	872.77	17.11		
Corrected Total	67	156799.42			

C 4.12 Analysis of variance for pectin lyase of *C. gloeosporioides* using low molecular weight chitosan

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Chitosan (Conc)	3	2.84	0.711	49.78	<.0001
Droplet size (Ds)	3	2.30	0.769	53.87	<.0001
Conc x Ds	9	017	0.019	1.34	<.0001
Error	51	0.72	0.014		
Corrected Total	67	7.09			

C 4.13 Analysis of variance for disease incidence of dragon fruit plants 90 days after inoculation treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Chitosan (Conc)	3	15.85	5.28	264.0	<.0001
Droplet size (Ds)	4	28.60	7.15	357.5	<.0001
Conc x Ds	9	1.89	0.21	10.5	<.0001
Error	34	0.94	0.02		
Corrected Total	50	47.28			

C 4.14 Analysis of variance for area under disease progress curve (AUDPC) of dragon fruit plants 90 days after treatment.

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Chitosan (Conc)	3	1507.01	502.3	22.99	<.0001
Droplet size (Ds)	4	1662.69	415.6	19.02	<.0001
Conc x Ds	9	687.44	76.38	3.49	0.003
Error	34	742.81	21.847		
Corrected Total	50	4599.94			

C 4.15 Analysis of variance for disease severity (score) of dragon fruit plants 90 days after treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Chitosan (Conc)	3	0.889	0.29	9.66	<0.0001
Droplet size (Ds)	4	1.967	0.49	16.33	<0.0001
Conc x Ds	9	1.187	0.131	4.36	0.0007
Error	34	1.237	0.03		
Corrected Total	50	5.28			

C 4.16 Analysis of variance for disease severity (%) of dragon fruit plants 90 days after treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Chitosan (Conc)	3	925.89	308.63	39.64	<0.0001
Droplet size (Ds)	4	3751.87	937.96	120.48	<0.0001
Conc x Ds	9	1234.93	137.21	17.63	<0.0001
Error	34	264.69	7.78		
Corrected Total	50	6300.04			

C 5.1 Analysis of variance for disease incidence (%) of dragon fruit plants during 30 days of treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	96.76	24.19	83.23	<0.0001
Days	10	216.06	21.60	74.34	<0.0001
Trt x Days	40	85.67	2.14	7.37	<0.0001
Error	110	31.97	0.29		
Corrected Total	164	430.48			

C 5.2 Analysis of variance for disease severity (%) of dragon fruit plants 30 days after treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	57.67	14.41	20.62	<0.0001
Error	10	6.99	0.69		
Corrected Total	14	64.66			

C 5.3 Analysis of variance for area under disease progress curve (AUDPC) of dragon fruit plants 30 days after treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	24836.50	6209.15	1530.21	<.0001
Error	10	40.57	4.05		
Corrected Total	14	24877.07			

C 5.4 Analysis of variance for lignin contents of uninoculated dragon fruit plants during 30 days of treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	12.17	3.04	74.12	<0.0001
Days	10	6.44	0.64	15.68	<0.0001
Trt x Days	40	9.06	0.22	5.52	<0.0001
Error	110	4.51	0.04		
Corrected Total	164	32.20			

C 5.5 Analysis of variance for lignin contents of dragon fruit plants inoculated with *C. gloeosporioides* during 30 days of treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	23.51	5.87	92.37	<0.0001
Days	10	14.47	1.44	22.75	<0.0001
Trt x Days	40	16.29	0.40	6.40	<0.0001
Error	110	7.00	0.06		
Corrected Total	164	61.28			

C 5.6 Analysis of variance for total phenolics of uninoculated dragon fruit plants during 30 days of treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	0.38	0.095	12.54	<0.0001
Days	10	0.29	0.029	3.87	0.0002
Trt x Days	40	0.72	0.018	2.40	0.0002
Error	110	0.83	0.007		
Corrected Total	164	2.23			

.7 Analysis of variance for total phenolics of dragon fruit plants inoculated with *C. gloeosporioides* during 30 days of treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	3.89	0.97	76.84	<0.0001
Days	10	6.96	0.69	54.94	<0.0001
Trt x Days	40	2.24	0.05	4.43	<0.0001
Error	110	1.39	0.012		
Corrected Total	164	14.49			

C 5.8 Analysis of variance for PO activity of uninoculated dragon fruit plants during 30 days of treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	0.73	0.033	360.46	<0.0001
Days	10	0.68	0.068	134.10	<0.0001
Trt x Days	40	0.404	0.010	19.94	<0.0001
Error	110	0.05	0.0005		
Corrected Total	164	1.87			

C 5.9 Analysis of variance for PO activity of dragon fruit plants inoculated with *C. gloeosporioides* during 30 days of treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	1.39	0.347	51.62	<0.0001
Days	10	0.74	0.074	11.09	<0.0001
Trt x Days	40	0.70	0.017	2.60	<0.0001
Error	110	0.74	0.006		
Corrected Total	164	3.58			

C 5.10 Analysis of variance for PPO activity of uninoculated dragon fruit plants during 30 days of treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	0.076	0.019	5.83	<0.0001
Days	10	0.133	0.013	4.06	<0.0001
Trt x Days	40	0.277	0.006	2.11	0.0012
Error	110	0.361	0.003		
Corrected Total	164	0.848			

C 5.11 Analysis of variance for PPO activity of dragon fruit plants inoculated with *C. gloeosporioides* during 30 days of treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	0.488	0.122	95.49	<0.0001
Days	10	0.388	0.038	30.36	<0.0001
Trt x Days	40	0.309	0.007	6.05	<0.0001
Error	110	0.140	0.001		
Corrected Total	164	1.327			

C 5.12 Analysis of variance for PAL activity of uninoculated dragon fruit plants during 30 days of treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	1.706	0.426	63.17	<0.0001
Days	10	1.813	0.181	26.85	<0.0001
Trt x Days	40	1.691	0.042	6.26	<0.0001
Error	110	0.743	0.006		
Corrected Total	164	5.954			

C 5.13 Analysis of variance for PAL activity of dragon fruit plants inoculated with *C. gloeosporioides* during 30 days of treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	1.798	0.448	20.69	<0.0001
Days	10	1.829	0.182	8.45	<0.0001
Trt x Days	40	2.603	0.065	3.01	<0.0001
Error	110	2.32	0.021		
Corrected Total	164	8.608			

C 5.14 Analysis of variance for *β*-1,3-glucanase activity of uninoculated dragon fruit plants during 30 days of treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	11.24	2.81	153.01	<0.0001
Days	10	11.69	1.16	63.65	<0.0001
Trt x Days	40	6.49	0.16	8.84	<0.0001
Error	110	2.02	0.01		
Corrected Total	164	31.45			

C 5.15 Analysis of variance for *β*-1,3-glucanase activity of dragon fruit plants inoculated with *C. gloeosporioides* during 30 days of treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	13.68	3.42	93.68	<0.0001
Days	10	20.70	2.07	56.68	<0.0001
Trt x Days	40	8.77	0.21	6.00	<0.0001
Error	110	4.01	0.03		
Corrected Total	164	47.18			

C 5.16 Analysis of variance for chitinase activity of uninoculated dragon fruit plants during 30 days of treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	0.239	0.059	3.83	0.0059
Days	10	0.660	0.066	4.22	<0.0001
Trt x Days	40	1.145	0.029	1.93	0.003
Error	110	1.720	0.015		
Corrected Total	164	3.764			

C 5.17 Analysis of variance for chitinase activity of dragon fruit plants inoculated with *C. gloeosporioides* during 30 days of treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	0.548	0.137	3.15	0.017
Days	10	0.854	0.085	1.96	0.044
Trt x Days	40	3.261	0.082	1.91	0.004
Error	110	4.794	0.043		
Corrected Total	164	9.457			

C 5.18 Analysis of variance for number of shoots of uninoculated and inoculated dragon fruit plants after 30 days of treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	50.04	12.51	4.81	0.006
Inoculation (Inoc)	1	13.33	13.33	5.13	0.034
Trt x Inoc	4	30.32	7.58	2.91	0.047
Error	20	52.00	2.60		
Corrected Total	29	145.69			

C 5.19 Analysis of variance for number of shoots of uninoculated and inoculated dragon fruit plants after 60 days of treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	100.2	25.05	10.16	0.0001
Inoculation (Inoc)	1	13.33	13.33	5.41	0.0307
Trt x Inoc	4	28.48	7.12	2.89	0.0487
Error	20	49.33	2.46		
Corrected Total	29	191.34			

C 5.20 Analysis of variance for number of shoots of uninoculated and inoculated dragon fruit plants after 90 days of treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	61.8	15.46	3.36	0.02
Inoculation (Inoc)	1	36.3	36.3	7.89	0.01
Trt x Inoc	4	54.7	13.69	2.96	0.04
Error	20	92.0	4.60		
Corrected Total	29	244.9			

C 5.21 Analysis of variance for stem diameter of uninoculated and inoculated dragon fruit plants after 30 days of treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	0.661	0.165	5.84	0.002
Inoculation (Inoc)	1	0.124	0.124	4.42	0.048
Trt x Inoc	4	0.392	0.098	3.50	0.025
Error	20	0.566	0.028		
Corrected Total	29	1.743			

C 5.22 Analysis of variance for stem diameter of uninoculated and inoculated dragon fruit plants after 60 days of treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	5.83	1.45	32.40	0.0002
Inoculation (Inoc)	1	0.97	0.97	21.60	<.0001
Trt x Inoc	4	0.592	0.148	3.28	0.034
Error	20	0.90	0.045		
Corrected Total	29	8.29			

C 5.23 Analysis of variance for stem diameter of uninoculated and inoculated dragon fruit plants after 90 days of treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	5.65	1.41	22.70	<.0001
Inoculation (Inoc)	1	2.24	2.24	35.96	<.0001
Trt x Inoc	4	0.78	0.19	3.25	0.033
Error	20	1.24	0.06		
Corrected Total	29	9.91			

C 5.24 Analysis of variance for shoot length of uninoculated and inoculated dragon fruit plants after 30 days of treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	2.24	0.56	5.09	0.0053
Inoculation (Inoc)	1	6.53	6.53	55.37	<.0001
Trt x Inoc	4	3.76	0.94	8.54	0.0003
Error	20	2.36	0.11		
Corrected Total	29	14.89			

C 5.25 Analysis of variance for shoot length of uninoculated and inoculated dragon fruit plants after 60 days of treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	1.36	0.34	3.09	0.039
Inoculation (Inoc)	1	8.64	8.64	77.15	<.0001
Trt x Inoc	4	1.28	0.32	2.90	0.0481
Error	20	2.24	0.11		
Corrected Total	29	13.52			

C 5.26 Analysis of variance for shoot length of uninoculated and inoculated dragon fruit plants after 90 days of treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	1.55	0.38	7.67	<.0001
Inoculation (Inoc)	1	8.42	8.42	166.32	0.0006
Trt x Inoc	4	2.71	0.67	13.39	<.0001
Error	20	1.01	0.05		
Corrected Total	29	13.70			

C 5.27 Analysis of variance for chlorophyll a of uninoculated and inoculated dragon fruit plants after 30 days of treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	25.14	6.28	21.93	<.0001
Inoculation (Inoc)	1	3.73	3.73	13.02	0.0018
Trt x Inoc	4	4.08	1.02	3.64	0.021
Error	20	5.73	0.28		
Corrected Total	29	38.68			

C 5.28 Analysis of variance for chlorophyll a of uninoculated and inoculated dragon fruit plants after 60 days of treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	52.67	13.16	39.93	<.0001
Inoculation (Inoc)	1	9.95	9.95	30.18	<.0001
Trt x Inoc	4	4.58	1.14	3.47	0.0261
Error	20	6.59	0.32		
Corrected Total	29	73.80			

C 5.29 Analysis of variance for chlorophyll a of uninoculated and inoculated dragon fruit plants after 90 days of treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	78.64	19.66	32.65	0.0100
Inoculation (Inoc)	1	4.88	4.88	8.11	<.0001
Trt x Inoc	4	10.92	2.73	4.53	0.0091
Error	20	12.04	0.60		
Corrected Total	29	106.48			

C 5.30 Analysis of variance for chlorophyll b of uninoculated and inoculated dragon fruit plants after 30 days of treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	19.93	4.98	20.06	<.0001
Inoculation (Inoc)	1	1.16	1.16	4.69	0.0427
Trt x Inoc	4	5.20	1.30	5.24	0.0047
Error	20	4.96	0.24		
Corrected Total	29	31.27			

C 5.31 Analysis of variance for chlorophyll b of uninoculated and inoculated dragon fruit plants after 60 days of treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	34.48	8.62	63.53	<.0001
Inoculation (Inoc)	1	7.70	7.70	56.75	<.0001
Trt x Inoc	4	3.41	0.85	6.29	0.0019
Error	20	2.71	0.13		
Corrected Total	29	48.31			

C 5.32 Analysis of variance for chlorophyll b of uninoculated and inoculated dragon fruit plants after 90 days of treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	56.87	14.21	16.36	0.5908
Inoculation (Inoc)	1	0.25	0.25	0.30	<.0001
Trt x Inoc	4	12.19	3.04	3.51	0.0251
Error	20	17.38	0.86		
Corrected Total	29	86.71			

C 5.33 Analysis of variance for total chlorophyll of uninoculated and inoculated dragon fruit plants after 30 days of treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	86.49	21.73	24.01	<.0001
Inoculation (Inoc)	1	4.56	4.56	5.03	0.036
Trt x Inoc	4	10.96	2.74	3.02	0.042
Error	20	18.10	0.905		
Corrected Total	29	113.38			

C 5.34 Analysis of variance for total chlorophyll of uninoculated and inoculated dragon fruit plants after 60 days of treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	165.72	41.43	104.24	<.0001
Inoculation (Inoc)	1	35.16	35.16	88.47	<.0001
Trt x Inoc	4	10.42	2.606	6.56	0.0015
Error	20	7.94	0.397		
Corrected Total	29	219.26			

C 5.35 Analysis of variance for total chlorophyll of uninoculated and inoculated dragon fruit plants after 90 days of treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	263.59	65.89	30.47	<.0001
Inoculation (Inoc)	1	7.39	7.39	3.42	0.079
Trt x Inoc	4	32.39	8.09	3.75	0.019
Error	20	43.25	2.16		
Corrected Total	29	346.63			

C 6.1 Analysis of variance for percent inhibition in radial mycelial growth of *C. gloeosporioides* isolated from dragon fruit using low molecular weight chitosan

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Chitosan (Conc)	3	15322.26	5107.42	249.04	<.0001
Droplet size (Ds)	5	8085.10	1617.02	786.60	<.0001
Conc x Ds	15	2526.63	168.44	25.94	<.0001
Error	87	564.89	6.49		
Corrected Total	111	88104.44			

C 6.2 Analysis of variance for conidial germination inhibition of *C. gloeosporioides* isolated from dragon fruit using low molecular weight chitosan

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Chitosan (Conc)	3	16660.56	2548.33	294.53	<.0001
Droplet size (Ds)	5	12741.68	5553.52	641.85	<.0001
Conc x Ds	15	1281.56	85.43	9.87	<.0001
Error	87	752.75	8.65		
Corrected Total	111	88827.50			

C 6.3 Analysis of variance for disease incidence on dragon fruit after 28 days of treatment.

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Chitosan (Conc)	3	312.50	104.1	2602.5	<.0001
Droplet size (Ds)	4	277.19	69.29	1732.2	<.0001
Conc x Ds	9	10.95	1.21	30.25	<.0001
Error	51	2.37	0.04		
Corrected Total	67	603.01			

C 6.4 Analysis of variance for disease severity on dragon fruit after 28 days of treatment.

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Chitosan (Conc)	3	6.11	2.03	67.66	<.0001
Droplet size (Ds)	4	5.84	1.46	48.66	<.0001
Conc x Ds	9	3.65	0.405	13.5	<.0001
Error	51	1.97	0.03		
Corrected Total	67	17.57			

C 7.1 Analysis of variance for weight loss in dragon fruit during 28 days of storage after treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	544.63	136.15	575.54	<.0001
Days	4	5235.65	1308.91	5532.78	<.0001
Trt x Days	16	252.27	15.76	66.65	<.0001
Error	75	17.74	0.23		
Corrected Total	99	6050.31			

C 7.2 Analysis of variance for fruit firmness in dragon fruit during 28 days of storage after treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	1096.06	274.01	38.57	<.0001
Days	4	4377.54	1094.38	154.05	<.0001
Trt x Days	16	263.02	16.43	2.31	0.008
Error	75	532.63	7.10		
Corrected Total	99	6269.45			

C 7.3 Analysis of variance for soluble solids concentration in dragon fruit during 28 days of storage after treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	41.58	10.39	3.12	0.019
Days	4	302.78	75.69	22.74	<.0001
Trt x Days	16	42.34	2.64	0.80	0.686
Error	75	249.62	3.32		
Corrected Total	99	636.33			

C 7.4 Analysis of variance for titratable acidity in dragon fruit during 28 days of storage after treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	0.562	0.140	71.13	<.0001
Days	4	2.022	0.505	255.85	<.0001
Trt x Days	16	0.237	0.014	7.51	<.0001
Error	75	0.148	0.001		
Corrected Total	99	2.970			

C 7.5 Analysis of variance for total phenolic contents in dragon fruit during 28 days of storage after treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	0.070	0.017	12.38	<.0001
Days	4	0.182	0.045	32.10	<.0001
Trt x Days	16	0.055	0.003	2.44	0.005
Error	75	0.106	0.001		
Corrected Total	99	0.41			

C 7.6 Analysis of variance for total flavnoids in dragon fruit during 28 days of storage after treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	3.17	0.792	225.60	<.0001
Days	4	4.96	1.242	353.40	<.0001
Trt x Days	16	1.38	0.086	24.57	<.0001
Error	75	0.26	0.003		
Corrected Total	99	9.78			

C 7.7 Analysis of variance for lycopene in dragon fruit during 28 days of storage after treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	1.93	0.483	98.39	<.0001
Days	4	5.33	1.333	271.21	<.0001
Trt x Days	16	1.32	0.082	16.88	<.0001
Error	75	0.36	0.004		
Corrected Total	99	8.96			

C 7.8 Analysis of variance for total antioxidant activity in dragon fruit during 28 days of storage after treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	4.75	1.18	62.50	<.0001
Days	4	12.85	3.21	168.85	<.0001
Trt x Days	16	2.04	0.12	6.72	<.0001
Error	75	1.42	0.01		
Corrected Total	99	21.08			

C 7.9 Analysis of variance for respiration rate in dragon fruit during 28 days of storage after treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	192.19	48.04	7.21	<.0001
Days	4	4376.85	1094.21	164.19	<.0001
Trt x Days	16	2331.33	145.70	21.86	<.0001
Error	75	499.82	6.66		
Corrected Total	99	7400.21			

C 7.10 Analysis of variance for ethylene evolution in dragon fruit during 28 days of storage after treatment

S.O.V	d.f	S.S	M.S	F. Value	Pr > F
Treatment (Trt)	4	0.103	0.025	14.01	<.0001
Days	4	5.906	1.476	797.97	<.0001
Trt x Days	16	0.295	0.018	9.97	<.0001
Error	75	0.138	0.001		
Corrected Total	99	6.444			

BIODATA OF AUTHOR

Noosheen Zahid was born in Multan, Punjab, Pakistan on 31st December, 1980. She had her primary education at The Junior School Multan and continued her secondary education at Nishat High School Multan. After completing secondary education, she continued her intermediate studies at Govt. Degree College for Women Multan.

In October, 2000, she joined University College of Agriculture, Bahauddin Zakariya University Multan, Pakistan and completed her B.Sc. (Hons.) Agriculture with specialization in Plant Breeding and Genetics as a major subject. For her M.Sc. (Hons.) Agriculture (Plant Breeding and Genetics), she joined Department of Plant Breeding and Genetics, Faculty of Agriculture, University of Agriculture Faisalabad, Pakistan and completed her degree in August, 2006, with 71.0% aggregate marks.

On October 2006, she was offered to join Agricultural Biotechnology Reseach Institute, (AARI), Faisalabad as an Assistant Research Officer. During her tenure she worked on Genome mapping study for stripe and leaf rust in bread wheat and also worked on Wheat maize hybridization. She resigned from here job on 15th April 2007 and joined as a Scientific officer in Biotechnolgy at Central cotton Research Institiute (CCRI), Multan, Pakistan on 16th April 2007. During her stay at CCRI she worked on inter-specific hybridization to transfer desirable characters of wild species of cotton to cultivated cotton for commercial exploitation. She was happily married in year 2009, to Mehdi Maqbool which is still continued.

In August, 2010 she was offered a scholarship for her PhD studies from The School of Biosciences, Faculty of Science, The University of Nottingham, Malaysia Campus, which she accepted and continued her postgraduate studies. During her research period she has published a few research papers in highly reputable journals and proceedings and also presented (oral, poster) in many conferences, seminars and symposiums.

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