ผลของไคโทซานต่อการเติบโตและปริมาณผลผลิตในข้าว *Oryza sativa* L. พันธุ์ปทุมธานี 1

นายไพฑูรย์ แสนบัวหลวง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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EFFECTS OF CHITOSAN ON GROWTH AND PRODUCTION OF RICE *Oryza sativa* L. cv. PATHUM THANI 1

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ไพฑูรย์ แสนบัวหลวง : ผลของไคโทซานต่อการเติบโตและปริมาณผลผลิตในข้าว *Oryza sativa* L. พันธุ์ปทุมธานี 1. (EFFECTS OF CHITOSAN ON GROWTH AND PRODUCTION OF RICE *Oryza sativa* L. cv. PATHUM THANI 1) อ. ที่ปรึกษา : อ.ดร. รัฐ พิชญางกูร, 60 หน้า.

ไคโทซานเป็นอนุพันธ์ของไคทิน ซึ่งไคทินเป็นองค์ประกอบหลักของเปลือกกุ้ง ปู แมง และแมลง ไคโทซานได้รับความสนใจเป็นอย่างมาก เพราะนอกจากจะมีวัตถุดิบในประเทศที่สามารถใช้เพื่อผลิตในปริมาณมากจากอุตสาหกรรมแปรรูปอาหารทะเลแล้ว เรายังสามารถนำไคโทซานไปประยุกต์ใช้ในงานด้านต่างๆ ได้มากมาย อาทิ ในด้านการเกษตร ไคโทซานสามารถยับยั้งการเจริญของแบคทีเรีย และเชื้อรา ที่เป็นสาเหตุของโรคพืช กระตุ้นให้พืชสร้างภูมิคุ้มกันเพื่อต้านทานต่อเชื้อโรคและแมลงที่เป็นศัตรู รวมทั้งกระตุ้นการเจริญเติบโตและช่วยเพิ่มผลผลิตในพืชหลายชนิด วัตถุประสงค์ของการทดลองนี้ คือ ศึกษาผลของระดับความเข้มข้นของไคโทซานต่อการเติบโตและปริมาณผลผลิตในข้าว *Oryza sativa* L. พันธุ์ปทุมธานี 1 โดยสกัดไคโทซานจากเปลือกปูแล้วนำไปย่อยด้วยไคทิเนสจาก *Bacillus licheniformis* SK-1 เพื่อลดขนาดโมเลกุลแล้วทำการวัดเปอร์เซ็นต์การกำจัดหมู่แอซิทิลและน้ำหนักโมเลกุลของไคโทซาน พบว่าไคโทซานที่ได้มีเปอร์เซ็นต์การกำจัดหมู่แอซิทิลเท่ากับ 91% และน้ำหนักโมเลกุลเฉลี่ย (MW) เท่ากับ 46 kDa เมื่อนำสารละลายไคโทซานความเข้มข้นต่างๆ 10, 20, 40, และ 80 ppm ไปทดสอบผลของไคโทซานต่อการงอกของเมล็ดข้าว การเจริญเติบโต และปริมาณผลผลิตข้าว โดยใช้น้ำกลั่นเป็นชุดควบคุม พบว่าไคโทซานไม่มีผลต่อเปอร์เซ็นต์การงอกของเมล็ดข้าว แต่พบว่าไคโทซานมีผลต่อการเติบโตของต้นข้าวโดยไคโทซานความเข้มข้น 20 และ 40 ppm ทำให้ความสูง ความยาวใบ พื้นที่ใบ และน้ำหนักแห้งของต้นข้าวเพิ่มมากขึ้น การศึกษาผลของไคโทซานต่อการแตกกอ จำนวนรวง และปริมาณผลผลิต พบว่าไคโทซานความเข้มข้น 20, 40, และ 80 ppm ทำให้ต้นข้าวแตกกอเพิ่มมากขึ้น และมีจำนวนรวงเพิ่มมากขึ้นอย่างมีนัยสำคัญ ($P<0.05$) และเมื่อนับจำนวนเมล็ดข้าวรวมทั้งหมด และหาน้ำหนักเฉลี่ยต่อ 100 เมล็ด พบว่า ที่ความเข้ม 20 และ 40 ppm ทำให้มีจำนวนเมล็ดข้าวเพิ่มขึ้นและไคโทซานทุกความเข้มข้นมีผลทำให้น้ำหนักเมล็ดข้าวเพิ่มมากขึ้น 3-5% เมื่อเทียบกับชุดควบคุม ผลที่ได้จากการศึกษาครั้งนี้แสดงให้เห็นว่าไคโทซานความเข้มข้น 40 ppm มีแนวโน้มเพิ่มการเติบโตและปริมาณผลผลิตข้าว

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต.....

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PAITOON SEANBUALUANG : EFFECTS OF CHITOSAN ON GROWTH AND PRODUCTION OF RICE *Oryza sativa* L. cv. PATHUM THANI 1. THESIS ADVISOR : RATH PICHYANGURA, Ph. D. 60 pp.

Chitosan is a derivative of chitin (produced by deacetylation of the aceta amido group). Chitin is the principal component of the hard exoskeleton of shrimp, crabs and insects. Chitosan has become interesting not only because the abundant of crustaceans shells available from fishery industry, but also as a high potential natural material which can be used in various applications. Chitosan has ability to inhibit growth of certain bacteria and fungi which cause diseases in plants, induces plant defense mechanism, stimulates plant growth and enhances yield production. The objective of this study is to investigate the effects of chitosan concentrations on growth and yield production of rice *Oryza sativa* L. cv. Pathum Thani 1. Chitosan was prepared from crab shells and hydrolyzed by chitinase produced from *Bacillus licheniformis* SK-1. The percent of degree of deacetylation (%DD) and molecular weight of chitosan was determined. The chitosan prepared has 91 %DD and the molecular weight of 46 kDa. The effects of chitosan on seed germination, growth and yield production were investigated. Chitosan treatments consisted of four concentrations at 10, 20, 40, and 80 ppm, distilled water as a control. Chitosan did not affect seed germination. The difference among treatment was not significant. Chitosan increased plant height, leaf blade length and area, and rice straws dry weight. The most effective concentrations were 20 and 40 ppm. The study also found that chitosan treatment at 20, 40, and 80 ppm significantly increase panicles per plant when compared to the control ($P<0.05$). Other results showed that total grain number and grain weight were increased of 3 to 5 % by chitosan treatment, where 40 ppm was the most effective concentration. This study suggests that chitosan at the concentration of 40 ppm had a potency to stimulate growth and increase rice yield. The effective application of chitosan on rice plants is dependent on the concentration and the application methods of chitosan.

Field of Study	Biotechnology	Student's Signature Paitoon S.
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CHAPTER I

INTRODUCTION

Background

Chitin is natural biopolymer, abundant next to cellulose. This polymer is the principal component of the hard exoskeleton of million species of arthropods; shrimp, crabs and insects. It is also forming structural components in the cell wall of fungi, bacteria and some algae.

Chitosan is the *N*-deacetylated derivative of chitin. Chitin polymer consists of mostly 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc) and small amount, less than 50%, of 2-amino-2-deoxy-β-D-glucopyranose (GlcN) residues, linked by β-1,4 glycosidic bond. In contrast, chitosan has GlcN in its polymer chain more than 50% (Fig 1.1).

Despite the wide spread occurrence of chitin, most chitin and chitosan are manufactured from the shells of shrimps and crabs, and sometimes are produced from squid pens. Moreover, it has also been isolated from fungal mycelia (New *et al*., 2002; Nwe and Stevens, 2003). In industrial processing, chitin and chitosan production normally begins with removal of the mineral salt from the raw materials. This demineralization process is achieved by extraction with low concentration, 1-1.5 M of strong acid such as hydrochloric acid. Secondly, a deproteinization process, the protein contents are eliminated either by alkaline solution or by digestion with proteolytic enzymes such as trypsin, papain, pepsin, and alcalase. Lastly, pigments are sometimes removed by using organic solvents; acetone, chloroform, ethyl acetate or ethanol. The product yield from these procedures can be considered as chitin. Chitosan can be obtained by heterogeneously deacetylation of chitin. This process is accomplished in high concentration alkaline solution such as 50% (w/w) sodium hydroxide (NaOH) or 50% (w/w) or potassium hydroxide (KOH). Chitosan becomes soluble in weak

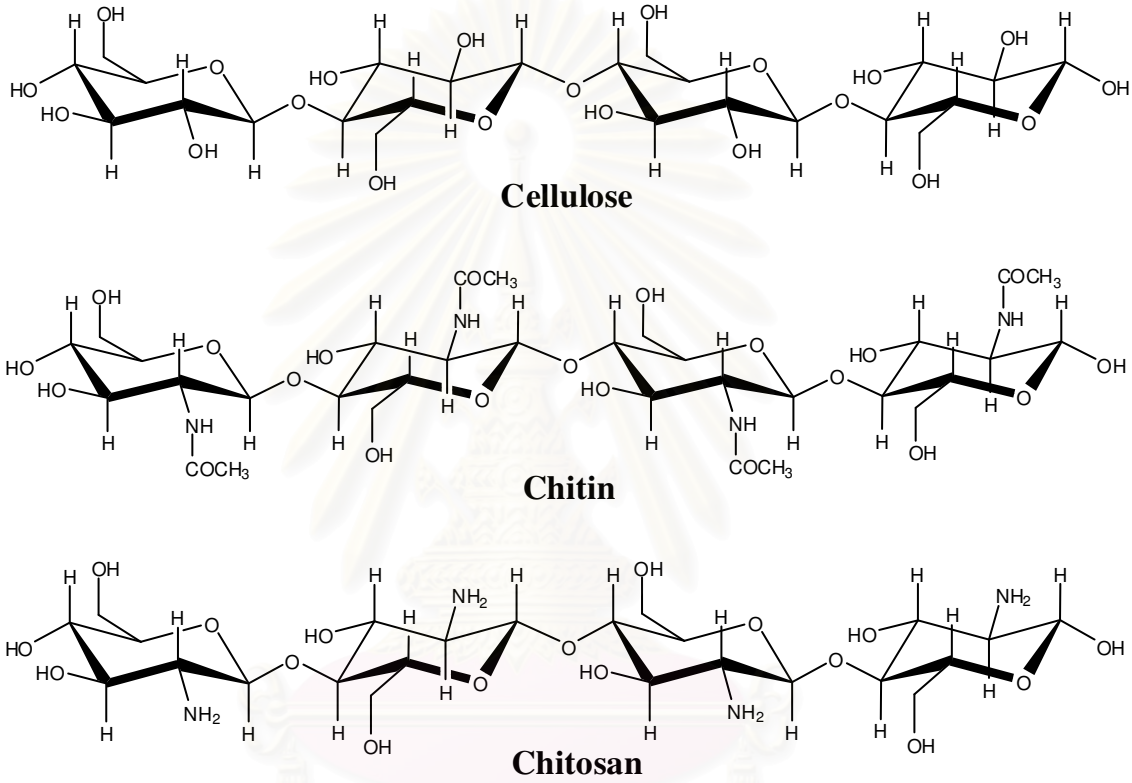


Figure 1.1 Chemical structures of chitin and chitosan compared to cellulose

organic acidic solution, acetic acid, citric acid, lactic acid or formic acid, when the degree of deacetylation of chitin reaches of above 80%. The degree of deacetylation varies according to the duration, the temperature and the concentration of the sodium hydroxide used in the deacetylation process. Furthermore, many chemical characteristics of the chitosan; molecular weight, polydispersity, and purity are greatly dependent on the method and equipment used, as well as the source of chitinous material used. It is; therefore, crucial to control precisely methods of production of chitosan to obtain the exact characteristics needed for each application of the end product.

Chemical and Physical Characteristics of Chitosan

As previously mentioned, chitosan is a heteropolymer which composed of GlcNAc and GlcN. Each monomer in the polymer chain has reactive hydroxyl and amino groups. The hydroxyl group can form both intramolecular hydrogen bonding and intermolecular hydrogen bonding when chitosan is pack in the crystalline form. For instance, intramolecular hydrogen bonding can forms between hydroxyl group at C-3 and oxygen atom, and C-6 and amino group at C-2 whereas intermolecular hydrogen bonding, the interaction among chains can occurs at C-6 of each chain. Chitosan is insoluble in water and most organic solvents. However it is soluble in strong acid such as hydrochloric acid and sulfuric acid, and weak organic acid, such as acetic acid, phosphoric acid, and formic acid. Moreover, when the amino or acetamido groups are considered; they have a lone pair electron which can form ionic bond with alkaline metals, alkaline earth metals, and transition metals. Amino group of chitosan monomer presents positive charge when dissolved in such acids.

Chitosan Applications

Chitosan is a biodegradable natural compound, nontoxic, and have unique physiological and biological properties. Especially cationic nature of chitosan, it is of great interest because most polysaccharides or more of the same types are

Table 1.1 Principle applications of chitosan

Cosmetics & toiletries	Maintain skin moisture, treat acne, tone skin, oral care, improve suppleness of hair, reduce static electricity in hair
Food & beverages	Dietary fiber, reduce cholesterol, preservative, thickener and stabilizer, protective, fugistatic, antimicrobial, coating fruits and foods, antioxidant
Water & waste treatment	Flocculant to clarify water, removal of metal ion, chelator, emulsifier, membrane
Biopharmaceutics	Immunologic, antitumoral, healing, hemostatic and anticoagulant, bacteriostatic, membrane, artificial organs, fabrics, fibers and drug deriver
Agriculture	Plant elicitor, stimulation of plant growth, seed coating, improvement of yield, fungicide, bactericide, antivirus, frost protection

neutral or negatively charged. By controlling the molecular weight, the degree of deacetylation and purity of chitosan, so it is possible to produce a broad range of chitosan and its derivatives that can be used for applications in many industries (Table 1.1). For example, chitosan can be use in cosmetics - lotions, facial and body creams, and hair additives (Rinaudo, 2006), dietary - coating, preservative, antioxidant and antimicrobial (Roller and Covill, 1999; Benjakul *et al*., 2000; Shahidi *et al*., 2001; Rinaudo, 2006), water and waste treatment - chelator, emulsifier and flocculent, pharmacology and medicine - fibers, fabrics, drugs, membranes, artificial organ, immunologic and healing (Liu *et al*., 2001; Rinaudo, 2006) and agriculture – fungicide, bactericide, antivirus and elicitor (Surguchova *et al.,* 2000; Ren *et al*.,2001; Agrawal *et al*., 2002; Bautista-Baños *et al.,* 2006). Thailand is an agricultural country producing various agricultural products. Moreover, chitin and chitosan is a by product of Thailand's shrimp and crab industry. Therefore in this work we will concentrate on the application of chitosan in agriculture as an antimicrobial substance, an elicitor of defense response system and a stimulant for plant growth.

Antimicrobial Activity of Chitosan

- ***Fungicidal Activity***

The fungicidal activity of chitosan has been well reported both in *in vitro* and *in situ* studies. Generally, most study showed that the inhibition level of fungi is highly correlated to concentration of chitosan. The polycationic nature of this polymer is the key to its antifungal properties (Hirano and Nagao, 1989; Bautista-Baños *et al.,* 2006). Chitosan also exert effects on the synthesis of certain fungal enzyme (El Ghaouth *et al*., 1992; Bautista-Baños *et al.,* 2006). There is strong evidence that mycelial growth can be inhibited when the growth media of fungi are amended with chitosan. The radial growth of *Alternaria alternate, Botrytis cinerea, Colletrotichum gloesoporioides* and *Sclerotenia sclerotiorum* decreased by which increased chitosan concentration (0.75-6.0 mg ml^{-1}). The potent of chitosan in inhibiting growth of *B. cinerea* which cause gray-

mold diseases in grapevine was confirmed. The chitogel, a chitosan formulate solution, was supplemented in the culture medium. The percentage of *B. cinerea* inhibition expresses the reduction in fungal growth on chitogel-amended plates relative to fungal growth on chitogel-free plates. As a result, maximum growth inhibition was 64% at 5% (v/v) chitogel and 44% at 1.75% (v/v) chitogel. The inhibition was dependent on chitogel concentration (Barka *et al*., 2004). In cucumber plants, chitosan with concentration of 50 ppm affected the growth of *B. cinerea* by inhibition conidia germination. It was found that spraying chitosan either before inoculation with *B. cinerea* or after inoculation; it can reduce disease development (Ben-Shalom *et al*., 2003). Furthermore chitosan was able to inhibit the radial growth and submerged growth of *Fusarium solani* f. sp. *glycines*, which cause sudden death syndrome in soybeans. The effective concentration of chitosan was up to 1 mg ml^{-1} (Prapagdee *et al.*, 2007)

Recently, some studies have shown that chitosan not only inhibits growth of the pathogen but also induces morphological changes, structural alterations and molecular disorganization of the fungal cell (El Ghaouth *et al*., 1999). For example, microscopic observations indicate the coagulation in the fungal cytoplasm characterized by the appearance of both small and large vesicles in mycelium treated with chitosan (Barka *et al.,* 2004). These results are in line with earlier study of several sections of invading hyphae that displayed wall loosening and cytoplasm disintegration as a result of chitosan treatment (Benhamou *et al*., 1998). Chitosan can affect the morphology of hyphae of *Trichoderma longibrachiatum*. Others study reported that chitosan caused mycelial branching, abnormal shapes, swelling and hyphae size reduction which carried out on *Fusarium oxysporum* f. sp. *radicis-lycopersici, Rhizopus stolonifer* and *S. sclerotiorum* (Benhamou, 1992; El Ghaouth *et al*., 1992; Cheah *et al*., 1997).

- ***Bactericidal and Antiviral Activities***

There are several studies have documented bactericidal or bacteriostatic effects of chitosan on plant diseases. Bautista-Baños and colleagues (2006), studied the efficacy of chitosan at various concentrations in inhibiting growth of

certain bacterial strains; *Erwinia amylovora* and *Agrobacterium tumefaciens*. The result showed that chitosan concentration had a direct effect on bacterial growth. In addition some studies which carried out on food borne pathogens; it is showed that chitosan was disrupting bacterial outer membrane properties and increasing cellular leakage (Helander *et al.,* 2001)

Chitosan also induces resistance to viral infection on many plants. For example, mung bean and pea plants were locally infected by alfalfa mosaic, bean golden mosaic, and peanut stunt viruses. They were completely controlled with the highest chitosan concentration (0.1%) either sprayed or added to the inoculum (Pospiezny *et al*., 1991). In tomato plants, chitosan inhibited the development of systematic infection caused by tobacco mosaic virus and potato virus X (PVX) as well as potato spindle tuber viroid with the same concentration of chitosan (Pospiezny, 1997). Chitosan treatment (1 mg ml^{-1}), prevented infection of potato plants with PVX and potato virus Y (Surguchova *et al.,* 2000). Other study; for instance, indicated that the maximum resistance to viral infection which chitosan-induced in tomato plants a day after treatment. However the antiviral activity is regard to the molecular weight of chitosan. The greatest antiviral activity was shown by 120 kDa (Cherkov *et al*., 2001).

Chitosan as an Elicitor of Defense Response Mechanisms in Plants

Plants are well adapted to cope with definite pathogens; the plant's defense response against pathogens can be elicited by numerous external signals. Chitosan is one of the biotic elicitors that can induce defense response mechanism in plants. The early phase of plant response to chitosan is usually accompanied by the production of reactive oxygen species (ROS) such as superoxide and hydrogen peroxide (H_2O_2). This H_2O_2 can damages pathogens directly, restrains its propagation, and causes pore formation by the oxidation of biomolecules including cell membranes. In addition, H_2O_2 can reinforce plant cell wall by eliciting lignification and inducing associated protein cross-linking (Lesney, 1990; Levine *et al*., 1994; Alvarez *et al*., 1998; Vander *et al*., 1998).

H_2O_2 evolution was evident throughout the entire surface of tomato guard cells treated with chitosan. The study found that chitosan also inhibited stomatal opening in tomato epidermis. This result suggests that H_2O_2 probably mediate the chitosan-induced decrease of stomatal aperture (Lee *et al*., 1999); alternatively, chitosan induced a decrease of potassium (K) in the guard cell which studied in pepper plants (Bittelli *et al*., 2001). Chitosan-induced generation of H_2O_2 was also observed in rice cell in suspension culture; this study found that the generation of H_2O_2 reached a maximum at 60 μg ml^{-1} at 50 minutes post treatment. The molecular weight of chitosan significantly affects the production of H_2O_2, shown to be highest in for chitosan with low molecular weight (1335 Da) (Lin *et al.*, 2005). This ROS can also act as a secondary messenger to induce the expression of defense-related genes and stimulate synthesis of phytoalexins (Levine et al., 1994; Jabs *et al*., 1997; Orozco-Cardenas and Ryanand, 1999).

Chitosan has been shown to be a potent inducer for transcription and translation of genes related to self defense response of plants. In general, induction of defence reaction in plants is mostly correlated with enzymatic responses. There are several studies demonstrating that chitosan has the ability to elicit protein synthesis involved in plant defense responses. For example chitosan was found to possess proteinase inhibitor inducing activity when supplied to young excised tomato plants (Walker-Simmons and Ryan, 1984). Accumulation of this protein also found in tomato suspension culture when adding chitosan in the culture medium (Walker-Simmons and Ryan, 1986). The chitosan induced accumulation of proteinase inhibitor mimics the response to pathogens or herbivores attack. Chitosan was proposed to induce expression of proteinase inhibitor gene through octadecanoid pathway. This lipid-based octadecanoid pathway leads to jasmonic acid (JA) synthesis has been implicated as an integral part of the signal transduction pathway leading to the activation of proteinase inhibitor gene (Doares *et al*., 1995). There are several studies reporting that chitosan could stimulate other systems that are involved in plant defense such as pathogenesis-related proteins (PRs), namely OsPR5, OsPR10, chitinase, β-1, 3-

glucanase, peroxidase (Kauffman *et al*., 1987; Legrand *et al*., 1987; Vigers *et al*., 1991; Mason and Davis, 1997; Vander *et al*., 1998; van Loon and Strien 1999; Agrawal *et al*., 2002; Ben-Shalom *et al*., 2003; Lin *et al*., 2005). Moreover, chitosan also increases phenylalanine ammonia-lyase (PAL) activity, a key enzyme involve in the biosynthesis of phenolic compounds such as flavonoids and phytoalexins. For example, Vander *et al.* (1998) showed that both PAL and peroxidase activities were activated when chitosan was injected into the intercellular space of wheat leaves using a hypodermic syringe. Similarly in soybeans, the application of chitosan to soybeans leaves caused an increased in PAL activity. This study also reported that chitosan can increase tyrosine ammonia-lyase (TAL) activity, which is a key enzyme in the phenylpropanoid pathway. The study suggested that the elevation of enzyme activity was dependent on the size of the chitosan oligomers and time post treatment (Khan *et al*., 2003). In rice plants, PAL activity significant increased after the cell suspension was treated with 50 μg $ml^{-1}$of chitosan molecular weight of 1335 Da (Lin *et al*., 2005). Other study was performed in the Chinese herb *Cistanche deserticola* Y.C. Ma, cell suspension culture. The PAL activity in the treated cell culture reached the highest activity at the first day after elicitation, and then decreased to the control level after 3 days (Cheng *et al*., 2006).

Further defense mechanism of plants is the production of variety of secondary metabolites including phytoalexins. Phytoalexins show high antifungal activity and accumulate around the infection sites soon after pathogen attack. In rice plant, momilactone A and sakuranetin have been identified as the major phytoalexins (Obara *et al*., 2002) and are induced under a variety of both abiotic and biotic stimuli, including chitosan. Accumulation of phytoalexin sakuranetin and momilactone A by chitosan treatment has been reported in rice leaves. The amount of these two phytoalexins dramatically increased at 72 hours after treatment (Agrawal *et al*., 2002). Several studies were conducted in Leguminosae, plants of the pea or bean family. Seven-day old Colombian bean seedlings were treated with chitosan, and the accumulation of phytoalexins was

determined. The result showed that chitosan was to stimulate accumulation of phytoalexins daidzein, genistein, coumestrol, and phaseollin. However the accumulation of these compounds was nearly 50% of the response obtained with $CuCl_2$ (Durango *et al*., 2002). Soybean leaves were treated with different size of chitosan oligomers. It was found that increase in phenolic content correlated to chain length, pentamer and hexamer forms of chitosan showed higher phenolic content than tetramer (Khan *et al*., 2003).

Chitosan as a Growth Stimulator and Yield Enhancer

Recently, not only the defense responses induced by chitosan have been reported, but the plant growth promotion activity has also been studied in some species. It has been shown that chitosan promotes various processes in developing flower buds of lisianthus (*Eustoma grandiflorum*) (Jamal Uddin *et al*., 2004). Chitosan also accelerated the flowering period and also increased the mass and number of flowers in *E. grandiflorum*. Additional the plant showed a significant higher fresh and dry mass of leaves in the chitosan treated soil than the untreated (Ohta *et al*., 1999). Chitosan has potential to stimulate grapevine (*Vitis vinifera*) plantlet development and induce protection from *B. cinerea*. In this study, various concentrations of chitogel and formulated chitosan solution were added to the nutrient medium range from 0.5% to 10% (v/v). The results showed that chitogel at low concentrations enhance the plantlets growth compared to the control. However, at concentrations above 2.0% (v/v) of chitogel had negatively effect on the plantlets growth, based on shoot length measurement. The optimal concentration of chitogel for allowing the best growth of grapevine was 1.75% (v/v); in addition, shoot and root dry weights increased as well as stem length and the number of nodes was significantly enhanced. These results reflect a dose-dependent decrease in shoot length at higher concentrations of chitogel. The study also showed capability of chitogel to promote growth of grapevine plantlet according to the faster growth rate and number of secondary root (Barka *et al*., 2004). The effect of chitosan on the

formation of protocorm-like bodies in meristem buds and the growth and differentiation of orchid protocorm was observed. The growth of meristem explants into protocorm-like bodies was accelerated up to 15 times in the presence of chitosan oligomer, and the optimum concentration was 15 ppm. The differentiation of orchid tissue in culture solid medium supplied with 20 ppm of either 10 kDa fungal or 1 kDa shrimp chitosan gave the best result compared with the control. This study suggest that minute amount of chitosan has a profound effect on the growth and development of orchid plant tissue (Nge *et al*., 2006). Chitosan stimulates renewed orchid flower production and have positive effect on the growth of root, shoot and leaves of Gerbera flower plant (Wanichpongpan *et al*., 2001; Chandrkrachang, 2002). Similar result was obtained in experiment with soybean (*Glycine max* L. Merrill). Soybean seeds were coat with 0.4% chitosan oligomer; seed germination and growth rates were observed. The result showed that germination and growth rate of soybean plant was higher than those of the control; moreover, the crop yield was 20% to 30 % higher than that of the control (Dzung and Thang, 2002). Other studies reported that chitosan affected growth and quality of soybean sprouts. In other words, total weight, vitamin C content, and hardness of soybean sprouts was increased by chitosan treatment (Lee *et al*,. 1999; Choi *et al*,. 2000; No *et al*., 2003). In rice plants, there are few publications related to the effect of chitosan on the subsequent yield, but these reports indicated that chitosan improved rice yield at harvest. Chitosan was applied by spraying 2-3 times during cultivation. The experiment was conducted in Ubonratchathani, Chacheongsao and Petchaboon areas of Thailand. The result reported that rice yield increased 40 to 60% compared control (Chandrkrachang, 2002). Similar result was observed in experiment with indica rice cultivars, IR24, IR50 and Jyothi where soil amended with *Bacillus* and chitosan increased in growth; root and shoot length, and grain yield of 10 to 20% (Vasudevan *et al*., 2002). This result was nearby with Hong's study (1998) which showed that a 17.6% yield above control was achieved in indica rice after treatment with chitosan. Furthermore, in 2005, a study was conducted in rice c.v Suphanburi 1 with four levels chitosan concentration and

four frequency of foliar spray. The rice yield increased as a result of chitosan treatment compared to the control. Chitosan concentration at 20 ppm achieved a higher yield than those of the other concentrations tested. This experiment also demonstrated that four times of foliar spray in each concentration significantly affected the total rice yield, panicles per plant, and dry matter accumulation (Boonlertnirun *et al*., 2005). Nevertheless, Boonlertnirun *et al*. did not describe the sources and molecular weight of chitosan used in their experiment.

Rice (*Oryza sativa* L.)

Rice (*Oryza sativa* L.) is one of the principal cereals used by world's populations. Especially in the densely populated countries of Asia rice is the most important staple food. As much as 80% of the dietary calorie intake of people of Asia is derived from rice. There are many rice varieties, which have been developed and grown in more than 100 countries.

The study of rice morphology and development is basically for rice cultivation and for selection the criteria in crop improvement. The morphological characters are used to monitor plant development. Monitoring is determined by observation of growth stages; emergence, tillering, panicle initiation, booting, heading, and maturation. This observation helps predict cultivar growth stages and final yield production.

Morphology of Cultivated Rice

Rice morphology is basically study of plant anatomy. It has application in rice research and crop production.

- ***Leaves***: each leaf consists of a leaf sheath and a leaf blade connecting by junction or collar where a pairs of auricles and a ligule present. Leaf sheath is an elongated leaf rolled into cylinder that encloses developing new leaves and supporting the stem during vegetative growth. It also acts as a storage site for

starch and sugar before heading. Leaf blade is long and lanceolate and has a midrib with large and small parallel veins on each side. It is the principle organ for photosynthesis. Ligule is a thin, triangular membrane at the base of the leaf blade. It is located between the leaf blade and the sheath. Auricles are located at the boundary between the leaf sheath and collar. They are sickle shape and hairy.

• ***Culm***: is the plant stem and is composed of a series of nodes and internodes. There are two types of culm. Main culm is the first plant stem, developing during early vegetative growth and prior to tillering. Tillers are culm that develops form the main culm.

• ***Root***: the rice plant develops three distinct root types; seminal root or radicle, mesocotylar roots, and adventitious roots or crown. The roots develop from different parts or tissues. The radicle is the first to emerge from the seed at germination. Mesocotylar roots usually do not form, but can form under condition of deep sowing or chemical seed treatment. These roots are thin, unbranched, and develop from the lower to the upper part of mesocotyl. Adventitious or nodal or crown roots is emerged from each node as a crown around the culm.

• ***Panicle***: is composed of a panicle neck node (base), rachis (axis), primary and secondary branches, pedicel, and spikelets.

• ***Flower***: or spikelet has a pairs of rudimentary glumes and a lemma and palea that enclose the flora organs. The rice flower is perfect flower composed of six stamens (anther and filament) and on pistil (two stigmas, two styles and one ovary).

• ***Grain***: the rice kernel is composed of hull and caryopsis. The hull is composed of sterile lemmas, rachilla, palea and lemma. The caryopsis contains the embryo and starchy endosperm, surrounded by seed coat (tegmen) and the pericarp.

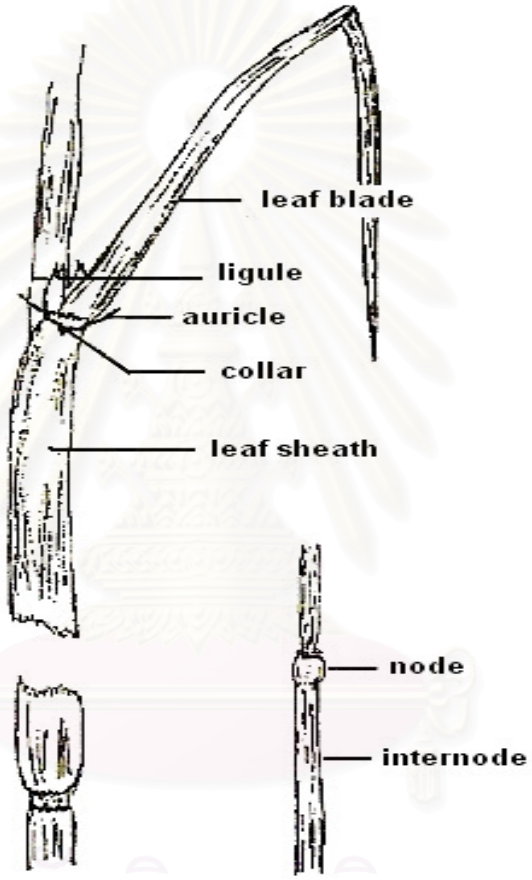


Figure 1.2 Stem and leaf showing leaf part and an external view of culm (Smith and Dilday, 2003)

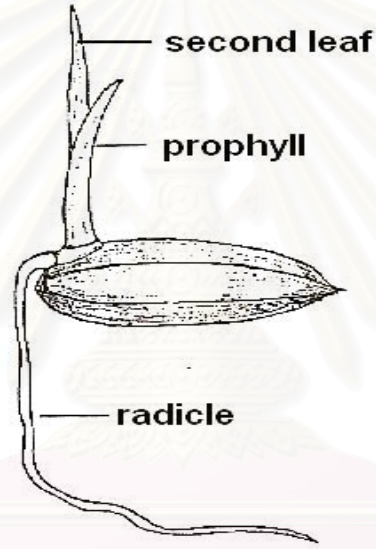


Figure 1.3 Rice seed germination (Smith and Dilday, 2003)

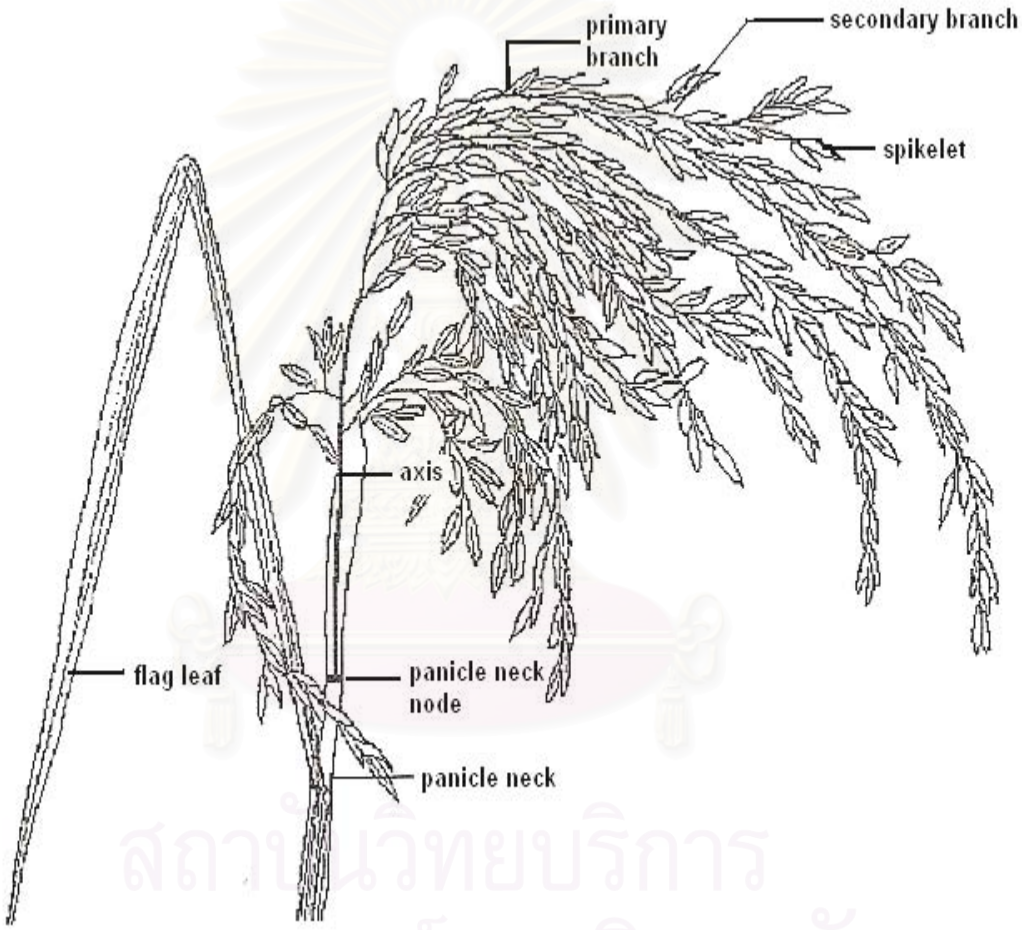


Figure 1.4 Panicle of the rice plants (Smith and Dilday, 2003)

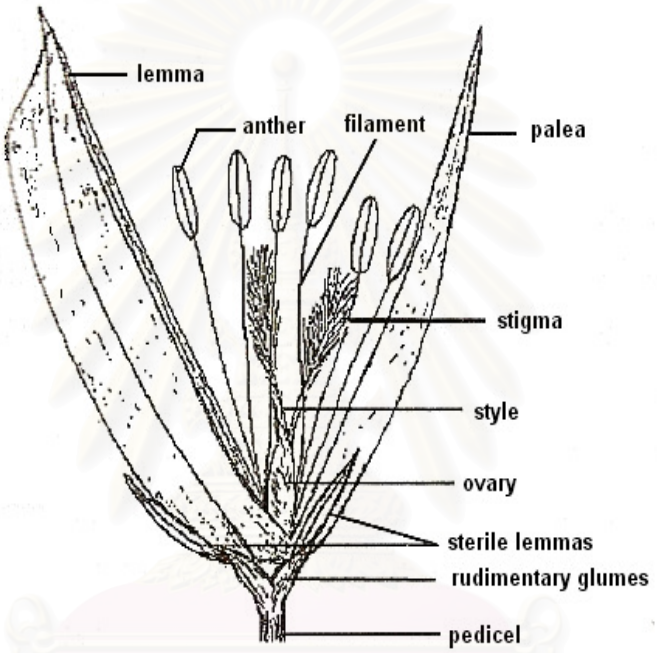


Figure 1.5 Spikelet or rice flower components (Smith and Dilday, 2003)

Development of Cultivated Rice

The growth duration of cultivated rice ranges from less than 80 days to 280 days. Cultivars can generally be divided into three groups: early-maturing cultivars (80 to 130 days), intermediate-maturing cultivars (130 to 160 days), and late-maturing cultivars days (more than 160 days) (Yoshida, 1981).

The growth duration can be divided in many stages, but the most basic division is into three phases: the vegetative growth phase, reproductive phase, and ripening phase (Smith and Dilday, 2003). The vegetative phase begins with germination and ends at panicle initiation, when the plant begins to partition assimilate to the developing panicle. Next, reproductive phase, the panicle form within the leaf sheath, is exerted, and undergoes anthesis (flowering). Last stage, ripening or grain-filling phase begins after anthesis and ends at maturation.

The duration of vegetative phase (germination to panicle initiation) is generally considered the most variable of all the growth phases (Yoshida, 1981; Vergara *et al*., 1991). It can vary from 25 to 90 days. The duration of reproductive phase (panicle initiation through flowering) is generally considered to be 30 days for most cultivars (Yoshida, 1981; Smith and Dilday, 2003). Nevertheless, it can rage from 15 to 45 days, depending on cultivar and temperature (Blanco, 1982). Early-maturing cultivars also may have a shorter reproductive phase, so they are faster panicle formation. The duration of ripening phase (flowering through maturation) varies from 25 to 45 days. The ripening phase duration is also depend on rice cultivar; thus, long-grain cultivars fill in approximately 35 days compared to medium-grain cultivars, which required 45 days, and short-grain cultivars, which may require 50 days (Smith and Dilday, 2003).

- ***Vegetative phase***: this phase begins with seed germination and proceeds with repetitive production of shoot units (defined as an internode that produces a leaf at its upper end, a tiller bud on it lower end, and a root band on both its upper and lower ends, finish when panicle) until panicle initiation. Each shoot unit

produces a leaf, tiller and root primordia. After germination seedling growth continues with extension of the coleoptile and emergence of prophyll and radicle**.** The prophyll, first leaf, is not true leaf since it lacks a blade. In germination test, the emergence of coleoptile and of radicle is used to indicate seed germination. Seedling stage can be determined by marking and counting leaves as they emerge. So it is starting from prophyll emerge and generally end at the fourth true leaf emerge that is, the prophyll is not considered as the first leaf. Duration of this seedling stage is varied from 25 to 35 days. The tillering stage usually occurs at, or after the end of seedling stage. In fact, first tiller of the plants will be initiated from the third of shoot units, and it becomes visible protrusion when the given leaf begins to emerge. Tillers can potentially emerge three nodes below each emerging leaf in continuous pattern up the culm. Thus for a given leaf that is emerging at the *n*th node of the plant, there is a tiller bud potentially emerging at the (*n*-3)th node. Under field condition, cultivars have a maximum tiller number and are also observed to have a termination point for effective tillering. This is a point which tiller number equals the number of panicles at maturity. Rice cultivars vary in tiller number as well as in earliness of tillering. Tillering is also affected by plant spacing. When seeds are drilled densely, and plant density is high so that maximum tiller number is low and vice versa.

• ***Reproductive phase***: starts from panicle initiation through anthesis and is characterized by change in vegetative growth and formation of panicle. Increase in plant height is a result from internode elongation, reduction in tillering and root growth. Panicle formation is synchronized with development of the upper most four leaves on the culm. At first, panicle formation is not visible with naked eye, but it can be seen at booting period, where the leaf sheath visibly thickens. The panicle double in size every 3 days its formation and booting generally defined as the first visual evidence of panicle swelling within the leaf sheath (Moldenhauer *et al*., 1994). Then heading, panicle exerts from the flag leaf sheath. Finally anthesis or flowering period begins with flowering day. As panicle emerges, spikelets at the uppermost tip of the panicle begin to undergo

anthesis and proceed in descending order down the panicle. It can take 7 to 10 days for all spikelets on the panicle to complete anthesis. Anthesis refers to events between the opening and closing of the spikelet. It usually lasts 1 to 2.5 hours, and occurs between 9:00 A.M. to 2:00 P.M. At lower temperature and on cloudy day, anthesis may begin later and take longer. It can be inhibited by temperature below 22 °C or above 32 °C, causing sterility (Vergara *et al*., 1970). During anthesis, the spikelet opens by movement of lemma. Anther filaments elongate and are exerted, and the tip of the feathery stigma becomes visible. Anther filament continues to elongate to bring the anther completely pass the tip of lemma and palea. Anthers dehiscence occurs just before or as the palea and lemma open. Pollen grains thus fall onto the stigma; as a result, rice being predominantly self-pollinated. After self-pollination, fertilization is began and completed within 5 to 6 hours (Smith and Dilday, 2003).

- ***Ripening phase***: grain ripening begins 3 weeks after fertilization and usually take 25 to 50 days. There are four steps in the ripening process. Firstly, milky stage: developing starch grain in the kernel is soft and interior of the kernel is filled with white liquid resembling milk. Next soft dough stage: starch is beginning to firm, but is still soft. Then hard dough stage: whole kernel is firm, moisture content is greater that 20%. Finally maturation: whole kernel is hard and moisture content is less than 20% (Yoshida, 1981). Most important suppliers which provide photosynthate to the ripening panicle are the five uppermost leaves. The flag leaf which being the primary supplier. These leaves have the longest physiological lifespan on the plant. They remain green throughout the ripening phase.

Table 1.2 The growth duration of cultivated rice. There are three phases consist of vegetative, reproductive and ripening phase.

Development of cultivated rice						
Vegetative phase			**Reproductive phase**		**Ripening phase**	
Seedling	Tillering	Panicle initiation	Panicle formation	Flowering	Grain filling	Maturation
30 days	**45- 60 days**		**25 - 30 day**		**30 days**	

150 days

Table 1.3 Thai rice exports by region in 2003-2007

Region/Year	**2003**	**2004**	**2005**	**January-June**	
				2006	**2007**
Asia	2,584,491	2,057,800	2,069,285	1,012,677	1,289,250
Middle East	1,610,267	906,178	1,577,274	893,572	545,332
Europe	441,633	332,282	404,469	178,357	281,829
Africa	4,757,263	3,450,533	2,736,271	1,349,875	1,674,399
America	613,534	414,293	483,163	210,513	213,745
Oceania	133,036	143,261	150,193	69,982	69,914
Total	10,140,224	7,304,346	7,420,656	3,714,978	4,074,472
Value (M. Baht)	110,376	90,874	97,349	46,983	52,984
Value (M. US$)	2,749	2,278	2,553	1,205	1,508
Private Export	10,117,574	7,304,346	7,420,656	3,714,978	3,746,093
Govt. Export	22,651	-	-	-	328,379

Units: Metric Ton

Source: Department of Foreign Trade (available online at: *http://www.dft.moc.go.th*)

Although Thailand is a major rice producers worldwide and tend to increase in export annually (Table 1.3). In reality rice production is usually limited by cultivation area, technology and cost. Farmer usually suffers from yield per unit area is low as well as pests and diseases spread out which cause yield decrease resulting in income decrease. The conventional ways to solve these problems are supplement with fertilizer in high dose and chemical pesticides treatment. Therefore the production cost is higher and chemical pesticides remain in an environment. Still chitosan is a natural material and there is a variety of benefits; inhibit growth of certain microorganisms result in plant diseases, induce defense response, stimulate growth, and enhance yield production in several plants such as potato, soybeans, and rice. Furthermore chitosan is produced from the waste of fishery (shrimp and crab shells), so there is sufficiently raw material.

The purpose of this study was to investigate effect of chitosan at different concentrations on seed germination, rice growth and production of cultivated rice *Oryza sativa* L. c.v Pathum Thani 1.

CHAPTER II

MATERIALS AND MATHODS

Equipments

- Autoclave; Model MLS-3020, Sanyo, Japan
- Auto pipette; Pipetman, Gilson, France
- Centrifuge; Model 5174C, Eppendorf, Germany
- Centrifuge; Model H-103N, Kokusan Enshinki, Japan
- Centrifuge; Model J-301, Beckman Coulter, U.S.A.
- Shaking incubator; Model 4000, New Brunswick Scientific, U.S.A.
- Incubator; Model OB-28L, Fisher Scientific, U.S.A.
- Laminar flow; Model MCV-B131S(T), Sanyo, Japan
- Magnetic stirrer; Model M21/1, Fisher Scientific, U.S.A.
- pH meter; Model PHM83, Radiometer, Denmark
- Spectrophotometer; Model 6400, Jenway, U.K.
- UV-VIS Spectrophotometer, Model: G1103A, Agilent, U.S.A.

Chemicals

- Acetic acid (CH_3COOH), Lab-Scan, Ireland
- Agar, Scharlau, Spain
- Ammonium sulphate ($(NH_4)_2SO_4$), Merck, Germany
- Hydrochloric acid (HCl), Lab-Scan, Ireland
- N-acetyl-D-glucosamine, Sigma, U.S.A.
- Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$), BDH, England
- Di-Potassium hydrogen phosphate (K_2HPO_4), Fisher Scientific, U.K.
- Potassium dihydrogen orthophosphate (KH_2PO_4), Fisher Scientific, U.K.
- Potassium ferricyanide ($K_3Fe(CN)_6$), BDH, England
- Sodium carbonate, (Na_2CO_3), Fisher Scientific, U.K.
- Sodium chloride (NaCl), Ajax Finechem, New Zealand
- Sodium hydroxide (NaOH), Carlo Erba, Italy

- Shrimp chitin, Taming Enterprises, Thailand
- Tryptone, Scharlau, Spain
- Yeast extract, Scharlau, Spain

Preparation of Chitosan

Crab shells were used for chitosan production. The demineralization step was accomplished by soaking 600 g of crab shells in 10 litters of 1.5 M hydrochloric acid (HCl) for 24 hours. The acid solution was replaced with a freshly prepared solution every 8 hours. The deproteinization step was accomplished by soaking the protein containing crab shells, from the demineralization step, in 10 liters of 1.5 M sodium hydroxide (NaOH) for 24 hours. The sodium hydroxide solution was replaced with freshly prepared solution every 8 hours. The resulted chitin product was then deacetylated. Deacetylation of chitin was performed in 50% (w/w) sodium hydroxide (NaOH) for 48 hours, the NaOH solution was changed after the first 24 hours. The chitosan product was washed in distilled water until the pH was neutralized.

Determination of Degree of Deacetylation

The degree of deacetylation (DD) of chitosan was determined by the first derivative UV-spectrophotometry with modified version of the method described by Muzzarelli and Rocchetti (1985). Briefly, 0.2 g chitosan was dissolved in 100 ml of 0.2 M acetic acid and stirred at 100 rpm for 24 hours following by filtration with filter paper (Whatman no. 5). The chitosan solution (5 ml) was diluted in 100 ml distilled water to get the final concentration of 0.1 mg ml^{-1}. The solution was transferred into 1-cm cuvette for the determination using a UV-VIS spectrophotometer (Agilent G1103A, U.S.A.). The DD was calculated as following formula:

$$\%DD = [1-\{A/((10xW)-204A) + A\}/161] \times 100$$

Where:

A = Concentration of N-acetyl-D-glucosamine (g/L) divide by 204

W = Chitosan weight (g) in 100 ml 0.01 M acetic acid

204 = Molecular weight of N-acetyl-D-glucosamine

161 = Molecular weight of D-glucosamine

Bacterial Strain and Enzyme Production

Bacillus licheniformis SK-1 was used for producing chitinase. *B. licheniformis* was cultured in colloidal chitin minimum medium (CCMM) containing 1.5% (dry weight) colloidal chitin, 0.25% yeast extract, 0.1% $(NH_4)_2SO_4$, 0.03% $MgSO_4.7H_2O$, 0.6% KH_2PO_4, 1.0% K_2HPO_4 and 2.0% agar, pH 7.5. A single colony was collected and inoculated into 3 ml. of Luria-Bertani (LB) medium consists of 1.0% tryptone, 0.5% yeast extract and 1.0% sodium chloride, pH 7.5. *B. licheniformis* was grown in shaking flasks at 250 rpm, 37 °C overnight. This starter (2.5 ml) was inoculated into 250 ml of chitin powder minimum medium (CPMM) composed of 1.5% (dry weight) shrimp chitin, 0.25% yeast extract, 0.1% $(NH_4)_2SO_4$, 0.03% $MgSO_4.7H_2O$, 0.6% KH_2PO_4 and 1.0% K_2HPO_4, pH 7.5, for enzyme production. *B. licheniformis* was cultured at 250 rpm, 50 °C for 6 days.

Preparation of Crude Enzyme

At day 6 after inoculation, crude enzyme was collected and centrifuged at 8,000 rpm, for 10 minute. Supernatant containing chitinase was collected and stored at 4 °C for further biochemical analysis.

Enzyme Assay

Chitinase activity was assayed by colorimetric method (Imoto, 1971) based on the Shale's method. In brief, the reaction mixture consists of 550 µl of 100 mM phosphate buffer pH 6.0, 100 µl of 1% colloidal chitin and 100 µl of

crude enzyme. After 15 minute of incubation at 50 °C, the release of *N*-acetyl-D-glucosamine was determined by adding 1 ml ferric cyanide reagent and boiled for 15 minutes. Then stop reaction by cooling down in cool water and centrifuged at 3,000-4,500 rpm for 10 minutes to remove the undigested substrate. The absorbance at 420 nm was measured by spectrophotometer (Jenway 6400, U.K.) against distilled water (A_1). A blank value (A_0) was obtained by using denatured the enzyme instead of the enzyme in previous reaction. The difference of A_0 and A_1 was used to determine the amount of *N*-acetyl-*D*-glucosamine from standard curve. (See Appendix A). A unit (U) of enzymatic activity is defined as the amount of enzyme releasing 1 µmole *N*-acetyl-D-glucosamine min^{-1}.

Preparation of Chitosan Solution

Dissolve chitosan of 20 g in 1.5 litters of 1.5 M acetic acid and adjust pH with sodium hydroxide (half equivalent). Add 100 U chitinase from *B. licheniformis* SK-1. The reaction was incubated at 50 °C, overnight. Chitosan solution was freshly prepared in distilled water at concentrations of 10, 20, 40, and 80 ppm, before use.

Determination of Molecular Weight of Chitosan

Molecular weight of chitosan was determined using Gel Permeation Chromatography (Waters 600E, U.S.A.). Test conditions; eluent: 0.5 M acetate buffer pH 4, flow rate 0.6 ml min^{-1}, injection volume: 20 µl, temperature 30 °C, column set: ultrahydrogel linear 1 column (MW resolving range 1,000-20,000,000) + guard column, polymer standard: polysaccharide (pullulan: MW 5,900-788,000), calibration method: polysaccharide standard calibration. Chitosan (2 mg ml^{-1}) was dissolved in eluent and filtered using nylon 66 membrane (pore size 0.45 µm) before injection.

Germination Test

Rice (*Oryza sativa*, cv. Pathum Thani 1) was used in this study. The experiment was designed into five treatments. There are four concentrations of chitosan; 10, 20, 40 and 80 ppm and distilled water used as control. One-hundred rice seeds were sown on two sheets of square cloths (blotters) evenly and rolled, then soaked in appropriate chitosan solution for 12 hours. The rice seeds were placed inside the plastic box to prevent moisture loss. Each treatment was incubated at 28 °C, for 7 days. The numbers of germinated seeds were recorded. Seed germination is defined as the growth process of a mature seed, characterized by the emergence of both radicle and coleoptile (embryonic shoot) from the ruptured seed coat.

Effect of Chitosan on Rice Growth

The experiment was conducted at Chulalongkorn University, Bangkok, from January 19, 2007 to May 25, 2007 totally 126 days. First, one hundred rice seeds were soaked in chitosan solution at 10, 20, 40, and 80 ppm, for 48 hours and leaved them at room temperature for 12 hours. Distilled water was used as a control. Then rice seeds were sown in pots, and five day after seeding, 10 ml of chitosan was secondly applied in each pot. Parameters used for measurement plant growth were plant height, leaf blade length, width, and area of the second, third and fourth leaves and shoot dry weight. The plant height was measured from the first node of rice stem through the tip of the longest leaf. The plant height was recorded every 5 days for thirty-days. The leaf blade length was measured from collar through the tip of individual leaf and the leaf blade width was measured at the central of the leaf. The measurements were carried out after the leaves completely emerging from the rice culm and recorded every five days. Leaf area was calculated by using Yoshida's formula (Yoshida, 1981) as follow:

$$\text{Leaf area } (cm^2) = \text{leaf blade length (cm)} \times \text{leaf blade width (cm)} \times 0.75$$

Finally, determinations of shoot dry weight; the rice grains were harvested, and rice straws were collected. The rice straws were dried out for 3 days and dried at 80 °C, for overnight. The rice straw dry weight was recorded.

Effect of Chitosan on Rice Tillering

Thirty days after seeding, the end of seedling stage, a healthiest rice paddy was transplanted to other new pot (Figure 2.1). Chitosan solution (10 ml) was thirdly applied together with 1.11 g of fertilizer one day after transplantation. The rice paddies were grown in natural light condition and supplied with water during the entire cultivation period. The number of tillers per plant was recorded thirty days after transplantation.

Effect of Chitosan on Rice Yield

Rice plants were fourthly supplied with 10 ml of each chitosan concentration together with 0.67 g of fertilize forty days after transplantation. Rice plants proceeded through panicle initiation, flowering, and ripening phase until maturation. Panicles per plant, grains per panicle and plant were recorded. Total grain dry weight and a hundred grain dry weight were measured.

Data Analysis

The experiment was set up in a completely randomized design (CRD) and was carried out in 5 replications. The mean differences in percent of seed germination, plant growth, number of tillers, and rice yield among treatments were compared by one-way ANOVA ($P<0.05$), using the SAS system version 9.0 (SAS Institute Inc., U.S.A.). Duncan's' Multiple Range Test (DMRT) was performed for mean comparison.

Figure 2.1 Rice transplantation

CHAPTER III

RESULTS

Chitosan Characteristics

Chitosan was extracted from crab shells by following process: demineralization in hydrochloric acid, deproteinization in sodium hydroxide, resulting in chitin. The chitin was deacetylated in 50% (w/w) sodium hydroxide. Chitosan obtained from this process was 40 g of white solid flakes. The percent degree of deacetylation (%DD) of chitosan was determined by modifying Muzzarelli and Rocchetti's method (1981). The %DD of the chitosan prepared was 91%. Chitinase, produced from *Bacillus licheniformis* SK-1, with enzymatic activity of 150 mU ml^{-1}, was used to hydrolyze chitosan. The reaction was carried out at 50 °C, overnight to allow complete hydrolysis. Chitosan was dissolved in eluent and filtered before injection through the column of gel permeation chromatography. The average molecular weight of obtained chitosan was 46 kDa with polydispersity of 2.2.

Effect of Chitosan on Seed Germination

Rice seeds were rolled inside two layers of cloth and soaked with the chitosan solution for 12 hours. The treated rice seeds were incubated at 28 °C for 7 days. Germinating seeds that both radicle and coleoptile emerged from ruptured seed coat were counted. The result revealed that percents of seed germination in each treatments were not significantly different, and percent of germination was 94 to 95% (Table. 3.1).

Effect of Chitosan on Growth

Rice seeds were soaked in chitosan solution for 48 hours before sown into pots. Five days after seeding (DAS) rice paddies were secondly supplied with 100 ml of chitosan solution together with fertilizer, and plant height was recorded

Table 3.1 Percent of seed germination of rice seed treated with various chitosan concentrations for 7 day at 28 °C.

Treatments	control	10 ppm	20 ppm	40 ppm	80 ppm
% Germination^{ns}	94±2.0	94±2.1	95±3.3	94±2.6	94±1.4

***Note**: ns, non-significant*

Table 3.2 Effect of chitosan concentrations on plant height at seedling stage

Treatments/ Days	Plant height (cm)					
	5	10	15^{ns}	20^{ns}	25^{ns}	30^{ns}
control	7.59±0.25^{b}	19.04±0.44^{ab}	22.36±0.77	24.62±0.88	25.72±0.84	27.88±1.4
10 ppm	7.63±0.31^{b}	18.74±0.42^{b}	22.50±0.57	24.59±1.2	26.40±1.8	28.52±2.4
20 ppm	7.60±0.46^{b}	18.98±0.26^{ab}	22.52±0.79	24.16±1.1	25.91±1.3	28.48±0.83
40 ppm	7.69±0.37^{b}	19.64±0.71^{a}	22.81±0.89	24.55±1.5	26.10±1.6	28.19±1.6
80 ppm	8.64±0.31^{a}	18.67±0.87^{b}	22.08±0.60	24.64±0.86	26.42±1.3	28.43±1.3

***Note**: means ± SD followed by the same letters in the same column show no significant difference (P<0.05); ns, non-significant*

every five days for thirty days. The result showed that the plant height was significantly increased 5 DAS compared with control. In contrast, plant height was not significantly different 10 DAS, but chitosan treatment at 40 and 80 ppm showed the tendency to increase plant height (Table 3.2). The effects of chitosan concentration on leaf blade length and width of the second, third, and fourth leaves were also investigated. The leaf blade length of all selected leaves was highest in the treatment where rice seeding was supplied with 40 ppm chitosan; however, it was not significantly different when compared to the control (Table 3.3). The leaf blade width was totally non-significant difference when compared to other treatments (Table 3.4). Leaf area was calculated according to Yoshida's formula (1981). The result showed that chitosan concentration of 40 ppm resulted in the highest leaf area compared with other treatments. However; the difference among treatments was found in the second leaf. For the third and fourth leaves, leaf area did not significantly difference when compared to other treatments (Table 3.5).

Rice straws were collected, air-dried and then dried at 80 °C. The shoot dry weight was recorded. Chitosan treatments increased the shoot dry weight compared to control (Table 3.6), and it was found that the dry weight of rice straws which supplied with chitosan concentrations of 20 and 40 ppm was higher than other treatments. However, the difference in rice straws dry weight among treatments were not significant at $P<0.05$.

Figure 3.1 Rice paddies (30 days) treated with chitosan concentrations at concentration of 10, 20, 40, and 80 ppm compared with untreated control. The plant height of rice paddies treated chitosan was higher than the plant height of untreated control. (From left; control, 10, 20, 40 and 80 ppm respectively)

Table 3.3 Effect of chitosan concentrations on leaf blade length of the second, third, and fourth leaves at seedling stage.

Treatments/ days	**Leaf blade length (cm)**									
	2nd leaf			***3rd leaf***			***4th leaf***			
	Day 07^{ns}	Day 12	Day 17	Day 10	Day 15	Day 20	Day 13^{ns}	Day 18^{ns}	Day 23^{ns}	Day 28^{ns}
control	3.92±0.17	3.92±0.10^{ab}	3.94±0.11^{ab}	12.75±0.46^{ab}	12.71±0.44^{ab}	12.46±0.09^{ab}	15.28±0.71	15.31±0.69	15.27±0.68	15.22±0.68
10 ppm	3.84±0.30	3.76±0.29^{ab}	3.84±0.31^{ab}	12.62±0.43^{ab}	12.28±0.28^{b}	12.24±0.50^{b}	15.31±0.49	15.37±0.49	15.33±0.48	15.26±0.48
20 ppm	3.82±0.14	3.86±0.17^{ab}	3.81±0.13^{ab}	12.81±0.21^{ab}	12.79±0.18^{ab}	12.69±0.18^{ab}	15.44±0.65	15.42±0.67	15.42±0.68	15.35±0.57
40 ppm	4.07±0.22	4.03±0.19^{a}	3.76±0.19^{b}	13.21±0.55^{a}	13.18±0.53^{a}	12.91±0.51^{a}	15.89±0.78	15.86±0.76	15.80±0.76	15.76±0.74
80 ppm	3.75±0.25	3.68±0.24^{b}	3.76±0.19^{b}	12.51±0.49^{b}	12.50±0.51^{b}	12.31±0.51^{ab}	15.14±0.66	15.06±0.45	15.06±0.46	14.96±0.45

***Note**: means ± SD followed by the same letters in the same column show no significant difference (P<0.05); ns, non-significant*

Table 3.4 Effect of chitosan concentrations on leaf blade width of the second, third, and fourth leaves at seedling stage.

Treatments/ days	**Leaf blade width (cm)**									
	2nd leaf			***3rd leaf***			***4th leaf***			
	Day 07^{ns}	Day 12^{ns}	Day 17^{ns}	Day 10^{ns}	Day 15^{ns}	Day 20^{ns}	Day 13^{ns}	Day 18^{ns}	Day 23^{ns}	Day 28^{ns}
control	0.30±0.02	0.30±0.01	0.30±0.01	0.31±0.01	0.31±0.01	0.31±0.01	0.39±0.01	0.39±0.02	0.40±0.01	0.39±0.03
10 ppm	0.29±0.01	0.30±0.01	0.30±0.00	0.32±0.01	0.32±0.01	0.32±0.01	0.38±0.02	0.40±0.01	0.40±0.00	0.39±0.01
20 ppm	0.30±0.01	0.30±0.00	0.30±0.01	0.31±0.01	0.32±0.02	0.31±0.01	0.39±0.01	0.39±0.01	0.40±0.01	0.40±0.01
40 ppm	0.31±0.01	0.30±0.00	0.30±0.01	0.31±0.01	0.31±0.01	0.30±0.03	0.39±0.01	0.39±0.01	0.39±0.01	0.39±0.02
80 ppm	0.30±0.01	0.30±0.00	0.30±0.01	0.31±0.01	0.31±0.01	0.30±0.06	0.38±0.01	0.40±0.01	0.40±0.00	0.39±0.01

***Note**: means ± SD followed by the same letters in the same column show no significant difference (P<0.05); ns, non-significant*

Table 3.5 Effect of chitosan concentrations on leaf area of the second, third, and fourth leaves at seedling stage.

Treatments/ days	**Leaf blade width (cm)**									
	2nd leaf			***3rd leaf***			***4th leaf***			
	Day 07	Day 12	Day 17	Day 10^{ns}	Day 15^{ns}	Day 20^{ns}	Day 13^{ns}	Day 18^{ns}	Day 23^{ns}	Day 28^{ns}
control	0.87±0.08^{ab}	0.89±0.04^{ab}	0.89±0.04^{ab}	2.97±0.10	2.98±0.08	2.92±0.03	4.48±0.27	4.52±0.24	4.56±0.16	4.42±0.20
10 ppm	0.84±0.07^{b}	0.84±0.05^{bc}	0.86±0.07^{ab}	2.99±0.18	2.90±0.14	2.87±0.17	4.35±0.36	4.56±0.22	4.60±0.14	4.51±0.27
20 ppm	0.86±0.03^{ab}	0.87±0.04^{abc}	0.86±0.02^{ab}	2.96±0.17	3.03±0.21	2.95±0.16	4.52±0.25	4.56±0.24	4.63±0.27	4.28±0.25
40 ppm	0.94±0.05^{a}	0.91±0.04^{a}	0.91±0.04^{a}	3.04±0.13	3.07±0.19	2.98±0.93	4.61±0.13	4.67±0.22	4.65±0.20	4.56±0.20
80 ppm	0.83±0.08^{b}	0.83±0.06^{c}	0.84±0.06^{b}	2.89±0.19	2.90±0.09	2.99±0.14	4.36±0.31	4.47±0.13	4.49±0.15	4.56±0.21

***Note**: means ± SD followed by the same letters in the same column show no significant difference (P<0.05); ns, non-significant*

Table 3.6 Effect of chitosan concentrations on rice straw dry weight

Treatments	control	10 ppm	20 ppm	40 ppm	80 ppm
Rice straw dry weight (g)^{ns}	31.68±2.0	32.37±7.3	37.32±8.2	37.59±4.3	32.15±4.3

***Note**: ns, non-significant*

Effect of Chitosan on Rice Tillering

A rice paddy was transplanted to other new pot and thirdly supplied with chitosan solution. Thirty days after transplantation, the number of tillers per plant was recorded. We found that the number of tiller per plant was 16 to 26 tillers. The rice plant which treated with chitosan at 20, 40, and 80 ppm tend to increase tiller number per plant compared with control at $P<0.05$ (Table 3.7).

Effect of Chitosan on Rice Yield

Growth duration of this rice cultivar Pathum Thani 1 was totally 126 days. Chitosan were totally supplied four times. At the ripening phase, maturation stage, rice yield was harvested. Panicles per plant and grains per panicle were recorded. We found that panicle number per plant in the rice which supplied with chitosan at 20, 40, and 80 ppm significantly increased compared with control, whereas grain number per plant was not difference ($P<0.05$). Total rice grains were counted. Chitosan concentrations at 20 and 40 ppm tended to increase grain number per plant as well as total rice grain number. Other result showed that in rice treated with chitosan, grain weight increased by 3 to 5% compared with control. The difference; however, on rice yield among treatments was not significant at $P<0.05$.

Table 3.7 Effect of chitosan concentration on tillers, panicles, and rice yield

Treatments	Tiller/ plant	Panicle/ plant	Grains/ panicle ^{ns}	Grains/ plant ^{ns}	Total grain/ 5 pots (seeds)	Total grain weight/ 5 pots (g)	100-grains weight (g) ^{ns}
control	20±1^{ab}	18±1^{b}	94±8	1763±212	8816	145.99	2.28±0.2
10 ppm	19±2^{b}	17±1^{b}	91±9	1684±299	8420	144.21	2.36±0.2
20 ppm	24±3^{a}	21±1^{a}	94±14	1846±330	9230	140.76	2.37±0.2
40 ppm	22±1^{a}	20±1^{a}	93±6	1854±115	9268	138.68	2.40±0.2
80 ppm	23±1^{a}	21±1^{a}	90±6	1746±243	8728	145.85	2.38±0.2

***Note**: means ± SD followed by the same letters in the same column show no significant difference (P<0.05); ns, non-significant*

CHAPTER IV

DISCUSSION

Chitosan Preparation

There are several methods which used to extract chitosan. The initial step may begin with eliminating protein contents and then demineralization or vice versa. The degree of deacetylation can be varied dependent on duration, temperature as well as concentration of sodium hydroxide. In this experiment chitosan with %DD of 91 and molecular weight of 46 kDa was prepared from crab shells using 1.5 M hydrochloric acid for demineralization step, 1.5 M sodium hydroxide for deproteinization step, and then deacetylated in 50% (w/w) sodium hydroxide. Low molecular weight chitosan can be prepared with non-specific enzyme like papain, amylase, lysozyme, cellulase, and glucanase (Muzzarelli, 1997; Lin *et al*., 2002; Lin *et al*., 2005; Feng *et al*., 2005), acid hydrolysis (Chen and Hwa, 1996; Liu *et al*., 2006). Chitinase is specific to hydrolysis β-1,4 glycosidic bond which connecting *N*-acetyl-D-glucosamine and D-glucosamine. Therefore, if the *N*-acetyl-D-glucosamine monomer distributes regularly, the chitosan molecules are quite homogenous.

Effect of Chitosan on Seed Germination

Chitosan solution at various concentrations; 10, 20, 40, and 80 ppm did not significantly affect rice seed germination. The percents of seed germination were 94% to 95%. The germination test was conducted at 28 °C, for 7 days. Previous reports revealed that chitosan improved seed germination in some plants such as wheat, but the germination percentage was also not different (Reddy *et al.,* 1999). Germination was observed in pearl millet seeds soaked in different dilution of Elexa™ which contains 4% chitosan as its active ingredient. The Elexa™ dilution of 1: 19 showed maximum germination percentage, 91%

whereas untreated seed germination percentage was 83%. However, the difference among treatments was not significantly different at $P<0.05$. Generally, the germination process begins with absorption of water then initiation of the biochemical process involved in embryo growth and ends with growth and emergence of radicle and coleoptile protrusion (Smith and Dilday, 2003). During biochemical process, α-amylase in aleurone layer plays an important role in hydrolyzing the endosperm starch into metabolizable sugars, which provide the energy for the growth of roots and the shoots (Beck and Ziegler, 1989). The expression of α-amylase is triggered by active gibberellin synthesized in epithelium of embryo (Haneko *et al*., 2002). Nevertheless, there is report that chitosan can repress the gibberellin signaling pathway leading to the induction of α-amylase in barley embryos (Loreti *et al*., 2002). Other factors affect seed germination is dormancy and temperature (Yoshida, 1981). The germination percentages of 90 to 97% occur if temperatures are between 27 and 37 °C. However, we did not observe an improvement in rice seed germination in our experiments. We were also using traditional method for investigation of seed germination percentage. The germination test was conducted by soaking rice seeds in chitosan solution with various concentrations range from 10, 20, 40 and 80 ppm (distilled water was used as control) for 48 hours and leaving them at room temperature for 24 hours. The presence of radicle was used to indicating seed germination. The result revealed that percent of seed germination among treatments did not different at $P<0.05$. We also found that emerging radicle was thin and long when rice seeds were treated with low concentrations of chitosan solution (10 and 20 ppm) as well as the control. In contrast, at high concentrations of chitosan solution (40 and 80 ppm), the radicle was large and short (data not show).

Effect of Chitosan on Growth

Rice seeds were grown in pot containing soil with pH 6.2 and salinity of 842 micromho. Rice paddies were treated with chitosan five days after seeding.

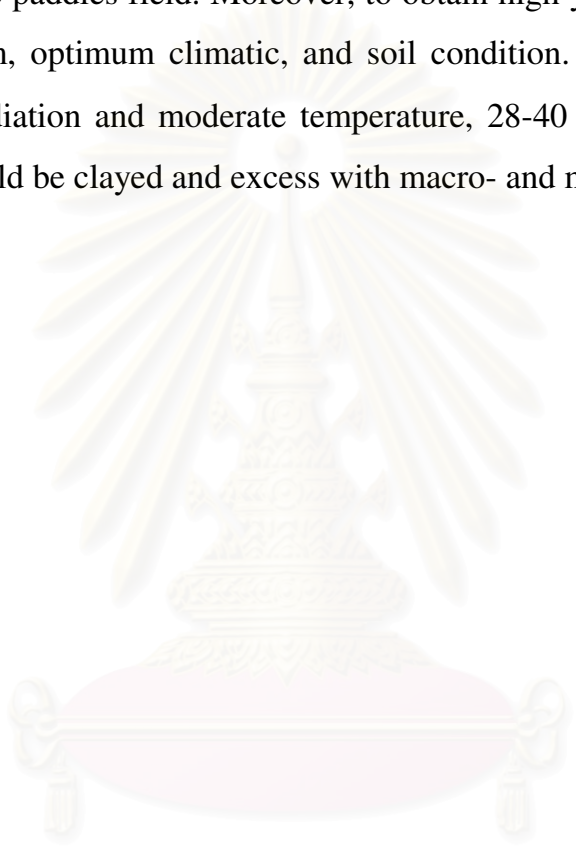
The plant growth parameters were recorded. Chitosan with concentration of 40 ppm showed a tendency to accelerate growth by increasing plant height, leaf blade length and area, although the difference on the rice growth was not significant statistically. There was a positive effect of chitosan treatment on rice growth where the dry weight of the rice straws was recorded. All chitosan treatments increased rice straws dry weight where the most effective concentrations were 20 and 40 ppm. These results; in addition, were supported by previous studies. There are several publication reported that chitosan stimulated growth of certain plants; for examples, sweet basil *Ocimum basilicum*, pearl millet, soybeans, grapevine, and *Eustoma grandiforum* (Dzung and Thang, 2002; Barka *et al*., 2004; Jamal Uddin *et al*., 2004; Sharathchandra *et al*., 2004; Kim *et al*., 2005). In orchid tissue culture, chitosan with low concentrations of 10 to 20 ppm enhanced protocorm-like body production, and accelerated explants growth (Pornpeanpakdee *et al*., 2002; Nge *et al*., 2006). In rice plants; for instance, the study was conducted in indica rice cultivars; IR24, IR25, and Jyothi. It was found that rice seedling grown in soils amended with *Bacillus* and chitosan increased shoot lengths in all the three cultivars (Vasudevan *et al.,* 2002). This study was supported by Boonlertnirun and colleagues' experiment (2005), the effect of chitosan on growth was defined by the dry matter accumulation. Dry weight was significantly increased with chitosan treatment by four times of foliar spray. According to the result, the experiment should be further explored in paddy field. The application method of chitosan may also be changed by spraying through the rice plants directly or mixing to fertilizer then spread through field, instead of directly addition in the water supply.

Effect of Chitosan on Rice Tillering and Yield

Rice plants were totally supplied with chitosan for 4 times, before germination, 5 day after seedling, a day after transplantation, and before panicle initiation stages (50 days after transplantation). After seedling stage, young plants were grown in pots under natural light condition and supplied with

chitosan together with fertilizer. Chitosan with concentrations of 20, 40 and 80 ppm increased tiller number per plant. Normally, when tiller number per plant increases the number of panicle should increase as well. The result revealed that the panicle number correlated to number of tillers that is the rice treated with chitosan at the same concentration showed significantly increase panicle numbers per plant compared with the control at $P<0.05$. The effect of chitosan on rice yield was also studied. There was a positive effect of chitosan treatment on grain number and grain weight. The grain number per plant was higher where the rice was treated with chitosan concentrations of 20 and 40 ppm than the control. Although the total grain weight was lower than the control. From this result, it is indicated that the rice which was treated with chitosan concentrations of 20 and 40 ppm has a number of un-filled grains more than untreated plants. It may due to limitation of nutrients during grain filling stage which result from there was higher numbers of tiller and panicle per plant than the control. However, when grain weight was determined; it increased by 3 to 5% compared with untreated plants. These results correlate to previous reports (Chandrkrachang, 2002; Vasudevan *et al.,* 2002; Boonlertnirun *et al*., 2005) that rising yield result from chitosan treatment. Chitosan was sprayed for 2-4 times during cultivation, and rice yield was increased 40-60% compared to control (Chandrkrachang, 2002). Similar to the experiment which conducted indica rice cultivars, IR24, IR50 and Jyothi where soil amended with *Bacillus* and chitosan, grain yield was increased 10-20% (Vasudevan *et al*., 2002). This result was nearby with Hong's study (1998), which showed that yield of indica rice was achieved by 17.6% above control after chitosan treatment. Furthermore, in 2005, the study was conducted in rice cv. Suphanburi 1 with four concentrations of chitosan concentration and four frequencies of foliar spray. The rice yield increased resulting from chitosan treatment when compared to control, and treatment of 20 ppm chitosan achieved highest yield. These results also reported that four times of foliar spray in each concentration significantly affected total rice yield, panicles per plant, and dry matter accumulation (Boonlertnirun *et al*., 2005).

It is interesting that chitosan has potent to enhance yield production; although, the rice yields among treated and untreated rice did not significantly different. The study suggests that further experiments should be conducted in field and an alternate chitosan application method and frequency of chitosan treatment should be tested. Instead of apply chitosan through the water supply, chitosan may be directly sprayed to the rice plants or mix with fertilizer then inoculated in the paddies field. Moreover, to obtain high yield also requires good irrigation system, optimum climatic, and soil condition. Rice is grown in high rate of solar radiation and moderate temperature, 28-40 °C (Smith and Dilday, 2003). Soil should be clayed and excess with macro- and micronutrients.



CHAPTER V

CONCLUSION

The chitosan was isolated from crab shells. The degree of deacetylation and molecular weight of chitosan were 84 %DD and 46 kDa respectively. Chitosan did not affect the percent of seed germination after the germination test was conducted for 7 day, temperature of 28 °C under dark condition. The study investigated the effects of chitosan concentration on rice growth. Treatment of chitosan 100 ml at 40 and 80 ppm have a tendency to increase plant height. The most effective chitosan concentration which accelerated the second, third, and fourth leaf blade lengths and areas was 40 ppm. Dry matter accumulation has a tendency to increase to increase with 20 and 40 ppm chitosan treatments.

Chitosan concentrations at 20, 40, and 80 ppm increased panicle per plant significantly. Chitosan did affect neither total rice grains nor grains weight; but treatment at 40 ppm has a tendency to increase the number of rice grains and grain weight (weight per a hundred grains).

In conclusion, chitosan concentration of 40 ppm was the most effective concentration which affected both growth and yield production of rice *Oryza sativa* cv. Pathum Thani 1.

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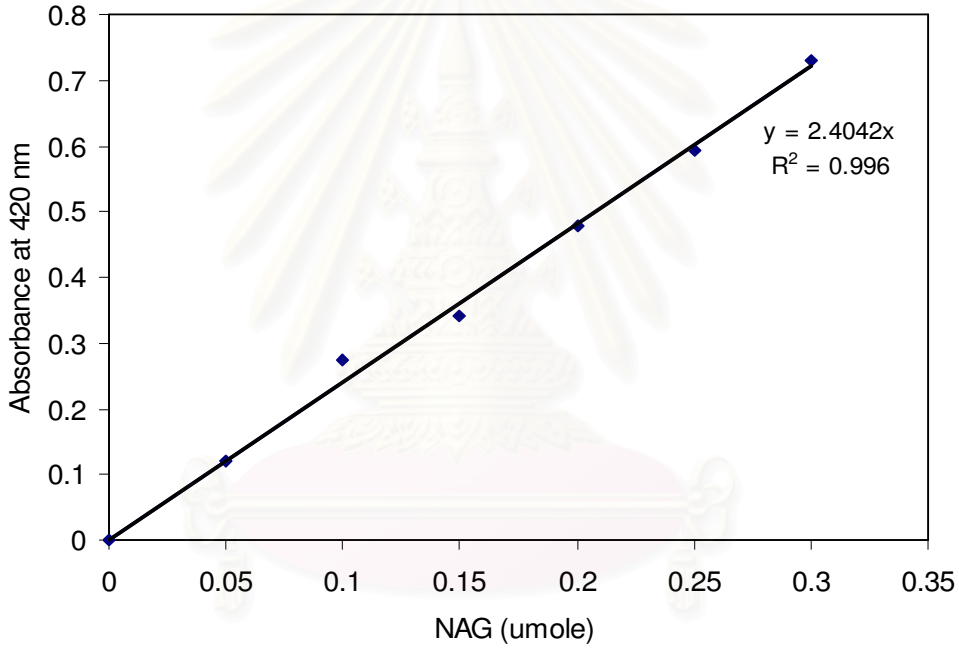
APPENDICES

APPENDIX A

Standard curve of *N*-acetyl-*D*-glucosamine for chitinolytic enzyme assay by colorimetric method

Standard curve for *N*-acetyl-*D*-glucosamine (GlcN) was made by monitoring the absorbance at 420 nm of standard concentration of GlcN according to the Shale's method



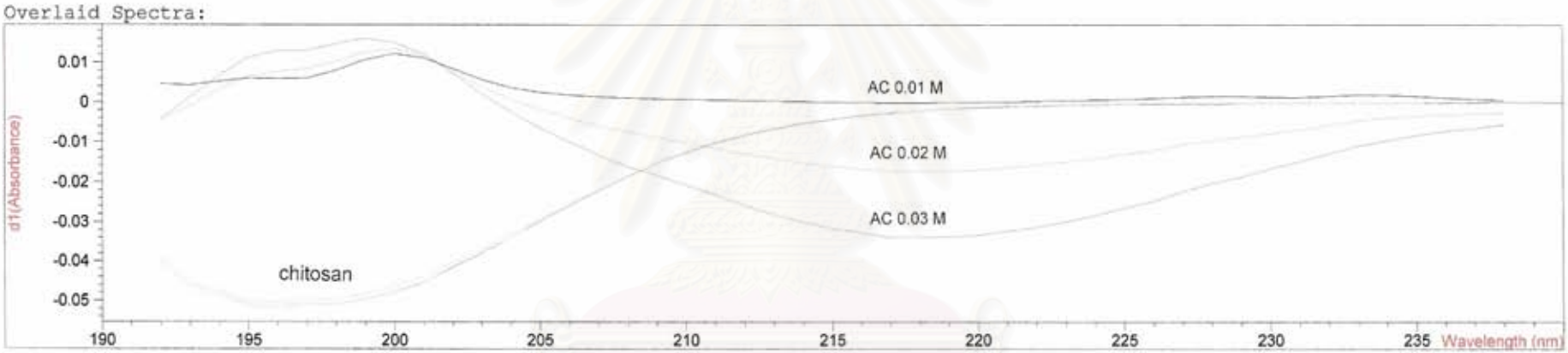
APPENDIX B

Preparation of colloidal chitin

Colloidal chitin was prepared from shrimp chitin by following process: add cool hydrochloric acid (12 M) of 400 ml into 10 to 20 g chitin and stirred for 2-4 hours on ice. The mixture was heated at 37 °C for 10 to 15 minutes with moderate shaking allow acid hydrolyzes chitin. The viscosity of the solution was rapidly increased and decreased within a few minutes, and the appearance of the mixture becomes clearer. The hydrolyzed chitin was filtered through 4 litters distilled or deionized water below 4 °C. The solution becomes turbid because of reprecipitation of chitin, and then kept the solution at 4 °C for overnight. The supernatant was decanted out, and remaining colloidal chitin was collected by centrifugation at 80,000 -12,000 rpm for 15 minutes. Wash pellet with distilled water then centrifuge at 80,000 rpm until pH 7. Resuspend pellet in 200 ml distilled water then add 0.02 % (w/v) NaN_3 and keep the solution at 4 °C. Chitin content was determined by percent dry weight.

APPENDIX C

First derivative spectra of chitosan and acetic acid solution with various concentrations. Chitosan (0.01 g) was diluted in 100 ml of 0.01 M acetic acid and acetic acid (AC) was varied from 0.01, 0.02, and 0.03 M.



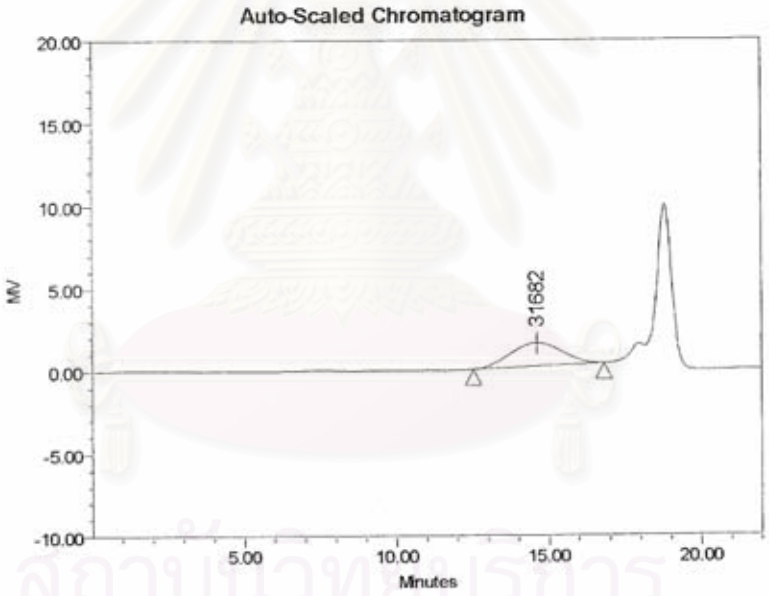
#	Name	d1(Abs)<201.4nm>	#	Name	d1(Abs)<201.4nm>	#	Name	d1(Abs)<201.4nm>
1	0.01M	1.0090E-2	3	0.03M	1.0261E-2	5	Sample2	-4.3778E-2
2	0.02M	1.0173E-2	4	Sample1	-4.3808E-2	6	Sample3	-4.2441E-2

APPENDIX D

Report for molecular weight determination of chitosan

SAMPLE INFORMATION

Sample name:	chitosan	Acquired By:	TEMSIRI
Sample type:	Broad Unknown	Date Acquired:	01/11/2006 13: 49: 28
Vial:	9	Acq. Method Set:	GPC_F06
Injection #:	1	Date Processed:	01/11/2006 14: 33: 57
Injection volume:	20.00 ul	Processing Method:	Y2007_F06_acetate_1
Run time:	22.0 minutes	Channel Name:	410
Sample Set Name:		Proc. Chnl. Descr.:	



GPC Results

	Mn	Mw	MP	Mz	Mz+1	Polydispersity
1	20825	46428	31682	89536	139912	2.229425

BIOGRAPHY

Mr. Paitoon Seanbualuang was born on October 30, 1979 at Chiang Rai province. In 2002, he received Bachelor's degree of Science in Biology from Department of Biology, Faculty of Science, Burapha University. In 2005, he applied for Program in Biotechnology, Faculty of Science, Chulalongkorn University and worked under supervision of Dr. Rath Pichyangura.