ผลของไคโทซานและกากไคโทซาน O-80 ต่อการเติบโต การสังเคราะห์ด้วยแสง ผลได้และการแสดงออกของยีนของข้าว *Oryza sativa* L.

นางสาวหนึ่งฤทัย คณานนท์

จุฬาลงกรณ์มหาวิทยาลัย
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EFFECTS OF CHITOSAN AND O-80 CHITOSAN RESIDUE ON GROWTH, PHOTOSYNTHESIS, YIELD AND GENE EXPRESSION OF RICE *Oryza sativa* L.

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หนึ่งฤทัย คณานนท์ : ผลของไคโทซานและกากไคโทซาน O-80 ต่อการเติบโต การสังเคราะห์ด้วยแสง ผลได้และการแสดงออกของยีนของข้าว *Oryza sativa* L. (EFFECTS OF CHITOSAN AND O-80 CHITOSAN RESIDUE ON GROWTH, PHOTOSYNTHESIS, YIELD AND GENE EXPRESSION OF RICE *Oryza sativa* L.) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.ศุภจิตรา ชัชวาลย์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร.บุญธิดา โฆษิตทรัพย์, 145 หน้า.

งานวิจัยนี้มีวัตถุประสงค์เพื่อเพิ่มผลผลิตข้าวโดยใช้ข้าวพันธุ์ปทุมธานี1เป็นต้นแบบสำหรับการประยุกต์ผลิตภัณฑ์ที่เกี่ยวข้องกับไคทิน/ไคโทซาน สำหรับการใช้ไคโทซานเพื่อเพิ่มประสิทธิภาพในการเจริญเติบโตของกล้าข้าว ได้ทำการทดสอบไคโทซานชนิดพอลิเมอร์ (P) และโอลิโกเมอร์ (O) ที่มี %DD ประมาณ 80% และ 90% ใน 1% (v/v) กรดอะซิติก ที่ความเข้มข้น 5 ถึง 80 mg/L พบว่าไคโทซานทุกแบบยับยั้งการเจริญเติบโตของกล้าข้าวเนื่องจากผลเชิงลบของกรดอะซิติก ซึ่งเป็นตัวทำละลายของไคโทซาน แต่กรดแลกติกมีความเป็นพิษต่อกล้าข้าวน้อยกว่ากรดอะซิติกและกรดซิตริก และเมื่อทำละลายไคโตซานด้วยกรดแลกติก พบว่าไคโตซานชนิดพอลิเมอร์ ที่มี 90% DD ความเข้มข้น 40 mg/L (P90-40) มีความเหมาะสมที่สุดในการใช้เพื่อกระตุ้นการเจริญเติบโตของกล้าข้าว การศึกษานี้ชี้ให้เห็นว่าควรมีการศึกษาถึงชนิด ความเข้มข้น และตัวทำละลายที่เหมาะสมของไคโทซาน ก่อนนำไคโทซานไปใช้ทางการเกษตร สำหรับวัสดุที่มีไคทินเป็นองค์ประกอบหลัก เปลือกกุ้ง (SS) หรือ กากไคทิน (FCW) ที่ 3 ระดับความเข้มข้น คือ 0.25, 0.5 และ 1% (w/w) ถูกนำมาใช้เป็นสารปรับปรุงดิน ก่อนการย้ายกล้าข้าวเป็นเวลา 7 วัน และศึกษาการเจริญเติบโต การสังเคราะห์แสงและผลผลิตของข้าว โดยการใช้ปุ๋ยเคมีหรือปุ๋ยอินทรีย์เป็นชุดการทดลองควบคุม พบว่าการใช้เปลือกกุ้ง (SS) และกากไคทิน (FCW) ทำให้ข้าวมีความสามารถในการสังเคราะห์แสงเพิ่มขึ้น ซึ่งส่งผลให้มีจำนวนหน่อ ชีวมวลส่วนต้นและผลผลิตข้าวสูงขึ้นอย่างมีนัยสำคัญทางสถิติ โดยเฉพาะอย่างยิ่งการเติม 1% (w/w) เปลือกกุ้ง (SS) หรือกากไคทิน (FCW) ทำให้น้ำหนักเมล็ดต่อกระถางเพิ่มขึ้น 4.9 เท่า และ 4.3 เท่า ตามลำดับ เมื่อเทียบกับชุดควบคุมที่ใช้ปุ๋ยเคมี ในการศึกษาผลของเปลือกกุ้ง (SS) และกากไคทิน (FCW) ในระดับยีนที่เกี่ยวข้องกับกระบวนการสังเคราะห์แสง ได้เลือกศึกษาการแสดงออกของยีนที่เกี่ยวข้องกับกระบวนการสังเคราะห์ด้วยแสง คือ *Oxygen-evolving enhancer protein 1 (OEE1)*, *Chlorophyll a-b binding protein (PsbS1)* และ *Ribulose bisphosphate carboxylase small chain* (*rbcS)* หลังจากได้รับเปลือกกุ้ง (SS) และกากไคทิน (FCW) พบว่าระดับการแสดงออกของยีน *OEE1* และ *rbcS* ในกล้าข้าวหลังจากเติมกากไคทิน (FCW) มีระดับเดียวกับการแสดงออกของยีนในชุดการทดลองควบคุม ขณะที่ระดับการแสดงออกของยีน *PsbS1* เพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติหลังจากทำการทดลองเป็นเวลา 14 วัน ในขณะที่ชุดการทดลองที่มีการเติมเปลือกกุ้ง กล้าข้าวมีการแสดงออกของยีน *rbcS* ลดลงอย่างมีนัยสำคัญทางสถิติ แต่ระดับการแสดงออกของยีน *OEE1* and *PsbS1* ไม่แตกต่างจากชุดควบคุม จากการทดลองนี้ชี้ให้เห็นว่า การกระตุ้นการเจริญเติบโตของกล้าข้าวด้วยเปลือกกุ้ง (SS) และกากไคทิน (FCW) เป็นผลมาจากกลไกที่แตกต่างกัน จากการทดลองทั้งหมดสรุปได้ว่า ไคโตซานชนิดพอลิเมอร์ที่ 90% DD ที่ระดับความเข้มข้น 40 mg/L (P90-40) ละลายในกรดแลกติก มีศักยภาพในการเพิ่มการเจริญเติบโตของกล้าข้าวพันธุ์ปทุมธานี 1 สำหรับเปลือกกุ้ง (SS) และกากไคทิน (FCW) สามารถนำมาใช้เป็นสารกระตุ้นการเจริญเติบโตของกล้าข้าว เพื่อที่จะพัฒนาการใช้กากไคทินสำหรับการผลิตข้าวแบบยั่งยืน

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NUNGRUTHAI KANANONT: EFFECTS OF CHITOSAN AND O-80 CHITOSAN RESIDUE ON GROWTH, PHOTOSYNTHESIS, YIELD AND GENE EXPRESSION OF RICE *Oryza sativa* L.. ADVISOR: ASSOC. PROF. SUPACHITRA CHADCHAWAN, Ph.D., CO-ADVISOR: ASST. PROF. BOONTHIDA KOSITSUP, Ph.D., 145 pp.

This research was aimed to increase rice production, using 'Pathumthani1' rice as a model, by the application of chitin/chitosan related products. For chitosan application, polymeric (P) and oligomeric (O) chitosan with 80% and 90% deacetylation in 1% (v/v) acetic acid at 5 – 80 mg/L were tested for the enhancement of seedling growth. It was found that all chitosan treatments inhibited seedling growth due to the negative effects of the chitosan solvent, acetic acid. Lactic acid was shown to have less toxicity to rice seedlings in comparison with acetic acid and citric acid. When lactic acid was used as chitosan solvent, 40 mg/L polymeric chitosan with 90% deacetylation (P90-40) was found to be the most appropriate plant growth stimulator. Based on these data, it should be noted that the appropriate chitosan types, concentration and solvent should be under consideration prior to agricultural application. For chitin rich material, three levels of shrimp shell (SS) or fermented chitin waste (FCW), 0.25, 0.5 and 1 % (w/w) were applied as soil supplement 7 days before seedling transplanting and rice growth, photosynthesis parameters and yield were investigated. Addition of either chemical or organic fertilizer was used as controls. It was found that SS and FCW application resulted in the increase of photosynthesis ability leading to the significantly higher tiller numbers, shoot biomass and grain yields. Particularly, addition of 1% SS or FCW led to the increase of grain weight/pot by 4.9 and 4.3 fold compared with the chemical fertilizer control. To investigate the SS and FCW effects at the molecular level, the genes involving in photosynthesis process, *Oxygen-evolving enhancer protein 1 (OEE1)*, *Chlorophyll a-b binding protein (PsbS1)* and *Ribulose bisphosphate carboxylase small chain* (*rbcS)* were studied for gene expression after SS or FCW treatment. The gene expression levels of *OEE1*, and *rbcS* in the seedlings after FCW addition were found to be similar to the controls, while the gene expression levels of *PsbS1* significantly increased on day 14 after treatment. Meanwhile in SS treatment, treated seedlings significantly had the lower level of *rbcS* gene expression but showed the similar level of *OEE1 and PsbS1* gene expression, compared to the controls. These suggested that the rice seedling growth stimulation by SS and FCW resulted by different mechanisms. In conclusion, 40 mg/L of P90 chitosan in lactic acid had a potential to enhance 'Pathumthani1' rice seedling growth, while SS and FCW can be considered as rice seedling growth stimulants in order to develop chitin-rich residues for sustainable rice production.

Department:	Botany	Student's Signature	.....
Field of Study:	Botany	Advisor's Signature	.....
Academic Year:	2014	Co-Advisor's Signature	.....

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LIST OF ABBREVIATIONS

μl	microliter
μmol m^{-2} s^{-1}	Photosynthetic Photon Flux (PPF)
℃	The *degree* Celsius
aa	acetic acid
A_{max}	maximum *photosynthesis rate*
ca	citric acid
CC	conventional chitosan
CF	chemical fertilizer
C_i	intercellular CO_2 concentration
cm	centimeter
cm^2	square centimeter
cm^3	cubic centimeter
cv.	*cultivar*
DD	Degree of deacetylation
E	transpiration rate
EC	Electric Conductivity
EF1-α	*Elongation factor 1 alpha*
FCW	fermented chitin waste
FW	fresh weight
g	gram
g/L	weight per volume (gram/liter)
g_s	stomata conductance
h	hour
kDa	kiloDalton
kGy	kiloGray
la	Lactic acid
mg/g Fw	milligram per gram fresh weight
mg/L	weight per volume (milligram/liter)
mg/ml	weight per volume (milligram/milliliter)
MW	molecular weight
Ø	diameter
O80	Oligomeric chitosan with 90% DD
O90	Oligomeric chitosan with 90% DD

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OC	Organic carbon
OEE1	*Oxygen-evolving complex protein 1*
OF	Organic fertilizer
OM	Organic matter
P80	Polymeric chitosan with 80-90% DD
P90	Polymeric chitosan with more than 90% DD
PH	Plant height
PLB	protocorm-like body
P_n	net photosynthesis rate
PsbS1	*Chlorophyll a-b binding protein*
RDW	Root dry weight
RFW	Root fresh weight
RL	Root length
rsbS	*Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit*
SCD	submicron chitosan dispersions
SDW	Shoot dry weight
SFW	Shoot fresh weight
SS	Shrimp shell
v/v	volume per volume
w/v	weight per volume

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CHAPTER I

INTRODUCTION

Rationale

Rice (*Oryza sativa* L.) is one of the most important cereal crops in the world, especially in Asia. Although Thailand has a strong tradition background of rice production, the yield per unit area is less than those in other major exporting countries, such as China, India and Vietnam. Thus, it is possible that Thailand will lose the market share and the competiveness in the rice market in the future. Therefore, the improvement of rice yield has been investigated continuously.

Chitosan consists of glucosamine residue biopolymer. Its characteristics are non-toxic, biodegradable and environmental friendly. It has been adopted in several applications such as food, cosmetic, biomedical and also agricultural applications. In agriculture, chitosan not only has eliciting activities leading to the induction of defense responses in plants, but also stimulates plant growth and yield. From previous study, researchers found that leaf area and dry matter weights of soybean, mini-tomato, upland rice and lettuce were increased when mixing chitosan into soil before planting. The results also demonstrated that the color of chitosan-treated leaves of those plants was darker green than control (Chibu and Shibayama, 1999). Moreover, the foliar application of a chitosan pentamer affected the net photosynthetic rate of soybean and maize one day after application (Khan *et al.*, 2002). Chitosan significantly increased the chloroplast diameter of *Dendrobium* after regularly application for a year and the gene expression of *ycf2*, a chloroplast gene was decreased after treated with chitosan for 12 hours (Limpanavech *et al.*, 2008). In addition, it also enhanced chlorophyll and carotenoid contents and mineral nutrient uptake, especially the increase in Mg leading to increase of chlorophyll content in coffee leaves (Dzung *et al.*, 2011). These results indicated that chloroplast was one of the target sites for chitosan action and led to plant growth stimulation.

The effects of chitosan application on photosynthesis characteristics and gene expression in rice leading to the enhancement of plant growth and productivity are still unclear. Therefore, the aim of this research is to investigate effects of chitosan types and concentrations on growth, photosynthesis, yield and some gene expression of rice (*Oryza sativa* L.). In addition, this research will also develop the use of waste obtained from chitosan fermentation to be used as stimulants for organic/semi-organic farming.

Objectives

1. To determine the effects of different types and concentrations of chitosan molecules on rice growth.
2. To determine the effects of different solvents of chitosan on rice growth in order to find suitable chitosan solvent with no effects on 'Patumthani1' rice growth.
3. To determine the effects of dried shrimp shell or O-80 chitosan residue (fermented chitin waste; FCW) on rice growth enhancement for organic/semi-organic farming.
4. To compare gene expression induced by shrimp shell and O-80 chitosan residue (fermented chitin waste; FCW).

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Expecting benefits

The understanding of the chitosan effects on rice photosynthesis, growth, yield, and gene expression will lead to the appropriate application of these products to increase the rice production in Thailand.

Contents of the thesis:

1. Literature review
2. Study of the effects of different types and concentrations of chitosan molecules on rice growth
    2.1. To determine the appropriate types and concentrations of chitosan molecules on rice seedling
    2.2. To study the effects of different solvents of chitosan on rice seedling
        2.2.1. To study the effects of three different solvents of chitosan on rice seedling in order to find suitable chitosan solvent with no effects on 'Patumthani1' rice growth
        2.2.2. To study the effect of the difference types and concentrations of chitosan molecules in the suitable chitosan solvent on rice seedlings growth
3. Study of the effects of shrimp shell and O-80 chitosan residue (fermented chitin waste; FCW) on rice growth, photosynthesis and yield
4. Investigation of the gene expression induced by shrimp shell and O-80 chitosan residue (fermented chitin waste; FCW)
5. Results and discussion
6. Conclusions

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CHAPTER II
LITERATURE REVIEWS

2.1. Rice (*Oryza sativa* L.)

2.1.1. Rice situation

Rice is consumed as staple food for human in various region of the world, especially Asia. In Africa, rice consumption has been increased due to the increase of their population. It has been concerned as one of the important food crops (Mohanty, 2013). According to USDA reports on February 2015, worldwide rice consumption for 2014/15 increased 0.4 million tons, while global rice production was 1.72 million tons lower than last year (USDA, 2015). Rice production is firstly limited by land and water resources. In order to increase rice production, the improvement of rice yield per unit area is one of good ways to solve this problem (Kubo and Purevdorj, 2004).

Thailand is one of rice producers of the world and also listed in top five rice exporters, accounting for 80% of global net trade, together with Vietnam, India, Pakistan, and the United States (Food and Agriculture Organization, 2014). In 2013, Thailand exported rice about 6.61 million tons which was approximatively 134 billion baht (Office of Agricultural Economics, 2015). However, the main problem of rice trade in Thailand is the cost of rice production. It is higher than the important competitive country, especially Vietnam, whereas rice yield per area is lower (Pisanwanich, 2011). This problem leads to the higher price of Thai rice in the market which consequently cause Thailand to lose the market share in the future. In order to strengthen competitiveness in rice market, the improvement of rice productivity need to be continuously developed

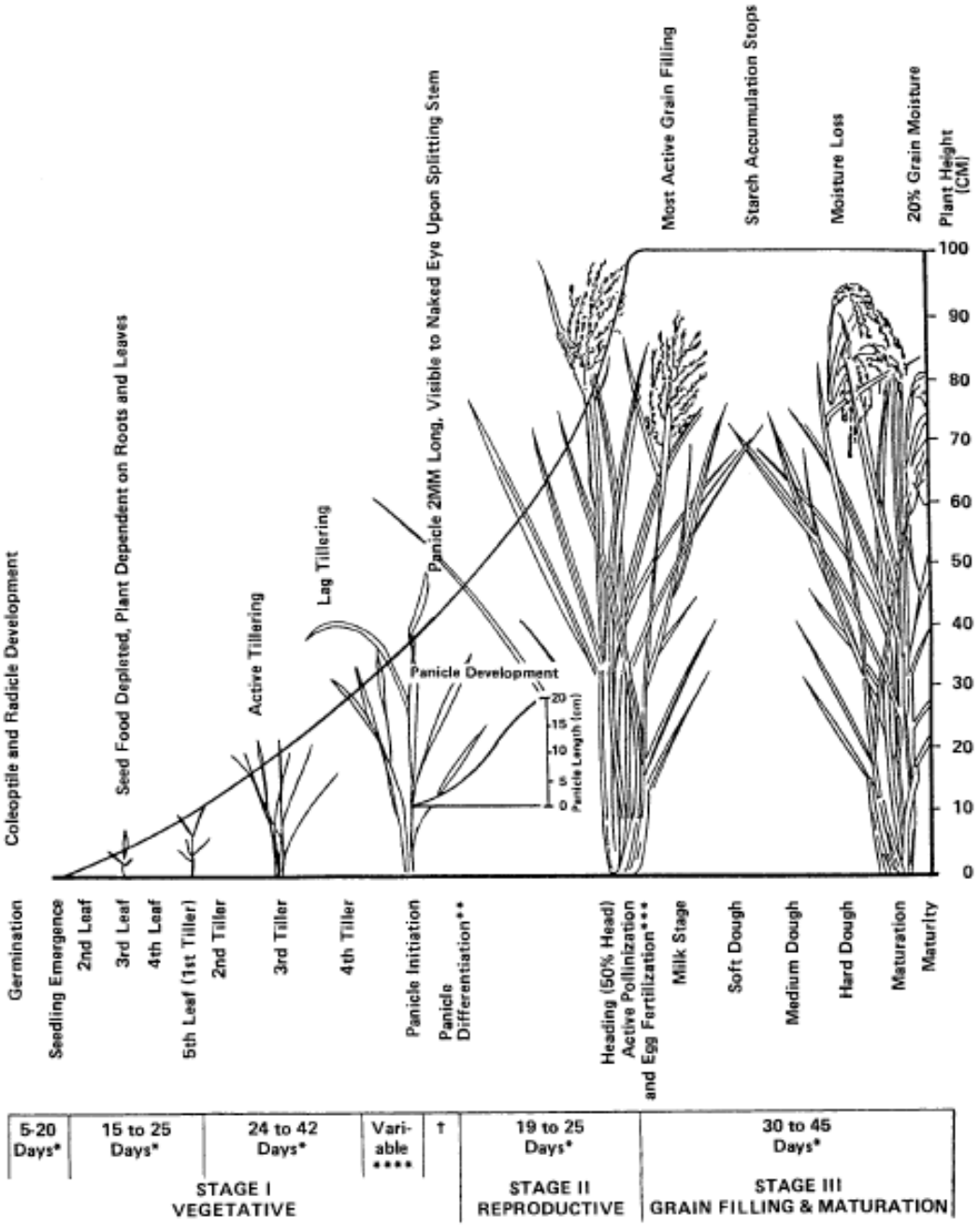
2.1.2. Growth and development

Rice is a monocot plant and generally grown as an annual plant. The growth duration depends on rice variety and the environment (Moldenhauer *et al.*, 2013). Growth of rice can be divided into three main phases (Figure 1):

I. Vegetative phase (seed germination to panicle initiation)
II. Reproductive phase (panicle initiation to flowering)
III. Ripening phase (flowering to maturity)

Vegetative phase

The vegetative phase begins with seed germination. When seed imbibes moisture and oxygen at an optimum temperature, the radicle root primarily emerges and becomes anchored in the soil. Then, the coleoptile which encloses the primary leaf appears from the germinating embryo (Figure 2A). Pre-tillering stage is the development of seminal roots and the first four leaves by using nutrition from the disintegration of endosperm (Figure 2B). Tillering stage begins with the appearance of the first tiller which originates from the axillary bud in one of the lowermost nodes until reaching the maximum tiller number. The growth of rice in this stage not only expands the number of tiller but also increases plant height. After maximum tillering, no more efficient tillers are produced and some tillers die due to competitive effects leading to the decline of tiller number. Active tillers continuously develop in term of stem elongation which is used as the signal of the end of vegetative growth. However, the internode elongation or panicle initiation (PI) may overlap with the beginning of reproductive growth (Moldenhauer *et al.*, 2013).



†3 to 5 days.
*Under warm conditions use the lower number of days and for cool conditions use the larger number of days.
**The reproductive stage begins with panicle initiation.
***Stage III begins when 50% of the florets are pollinated.
****Variable time – 0 to 25 days (dependent upon variety).

Figure 1. Growth stage of rice (Moldenhauer *et al.*, 2013)

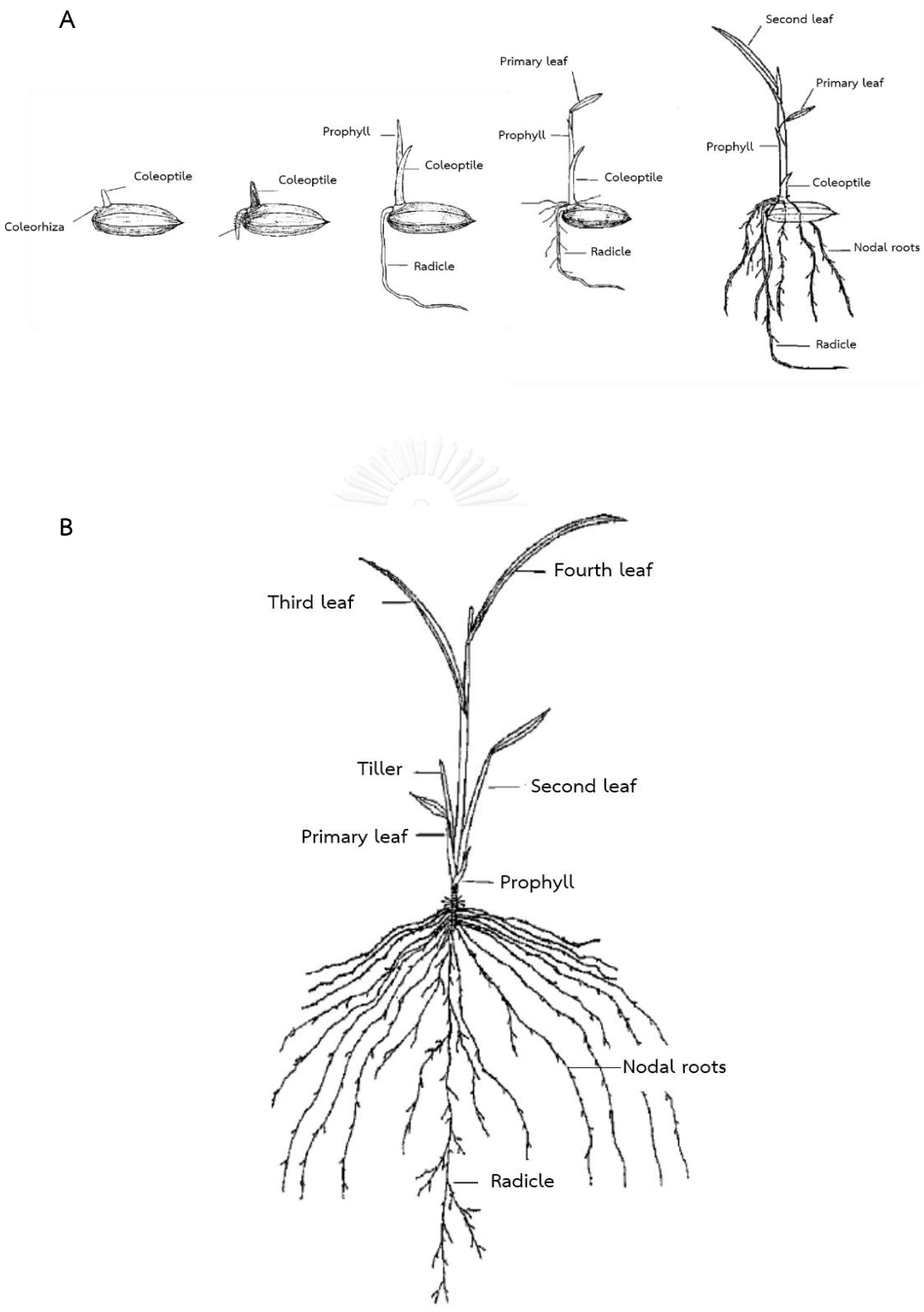


Figure 2. The development of seedling; germinated seed (A) and seedling (B) (modified from Moldenhauer and Gibbons, 2002)

Reproductive phase

Panicle initiation (PI) refers to the initiation of the panicle primordium at the shoot apex which can be visible only with microscopic techniques. When the panicle is differentiated, growing panicle can be seen with the naked eye about 10 days after initiation. Then, the panicle completely grows and develops within the flag leaf sheath. The swollen of flag leaf sheath is called the booting stage. Subsequently, the panicle will emerges from the flag leaf sheath which is called the heading stage. At last, the final stage of this phase is the flowering stage which begins with the emergence of anthers from the spikelet. As the panicle emerges, spikelets in the uppermost part of the panicle firstly bloom and then the flowering of spikelets move down to the panicle base (Figure 3). Moreover, the fertilization of rice is self-pollination, because it is usually pollinated inside lemna and palea before pollens are released to the air (Moldenhauer and Gibbons, 2002).

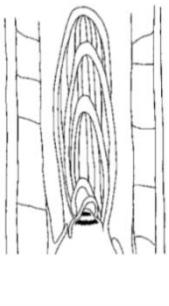
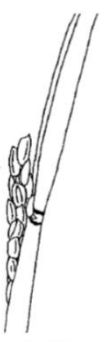
Growth Stage	R0	R1	R2	R3	R4
Morphological Marker	Panicle development has initiated	Panicle branches has formed	Collar formation on flag leaf	Panicle exsertion from boot, tip of panicle is above collar of flag leaf	One or more florets on the main stem panicle has reached anthesis
Illustration					

Figure 3. Reproductive phase with morphological marker (modified from Moldenhauer and Gibbons, 2002)

Ripening phase

After completion of fertilization, the grain is mainly formed from accumulation of carbohydrate which is produced by photosynthesis in the upper leaves. The initiating grain formation starts with the milk grain stage. During this stage, grain is filled by the developing starch which is white, milky liquid and soft similar to milk. Then, the milky liquid starch in the endosperm begins to solidify by loss of moisture. Finally, whole kernel becomes hard and moisture content is less than 20% (Figure 4). In addition, senescence of leaves is observed in a descending order and the non-functioning tillers which become straw (Moldenhauer and Gibbons, 2002).

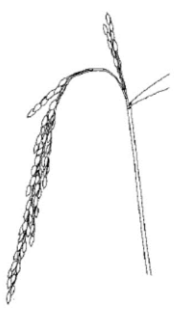
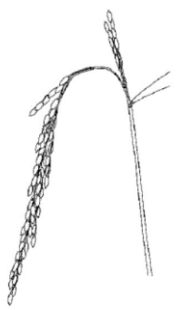
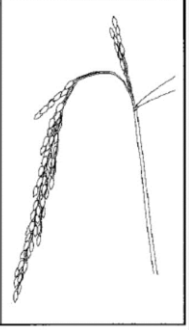
Growth Stage	R5	R6	R7	R8	R9
Morphological Marker	At least one caryopsis on the main stem panicle is elongating to the end of the hull	At least one caryopsis on the main stem panicle has elongated to the end of the hull	At least one grain on the main stem panicle has a yellow hull†	At least one grain on the main stem panicle has a brown hull‡	All grains which reached R6 have brown hulls
Illustration					

Figure 4. Ripening phase with morphological marker (modified from Moldenhauer and Gibbons, 2002)

2.1.3. Characteristics of ‘Pathumthani1’ rice

Thai Pathumthani Fragrant Rice (‘Pathumthani1’ rice) is the new fragrant cultivar and grain quality is similar to Thai Jasmine Rice (Thai Hom Mali Rice: KDML 105). It is derived from cross fertilization between BKNA6-18-3-2 and PTT85061-86-3-2-1. Furthermore, it is a photoperiod insensitivity and popularly grown in the central region of Thailand due to abundance of the irrigation system in this region.

In general, ‘Pathumthani1’ rice is approximately 104-133 cm. in plant height and harvesting time is about 104 - 126 days. Moreover, it has high yield productivity about 650 - 774 kg/rai. For grain quality, its aroma and tender texture are not much different from Thai Jasmine Rice.

In term of resistance, this cultivar is resistant to both brown and white-backed planthoppers and also resistant to rice blast disease and bacterial leaf blight disease. However, it is susceptible to green rice leafhopper, ragged stunt and yellow orange leaf disease. This cultivar was developed by Pathumthani Rice Research Center and submitted PTT90071-93-8-1-1 line to the Research and Development Committee, Department of Agriculture. Finally, it was named as ‘Pathumthani1’ and recommended to grow in irrigation service areas (Rice Department, 2014).

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2.2. Chitin-Chitosan

2.2.1. Definition, structure and properties

Chitin is the second most important natural polymer in the world after cellulose. It can be found in the component of arthropod exoskeletons, particularly crustaceans, as well as in fungal cell wall. It consists of N-acetyl-D-glucosamine (acetylated unit) and D-glucosamine (deacetylated unit) linked together with β-(1-4) glycosidic bond. Chitin has the predominant acetylated units in the polymeric chain, whereas chitosan is the predominant deacetylated units in the polymeric chain (Figure 5) (Nwe *et al.*, 2011).

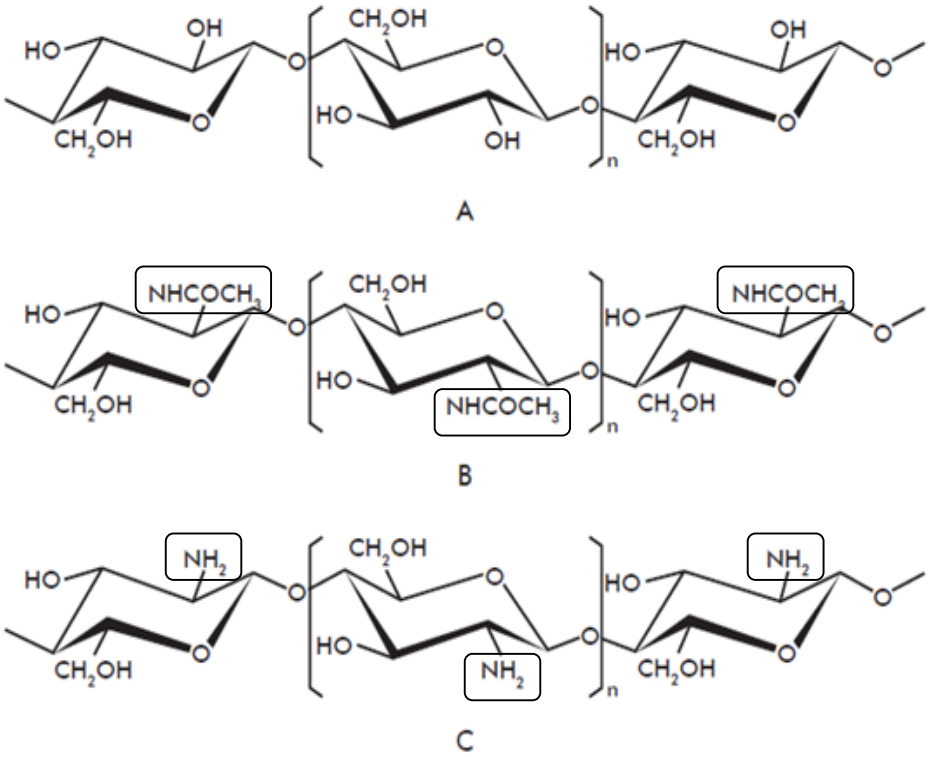

Figure 5. The structural of cellulose (A), fully acetylated chitin (B), and fully deacetylated chitosan (C) (modified from Sharp, 2013)

Chitin and chitosan have similar properties such as biodegradable, non-toxic and antimicrobial activity. Interestingly, the positive charges on these deacetylated units in biopolymer result in some unique properties in chitosan (Rinaudo, 2006). Furthermore, the solubility is one of the most important characteristic in order to distinguish chitin and chitosan molecules (Hayes, 2012).

In general, chitin is hard, inelastic, nitrogenous polysaccharide and insoluble in water as well as many organic solvents; however, when the degree of deacetylation (DD) over 50%, it becomes chitosan which can dissolve in weak organic acids such as acetic, citric and lactic acids (Rinaudo, 2006).

2.2.2. Shrimp shell

Thailand is one of the world leading exporters of frozen shrimp (Asche, 2014). Consequently, there are a lot of shrimp shell wastes obtained from seafood processing industry. The excessive shrimp shell wastes might lead to environmental pollution. However, this waste consisting of chitin, protein, mineral salts (calcium carbonate), lipid compounds and carotenoids have been being investigated in sustainable development and recycling for value-added material such as chitin and chitosan (Table 1.) (Francisco *et al.*, 2015; Hossain and Iqbal, 2014).

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Table 1. Valuable components of shrimp heads (*Penaeus vannamei*) (Trung and Phuong, 2012)

Components	Contents*
Chitin (%)	9.3 ± 0.8
Protein (%)	54.4 ± 1.8
Minerals (%)	21.2 ± 1.6
Lipid (%)	11.9 ± 1.4
Carotenoids (mg/kg)	206 ± 14

*based on dry basis

The quantity of constituents varies from species and individuals due to growth stage, feeding and environmental conditions (Diaz-Rojas *et al.*, 2006)

2.2.3. Fermented chitin waste

The fermented chitin waste (FCW) is a by-product from chitinase production via *Bacillus licheniformis* SK-1 fermentation using shrimp shell as a chitin source (Kudan and Pichyangkura, 2009). Chitinase is a hydrolytic enzyme which breaks down glycosidic bonds in polymeric chitin/chitosan into low-molecular-weight products or oligomeric chitin/chitosan. In addition, this enzyme is used for the O-80 chitosan production from OliZac Technologies Company Ltd. Bangkok, Thailand.

FCW also has chitosan functions, high nitrogen (N) content and microbes. Muymas *et al.* (2014) reported that the appropriate concentration of FCW when used as soil supplement could promote growth and yield of lettuce. Therefore, FCW has potential to be plant growth stimulator.

2.2.4. Chitosan

In commercial processing, chitosan can be produced from shrimp shell waste by chemical treatments. The process of chitosan production is divided into three main steps. Firstly, shrimp shell waste is converted into chitin by deproteinization with weak base (1-2 N NaOH) and then demineralization with weak acid (1-2 N HCl). Finally, chitin is deacetylated by strong base (40-50% NaOH) in order to transform to chitosan (Figure 6) (Dutta *et al.*, 2004). Moreover, the distribution of the degree of deacetylation (DD) and molecular weight of chitosan is varied, according to processing conditions. These properties also affect solubility and viscosity of dilute acid solvent as well as their biological activity (Synowiecki and Al-Khateeb, 2003).

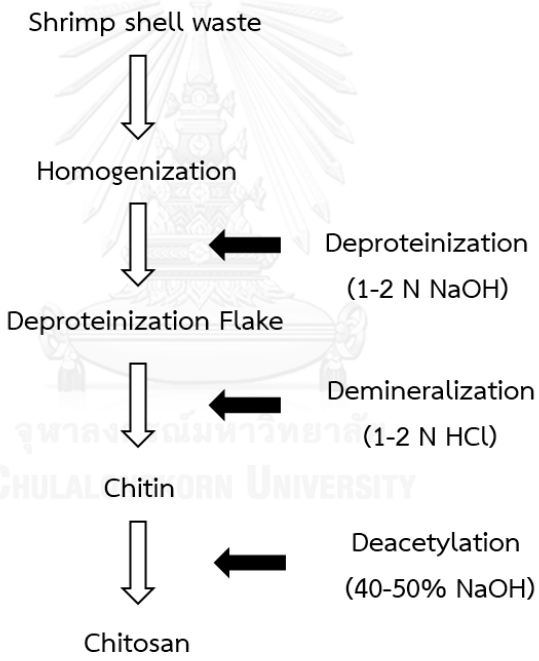

Figure 6. Production of chitin and chitosan by the chemical method (modified from Kandra *et al.*, 2012)

2.2.5. Utilizations of shrimp shell waste, chitin and chitosan

Utilization of shrimp shell waste is not only for animal feed or soil amendment but also for chitin/chitosan production (Coward-Kelly *et al.*, 2006; Nemec and Lee, 1992; No and Meyers, 1995). Chitin and chitosan are versatile materials with evident properties, for example, cationic polymer, biocompatible with animal/human cells and tissues, biodegradable, less toxicity, adsorption capacity, gel forming ability and antimicrobial activity. These materials are used in several applications such as medical and material sciences, biotechnology, food and also agriculture (Table 2) (Prashanth and Tharanathan, 2007).

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Table 2. Application potential of chitin/chitosan (modified from Prashanth and Tharanathan, 2007)

Sector	Application
Medical science (Drugs and Pharmaceuticals)	Hemostasis Controlled drug release Oral hygeine, Periodontal use Antitumour, Antiulser, Anticoagulant Wound healing, Wound dressing Suture threads, Contact lens
Material sciences	Hydrocolloid Electrochemistry (biosensors) Cosmetics (moisturizer, skin care, products) Packaging films/Composite coating formulations Textile finishing (dye binding) Polymeric membranes
Biotechnology	Flocculating agent Reverse osmosis membranes Polymeric nanoparticles Synthetic polymer blends Degradation products (low MW chitosan, chitooligomers, monomers) – value addition
Food and Nutrition	Food preservation, Water purification Biotechnology (immobilization matrix) Dietary supplements, Functional foods Hypocholesterolemic Antioxidant, Prebiotics
Agriculture	Animal feed Soil enrichment Increase of crop production

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2.2.6. Agricultural applications

Chitin and chitosan have been experimentally investigated on many plants with a broad range of beneficial purposes. These purposes can be widely divided into three major areas, including plant protection, postharvest technology and also plant growth stimulation (Dong *et al.*, 2004; El Hadrami *et al.*, 2010; Pornpienpakdee *et al.*, 2010). Chitin and chitosan offer natural alternatives to the use of chemical products due to non-toxic and eco-friendly properties. However, plant responses to chitin and chitosan varies according to their type and degree of deacetylation, concentration, plant species, environmental conditions as well as application procedures (i.e., foliar application, soil amendment and seed soaking) (Sharp, 2013). For both plant protection and postharvest purposes, the important properties are antiviral, antibacterial and antifungal activities.

Applications of chitin and chitosan in plant protection

Chitin, chitosan and their derivatives can affect plant pathogen directly on growth and development. They have been well known as plant elicitors which induce plant defense responses against viruses, bacteria, fungi and other pests (El Hadrami *et al.*, 2010). As *in vitro* study, Xu *et al.* (2007) reported that oligochitosan could inhibit mycelial growth and developmental stages in life cycle of *Phytophthora capsici* which leads to blight and fruit rot disease on pepper and economic crops. These results suggested that polycationic properties of oligochitosan directly obstructed endomembrane system of *P. capsici*. For plant response, chitosan oligomer which acted like plant pathogenic fungi could activate plant defensive genes through the octadecanoid pathway in tomato leaf (Doares *et al.*, 1995).

Moreover, the addition of modified chitosan on nutrient agar (NA) medium could noticeably inhibit the growth of the bacteria; *Agrobacterium tumefaciens* (crown gall disease) and *Erwinia carotovora* (soft mould disease) (Rabea and Steurbaut, 2010). Goy *et al.* (2009) proposed three hypotheses in antibacterial mechanisms of chitosan. First, positive charge of chitosan could changes the permeability of bacterial cell wall,

leading to leakage of intracellular electrolytes and internal osmotic imbalances. Moreover, the microbial mRNA and protein synthesis were directly inhibited by chitosan binding. Finally, chitosan could act as chelating agents in order to block essential nutrients for microbial growth.

When chitosan was applied as seed coating followed by soil amendment, chitosan-treated tomato seedling showed healthier appearance of the root system than untreated plant and the incidence of a disease which was caused by *Fusarium oxysporum* also reduced. On the other hand, seed treatment alone was less effective than the combination of chitosan application (Benhamou *et al.*, 1994) In addition, foliar application of chitosan stimulated both callose formation and abscisic acid accumulation in leaf tissues of bean (*Phaseolus vulgaris* L. cv. *Borlotto Nano Lingua di Fuoco*). It also induced a high level of plant resistance against tobacco necrosis virus (TNV) (Iriti and Faoro, 2008).

Applications of chitin and chitosan in postharvest technology

In climacteric fruit, the effects of chitosan on ripening, enzymatic activity, and disease development in 'Nam Dok Mai' mango (*Mangifera indica* L.) fruit were investigated. The chitosan coating at concentration of 0.5% to 2.0% inhibited growth of *Colletotrichum gloeosporioides* causing anthracnose disease on mangoes whereas chitosan at concentration of 1.0% could delay the physico-chemical changes including delayed ripening, reduced respiration rate as well as ethylene production, and also the decrease of weight loss, ascorbic acid, and total titratable acidity (Jitareerat *et al.*, 2007). In Cavendish banana, the effects of degree of deacetylation (DD) (70%, 80%) and concentrations (1, 1.5, 2 % w/w) of chitosan were determined on vitamin C and weight loss. Sensory tests including the changes in color, texture, and aroma were also examined after 7 days of storage at 30±2°C. The result showed that chitosan coating could delay ripening processes and the reduction of vitamin C and weight loss were correlated with the increase of DD and concentration of chitosan. The most suitable condition that showed acceptable appearance and quality of banana was chitosan

coating at concentration of 2% (w/w) with 80% degree of deacetylation (Suseno *et al.*, 2014).

Chitosan application as a pre-harvest treatment can maintain postharvest quality of agricultural products. Muymas *et al.* (2014) reported that the application of fermented chitinous material (FCM) as soil supplement could maintain postharvest quality of 'Red Oak' lettuce during storage by increasing chlorophyll and antioxidant contents. Furthermore, Reddy *et al.* (2000) investigated postharvest decay and quality of pre-harvest chitosan sprayed strawberry and found that chitosan spraying at the concentration of 2, 4 and 6 g/L significantly reduced gray mold rot caused by *Botrytis cinerea*. Moreover, the fruit sprayed twice with chitosan at the concentration of 6 g/L had the highest fruit firmness and the slowest ripening process which was indicated by the reduction of anthocyanin synthetic rate and titratable acidity when compared with other chitosan treatments and non-treated fruit. In summary, this study suggested that chitosan may act as a barrier to plant pathogen infection, moisture evaporation and O_2 uptake, which led to extended shelf life.

Applications of chitin and chitosan in plant growth

Enhancement of plant growth after chitin and/or chitosan treatments has been reported in several methods such as foliar spraying, seed soaking, soil supplement, hydroponic and plant tissue culture in both monocot and dicot plants (Asghari-Zakaria *et al.*, 2009; Suchada Boonlertnirun *et al.*, 2008; Chibu and Shibayama, 1999; Jirapornprasert *et al.*, 2011; Mondal *et al.*, 2012; Ohta *et al.*, 1999; Pornpienpakdee *et al.*, 2010)

Chitin/chitosan application by seed soaking

The influences of seed soaking in chitosan solution on prevention of pest and pathogen infection, improving seed germination, reducing time of germination, stimulating plant growth and increasing yield have been reported in dicot and monocot plants. In dicot plant, Zeng *et al.* (2012) investigated the effects of chitosan coating at different concentrations on plant protection, growth and yield in soybean. The study

revealed that chitosan at concentration of 5% (w/v) could boost plant defense against the attacks of black cutworm, soybean pod borer and soybean aphid and also enhanced seed germination, growth and yield components up to approximately 20% higher than that of control treatment. Moreover, the response to different molecular weights of chitosan on growth of soybean sprouts was evaluated. Soaking soybean seeds with high molecular weights (> 1,000 kDa) of chitosan significantly increased hypocotyl length, root length, total length, hypocotyl thickness and also fresh weight when compared to water-treated plant (Y. S. Lee *et al.*, 2005).

In sunflower sprouts, the effects of seed soaking times, molecular weights, solvent types and concentrations of chitosan solution on plant growth were examined. This research concluded that the best condition for stimulating sunflower sprouts growth was seeds soaking with 28 kDa chitosan dissolved in lactic acid at the concentration of 0.5% for 18 hours, because it significantly increased total weight and germination rate by 12.9% and 16.0%, respectively comparing to control (Cho *et al.*, 2008).

In Chinese cabbage and rapeseed, the suitable concentration and type of chitosan for seed germination and seedling growth improvement have been evaluated. Two types, 85% DD and 75% DD, and different concentrations of chitosan ranging from 0.02 – 1.60 mg/ml were used in this study. The result showed that 85% DD chitosan at 0.10 - 0.40 mg/ml and 75% DD chitosan at 0.10 mg/ml could increase rapeseed germination. On the contrary, the enhancement of seed germination in Chinese cabbage was not observed in all chitosan treatments. The presence of chitosan at concentration of 0.80 mg/ml initially produced abnormal appearance of Chinese cabbage seedling (Y. L. Wang *et al.*, 2012).

In monocot plant, the effects of chitosan (Elexa^{TM}) concentrations and seed soaking time on germination and vigor index of pearl millet were tested. Seeds soaked in 1:19 dilution of chitosan for 6 hours showed the maximum germination and seedling vigor (Sharathchandra *et al.*, 2004). In ‘Pathumthani1’ rice, the seed quality after storage using seed coating with polymeric and oligomeric chitosan solutions at the

concentrations of 50, 100, 150 ppm were investigated. The research indicated that seed coating with 50 ppm of polymeric chitosan was the most effective treatment for maintaining 'Pathumthani1' rice seed storability because it showed the highest rice seed quality, especially germination percentage and shoot length of seedling after storage (Suvannasara and Boonlertnirun, 2013).

Chitin/chitosan application as foliar treatment

Influences of chitosan application as a foliar spray have been reported not only on plant protection but also on plant growth and yield. The chitosan treatment affected morphological, physiological and yield characters as well as biochemical parameters such as photosynthetic system in Indian spinach, lady's finger and maize (Mondal *et al.*, 2012; Mondal *et al.*, 2013; Mondal *et al.*, 2011). However, the optimum chitosan concentration for each plant species is different.

In Indian spinach, its maximum growth and development could be approached at the 5 ppm chitosan (Mondal *et al.*, 2011), whereas the highest yield in both lady's finger and maize was recorded at 100 ppm - 125 ppm of chitosan solution (Mondal *et al.*, 2012; Mondal *et al.*, 2013). Moreover, the effects of different concentrations and types of chitosan, including conventional chitosan (CC) and submicron chitosan dispersions (SCD), were investigated on the vegetative growth of dragon fruit plant. The plants sprayed with 600 nm SCD at concentration of 1.0% showed the highest shoot number, stem diameter, length diameter and also chlorophyll content among other treatments (Zahid *et al.*, 2014). Furthermore, foliar application with 80% DD of oligomeric chitosan (O-80) at 1 ppm – 100 ppm could induce early flowering and increase the inflorescence number of *Dendrobium* orchid. Particularly, 50 ppm of O-80 chitosan could increase chloroplast size, and 10 ppm of O-80 chitosan was shown to affect chloroplast gene expression (Limpanavech *et al.*, 2008).

Furthermore, Khan *et al.* (2002) studied impacts of foliar application of pentameric chitosan (CH5), pentameric chitin (CHIT5) and high molecular mass chitin in maize and soybean. It was found that these treatments did not affect plant growth

parameters consisting of plant height, root length, leaf area, shoot dry mass, root dry mass and total dry mass. On the other hand, the different responses of photosynthetic parameters to these chitin and chitosan molecules were observed in treated leaves.

Moreover, the applications of chitin and chitosan not only use as a foliar spray but also combine with other methods. S Boonlertnirun *et al.* (2006) investigated the chitosan types and the appropriate method for 'Suphan Buri 1' rice yield improvement. The result showed that the combination of seed soaking and foliar sprayings with polymeric chitosan significantly increased numbers of tiller per plant and dry matter accumulation, whereas this combination was a tendency to achieve higher yield than individual application of either seed soaking or foliar spraying.

In Malabar spinach, plant growth was observed in plant treated with irradiated chitosan solution which was applied as foliar spraying together with pouring the remainder to neighboring soil. As a result, plant growth parameters including root weight, leaf and stem weight, total fresh weight and total dry weight were highest with the 30 kGy irradiated chitosan treatment (Rahman *et al.*, 2013).

Chitin/chitosan application as soil supplement

Due to insoluble property of chitin, soil application may be an appropriate way to study the effect of chitin on plant growth. Furthermore, the effect of chitin and chitosan as soil supplemented for plant growth has been hypothesized that it not only increased plant nutrition in soil but also promoted the growth of advantageous soil microbes (Sharp, 2013). Therefore, the stimulation of plant growth *via* soil supplemented may occur by various mechanisms in both plants and microbes.

Kavino *et al.* (2010) revealed that the soil mixed with chitin and *Pseudomonas fluorescens* strains CHA0 could increase growth, yield and leaf nutrient contents of banana compared to untreated treatment. In addition, Muymas *et al.* (2011) reported that the 20% (w/w) fermented chitin incorporated into soil showed the highest growth of lettuce cv. 'Red Oak' and also increased soil microbial populations which could degrade chitin to nitrogen source.

Moreover, growth of soybean, upland rice, mini-tomato and lettuce grown in soil mixed with chitosan powder at 0.1% - 0.5% (w/w) before planting was evaluated. Among these crops, dry weight and leaf area of soybean, upland rice and lettuce were increased with all concentrations, whereas chitosan at 0.1% (w/w) enhanced leaf area, leaf length and dry weight of mini-tomato. Besides, the darker green leaves in chitosan treatments were observed by SPAD value as compared to control (Chibu and Shibayama, 1999). The addition of chitosan powder at 1% (w/w) during sowing time clearly promoted vegetative and reproductive growth of *Eustoma grandiflorum* such as increased dry weight, reduced flowering times, and improved cut-flower quality as measured by leaf size, shoot length, and flower number (Ohta *et al.*, 1999). In addition, Suchada Boonlertnirun *et al.* (2008) investigated appropriate applications of chitosan for increasing the rice yield. It was found that application of chitosan by seed soaking and four times of soil application throughout cropping season significantly increased rice yield over the other treatments whereas application of seed soaking followed by four times of foliar spraying tended to show an ability on disease control.

Noteworthy, there are some reports about the effect of chitosan on plant growth under aseptic condition. In *Dendrobium* orchids, the suitable chitosan concentration in solid and liquid medium could enhance growth and development of orchids; however, too high concentration of chitosan had a negative effect on growth (Nge *et al.*, 2006; Pornpienpakdee *et al.*, 2010). Furthermore, Barka *et al.* (2004) reported that 1.75 % (v/v) of chitogel supplemented medium increased root and shoot biomass of grapevine by the stimulation of CO_2 fixation. As mentioned above, it indicated that the efficient chitosan molecules acted through unknown mechanisms for stimulating of plant growth in cellular level (Sharp, 2013).

2.2.7. Mechanisms of chitin and chitosan in plant growth

Currently, the exact mechanisms of the plant growth simulation by chitin, chitosan, and their derivatives are still incompletely understood. However, there are

several experimental evidences to describe these mechanisms in different stages of plant growth.

Improvement of germination

Because of its film-forming property which is a semi-permeable membrane, chitosan coating on seed surface could maintain the seed moisture and absorb the soil moisture which directly promoted seed germination (Zeng *et al.*, 2012). During germination process, chitinase activity was found in the exo-tissues of chitosan coated-seeds. This enzyme is a key factor against plant pathogenic microorganisms (Hirano *et al.*, 1990). Moreover, hormone and enzyme activities, involving in the degradation and mobilization of food reserves are observed. Zhou *et al.* (2002) reported that in peanut seeds, chitosan-coating raised germination percentage, germinating energy, lipase activity, gibberellic acid (GA_3) and indole acetic acid (IAA) contents. In addition, Hameed *et al.* (2013) reported that chitosan priming improved both of hydrolytic and antioxidant enzymes activities including α-amylase, α-esterase, β-esterase, catalase, peroxidase, superoxide dismutase, as well as protease in wheat seeds. As a result, the improvement of seed germination resulted from the increase of soluble sugar and free amino acid contents together with the enhancement of free radical-scavenging capacity.

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Improvement of photosynthesis

Photosynthesis is the fundamental metabolic process of plant growth. This process primarily occurs in chloroplast which contains chlorophyll *a*, *b* and carotenoid. These pigments play important roles in photosynthesis by harvesting light energy and then converting it into chemical energy in form of organic molecules (Taiz and Zeiger, 2002). Chitin/chitosan-induced responses of photosynthesis have been established in various targets such as chloroplast size (Limpanavech *et al.*, 2008), photosynthetic pigment content (Dzung *et al.*, 2011), photosynthetic rate (Mondal *et al.*, 2012) and also number and size of leaf (Mondal *et al.*, 2011).

When *Dendrobium* orchid leaves were sprayed with 90% DD chitosan at molecular weight of 45 kDa, Limpanavech *et al.* (2008) found that chitosan significantly increased the chloroplast diameter at concentrations of 10 - 50 ppm in young leaves and at 50 ppm in old leaves. After 24 h of chitosan treatment, it was found that the application of chitosan affected on *ycf2* gene expression in chloroplast. Mondal *et al.* (2011) also reported that the foliar application of chitosan influenced on photosynthetic characteristics of Indian spinach such as chlorophyll content, nitrate reductase activity (NRA) in leaf, leaf number per plant, leaf area and also specific leaf weight (SLW). In coffee, chitosan oligomers not only significantly increased the content of photosynthetic pigments, but also enhanced mineral nutrient uptake, especially magnesium (Mg) which is an important element in chemical structure of a chlorophyll molecule. It also reduced transpiration of the coffee leaves (Dzung *et al.*, 2011).

Moreover, soil treatment with chitin and/or chitosan has been shown the positive effect on chloroplast. The addition of fermented chitin with *P. fluorescens* strains CHA0 into soil significantly enhanced total chlorophyll and chlorophyll stability index of banana leaves leading to increase biomass and yield of banana (Kavino *et al.*, 2010). Similarly, beans which were watered with the 2.5% chitosan solution once at the beginning of the experiment showed the highest level of chlorophyll *a*, *b* and total chlorophyll when compared to control. However, the effective effect depended on concentrations of chitosan (Sheikha and Al-Malki, 2011).

In the photosynthetic process, coffee seedlings sprayed with chitosan nanoparticles significantly increased chlorophyll content, nutrient uptake and net photosynthesis rate (P_n). This result indicated that the increase of nutrient uptake, especially Mg in the coffee leaves led to the enhancement of chlorophyll contents and (Van *et al.*, 2013). Mondal *et al.* (2012) also reported that the foliar application of chitosan significantly increased net photosynthesis rate (P_n) and nitrate reductase activity (NR) which were involved in nitrogen assimilation of treated-okra plants but did not significantly enhance chlorophyll content when compared to control. However, non-treated plants showed the lowest NR, P_n and chlorophyll content.

Furthermore, the addition of 1.75% (v/v) chitogel to culture medium increased the growth of grapevine plantlets. This increase was confirmed by photosynthetic measurements. It was found that the average of O_2 production and CO_2 fixation of chitogel treated-plantlets increased 2-fold and 1.5-fold compared to control plantlets, respectively. Moreover, this treatment also increased the ratio of photochemical (qP) and non-photochemical quenching (qN). Therefore, it indicated that most of light energy which was absorbed by chotogel-treated plants was utilized in photochemistry (Barka *et al.*, 2004).

Even though chitosan pentamer (CH5) and chitin pentamer (CHIT5) decreased net photosynthetic rate (P_n) of soybean and maize after the first day of foliar application, the increase of P_n was found on the second day in CH5 treatment of soybean. Meanwhile, both of CH5 and CHIT5 treatments in maize could increase P_n on day 3 and day 5, respectively. This increase was associated with the increase of stomatal conductance (g_s) and transpiration rate (E), while the intercellular CO_2 concentration (C_i) was not different from the control plants. On the other hands, the decrease of P_n at the earliest day resulted from the decrease of stomatal aperture because CH5 and CHIT5 might produce H_2O_2 which is an elicitor in stomatal aperture. However, the growth of maize and soybean did not significantly differ among control and foliar application of chitosan and chitin oligomer (Khan *et al.*, 2002). Iriti *et al.* (2009) also reported that the foliar application of chitosan on common bean decreased

transpiration rate (E) and induced stomatal closure which was activated by chitosan elicitation via a H_2O_2-mediated process. Moreover, chitosan could stimulate the xanthophyll cycle towards de-epoxidation state in order to protect the photosynthetic apparatus against photodamage.

However, chitosan can be used as an anti-transpirant for plants. The foliar spray with chitosan was shown to reduce transpiration rate, while the biomass and yield were maintained in pepper (Bittelli *et al.*, 2001) and common bean (Iriti *et al.*, 2009).

Improvement of plant development

Improvement in plant development after the application of chitin/chitosan treatment has been reported at differential stages of plant growth.

At vegetative stage

Nge *et al.* (2006) reported that addition of chitosan into plant tissue culture media induced fresh-green differentiated tissue on protocorms of *Dendrobium phalaenopsis* within 2-3 weeks after treatment, while the development of the green tissues was observed in control treatment after 6 weeks of cultivation. In *Dendrobium bigibbum* var. *compactum*, germinating seeds in modified VW medium containing chitosan at 10 mg/L of P70, P80, P90 or O70, 20 mg/L of O70 or O80, or 40 mg/L of O90 reached the fourth stage of protocorm development within 8 weeks, while 6 weeks in the medium without chitosan supplement, 77% of protocorms were developed to the third stage and some showed irregular globular green structure. Up to 8 weeks in the control medium, no fourth stage protocorms were found. Protocorms at this stage showed the first signs of a developed leaf primordium. However, the addition of 20 mg/l of O-80 chitosan could increase this proportion up to 91% (Kananont *et al.*, 2010). Pornpienpakdee *et al.* (2010) studied effect of chitosan on protocorm-like body (PLB) of *Dendrobium* 'Eiskul' which was cultured in the shoot differentiation medium and found that the number of new shoots in the medium supplemented with 20 mg/L of O80 chitosan was 3-fold higher than that in the medium

without chitosan during 4 months of the experiment. Therefore, the suitable concentration and type of chitosan has the potential to stimulate the differentiation of orchid tissue.

At reproductive stage

The effects of chitosan application at reproductive stage have been reported in many methods and plant species. Sharathchandra *et al.* (2004) reported that pearl millet seed soaked with a 20 fold dilution of commercially chitosan formulation, Elexa, for 6-hours had increased number, length and girth of earheads as well as seed weight at the seed maturation stage when compared to non-treated plants. In addition, *Eustoma grandiflorum* (Raf.) Shinn. grown in 1% (w/w) chitosan-supplemented soil flowered 15 days earlier than untreated plants, and cut-flower qualities, in term of numbers and weight were greater than control plants (Ohta *et al.*, 1999). Limpanavech *et al.* (2008) found that the foliar application of chitosan O-80 at all concentrations tested, 1, 10, 50, and 100 ppm could induce early flowering and increase the accumulative inflorescence number of *Dendrobium* 'Eiskul' during the 68 weeks of the experimental period, when compared to the non-chitosan-treated plants. Moreover, study of Dzung *et al.* (2011) showed that the foliar application of chitosan oligomer at concentration of 60 ppm under field condition could reduce the rate of fallen fruit of coffee when compared to untreated plants. In 'Suphunburi 1' rice, S Boonlertnirun *et al.* (2005) determined the optimum concentration and frequency of foliar application of polymeric chitosan on rice yield. The results showed that four times of foliar spraying with 20 ppm of chitosan increased dry matter accumulation, panicle per plant, and yield over control plants. Unfortunately, there is obviously no information on the role of chitosan on reproductive growth based on biochemical and molecular responses in plants.

Effect of chitosan on expression of genes involving in plant growth

Although the enhancement of plant growth and yield by chitin/chitosan application has been reported in many plant species, there is only few information about the mechanism of plant growth induction at molecular level.

Chamnanmanoontham *et al.* (2015) studied the growth promoting responses induced by chitosan at molecular level in rice (*Oryza sativa* L. cv. *Leung Pra Tew 123*) seedlings. Rice seedlings treated with or without chitosan O80 at 40 mg/L by seed soaking and foliar spraying were used for proteomic analysis together with co-expression network analysis. It was found that eight loci in rice genome are involved in one of three main processes in plants; photosynthesis, carbohydrate metabolism and cell redox homeostasis. In addition, this proteomic data about photosynthesis was supported by the significant increase of photosynthetic pigment content in chitosan treated plants compared to control plant. This is similar to the research of Limpanavech *et al.* (2008) which found that chitosan not only significantly increased the chloroplast diameter but also directly affected the expression of chloroplastic gene, *ycf2,* which encoded essential products for cell survival after 24 h of foliar spraying with chitosan O-80 on *Dendrobium* 'Eiskul' leaves. These results indicated that chloroplast was one of the target sites for chitosan action leading to plant growth stimulation.

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CHAPTER III
MATERIALS

3.1. Materials

3.1.1. Plant material

'Pathumthani 1' rice seeds used in all experiments were obtained from the Pathumthani Rice Research Center, Bureau of Rice Research and Development, Pathum Thani, Thailand.

3.1.2. Chitosan molecule

The polymeric (P) and oligomeric (O) forms of chitosan with an 80 or 90% degree of deacetylation (DD) (P80, O80, P90 and O90) were obtained from Assistant Prof. Dr. Rath Pichyangkura, Department of Biochemistry, Faculty of Science, Chulalongkorn University.

3.1.3. Dried shrimp shell

Dried shrimp shell waste was purchased from local market at Chon Buri province, Thailand.

3.1.4. O-80 chitosan residues or the fermented chitin waste (FCW)

O-80 chitosan residues or the fermented chitin waste (FCW) was obtained from OliZac Technologies Company Ltd. Bangkok, Thailand.

3.2. Instruments

3.2.1. Instruments for studying the effects of chitosan and their solvents on rice seedling growth

Equipment for planting

1. Barrel
2. Beaker
3. Balance: PG503-S (Mettler Toledo, USA)
4. Balance: U 4600 P+ (Scientific Promotion Co., Ltd., Thailand)
5. Cylinder

6. Hand sprayers
7. Handheld pH meter: -pHTestr30 (Eutech Instruments Pte Ltd., Singapore)
8. Micro pipette: Pipetman (Gilson, France)
9. Pipette tip
10. Nylon net (9×9 cm^2)
11. Plastic pot and punnet (11×11×6 cm^3)

Equipment for collecting plant growth

1. Balance: PG503-S (Mettler Toledo, USA)
2. Balance: AG285 (Mettler Toledo, USA)
3. Forceps
4. High performance lab oven (Binder®, Germany)
5. Paper bag
6. Ruler
7. Scissors

3.2.2. Instruments for studying the effects of shrimp shell and fermented chitin waste (FCW) on rice growth, photosynthesis and yield

Equipment for planting

1. Cylinder
2. Mechanical scale (Horse, Thailand)
3. Plastic pot (Ø 10 inch.)
4. Digital conductivity meter: S230 SevenCompact™ conductivity (Mettler Toledo, USA)

Equipment for collecting plant yield

1. Balance: PG503-S (Mettler Toledo, USA)
2. Balance: AG285 (Mettler Toledo, USA)
3. Forceps
4. High performance lab oven (Binder®, Germany)
5. Paper bag
6. Scissors

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Equipment for photosynthetic measurement

1. Portable photosynthesis system: LI-6400XT (LI-COR, USA)
2. Ruler
3. Timer

Equipment for chloroplast diameter measurement

1. 5 mm thickness Expanded Polyethylene (EPE) foam sheet
2. Plant microtome automatic: Mt-3 (NK system, Japan)
3. Microtome blades: Low profile (DURAEDGE®, USA)
4. Slides
5. Cover slips
6. Forceps
7. Needles
8. 15 x 60 mm plates (Anumbra, Czech Republic)
9. Olympus BH-2 microscope (Olympus America Inc., USA)
10. Olympus DP70 camera (Olympus America Inc., USA)
11. Image Pro-Plus 5.1 program (Media Cybernetics, Inc., USA)

Equipment for chlorophyll content measurement

1. Scissors
2. Balance: PG503-S (Mettler Toledo, USA)
3. Balance: AG285 (Mettler Toledo, USA)
4. Proline prospenser bottle-top dispenser (BIOHIT PROLINE®,Finland)
5. Glass tube 15 ml.
6. Plastic caps
7. Parafilm
8. Micro pipette (Gilson, France)
9. Pipette tips
10. Spectrometer: 8453E UV-Vis (Agilent Technologies Inc., Germany)
11. Cuvettes

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Equipment for RNA extraction

1. Aluminum foil
2. Flask
3. Cylinder
4. Beaker
5. Deep freezer (-80° C) (New Brunswick Scientific, Belgium)
6. Freezer (-20° C) (Sanyo Electric Co., Ltd, Japan), Japan)
7. pH meter (Denver Instrument Company, USA)
8. Refrigerated centrifuge: Universal 2R (Hettich, Germany)
9. Microcentrifuge tubes
10. Vortex mixture: Vortex-Genie 2 (Scientific Industries, Inc., USA)
11. Spectrometer: 8453E UV-Vis (Agilent Technologies Inc., Germany)
12. Dry bath incubator (Major Scientific Products Co. Ltd, USA)
13. Timer
14. Spatulas
15. Mortars and pestles
16. Micro pipette (Gilson, France)
17. Pipette tips (10, 200 and 1,000 μl)
18. Aluminum foil
19. Liquid nitrogen container
20. Handed sprayers for hydrogen peroxide (H_2O_2) and 70% (v/v) ethanol
21. Latex gloves
22. Cooler
23. Autoclave (Taichung, Taiwan)
24. Hot air oven (Memmert, Germany)

Equipment for gel electrophoresis

1. Flasks
2. Cylinders

3. Microwave: ER-D23SC (S) (Toshiba, Japan)
4. Gel electrophoresis system: minirun GE-100 (Hangzhou BIOER Technology Co. Ltd., China)
5. Gel Doc™ 2000 and UV transilluminator (Bio-Rad, USA)

Equipment for real-time polymerase chain reaction (real-time PCR)

1. Pipette (Gilson, France)
2. Pipette tips
3. PCR machine: PTC-100™ programmable thermal controller (MJ Research Inc., USA)
4. Real-time PCR: CFX96 Touch™ (Bio-Rad Laboratories, Inc., USA)
5. Dry bath incubator (Major Scientific Products Co. Ltd, USA)
6. Deep freezer (-80° C) (New Brunswick Scientific, Belgium)
7. Freezer (-20° C) (Sanyo Electric Co., Ltd, Japan)
8. pH meter (Denver Instrument Company, USA)
9. Refrigerated centrifuge: Universal 32R (Hettich, Germany)
10. Microcentrifuge (Sorvall Biofuge Pico, Germany)
11. Microcentrifuge tube
12. Individual PCR Tubes 8-tube strip, clear (Bio-Rad Laboratories, Inc., USA)
13. Optical flat 8-cap strips for 0.2 ml tube strips/plates (Bio-Rad Laboratories, Inc., USA)
14. Autoclave: TC-459 (Taichung, Taiwan)
15. Vortex mixture: Vortex-Genie 2 (Scientific Industries, Inc., USA)
16. Spectrometer: 8453E UV-Vis (Agilent Technologies Inc., Germany)
17. Real time gel electrophoresis system: minirun GE-100 (Hangzhou BIOER Technology Co. Ltd., China)

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3.3. Chemicals and reagents

3.3.1. Chemicals and reagents for studying the effects of chitosan and their solvents on rice seedling growth

1. Modifield WP no.2 solution (see in Appendix A-1)
2. Distilled water
3. Acetic acid (LAB-Scan, Ireland)
4. Lactic acid
5. Citric acid
6. Four types of chitosan molecules (Limpanavech *et al.*, 2008)
   - 6.1 P80: Polymeric chitosan with 80-90% DD (MW = 530,000 Da)
   - 6.2 P90: Polymeric chitosan with more than 90% DD (MW = 450,000 Da)
   - 6.3 O80: Oligomeric chitosan with 90% DD (MW = 45,000 Da)
   - 6.4 O90: Oligomeric chitosan with 90% DD (MW = 110,000 Da)
7. Tween 20

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3.3.2. Chemicals and reagents for studying the effects of shrimp shell and fermented chitin on rice growth, photosynthesis and yield

Chemicals for planting

1. Clay soil (local market, Bangkok)
2. Chemical fertilizer (16-0-0 and 46-0-0; Viking Fertilizer Co Ltd., Bangkok)
3. Organic fertilizer (chicken manure: brand; puiinsee-ttv, Nakhon Ratchasima)

Chemicals for photosynthetic measurement

1. CO_2 cylinders (LI-COR, USA)
2. Anhydrous calcium sulfate or Drierite desiccant (W.A. Hammond Drierite Co., Ltd., USA)

Chemicals for chloroplast diameter measurement

1. Safranin O solution

Chemicals for chlorophyll content measurement

1. 80% (v/v) Acetone
2. Distilled water

Chemicals for RNA extraction

1. Liquid nitrogen
2. Hydrogen peroxide (H_2O_2)
3. 80% (v/v) Ethyl alcohol (Liquid Distillery Organization Excise Dept, Thailand)
4. RNA extraction buffer (see in appendix A-2)
5. Phenol: chloroform: isoamyl alcohol (25:24:1) v/v
6. Absolute ethanol (Liquid Distillery Organization Excise Dept, Thailand)
7. Ethyl alcohol (C_2H_6O) (Merck, Germany)
8. TE buffer (see in appendix A-3)
9. Lithium chloride ($LiCl_2$) (Sigma Chemical Company, USA)
10. Diethyl pyrocarbonate (DEPC) (Sigma Chemical Company, USA)
11. DEPC-treated TE Buffer (see in appendix A-4)
12. DEPC-treated Water (see in appendix A-5)
13. Chloroform (Merck, Germany)
14. Sodium acetate (CH_3COONa) (Sigma Chemical Company, USA)
15. 2-Mercaptoethanol (Merck, Germany)

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Chemicals for gel electrophoresis

1. Agarose (Research Organics, USA)
2. 5X TBE buffer (Tris Borate EDTA) (see in appendix A-6)
3. Ethidium bromide (Gibco BRI, USA)
4. DNA/RNA loading dye (see in appendix A-7)

Chemicals for DNase I treatment

1. Recombinant DNase I (Takara, Japan)
2. DNase I Buffer
3. DEPC-treated water

Chemicals for Reversed transcription PCR (RT- PCR)

1. 5X iScript reverse transcription supermix (Bio-Rad, USA)
2. Nuclease-free water

Chemicals for real-time polymerase chain reaction (real-time PCR)

1. RQ1 Rnase-free *DNasel* (Takara Bio Inc., Japan)
2. M-MLV reverse Transcriptase (Promega, USA)
3. SsoFast^{TM} EVagreen® Supermix (Bio-Rad, USA)
4. Phenol: chloroform: isoamyl alcohol (25:24:1) v/v
5. DEPC-treated TE buffer (see in appendix A-4)
6. Ethyl alcohol (C_2H_6O) (Merck, Germany)
7. Sodium acetate (Sigma-Aldrich Co., USA)

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CHAPTER IV

METHODS

4.1. To study the effects of different types and concentrations of chitosan molecules on rice growth, photosynthesis and yield

4.1.1. To determine the appropriate type(s) and concentration(s) of chitosan molecules for rice seedling growth enhancement

4.1.1.1. Plant material, chitosan treatments and the experimental design

'Pathumthani1' rice and 4 types of chitosan molecules, polymeric (P) and oligomeric (O) forms of chitosan, with a degree of deacetylation of 80% and 90% (designated P80, P90, O80 and O90, respectively) were used in this study. Each chitosan type was tested at 5 concentrations; 5, 10, 20, 40 and 80 mg/L. The solvent of chitosan stock solution, 1% (v/v) acetic acid, at the same concentrations as chitosan treatment and distilled water were used as control conditions. Therefore, the total of 26 treatments was performed as described below:

Treatment 1: soaking and spraying with distilled water
Treatment 2: soaking and spraying with acetic acid at 5 mg/L
Treatment 3: soaking and spraying with acetic acid at 10 mg/L
Treatment 4: soaking and spraying with acetic acid at 20 mg/L
Treatment 5: soaking and spraying with acetic acid at 40 mg/L
Treatment 6: soaking and spraying with acetic acid at 80 mg/L
Treatment 7: soaking and spraying with P80 at 5 mg/L
Treatment 8: soaking and spraying with P80 at 10 mg/L
Treatment 9: soaking and spraying with P80 at 20 mg/L
Treatment 10: soaking and spraying with P80 at 40 mg/L
Treatment 11: soaking and spraying with P80 at 80 mg/L
Treatment 12: soaking and spraying with O80 at 5 mg/L
Treatment 13: soaking and spraying with O80 at 10 mg/L
Treatment 14: soaking and spraying with O80 at 20 mg/L

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Treatment 15: soaking and spraying with O80 at 40 mg/L

Treatment 16: soaking and spraying with O80 at 80 mg/L

Treatment 17: soaking and spraying with P90 at 5 mg/L

Treatment 18: soaking and spraying with P90 at 10 mg/L

Treatment 19: soaking and spraying with P90 at 20 mg/L

Treatment 20: soaking and spraying with P90 at 40 mg/L

Treatment 21: soaking and spraying with P90 at 80 mg/L

Treatment 22: soaking and spraying with O90 at 5 mg/L

Treatment 23: soaking and spraying with O90 at 10 mg/L

Treatment 24: soaking and spraying with O90 at 20 mg/L

Treatment 25: soaking and spraying with O90 at 40 mg/L

Treatment 26: soaking and spraying with O90 at 80 mg/L

The experiment was performed with a randomized complete block design (RCBD) with 4 replications/treatment. All of water, acetic acid and chitosan treatments were applied twice by seed soaking and foliar spraying on 14-day-old seedlings.

4.1.1.2. Plant cultivation and growth measurement

1. For each treatment, seeds were soaked with distilled water, acetic acid or chitosan solution for 2 days as indicated in 4.1.1.1 and then, were sown on plastic net in a basket (40 seeds/121 cm^2 planting area) containing 700 ml of hydroponic solution WP No.2 (Figure 7A) (Vajrabhaya andVajrabhaya, 1991).
2. Seedlings were grown under natural condition in the greenhouse at Department of Botany, Faculty of Science, Chulalongkorn University. The hydroponic solution in each container was refreshed every 7 days until the end of experiment.

3. For the second application of chitosan, 14-day-old seedlings were sprayed with varied concentrations of chitosan solution as indicated in 4.1.1.1. Meanwhile, distilled water and 5 concentrations of acetic acid were used in control treatments.
4. Plant growth parameters including plant height (PH), root length (RL), shoot and root fresh weight (SFW, RFW), shoot and root dry weight (SDW, RDW) were collected on 7, 14 and 21 days after germination. At least 3 plants/replication were used for each time point.

Figure 7. The growing rice in hydroponic technique, germinating seeds (A) and 21-day-old seedlings (B)

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According to the result of this experiment, the negative effect of acetic acid using as chitosan solvent was observed on rice seedling growth. Therefore, it was necessary to examine the most appropriate solvent which showed the minimal inhibitory effect on plant growth and then this solvent was used in all subsequent assays.

4.1.2. To study the effects of different solvents of chitosan on rice seedling growth

4.1.2.1. To study the effects of three different chitosan solvents on growth of seedling in order to find a suitable solvent without negative effects.

4.1.2.1.1. Plant material, solvent treatments and experimental design

'Pathumthani1' rice and 3 different chitosan solvents including 1% (v/v) acetic acid, 1% (v/v) citric acid and 1% (v/v) lactic acid were used in this study. Each solvent was diluted to 5 concentrations, 5, 10, 20, 40 and 80 mg/L. Distilled water was used as a control and the 16 treatments were performed as described below:

Treatment 1: soaking with distilled water
Treatment 2: soaking with acetic acid at 5 mg/L
Treatment 3: soaking with acetic acid at 10 mg/L
Treatment 4: soaking with acetic acid at 20 mg/L
Treatment 5: soaking with acetic acid at 40 mg/L
Treatment 6: soaking with acetic acid at 80 mg/L
Treatment 7: soaking with citric acid at 5 mg/L
Treatment 8: soaking with citric acid at 10 mg/L
Treatment 9: soaking with citric acid at 20 mg/L
Treatment 10: soaking with citric acid at 40 mg/L
Treatment 11: soaking with citric acid at 80 mg/L
Treatment 12: soaking with lactic acid at 5 mg/L
Treatment 13: soaking with lactic acid at 10 mg/L
Treatment 14: soaking with lactic acid at 20 mg/L
Treatment 15: soaking with lactic acid at 40 mg/L
Treatment 16: soaking with lactic acid at 80 mg/L

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The experiment was carried out with a randomized complete block design (RCBD). Each treatment consisted of 4 replications, each of which consisted of 3 samples.

4.1.2.1.2. Plant cultivation and growth measurement

1. Rice seeds were soaked with distilled water and 3 solvents for 2 days as indicated in (4.1.2.1.1.). Then, seeds were sown on plastic net in each basket (40 seeds/121 cm^2 planting area) containing WP No.2 hydroponic solution (Vajrabhaya and Vajrabhaya, 1991).

2. Seedlings were grown under natural condition in the greenhouse at Department of Botany, Faculty of Science, Chulalongkorn University. The hydroponic solution in each container was refreshed every 7 days until the end of experiment.

3. Plant height (PH), root length (RL), shoot and root fresh weight (SFW, RFW), shoot and root dry weight (SDW, RDW) were collected at 7 and 14 days after germination at least 3 plants per replication for each time point.

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4.1.2.2. To study the effect of the different types and concentrations of chitosan molecules in the suitable chitosan solvent on rice seedlings growth

4.1.2.2.1. Plant material, solvent treatments and experimental design

Lactic acid was used as the appropriate chitosan solvent in this experiment because it showed a markedly lower toxicity to the rice seedlings growth than acetic acid and citric acid. Therefore, the P80, O80, P90 and O90 forms of chitosan in 1% (v/v) lactic acid at 3 concentrations; 10, 20 and 40 mg/L were used to study the effects of different types and concentrations of chitosan molecules on rice seedling growth. Meanwhile, distilled water and lactic acid alone was used as controls. Therefore, the 16 treatments were performed as described below:

Treatment 1: soaking and spraying with distilled water
Treatment 2: soaking and spraying with lactic acid at 10 mg/L
Treatment 3: soaking and spraying with lactic acid at 20 mg/L
Treatment 4: soaking and spraying with lactic acid at 40 mg/L
Treatment 5: soaking and spraying with P80 at 10 mg/L
Treatment 6: soaking and spraying with P80 at 20 mg/L
Treatment 7: soaking and spraying with P80 at 40 mg/L
Treatment 8: soaking and spraying with O80 at 10 mg/L
Treatment 9: soaking and spraying with O80 at 20 mg/L
Treatment 10: soaking and spraying with O80 at 40 mg/L
Treatment 11: soaking and spraying with P90 at 10 mg/L
Treatment 12: soaking and spraying with P90 at 20 mg/L
Treatment 13: soaking and spraying with P90 at 40 mg/L
Treatment 14: soaking and spraying with O90 at 10 mg/L
Treatment 15: soaking and spraying with O90 at 20 mg/L
Treatment 16: soaking and spraying with O90 at 40 mg/L

The experiment was performed in a randomized complete block design (RCBD) with 4 replications/treatment, each of which consisted of at least 3 samples.

4.1.2.2.2. Plant cultivation and growth measurement

Plant cultivation and growth measurement were performed as described in 4.1.1.2.

4.1.3. Statistical analysis

Data of each parameter in the RCBD experiment were analyzed using one-way analysis of variance (ANOVA), following by Duncan's Multiple Range Test (DMRT) using the IBM SPSS Statistic software and using significance at the $P<0.05$ level.

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4.2. To study the effects of shrimp shell (SS) and O-80 chitosan residue (FCW) on rice growth, photosynthesis and yield

4.2.1. Plant materials, shrimp shell (SS) and O-80 chitosan residue (FCW) treatments, and experimental design

'Pathumthani1' rice was used in this study. Three levels of dried shrimp shell (SS) or O-80 chitosan residue (fermented chitin waste; FCM), 0.25%, 0.50%, and 1.0% (w/w), were applied as soil supplement for 7 days before rice seedling transplantation as described below:

Treatment 1: Chemical fertilizer only (0.2 g/kg soil of chemical fertilizer-16:20:0 and 0.132 g/kg soil of chemical fertilizer-46:0:0)
Treatment 2: Organic fertilizer only (0.662 g/kg soil of chicken manure)
Treatment 3: 0.25 % (w/w) of ground shrimp shell and organic fertilizer
Treatment 4: 0.5 % (w/w) of ground shrimp shell and organic fertilizer
Treatment 5: 1 % (w/w) of ground shrimp shell and organic fertilizer
Treatment 6: 0.25 % (w/w) of O-80 chitosan residue and organic fertilizer
Treatment 7: 0.5 % (w/w) of O-80 chitosan residue and organic fertilizer
Treatment 8: 1 % (w/w) of O-80 chitosan residue and organic fertilizer

The experiment was performed in a randomized complete block design (RCBD) with 4 replications, each of which consisted with 3 seedlings.

4.2.2. Soil preparation, plant cultivation, fertilizer and water management

4.2.2.1. Clay soil, composed of sand, slit and clay at 16%, 26% and 58%, respectively was used in this experiment. Each pot contains 5 kg of clay soil (local market, Bangkok), which was thoroughly mixed with 3 concentrations of SS or FCW as indicated in 4.2.1. for 7 days before seedling transplantation. Soil supplemented with chemical fertilizer alone (CF) or chicken manure alone, considered as organic fertilizer (OF), was used as a control. The quantity and time to apply fertilizer were used as indicated from the recommendation of the company (Figure 8).

- In case of chemical fertilizer, 1 g of chemical fertilizer (16-20-0) was added to each pot at 20 days after transplantation and 0.66 g of chemical fertilizer (46-0-0) was added to each pot again after 60 days of transplantation.

- In case of chicken manure fertilizer, 3.31 g of chicken manure was added to the pot at 20 days after transplanting and the same amount of the chicken manure was added again after 60 days of transplantation.

4.2.2.2.The physicochemical properties of soil including pH, electrical conductivity (EC), organic matters (OM), contents of nitrogen (N), phosphorus (P), potassium (K), calcium (Ca) and magnesium (Mg) were determined before planting and after the harvest (see in appendix B-1).

4.2.2.3 For seedling preparation, rice seeds were soaked with distilled water for 2 days and then germinated on a pot containing clay soil in the greenhouse.

4.2.2.4. Three 28-day-old seedlings were transplanted to each pot and cultured under natural condition in the greenhouse during rainy season (March to August 2012) at Department of Botany, Faculty of Science, Chulalongkorn University. Water level in the pot was maintained at 5 cm above soil surface throughout the experimental period.

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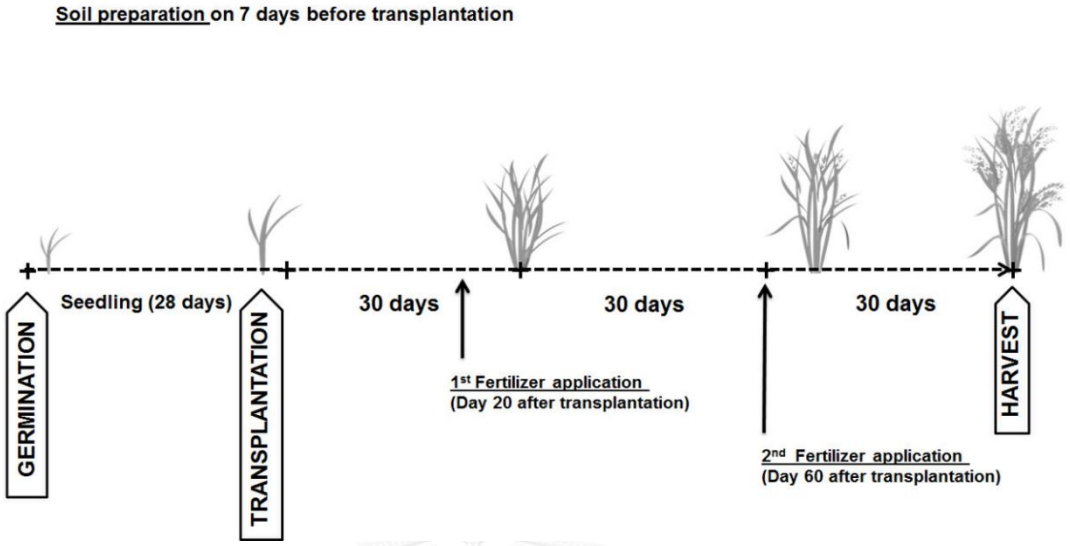

Figure 8. The procedure of transplanting rice cultivation

4.2.3. Determination of plant growth and yield

4.2.3.1. Tillers numbers/plant in each pot at 0, 15, 30, 45 and 60 days after transplantation and the dry weight of shoot biomass/pot at the end of the experiment were recorded for plant growth determination.

4.2.3.2. Flowering time (days of flowering; DOF) was recorded when rice plants initially bloomed (flowering stage). Moreover, yield components including panicle numbers/pot, spikelets/panicle, filled grains/panicle, 100-grain-weight and the weight of total seeds/pot were determined after harvest.

4.2.4. Determination of photosynthetic characteristics

4.2.4.1. At vegetative stage, net photosynthetic rate (A_{max}), stomatal conductance (g_s), internal concentration of CO_2 (C_i) and transpiration rate (E) of the three uppermost fully expanded leaves/pot were measured after 30 days of transplantation with a LI-6400 XT portable photosynthesis system (LI-COR, Lincoln, NE, USA).

4.2.4.2. At reproductive stage, net photosynthetic efficiency of the second leaf and flag leaf was measured after 60 and 67 days of transplantation, respectively.

4.2.4.3. The measurement was performed at a photosynthetic photon flux density (PPFD) of 1,200 μmol/m^2s and CO_2 concentration of 380 μmol/mol.

4.2.5. Determination of leaf chloroplast diameter and chlorophyll content

4.2.5.1. After 35 days of transplantation, three fully expanded leaves from each pot were used to study the effects of chitosan at cellular level.

4.2.5.2. In order to measure chloroplast diameter, three leaves/pot were sectioned by vibratome (Automatic plant microtome, Mt-3, NK system) and then digitally recorded with an Olympus BH-2 microscope (Olympus America, Inc.) equipped with Olympus DP70 camera and DP controller software. Then, chloroplast diameter was measured according to Limpanavech *et al.* (2008) and at least 120 chloroplasts per treatment were used for the statistical analysis.

4.2.5.3. Photosynthetic pigment contents of the same leaves used for 4.2.5.2 were extracted by using 80% acetone solution as described in Porra (2002). In brief, 10 ml of 80% acetone solution were added to 25 mg of rice leaves and kept in the dark for 24 hours. Afterward, chlorophyll *a*, chlorophyll *b* and carotenoid contents were measured at 663.2, 646.8 and 470 nm by a spectrometer, respectively and the photosynthetic pigment concentrations were calculated from the following equation:

$$\text{Chlorophyll } a \ (C_a) = (12.25A_{663.2} - 2.79A_{646.8})/FW$$

$$\text{Chlorophyll } b \ (C_b) = (21.5A_{646.8} - 5.10A_{663.2})/FW$$

$$\text{Carotenoids} = [(1000A_{470} - 1.82\ C_a - 85.02\ C_b)/198]/FW$$

The chlorophyll *a*, chlorophyll *b* and carotenoid contents were expressed as mg/g Fw (Lichtenthaler and Buschmann, 2001).

4.2.6. Statistical analysis

Data of each parameter which obtained from 4 replications of 8 treatments in the RCBD experiment were analyzed using a one-way analysis of variance (ANOVA), followed by the Duncan's Multiple Range Test (DMRT) using the IBM SPSS Statistic software and accepting significance at the $P<0.05$ level.

4.3. To study the gene expression induced by shrimp shell (SS) and O-80 chitosan residue (FCW) when these substances were used as stimulants.

At vegetative stage, the addition of 0.25% SS and 0.25% FCW was the suitable treatments to investigate chitin/chitosan-induced gene expression which involved in photosynthesis because these treatments showed the highest tiller number/plant at 15 and 30 days after transplantation. Moreover, three genes including *Oxygen-evolving enhancer protein 1 (OEE1*: *LOC_Os01g31690*) and *Chlorophyll a-b binding protein (PsbS1: LOC_Os01g64960)* in light reaction together with *Ribulose bisphosphate carboxylase small chain (rbcS: LOC_Os12g19381)* in carbon fixation were selected as the representative genes from photosynthesis process in order to evaluate the effect of 0.25% SS and 0.25% FCW using as soil supplement on gene expressions by quantitative RT-PCR technique (Chamnanmanoontham *et al.*, 2015). In addition, the rate of photosynthesis and photosynthetic pigment content were also determined.

4.3.1. Shrimp shell (SS) and O-80 chitosan residue (FCW) treatments

The SS and FCW at concentration of 0.25% (w/w) were used in this experiment. The experiment was performed in the same way as indicated in 4.2. Photosynthetic parameters and photosynthetic pigment contents were determined at 0, 7 and 14 days after transplanting. Moreover, rice leaf tissues were harvested on the same days of tissue collection for photosynthetic pigment content determination in order to investigate gene expression.

4.3.2. The specific primer design for quantitative RT-PCR technique

The specific primers of *Oxygen-evolving complex protein 1* gene (*OEE1)* and *Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit gene (rbcS)* were manually designed, and then were confirmed with the analysis of Primer3 and BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Meanwhile, the specific primers of *Chlorophyll a-b binding protein gene (PsbS1)* and a reference gene, *Elongation factor 1 alpha gene (EF1-*α) were designed as described in Chutimanukul (2013). The sequences of specific real-time PCR primer were described in Table 3.

Table 3 Primer pairs and Tm (°C) for determination of chitosan-inducible gene expression *via* real-time PCR technique. The *EF1-α* gene was used as a reference gene.

Primer Name	Sequence	Tm (°C)
OEE1-F	5'-GTCCTTCCTCGACCCAAAGG-3'	57.6
OEE1-R	5'-TCTCTGGCTTGCTCTTGGTG-3'	57.3
rbcS -F	5'-ACTACGATGGCAGGTATTGG-3'	54.3
rbcS- R	5'-GCCGATGATACGGACAAAGG-3'	55.9
PsbS1- F	5'-GCATCGCCTTCTCCATCA- 3'	55
PsbS1- R	5'-GAAGACGACGTTGAAGAGGA- 3'	54.2
EF1-α - F	5'-ATGGTTGTGGAGACCTTC- 3'	53.7
EF1-α - R	5'-TCACCTTGGCACCGGTTG- 3'	58.2

4.3.3. RNA extraction

Total RNA was extracted by the standard hot-phenol procedure (Thikart et al., 2005) as described in appendix B-2.

4.3.4. The DNase I treatment and the first strand cDNA synthesis

The *DNase* I treatment and the first strand cDNA synthesis were done as described in appendix B-3 and B-4.

4.3.5. The expression level of chitosan-induced gene involving in photosynthesis

After cDNA synthesis, the quantitative real-time PCR was carried out on the Real-time PCR: CFX96 Touch™ (Bio-Rad Laboratories, Inc., USA) according to the SYBR green detection protocol (SsoFast^{TM} EVagreen® Supermix (Bio-Rad, USA)). The components of each real-time PCR reaction and the thermal cycle were described in appendix B-7.

4.3.6. Calculation of relative gene expression level

The expression level of these three genes was determined in comparison with a housekeeping gene, *EF1-*α, in reference to the expression on day 0 of the control. For the expression of each gene, at least 3 independent real-time PCR reactions of the same cDNA preparation were performed. The calculation of the relative expression ratio of target gene was done as demonstrated in the appendix B-8 (Pfaffl, 2001).

4.3.7. Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA), followed by Duncan's Multiple Range Test (DMRT) using the IBM SPSS Statistic software and accepting significance at the $P<0.05$ level.

CHAPTER V
RESULTS

5.1. Effects of different types and concentrations of chitosan molecules on rice seedling growth

5.1.1. Effects of different types and concentrations of chitosan molecules on 'Patumthani1' rice seedlings

An overview of the effects of different chitosan types, P-80, O-80, P-90, and O-90, dissolved in acetic acid on growth of 'Patumthani1' seedling applied by seed soaking and then foliar spraying on 14-day-old seedlings are shown in Figure 9. Plant growth parameters in term of plant height (PH), shoot fresh weight (SFW), shoot dry weight (SDW), root length (RL), root fresh weight (RFW) and root dry weight (RDW) were determined at 7, 14 and 21 days after germination and the detail of each parameter are shown in Table C-1 to C-6 in Appendix C, respectively.

It was found that none of chitosan treatments significantly increased the growth of seedling over 21 days after germination when compared to water control. Noteworthy, the solvent of chitosan stock solution, 1% (v/v) acetic acid also showed the negative effects on seedling growth in comparison with water (Figure 9). Particularly, PH, SFW, SDW and RL of seedling treated with acetic acid were significantly lower than non-treated seedling (Table C-1 – C-4; Appendix C).

On the other hand, RL of 7-day-old seedlings treated with 80% or 90% DD oligomeric chitosan at concentration of 40 mg/L (O80-40 and O90-40) was significantly longer than control seedling. However, the positive effect on seedling growth was not observed after 14 days of germination (Figure 9, Table C-4: Appendix C).

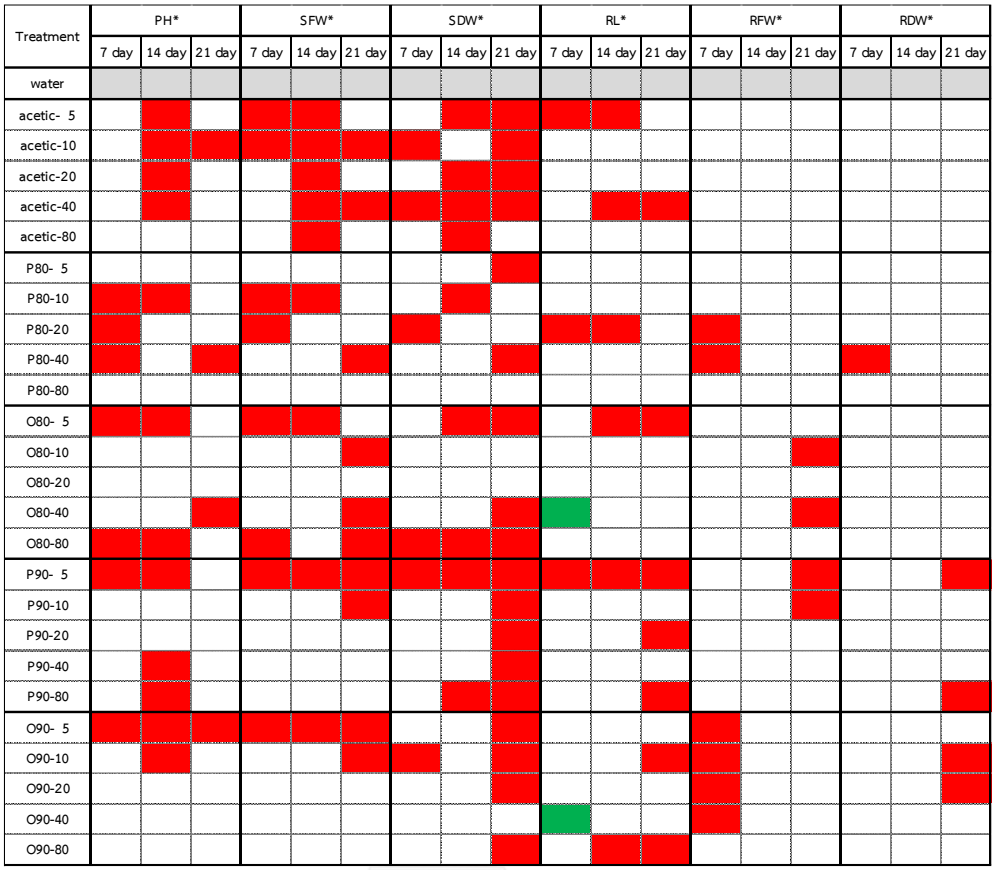

Figure 9. The effects of chitosan treatments and acetic acid as chitosan solvent in comparison with water on rice seedlings growth including plant height (PH), shoot fresh weight (SFW), shoot dry weight (SDW), root length (RL), root fresh weight (RFW) and root dry weight (RDW) at 7, 14 and 21 days after germination.

Column with * are significant difference with respect to the control (water), according to Duncan's multiple range tests ($P<0.05$). Red color displayed a significantly decreased value when compared with the control (water), whereas the green color showed a significantly increased value. The white color showed no significant difference.

Negative effect of acetic acid used as chitosan solvent on SDW.

The dry weight of shoots was used as a representative of plant growth parameters in order to clearly show the growth inhibitory effect of acetic acid as a chitosan solvent on growth of 'Patumthani1' rice seedling. It was found that all concentrations of acetic acid completely inhibited rice seedling growth by decreasing SDW after 14 days of germination (Figure 10). The most reduction of SDW ranged from 10 to 23% compared with that of the control at 21 days after germination (Table C-3).

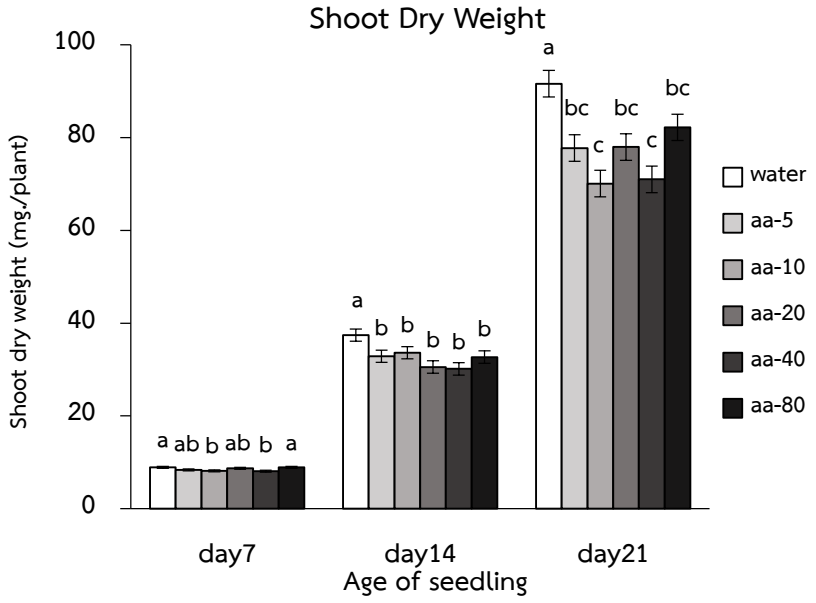

Figure 10. Effect of water and acetic acid at 5, 10, 20, 40, and 80 mg/L on SDW of rice seedlings. Bar with different letters denote significant differences according to Duncan's multiple range tests (P<0.05)

However, according to the data from Figure 9, treatments with 80% DD both of polymeric and oligomeric chitosan at concentration of 80 and 20 mg/L (P80-80 and O80-20) showed no significant differences in all parameters of seedling growth between water and chitosan throughout the experiment. Therefore, the negative effects of chitosan on rice seedling growth probably caused by the combination between acetic acid's effect and chitosan's effect (Figure 9).

5.1.2. The effects of three solvents of chitosan on 'Patumthani1' rice growth in order to find a suitable chitosan solvent without negative effects.

A summary of the effects of chitosan solvents including acetic, citric and lactic acid on seedling growth are shown in Figure 11. Seedling growth analysis was evaluated in terms of plant height (PH), shoot fresh weight (SFW), shoot dry weight (SDW), root length (RL), root fresh weight (RFW) and root dry weight (RDW). The in-depth data of each shoot and root parameter are separately described in Table C-7 and C-8 (Appendix C), respectively.

The negative effects on seedling growth including PH, SFW, SDW, RL and RFW were found in both of citric and lactic acid treatments (Figure 11, Table C-7 and C-8). These negative results were similar to acetic acid treatments. However, the root weight of rice seedling treated with lactic acid at concentration of 20 mg/L increased by 13.8% compared with that of water control after 7 days of germination (Figure 11, Table C-8).

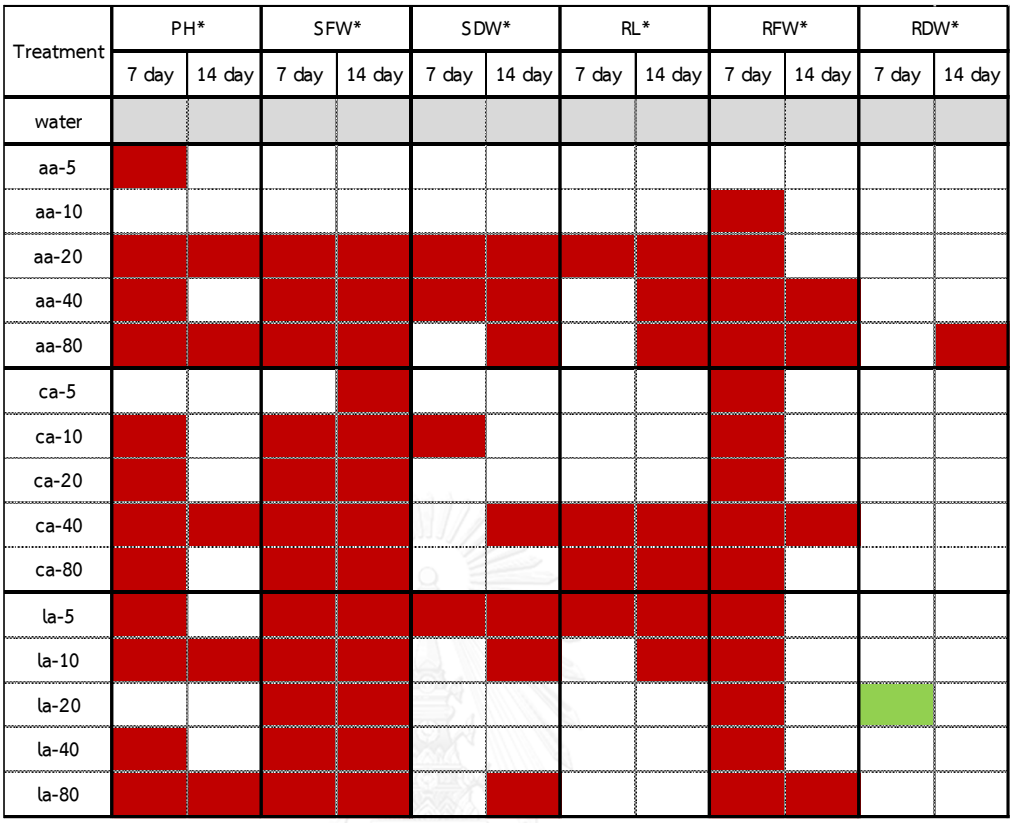

Figure 11. The effects of three solvents of chitosan; acetic acid, citric acid and lactic acid, on rice seedlings growth in term of plant height (PH), shoot fresh weight (SFW), shoot dry weight (SDW), root length (RL), root fresh weight (RFW) and root dry weight (RDW) on day 7, 14 and 21 after germination

Column with * represented significant difference with respect to the control (water), according to Duncan's multiple range tests ($P<0.05$). Red color displayed a significantly decreased value when compared with the control (water), whereas the green color showed a significantly increased value. The white color showed no significant difference.

Consequently, in order to investigate growth inhibitory effects by their concentrations in terms of SDW, citric acids and lactic acids were evaluated separately as alternative chitosan solvents at the same concentrations of acetic acid (5, 10, 20, 40 and 80 mg/L) (Figure 12 and Figure 13).

Effects of various concentrations of citric acid on rice seedling growth

After seed treatment, seedling in the citric acid treatment at concentration of 10 mg/L showed the significant decrease of SDW when compared to non-treated seedlings after 7 days of germination. Moreover, the application of high concentrations of citric acid (40 and 80 mg/L) tended to slightly decrease SDW of 7-day-old seedlings compared to water treatment.

Similarly, the negative effect of high concentrations of citric acid on SDW was also found in 14-day-old seedlings. Moreover, SDW of 14-day-old seedlings treated with 5 mg/L citric acid significantly decreased, compared with that of non-treated seedlings. However, SDW of 14-day-old seedlings treated with citric acid at 10 and 20 mg/L was not different from non-treated control. Especially, SDW resulted from 20 mg/L of citric acid treatment showed no significant difference from non-treated ones throughout the experiment (Figure 12).

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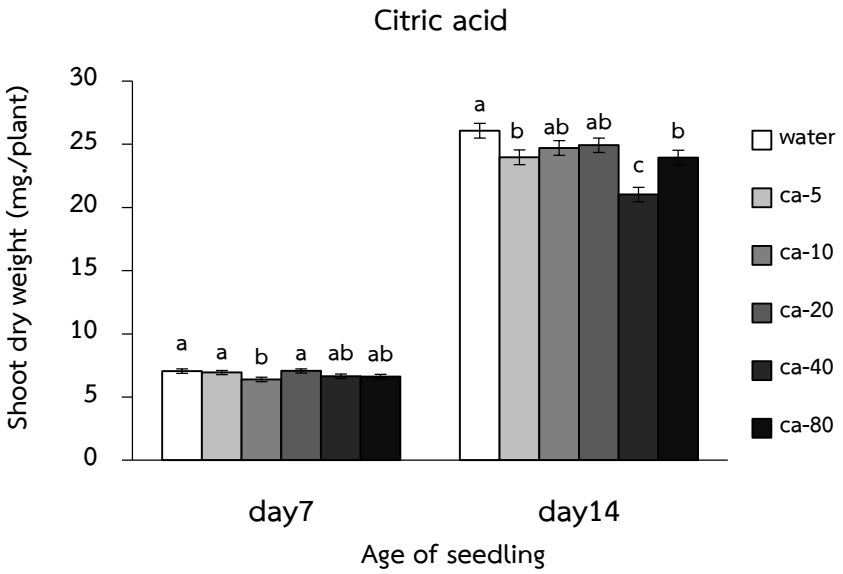

Figure 12. Effect of water and citric acid at 5, 10, 20, 40, and 80 mg/L on SDW of rice seedling. Bar with different letters denote significant differences according to Duncan's multiple range tests ($P<0.05$)

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Effects of various concentrations of lactic acid on rice seedling growth

There was no significant difference in SDW among treatments with the difference of lactic acid concentrations after 7 days of germination. On the other hand, the inhibitory effect of the lactic acid in seedling growth in term of SDW was found after 14 days of germination. SDW of seedlings treated with lactic acid at concentration of 5, 10 and 80 mg/L were significantly decreased, when compared to water control. However, seedlings that treated with lactic acid at concentrations of 20 and 40 mg/L showed the less negative effects on seedling growth. As a result, SDW in these two treatments was not significantly different from water control at the beginning of germination (Figure 13).

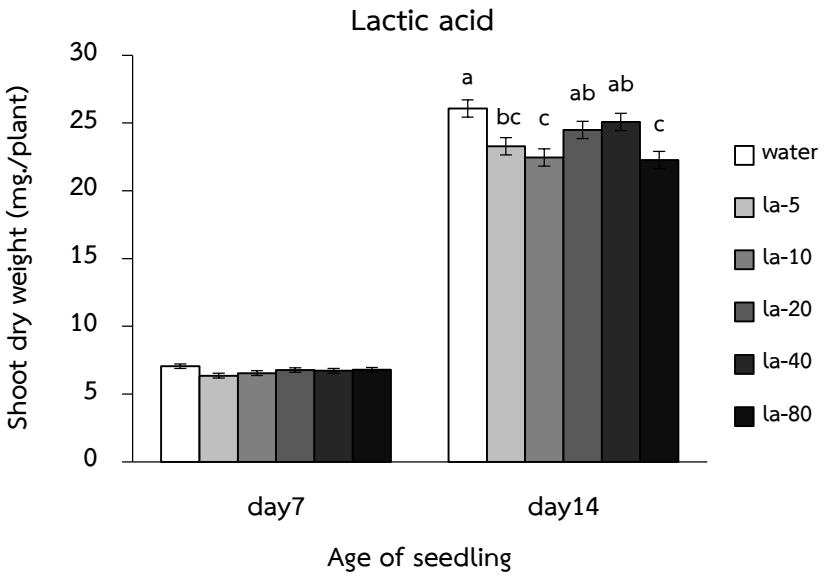


Figure 13. Effect of water and lactic acid at 5, 10, 20, 40, and 80 mg/L on SDW of rice seedling. Bar with different letters denote significant differences according to Duncan's multiple range tests (P<0.05)

Interestingly, lactic acid at concentrations of 20 mg/L not only showed the lowest growth inhibition on SDW, but also showed the significant increase of RL compared to control (Figure 11 and Figure 13).

Consequently, lactic acid was selected to use as an alternative chitosan solvent for the next experiment in order to determine the suitable types and concentrations of chitosan molecules on 'Patumthani1' rice seedlings growth.

5.1.3. The effects of different types and concentrations of chitosan molecules in lactic acid on rice seedlings growth

After seeds soaking followed by foliar spraying with various chitosan molecules on 14-day-old seedlings, the effects of different chitosan molecules, dissolved in lactic acid on seedling growth are shown in Figure 14. Plant growth parameters, including plant height (PH), shoot fresh weight (SFW), shoot dry weight (SDW), root length (RL), root fresh weight (RFW) and root dry weight (RDW) were determined at 7, 14 and 21 days after germination (Figure 14). The in-depth data of seedling in shoot and root part are described in Table C-9 and C-10 (Appendix C), respectively.

Overall, the negative effect on seedling growth was observed in seedlings treated with chitosan dissolved in lactic acid when compared to water control, especially in shoot part. On the contrary, some chitosan treatments such as treatments with 90% DD chitosan positively affected shoot and root part of seedling in term of PH, RL, RFW and RDW. When considered with the seedlings treated with chitosan P90 at 40 mg/L, RDW significantly increased by 16% compared with that of water control after 14 days of germination. In addition, this treatment showed the lowest inhibitory effect on 21-day-old seedling growth.

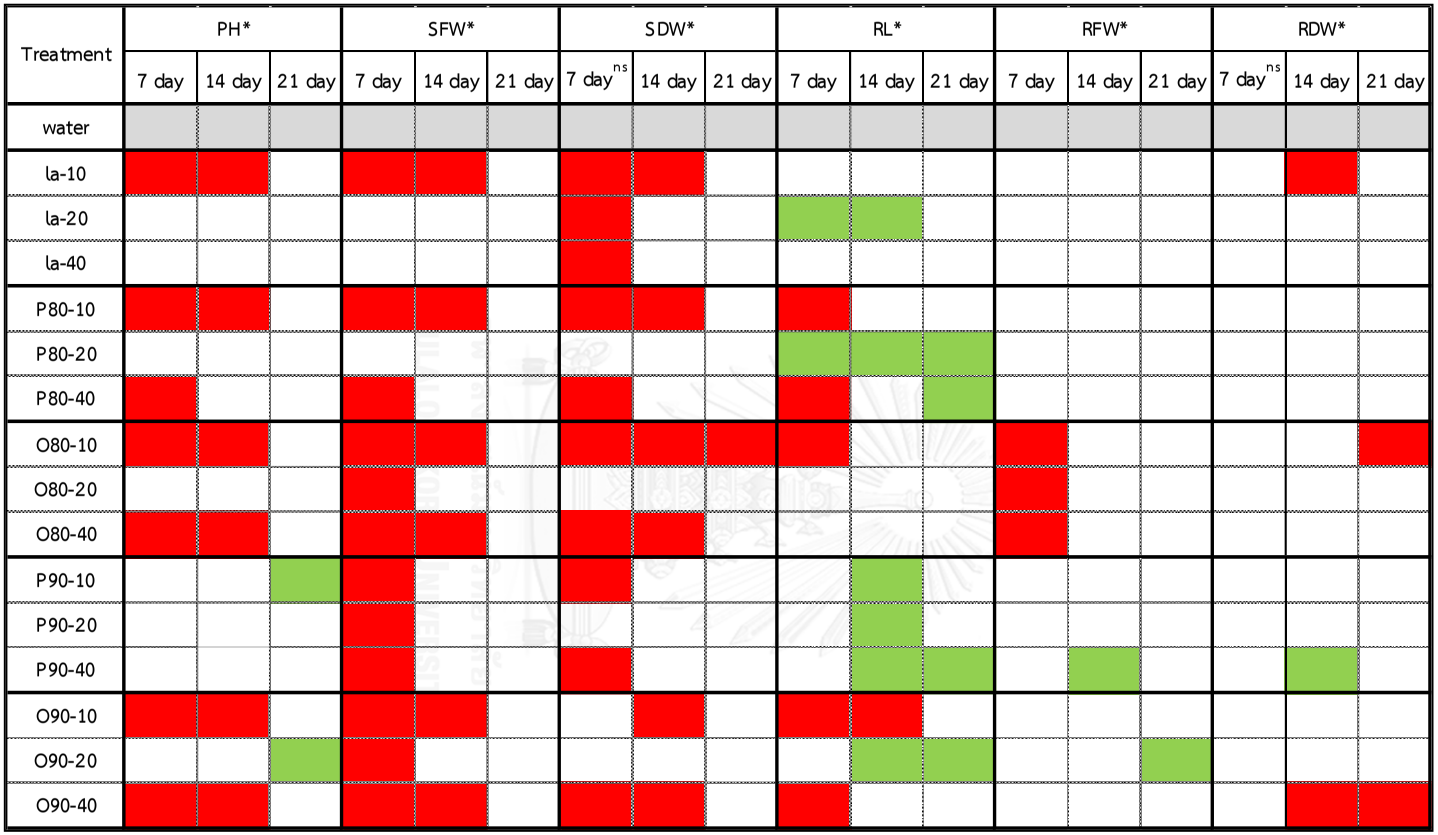
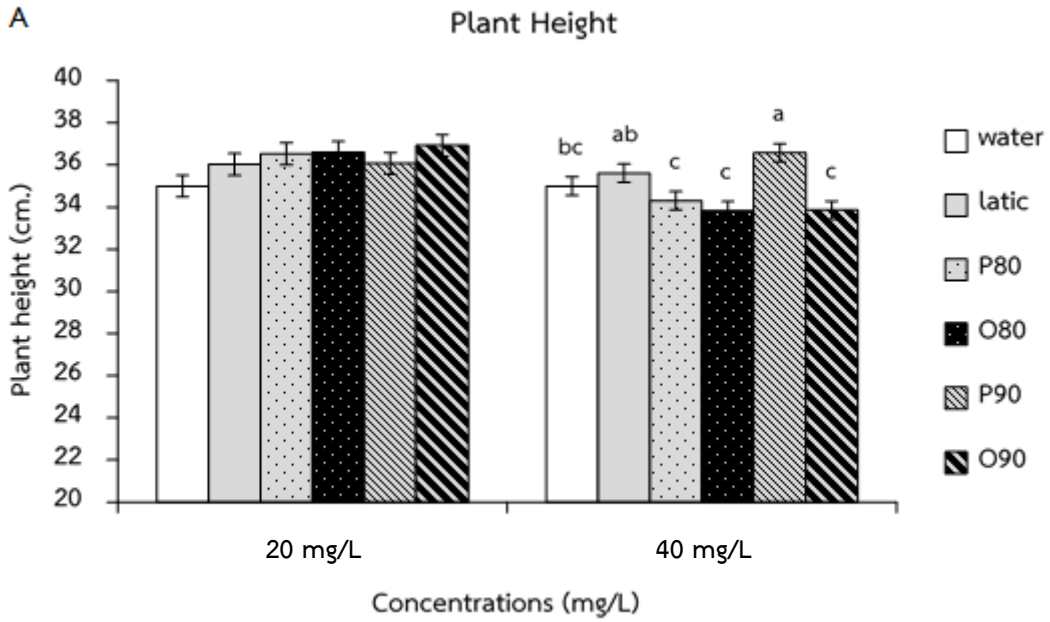
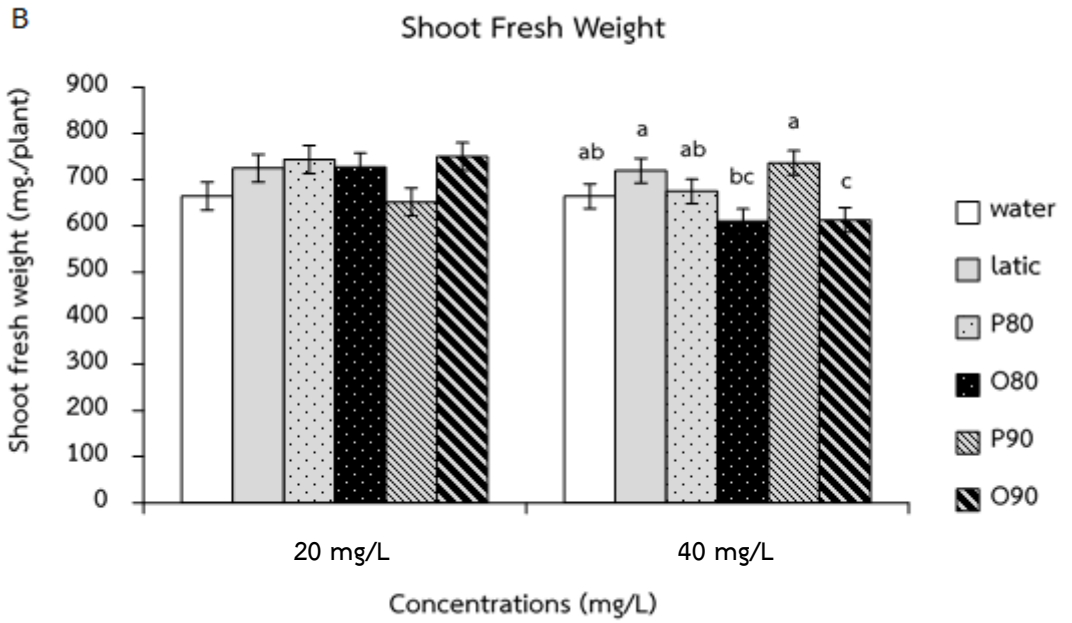


Figure 14. The effects of chitosan and lactic acid used as chitosan solvent on rice seedlings growth; plant height (PH), shoot fresh weight (SFW), shoot dry weight (SDW), root length (RL), root fresh weight (RFW) and root dry weight (RDW) on day 7 and 14 after germination.

Column with star (*) are significantly different with respect to the control (water), according to Duncan's multiple range tests ($P<0.05$). The red color displayed a significantly decreased value when compared with the control (water), whereas the green color showed a significantly increased value. The white color showed no significant difference.

Consequently, 21-day-old ‘Pathumthani1’ rice seedlings that treated with different chitosan types at concentration of 20 mg/L and 40 mg/L and dissolved with 1% (v/v) lactic acid were determined growth parameters; plant height, shoot fresh weight, shoot dry weight, root length, root fresh weight and root dry weight as shown in Figure 15A – C and Figure 16A – C.





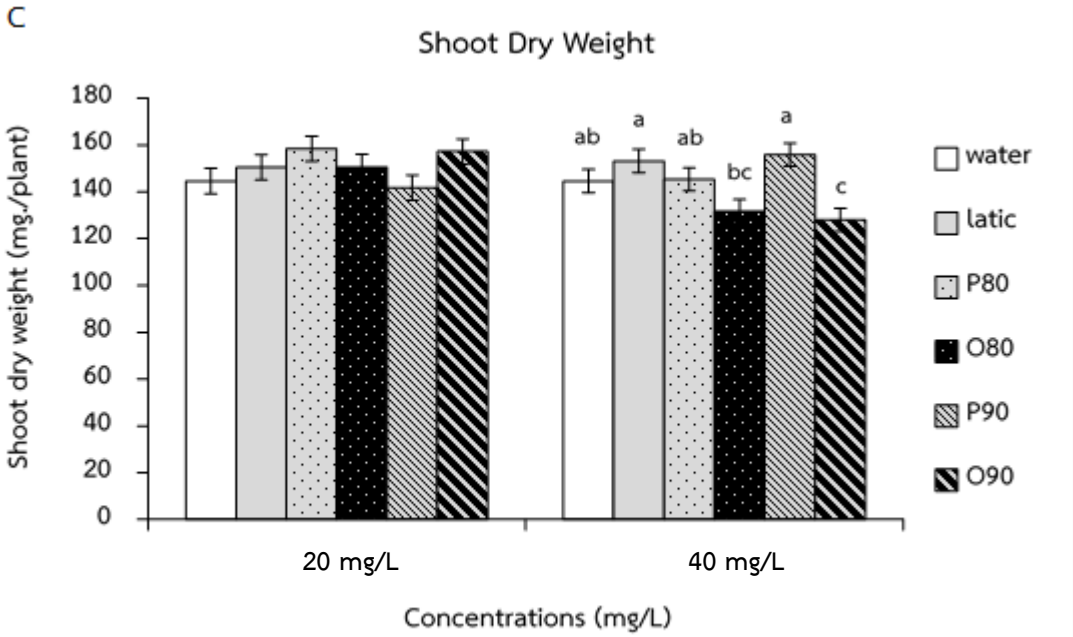
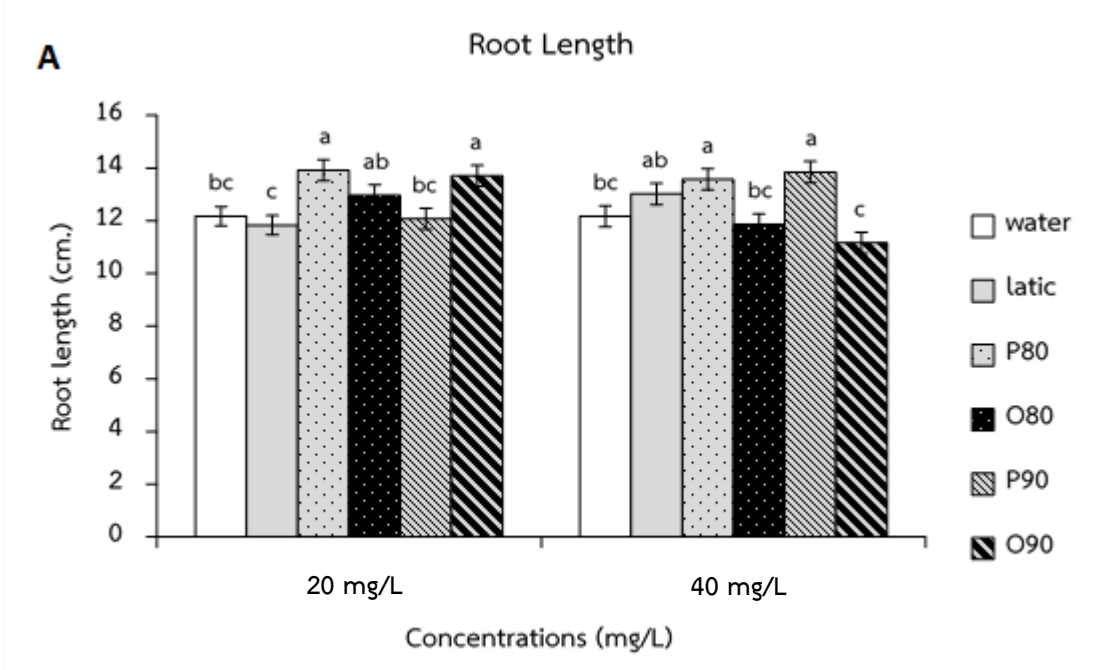


Figure 15. Effect of water, lactic acid and different chitosan types at 20 and 40 mg/L on shoot part of 21-day-old seedling including plant height shoot (A), shoot fresh weight (B), shoot dry weight (C). Bar with different letters denote significant differences according to Duncan's multiple range tests (P<0.05)



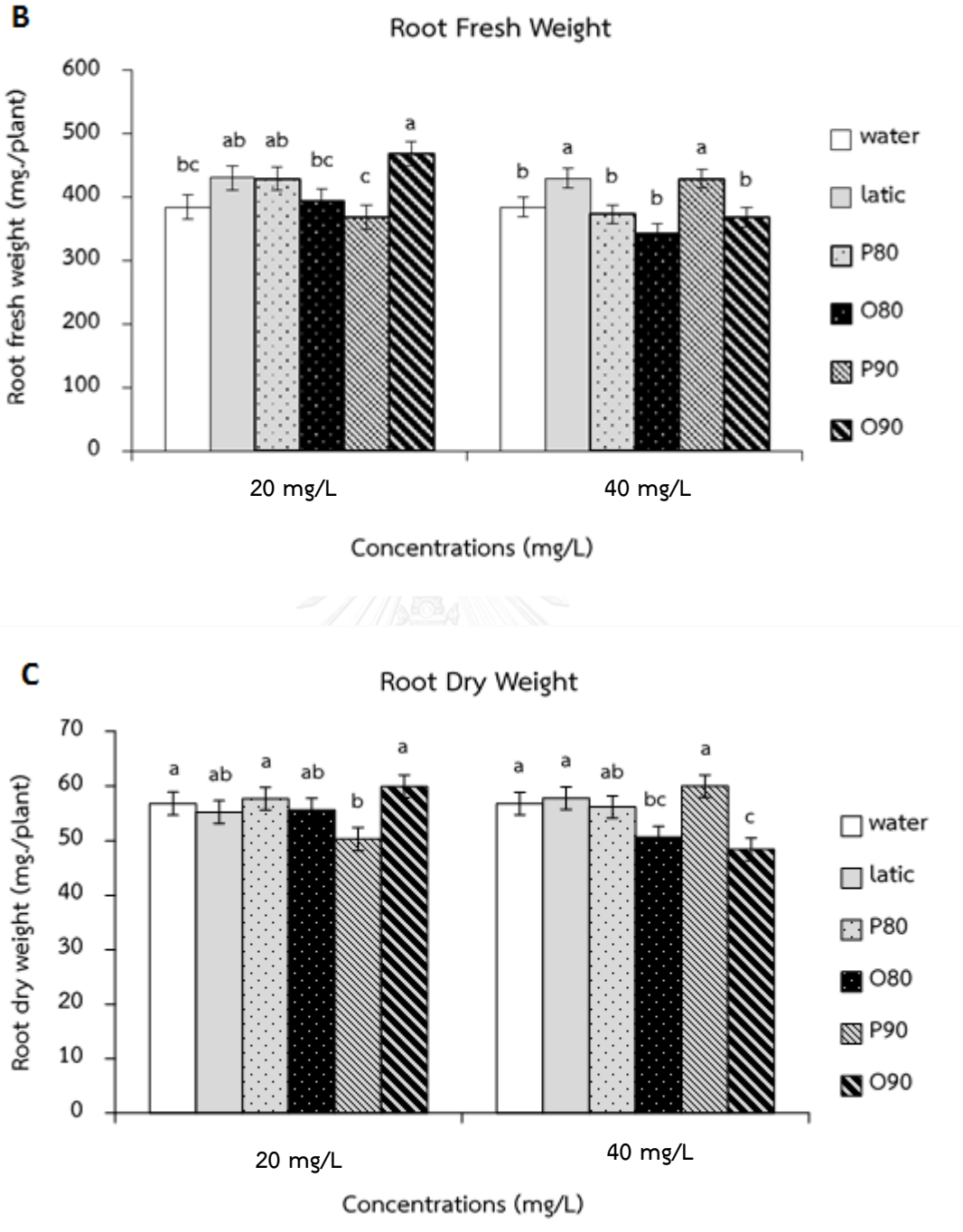


Figure 16. Effect of water, lactic acid and different chitosan types at 20 and 40 mg/L on root part of 21-day-old seedling including root length (A), root fresh weight (B), and root dry weight (C). Bar with different letters denote significant differences according to Duncan's multiple range tests ($P<0.05$)

After seed soaking for 2 days and followed by foliar spraying with 20 mg/L of various chitosan types on 14-day-old seedlings, it was found that there were no significant differences on shoot growth of seedling (PH, SFW and SDW) (Figure 15A – C). Meanwhile, RL of seedling treated with P80 and O90 at 20 mg/L was significantly longer than seedlings treated with water and lactic acid controls (Figure 16A). However, these treatments did not significantly increase RDW when compared to water and lactic acid. Only O90 treatment significantly increased RFW (Figure 16B and C).

On the other hand, the significant differences on the shoot and root growth due to the different types of chitosan were observed in 40 mg/L of chitosan treatments. In comparison with water control, only P90 treatment significantly increased PH, RL and the RFW (~12% increase) but the treatment was not a statistically significant increase when compared to lactic acid control (Figure 15A, Figure 16A and B). Moreover, P90 treatment tended to increase the SFW (11% increase) and SDW (Figure 15C and Figure 16C). In contrast, both 80% and 90% DD of oligomeric chitosan at 40 mg/L (O80-40 and O90-40) showed negative effects on the shoot and root growth (Figure 15A – C and Figure 16A – C). Summarily, P90 chitosan at concentration of 40 mg/L in 1% (v/v) lactic acid was the most effective treatment to increase ‘Pathumthani1’ rice seedling growth.

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5.2. Effects of shrimp shell (SS) and fermented chitin waste (FCW) on rice growth, photosynthesis and yield

5.2.1 The effects of shrimp shell (SS) and fermented chitin waste (FCW) on rice growth and yield

Rice growth

The effects of shrimp shell (SS) and fermented chitin waste (FCW) at concentrations of 0.25%, 0.5% and 1% (w/w) on growth on tiller numbers/plant was elucidated by addition of SS and FCW into soil mixture 7 days before transplantation was shown in Figure 17.

It was found that the significant increase of tiller numbers/plant was observed in SS and FCW treatments at concentration of 0.25% and 0.5% within 30 days after transplanting when compared to chemical (CF) and organic fertilizers (OF) (Figure 17 and Table C-11 see in Appendix C). In addition, these four treatments of SS and FCW showed the maximum tiller numbers/plants after 45 days of transplanting, especially tiller numbers/plants resulted from 0.5% SS and 0.5% FCW treatments showed approximately 3.5-fold higher than CF control. However, tiller numbers/plants in these treatments slightly declined after 60 days of transplantation due to some tiller death (Figure 17 and Table C-11).

Although the application of 1.0% SS or 1.0% FCW caused the delay of tillering stage in rice, it increased tiller numbers/plant after 60 days of transplanting. Particularly, the addition of 1.0% FCW showed the most tiller numbers/plants with 17 tillers/plants after 60 days of transplantation (Figure 17 and Table C-11).

Likewise, varied concentrations of SS and FCW significantly affected the reproductive stage of rice. Generally, the flowering day of ‘Pathumthani1’ rice is approximately 90-95 days after germination. However, in our observation, the low concentrations of SS and FCW addition rapidly induced early flowering in rice. On the other hand, the high concentration of SS and FCW could delay the flowering period (Table C-11).

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At low concentrations of SS and FCW, rice plants grown in soil with the addition of 0.25% SS and 0.25% FCW started flowering around 91 - 93 days of germination, whereas the days of flowering of plants grown in 0.5% SS or FCW treatments were approximately 95 and 93 days after germination, respectively. At high concentration, 1.0% SS and FC application could delay the flowering day to 99 days after germination. While in the control treatments, CF and OF, a flowering-time of rice was about 96.5 and 102 days after germination, respectively (Table C-11).

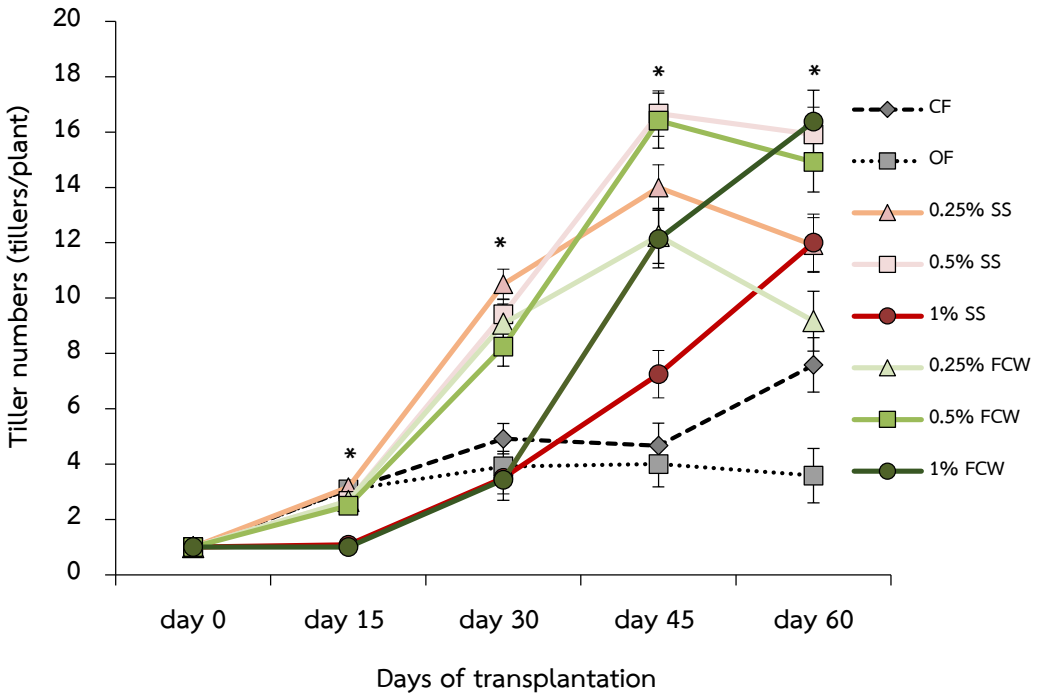

Figure 17. The effect of 0.25, 0.5 and 1% (w/w) shrimp shell (SS) or fermented chitin waste (FCW) as soil supplemented on tiller numbers/plant at 5 timing; 0, 15, 30, 45 and 60 days after transplantation. Bar with star (*) indicated significant differences according to Duncan's multiple range tests (P<0.05).

Table 4. The effects of shrimp shell (SS) and fermented chitin waste (FCW) as soil supplement at concentration of 0.25, 0.5 and 1% (w/w) on days of flowering, yield components consisting of panicle/pot, spikelets/panicle, filled grain/panicle, grain weight/pot, 100-grain-weight and dry weight of shoot biomass after harvest

Treatment	Conc. (% w/w)	Day of flowering	Dry weight of shoot biomass	Yield component				
				Number of panicle/pot	Spikelets/ panicle	Filled grain/panicle (seed)	100-grain-weight	Grain weight/pot
CF		96.5 ± 2.4 ^{bc}	17.5 ± 1.6 ^{e}	19.0 ± 4.1 ^{d}	51.2 ± 28.9 ^{e}	40.8 ± 25.0 ^{e}	2.20 ± 0.04 ^{de}	18.4 ± 3.0 ^{f}
OF		102.0 ± 4.3 ^{a}	6.5 ± 1.0 ^{f}	9.0 ± 1.4 ^{e}	16.3 ± 21.2 ^{d}	12.7 ± 17.5 ^{d}	2.14 ± 0.03 ^{e}	5.2 ± 1.2 ^{g}
SS	0.25	91.5 ± 2.4 ^{d}	33.6 ± 3.0 ^{d}	28.0 ± 2.9 ^{c}	69.5 ± 21.1 ^{c}	57.5 ± 19.2 ^{c}	2.24 ± 0.03 ^{bcd}	33.5 ± 4.9 ^{e}
	0.5	95.5 ± 1.9 ^{bcd}	56.0 ± 3.2 ^{b}	41.0 ± 2.7 ^{a}	80.0 ± 19.6 ^{b}	65.1 ± 18.6 ^{b}	2.27 ± 0.02 ^{abc}	59.6 ± 3.0 ^{c}
	1	102.8 ± 4.5 ^{a}	66.9 ± 8.2 ^{a}	44.5 ± 4.7 ^{a}	117.9 ± 38.0 ^{a}	97.6 ± 34.5 ^{a}	2.30 ± 0.04 ^{ab}	90.4 ± 10.0 ^{a}
FCW	0.25	93.0 ± 1.8 ^{cd}	28.4 ± 1.2 ^{d}	21.3 ± 1.3 ^{d}	68.2 ± 21.9 ^{c}	57.2 ± 19.6 ^{c}	2.25 ± 0.03 ^{bcd}	28.0 ± 2.3 ^{e}
	0.5	93.5 ± 1.9 ^{cd}	49.0 ± 2.7 ^{c}	36.0 ± 0.8 ^{b}	76.4 ± 21.9 ^{b}	65.0 ± 20.5 ^{b}	2.33 ± 0.06 ^{a}	49.9 ± 6.1 ^{d}
	1	99.5 ± 1.7 ^{ab}	67.1 ± 9.5 ^{a}	36.8 ± 1.5 ^{b}	110.7 ± 31.6 ^{a}	92.9 ± 26.0 ^{a}	2.22 ± 0.05 ^{cd}	79.4 ± 11.1 ^{b}

Data are shown as means of 12 replicated plants/treatment ± 1 SD. Means within a column followed by a different letter are significantly different (P<0.05) according to Duncan's multiple range test.

Addition of SS and FCW at all concentrations, 0.25%, 0.5% and 1.0% (w/w), significantly increased shoot dry weight. The increase of plant biomass was associated with the increasing concentrations of these materials.

The 1.0% SS or 1.0% FCW treatments displayed the highest degree of shoot biomass accumulation but no significant difference between the effects on growth caused by these two types of materials was found. Shoot biomasses in these two treatments were approximately 3.8 times higher than the biomass obtained from CF control (Table 4).

Rice yield

Interestingly, SS and FCW supplement increased yield components in dose-dependent manner. All concentrations of SS and FCW addition to soil (0.25 %, 0.5 % and 1.0 %) significantly enhanced yield components including numbers of panicle/pot, spikelets/ panicle, filled grains/panicle as well as grain weight/pot, when compared to CF and OF controls (Table 4).

In term of panicle numbers/pot, rice plants grown in soil with addition of 0.5% - 1.0% SS produced the significantly increased panicles numbers/pot by 2.2-fold and 2.3-fold when compared to CF control, respectively. Meanwhile in FCW treatments, panicles numbers/pot in the addition of 0.5% - 1.0% FCW treatments increased 1.8- and 1.9-fold when compared to CF control, respectively. However, there was no significant differences between two concentrations in both SS and FCW additions. On the other hand, varied concentrations of each material affected spikelets/panicle and filled grain/panicle. The supplementation with 1.0% SS and 1.0% FCW significantly increased spikelets/panicle and filled grains/panicle approximately 2.3-fold which was the highest value among all experiments when compared to CF control. However, there was no significant difference when compared between the effects caused by these two types of materials (Table 4).

For rice productivity, soil supplementation of 1.0% SS or 1.0% FCW significantly improved grain yield/pot, which were 4.9 and 4.3 times, respectively higher than the grain yield/pot obtained from CF control (Table 4).

5.2.2 Effects of shrimp shell (SS) and fermented chitin waste (FCW) on photosynthetic characteristics

To explain how SS and FCW enhanced rice growth and productivity, the chloroplast size, photosynthetic pigment contents and net photosynthesis rate were examined at vegetative and reproductive stages.

Chloroplast diameter and photosynthetic pigment contents

At vegetative stage (35 days after transplantation), the effects of shrimp shell (SS) and fermented chitin waste (FCW) at concentrations of 0.25%, 0.5% and 1% on chloroplast size and photosynthetic pigment contents were investigated (Table 5).

For chloroplast diameter, the addition of 0.25% SS was significantly enhanced chloroplasts diameter when compared to other treatments, whereas the smallest chloroplasts size was found in the treatments of 0.5% SS or FCW soil supplementation. The average chloroplast size was ranged from 2.9 – 3.1 μm among all treatments. (Table 5).

For photosynthetic pigment contents, both of SS and FCW applications significantly increased photosynthetic pigment contents. Although the addition of 0.5% SS and 0.5% FCW resulted in the smallest chloroplasts size, these treatments showed the significantly highest photosynthetic pigment contents including chlorophyll *a*, chlorophyll *b* and carotenoid. The level of pigment contents in plants grown in soil with addition of 0.5% SS was 1.4 - 1.7 times, whereas 0.5% FCW addition was 1.5 – 1.8 times higher than the level found in plant grown in soil supplemented with CF control. The lowest photosynthetic pigment content was found in OF control (Table 5).

Table 5. The effect of shrimp shell (SS) and fermented chitin waste (FCW) concentrations on chloroplast diameter and photosynthetic pigment contents: chlorophyll *a*, *b* and carotenoid at 35 days after transplantation

Treatment	Conc. (% w/w)	Chloroplast diameter (μm)	Photosynthetic pigment contents (mg/g FW)		
			Chl *a*	Chl *b*	carotenoid
CF		3.1 ± 0.37^{bc}	1.43 ± 0.31^{b}	0.50 ± 0.12^{bc}	0.56 ± 0.14^{bc}
OF		3.0 ± 0.44^{c}	1.30 ± 0.12^{b}	0.46 ± 0.04^{c}	0.48 ± 0.05^{c}
SS	0.25	3.2 ± 0.41^{a}	1.94 ± 0.25^{ab}	0.71 ± 0.10^{ab}	0.74 ± 0.1^{ab}
	0.5	2.9 ± 0.39^{d}	2.41 ± 0.26^{a}	0.80 ± 0.15^{a}	0.79 ± 0.15^{ab}
	1	3.1 ± 0.44^{b}	2.11 ± 0.79^{a}	0.65 ± 0.31^{abc}	0.65 ± 0.32^{abc}
FCW	0.25	3.1 ± 0.37^{bc}	2.27 ± 0.64^{a}	0.67 ± 0.05^{abc}	0.72 ± 0.06^{abc}
	0.5	2.9 ± 0.31^{d}	2.57 ± 0.50^{a}	0.84 ± 0.11^{a}	0.84 ± 0.11^{a}
	1	3.1 ± 0.30^{bc}	2.32 ± 0.20^{a}	0.79 ± 0.25^{a}	0.80 ± 0.25^{ab}

Data are shown as the mean ± 1 SD and are derived from 4 independent repeats. Means were compared by using ONEWAY-ANOVA and different letter represented significantly different (P<0.05).

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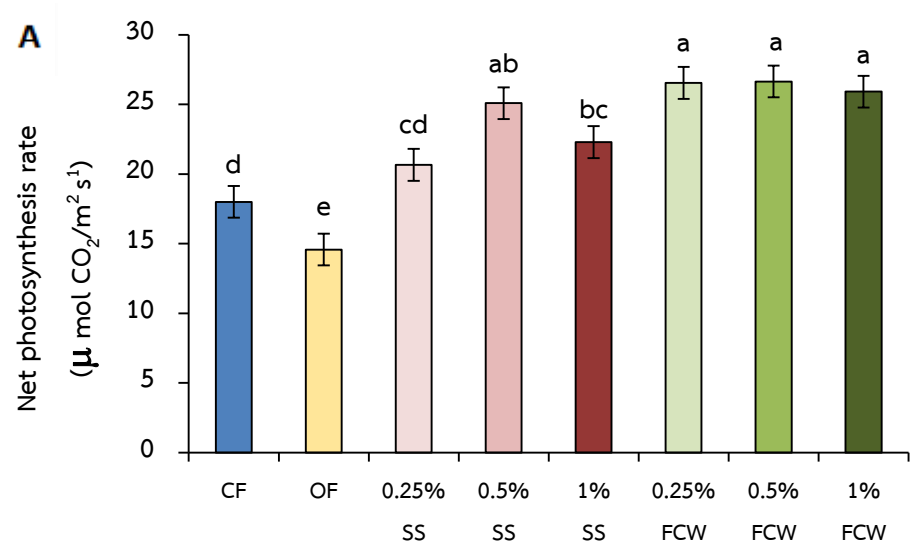
Net photosynthesis rate

In order to understand the effect of SS and FCW application on plant growth and photosynthetic activity, net photosynthesis rate (A_{max}), stomatal conductance (g_s), internal concentration of CO_2 (C_i) and transpiration rate (E) were determined in 'Pathumthani1' rice after 30 days of SS or FCW addition to soil supplemented (Figure 18 and Table C-12 see in Appendix C).



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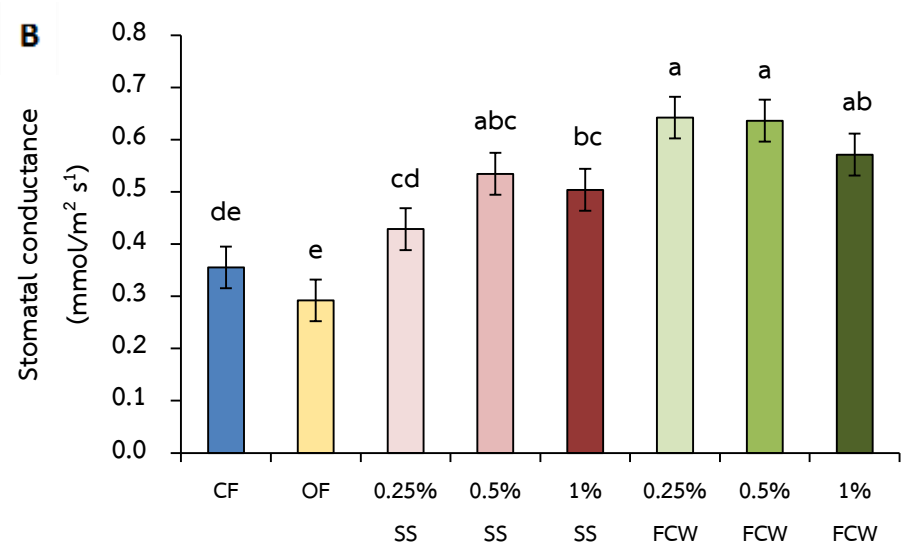


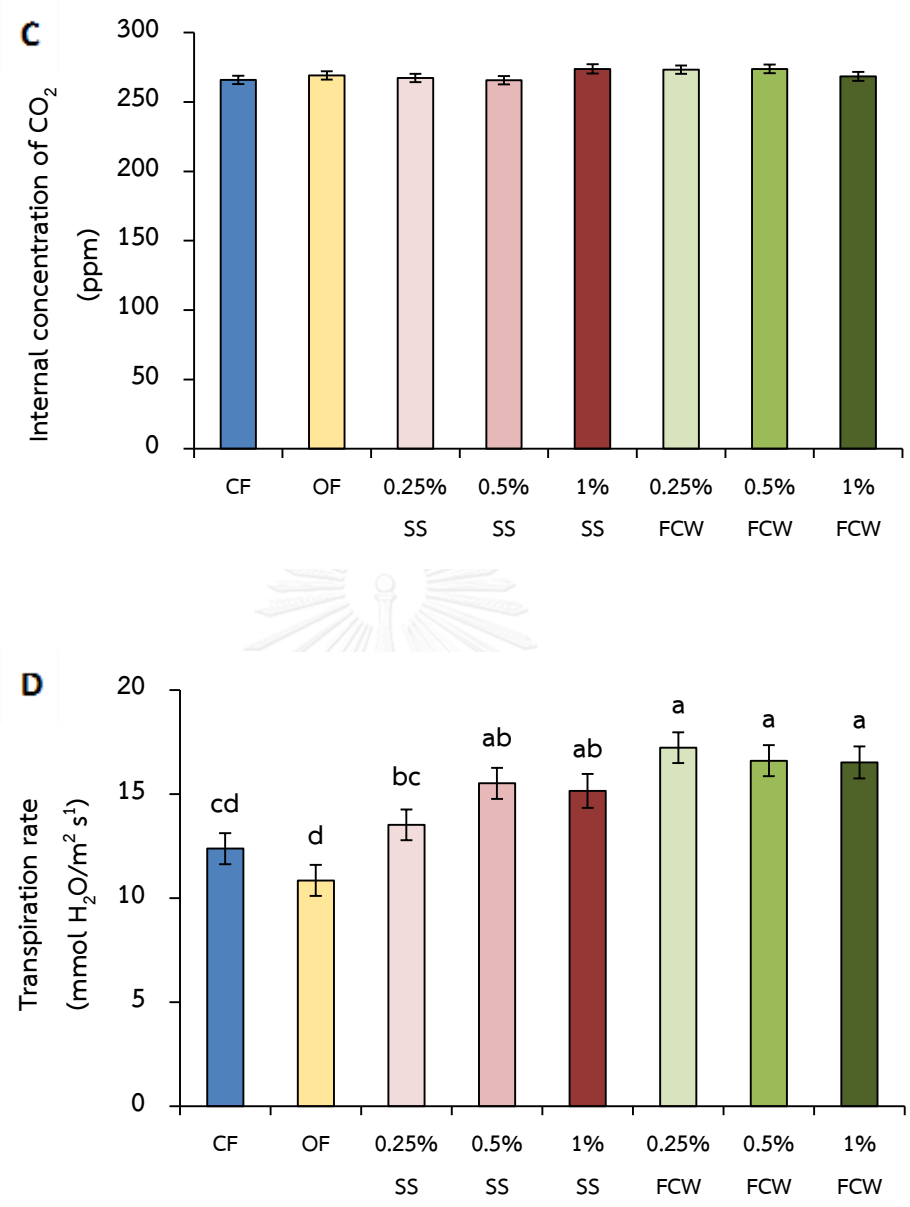
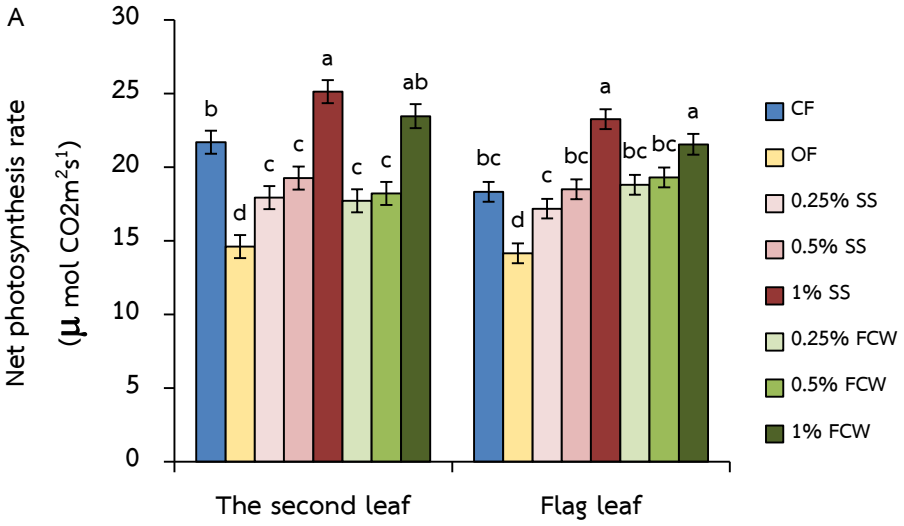


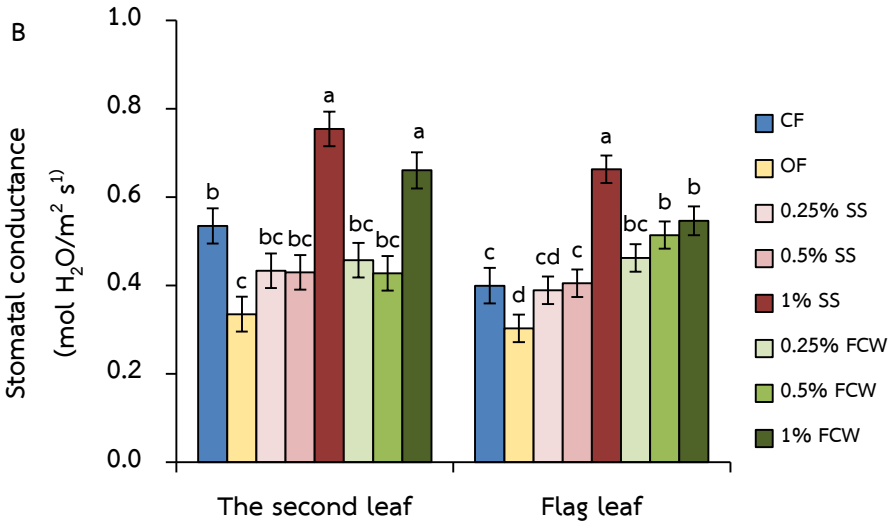
Figure 18. The effect of 0.25, 0.5 and 1% (w/w) shrimp shell (SS) or Fermented chitin waste (fermented chitin: FCW) as soil supplemented on net photosynthesis rate (A), stomatal conductance (B), Internal concentration of CO_2 (C), and transpiration rate (D) at vegetative stage after 30 days of transplantation. Bar with different letters stand for significant differences according to Duncan's multiple range tests (P<0.05).

Rice plants grown in soil supplemented with SS and FCW not only had the higher concentration of photosynthetic pigment contents but also had the higher net photosynthetic rate at vegetative stage. After 30 days of transplantation, net photosynthesis rate (A_{max}) of all SS and FCW treated plants were significantly higher than CF and OF controls (Figure 18A). Particularly, all FCW treatments, which were no significantly different among concentrations, showed the highest A_{max} about 1.4 fold compared to CF control, while the varied responses of A_{max} in SS treatments depended on their concentrations (Figure 18A). Moreover, the increase of A_{max} in both SS and FCW treatments was correlated to the increase of stomata conductance (g_s) and transpiration rate (E), whereas the intercellular CO_2 concentration (C_i) was not different (Figure 18).

At reproductive stage, the second leaf at day 60 after transplantation and the flag leaf at booting period were used to investigate the effect of SS and FCW additions on photosynthetic parameters such as net photosynthesis rate (A_{max}), stomatal conductance (g_s), internal concentration of CO_2 (C_i), and also transpiration rate (E) (Figure 19A-D and Table C-13, C-14 see in Appendix C). The highest A_{max} was observed in both the second leaf and the flag leaf of plants grown in soil supplemented with 1% SS or 1% FCW (Figure. 19A). On the other hand, A_{max} of the second leaf from plants treated with 0.25 – 0.5% SS or FCW was significantly lower than that of CF control at reproductive stage (60 days after transplantation) (Figure 19A). The similar phenomenon was also observed in stomatal conductance (g_s) and transpiration rate (E) (Figure 19B and 19D). Similar to the vegetative stage, there was no significant difference among treatments in internal CO_2 concentration (C_i) of the second leaf (Figure 19 C).

Moreover, net photosynthesis rate (A_{max}), stomatal conductance (g_s) and transpiration rate (E) of flag leaf had the similar trend to the second leaf in both SS and FCW treatments (Figure 19A-B and 19D). However, a significant difference of internal CO_2 concentration (C_i) was observed (Figure 19C).


A
Net photosynthesis rate
(μ mol CO2m-2s-1)
30
25
20
15
10
5
0
b
d
c
c
a
c
c
ab
bc
d
c
bc
a
bc
bc
a
The second leaf
Flag leaf
CF
OF
0.25% SS
0.5% SS
1% SS
0.25% FCW
0.5% FCW
1% FCW


B
Stomatal conductance
(mol H2O/m2 s1)
1.0
0.8
0.6
0.4
0.2
0.0
b
c
bc
bc
a
bc
bc
a
c
d
cd
c
a
bc
b
b
The second leaf
Flag leaf
CF
OF
0.25% SS
0.5% SS
1% SS
0.25% FCW
0.5% FCW
1% FCW

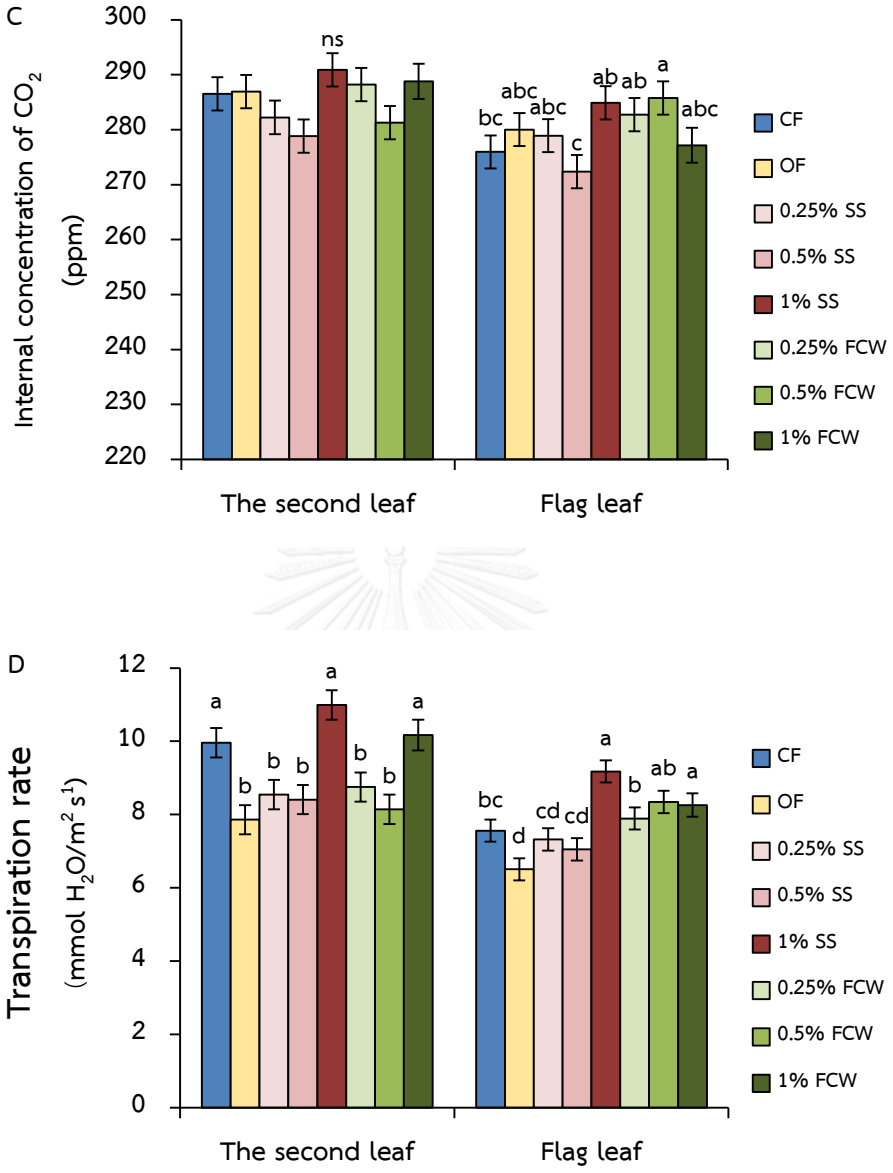


Figure 19. The effect of 0.25, 0.5 and 1% (w/w) shrimp shell (SS) or fermented chitin waste as soil supplemented on net photosynthesis rate (A), stomatal conductance (B), Internal concentration of CO_2 (C), and transpiration rate (D) of the second leaf and flag leaf at reproductive stage on 60 days after transplantation and booting period. Bar with different letters stand for significant differences according to Duncan's multiple range tests (P<0.05).

5.2.3 Effects of shrimp shell (SS) and fermented chitin waste (FCW) on soil pH and nutrition component

The chemical characteristics of soil before transplanting and after harvest were analyzed in term of pH, electrical conductivity (EC), organic matters (OM) and contents of nitrogen (N), phosphorus (P), potassium (K), calcium (Ca) and magnesium (Mg) (Table 6 and Table 7).

Before transplantation

Addition of SS and FCW resulted in the increase of soil pH, total nitrogen, contents of phosphorus and organic matter. The increase in soil nutrient was correlated with the increasing of SS and FCW levels.

In term of pH, soil pH in CF and OF controls before transplantation were 5.0 and 5.1, respectively, whereas soil with SS addition ranged from pH 6.2 - 7.3. In FCW treatments, soil pH was lower than SS treatments and ranged from 5.3 - 5.9. Furthermore, addition of 1% SS and 1% FCW significantly increased total nitrogen in soil from 0.16% in CF control to 0.25% and 0.21%, respectively. For P availability, soil treated with 1% SS was 5.7 times higher than soil with CF control, while 1% FCW treatment resulted in 3.6 times higher in P availability, compared to CF control. Moreover, the highest organic matter (OM) was observed in soil treated with the highest concentration of each SS and FCW addition, approximately 2.4% increasing from 1.8% in CF control. Interestingly, the significant increase of K availability in soil was only observed in FCW addition, whereas the significant increase of calcium content was only found in SS addition (Table 6).

Table 6. Chemical characteristics of soil before transplanting including pH, electrical conductivity (EC), total nitrogen (N), contents of phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), organic matters (OM) and also organic carbon (OC)

Treatment	Conc. (% w/w)	Before transplanting								
		pH	EC	Total N (%)	P (mg/Kg)	K (mg/Kg)	Ca (mg/Kg)	Mg (mg/Kg)^{ns}	OM (%)	OC (%)
CF		5.0 ± 0.2^{g}	0.40 ± 0.02^{bc}	0.16 ± 0.02^{d}	20.43 ± 1.81^{d}	93.5 ± 4.1^{d}	905.4 ± 82.3^{b}	472.8 ± 35.9	1.8 ± 0.2^{c}	1.1 ± 0.1^{c}
OF		5.1 ± 0.1^{fg}	0.44 ± 0.01^{a}	0.17 ± 0.01^{cd}	20.55 ± 2.17^{d}	92.2 ± 0.0^{d}	952.9 ± 29.0^{b}	490.9 ± 34.2	1.9 ± 0.2^{c}	1.1 ± 0.1^{c}
SS	0.25	6.2 ± 0.2^{c}	0.34 ± 0.05^{de}	0.15 ± 0.02^{d}	30.40 ± 5.93^{d}	98.5 ± 5.3^{d}	1002.5 ± 109.9^{b}	484.9 ± 36.2	2.0 ± 0.1^{bc}	1.2 ± 0.1^{bc}
	0.5	7.1 ± 0.1^{b}	0.32 ± 0.03^{e}	0.21 ± 0.02^{b}	66.45 ± 8.21^{b}	97.0 ± 2.6^{d}	1177.1 ± 170.2^{a}	473.3 ± 35.3	2.2 ± 0.1^{ab}	1.3 ± 0.1^{ab}
	1	7.3 ± 0.0^{a}	0.50 ± 0.05^{a}	0.25 ± 0.01^{a}	116.75 ± 19.94^{a}	98.5 ± 2.5^{d}	1274.2 ± 63.6^{a}	465.4 ± 20.2	2.4 ± 0.2^{a}	1.4 ± 0.1^{a}
FCW	0.25	5.3 ± 0.1^{ef}	0.40 ± 0.03^{bc}	0.19 ± 0.02^{bc}	32.30 ± 2.79^{cd}	122.5 ± 3.0^{c}	873.2 ± 15.4^{b}	476.5 ± 21.5	2.0 ± 0.1^{bc}	1.1 ± 0.0^{bc}
	0.5	5.5 ± 0.1^{e}	0.39 ± 0.01^{bcd}	0.17 ± 0.01^{cd}	47.79 ± 5.60^{c}	150.5 ± 3.8^{b}	877.0 ± 79.2^{b}	483.2 ± 19.4	2.1 ± 0.0^{b}	1.2 ± 0.0^{b}
	1	5.9 ± 0.3^{d}	0.36 ± 0.07^{cde}	0.21 ± 0.02^{b}	73.33 ± 8.66^{b}	218.2 ± 19.4^{a}	871.5 ± 93.6^{b}	481.4 ± 43.8	2.4 ± 0.1^{a}	1.4 ± 0.1^{a}

Data are shown as means of 4 replicated soils per treatment ± 1 SD. Means within a column followed by a different letter are significantly different (P<0.05) according to Duncan's multiple range test. ns = no significant difference.

After harvest

After rice harvest, the range of soil pH in both of SS and FCW treatments tended to deceased when compared to the soil before transplantation. In detail, soil with SS addition ranged from pH 5.4 - 5.9, while soil supplemented with FCW has pH ranging from pH 5.0 - 5.2. In comparison with soil pH before transplantation, soil treated with chemical fertilizer alone increased from pH 5.0 to pH 5.2, while soil treated with chicken manure fertilizer alone dramatically increased from pH 5.1 to pH 5.7. (Table 6 and Table 7).

In addition, the highest organic matter still remained in soil after harvest about 24% in 1% SS and 1% FCW treatments. For total nitrogen, soil treated with 1% SS and 1% FCW showed the highest total nitrogen, approximately 0.18% and 0.19%, respectively. Whereas, total nitrogen in CF control was 0.13%. Moreover, soil treated with 1% SS increased 5.5-fold of P availability compared to soil in CF control, while 1% FCW treatment was 2.1-fold increase. There were no significant differences in EC and Mg availability (Table 7).

The increasing of the soil nutrition component after harvest can occurred by the addition of organic fertilizer and chemical fertilizer on day 20 and day 60 after transplantation.

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Table 7. Chemical characteristics of soil after harvest including pH, electrical conductivity (EC), total nitrogen (N), contents of phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), organic matters (OM) and also organic carbon (OC)

Treatment	Conc. (% w/w)	After harvest								
		pH	EC ^{ns}	Total N (%)	P (mg/Kg)	K (mg/Kg)	Ca (mg/Kg)	Mg (mg/Kg)^{ns}	OM (%)	OC (%)
CF		5.2 ± 0.3^{cd}	0.99 ± 0.28	0.13 ± 0.02^{c}	31.16 ± 9.27^{c}	68.0 ± 5.2^{bc}	2251.1 ± 350.4^{c}	506.3 ± 44.6	1.9 ± 0.2^{b}	1.1 ± 0.1^{b}
OF		5.1 ± 0.2^{b}	0.99 ± 0.13	0.15 ± 0.01^{bc}	40.20 ± 9.14^{bc}	86.5 ± 7.9^{a}	3079.8 ± 840.5^{bc}	548.7 ± 61.7	1.9 ± 0.2^{b}	1.1 ± 0.1^{b}
SS	0.25	6.2 ± 0.1^{bc}	1.24 ± 0.31	0.15 ± 0.01^{bc}	55.36 ± 12.93^{bc}	59.5 ± 8.9^{cd}	3939.8 ± 1050.6^{ab}	516.5 ± 48.4	2.3 ± 0.2^{a}	1.3 ± 0.1^{a}
	0.5	7.1 ± 0.1^{b}	1.19 ± 0.43	0.16 ± 0.02^{ab}	46.11 ± 13.96^{bc}	48.0 ± 2.8^{e}	2329.4 ± 676.2^{c}	491.8 ± 58.1	2.0 ± 0.1^{b}	1.2 ± 0.1^{b}
	1	7.3 ± 0.1^{a}	1.14 ± 0.20	0.18 ± 0.03^{ab}	172.83 ± 37.36^{a}	52.5 ± 7.0^{de}	3193.8 ± 427.7^{abc}	476.3 ± 52.8	2.4 ± 0.1^{a}	1.4 ± 0.1^{a}
FCW	0.25	5.3 ± 0.2^{cd}	1.22 ± 0.48	0.16 ± 0.02^{abc}	30.33 ± 5.77^{c}	73.0 ± 2.0^{b}	4153.5 ± 680.8^{a}	503.5 ± 32.1	2.1 ± 0.1^{b}	1.2 ± 0.1^{b}
	0.5	5.5 ± 0.1^{d}	1.31 ± 0.06	0.16 ± 0.03^{ab}	43.76 ± 7.40^{bc}	67.0 ± 6.0^{bc}	3715.6 ± 649.7^{ab}	519.9 ± 12.4	2.4 ± 0.1^{a}	1.4 ± 0.1^{a}
	1	5.9 ± 0.2^{cd}	1.25 ± 0.16	0.19 ± 0.02^{a}	66.96 ± 22.10^{b}	70.0 ± 4.3^{b}	3196.1 ± 301.9^{abc}	497.0 ± 38.7	2.3 ± 0.1^{a}	1.3 ± 0.1^{a}

Data are shown as means of 4 replicated soils per treatment ± 1 SD. Means within a column followed by a different letter are significantly different (P<0.05) according to Duncan's multiple range test. ns = no significant difference.

5.3. The gene expression induced by shrimp shell and fermented chitin waste when these substances are used as stimulants.

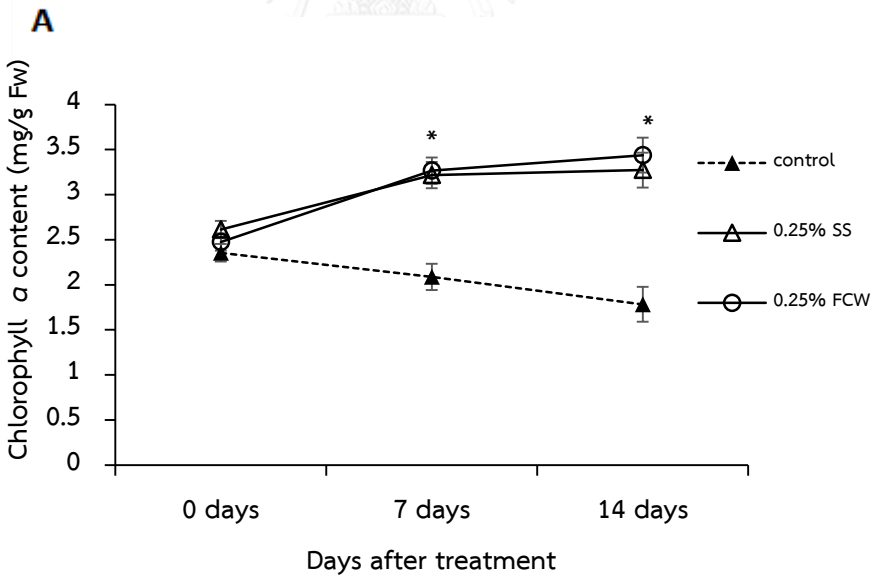
According to the result from session 5.2., 1% SS or 1% FCW application showed the highest yield components at reproductive stage but delayed tiller productivity at vegetative stage. Meanwhile, 0.25% SS and FCW additions showed the highest tiller number/plant on day 15 and day 30 after transplantation (Figure 17 and Table C-11 see in Appendix C). Photosynthetic pigment contents also numerically increased when compared to controls at 35 days after transplantation (Table 5) Therefore, 0.25% SS and FCW addition were chosen to investigate chitin/chitosan-induced gene expression which involved in photosynthesis.

Chamnanmanoontham et al. (2015) reported that foliar application of 40 mg/L chitosan oligomer could stimulate vegetative growth at seedling stage of rice cultivar LPT 123. In addition, from proteomic and co-expression network analysis reveal that chitosan can up-regulated proteins involving photosynthesis process. From the research, *Oxygen-evolving enhancer protein 1* gene (*OEE1*) and *Chlorophyll a-b binding protein gene (PsbS1)* in light reaction together with *Ribulose bisphosphate carboxylase small chain gene (rbcS)* in carbon fixation were selected to determine the effect of 0.25% SS and FCW as soil supplement on the net photosynthesis rate, photosynthetic pigment content and also gene expression.

Photosynthetic pigment contents

Before starting the experiment, no significant differences in photosynthetic pigment contents among treatments were found. However, after treatment, the addition of 0.25% SS and 0.25% FCW significantly induced photosynthetic pigment contents including chlorophyll *a*, chlorophyll *b* and carotenoid, compared to control within 7 days after transplantation. Particularly, plants grown in soil supplemented with 0.25% FCW had the significant highest quantity in all types of photosynthetic pigment.

After 7 days of treatment, chlorophyll *a* and *b* content in seedlings treated with 0.25% FCW was approximately 1.6 and 1.5 times higher than non-treated ones, respectively, and carotenoid content was also increased by 1.4 fold. Moreover, both of chlorophyll *a* and chlorophyll *b* contents in 14-day-old seedlings were approximately 1.9 times higher than non-treated plants, whereas carotenoid content was increased by 1.8-fold (Figure 20 and Table C-15 see in Appendix C).



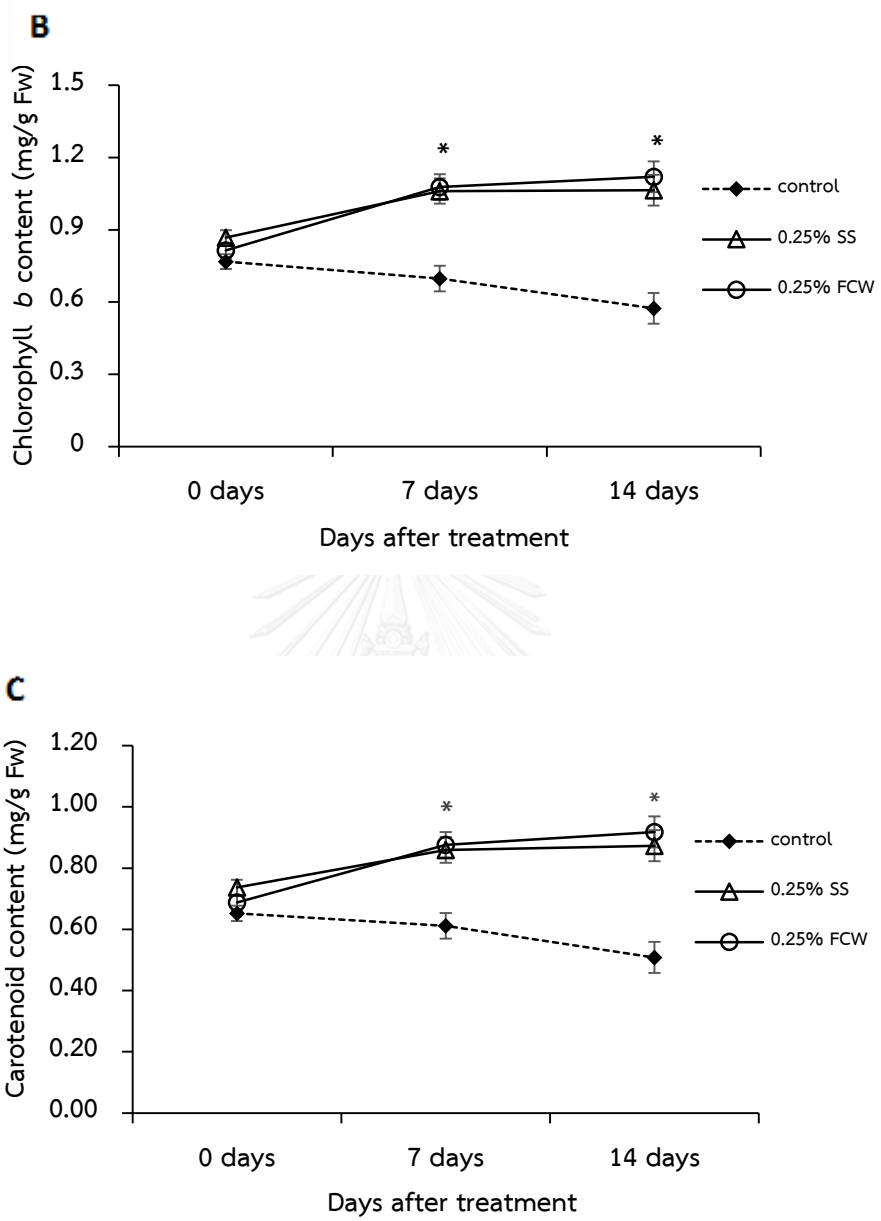
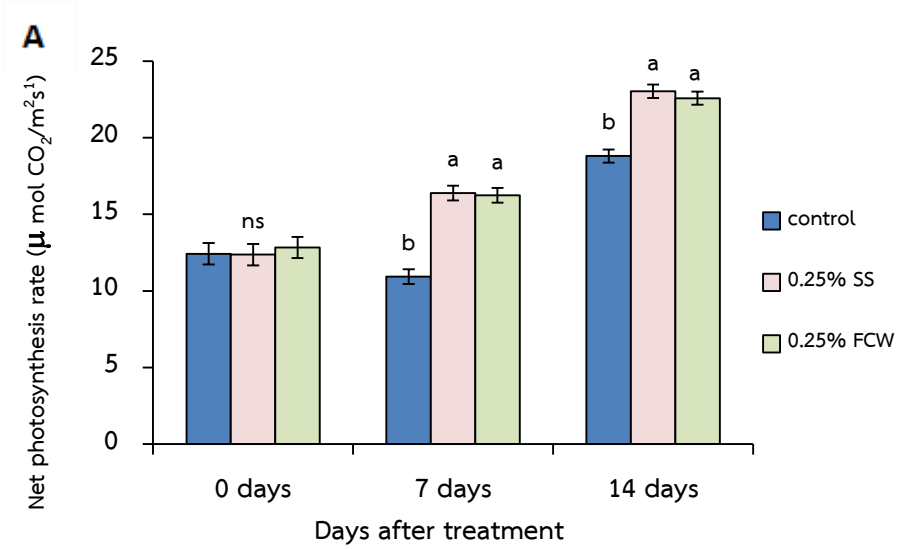


Figure 20. The effect of 0.25% (w/w) shrimp shell (SS) or fermented chitin waste (FCW) as soil supplemented on contents of chlorophyll *a* (A), chlorophyll *b* (B) *and* carotenoid (C) on day 0, 7 and 14 after treatment. Bar with star (*) represented for significant differences according to Duncan's multiple range tests ($P<0.05$).

Net photosynthesis rate

There were no significant differences in photosynthetic parameters in seedlings before treatments. After soil supplemented with SS and FCW, seedlings treated with 0.25% SS or 0.25% FCW significantly increased in A_{max}, stomatal conductance (g_s), and transpiration rate (E) when compared to non-treated plants. The increasing of stomatal conductance (gs) and transpiration rate (E) were observed only at day 7 after transplantation. In contrast, internal concentration of CO_2 (C_i) of both SS and FCW treated-seedling significantly decreased throughout the experiment (Figure 21 and Table C-16 – C-19 see in Appendix C).



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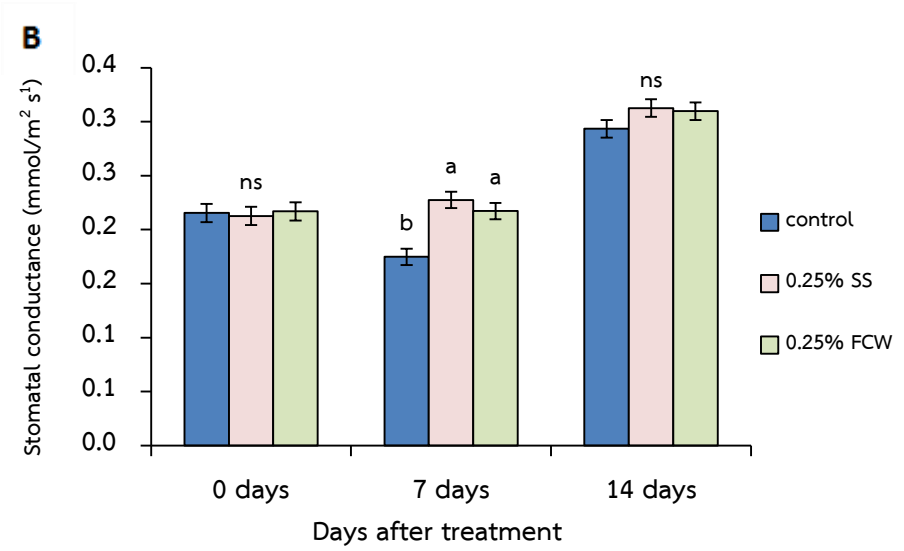

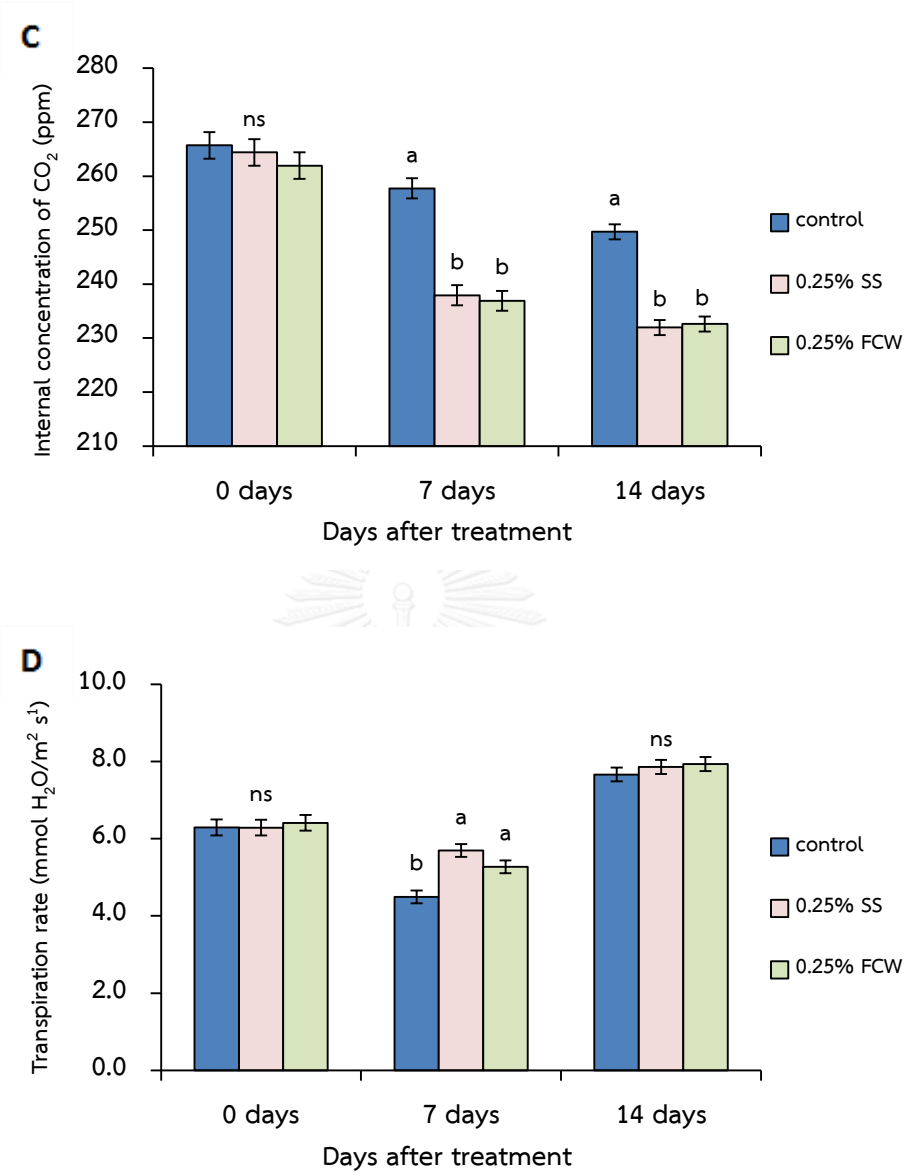

Figure 21. Effects of 0.25% (w/w) shrimp shell (SS) or fermented chitin waste (FCW) as soil supplemented on net photosynthesis rate (A), stomatal conductance (B), Internal concentration of CO_2 (C), and transpiration rate (D) of seedling at 0, 7 and 14 days after treatment. Bar with different letters stand for significant differences according to Duncan's multiple range tests (P<0.05).

Gene expressions

In order to investigate the expression of genes involving photosynthesis process leading to plant growth enhancement, three genes, previously shown to respond to chitosan elicitation in proteomic experiments, were used to examine for the gene expression response in 'Pathumthani1' rice to SS and FCW addition. The gene expression of *OEE1* and *PsbS1* gene which encoded protein in light reaction was analyzed together with *rbcS* gene in CO_2 fixation by using quantitative RT-PCR technique (Figure 22 - 24).

Oxygen-evolving enhancer protein 1 (OEE1) in light reaction

The gene expression of *OEE1* gene encoding Oxygen-evolving enhancer protein1 in light reaction was not significantly different among all treatments. However, in FCW treatment, it showed highest level of gene expression 7 days after treatment. In contrast, the level of *OEE1* transcript in SS treatment tended to lower than non-treated plants (Figure 22).

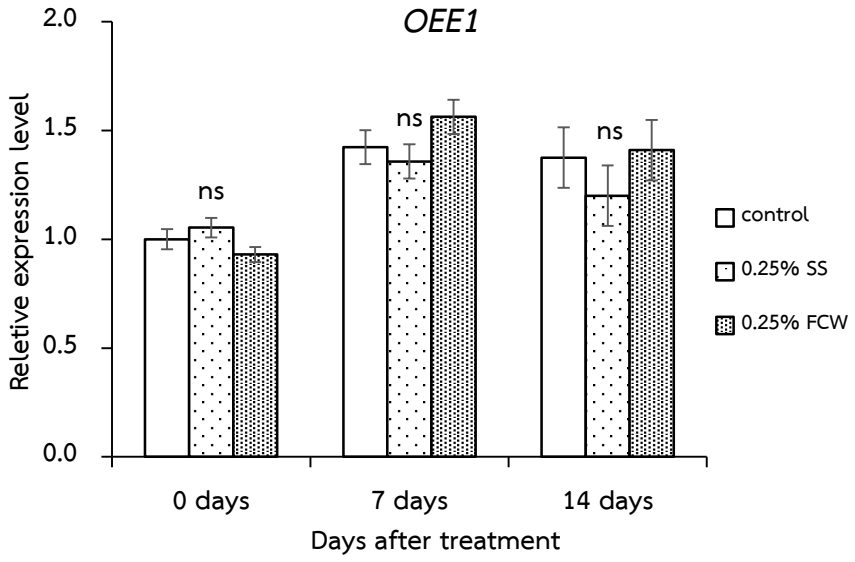

Figure 22. Relative gene expression of *OEE1* on seedling treated with SS and FCW as soil supplement at days 7 and 14 after treatment. Bar with different letters stand for significant differences according to Duncan's multiple range tests (P<0.05).

Chlorophyll a-b binding protein gene (PsbS1) in light reaction

In SS treatment, *PsbS1* transcript was down-regulated after 7 days of SS application. However, after 14 days of treatment, the expression level of *PsbS1*gene in SS treatment and control treatment were similar. Although the transcript of *PsbS1* in FCW treatment 7 days after treatment was not significantly different from non-treated plants, the level of *PsbS1* transcript significantly increased, approximately 2.4 times within 14 day after treatment when compared to control plants, approximately 1.4 times (Figure 23).

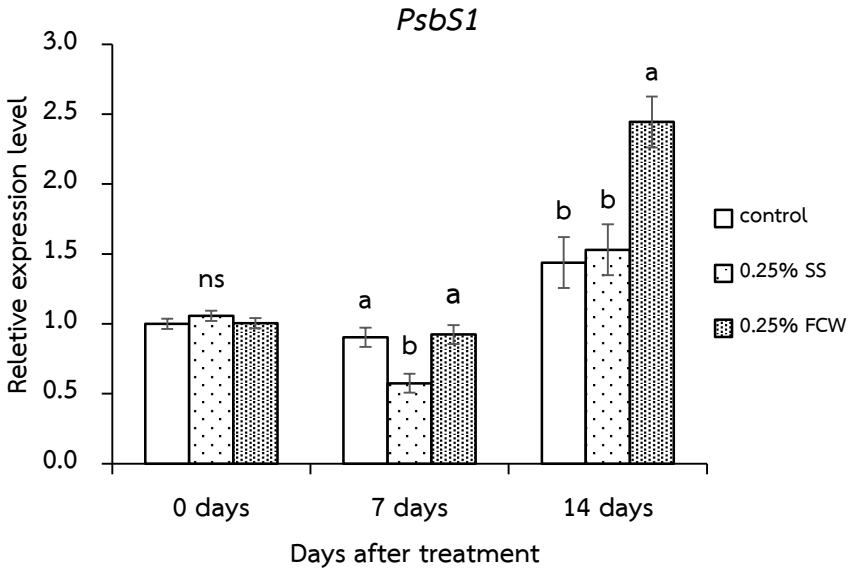

Figure 23. Relative gene expression of *PsbS1* on seedling treated with SS and FCW as soil supplement at days 7 and 14 after treatment. Bar with different letters stand for significant differences according to Duncan's multiple range tests ($P<0.05$)

Ribulose bisphosphate carboxylase small chain gene (rbcS) in carbon fixation

The expression level of *rbcS* mRNA in non-treated plants slightly increased over time and the level of *rbcS* transcript in FCW treatment was the same level as control treatment. Meanwhile in SS treatment, the transcript of *rbcS* significantly decreased at 7 days after treatment and was maintained until day 14 after treatment (Figure 24).

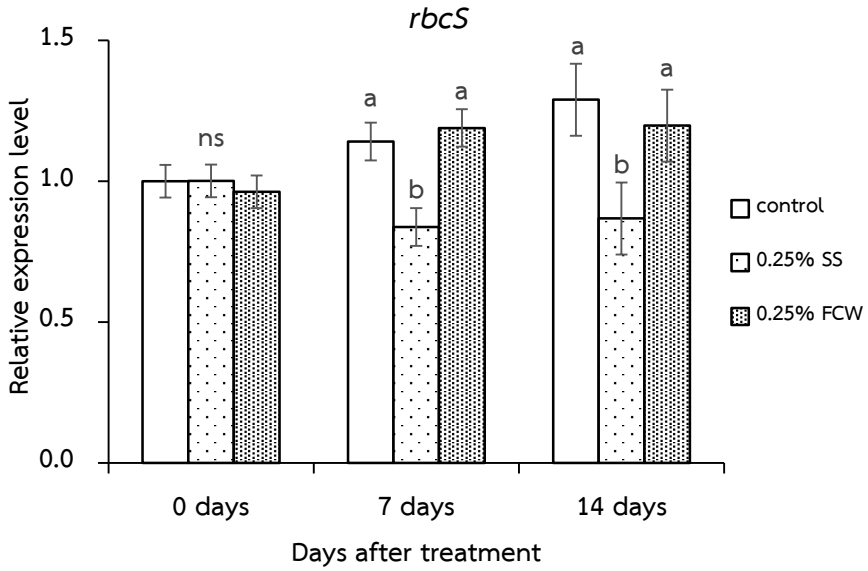

Figure 24. Relative gene expression of *rbcS* on seedlings treated with SS or FCW as soil supplement 7 and 14 days after treatment. Bar with different letters stand for significant differences according to Duncan's multiple range tests ($P<0.05$).

CHAPTER VI
DISCUSSION

6.1. Effects of different types and concentrations of chitosan molecules on rice seedling growth

The negative impact on growth of 'Pathumthani1' rice seedlings when applied with all four types of chitosan in 1% (v/v) acetic acid (Table C-1 to C-6 in Appendix C), and also with the O80 and O90 in 1% (v/v) lactic acid (Figure 15 and Figure 16) suggested the caution in chitosan application in agriculture. Only O90 at 40 mg/L in 1% (v/v) lactic acid treatment showed the lowest growth responses (Figure 15 and Figure 16). Therefore, these results are the opposite of publications that are previously reported in many plant such as artichoke, cowpea, oregano and pearl millet (Farouk and Ramadan, 2012; Sharathchandra *et al.*, 2004; Yin *et al.*, 2012; Ziani *et al.*, 2010).

In rice, S Boonlertnirun *et al.* (2006) found that the application of polymeric chitosan (96.62% DD, MW ~100,000 KDa) at 20 mg/L by seed soaking together with four times of foliar sprayings was the most effective treatment for 'Suphan Buri 1' rice growth because it tended to increase rice yield. Moreover, Chamnanmanoontham *et al.* (2015) reported that treatment with 80% DD of oligomeric chitosan (O80) at 40 mg/L significantly enhanced the growth of '*Leung Pra Tew 123'* rice seedling when chitosan was applied by seed soaking and foliar spraying. In addition, Seanbualuang (2007) also investigated the effects of chitosan's concentration on rice growth and yield by seed soaking and soil treatment. He reported that there was no significantly different in rice yield between treated and untreated plant. However, the application of polymeric chitosan (84% DD, MW 46 kDa) at 40 mg/L was the most effective treatment since it had a tendency to increase growth and yield of 'Pathumthani1' rice. The different response of appropriate chitosan treatments for plant growth seems to lie in part in the cultivar specific sensitivity to the solvent of chitosan, as well as to the chitosan types and concentrations. Therefore, in our experiment, we found that the toxicity of

acetic acid to the ‘Pathumthani1’ rice cultivar overcomes any (relatively weak) growth improvement from the chitosan.

The growth inhibitory effect of acetic acid have been previously reported. Rao and Mikkelsen (1977) reported that acetic acid caused the growth retardation of rice seedling cv. ‘*Earlirose*’. Meanwhile in sunflower sprouts, Cho *et al.* (2008) investigated the effects of different molecular-weight chitosan (22, 59, 224, 493, and 746 kDa) by dissolved in 0.1% acetic acid on sunflower sprouts. All chitosan treatment improved total weight, germination rate, length and thickness of hypocotyl of sunflower sprouts when compared to control treatments (water or acetic acid). However, 0.1% acetic acid alone dramatically reduced total weight by 14.9% compared to water control. In conclusion, chitosan/solvent responses depended on both species and cultivar. In contrast to acetic acid, citric acid and lactic acid were less inhibitory to the growth of the ‘Pathumthani1’ rice (Figure 11, Table C-7 and C-8 in Appendix C), and lactic acid alone slightly enhanced to root dry weight of seedling (Figure 11).

The use of lactic acid as a chitosan solvent has previously been reported to enhance plant growth. In sunflower sprouts, Cho *et al.* (2008) compared the effects of 28 kDa chitosan in 4 different types of chitosan solvents including water, acetic, lactic, and ascorbic acid on growth of sunflower sprouts. The results indicated that there were no significant differences in total weight of sprouts among chitosan in 4 solvent types. Nevertheless, sprouts treated with chitosan in lactic acid showed the significant highest total weight, approximately 10.5% higher than the water control. Whereas, No et al., (2003) reported that the total weight of soybean sprouts showed similar response among water, acetic and lactic acids alone. On the other hand, when 493 kDa chitosan was dissolved in 1% acetic or 1% lactic acid, total weight of chitosan-treated soybean sprouts were increased by 21.8% and 18.8%, respectively compared to water control.

The evidence that the ‘Pathumthani1’ cultivar responded to various types and doses of chitosan differently when it was dissolved in lactic acid here is compatible with previous studies in *Dendrobium* orchids (Kananont *et al.*, 2010; Limpanavech *et*

al., 2008; Pornpienpakdee *et al.*, 2010) and rice (S Boonlertnirun *et al.*, 2006; W. Lin *et al.*, 2005), which acetic acid was used as the solvent.

Therefore, the suitable solvent together with the type and concentration of chitosan should be specifically determined for each cultivar as well as plant species before the application in order to obtain the most productive responses. Noticeably, for the chitosan application in rice agriculture, the polymeric chitosan are potentially more suitable than the oligomeric forms according to this results and those previously reported (S Boonlertnirun *et al.*, 2006; Seanbualuang, 2007).

6.2. Effects of shrimp shell (SS) and fermented chitin waste (FCW) on rice growth, photosynthesis and yield

Enhancement of growth and productivity of 'Pathumthani1' rice by SS and FCW soil supplements

The applications of chitin-rich residues including SS and FCW can significantly increase rice growth and productivity (Table 4). This increase in rice growth was directly resulted from the increase of organic matter (OM) and soil nutrients when SS and FCW were applied as soil amendment before rice transplantation (Table 6). Moreover, organic matter (OM) has been reported to improve soil physical, chemical and biological properties by enhanced soil aggregation and available water content, as well as enhances cation exchange capacity, leading to the soil fertility improvement (Khaleel *et al.*, 1981; Matsumoto *et al.*, 1999; Metzger and Yaron, 1987). The increase of N and P content in soil supplemented with SS and FCW related to yield components, especially P which was considered to be important for rice productivity (Rehim *et al.*, 2014). However, in this study, the pattern of rice growth and yield when using two chitin types (SS or FCW) at the same level was not different.

The effect of OM on plant growth was previously investigated in lettuce (Muymas *et al.*, 2014). The addition of 20% (w/w) fermented chitin as soil supplement significantly increased growth of lettuce cv. 'Red Oak' and also the improved soil microbes populations which can degrade chitin to an efficient nitrogen source. These were compatible with our results that the application of SS and FCW were also able

to increase biomass accumulation and yield of ‘Pathumthani1’ rice. However, the amount of SS and FCW using in rice cultivation was lower than lettuce. On the contrary, Chibu and Shibayama (1999) reported that soil supplemented with 0.1% (w/w) chitosan before planting improved growth of lettuce cv. *Melbourne MT*, whereas the increase of rice cv *misatohatamochi* was found in soil treated with 0.5% (w/w) chitosan. Therefore, the appropriate level of chitin rich material to enhance plant growth and yield are varied from species to cultivars.

In addition, chitin-rich materials incorporated into the soil could promote soil microbe diversity (Cretoiu *et al.*, 2014; Muymas *et al.*, 2011). Therefore, several substances including N-acetyl-glucosamine (chitin), glucosamine, acetic acid and ammonia were released after the degradation of chitin-rich materials. Moreover, the polymer form of chitin is probably hydrolyzed to oligomeric form by chitinolytic organisms (Muzzarelli, 1977). In addition, banana trees that were grown in soil mixed with chitin and rhizosphere colonizing bacteria had the highest increase of growth, leaf nutrient contents and yield when compared to other treatments (Kavino *et al.*, 2010). Besides, amendment of soil with chitin was offered as a good agricultural practice for plant protection against soil pathogen and nematodes (Cretoiu *et al.*, 2013; Kobayashi *et al.*, 2002). Soil supplemented with chitin averagely increased the growth of red pepper in both normal and diseased conditions (Rajkumar *et al.*, 2008).

Although the vegetative stage and flowering time of plants can be delayed by the high nitrogen concentration in plants due to the excess nitrogen in soil (Hodges, 2010), it was also found in this experiment that the application of SS and FCW at concentration of 1.0 % (w/w) resulted in the delay of tillering stage and flowering date of ‘Pathumthani1’ rice compared to other treatments (Figure 17 and Table 4). On the other hand, the application of chitin and chitosan at the suitable concentration could induce early flowering time. Ohta *et al.* (1999) reported that the addition of chitosan powder at 1% (w/w) during sowing time reduced date of first flowering and also improved cut-flower quality of *Eustoma grandiflorum* ‘Kairyou Wakamurasaki’ (Ohta *et al.*, 1999). In other ornamental plants such as *Begonia hiematis* Fotsch., *Lobelia*

erinzas L., *Calceolaria herbeohybrida* Voss and *Campanula fragilis* L., growths of eight flowering plants grown in soil mixed with 1% (w/w) chitosan exhibited the early flowering when compared to untreated plants (Ohta *et al.*, 2004).

In the similar to soil amendment, the early flowering time of plant was also observed by foliar application of chitosan. Limpanavech *et al.* (2008) found that the foliar application of O-80 chitosan reduced flowering period and increased numbers of inflorescence in *Dendrobium* 'Eiskul'. Based on these information, it indicated that growth response in treated-plant not only caused by the nutrient contents, especially P and N, but also probably occurred by some effective substances from chitin and chitosan through unclear mechanisms for stimulating of plant growth in cellular level (Sharp, 2013).

Under *in vitro* condition, there are some reports about the effect of chitosan on plant growth. In *Dendrobium* orchids, the suitable chitosan concentration in solid and liquid medium could enhance growth and development of orchids. However, high concentration of chitosan (80 mg/L) had a negative effect on growth (Nge *et al.*, 2006; Pornpienpakdee *et al.*, 2010).

Improvement of photosynthetic pigment contents and photosynthesis parameters by SS and FCW soil supplements

Under nitrogen enrichment, the increase in the nitrogen source leads to the increase in photosynthetic pigments in photosynthetic organisms such as algae (Zubia (Pancha *et al.*, 2014; Zubia *et al.*, 2014) and plants (W. Wang *et al.*, 2014). Nitrogen is the essential element for chlorophyll synthesis. Therefore, the increase in nitrogen supply could increase photosynthetic pigment contents consisting of chlorophyll *a*, chlorophyll *b* and carotenoid until it reaches the optimal concentration in the medium (W. Wang *et al.*, 2014). This was consistent with our experiment in application of SS and FCW for rice plants. Particularly, the highest content of photosynthetic pigments was found in rice treated with 0.5% SS and 0.5% FCW (Table 5).

Similarly, soil amendment with chitin and plant growth-promoting rhizobacteria (PGPR) significantly increased N, P and K contents in banana leaves leading to enhance total chlorophyll contents when compared to untreated control (Kavino *et al.*, 2010). Moreover, Chibu and Shibayama (1999) found that soybean, upland rice, mini-tomato and lettuce grown in soil mixed with chitosan powder ranged from 0.1% to 0.5% (w/w) before planting had the darker green leaves observed by SPAD value when compared to control. In addition, the highest photosynthetic pigment content was observed in treated-bean by watering with 2.5% chitosan solution once at the beginning (Sheikha and Al-Malki, 2011).

Since photosynthetic pigments are the composition in light harvesting complex, the increase of photosynthetic pigment contents lead to enhancement of photosynthesis capacity in plants. This was consistent with SS treatments at vegetative stage. In this experiment, rice grown in the soil supplied with 0.5% SS showed the highest level of net photosynthesis rate with slight difference of that among varied concentrations leading to the enhancement of net photosynthesis rate (Table 5 and Figure 18). Meanwhile in FCW addition, although soil supplied with 0.5 % FCW increased level of photosynthetic pigments in FCW-treated plants at vegetative stage (Table 5), there was no significant difference in net photosynthesis rate among the treatments of FCW application at various concentrations (Figure 18). Although the photosynthetic pigment contents in plants with SS and FCW treatments were not investigated at reproductive stage, soil added with 1.0% SS and 1.0% FCW resulted in the highest level of net photosynthesis rate. According to amount of C_i within cell after treatment, it indicated that the improvement of net photosynthesis rate in flag leaf probably achieved by improving the carboxylation rate due to increase C_i usage within the leaf cell (Figure 16). Consequently, these treatments also showed the highest grain weight/pot (Table 4). Moreover, this result was consistent with Barka *et al.* (2004). They reported that grapevine grown in medium supplemented with 1.75 % (v/v) chitogel resulted in the stimulation of 2-fold O_2 production and 1.5-fold CO_2 fixation leading to enhance growth of plantlet. This information indicated that there are other factors collaborating with photosynthetic pigments to contribute the photosynthesis ability

In addition, not only nitrogen (N) but also phosphorus (P) as well as potassium (K) could influence photosynthesis capacity. It has been shown in tea leaves that phosphorus supplies affect photosynthesis process. P deficiency impaired the electron transport chain from photosystem II (PSII) to photosystem I (PSI), which resulted in the ATP production by light reaction and then limited RuBP regeneration, and hence, the rate of CO_2 assimilation (Z. H. Lin *et al.*, 2009). The increase in P with the SS and FCW applications could contribute to the increase in net photosynthesis rate *via* the electron transport flow enhancement. Besides, K is also used in photosynthesis and involved in water regulation (Hodges, 2010). In this experiment, although the increase of net photosynthesis rate were found in both of SS and FCW treatments, only FCW-soil supplement significantly increase the soil K before planting leading to the differential response of stomatal conductance between SS and FCW additions (Figure 19B). Summarily, the action of SS and FCW might be different in photosynthesis response of rice.

However, effects of chitin and chitosan on photosynthetic process when using foliar application were reported in many plant such as coffee, pepper, common bean and strawberry (Bittelli *et al.*, 2001; Dzung *et al.*, 2011; El-Miniawy *et al.*, 2013; Iriti *et al.*, 2009). The differential responses on net photosynthetic rate, stomata conductance (g_s) and transpiration rate (E) between foliar application of chitosan pentamer (CH5) and chitin pentamer (CHIT5) were observed in soybean and maize (Khan *et al.*, 2002). Although there was no significantly difference in chlorophyll content between untreated okra plants and sprayed-okra plants with chitosan, net photosynthesis rate (P_n) and nitrate reductase activity (NR) involving in nitrogen assimilation were increased (Mondal *et al.*, 2012). Iriti *et al.* (2009) reported that chitosan elicitation with 0.15% (w/v) chitosan in bean resulted in the decrease of transpiration rate (E) and this also induced stomatal closure *via* a H_2O_2-mediated process. In addition, foliar application of chitosan also stimulate the xanthophyll cycle towards de-epoxidation state in order to protect the photosynthetic apparatus.

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6.3. The expression of gene involving photosynthesis induced by shrimp shell (SS) and fermented chitin waste (FCW) when these substances are used as stimulants

According to photosynthetic results in previous experiment, suitable concentration of SS and FCW could increase photosynthetic pigment contents and photosynthetic efficiency in rice (Table 5, Figure 18 and 19). From the results, it indicated that the increase of net photosynthesis rate probably occurred by the increase of photosynthetic pigment contents and the enhancement of carboxylation rate *via* the increase of C_i usage within the leaf cell (Figure 19C). Moreover, the photosynthetic responses to addition of two chitin types (SS and FCW) were supposed to be different through stomatal conductance of flag leaf (Figure 19B).

Previous results were supported in this experiment that the application of 0.25% SS and 0.25% FCW also increased the photosynthetic pigment contents and net photosynthesis rate, but decreased C_i within cell (Figure 20 and Figure 21). For these reasons, we investigate the differential effects of SS and FCW on photosynthesis at the molecular level. In addition, the expression of rice genes involving photosynthesis both of light reaction and carbon fixation in response to SS and FCW treatments by real time RT-PCR was examined.

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In light reaction, we examined the *Oxygen-evolving enhancer1* gene (*OEE1*), the gene involving the photolysis process in the photosystem II complex (Thornton *et al.*, 2004) and the *chlorophyll a/b binding protein* gene (*PsbS1*) which is a component in light harvesting system (LHCII) and regulates non-photochemical quenching (qN) (Kereïche *et al.*, 2010). It was subjected to investigate the gene activity in SS and FCW treated plants, compared to the control group. The results showed that there was no significant difference in the relative expression level of the *OEE1* gene among treatments throughout the experiment (Figure 22). Meanwhile in *PsbS1* gene, the relative expression level of FCW-treated plant was significantly increased when compared to control plant, while the relative expression level of that in SS-treated plants was the same level as control after 14 days of treatment (Figure 23). These data

suggest that light reaction showed differently photoprotective mechanisms between SS-treated plant and FCW-treated plant. This is similar to Iriti *et al.* (2009) experiment. They reported that foliar application of chitosan could stimulate the xanthophyll cycle towards de-epoxidation state in order to protect the photosynthetic apparatus against photodamage. The higher level of *PsbS1* gene expression may contribute to the photosynthesis enhancement by FCW.

In carbon fixation, the expression pattern of *ribulose bisphosphate carboxylase small chain* (*rbcS*) gene which encodes a subunit of key enzyme in the first step of carbon fixation was investigated (Kanevski *et al.*, 1999). This result showed that the different pattern of relative expression level of the *rbcS* gene was found in the addition of two chitin types (SS and FCW) in spite of having the similar pattern of net photosynthesis rate and C_i responses (Figure 21A and 21C). In FCW treatment, there was no significant difference in the relative expression level of the *rbcS* gene between treated and untreated plant throughout the experiment. However, the relative expression level of *rbcS* gene in SS-treated plant was significantly decreased when compared to control plant (Figure 24). In conclusion, our results indicated that the action of SS and FCW acted on different response to photosynthesis process.

Suzuki *et al.* (2007) reported the information about RuBisCO activity in rice. They found that when overexpressed *rbcS* gene, the transgenic plant dramatically increased RuBisCO content. However, the massive increase of RuBisCO content did not affect the photosynthetic capacity in transgenic plant. On the other hand, Parry *et al.* (2012) suggested that the factor such as RuBisCO turnover rate, affinity, or specificity for CO_2 enabled were the key factors that strongly improved photosynthetic activity and also crop improvement. Therefore, it was likely that the action of these two supplements in carbon fixation were at translation or post-translation levels of this genes or may affect in turnover rate or CO_2 affinity of RuBisCO rather than increased RuBisCO content in order to increased net photosynthesis rate.

Furthermore, according to report of Chamnanmanoontham *et al.* (2015), she found that. chitosan application on rice seedling could affect nine proteins which had co-expression network with other genes in three necessary processes; photosynthesis, carbohydrate metabolism and cell redox homeostasis. For photosynthesis, there were five up-regulated proteins including Oxygen-evolving enhancer protein 1, Chlorophyll A-B binding protein, Glyceraldehyde-3-phosphate, and two of Ribulose bisphosphate carboxylase small chain. However, this research investigated only three representative chitosan-inducible genes in photosynthesis. Therefore, the rest of genes are also interesting to be investigated.

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CHAPTER VII
CONCLUSIONS

7.1. Effects of different types and concentrations of chitosan molecules on rice seedling growth

Among acetic, citric and lactic acids as chitosan solvents, lactic acid showed the lowest growth inhibition of 'Pathumthani1' rice seedlings. P90 at 40 mg/L in 1% (v/v) lactic acid increased shoot and root growth of 21-day-old seedlings more than 10%. Therefore, lactic acid could be an alternative chitosan solvent for agriculture applications. However, the suitable chitosan type, concentration and solvent should be under consideration for each plant species as well as cultivar prior to agricultural application.

7.2. Effects of shrimp shell (SS) and fermented chitin waste (FCW) on rice growth, photosynthesis and yield

In order to develop chitin-rich residues for sustainable rice production, shrimp shell (SS) and fermented chitin (FCW) from Agro-industrial wastes have potential to be a growth stimulant since 1% (w/w) SS and 1% (w/w) FCW soil amendment could promote 'Pathumthani1' rice growth by increase of photosynthetic pigments and photosynthesis ability at vegetative stage led to enhance rice productivity.

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7.3. The gene expression induced by shrimp shell (SS) and fermented chitin waste (FCW) when these substances are used as stimulants

Both 0.25% (w/w) shrimp shell (SS) and FCW significantly increased photosynthetic pigments and photosynthesis ability. On the contrary, at the molecular level, SS and FCW amendment had different patterns of *PsbS1* gene expression in light reaction and *rbcS* gene expression in carbon fixation. Consequently, this is evidence that the rice seedling growth stimulation by SS and FCW resulted by different mechanisms in order to enhance photosynthesis efficiency.

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APPENDIX

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APPENDIX A

Chemical solutions

1. **Modifield WP no.2 solution** (Vajrabhaya and Vajrabhaya, 1991)

Macroelements:

KNO_3	580	mg
$CaSO_4$	500	mg
$MgSO_4.7H_2O$	450	mg
Triple super phosphate	250	mg
$(NH_4)_2SO_4$	100	mg

Microelements:

Na_2EDTA[a]	160	mg
$FeSO_4.7H_2O$[a]	120	mg
$MnSO_4.H_2O$	15	mg
H_3BO_3	5	mg
$ZnSO_4.7H_2O$	1.5	mg
KI	1	mg
$Na_2MoO_4.2H_2O$	0.1	mg
$CuSO_4.5H_2O$	0.05	mg
$CoCl_2.6H_2O$	0.05	mg
H_2O	800	mg

Stir with a magnetic stirrer, add 2 ml of $FeSO_4.7H_2O$ and adjust the volume to 1 L with water.

[a]Preparation of 30 g/L FeSO4 stock

Na_2EDTA	40	g
$FeSO_4.7H_2O$	30	g

Stir each chemical solution with a magnetic stirrer and adjust the volume to 1 L with water.

2. **RNA extraction buffer**

Tris pH 9.0	100	mM
NaCl	100	mM

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EDTA	20	mM
Lauryl sarcosinate	1.0%	(w/v)
2-mercaptoethanol	0.1%	(v/v)
DEPC (diethyl pyrrocarbonate)	0.1%	(v/v)

3. TE buffer

Tris base (pH 8.0)	10	mM
EDTA (pH 8.0)	1	mM

4. DEPC-treated TE Buffer

Tris base (pH 8.0)	10	mM
EDTA (pH 8.0)	1	mM
DEPC (diethyl pyrrocarbonate)	0.1%	(v/v)

5. DEPC-treated water

DEPC (diethyl pyrrocarbonate)	0.1%	(v/v)
Distilled water		

6. 5X TBE buffer (Tris Borate EDTA)

Tris base	54	g
Boric acid	27.5	g
0.5 M EDTA (pH 8.0)	20	ml

7. DNA/RNA loading dye

Glycerol in water	30%	(v/v)
Bromophenol bule	0.25%	(v/v)
Xylene cyanol	0.25%	(v/v)

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APPENDIX B

Protocols

1. The physicochemical properties of soil

For soil sample preparation, litters and gravels were removed from the 64 soil samples which obtained from 8 treatments with 4 replications before planting and after harvest period. Then, these soil samples were dried at room temperature.

For soil pH and EC analysis, dried soil sample were grinded into 2 mm. in diameter of soil particles by mortar and pestle, whereas 0.5 mm. soil particles were used for chemical soil analysis.

1.1. pH analysis

The analysis of soil pH was examined by using a 1:1 mixture of soil and distilled water. The mixture was stirred for 30 minutes and then the pH was read by a pH meter using a glass electrode (Peech, 1965).

1.2. Electrical conductivity (EC) of soil analysis

Soil EC was analyzed by using a 1:5 mixture of soil and distilled water. The mixture was shaken for 2 hours and soil particle deposited at the bottom of a water body overnight. The supernatant was read with a digital conductivity meter (Digital conductivity meter, Fisher Scientific) (J. J. Lee *et al.*, 2004).

1.3. Soil organic matter (OM) analysis

One gram of soil was used for determining total soil organic matter (OM) by Walkley and Black method (Nelson and Sommers, 1996) and O-phenanthroline was used as an indicator. The solution was titrated against 0.5 N $FeSO_4.7H_2O$ for green to red-brown end point, whereas blank was prepared in the same manner without adding a soil sample.

1.4. Total nitrogen (N) in soil analysis

Kjeldahl method was used for analyzing total nitrogen (Bremner, 1965) and a bromocresol-green methyl-red was used as the indicator. The titration used 0.1 N HCl for the green to red-brown end point. Blank was prepared in the same manner without adding a soil sample.

Calculation:

$$\% \text{ nitrogen} = \frac{\text{ml } H_2SO_4 \text{ used (soil sample – blank titration)} \times N \times 0.14 \times 100}{\text{Weight of soil sample (g)}}$$

1.5. Availability phosphorus (P) in soil analysis

Soil available P was extracted by with NH_4F and HCl (Bray and Kurtz, 1945) and the absorbance of the filtrate was read at 882 nm by spectrophotometer (Lambda 35 UV/VIS Spectrometer, PerkinElmer).

Calculation:

$$\text{Available P} = \frac{B \times df \text{ (sample)} \times R \text{ mg kg}^{-1}}{A \times df \text{ (standard)}}$$

A = weight of soil sample (g)

B = 0.03 N NH_4F, 0.1 N HCl (ml)

R = standard set

df = dilution factor

1.6. Availability potassium (K) in soil analysis

Soil available K were extracted by NH_4OAc (Jackson, 1958) . The absorbance of the filtrate was examined at 383 nm by flame photometers (Corning 410, Sherwood Scientific, Ltd., UK).

Calculation:

$$\text{Available K} = \frac{D \times df \text{ (sample)} \times R \text{ mg kg}^{-1}}{A \times df \text{ (standard)}}$$

A = weight of soil sample (g)

B = 1 N ammonium acetate solution of pH 7 (ml)

df = dilution factor

D = standard solution for KCl mg kg^{-1}

1.7. Availability of calcium (Ca) and magnesium (Mg) in soil analysis

Soil available Ca and Mg were extracted by NH_4OAc (Jackson, 1958). The absorbance of the filtrate was investigated at 422 nm and 285 nm, respectively by flame atomic absorption spectrophotometer (GBC, AA S/N 6360).

Calculation:

$$\text{Available Ca} = \frac{D \times df \times B \text{ mg kg}^{-1}}{A}$$

A = weight of soil sample (g)

B = 1 N ammonium acetate solution of pH 7 (ml)

df = dilution factor

D = standard solution for Ca (mg ml^{-1})

Calculation:

$$\text{Available Mg} = \frac{D \times df \times B \text{ mg kg}^{-1}}{A}$$

A = weight of soil sample (g)

B = 1 N ammonium acetate solution of pH 7 (ml)

df = dilution factor

D = standard solution for Mg (mg ml^{-1})

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2. RNA extraction

2.1. The standard hot-phenol method was used for total RNA extraction as described by Thikart *et al.* (2005). Rice tissues were ground into a fine-powder in liquid nitrogen using chilled mortars and pestles.

2.2. The ground tissues were added into 500 μl of hot RNA extraction buffer (80°C) together with 500 μl of phenol: chloroform: isoamyalcohol (25:24:1) and 0.5 μl of β-mercaptoethanol. The mixtures were well homogenized and kept at 4°C on ice for an hour.

2.3. A homogenized sample was centrifuged at 14,000 rpm for 10 minutes at 4°C. The approximately 400 - 500 μl of supernatant was transferred to a fresh microcentrifuge tube.

2.4. Total RNA was precipitated by addition of double volumes of cool absolute ethanol and stored at -20°C for 30 minutes. The mixture was centrifuged at 14,000 rpm for 10 minutes at 4°C.

2.5. After centrifugation, the pellet was optically observed and then it was washed with cooled 80% ethanol followed by air-dried at room temperature.

2.6. The pellet was dissolved in 160 μl of DEPC-treated TE buffer. RNA solution was added 40 μl of 10 M $LiCl_2$ and kept overnight at -20°C.

2.7. After thawing RNA on ice, RNA solution was centrifuged at 14,000 rpm for 10 minutes at 4°C and then the pellet was washed with cooled 80% ethanol followed by air-dried at room temperature again.

2.8. The pellet was dissolved in 20 μl DEPC-treated TE buffer and stored at -20°C

2.9. The quality and quantity of RNA were performed by agarose electrophoresis and measuring the absorbance at 260 with a spectrophotometer. The concentration of RNA was calculated in μg/ml unit, using the following equation:

$$[RNA] = 40^{*} \times A_{260} \times \text{dilution factor}$$

* The absorbance at 260 nm (A_{260}) of 1.0 corresponds to the RNA of approximately 40 μg/ml (Sambrook *et al.*, 2001)

3. DNase I treatment

DNase I treatment reaction

Total RNA	20 μg
DNase I Buffer	1X
Recombinant DNase I	10 U
DEPC-treated water	variable μl
Total volume	50 μl

Protocol

3.1. Ten micrograms of the total RNA sample were added to the DNase I treatment mixture (as described above) and the mixture was incubated at 37°C for an hour.

3.2. After incubation, the mixture was added 100 μl of DEPC-treated water together with 150 μl of phenol: chloroform: isoamylalcohol (25:24:1) and mixed well.

3.3. Then, the mixture was centrifuged at 12,000 rpm for 10 minutes at 4°C. The upper aqueous phase was transferred to a fresh microcentrifuge tube in order to precipitate RNA pellet by the addition of 0.1 volumes of 3M NaOAc (pH 5.2) and 0.6 volumes of cooled isopropanol.

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3.4. The mixture was kept at -80°C for 30 minutes and then centrifuged at 12,000 rpm for 10 minutes at 4°C.

3.5. After centrifugation, the pellet was washed with cooled 80% ethanol and shortly air-dried at room temperature.

3.6. Finally, the DNA-free RNA pellet was suspended in 10 μl DEPC-treated TE buffer (Chutimanukul, 2013).

4. cDNA synthesis using iScript™ Reverse Transcription Supermix

iScript™ RT Supermix reaction

iScript™ Reverse Transcription Supermix	4 μl
RNA templete (2 μg total RNA)	variable μl
Nuclease free water	variable μl
Total volume	20 μl

Protocol

4.1. Reverse transcription PCR was performed in a 20 μl solution as described above.

4.2. The first strand cDNA was synthesized by PCR technique. This reaction included 3 steps;

Priming	at 25°C for 5 minutes
Reverse transcription	at 42°C for 30 minutes
RT inactivation	at 85°C for 5 minutes

5. Detection of gene expression using quantitative Real-time PCR

Real-time polymerase chain reaction

SsoFast™ EVagreen® Supermix	5 μl
5 μM Forward primer	0.25 μl
5 μM reverse primer	0.25 μl
Sterile water	3.5 μl

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cDNA	1	μl
Total volume	10	μl

Protocol

5.1. The cDNA was used as template to quantify the level of gene expression by quantitative real-time PCR (Real-time PCR: CFX96 Touch™ (Bio-Rad Laboratories, Inc., USA)).

5.2. The specific primer sequences for each gene including were designed for quantitatively determine the expression**.** Meanwhile, primers of elongation factor 1-α (EF1-α), a housekeeping gene, were used as a reference gene in order to normalize gene expressions

5.3. At least 3 independent real-time PCR reactions were performed on the same cDNA preparation.

5.4. The thermal cycle used was as follows:

5.4.1. enzyme activation 95℃ for 20 second

5.4.2.Than 40 cycles of 95℃ for 5 minutes (denaturation), 54℃ for 20 seconds (annealing) and 72℃ for 20 seconds (extension).

5.4.3.Finally, melting curve of each product was considered by a final extension at 70-90℃ for 5 seconds per 0.5℃.

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6. Calculation of relative gene expression level

6.1. The level of gene expression was determined in comparison with the reference gene expression in reference to the expression on Day 0 of the control. The *OEE1*, *PsbS1* and *rbcS* at 0 hour were set as controls of interested gene, whereas *EF-1*α at 0 hour were set as controls of reference gene

6.2. The relative expression ratio of target gene was calculated based on PCR efficiency (E) and the CP deviations. CP was defined as the point at which the fluorescence rises appreciably above the background fluorescence (Pfaffl, 2001).

$$\text{Relative gene expression level} = \frac{(E_{target})^{\Delta CP\,target\,(control-sample)}}{(E_{ref})^{\Delta CP\,ref\,(control-sample)}}$$

R = relative expression ratio of target gene

E_{target} = $10^{-1/slope}$ of interested gene

E_{ref} = $10^{-1/slope}$ of reference gene

ΔCP_{target}(control-sample) = $CP_{0\ hour}$ – $CP_{\text{any time point of interested gene}}$

ΔCP_{ref}(control-sample) = $CP_{0\ hour}$ – $CP_{\text{any time point of reference gene}}$

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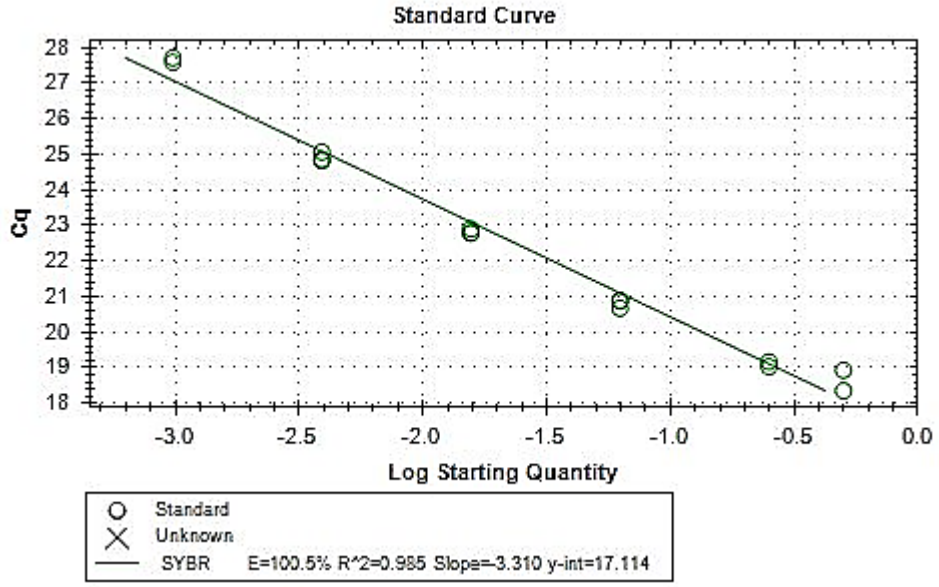

Figure B-1 Standard curve of *Elongation factor 1 alpha* (*EF1-**α***)

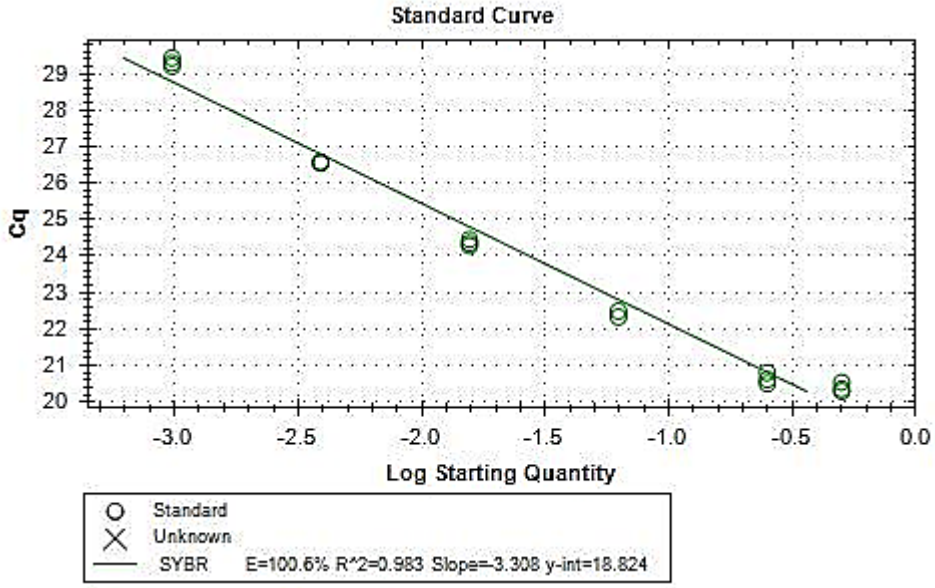

Figure B-2 Standard curve of *Oxygen-evolving complex protein 1 (OEE1)*

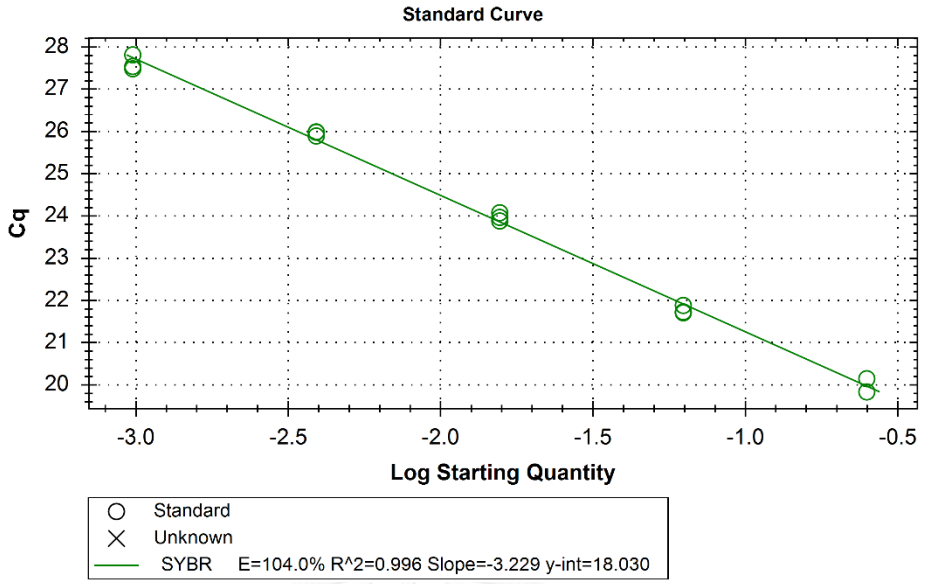

Figure B-3 Standard curve of *Chlorophyll a-b binding protein (PsbS1)*

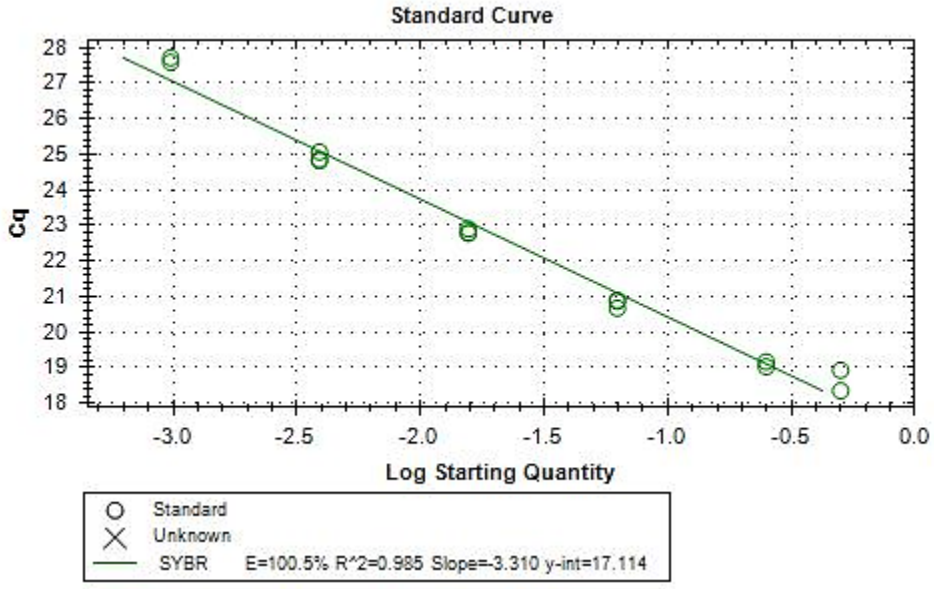

Figure B-4 Standard curve of *Ribulose-1,5-bisphosphate carboxylase/ oxygenase small subunit (rbcS)*

APPENDIX C

Data tables

Table C-1. The effect of chitosan treatments, water and acetic acid as chitosan solvent on plant height (cm) of 7, 14 and 21 days old seedlings.

Treatment	Conc. (mg/L)	Plant height (cm)		
		7 days	14 days	21 days
Water		13.86 ± 0.77^{abcdef}	26.84 ± 1.62^{a}	37.39 ± 4.10^{abc}
Acetic acid	5	13.38 ± 0.96^{defghi}	25.47 ± 2.08^{bcde}	34.95 ± 4.48^{abcdef}
	10	13.10 ± 0.87^{fghij}	25.12 ± 1.40^{de}	34.18 ± 3.36^{ef}
	20	14.03 ± 1.08^{abcde}	24.85 ± 1.27^{ef}	35.97 ± 1.86^{abcdef}
	40	13.25 ± 0.39^{efghi}	23.76 ± 1.37^{f}	34.65 ± 3.52^{bcdef}
	80	14.23 ± 0.77^{abc}	25.68 ± 0.78^{abcde}	37.43 ± 2.74^{abc}
P80	5	13.80 ± 0.98^{abcdef}	26.68 ± 0.95^{ab}	37.39 ± 2.71^{abc}
	10	12.80 ± 0.82^{hijk}	25.18 ± 0.82^{de}	36.94 ± 1.84^{abcde}
	20	12.40 ± 0.97^{jk}	25.71 ± 0.86^{abcde}	37.57 ± 3.72^{ab}
	40	12.62 ± 1.06^{ijk}	26.55 ± 1.36^{abc}	34.29 ± 3.61^{def}
	80	13.98 ± 0.87^{abcde}	26.47 ± 1.39^{abc}	36.59 ± 3.94^{abcde}
O80	5	12.76 ± 0.48^{hijk}	24.86 ± 0.67^{ef}	36.48 ± 3.35^{abcde}
	10	13.64 ± 0.94^{cdefg}	26.78 ± 0.81^{a}	36.16 ± 2.4^{abcdef}
	20	13.93 ± 0.42^{abcde}	26.63 ± 1.51^{ab}	36.88 ± 1.89^{abcde}
	40	13.99 ± 0.61^{abcde}	25.77 ± 0.65^{abcde}	34.06 ± 3.41^{ef}
	80	12.98 ± 1.41^{ghijk}	24.96 ± 1.31^{de}	35.08 ± 2.6^{abcdef}
P90	5	12.30 ± 0.91^{k}	25.04 ± 1.9^{de}	37.15 ± 1.18^{abcd}
	10	13.68 ± 0.79^{bcdefg}	26.58 ± 1.34^{abc}	35.83 ± 4.58^{abcdef}
	20	14.48 ± 0.81^{ab}	26.62 ± 1.18^{ab}	36.25 ± 4.56^{abcdef}
	40	13.79 ± 0.86^{abcdef}	25.43 ± 1.85^{bcde}	35.63 ± 3.21^{abcdef}
	80	13.48 ± 0.72^{cdefgh}	25.46 ± 1.84^{bcde}	36.13 ± 4.09^{abcdef}
O90	5	12.63 ± 1.00^{ijk}	24.82 ± 0.86^{ef}	33.48 ± 1.56^{f}
	10	13.29 ± 1.09^{efghi}	25.33 ± 2.08^{cde}	34.60 ± 3.20^{cdef}
	20	13.91 ± 0.67^{abcdef}	26.14 ± 1.12^{abcd}	36.61 ± 2.05^{acde}
	40	14.19 ± 0.72^{abcd}	26.75 ± 1.24^{a}	37.83 ± 1.39^{a}
	80	14.52 ± 0.81^{a}	26.03 ± 1.04^{abcde}	34.66 ± 2.50^{bcdef}

Column with different letters denote significant differences according to Duncan's multiple range tests (P<0.05)

Table C-2. The effect of chitosan treatments, water and acetic acid as chitosan solvent on shoot fresh weight (mg) of 7, 14 and 21 days old seedlings.

Treatment	Conc. (mg/L)	Shoot fresh weight (mg)		
		7 days	14 days	21 days
Water		59.8 ± 2.8^{abcd}	228.1 ± 37.5^{abcd}	527.0 ± 116.9^{abc}
Acetic acid	5	54.6 ± 3.9^{fghi}	200.0 ± 30.2^{efghij}	484.0 ± 104.1^{bcdefg}
	10	53.0 ± 6.1^{ghi}	196.7 ± 45.5^{fghij}	429.1 ± 67.7^{fg}
	20	57.3 ± 5.5^{bcdefg}	181.3 ± 18.0^{ij}	464.0 ± 59.5^{cdefg}
	40	56.8 ± 2.2^{cdefg}	176.4 ± 39.0^{j}	441.9 ± 68.4^{fg}
	80	58.8 ± 5.2^{abcdef}	200.5 ± 17.3^{efghij}	524.9 ± 56.5^{abcd}
P80	5	60.1 ± 4.3^{abcd}	239.1 ± 38.9^{a}	555.0 ± 35.0^{a}
	10	54.7 ± 3.8^{fghi}	196.9 ± 30.1^{fghij}	496.0 ± 50.0^{abcdef}
	20	52.2 ± 4.1^{i}	209.9 ± 19.6^{bcdefgh}	524.8 ± 90.5^{abcd}
	40	56.5 ± 5.0^{cdefgh}	209.4 ± 32.3^{bcdefgh}	445.6 ± 75.8^{efg}
	80	58.3 ± 4.0^{abcdef}	229.1 ± 40.4^{abc}	512.6 ± 104.8^{abcde}
O80	5	55.0 ± 5.7^{fghi}	192.5 ± 14.8^{hij}	491.9 ± 59.9^{abcdef}
	10	56.0 ± 3.6^{defghi}	210.5 ± 24.7^{bcdefgh}	457.4 ± 66.2^{defg}
	20	56.7 ± 3.8^{cdefg}	232.4 ± 30.3^{ab}	484.7 ± 85.9^{bcdefg}
	40	62.1 ± 5.0^{a}	203.6 ± 24.7^{cdefghi}	420.4 ± 86.3^{g}
	80	53.0 ± 4.0^{ghi}	203.2 ± 22.9^{cdefghi}	446.6 ± 81.8^{efg}
P90	5	52.4 ± 6.3^{hi}	198.0 ± 33.4^{fghij}	452.9 ± 61.2^{efg}
	10	57.1 ± 4.7^{bcdefg}	226.2 ± 47.9^{abcde}	439.7 ± 90.9^{fg}
	20	59.4 ± 4.8^{abcde}	227.8 ± 30.8^{abcd}	491.3 ± 93.1^{abcdef}
	40	58.2 ± 4.3^{abcdef}	207.6 ± 36.4^{bcdefgh}	477.1 ± 81.2^{bcdefg}
	80	57.1 ± 4.1^{bcdefg}	202.4 ± 29.5^{defghi}	472.9 ± 92.6^{bcdefg}
O90	5	55.3 ± 4.1^{efghi}	194.6 ± 23.8^{ghij}	426.6 ± 62.4^{fg}
	10	56.0 ± 5.3^{defghi}	208.6 ± 33.8^{bcdefgh}	430.2 ± 73.6^{fg}
	20	60.4 ± 2.9^{abc}	220.1 ± 26.1^{abcdefg}	486.0 ± 57.3^{bcdefg}
	40	60.0 ± 6.1^{abcd}	222.7 ± 29.1^{abcdef}	535.9 ± 41.8^{ab}
	80	61.2 ± 2.6^{ab}	215.4 ± 24.6^{abcdefgh}	462.5 ± 58.0^{cdefg}

Column with different letters denote significant differences according to Duncan's multiple range tests (P<0.05).

Table C-3. The effects of chitosan treatments, water and acetic acid as chitosan solvent on shoot dry weight (mg) of 7, 14 and 21 days old seedlings.

Treatment	Conc. (mg/L)	Shoot dry weight (mg)		
		7 days	14 days	21 days
Water		8.9 ± 0.7^{abcde}	37.4 ± 5.2^{abcd}	91.6 ± 13.9^{a}
Acetic acid	5	8.4 ± 0.6^{defgh}	32.9 ± 5.3^{efg}	77.7 ± 12.3^{efghi}
	10	8.1 ± 0.8^{fghi}	33.6 ± 6.5^{defg}	70.1 ± 12.5^{i}
	20	8.7 ± 0.8^{abcdefg}	30.5 ± 3.7^{fg}	78.0 ± 8.5^{defghi}
	40	8.1 ± 0.7^{ghi}	30.2 ± 6.7^{g}	71.0 ± 11.3^{hi}
	80	8.9 ± 0.8^{abcde}	32.7 ± 2.2^{efg}	82.2 ± 7.8^{abcdef}
P80	5	9.0 ± 0.7^{abcd}	38.6 ± 5.6^{a}	89.6 ± 7.2^{ab}
	10	8.4 ± 0.5^{defgh}	32.8 ± 4.2^{efg}	78.8 ± 9.1^{cdefghi}
	20	7.9 ± 0.8^{hi}	34.1 ± 3.0^{cdefg}	87.7 ± 14.4^{abcd}
	40	8.2 ± 0.8^{efgh}	35.0 ± 3.8^{abcde}	75.2 ± 10.1^{fghi}
	80	9.0 ± 0.6^{abcd}	37.5 ± 4.9^{abcd}	82.9 ± 15.8^{abcdef}
O80	5	8.5 ± 0.7^{cdefgh}	32.9 ± 2.9^{efg}	80.3 ± 9.1^{bcdefgh}
	10	8.6 ± 0.6^{bcdefg}	35.2 ± 4.7^{abcde}	84.4 ± 11.4^{abcdef}
	20	8.5 ± 0.5^{bcdefgh}	38.6 ± 3.9^{a}	85.9 ± 6.3^{abcde}
	40	9.2 ± 0.8^{ab}	34.2 ± 3.7^{bcdef}	76.9 ± 13.1^{efghi}
	80	8.0 ± 0.8^{ghi}	33.2 ± 2.7^{efg}	79.5 ± 9.1^{cdefghi}
P90	5	7.5 ± 0.8^{i}	33.1 ± 5.8^{efg}	76.7 ± 7.1^{efghi}
	10	8.6 ± 1.0^{bcdefg}	38.2 ± 6.2^{ab}	77.9 ± 9.1^{defghi}
	20	8.9 ± 0.8^{abcde}	37.8 ± 4.9^{abc}	81.1 ± 15.4^{bcdefg}
	40	8.8 ± 0.6^{abcdef}	33.5 ± 5.6^{defg}	79.0 ± 13.7^{cdefghi}
	80	8.6 ± 0.6^{bcdefgh}	32.9 ± 4.3^{efg}	75.1 ± 14.2^{fghi}
O90	5	8.2 ± 1.0^{efgh}	34.2 ± 6.1^{cdefg}	71.1 ± 11.9^{ghi}
	10	8.0 ± 1.0^{ghi}	34.3 ± 4.9^{bcdef}	70.6 ± 9.7^{hi}
	20	9.0 ± 0.5^{abcd}	35.2 ± 3.8^{abcde}	78.8 ± 8.2^{cdefghi}
	40	9.2 ± 0.9^{abc}	37.7 ± 4.8^{abc}	88.3 ± 9.4^{abc}
	80	9.4 ± 0.6^{a}	36.2 ± 4.0^{abcde}	76.6 ± 9.8^{efghi}

Data are shown as the mean ± 1 SD and column with different letters denote significant differences according to Duncan's multiple range tests (P<0.05).

Table C-4. The effect of chitosan treatments, water and acetic acid on root length (cm) of 7, 14 and 21 days old seedlings.

Treatment	Conc. (mg/L)	Root length (cm)		
		7 days	14 days	21 days
Water		10.97 ± 0.40^{cdefg}	12.08 ± 0.57^{abc}	17.87 ± 2.05^{abcd}
Acetic acid	5	10.31 ± 0.64^{hi}	11.06 ± 0.97^{defgh}	15.88 ± 2.80^{dfgh}
	10	11.05 ± 0.60^{bcdefg}	11.48 ± 1.38^{bcdef}	16.33 ± 2.58^{dfgh}
	20	10.93 ± 0.55^{cdefg}	11.16 ± 0.87^{cdefgh}	17.10 ± 1.87^{abcdef}
	40	10.63 ± 0.8^{fgh}	10.43 ± 1.52^{ghi}	14.53 ± 2.16^{h}
	80	10.96 ± 1.08^{cdefg}	12.03 ± 0.88^{abcd}	15.94 ± 3.06^{dfgh}
P80	5	11.21 ± 0.58^{abcdef}	11.95 ± 1.55^{abcd}	18.42 ± 1.96^{abc}
	10	11.08 ± 0.40^{bcdef}	11.32 ± 0.5^{cdefg}	17.80 ± 0.44^{abcde}
	20	9.84 ± 0.88^{i}	10.35 ± 0.79^{hi}	17.43 ± 1.76^{abcdef}
	40	10.78 ± 0.52^{efgh}	11.29 ± 0.58^{cdefg}	17.17 ± 1.73^{abcdef}
	80	11.3 ± 0.71^{abcde}	11.82 ± 1.31^{abcde}	16.18 ± 1.90^{dfgh}
O80	5	10.63 ± 0.49^{fgh}	10.94 ± 0.64^{efghi}	15.37 ± 1.31^{fgh}
	10	11.53 ± 0.35^{abc}	12.07 ± 1.32^{abc}	18.88 ± 1.43^{ab}
	20	11.12 ± 0.73^{abcdef}	12.57 ± 0.85^{a}	19.06 ± 1.25^{a}
	40	11.68 ± 0.69^{ab}	12.38 ± 1.08^{ab}	17.98 ± 2.02^{abcd}
	80	11.16 ± 0.66^{abcdef}	11.81 ± 0.72^{abcde}	16.93 ± 2.23^{bcdefg}
P90	5	9.83 ± 0.75^{i}	10.09 ± 1.11^{i}	15.72 ± 1.21^{efgh}
	10	11.48 ± 0.65^{abcd}	12.62 ± 0.85^{a}	16.06 ± 3.26^{dfgh}
	20	10.87 ± 0.77^{defgh}	11.86 ± 0.62^{abcde}	15.73 ± 2.96^{efgh}
	40	11.05 ± 0.84^{bcdefg}	11.41 ± 1.51^{bcdef}	16.88 ± 2.02^{bcdefg}
	80	10.44 ± 0.64^{gh}	11.12 ± 0.8^{cdefgh}	15.67 ± 3.06^{fgh}
O90	5	11.28 ± 0.74^{abcde}	12.06 ± 1.13^{abc}	15.93 ± 1.59^{dfgh}
	10	11.17 ± 0.82^{abcdef}	11.73 ± 0.51^{abcde}	14.93 ± 2.20^{gh}
	20	11.43 ± 0.40^{abcd}	11.56 ± 0.54^{bcdef}	16.68 ± 2.54^{cdefg}
	40	11.74 ± 0.75^{a}	11.84 ± 0.97^{abcde}	17.17 ± 2.01^{abcdef}
	80	11.22 ± 0.40^{abcdef}	10.77 ± 2.12^{fghi}	15.53 ± 1.96^{fgh}

Data are shown as the mean ± 1 SD and column with different letters denote significant differences according to Duncan's multiple range tests (P<0.05).

Table C-5. The effects of chitosan treatments, water and acetic acid on root fresh weight (mg) of 7, 14 and 21 days old seedlings.

Treatment	Conc. (mg/L)	Root fresh weight (mg)		
		7 days	14 days	21 days
Water		24.2 ± 6.6^{abc}	123.7 ± 23.1^{abcd}	159.2 ± 31.8^{abcd}
Acetic acid	5	20.4 ± 6.7^{cdef}	106.0 ± 26.1^{cd}	170.4 ± 38.4^{ab}
	10	21.5 ± 6.7^{bcdef}	116.0 ± 33.1^{abcd}	130.5 ± 16.9^{cdef}
	20	23.5 ± 6.1^{abc}	107.3 ± 26.9^{cd}	158.1 ± 31.9^{abcd}
	40	25.4 ± 3.5^{a}	106.9 ± 45.1^{cd}	157.3 ± 37.6^{abcd}
	80	23.7 ± 3.3^{abc}	112.1 ± 23.2^{bcd}	184.1 ± 27.0^{a}
P80	5	22.2 ± 3.4^{abcde}	136.3 ± 34.1^{a}	171.8 ± 66.1^{ab}
	10	20.9 ± 2.1^{bcdef}	106.6 ± 21.0^{cd}	161.0 ± 27.9^{abc}
	20	19.2 ± 1.7^{def}	114.0 ± 21.5^{bcd}	134.6 ± 41.9^{cdef}
	40	18.7 ± 1.8^{ef}	107.7 ± 21.1^{cd}	158.5 ± 40.9^{abcd}
	80	20.9 ± 3.4^{bcdef}	123.7 ± 10.4^{abcd}	150.2 ± 40.7^{bcde}
O80	5	22.1 ± 3.3^{abcde}	104.1 ± 17.5^{d}	158.3 ± 44.6^{abcd}
	10	22.9 ± 2.7^{abcd}	110.1 ± 25.1^{bcd}	118.2 ± 56.7^{ef}
	20	22.1 ± 2.3^{abcde}	120.9 ± 19.1^{abcd}	129.9 ± 63.6^{cdef}
	40	24.5 ± 2.1^{ab}	106.4 ± 16.5^{cd}	107.3 ± 50.2^{f}
	80	20.9 ± 3.8^{bcdef}	114.8 ± 17.6^{bcd}	140.7 ± 62.1^{bcdef}
P90	5	22.0 ± 3.9^{bcdef}	108.6 ± 19.5^{cd}	119.3 ± 58.5^{ef}
	10	21.4 ± 4.6^{bcdef}	126.9 ± 32.8^{abc}	116.9 ± 55.7^{ef}
	20	25.4 ± 2.8^{a}	127.1 ± 21.7^{abc}	142.4 ± 30.9^{bcde}
	40	20.8 ± 4.4^{bcdef}	117.2 ± 18.3^{abcd}	138.1 ± 22.3^{bcdef}
	80	20.5 ± 3.2^{cdef}	110.8 ± 27.1^{bcd}	133.1 ± 17.6^{cdef}
O90	5	18.5 ± 3.5^{ef}	112.1 ± 15.9^{bcd}	127.8 ± 39.1^{cdef}
	10	18.2 ± 6.1^{f}	112.6 ± 17.9^{bcd}	124.9 ± 24.7^{def}
	20	19.0 ± 4.7^{ef}	120.0 ± 24.2^{abcd}	133.1 ± 32.5^{cdef}
	40	19.1 ± 4.7^{def}	130.2 ± 19.8^{ab}	154.9 ± 28.3^{abcd}
	80	20.9 ± 3.7^{bcdef}	124.4 ± 22.8^{abcd}	155.3 ± 42.7^{abcd}

Data are shown as the mean ± 1 SD and column with different letters denote significant differences according to Duncan's multiple range tests (P<0.05).

Table C-6. The effects of chitosan treatments, water and acetic acid on root dry weight (mg) of 7, 14 and 21 days old seedlings.

Treatment	Conc. (mg/L)	Root dry weight (mg)		
		7 days	14 days	21 days
Water		3.8 ± 0.6^{abcdef}	16.1 ± 1.8^{abcdef}	29.2 ± 3.5^{ab}
Acetic acid	5	3.7 ± 0.4^{abcdef}	14.0 ± 2.9^{f}	28.5 ± 5.2^{abc}
	10	3.8 ± 0.4^{abcdef}	15.9 ± 3.2^{abcdef}	25.0 ± 4.4^{bcde}
	20	3.8 ± 0.4^{abcdef}	14.1 ± 2.2^{f}	28.5 ± 3.6^{abc}
	40	4.0 ± 0.4^{ab}	14.8 ± 3.7^{cdef}	25.8 ± 4.1^{bcde}
	80	4.0 ± 0.4^{abc}	14.6 ± 1.6^{def}	28.0 ± 3.8^{abcd}
P80	5	3.5 ± 0.5^{defg}	16.4 ± 2.1^{abcde}	28.8 ± 4.0^{abc}
	10	3.5 ± 0.4^{efg}	15.2 ± 1.2^{abcdef}	28.9 ± 4.2^{abc}
	20	3.6 ± 0.4^{cdefg}	14.7 ± 1.8^{cdef}	26.2 ± 3.4^{abcde}
	40	3.2 ± 0.3^{g}	15.0 ± 1.3^{cdef}	26.4 ± 3.4^{abcde}
	80	3.6 ± 0.3^{cdefg}	16.4 ± 2.0^{abcde}	26.3 ± 5.8^{abcde}
O80	5	3.9 ± 0.5^{abcd}	14.2 ± 1.7^{f}	26.0 ± 2.5^{abcde}
	10	3.6 ± 0.3^{bcdef}	14.3 ± 1.9^{f}	26.3 ± 3.7^{abcde}
	20	3.9 ± 0.3^{abcdef}	17.1 ± 1.5^{ab}	28.6 ± 5.4^{abc}
	40	4.1 ± 0.3^{a}	15.1 ± 1.4^{bcdef}	24.9 ± 3.7^{bcde}
	80	3.6 ± 0.5^{cdefg}	15.0 ± 1.7^{cdef}	30.3 ± 5.2^{a}
P90	5	3.7 ± 0.5^{abcdef}	14.8 ± 3.1^{cdef}	24.9 ± 2.4^{cde}
	10	3.8 ± 0.8^{abcdef}	16.6 ± 2.6^{abcd}	25.1 ± 4.2^{bcde}
	20	4.0 ± 0.4^{ab}	17.2 ± 2.5^{a}	28.8 ± 5.0^{abc}
	40	3.6 ± 0.5^{cdefg}	15.8 ± 1.8^{abcdef}	26.8 ± 4.6^{abcde}
	80	4.0 ± 0.4^{abc}	14.4 ± 2.2^{ef}	24.8 ± 2.0^{cde}
O90	5	3.9 ± 0.4^{abcde}	15.6 ± 2.2^{abcdef}	25.3 ± 7.4^{bcde}
	10	3.5 ± 0.6^{fg}	14.8 ± 1.3^{cdef}	23.4 ± 3.7^{e}
	20	3.8 ± 0.4^{abcdef}	15.1 ± 1.8^{bcdef}	24.2 ± 4.6^{de}
	40	4.1 ± 0.5^{a}	16.9 ± 2.6^{abc}	29.0 ± 5.5^{abc}
	80	3.9 ± 0.5^{abc}	15.8 ± 2.8^{abcdef}	26.2 ± 5.4^{abcde}

Data are shown as the mean ± 1 SD and column with different letters denote significant differences according to Duncan's multiple range tests (P<0.05)

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Table C-7. The effects of three solvents of chitosan including acetic, citric and lactic acid on shoot part of rice seedling

Treatment	Conc. (mg/L)	Plant height (cm)		Shoot fresh weight (mg)		Shoot dry weight (mg)	
		Day 7	Day 14	Day 7	Day 14	Day 7	Day 14
Water		11.5 ± 0.6 a	28.7 ± 1.0 abc	48.39 ± 4.36 a	173.03 ± 14.93 a	7.06 ± 0.44 ab	26.07 ± 3.59 a
Acetic acid	5	10.7 ± 0.7 bcd	29.0 ± 0.8 ab	45.59 ± 2.20 abcd	167.90 ± 24.63 ab	6.95 ± 0.41 abc	25.60 ± 1.93 ab
	10	11.6 ± 0.9 a	28.7 ± 0.8 abc	46.73 ± 2.62 ab	163.63 ± 26.90 abc	7.28 ± 0.52 a	26.16 ± 2.72 a
	20	10.3 ± 0.9 cde	27.5 ± 1.0 def	42.04 ± 2.12 defgh	147.15 ± 15.24 de	6.43 ± 0.39 bcd	20.93 ± 6.25 e
	40	10.3 ± 1.1 cde	28.3 ± 1.0 abcde	40.05 ± 4.68 gh	144.60 ± 10.01 def	6.28 ± 0.72 d	23.28 ± 3.05 bcde
	80	10.7 ± 0.8 bcd	27.0 ± 1.1 f	42.74 ± 6.47 cdefgh	137.61 ± 27.16 efg	6.71 ± 0.71 bcd	23.08 ± 2.98 bcde
Citric acid	5	11.2 ± 0.5 ab	28.7 ± 1.2 abc	45.83 ± 4.73 abc	149.71 ± 26.21 cde	6.94 ± 0.44 abc	23.97 ± 1.83 abcd
	10	10.5 ± 1.0 bcd	29.2 ± 1.6 a	41.33 ± 3.81 efgh	151.81 ± 34.55 cde	6.39 ± 0.64 cd	24.71 ± 1.84 abcd
	20	10.2 ± 1.0 cde	28.3 ± 1.0 abcd	43.97 ± 3.05 bcdef	151.93 ± 30.06 cde	7.07 ± 0.44 ab	24.92 ± 2.27 abc
	40	10.0 ± 1.3 de	25.9 ± 1.3 g	40.04 ± 8.78 gh	126.19 ± 16.22 g	6.65 ± 0.86 bcd	21.02 ± 2.59 e
	80	9.8 ± 0.9 e	27.8 ± 1.7 cdef	39.28 ± 4.42 h	146.59 ± 10.54 de	6.62 ± 0.78 bcd	23.94 ± 3.04 abcd
Lactic acid	5	10.3 ± 0.7 cde	27.9 ± 1.6 bcdef	40.39 ± 4.02 fgh	138.34 ± 40.18 efg	6.36 ± 0.48 d	23.29 ± 2.52 bcde
	10	10.5 ± 0.7 bcd	27.2 ± 0.9 ef	41.64 ± 3.70 efgh	138.86 ± 29.20 efg	6.54 ± 0.43 bcd	22.46 ± 1.82 cde
	20	11.2 ± 1.2 ab	28.6 ± 1.5 abc	44.68 ± 8.21 bcde	155.03 ± 30.54 bcd	6.78 ± 0.93 abcd	24.48 ± 1.75 abcd
	40	10.8 ± 0.4 bc	28.2 ± 1.0 abcde	41.10 ± 4.30 efgh	152.46 ± 35.21 cde	6.72 ± 0.50 bcd	25.08 ± 2.14 ab
	80	10.6 ± 0.7 bcd	26.9 ± 1.8 f	43.39 ± 5.87 bcdefg	130.09 ± 35.03 fg	6.80 ± 0.74 abcd	22.28 ± 2.20 de

Data are shown as the mean ± 1 SD and column with different letters denote significant differences according to Duncan's multiple range tests ($P<0.05$).

Table C-8. The effects of three solvents of chitosan including acetic, citric and lactic acid on root part of rice seedling

Treatment	Conc. (mg/L)	Root lenght (cm)		Root fresh weight (mg)		Root dry weight (mg)	
		Day 7	Day 14	Day 7	Day 14	Day 7	Day 14
Water		11.5 ± 0.6 a	28.7 ± 1.0 abc	13.43 ± 5.32 a	35.78 ± 11.50 abc	3.13 ± 0.38 bcd	6.38 ± 1.33 abc
Acetic acid	5	10.7 ± 0.7 bcd	29.0 ± 0.8 ab	10.72 ± 4.46 b	34.89 ± 15.42 abcd	2.85 ± 0.30 d	6.18 ± 0.64 abcd
	10	11.6 ± 0.9 a	28.7 ± 0.8 abc	8.61 ± 2.99 bcde	33.33 ± 14.41 abcd	3.01 ± 0.36 cd	6.33 ± 0.89 abc
	20	10.3 ± 0.9 cde	27.5 ± 1.0 def	8.15 ± 2.76 cde	30.09 ± 15.08 abcd	2.84 ± 0.45 d	5.56 ± 0.93 cd
	40	10.3 ± 1.1 cde	28.3 ± 1.0 abcde	8.85 ± 3.28 bcde	28.21 ± 12.57 def	3.04 ± 0.46 bcd	5.99 ± 1.52 bcd
	80	10.7 ± 0.8 bcd	27.0 ± 1.1 f	9.22 ± 2.44 bcde	22.09 ± 11.71 f	3.38 ± 0.32 abc	5.40 ± 0.81 d
Citric acid	5	11.2 ± 0.5 ab	28.7 ± 1.2 abc	9.65 ± 3.74 bcd	33.13 ± 19.94 abcd	2.95 ± 0.44 d	6.63 ± 0.86 ab
	10	10.5 ± 1.0 bcd	29.2 ± 1.6 a	7.64 ± 2.76 cde	34.18 ± 23.23 abcd	2.98 ± 0.32 cd	6.25 ± 1.18 abcd
	20	10.2 ± 1.0 cde	28.3 ± 1.0 abcd	6.93 ± 2.22 e	31.39 ± 16.32 abcd	3.08 ± 0.44 bcd	7.01 ± 1.29 a
	40	10.0 ± 1.3 de	25.9 ± 1.3 g	7.35 ± 1.89 de	27.47 ± 12.37 ef	3.10 ± 0.45 bcd	6.46 ± 1.00 abc
	80	9.8 ± 0.9 e	27.8 ± 1.7 cdef	7.23 ± 1.60 de	29.31 ± 13.76 cde	3.08 ± 0.32 bcd	6.50 ± 0.82 ab
Lactic acid	5	10.3 ± 0.7 cde	27.9 ± 1.6 bcdef	9.00 ± 5.12 bcde	31.60 ± 21.08 abcd	3.02 ± 0.30 cd	6.42 ± 1.50 abc
	10	10.5 ± 0.7 bcd	27.2 ± 0.9 ef	8.52 ± 4.79 bcde	29.80 ± 17.12 bcde	2.91 ± 0.32 d	6.19 ± 0.96 abcd
	20	11.2 ± 1.2 ab	28.6 ± 1.5 abc	9.98 ± 4.76 bc	36.66 ± 19.99 a	3.56 ± 0.70 a	7.03 ± 1.47 a
	40	10.8 ± 0.4 bc	28.2 ± 1.0 abcde	9.11 ± 1.82 bcde	36.30 ± 21.15 ab	3.43 ± 0.90 ab	7.03 ± 0.92 a
	80	10.6 ± 0.7 bcd	26.9 ± 1.8 f	8.33 ± 3.20 bcde	28.08 ± 16.71 def	3.05 ± 0.27 bcd	6.30 ± 0.79 abcd

Data are shown as the mean ± 1 SD and column with different letters denote significant differences according to Duncan's multiple range tests ($P<0.05$).

Table C-9. The effects of different types and concentrations of chitosan in 1% (v/v) lactic acid on shoot part of rice seedling

Treatment	Conc. (mg/L)	Plant height (cm)			Shoot fresh weight (mg)			Shoot dry weight (mg)		
		Day 7	Day 14	Day 21	Day 7	Day 14	Day 21	Day 7	Day 14	Day 21
Water		13.8 ± 0.6 ^{ab}	29.7 ± 1.8 ^{a}	35.0 ± 1.2 ^{bcde}	64.2 ± 4.1 ^{ab}	277.2 ± 23.3 ^{a}	664.2 ± 63.1 ^{abcdef}	10.3 ± 0.4 ^{a}	48.4 ± 3.7 ^{a}	144.6 ± 13.7 ^{abcd}
Lactic acid	10	12.5 ± 0.4 ^{cde}	27.9 ± 1.6 ^{bcdef}	34.2 ± 1.5 ^{de}	58.0 ± 4.5 ^{bcd}	235.2 ± 34.3 ^{cde}	635.7 ± 73.8 ^{def}	9.1 ± 0.6 ^{cde}	41.0 ± 5.2 ^{cd}	134.4 ± 15.7 ^{bcde}
	20	13.8 ± 0.9 ^{cdefg}	28.7 ± 2.3 ^{abcde}	36.0 ± 1.9 ^{abc}	61.2 ± 3.6 ^{ab}	255.4 ± 45.3 ^{abc}	724.5 ± 127.2 ^{abcde}	9.5 ± 0.3 ^{bcd}	45.1 ± 6.2 ^{ab}	150.5 ± 20.1 ^{ab}
	40	13.6 ± 0.9 ^{cdefg}	29.1 ± 2.0 ^{ab}	35.6 ± 2.0 ^{abcd}	61.3 ± 6.3 ^{ab}	256.0 ± 26.6 ^{abc}	719.6 ± 85.7 ^{abcde}	9.4 ± 0.8 ^{bcd}	44.8 ± 3.0 ^{abc}	153.2 ± 14.7 ^{a}
P80	10	12.7 ± 0.6 ^{abcdef}	28.0 ± 1.4 ^{bcdef}	34.6 ± 1.8 ^{cde}	55.2 ± 6.5 ^{cdef}	243.9 ± 24.1 ^{bcde}	669.6 ± 108.7 ^{abcdef}	9.2 ± 0.6 ^{efg}	42.9 ± 4.3 ^{bcd}	136.4 ± 18.5 ^{bcde}
	20	14.3 ± 0.6 ^{i}	28.9 ± 1.3 ^{abc}	36.5 ± 2.5 ^{ab}	60.7 ± 4.9 ^{ab}	265.5 ± 25.8 ^{ab}	743.5 ± 127.2 ^{ab}	9.7 ± 0.9 ^{abc}	47.2 ± 3.7 ^{a}	158.4 ± 22.8 ^{a}
	40	12.9 ± 0.8 ^{abcdef}	28.5 ± 1.6 ^{abcde}	34.3 ± 3.0 ^{de}	53.4 ± 4.5 ^{defg}	264.4 ± 27.6 ^{ab}	675.2 ± 164.7 ^{abcdef}	9.0 ± 1 ^{cde}	47.2 ± 2.9 ^{a}	145.4 ± 33.2 ^{abc}
O80	10	12.1 ± 0.6 ^{fgh}	26.9 ± 1.6 ^{f}	33.7 ± 1.9 ^{e}	51.4 ± 4.5 ^{fgh}	228.8 ± 33.7 ^{de}	630.0 ± 115.9 ^{ef}	8.5 ± 0.6 ^{fg}	40.4 ± 7.4 ^{d}	126.5 ± 19.2 ^{e}
	20	14.1 ± 0.6 ^{abcdef}	29.9 ± 1.6 ^{a}	36.6 ± 1.8 ^{ab}	57.0 ± 5.9 ^{bcde}	268.4 ± 24.2 ^{ab}	727.5 ± 120.2 ^{abcd}	9.7 ± 0.9 ^{ab}	46.1 ± 3.5 ^{ab}	150.6 ± 23.1 ^{ab}
	40	12.8 ± 0.9 ^{abcdef}	27.6 ± 1.2 ^{cdef}	33.8 ± 1.7 ^{e}	52.7 ± 7.8 ^{efg}	229.7 ± 25.1 ^{de}	610.3 ± 72.3 ^{f}	9.1 ± 0.6 ^{g}	42.6 ± 6.0 ^{bcd}	131.8 ± 14.1 ^{cde}
P90	10	14.2 ± 0.4 ^{i}	29.1 ± 0.9 ^{ab}	36.9 ± 1.5 ^{a}	58.9 ± 6.7 ^{bc}	252.5 ± 16.1 ^{abcd}	719.2 ± 52.9 ^{abcde}	9.9 ± 0.5 ^{cde}	44.7 ± 3.0 ^{abc}	155.4 ± 9.1 ^{a}
	20	13.9 ± 0.7 ^{defgh}	29.1 ± 1.5 ^{ab}	36.1 ± 2.7 ^{abc}	57.5 ± 7.2 ^{bcde}	256.8 ± 31.1 ^{abc}	651.7 ± 98.4 ^{bcdef}	9.7 ± 0.5 ^{abcd}	48.7 ± 3.0 ^{a}	141.8 ± 19.6 ^{abcde}
	40	14.2 ± 0.6 ^{gh}	28.8 ± 1.7 ^{abcd}	36.6 ± 1.4 ^{ab}	57.2 ± 4.8 ^{bcde}	270.6 ± 44.1 ^{a}	736.4 ± 75.0 ^{abc}	9.5 ± 0.5 ^{def}	48.4 ± 5.5 ^{a}	155.9 ± 16.8 ^{a}
O90	10	12.2 ± 0.7 ^{abcde}	27.4 ± 2.1 ^{def}	34.0 ± 1.5 ^{de}	50.0 ± 5.9 ^{gh}	225.3 ± 27.8 ^{e}	645.4 ± 92.8 ^{cdef}	8.4 ± 0.5 ^{abc}	40.7 ± 4.4 ^{d}	135.1 ± 13.4 ^{bcde}
	20	13.6 ± 0.4 ^{abcd}	29.5 ± 0.9 ^{a}	36.9 ± 1.6 ^{a}	58.1 ± 8.6 ^{bcd}	273.8 ± 18.0 ^{a}	750.0 ± 79.9 ^{a}	10.0 ± 0.7 ^{abc}	47.7 ± 3.4 ^{a}	157.2 ± 10.2 ^{a}
	40	12.1 ± 0.5 ^{abcdef}	27.3 ± 1.6 ^{ef}	33.9 ± 1.8 ^{e}	48.2 ± 6.2 ^{h}	224.1 ± 25.9 ^{e}	612.7 ± 92.1 ^{f}	8.2 ± 0.6 ^{bcd}	39.1 ± 4.1 ^{d}	128.1 ± 18.7 ^{de}

Data are shown as the mean ± 1 SD and column with different letters denote significant differences according to Duncan's multiple range tests ($P<0.05$).

Table C-10. The effects of different types and concentrations of chitosan in 1% (v/v) lactic acid on root part of rice seedling

Treatment	Conc. (mg/L)	Root lenght (cm)			Root fresh weight (mg)			Root dry weight (mg)		
		Day 7	Day 14	Day 21	Day 7	Day 14	Day 21	Day 7^{ns}	Day 14	Day 21
Water		9.5 ± 0.4 ^{cde}	9.4 ± 0.5 ^{de}	12.2 ± 1.2 ^{def}	16.9 ± 7.1 ^{abc}	100.7 ± 13.2 ^{bc}	384.5 ± 36.9 ^{bcd}	3.4 ± 0.605	17.5 ± 1.6 ^{bcd}	56.8 ± 6.9 ^{abc}
Lactic acid	10	9.0 ± 0.6 ^{efg}	8.9 ± 0.5 ^{ef}	12.8 ± 1.5 ^{abcde}	16.3 ± 6.3 ^{abc}	86.2 ± 16.5 ^{c}	403.0 ± 70.4 ^{bc}	3.5 ± 0.316	15.2 ± 2.0 ^{ef}	52.5 ± 5.7 ^{bcde}
	20	10.3 ± 0.3 ^{a}	10.3 ± 0.6 ^{a}	11.8 ± 1.5 ^{ef}	18.6 ± 3.3 ^{a}	95.1 ± 23.2 ^{bc}	430.6 ± 94.3 ^{ab}	3.6 ± 0.498	16.8 ± 3.5 ^{de}	55.2 ± 8.8 ^{abc}
	40	9.9 ± 0.8 ^{abc}	9.8 ± 0.7 ^{abcd}	13.0 ± 1.1 ^{abcde}	17.2 ± 3.1 ^{ab}	96.2 ± 26.6 ^{bc}	430.2 ± 69.2 ^{ab}	3.3 ± 0.457	17.7 ± 1.3 ^{bcd}	57.8 ± 6.4 ^{ab}
P80	10	8.6 ± 0.6 ^{gh}	8.9 ± 0.6 ^{ef}	12.4 ± 2.0 ^{cdef}	14.0 ± 1.9 ^{bcdef}	87.4 ± 21.4 ^{c}	387.6 ± 62.7 ^{bcd}	3.4 ± 0.456	15.9 ± 2.5 ^{def}	52.5 ± 7.9 ^{bcde}
	20	10.2 ± 0.9 ^{ab}	10.2 ± 0.9 ^{ab}	13.9 ± 0.9 ^{a}	16.7 ± 3.9 ^{abc}	112.5 ± 26.8 ^{ab}	429.5 ± 64.2 ^{ab}	3.5 ± 0.51	19.3 ± 2.6 ^{abc}	57.7 ± 7.9 ^{ab}
	40	8.4 ± 1.0 ^{ghi}	9.6 ± 0.5 ^{cd}	13.6 ± 2.0 ^{abc}	15.1 ± 5 ^{abcde}	112.4 ± 23.5 ^{ab}	373.3 ± 68.5 ^{bcd}	3.3 ± 0.336	19.5 ± 1.8 ^{ab}	56.2 ± 10.6 ^{abc}
O80	10	8.1 ± 0.6 ^{bcd}	9.0 ± 0.7 ^{ef}	12.6 ± 1.6 ^{bcde}	11.4 ± 4.4 ^{ef}	89.7 ± 21.0 ^{c}	365.8 ± 48.9 ^{cd}	3.4 ± 0.69	15.7 ± 3.4 ^{def}	47.3 ± 5.7 ^{e}
	20	9.2 ± 0.9 ^{abcd}	9.8 ± 0.4 ^{abcd}	13.0 ± 1.5 ^{abcde}	10.0 ± 3.8 ^{f}	88.1 ± 19.0 ^{c}	394.8 ± 78.8 ^{bcd}	3.5 ± 0.629	17.7 ± 1.5 ^{bcd}	55.7 ± 9.1 ^{abc}
	40	9.6 ± 0.6 ^{cde}	9.6 ± 0.8 ^{bcd}	11.9 ± 1.7 ^{def}	11.8 ± 4.7 ^{def}	82.4 ± 17.5 ^{c}	342.6 ± 35.1 ^{d}	3.5 ± 0.542	17.0 ± 2.8 ^{de}	50.6 ± 5.0 ^{cde}
P90	10	9.7 ± 0.5 ^{i}	10.3 ± 0.8 ^{a}	12.7 ± 2.0 ^{abcde}	16.0 ± 8.9 ^{abcd}	90.2 ± 16.2 ^{c}	386.9 ± 31.4 ^{bcd}	3.7 ± 0.291	17.3 ± 1.7 ^{cde}	56.2 ± 6.0 ^{abc}
	20	9.8 ± 0.5 ^{def}	10.3 ± 0.7 ^{a}	12.1 ± 1.5 ^{def}	13.8 ± 7.5 ^{bcdef}	96.8 ± 18.4 ^{bc}	369.0 ± 52.2 ^{cd}	3.5 ± 0.523	17.1 ± 2.0 ^{de}	50.3 ± 6.6 ^{cde}
	40	9.4 ± 0.5 ^{cd}	10.0 ± 0.6 ^{abc}	13.9 ± 1.2 ^{a}	13.3 ± 7.7 ^{bcdef}	122.0 ± 40.7 ^{a}	429.6 ± 42.4 ^{ab}	3.5 ± 0.55	20.3 ± 4.2 ^{a}	60.0 ± 7.1 ^{a}
O90	10	8.2 ± 0.5 ^{hi}	8.8 ± 0.9 ^{f}	13.2 ± 1.1 ^{abcd}	12.6 ± 6.5 ^{cdef}	95.9 ± 19.9 ^{bc}	417.8 ± 71.8 ^{abc}	3.4 ± 0.616	16.2 ± 2.3 ^{de}	54.4 ± 5.0 ^{abcd}
	20	9.9 ± 0.7 ^{abc}	10.3 ± 0.8 ^{a}	13.7 ± 1.7 ^{ab}	16.3 ± 9.5 ^{abc}	111.1 ± 14.3 ^{ab}	469.3 ± 56.2 ^{a}	3.8 ± 0.384	17.7 ± 2.2 ^{bcd}	59.9 ± 4.8 ^{a}
	40	8.7 ± 0.6 ^{fgh}	8.9 ± 0.4 ^{ef}	11.2 ± 1.4 ^{f}	13.3 ± 7.4 ^{bcdef}	93.5 ± 20.7 ^{bc}	368.2 ± 52.8 ^{cd}	3.4 ± 0.658	14.1 ± 3.1 ^{f}	48.4 ± 6.7 ^{de}

Data are shown as the mean ± 1 SD and column with different letters denote significant differences according to Duncan's multiple range tests ($P<0.05$).

Table C-11. The effects of shrimp shell (SS) and O-80 chitosan residue (fermented chitin waste: FCW) at concentrations of 0.25, 0.5 and 1% (w/w) on tiller numbers/plant at 0, 15, 30, 45 and 60 days after transplanting.

Treatment	Conc. (% w/w)	Tiller numbers/plant (tillers)				
		Day 0[ns]	Day 15	Day 30	Day 45	Day 60
CF		3.0 ± 0.0	3.1 ± 0.3 [a]	4.9 ± 1.6 [c]	4.7 ± 0.8 [cd]	7.6 ± 1.5 [c]
OF		3.0 ± 0.0	3.1 ± 0.5 [a]	3.9 ± 0.5 [c]	4.0 ± 0.6 [d]	3.6 ± 0.5 [d]
SS	0.25	3.0 ± 0.0	3.2 ± 0.4 [a]	10.5 ± 2.0 [a]	14.0 ± 3.0 [ab]	11.9 ± 2.2 [b]
	0.5	3.0 ± 0.0	2.7 ± 1.2 [ab]	9.4 ± 4.8 [ab]	16.7 ± 5.8 [a]	15.9 ± 6.0 [a]
	1	3.0 ± 0.0	1.1 ± 0.3 [c]	3.5 ± 1.8 [c]	7.3 ± 3.7 [c]	12.0 ± 4.0 [b]
FCW	0.25	3.0 ± 0.0	2.7 ± 0.8 [ab]	9.1 ± 1.9 [ab]	12.3 ± 1.9 [b]	9.2 ± 2.2 [c]
	0.5	3.0 ± 0.0	2.5 ± 0.7 [b]	8.3 ± 3.3 [b]	16.4 ± 3.6 [a]	14.9 ± 3.3 [ab]
	1	3.0 ± 0.0	1.0 ± 0.0 [c]	3.5 ± 1.5 [c]	12.2 ± 4.9 [b]	16.4 ± 6.5 [a]

Data are shown as the mean ± 1 SD and column with different letters denote significant differences according to Duncan's multiple range tests (P<0.05).

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Table C-12. The effects of shrimp shell (SS) and O-80 chitosan residue (fermented chitin waste: FCW) at concentrations of 0.25, 0.5 and 1% (w/w) on photosynthetic characteristics including net photosynthesis rate (A_{max}), stomatal conductance (g_s), internal concentration of CO_2, (C_i) and transpiration rate (E) at vegetative stage on days 30 after transplanting.

Treatment	Conc. (% w/w)	Photosynthetic characteristics			
		A_{max} (μmol $CO_2/m^2 s^1$)	g_s (mmol/m^2 s^1)	C_i ns (ppm)	E (mmol H_2O/m^2 s^1)
CF		18.01 ± 4.5 ^{d}	0.36 ± 0.10 ^{de}	265.9 ± 8.0	12.38 ± 2.56 ^{cd}
OF		14.58 ± 3.6 ^{e}	0.29 ± 0.08 ^{e}	269.2 ± 6.8	10.85 ± 2.80 ^{d}
SS	0.25	20.66 ± 3.0 ^{cd}	0.43 ± 0.08 ^{cd}	267.3 ± 7.5	13.52 ± 3.16 ^{bc}
	0.5	25.09 ± 2.5 ^{ab}	0.53 ± 0.13 ^{abc}	265.6 ± 11.6	15.51 ± 2.91 ^{ab}
	1	22.30 ± 7.6 ^{bc}	0.50 ± 0.20 ^{bc}	273.7 ± 13.5	15.15 ± 4.40 ^{ab}
FCW	0.25	26.54 ± 2.9 ^{a}	0.64 ± 0.17 ^{a}	273.2 ± 11.7	17.23 ± 3.49 ^{a}
	0.5	26.65 ± 3.3 ^{a}	0.64 ± 0.15 ^{a}	273.8 ± 11.8	16.60 ± 3.50 ^{a}
	1	25.92 ± 2.7 ^{a}	0.57 ± 0.14 ^{ab}	268.5 ± 11.7	16.52 ± 3.16 ^{a}

Data are shown as the mean ± 1 SD and column with different letters denote significant differences according to Duncan's multiple range tests (P<0.05).

Table C-13. The effects of shrimp shell (SS) and O-80 chitosan residue (fermented chitin waste: FCW) at concentrations of 0.25, 0.5 and 1% (w/w) on photosynthetic characteristics including net photosynthesis rate (A_{max}), stomatal conductance (g_s), internal concentration of CO_2, (C_i) and transpiration rate (E) of the second leaf at reproductive stage on days 60 after transplanting.

Treatment	Conc. (% w/w)	Photosynthetic characteristics			
		A_{max} (μmol $CO_2/m^2 s^1$)	g_s (mmol/m^2 s^1)	C_i (ppm)	E (mmol H_2O/m^2 s^1)
CF		21.70 ± 3.01 ^{b}	0.53 ± 0.13 ^{b}	286.5 ± 9.4	9.96 ± 1.54 ^{a}
OF		14.61 ± 3.11 ^{d}	0.33 ± 0.05 ^{c}	286.9 ± 9.5	7.86 ± 1.04 ^{b}
SS	0.25	17.94 ± 3.60 ^{c}	0.43 ± 0.15 ^{bc}	282.2 ± 12.5	8.54 ± 2.07 ^{b}
	0.5	19.27 ± 2.03 ^{c}	0.43 ± 0.07 ^{bc}	278.8 ± 6.5	8.41 ± 0.87 ^{b}
	1	25.14 ± 2.41 ^{a}	0.75 ± 0.26 ^{a}	290.9 ± 15.4	10.99 ± 1.42 ^{a}
FCW	0.25	17.72 ± 2.70 ^{c}	0.46 ± 0.16 ^{bc}	288.2 ± 11.3	8.75 ± 1.69 ^{b}
	0.5	18.21 ± 2.32 ^{c}	0.43 ± 0.13 ^{bc}	281.3 ± 12.0	8.14 ± 1.49 ^{b}
	1	23.47 ± 3.43 ^{ab}	0.66 ± 0.20 ^{a}	288.8 ± 11.1	10.17 ± 1.77 ^{a}

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Data are shown as the mean ± 1 SD and column with different letters denote significant differences according to Duncan's multiple range tests (P<0.05).

Table C-14. The effects of shrimp shell (SS) and O-80 chitosan residue (fermented chitin waste: FCW) at concentrations of 0.25, 0.5 and 1% (w/w) on photosynthetic characteristics including net photosynthesis rate (A_{max}), stomatal conductance (g_s), internal concentration of CO_2, (C_i) and transpiration rate (E) of the flag leaf at reproductive stage on days 67 after transplanting.

Treatment	Conc. (% w/w)	Photosynthetic characteristics			
		A_{max} (μmol $CO_2/m^2 s^1$)	g_s (mmol/m^2 s^1)	C_i (ppm)	E (mmol H_2O/m^2 s^1)
CF		18.33 ± 2.8 ^{bc}	0.40 ± 0.08 ^{c}	275.9 ± 11.6 ^{bc}	7.56 ± 1.31 ^{bc}
OF		14.15 ± 2.5 ^{d}	0.30 ± 0.07 ^{d}	280.0 ± 7.9 ^{abc}	6.51 ± 0.99 ^{d}
SS	0.25	17.19 ± 3.0 ^{c}	0.39 ± 0.11 ^{cd}	278.9 ± 10.5 ^{abc}	7.32 ± 1.40 ^{cd}
	0.5	18.50 ± 2.3 ^{bc}	0.40 ± 0.12 ^{c}	272.4 ± 11.4 ^{c}	7.05 ± 1.00 ^{cd}
	1	23.26 ± 2.2 ^{a}	0.66 ± 0.15 ^{a}	284.9 ± 11.8 ^{ab}	9.18 ± 0.73 ^{a}
FCW	0.25	18.80 ± 2.4 ^{bc}	0.46 ± 0.13 ^{bc}	282.7 ± 13.4 ^{ab}	7.89 ± 1.13 ^{bc}
	0.5	19.31 ± 1.4 ^{bc}	0.51 ± 0.11 ^{b}	285.7 ± 11.3 ^{a}	8.34 ± 0.71 ^{ab}
	1	21.55 ± 1.3 ^{a}	0.55 ± 0.13 ^{b}	277.2 ± 16.0 ^{abc}	8.26 ± 0.84 ^{a}

Data are shown as the mean ± 1 SD and column with different letters denote significant differences according to Duncan's multiple range tests (P<0.05).

Table C-15. The effect of 0.25% (w/w) shrimp shell (SS) or O-80 chitosan residue (fermented chitin: FCW) as soil supplemented on contents of chlorophyll *a*, *b* and carotenoid on day 0, 7 and 14 after treatment.

Treatment	Conc. (% w/w)	Photosynthetic pigment contents (mg/g FW)								
		chlorophyll *a*			chlorophyll *b*			carotenoid		
		day 0^{ns}	day 7	day 14	day 0^{ns}	day 7	day 14	day 0^{ns}	day 7	day 14
Control		2.36 ± 0.25	2.09 ± 0.33^{b}	1.78 ± 0.42^{b}	0.77 ± 0.09	0.70 ± 0.11^{b}	0.57 ± 0.12^{b}	0.65 ± 0.06	0.61 ± 0.09^{b}	0.51 ± 0.11^{b}
SS	0.25	2.61 ± 0.19	3.22 ± 0.22^{a}	3.27 ± 0.34^{a}	0.87 ± 0.06	1.06 ± 0.07^{a}	1.06 ± 0.12^{a}	0.74 ± 0.06	0.86 ± 0.05^{a}	0.87 ± 0.09^{a}
FCW	0.25	2.47 ± 0.13	3.27 ± 0.56^{a}	3.44 ± 0.25^{a}	0.81 ± 0.04	1.08 ± 0.20^{a}	1.12 ± 0.09^{a}	0.69 ± 0.03	0.88 ± 0.15^{a}	0.92 ± 0.07^{a}

Data are shown as the mean ± 1 SD and column with different letters denote significant differences according to Duncan's multiple range tests (P<0.05).

Table C-16. The effect of 0.25% (w/w) shrimp shell (SS) or O-80 chitosan residue (fermented chitin: FCW) as soil supplemented on net photosynthesis rate of seedling at 0, 7 and 14 days after treatment.

Treatment	Conc. (% w/w)	A_{max} (μmol $CO_2/m^2 s^1$)		
		day 0^{ns}	day 7	day 14
Control		12.43 ± 4.45	10.93 ± 2.09 ^{b}	18.8 ± 3.92 ^{b}
SS	0.25	12.37 ± 3.10	16.39 ± 3.38 ^{a}	23.0 ± 1.68 ^{a}
FCW	0.25	12.84 ± 3.14	16.25 ± 3.50 ^{a}	22.6 ± 2.42 ^{a}

Data are shown as the mean ± 1 SD and column with different letters denote significant differences according to Duncan's multiple range tests (P<0.05).

Table C-17. The effect of 0.25% (w/w) shrimp shell (SS) or O-80 chitosan residue (fermented chitin: FCW) as soil supplemented on stomatal conductance of seedling at 0, 7 and 14 days after treatment.

Treatment	Conc. (% w/w)	g_s (mmol/m^2 s^1)		
		day 0^{ns}	day 7	day 14^{ns}
Control		0.22 ± 0.06	0.17 ± 0.04 ^{b}	0.3 ± 0.08
SS	0.25	0.21 ± 0.03	0.23 ± 0.06 ^{a}	0.3 ± 0.03
FCW	0.25	0.22 ± 0.04	0.22 ± 0.05 ^{a}	0.3 ± 0.04

Data are shown as the mean ± 1 SD and column with different letters denote significant differences according to Duncan's multiple range tests (P<0.05).

Table C-18. The effect of 0.25% (w/w) shrimp shell (SS) or O-80 chitosan residue (fermented chitin: FCW) as soil supplemented on internal concentration of CO_2 of seedling at 0, 7 and 14 days after treatment.

Treatment	Conc. (% w/w)	C_i (ppm)		
		day 0^{ns}	day 7	day 14^{ns}
Control		265.70 ± 13.31	257.72 ± 14.94 ^{a}	249.7 ± 9.77
SS	0.25	264.41 ± 14.85	237.93 ± 13.52 ^{b}	232.0 ± 6.20
FCW	0.25	261.95 ± 12.18	236.90 ± 6.73 ^{b}	232.6 ± 10.30

Data are shown as the mean ± 1 SD and column with different letters denote significant differences according to Duncan's multiple range tests (P<0.05).

Table C-19. The effect of 0.25% (w/w) shrimp shell (SS) or O-80 chitosan residue (fermented chitin: FCW) as soil supplemented on transpiration rate of seedling at 0, 7 and 14 days after treatment.

Treatment	Conc. (% w/w)	E (mmol $H_2O/m^2 s^1$)		
		day 0^{ns}	day 7	day 14^{ns}
Control		6.29 ± 1.57	4.50 ± 0.82 ^{b}	7.7 ± 1.64
SS	0.25	6.29 ± 0.78	5.70 ± 1.25 ^{a}	7.9 ± 0.71
FCW	0.25	6.41 ± 0.95	5.27 ± 1.08 ^{a}	7.9 ± 0.88

Data are shown as the mean ± 1 SD and column with different letters denote significant differences according to Duncan's multiple range tests (P<0.05).

Table C-20. Relative gene expression of *OEE1* on seedling treated with SS and FCW as soil supplement at days 0, 7 and 14 after treatment.

Treatment	Conc. (% w/w)	Relative gene expression		
		OEE1		
		day 0^{ns}	day 7^{ns}	day 14^{ns}
Control		1.00 ± 0.00	1.42 ± 0.5	1.38 ± 0.4
SS	0.25	1.05 ± 0.21	1.36 ± 0.3	1.20 ± 0.4
FCW	0.25	0.93 ± 0.20	1.56 ± 0.5	1.41 ± 0.6

Data are shown as the mean ± 1 SD and column with different letters stand for significant differences according to Duncan's multiple range tests (P<0.05).

Table C-21. Relative gene expression of *PsbS1* on seedling treated with SS and FCW as soil supplement at days 0, 7 and 14 after treatment.

Treatment	Conc. (% w/w)	Relative gene expression		
		PsbS1		
		day 0[ns]	day 7	day 14
Control		1.00 ± 0.00	0.90 ± 0.20[i]	1.44 ± 0.44[b]
SS	0.25	1.06 ± 0.14	0.58 ± 0.36[l]	1.53 ± 1.01[b]
FCW	0.25	1.00 ± 0.26	0.92 ± 0.11[i]	2.44 ± 0.49[a]

Data are shown as the mean ± 1 SD and column with different letters stand for significant differences according to Duncan's multiple range tests (P<0.05).

Table C-22. Relative gene expression of *rbcS* on seedling treated with SS and FCW as soil supplement at days 7 and 14 after treatment.

Treatment	Conc. (% w/w)	Relative gene expression		
		rbcS		
		day 0[ns]	day 7	day 14
Control		1.00 ± 0.00	1.14 ± 0.24[i]	1.29 ± 0.67[a]
SS	0.25	1.00 ± 0.28	0.84 ± 0.32[l]	0.87 ± 0.27[b]
FCW	0.25	0.96 ± 0.28	1.19 ± 0.35[i]	1.20 ± 0.39[a]

Data are shown as the mean ± 1 SD and column with different letters stand for significant differences according to Duncan's multiple range tests (P<0.05).

VITA

Miss Nungruthai Kananont was born on November 15th, 1982 in Bangkok, Thailand. In 2004, she graduated with a second honor of bachelor's degree in genetic program from Department of Botany, Faculty of Science, Chulalongkorn University. Subsequently, she initially studied chitin-chitosan's application for stimulation of plant growth for master's degree of science in program in biotechnology, Chulalongkorn University with the thesis entitled "Improvement of Seed Propagation by Chitosan Treatment and Seed Transformation of Genus Dendrobium" and graduated in 2007. After that, she was granted from ChulalongKorn University Graduate Scholarship to Commemorate the 72nd Anniversary of His Majesty King Bhumibol Adulyadej in 2009 for doctor's degree and continuously worked with chitin-chitosan's application in botany program at the same university.

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